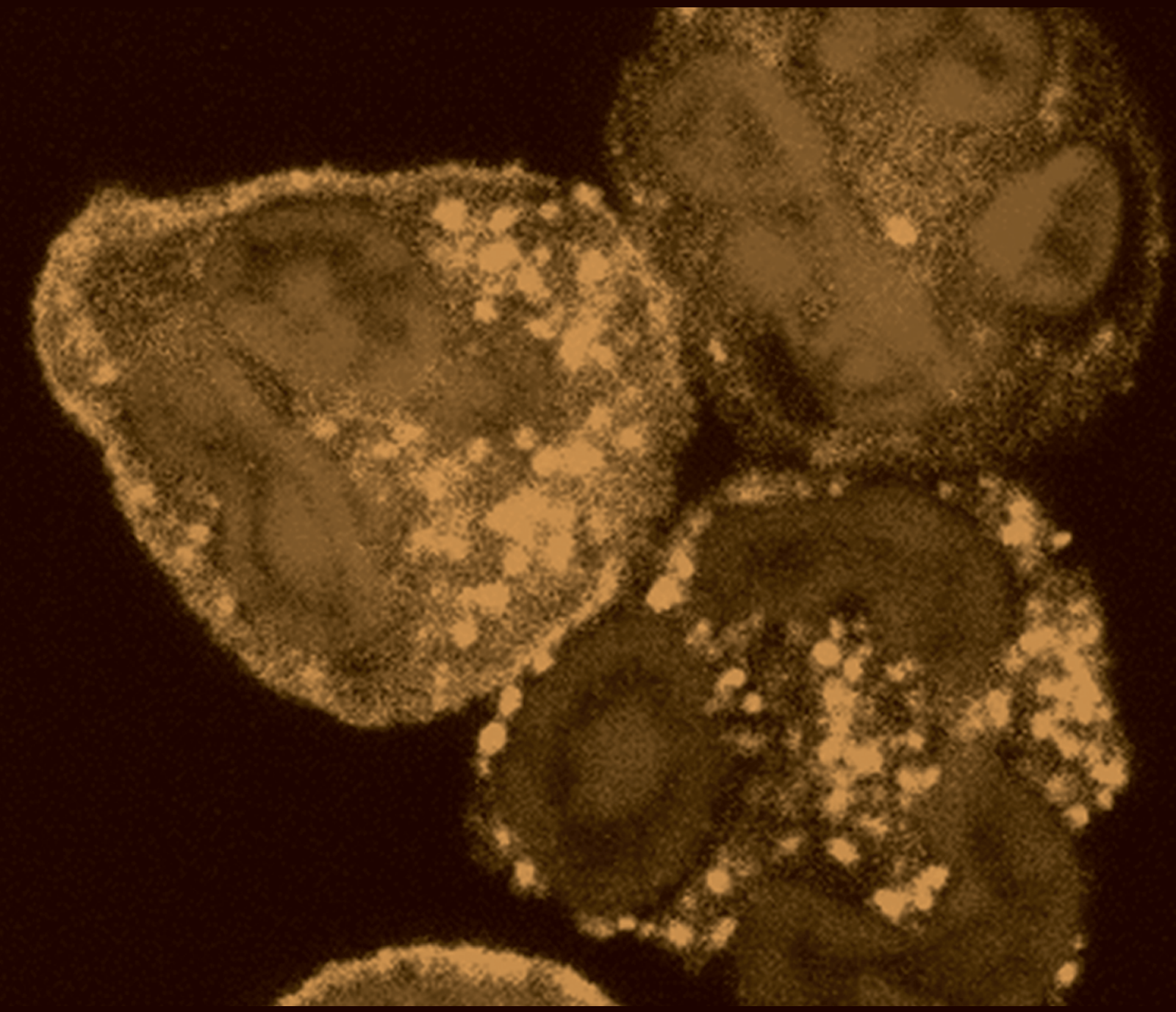
Mediators of Inflammation

Inflammation and Infection in Critical Care Medicine

Guest Editors: Jesús F. Bermejo-Martin, Ignacio Martín-Loeches, and Steven Bosinger



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Editorial Board

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Editorial

Inflammation and Infection in Critical Care Medicine

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Inflammation and infection are closely linked in critically ill patients. When adaptive immunity fails to prevent or control infection, an exacerbated/maintained inflammatory response emerges as consequence. It could be considered as an attempt of the immune system to fight the infection, which, in so many occasions, results insufficiently. In turn, uncontrolled inflammatory responses impair the development of specific and targeted responses against the infecting microbe, closing a vicious circle. The consequences of unbalanced inflammatory and adaptive responses to infection are microbial escape and tissue damage, contributing to the physiopathogenesis of sepsis, acute respiratory distress syndrome, or multiorganic failure. In this special issue, Steel C. et al. review the major virulence factors of *S. pneumoniae* and their role in triggering overexuberant inflammatory responses contributing to the immunopathogenesis of invasive disease in community-acquired pneumonia (CAP). These authors provided an insight into the pharmacological, anti-inflammatory strategies with adjunctive potential in the antimicrobial chemotherapy of CAP. In turn, Surbatovic M. et al. explored some of the more novel elements of immunoinflammatory response in severe sepsis and severe trauma, highlighting the role of Toll-like receptors, cytokines, and the genetic polymorphisms in the immune response to infection. Esteban E. et al. summarized the immune modulation actions of extracorporeal devices in the context of endotoxin removal, elimination of cytokines and inflammatory molecules, vascular and coagulation proteins, and removal of cells, which might play a role as an innovative coadjuvant treatment in sepsis or nonseptic respiratory failure. De Pascale G. et al. reviewed the role of mannose-binding lectin (MBL) in severe sepsis and septic shock. MBLs are serum proteins that recognize a wide range of pathogenic microorganisms and activate complement cascade via the antibody-independent pathway. While MBL-deficient patients are at increased risk of infection acquisition, an excess of MBL activation may drive unbalanced proinflammatory responses and additional host injury. MBL replacement therapy was also discussed. An original article from Rodriguez-Fernandez A. and colleagues revealed the protective role of eosinophils in *S. aureus* ventilator-associated pneumonia. Being a recognised actor in the pathophysiology of asthma, there is an increasing body of evidence on the antimicrobial roles played by eosinophils. This paper highlights the potential of an unexpensive tool such as the leukogram as biomarker in severe infections. Finally, Koch A. et al. investigated the regulation and prognostic relevance of symmetric dimethylarginine (SDMA) serum concentrations in critical illness and sepsis. SDMA impacts vascular tension and integrity via modulating nitric oxide (NO) pathways. These authors identified SDMA serum concentrations at admission as an independent prognostic biomarker in critically ill patients not only for short-term mortality at the ICU but also for unfavourable long-term survival, with patients with sepsis showing the highest SDMA levels. They proposed this molecule as a novel biomarker for mortality risk stratification for ICU patients.

We hope that this compilation will be of interest for both clinicians and researchers working in the field of host immune responses to infection in severely ill patients.

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Review Article

Overview of Community-Acquired Pneumonia and the Role of Inflammatory Mechanisms in the Immunopathogenesis of Severe Pneumococcal Disease

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Community-acquired pneumonia (CAP) remains a leading cause of morbidity and mortality among the infectious diseases. Despite the implementation of national pneumococcal polyvalent vaccine-based immunisation strategies targeted at high-risk groups, *Streptococcus pneumoniae* (the pneumococcus) remains the most common cause of CAP. Notwithstanding the HIV pandemic, major challenges confronting the control of CAP include the range of bacterial and viral pathogens causing this condition, the ever-increasing problem of antibiotic resistance worldwide, and increased vulnerability associated with steadily aging populations in developed countries. These and other risk factors, as well as diagnostic strategies, are covered in the first section of this review. Thereafter, the review is focused on the pneumococcus, specifically the major virulence factors of this microbial pathogen and their role in triggering overexuberant inflammatory responses which contribute to the immunopathogenesis of invasive disease. The final section of the review is devoted to a consideration of pharmacological, anti-inflammatory strategies with adjunctive potential in the antimicrobial chemotherapy of CAP. This is focused on macrolides, corticosteroids, and statins with respect to their modes of anti-inflammatory action, current status, and limitations.

1. Overview of Community-Acquired Pneumonia

1.1. Introduction. Community-acquired pneumonia (CAP) is commonly described as an acute infection of the lung parenchyma acquired in the community. It is most commonly bacterial in nature and is associated with clinical and/or radiological evidence of consolidation of part or parts of one or both lungs [1]. CAP is associated with a considerable burden of disease in most regions of the world [2–6]. It is one of the most important serious infectious diseases, accounting for a considerable number of hospital admissions, with an increasing incidence in many parts of the world and an increasing rate of serious complications [7]. As part of the burden of respiratory infections, CAP is well recognised to be a leading cause of death among the infectious diseases [6, 8]. The reason that CAP is so common relates to the very high prevalence of specific risk factors for this infection in patients worldwide [6]. While a myriad of microorganisms may cause CAP, in reality a relatively small number of pathogens predominate, in particular the bacteria, of which *Streptococcus pneumoniae* (pneumococcus) is by far the most common [7].

There is considerable concern about the emerging resistance among the usual CAP pathogens to the most commonly used antimicrobial agents. There are a number of important decisions that need to be made with regard to the assessment and management of patients with CAP, not least of which is

an evaluation of the severity of the infection [6]. A number of guidelines have been published worldwide, describing the optimal treatment of patients with CAP, with the aim of improving patient outcomes.

The remainder of this introductory overview of CAP will focus on (i) burden of disease, (ii) risk factors, (iii) microbiology, (iv) antimicrobial chemotherapy, (v) antibiotic resistance, and (vi) assessment of severity of illness using clinical scoring systems and laboratory biomarkers individually and in combination.

1.2. Burden of Disease. CAP continues to be a cause of considerable morbidity and mortality in most parts of the world, being the most frequent infectious cause of death in patients in the USA and throughout Europe [9]. Since aging is a significant risk factor for this infection and given that in many areas of the world, such as Europe, the population is aging, an increase in incidence in the next decades is anticipated [9]. The mechanism by which aging is associated with a risk for CAP is multifactorial, not simply related to chronological age, but frequently associated with (i) underlying comorbid conditions that more commonly occur in the aging population; (ii) a greater risk of being infected with antibiotic-resistant pathogens; (iii) social factors; and (iv) even place of residence [6]. Studies in the USA [2], Europe [5], Latin America [3], and the Asia-Pacific region [4] attest to the fact that CAP has a substantial clinical and economic burden, a high rate of antibiotic resistance among the pathogens, and a significant effect on both immediate and long-term prognosis, as well as on the quality of life of infected patients. Given this high burden of disease, it is recommended that steps be taken to ensure appropriate treatment, ongoing surveillance for antimicrobial resistance among the common pathogens, and strategies, including vaccination, to prevent these infections.

1.3. Risk Factors. As shown in the list below, there is a considerable number of risk factors for CAP that exist in populations all over the world, and most of these risk factors are associated with an impairment of the efficacy of host immune defence [7]. Many of these risk factors are also associated with a greater mortality risk [6]. In addition to aging, the common risk factors in adults are smoking, the presence of various underlying comorbid conditions, including chronic cardiorespiratory, renal and hepatic conditions, and, at least in some regions of the world, concomitant human immunodeficiency virus (HIV) infection [7]. There is also some evidence that male patients and those of certain racial or ethnic groups may be at greater risk of pneumonia [6].

Risk factors for community-acquired pneumonia are as follows:

(i) extremes of age (very young and the aging),

(ii) male gender,

(iii) certain populations (various racial or ethnic groups),

(iv) lifestyle factors (excessive alcohol consumption and smoking),

(v) underlying comorbid conditions such as

  (a) chronic cardiorespiratory illnesses,
  (b) chronic renal disorders,
  (c) hepatic conditions,
  (d) diabetes mellitus,
  (e) neoplastic diseases,
  (f) human immunodeficiency virus infection,

(vi) medications (e.g., inhaled corticosteroids, proton pump inhibitors),

(vii) additional risk factors associated with pneumococcal infections in particular (e.g., myeloma, hypogammaglobulinemia (such as IgG2 deficiency), surgical asplenia, or "functional" asplenia (such as in sickle cell disease).

Smoking, both active and passive (particularly in children), is a well-described risk factor for CAP, particularly in HIV-infected persons, as well as for many other infectious diseases, and this has been comprehensively reviewed recently [10]. The main mechanisms for this predisposition relate to the suppressive effect that smoking has on the protective actions of the airway mucociliary clearance mechanism, on the various components of the innate and adaptive immune systems of the host, as well as direct effects on microbial pathogens that promote their virulence, and possibly antibiotic resistance [10].

Several comorbid factors relate quite closely to the risk of CAP and the possibility of more severe illness, as well as to the likelihood of a worse outcome [6]. Among the most common comorbid predisposing factors to CAP, as mentioned above, are chronic obstructive pulmonary disease, congestive cardiac failure, diabetes mellitus, a high intake of alcohol, and smoking [6]. However, various other conditions, including those of the neurological system, the liver, the kidney, and also neoplastic disease, represent important risk factors. More recently, there has been considerable interest in the fact that inhaled medication (particularly inhaled corticosteroids) appears to be a risk factor for CAP and this has been reviewed elsewhere [11].

In a number of regions of the world, such as in sub-Saharan Africa, concomitant infection with HIV represents a major risk factor for CAP and has been extensively reviewed elsewhere [12–14]. The spectrum of bacterial pathogens causing CAP in HIV-infected patients is very similar to that in HIV-uninfected patients with *Streptococcus pneumoniae* (pneumococcus) predominating [13]. The clinical presentation of CAP in HIV-infected persons, particularly pneumococcal CAP, is similar to that among HIV-uninfected patients except that the patients are frequently younger, of the female sex, and have a greater frequency of respiratory symptoms [13, 14]. Furthermore, there is a similar spectrum of disease severities, with the commonly used severity of illness scores appearing to have an equivalent value in predicting outcome compared to HIV-uninfected individuals. However, when cases with bacteremic pneumococcal CAP are stratified according to age and severity of illness, HIV-infected persons

have been found to have a significantly higher mortality with an increasing trend as the CD4 cell count decreases [14]. Some investigators have therefore recommended that the decision to admit HIV-infected patients with CAP to hospital should be based on both the CD4 cell count and the severity of illness, those with a CD4 cell count <200/μL blood always being admitted to hospital, and those with a higher CD4 cell count being admitted to hospital if warranted according to the severity of illness [12].

Recent studies have identified several mechanisms, specifically the presence of gene polymorphisms, including single nucleotide polymorphisms (SNPs) in genes encoding proteins of the innate immune system, which contribute not only to susceptibility for development of CAP, but also to a worse outcome. For example, SNPs in the IL-6 gene, specifically IL-6 174 G/G, have been reported to protect patients with pneumococcal CAP against development of ARDS, septic shock, and multiple organ dysfunction syndrome, resulting in less severe disease and lower mortality [15]. In addition, investigation of the role of SNPs in the genes encoding the surfactant proteins (SP) A, B, C, and D revealed associations with both susceptibility for both development of CAP and more severe disease [16, 17]. Similar, albeit statistically insignificant, findings were reported for SP-A and SP-D in patients with pneumococcal CAP [17].

1.4. Microbiology of CAP. The most prominent organism causing CAP is the pneumococcus, and this remains true irrespective of the severity of infection across the spectrum of outpatients, inpatients not in the intensive care unit (ICU), and even cases with CAP requiring ICU admission [11, 18]. Studies in Europe, the USA, Latin America, the Asia-Pacific region, and elsewhere attest to the fact that the pneumococcus is consistently documented to be the most predominant pathogen [2–5, 9]. Interestingly, while it is well described that pneumococcal infections commonly complicate both seasonal and pandemic influenza infections, more recently it was documented that the pneumococcus was a common bacterial coinfection in patients with influenza A H1N1 infection who were admitted to hospital with CAP [19–21]. In the former two studies, the pneumococcus was the most common bacterial cause of bacterial co-infection, accounting for 62% and 54.8% of cases, respectively, and being associated with a greater risk of septic shock or need for vasopressors, as well as increased need for mechanical ventilation and a longer ICU stay [20, 21].

Many recent studies of pneumococcal infection have focussed on the issue of pneumococcal serotypes causing disease, and the possible association of different serotypes with disease severity, particularly in relation to the release of the newer pneumococcal conjugate vaccines and the extended indication for their use in adults [22–25]. While earlier studies suggested that host factors may be more important than isolate serotype in determining the severity and outcome of pneumococcal infections [22], suggesting that vaccination was unlikely to be associated with a change in these end-points, more recent studies suggest that IPD outcome is a serotype-related issue; for example, serotype 3 is more commonly associated with septic shock [24, 25]. When considering the likely benefit of vaccination, it also remains important to consider the serotypes implicated in nonbacteremic infections, which studies have suggested that due to greater serotype distribution are less comprehensively covered by currently available conjugate vaccines [26, 27].

After the pneumococcus, the next most common pathogens are the so-called atypical pathogens, the respiratory viruses, and *Haemophilus influenzae* [11]. Among the viruses, influenza predominates, but smaller numbers of various other respiratory viruses are also documented [11, 28, 29]. Less commonly, additional pathogens are documented, particularly in cases with respiratory-related comorbid illnesses. These include *Staphylococcus aureus, Pseudomonas aeruginosa*, and the Enterobacteriaceae [11]. The pathogens causing CAP have been extensively reviewed elsewhere [11].

1.5. Antimicrobial Chemotherapy of Bacterial CAP. On presentation of adults with suspected CAP, empiric antimicrobial chemotherapy is initiated according to the relevant national guidelines, with age, comorbidities, and disease severity being the primary determinants of the class of antibiotic(s) and route of administration. In the case of outpatient therapy, previously healthy patients who had not received antibiotics during the 3-month period prior to presentation, monotherapy with a macrolide or doxycycline is recommended by the Infectious Diseases Society of America (IDSA)/American Thoracic Society (ATS); in those with comorbidities and/or prior recent use of antibiotics, the recommendation is the combination of an antipneumococcal β-lactam and a macrolide, or alternatively, a respiratory fluoroquinolone [30]. In the case of inpatient therapy in the non-ICU setting, the guidelines advocate the combination of a β-lactam and a macrolide, or, alternatively, monotherapy with a respiratory fluoroquinolone. In the case of patients admitted to intensive care, the combination of a β-lactam with either a macrolide or a respiratory fluoroquinolone is recommended [30].

Clearly, these therapeutic strategies can be reevaluated on the basis of clinical response and acquisition of microbiological and other laboratory data.

1.5.1. Combination Antibiotic Therapy. The rationale for implementation of combination therapy (various combinations, but most frequently a β-lactam with a macrolide) in patients with severe CAP was largely based on a series of observational studies, both retrospective and prospective, conducted between 1999 and 2010, which demonstrated significantly lower in-hospital or ICU mortality [31]. Although the microbiological mechanisms underpinning the apparent benefit of combination antibiotic therapy remain unknown, activity of macrolides/fluoroquinolones/tetracyclines against the atypical pathogens *Chlamydia pneumoniae, Legionella pneumophila,* and *Mycoplasma pneumoniae*, as well as efficacy in eradicating polymicrobial sepsis, have been proposed [32]. When considering these possible mechanisms, it is also important to note that the benefit of adding a macrolide to standard β-lactam therapy is particularly evident in sicker

patients, such as those with severe sepsis due to pneumonia, and in intubated patients [31, 33]. Benefit also extends to cases infected with macrolide-resistant pathogens (e.g., macrolide-resistant pneumococcal infections and even to cases with gram-negative infections) [33]. The aforementioned studies, as well as several others which failed to confirm a survival benefit when comparing monotherapy with combination therapy, have recently been extensively reviewed elsewhere [34–37].

Clearly, the issue of optimum antimicrobial chemotherapy of CAP remains to be resolved and will be dependent on the acquisition of data from large, well-controlled multicentre, randomised clinical trials [37–39]. One such trial, the CAP-START study, is currently underway in Holland [38, 40]. This is a "multicentre, cluster randomised crossover trial" involving seven Dutch hospitals and 2100 hospitalised nonintensive care unit (ICU) patients. It is designed to compare the therapeutic efficacy of (i) β-lactam monotherapy; (ii) β-lactam/macrolide combination; and (iii) fluoroquinolone monotherapy. The primary outcome is all-cause mortality 90 days after hospital admission [38, 40].

1.6. Antimicrobial Resistance. There is a considerable body of literature devoted to the issue of antimicrobial resistance among the common pathogens causing CAP, especially with regard to the pneumococcus [41–44]. Many studies describing the burden of disease caused by CAP confirm that there is emerging and increasing resistance among many of the CAP pathogens, a phenomenon that is occurring worldwide and which involves all classes of antimicrobial agents, to a greater or lesser extent [2–5, 41]. Accordingly, it has been recommended that the choice of empiric antimicrobial therapy for patients with CAP must be based on prediction of the most likely infecting pathogens together with a full appreciation of the common antimicrobial susceptibility patterns of these pathogens in a given geographical area (unit, ward, or practice).

While it is clear that there is emerging resistance among the CAP pathogens, and particularly the pneumococcus [42], it remains unclear whether the presence of antimicrobial resistance alone is associated with unfavourable treatment outcomes [41]. There have been a number of studies and various reviews discussing the issue of antibiotic resistance in the management and outcome of pneumococcal CAP [41, 45–49]. While there have been some studies that have purported to document a higher mortality rate in patients infected with penicillin-resistant compared with penicillin-susceptible pneumococcal pneumonia [47], most other studies or reviews have not reached similar conclusions [41, 45, 46, 48, 49].

In a large study of 844 hospitalised cases with bacteremic pneumococcal CAP, the authors failed to document penicillin resistance as a risk factor for mortality [45]. Furthermore, these authors noted that discordant therapy (receipt for the first 2 days after the positive blood culture of a single antibiotic that was inactive against the pneumococcus isolated) with penicillin, cefotaxime, or ceftriaxone did not result in a higher mortality rate. Others has confirmed that resistance to penicillins and third-generation cephalosporins have not been associated with significant increased mortality [48]. One critical review of the literature documented only a single microbiological failure of a parenteral penicillin-class antibiotic in the treatment of a patient with pneumococcal pneumonia [49]. Some have suggested that the patients who are at risk for antibiotic-resistant pathogens (due to conditions such as advanced age, or underlying comorbid conditions) are those that may already have host risk factors for a higher mortality [42]. It is, however, important to recognise that changes in the breakpoint definitions for the penicillins and third-generation cephalosporins have occurred since that time [50] such that many of those pathogens previously described as penicillin-resistant would now be considered susceptible. Nevertheless, the previous recommendations that therapy in these patients should continue with high-dose penicillins and broad-spectrum cephalosporins is still recommended in current guidelines [46, 49].

The situation with macrolides and fluoroquinolones is less clear-cut [41, 46]. Macrolide resistance may be of low level, associated with an efflux mechanism (*mef* gene), or of high level, associated with ribosomal target site mutations (*erm* gene), and while the latter has clearly been associated with treatment failure, there have also been some cases of failure with the former, although relatively small in number [41, 46]. For this reason, it has been recommended that awareness of pneumococcal macrolide resistance levels and patterns in a given region, as well as the risk factors in individual patients for macrolide resistance, clearly determine the utility of macrolide monotherapy in the management of pneumococcal CAP. With the respiratory fluoroquinolones, it is clear that laboratory documented resistance is likely to be associated with clinical failure, but what is less well known is that organisms documented in the laboratory as being susceptible, sometimes harbour one-step mutations in their quinolone resistance-determining regions that may undergo further mutations on therapy that may render them resistant [41].

Clearly new options for the treatment of antibiotic resistant pneumococcal infections are desirable, and to this end several newer agents have recently been introduced which have enhanced activity against resistant pneumococcal infections. This topic has been reviewed elsewhere and includes a potential role for ceftaroline, linezolid, telavancin, and tigecycline [51].

1.7. Severity of Illness. The severity of the infection dictates a number of important issues in the management of patients with CAP. Severity of illness determines the site of care (in- or outpatient), the extent of the microbiological workup, and the choice of initial empiric antimicrobial therapy [6]. Increased severity of infection is associated with greater healthcare needs and costs. While to a large extent assessment of severity of infection is still based primarily on sound clinical judgment, researchers have been attempting to develop mechanisms by which severity may be objectively assessed, such as the use of clinical scoring systems, various biomarkers, or by measuring microbial load.

1.7.1. Severity of Illness Scoring Systems. A number of severity of illness scoring indices have been developed to assist in the evaluation of severity of pneumonia, of which the most commonly used are the Pneumonia Severity Index (PSI) and the CURB-65 [6, 7, 52, 53]. The PSI uses 20 variables which include patient age, gender, presence or absence of comorbid conditions, and/or vital sign abnormalities, as well as various laboratory and radiographic parameters [6, 54]. The CURB-65 uses only 5 variables, namely, presence or absence of confusion, urea >7 mmol/L, respiratory rate ≥30 breaths/minute, low blood pressure (systolic <90 mmHg or diastolic ≤60 mmHg), and age ≥65 years [6, 54]. With both scoring systems, cases can be stratified into low-, moderate-, or high-risk groups. The PSI was developed primarily to assist in identifying those patients who could safely be managed at home, whereas the CURB-65 was developed to document patients that were more severely ill, including those who needed to be flagged for ICU admission [55]. One of the advantages of the CURB-65 scoring system is that it is easy to use, but a limitation is that it may underestimate pneumonia severity in younger patients with comorbidities [55]. The PSI has been well validated and performs well in the assessment of low mortality risk patients, but it is complex to calculate and requires the evaluation of a number of laboratory parameters that potentially limits its use outside a hospital [55].

A number of additional scoring systems have also been developed, including those designed for the assessment of more severely ill cases, particularly those cases flagged for ICU admission, including the IDSA/ATS criteria, SMART-COP, PIRO-CAP, and SCAP, which appear to have better discriminatory values than PSI or CURB-65 in this situation [7, 54–56]. Each of the scoring systems that have been developed has various strengths and potential weaknesses so that no system is ideal, being unable to identify all patients at risk, or to replace clinical judgment; nonetheless, they are useful adjuncts for assessing cases [6, 53, 55]. Also of concern is that the scoring systems have been developed for assessment of patients on admission only and there is clearly a need for evaluating patients during the course of their hospitalisation, particularly in the setting of clinical deterioration [53].

1.7.2. Biomarkers. More recently, a number of biomarkers has been tested to determine their ability to stratify risk in patients with CAP, sometimes as an adjunct to clinical scoring systems [7, 55]. Among these are inflammatory markers, such as the white blood cell count, acute phase reactants, such as C-reactive protein (CRP), cytokines, such as interleukin-1 and tumour necrosis factor-α, stress hormones, and various other molecules [6, 53, 55]. Of these, C-reactive protein (CRP) and procalcitonin (PCT) have been particularly well studied and have been reported in many studies to be useful tools, sometimes with an accuracy similar to that of CURB-65 or one of the other scoring systems, increasing the accuracy of severity assessment when used in combination with these scoring systems [7, 55, 57]. CRP, for example, is universally available and in some studies has been shown to have value in site-of-care decisions in predicting 28-day mortality, and in the prediction of treatment failure [7, 55]. PCT has been shown in some studies to be more useful than CRP in predicting severity and outcome in patients with CAP but is not widely available in many healthcare systems, possibly because of its much higher cost [7, 55]. It has been said that PCT should be considered to be a prognostic indicator rather than a diagnostic factor, but there is evidence that it may safely help reduce the unnecessary use of antibiotics, reducing bacterial resistance and curbing healthcare costs without increasing mortality [57–60]. Other biomarkers include proatrial natriuretic peptide (proANP), B-type natriuretic peptide (BNP), provasopressin (proVP), adrenomedullin (ADM), proadrenomedullin (proADM), arginine vasopresine (AVP), cortisol, D-dimers, copeptin, and soluble triggering receptor expressed on myeloid cells-1 (TREM-1) [7, 53, 55].

1.7.3. Bacterial Load. A number of recent studies has documented that the pneumococcal bacterial DNA load correlates well with the severity of infection and has prognostic value, thus confirming a concept that has long been proposed, and which has become more accurate with the acquisition of more reliable assays [41, 47]. It has also been suggested that repeated measurements of the bacterial DNA load can accurately monitor treatment progress; however, more studies are needed to confirm these findings [41].

1.8. Clinical Outcome and Mortality

1.8.1. Clinical Stability/Failure. The evaluation of the clinical outcome of a patient with pneumonia is an important aspect of medical care and has been reviewed elsewhere [61]. Achievement of clinical stability dictates important management issues such as change from intravenous to oral therapy, the timing of hospital discharge, and assessment of likely patient outcome. A number of risk factors for clinical failure have been identified, but most do not recognize pneumococcal aetiology as an important issue [61]. For example, in one study even the presence of pneumococcal bacteraemia did not increase the risk of poor outcome [62].

1.8.2. Mortality. Respiratory tract infections, of which CAP plays a large role, are a major cause of death worldwide [8]. While many of the studies of CAP mortality have concentrated on in-hospital or 30 day mortality, others have evaluated long-term mortality (e.g., over a one-year period). One such study in older patients noted a hospital mortality of 11% versus 5.5% in controls (case controls matched for age, gender, race), while the one-year mortality was 40.9% versus 29.1%, respectively [52, 63]. Prior to that and subsequently, there has been a myriad of additional studies documenting high long-term mortality in patients with CAP [64]. One study documenting high long-term morbidity and mortality in CAP patients noted that it occurred particularly in those cases with high initial PSI scores [65]. Although many predisposing mechanisms have been documented, the presence of cardiovascular disease [66] and other comorbid conditions, including HIV infection, the subsequent documentation of primary or secondary neoplasms, and alterations in immune

function predominate [64]. Cardiovascular events are among the most studied, with a number of reports documenting an increased risk of cardiovascular events during and after serious infections, such as CAP [64, 67–69].

The remaining sections of this review are focused on (i) the major virulence determinants of the pneumococcus, the most common cause of CAP, and their involvement in triggering harmful inflammatory responses [70–113] and (ii) control of these using pharmacological, anti-inflammatory strategies.

2. Virulence Determinants of *S. pneumoniae*

S. pneumoniae colonises the human nasopharynx during the first few months of life. The human airway employs numerous mechanisms to protect the airways from colonisation and invasive pneumococcal infection. In the case of innate immune defences, these include the cough reflex, the mucociliary escalator, and a range of pattern recognition receptors (PRRs) [100]. The PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerisation domain receptors (NOD-like receptors, NLRs), RIG-1-like receptors (RLRs), and the manifold cytosolic DNA sensors (reviewed in [114]). PRRs recognize pathogen-associated molecular patterns (PAMPs). In addition, infected cells, or cells which are stressed, release host-derived molecules known as damage-associated molecular patterns (DAMPs) or alarmins. During an infection, DAMPs and PAMPs have been shown to synergize leading to synthesis and secretion of proinflammatory cytokines and chemokines, including IL-1β and IL-8, as well as stimulating cell differentiation and cell death [115].

Although these mechanisms protect the airways from *S. pneumoniae*, antibody-mediated mechanisms, and cell-mediated immunity are critical in clearing the lower airways of this pathogen. Cell-mediated resolution of nasopharyngeal colonisation involves both Th1 and Th17 responses [116, 117] triggered by a variety of surface virulence determinants such as the bacterial capsule, pili, and other adhesins, as well as the toxin, pneumolysin.

The major virulence determinants and mechanisms of subversion of host defences by *S. pneumoniae* are summarized in Table 1 [70–113]. The most significant of these virulence factors are described in more detail below.

2.1. Capsular Polysaccharide. The anti-phagocytic polysaccharide capsule is considered to be the main determinant of pneumococcal virulence and is essential for colonisation of the nasopharynx [71, 72, 84]. Ninety-two different capsular serotypes have been identified [100]. Capsular morphology alternates between two distinct phases known as the transparent and opaque phenotypes [118]. Relative to the opaque variant, the transparent phenotype has decreased capsular thickness and expresses less pneumococcal surface protein A (PspA) in the setting of a higher level of expression of the major adhesin, choline-binding protein A (CbpA), and the autolysin, LytA, favouring adhesion and colonisation [119]. A further decrease in capsular thickness precedes the transition from colonisation to invasion of the epithelium [120, 121]. The opaque form is the main variant found in the circulation. This highly phagocytosis-resistant variant has increased capsular thickness and expression of PspA, in the setting of decreased expression of CbpA, favouring extrapulmonary dissemination [119].

Cell-wall fragments and capsular polysaccharides of *S. pneumoniae* are recognised and bound by antibodies which in turn bind complement [84], with C1q binding correlating closely with the deposition of C3b and C3bi on the bacterial surface [122]. Capsular serotypes differ with respect to invasiveness, due mainly to differences in complement deposition on the capsular polysaccharide, as well as binding of complement factor H [123].

S. pneumoniae has been found to grow in chains of variable length and, in addition to capsular polysaccharide, longer chains appear to favour adherence and colonisation [124].

2.2. Pneumococcal Pilus. Pili also promote pneumococcal virulence. These are only expressed by certain strains and enable the bacteria to survive in the lung and to bind to epithelial cells [74]. Pileated strains also induce a greater TNF-dependent inflammatory response, increasing the potential to produce lung injury and invade host tissue [73].

2.3. Biofilm. Although the ability of *S. pneumoniae* to grow and persist as biofilms does not appear to reflect virulence potential [125], it remains advantageous, as biofilm-encased bacteria show a reduced susceptibility to antimicrobial agents and resistance to immune recognition. Domenesch and colleagues have shown that there is reduced phagocytosis of pneumococcal biofilms due to impaired deposition of C3b. Biofilm formation by *S. pneumoniae* is also effective in preventing not only activation of the classical complement pathway due to reduced binding of C-reactive protein and the complement component C1q, but also by suppressing the pneumococcal surface protein C-(PspC-) dependent activation of the alternative complement pathway [78].

Recent studies suggest that biofilms do not contribute to the development of invasive pneumococcal disease, but, rather appear to confer a quiescent mode of growth during colonisation [101]. The role of the capsular polysaccharide in biofilm formation has not yet been determined conclusively, although the absence of the capsular polysaccharide was reported in one study to favour biofilm formation [126]; however, another study showed that decreased capsular polysaccharide formation was associated with decreased biofilm production [78]. A recent study has shown that augmentation of pneumococcal biofilm formation due to cigarette smoking is likely to favour microbial colonisation and persistence [127].

2.4. Hydrogen Peroxide (H_2O_2). The pneumococcus is a major producer of H_2O_2 as a consequence of the activity of pyruvate oxidase, which is surprising, given that this catalase-negative pathogen is ill equipped to detoxify this

Table 1: Virulence determinants of *S. pneumoniae* and mechanisms of subversion of host defences.

Virulence factor	Function
Capsule	(i) Prevents entrapment in nasal mucus [70] (ii) Exhibits antiphagocytic activity [71] (iii) Facilitates adherence and colonisation of nasopharyngeal epithelial cells [72]
Pili	Enhances bacterial adhesion and ability to cause invasive disease [73, 74]
Pilus subunit (RrgA)	(i) Binds fibronectin, collagen I, and laminin [75] (ii) Prevents CR3-mediated phagocytosis [76] (iii) TLR2 agonist [77]
Biofilm	(i) Reduces susceptibility to antimicrobial agents [78] (ii) Prevents recognition and phagocytosis by the immune system [78]
H_2O_2	(i) Causes ciliary slowing and epithelial damage [79] (ii) Facilitates colonisation of nasopharyngeal epithelial cells [80] (iii) Bactericidal action against competing bacteria [81]
Pneumolysin	(i) Binds to cytoplasmic membrane cholesterol [82] (ii) Disrupts integrity of epithelial monolayer [83] (iii) Exhibits cytolytic activity [84] (iv) Modulates host inflammatory and immune responses [85]
Autolysin (LytA)	(i) Involved in autolysis resulting in the release of pneumolysin [86, 87] (ii) Facilitates colonisation of nasopharyngeal epithelial cells [88]
Choline binding protein A (CbpA)	(i) Promotes adhesion to human cell conjugates [83] (ii) Binds laminin [89]
Choline binding protein E (CbpE)	Mediates attachment to plasminogen [90]
Pneumococcal surface protein A (PspA)	(i) Inhibits complement-dependent phagocytosis [91] (ii) Binds lactoferrin [92]
Pneumococcal surface protein C (PspC)	Inhibits deposition of the terminal complement complex [93]
Pneumococcal adherence and virulence factor A (PavA)	Mediates attachment to plasminogen [94, 95]
Pneumococcal adherence and virulence factor B (PavB)	Mediates attachment to plasminogen and fibronectin [96]
Pneumococcal surface adhesin A (PsaA)	(i) Binds E-cadherin [97] (ii) Facilitates invasion of nasopharyngeal epithelial cells [98]
Plasmin and fibronectin binding protein A (PfbA)	Mediates attachment to plasminogen and fibronectin [99]
Pneumococcal serine-rich repeat protein (PsrP)	(i) Facilitates adherence to nasopharyngeal epithelial cells [100] (ii) Mediates biofilm production [101]
Putative histidine triad protein (PhpA)	Degrades C3 [102]
Neuraminidase (sialidase)	(i) Facilitates adherence and colonisation of nasopharyngeal epithelial cells [92, 103–105] (ii) Mediates biofilm production [103, 106]
Hyaluronidase	(i) Facilitates colonisation of nasopharyngeal epithelial cells [84] (ii) Aids the dissemination of the bacteria [84]
Endonuclease A (EndA)	Degrades neutrophil extracellular traps [107, 108]
Zinc metalloproteinase (ZmpB)	(i) Induces TNF production in the respiratory tract [109] (ii) Cleaves secretory IgA [110]
Streptococcus-specific glycosyl hydrolase (GHIP)	Facilitates invasion of nasopharyngeal epithelial cells [111]
ClpP protease	Induces apoptosis [112]
Ser/Thr kinase (StkP)	Regulator of cell division [113]

oxidant [128]. The pneumococcus not only utilises H_2O_2 as a virulence factor, causing significant damage to ciliated respiratory epithelium and impaired protective activity of the mucociliary escalator [79], but also to eliminate microbial competitors in the nasopharynx [81]. In addition, pyruvate oxidase has been reported to act as a sensor of the oxygenation status of the microbial environment, regulating both nutritional capability and thickness of the anti-phagocytic capsule [129].

2.5. Surface Proteins. The adherence and colonisation of host tissues by the pneumococcus is mediated by surface adhesins and enzymes [83, 84] which are also important virulence factors of *S. pneumoniae*. The most significant of these are described below.

2.5.1. Autolysins. Autolysins are cell-wall degrading proteases. These enzymes break down the peptidoglycan backbone enabling cell growth and cell division. However, excessive activity of autolysins results in the degradation of the cell-wall leading to cell lysis [86, 87]. *N*-acetylmuramic acid L-alanine amidase, also known as LytA amidase, is the major autolysin of *S. pneumoniae* [130]. The lysis of a portion of the bacterial cell-wall by LytA may increase the virulence of *S. pneumoniae* by promoting the release of potentially lethal toxins such as pneumolysin [84, 86].

Three other cell-wall hydrolytic enzymes have been identified *viz*. LytB, LytC, and choline binding protein E (CbpE) [84], all of which have been associated with nasopharyngeal colonisation [88].

2.5.2. Choline Binding Protein A. Choline binding proteins (Cbp) have a C-terminal binding module followed by a flexible proline-rich segment and a functional N-terminal module [83]. CbpA binds to terminal choline residues of teichoic or lipoteichoic acids present on the surface of *S. pneumoniae*, anchoring the pathogens to human cell glycoconjugates, favouring the transition from colonisation to invasion [83].

2.5.3. Pneumococcal Surface Protein A. Pneumococcal surface protein A (PspA) is also an important virulence factor of *S. pneumoniae*, inhibiting deposition of C3b on the bacterial surface, thereby interfering with complement activation and complement-dependent phagocytosis [91]. PspA also binds to and interferes with lactoferrin, increasing the availability of free iron required for bacterial growth [92].

2.5.4. Pneumococcal Adherence and Virulence Factors A and B. The adhesins, PavA and B of *S. pneumoniae*, promote the invasion of host cells and dissemination of the pneumococcus. PavA has been shown to bind to the extracellular matrix component, fibronectin, while PavB binds both fibronectin and plasminogen. These adhesive effects are mediated by repetitive sequences designated streptococcal surface repeats (SSURE) [95, 96]. It has been suggested that PavA may affect pneumococcal colonisation by modulating the expression or function of virulence factors of *S. pneumoniae* [95].

2.5.5. Neuraminidase. Three forms of neuraminidase have been identified in pneumococci, these being designated as NanA [105, 131], NanB [104, 131], and NanC [131]. NeuraminidaseA cleaves terminal sialic acid from cell surface glycans such as mucin, glycolipids, and glycoproteins [131], with resultant exposure of binding sites on the host cell surface contributing to pneumococcal adhesion and colonisation [83, 104, 105, 132]. NanA also plays an important role in biofilm formation [92, 95]. NanB is involved in the metabolic utilisation of sialic acid as a carbon and energy source by the pneumococcus, while NanC has a regulatory role [131, 133].

2.5.6. Hyaluronidase. Hyaluronidase is secreted by pneumococci and breaks down the hyaluronic acid component of host connective tissue and extracellular matrix [84]. Increased epithelial permeability caused by the action of hyaluronidase favours the spread and colonisation of *S. pneumoniae* [84], especially when acting in concert with pneumolysin [134].

2.5.7. Pneumolysin. The pneumococcal protein toxin, pneumolysin, is a member of the family of thiol-activated cytolysins and a critical virulence factor of the pathogen [85]. The toxin binds to cholesterol in the cytoplasmic membrane of eukaryotic cells, followed by insertion into the membrane, leading to the formation of large pores and cytolysis [83, 84]. In the early stages of infection, pneumolysin promotes nasopharyngeal colonisation via its inhibitory effects on ciliated respiratory epithelium [83]. In addition, the toxin has been shown to disrupt tight junctions thereby disrupting the integrity of the epithelial monolayer favouring invasiveness of the pathogen [82].

At high, cytotoxic concentrations, pneumolysin may also inhibit the protective functions of cells of both the innate and adaptive immune systems, as well as maturation of dendritic cells [135]. However, at lower noncytolytic concentrations, the toxin possesses proinflammatory activity as a consequence of sublytic pore formation and influx of Ca^{2+} into immune and inflammatory cells. This, in turn, causes hyperactivation of phagocytes, induction of proinflammatory cytokine production, and activation of the inflammasome, all of which are potentiated by the complement-activating properties of the toxin (as described below).

3. Harmful Effects of Excessive Activation of Antipneumococcal Host Defences

During pneumococcal CAP, a high bacillary load, aggravated by the implementation of antimicrobial chemotherapy with bactericidal agents which promote disintegration of the pathogen, results in excessive release of proinflammatory bacterial cell-wall products, toxins, and DNA. The consequence of these events is hyperactivation of host defence mechanisms, posing the potential threat of inflammation-mediated pulmonary damage and extrapulmonary spread of the pneumococcus. The major contributors to overexuberant inflammatory responses include (i) the proinflammatory, pore-forming interactions of pneumolysin

with neutrophils, macrophages, and epithelial cells, potentiated by complement-activating properties of the toxin; (ii) the interactions of pneumolysin, lipoteichoic acid, proteoglycan, and DNA with PRRs, especially on cells of the innate immune system and epithelial cells; (iii) inappropriate induction of neutrophil extracellular trap (NET) formation by pneumococcal α-enolase and possibly pneumolysin and H_2O_2; and (iv) possible inappropriate oxidative activation of redox signalling mechanisms in immune and inflammatory cells by pneumococcal H_2O_2.

3.1. Pneumolysin-Mediated Mechanisms. In addition to directly causing acute lung injury as a consequence of its cytotoxic effects on airway epithelium and endothelium [136], pneumolysin, via its complement-activating activities and sub-lytic, pore-forming interactions with neutrophils and macrophages, also potentiates the release of reactive oxygen species (ROS), granule proteases, leukotriene B4, and prostaglandin E2 by promoting the movement of extracellular Ca^{2+} into the cells [137, 138]. As a result of Ca^{2+} influx, several intracellular signalling cascades are activated. These involve p38 and mitogen-activated protein kinases, transforming growth factor-β-activated kinase 1-mitogen-protein kinase 3/6-p38 α/β, Ca^{2+}-calcineurin, nuclear factor kappa B (NFκB), and activator protein 1 (AP-1) [139–142]. The consequence is increased production of IL-8 and TNF, both of which promote neutrophil influx into the airways [140–143]. In addition, pneumolysin also activates the NLRP3 inflammasome in dendritic cells, and presumably other immune and inflammatory cell types, thereby potentiating caspase-1-mediated conversion of pro-IL-1β to the mature cytokine [144].

The aforementioned direct cytolytic actions of pneumolysin, acting in concert with the indirect proinflammatory activities of the toxin, promote the epithelial/endothelial damage which favours dissemination of the pneumococcus.

3.2. Pattern Recognition Receptors. The interactions of the pneumococcus with the various PRRs expressed by cells of the innate immune system, as well as epithelial cells, have been the subject of a recent review [114]. Pneumococcal cell wall components, pneumolysin, and DNA have all been reported to interact with, and activate, several different types of PRRs. In the case of the Toll-like receptors (TLRs), lipoteichoic acid, and, possibly proteoglycan, are TLR-2 ligands [114], pneumolysin has been reported to interact with and activate TLR-4 [145], while pneumococcal CpG-motif-containing DNA is detected by TLR-9 [114]. In each case, triggering of TLRs is accompanied by activation of NFκB and synthesis of proinflammatory chemokines/cytokines, especially IL-8 and TNF [114].

In addition, pneumococcal proteoglycans are recognised by and activate the nucleotide oligomerisation domain-like receptor, Nod2 [114], while microbial DNA is detected by the abundant cytosolic sensors of pathogen-derived nucleic acid [146, 147]. The consequence of these events is activation of NFκB, as well as the interferon regulatory transcription factors 3 and 7 (IRF 3/7) [114].

3.3. Induction of Neutrophil Extracellular Traps (NETs). NET formation is a postactivation strategy used mainly by dead and dying neutrophils and several other cell types including monocytes/macrophages and eosinophils, to isolate and kill microbial pathogens by trapping them in an extracellular matrix of citrullinated histones impregnated with antimicrobial granule proteins [148]. In the case of the pneumococcus, pathogen-derived α-enolase has been reported to induce NET formation following exposure of neutrophils to the pathogen [149]. Although unproven, it seems probable that pneumolysin and pathogen-derived H_2O_2 are also potential inducers of NET formation. However, as opposed to being an effective antipneumococcal host defence mechanism, the pneumococcus appears to be particularly adept at escaping from NETs [150], apparently as a consequence of production of the endonuclease, End A, which mediates degradation of NETs [107]. In addition to subverting NETs, the pneumococcus may also exploit poorly regulated NET formation as a strategy to promote invasion and dissemination due to epithelial and endothelial cytotoxicity mediated by the histone components of NETs [151].

3.4. Hydrogen Peroxide. While its role in microbial virulence is well established, the involvement of pneumococcus-derived H_2O_2 in activating harmful inflammatory responses during CAP and other infections remains to be established. This seems likely, however, as cell-permeable H_2O_2 is a potent activator of redox intracellular signalling mechanisms in many cell types, including those of the innate and adaptive immune systems [152, 153].

These various mechanisms of hyperactivation of inflammatory responses operative during pneumococcal CAP are summarised in Table 2.

4. Adjunctive Anti-Inflammatory Therapeutic Strategies in Severe CAP

The primary objective of adjunctive pharmacological strategies in severe CAP, especially severe pneumococcal disease, is to suppress overexuberant, harmful, pathogen-activated inflammatory responses, thereby attenuating inflammation-mediated pulmonary damage and dysfunction. In this setting, the three categories of anti-inflammatory agents which have attracted the greatest interest are macrolides, corticosteroids, and, more recently, statins [31, 32, 34–39]. Other categories of anti-inflammatory agent which remain largely untested, include the various types of $3'$-$5'$-cyclic adenosine monophosphate- (cAMP-) elevating agents, as well as non-steroidal anti-inflammatory agents (NSAIDs) [34].

4.1. Macrolides. Despite the absence of irrefutable proof of efficacy, the inclusion of macrolides in the current guideline recommendations for the therapy of CAP can be justified on several grounds. Notwithstanding their primary antimicrobial activities, which complement those of β-lactams in the treatment of CAP, macrolides possess well-recognised anti-inflammatory properties. These are both pathogen- and host-directed and have recently been reviewed in detail elsewhere

Table 2: Causes of overexuberant inflammatory responses during pneumococcal CAP.

Cause	Consequence
Excessive release of pneumolysin	Uncontrolled complement activation; hyperactivation of phagocytes and epithelial cells due to the noncytolytic, pore-forming actions of the toxin
Excessive release of bacterial cell-wall products (e.g., lipoteichoic acids and DNA), especially during chemotherapy with bactericidal agents	Sustained activation of various types of pathogen recognition receptors on/in cells of the innate immune system and epithelial cells, resulting in poorly regulated production of neutrophil-mobilising chemokines/cytokines
Poorly controlled formation of NETs with limited protective activity	Histone-mediated epithelial and endothelial toxicity, favouring extrapulmonary spread of the pneumococcus
Excessive release of cell-permeable, proinflammatory H_2O_2 by the pneumococcus	Uncontrolled activation of redox intracellular signalling mechanisms in cells of the innate and adaptive immune systems, as well as other cell types. The existence of this mechanism remains to be established

[34, 35]. Briefly, the pathogen-targeted anti-inflammatory activity of macrolides is achieved via the inhibitory effects of these agents on bacterial protein synthesis, thereby attenuating the production of pro-inflammatory toxins, such as pneumolysin in the case of the pneumococcus [35, 154]. In addition, abrupt bacteriolysis and accompanying excessive inflammation due to release of cell-wall components and endotoxins, as may occur with bactericidal antibiotics, are also countered by the predominantly bacteriostatic activity of macrolides [35].

The primary target of the secondary anti-inflammatory properties of macrolides, unrelated to antimicrobial activity, is neutrophil recruitment. Macrolide-mediated inhibition of neutrophil mobilisation is achieved predominantly via inhibition of production of the neutrophil-mobilising cytokines/chemokines IL-8, IL-17, and TNF, not only by cells of the innate immune system, but also by various types of structural cells [35, 155]. Although not fully understood, these inhibitory effects of macrolides on the synthesis of pro-inflammatory cytokines/chemokines appear to be achieved at the level of gene transcription. This results from antagonism of transcription factors such as nuclear factor kappa B (NFκB), possibly via (i) interference with redox signalling mechanisms; and (ii) enhancement of histone deacetylase activity [35, 156–158].

The beneficial therapeutic activities of these various pathogen- and host-directed anti-inflammatory activities of macrolides are evident in several chronic inflammatory diseases of the airways, particularly bronchiolitis obliterans, diffuse panbronchiolitis, and cystic fibrosis, and possibly chronic obstructive pulmonary disease (reviewed in [35]). Although difficult to prove conclusively, several lines of evidence, both clinical and experimental, also support the involvement of the anti-inflammatory activities of macrolides in controlling severe sepsis and/or inflammation-mediated lung injury in acute bacterial infection. This contention is supported by at least two noteworthy studies. The first of these reported that the use of macrolides was associated with decreased mortality in patients with pneumonia and severe sepsis, even in patients infected with macrolide-resistant pathogens, such as gram-negative organisms [33]. The second study, which was undertaken in patients with predominantly macrolide-resistant gram-negative sepsis and ventilator-associated pneumonia (VAP), reported that intravenous administration of clarithromycin (1 gram/daily) for 3 days resulted in accelerated resolution of VAP and earlier discontinuation of mechanical ventilation, as well as delaying, but not preventing, mortality [159]. More recently, administration of a macrolide within 24 hours of trial entry, but not β-lactam or fluoroquinolone antibiotics, to patients with acute lung injury secondary to pneumonia in most cases, was associated with significant decreases in both 180 day mortality and time to successful discontinuation of mechanical ventilation [160].

The beneficial anti-inflammatory activities of macrolides and macrolide-like antimicrobial agents have also been demonstrated in various animal models of experimental chemotherapy of acute bacterial infection (reviewed in [35]). In one such study reported by Karlström et al., using a murine model of pneumococcal pneumonia secondary to influenza virus infection, treatment of animals with either azithromycin or clindamycin alone or in combination with ampicillin resulted in significantly improved survival compared to animals treated with ampicillin only [161]. These beneficial effects of azithromycin/clindamycin were associated with decreased concentrations of airway pro-inflammatory cytokines and influx of inflammatory cells in the setting of less severe histopathological changes [161].

Because macrolides combine pathogen- and host-directed anti-inflammatory activities, therapy with these agents appears to be an ideal adjunctive strategy in severe CAP, possibly in a subset of patients at risk for development of ALI. Nonetheless, widespread acceptance of an adjunctive role for macrolides in this clinical setting is dependent on the acquisition of compelling data from prospective, randomised, controlled clinical trials [36–39, 162, 163].

4.2. Corticosteroids. Corticosteroids (CS) are broad-spectrum anti-inflammatory agents, but unlike macrolides, they are less effective in targeting neutrophils [164]. Although an adjunctive role for systemic CS in the clinical management of adults with penicillin-susceptible pneumococcal meningitis is well recognised [165], their role in the adjunctive therapy of severe CAP remains unproven. Several relatively small prospective/retrospective trials conducted between 2005 and 2007 in hospitalised patients with severe CAP, receiving

systemic CS at doses varying from 40 to 200 mg/daily for periods of 3 days and longer, reported beneficial effects of these agents on, amongst others, duration of hospital stay, and mortality [166–168]. However, this adjunctive promise of systemic CS was not confirmed in several more recent studies. In a large randomised, double-blinded, placebo-controlled trial, Snijders et al. failed to detect beneficial effects of systemic administration of prednisone (40 mg/daily for 7 days) on outcome in patients with CAP, with the frequency of late failure (>72 hours after hospital admission) being significantly ($P < 0.04$) more common in CS-treated patients [169]. In agreement with these findings, Polverino et al., in a retrospective study covering an approximately 10.5 year period involving 3257 patients who received a mean systemic CS daily dose of 45 mg for varying periods of time, reported that administration of CS did not influence either clinical stability or mortality [170]. Somewhat worryingly, however, administration of CS significantly prolonged hospital stay (9 versus 6 days, $P < 0.01$) [170].

In contrast, Meijvis et al. reported that systemic administration of dexamethasone, at the comparatively low dose of 5 mg/daily for 4 days from the time of admission, to nonimmunocompromised patients who did not require ICU admission, was associated with a significant decrease in length of hospital stay (7.5 versus 6.5 days $P < 0.048$) [171]. These observations suggest that the dose of the systemic CS and the immune status of the patient are potential determinants of the success of adjunctive therapy with CS in patients with CAP. In addition, the type of pathogen may also be a determinant of successful outcome of CS therapy, with infections caused by atypical pathogens, as opposed to those caused by *S. pneumoniae,* being more responsive to the beneficial actions of CS [172].

However, as concluded in a recent meta-analysis, conclusive proof of the role, if any, of systemic CS in the adjunctive therapy of severe CAP is dependent on the acquisition of compelling data from adequately powered randomised trials [173]. Three such studies are ongoing, one in the USA (Extended Steroids in CAPe-ESCAPe, projected completion date January 2017) [174] and two in Europe [38].

4.3. Statins. Statins is the collective term for a group of pharmacological inhibitors of the enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase, used to control hypercholesterolemia in the prevention of cardiovascular diseases and stroke. In addition to their cholesterol-lowering properties, statins have also been reported to possess significant anti-inflammatory activities which have been attributed to two major mechanisms. Firstly, interference with the prenylation of the small G-proteins Rac, Ras, and Rho, thereby attenuating G-protein-coupled receptor cellular signalling and activation in a variety of cell types, including immune and inflammatory cells [175–177]. The consequence is decreased activation of NFκB and resultant interference with the transcription of genes encoding various proinflammatory proteins such as inducible nitric oxide synthase, cyclooxygenase-2 and matrix metalloproteinase-9 [176, 178]. Secondly, via induction of heme oxygenase-1 expression, also resulting in attenuation of activation of NFκB, as well as decreased production of ROS in the setting of increased production of anti-inflammatory IL-10 [178, 179].

Interestingly, a number of predominantly retrospective studies conducted between 2005 and 2011 have reported that statin use in the prevention of cardiovascular diseases is associated with improved outcome of patients with CAP, possibly as a consequence of the anti-inflammatory activities of these agents. In the majority of these studies, which have recently been reviewed by Corrales-Medina and Musher [36], statin use was associated with an approximately 50% reduction in mortality. In addition to these, Doshi et al. in a retrospective study of patients ($n = 347$) with pneumococcal pneumonia, who presented at a single medical center between 2000 and 2010, reported that statin use, as opposed to administration of macrolides, was associated with decreased mortality 4, 14, 20, and 30 days after admission [39]. These findings suggest a mechanism in addition to those mentioned above by which statins may protect against invasive pneumococcal diseases. By decreasing plasma membrane concentrations of cholesterol in epithelial, endothelial, and immune/inflammatory cells, statins may restrict the binding of pneumolysin, thereby attenuating both the cytotoxic and pro-inflammatory activities of the toxin.

Although of considerable potential importance, the aforementioned studies have several significant limitations: (i) they do not address the adjunctive potential of statins administered at the time of presentation with CAP; (ii) concomitant use of statins may obscure the therapeutic potential of macrolides and CS; and (iii) statin use may be associated with a "healthy user" effect, distinguishing a subgroup of patients who are likely to have a better outcome [157]. Once again, large randomised, prospective, controlled clinical trials are necessary to evaluate the use of statins as a potential adjunctive therapy in CAP [36, 39].

4.4. Other Potential Adjunctive Therapies. Other largely untested adjunctive therapies in the clinical setting of CAP include (i) pharmacological agents which increase intracellular concentrations of the cyclic nucleotide, cAMP, in immune and inflammatory cells and (ii) NSAIDs. Cyclic AMP has been described as being "the master regulator of innate immune cell function" [180]; consequently pharmacological agents which increase the intracellular concentrations of this cyclic nucleotide possess broad-spectrum anti-inflammatory activities. Agents falling into this category include cAMP-specific and nonspecific phosphodiesterase inhibitors, as well as agonists of β_2-adrenoreceptors, adenosine $A2_A$ receptors, and the E-type prostaglandin receptors, EP_2 and EP_4. Although the potential of these agents is untested in CAP, it is noteworthy that the β_2-adrenoreceptor agonist, salbutamol, administered either intravenously or as an aerosol, has proved to be ineffective in the treatment of ALI [181, 182]. With respect to anti-inflammatory activity, salbutamol may not, however, be the most effective β_2-agonist to use in this clinical setting [183].

Administration of the NSAID, naproxen, to healthy individuals has been reported to augment the bactericidal action

Table 3: Adjunctive anti-inflammatory therapies in CAP.

Type of adjunctive therapy	Current status
Macrolide antibiotics	Recommended in current guidelines primarily for antimicrobial activity. The clinical relevance of anti-inflammatory activity remains to be conclusively established
Corticosteroids	Remains controversial and is the subject of several ongoing randomised, prospective, controlled trials
Statins	Show promise, but therapeutic efficacy of initiation at the time of diagnosis of CAP remains to be established
cAMP-elevatory agents	Theoretically promising, although few safe and effective agents currently available; salbutamol found ineffective in the treatment of ALI
NSAIDs	Of questionable value

of whole blood against a penicillin-resistant strain of *S. pneumoniae ex vivo* [184]. These potentiating effects of the NSAID were associated with increased phagocytic activity of blood phagocytes, as well as increased generation of antimicrobial ROS by these cells, due, presumably, to inhibition of production of immunosuppressive prostaglandin E_2 and activation of adenylyl cyclase via EP_2/EP_4 receptors [184]. Somewhat paradoxically, however, these antipneumococcal actions of NSAIDs may predispose to inflammation-mediated tissue damage via interference with cAMP-mediated immunoregulatory activity.

The aforementioned adjunctive anti-inflammatory therapies in CAP are summarised in Table 3. These and other types of adjunctive therapies, largely unsuccessful, have also been the subject of several recent reviews and include (i) intravenous gammaglobulin (of unproven benefit); (ii) monoclonal antibodies targeted against the IL-1 or PAF receptors, as well as TNF; (iii) recombinant human activated protein C (drotrecogin alfa); and (iv) the recombinant tissue factor pathway inhibitor, tifacogin [34, 162].

5. Conclusion

It is quite evident from a review of the scientific literature that CAP, and in particular infection due to *Streptococcus pneumoniae,* carries a considerable burden of disease among the world's population. The pneumococcus is the most common microbial cause of CAP, not only in mild infections, but also among patients requiring hospitalisation and even among critically ill cases. The reason that pneumococcal infection and CAP remain so common throughout the world relates to the high prevalence of risk factors for this infection in the general population, which includes aging, lifestyle factors, and underlying comorbid illnesses and, at least in some parts of the world, concomitant HIV infection. Pneumococcal pneumonia causes considerable morbidity and mortality and treatment of these infections is potentially being compromised by the emergence of resistance in this microorganism to the commonly used antibiotics. The pneumococcus expresses a large number of virulence factors, which not only render the microorganism very effective in causing infection, but also contribute to disease pathogenesis via their cytotoxic and proinflammatory activities. Targeting these virulence factors additionally as part of overall therapy has the potential for improving the outcome of such infections.

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Review Article

Immunoinflammatory Response in Critically Ill Patients: Severe Sepsis and/or Trauma

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Immunoinflammatory response in critically ill patients is very complex. This review explores some of the new elements of immunoinflammatory response in severe sepsis, tumor necrosis factor-alpha in severe acute pancreatitis as a clinical example of immune response in sepsis, immune response in severe trauma with or without secondary sepsis, and genetic aspects of host immunoinflammatory response to various insults in critically ill patients.

1. Some of the New Elements of Immunoinflammatory Response in Severe Sepsis

Infection has been the leading cause of death in humans, since the earliest written sources. In the early 15th century, the black death plague wiped out between one-third and one-half of the entire European and Asian populations. The Greek word "sepo," from which the term "sepsis" derives, means "decomposition of animal, or vegetable or organic matter in the presence of bacteria" [1]. Modern cytokine research began in 1932, with the pioneer work of Rich and Lewis, who first observed antigen-mediated inhibition of leukocyte migration in tuberculin-sensitized tissue. Cytokine biology expanded 30 years ago, and the term "cytokine" was first used by Cohen, referring to the variety of soluble factors, with wide range of biological activities related to immune system, produced by wide range of cell types [2]. In 1975, Carswell described the pivotal role of tumor necrosis factor, as one of the earliest monokines, in severe sepsis [3]. At first, this cytokine was also called "cahectin," which describes its ability to suppress lipoprotein lipase activity, leading to hypertriglyceridemia and rapid weight loss in experimental animals [4]. Interactions between infecting microorganisms and host response can lead to severe sepsis and septic shock. In response to pathogen adherence to an epithelial surface, the host initiates specific mucosal defense mechanisms, in order to prevent microbial invasion. The critical bacterial density needed to initiate an infection is called quorum. Bacterial cell-to-cell communication enables them to assess their population density and interact with the host as a population (quorum-sensing systems). Innate immunity—representing early non-specific response system—and adaptive immunity—representing more pathogen-specific response system—are parts of immune system as a whole [5]. The inflammatory response of the host is similar, regardless of the nature of the stimuli (infectious or noninfectious, like tissue injury). Initially, microorganisms bind to surface Toll-like receptors (TLRs) on phagocytic cells. These receptors are homologues of the *Drosophila* Toll protein. This binding

initiates a series of intracellular events resulting in the release of cytokines. TLR-2 type of these receptors reacts with Gram-positive bacterial cell wall antigens, such as peptidoglycans and lipoteichoic acid, while TLR-4 form reacts with lipopolysaccharide (LPS) and endotoxin of Gram-negative bacteria [6–8]. TLRs may recognize either pathogens or endogenous danger signals released by stressed or damaged cell and consequently alert the host by activating the innate immune system. Some molecular fragments from pathogens, such as LPS and bacterial DNA, may induce an immune response and are known as specific patterns called pathogen associated molecular patterns (PAMPs). These patterns are recognized by cellular receptors termed pattern recognition receptors (PRRs). Besides PAMPs, there are also several endogenous molecules, such as high-mobility group box- (HMGB-) 1, hyaluronan, and heat-shock proteins (HSPs) that are also able to trigger the immune response through PRRs. These signals are normal cell constituents, which may be released either passively (by necrotic cells) or actively (by stressed cell, in response to cellular injury). Endogenous analogues of PAMPs are called alarmins. These endogenous alarmins and exogenous PAMPs represent two subgroups of the larger category of danger signals termed damage associated molecular patterns (DAMPs) [9]. The explanation of SIRS in absence of obvious microbial infection was provided by Matzinger [10], thus elucidating host response to DAMPs that can activate innate immunity through, among others, TLRs.

Severe sepsis and/or trauma complicated with multiple organ dysfunction syndrome (MODS) are leading causes of death in intensive therapy units with mortality rate exceeding 50%. Besides the infection, the intensity of immunoinflammatory response also influences the outcome because it is essential for host defense. Unfortunately, if this reaction is uncontrolled, it can lead to the MODS [11]. Resident macrophages and polymorphonuclear cells (PMCs) initiate the primary host response to the invading microorganisms, for they are responsible for the primary phagocytosis and subsequent activation and recruitment of polymorphonuclear granulocytes and monocytes. The macrophage population increase by rapid differentiation of monocytes. This concerted action which constitutes the innate response to infections and tissue damage is mediated by various soluble and membrane-bound factors. Cytokines are potent, low molecular weight proteins produced by nucleated cells, particularly those of the immune system, which exert control over the duration and amplitude of the immune/inflammatory response. They are the main positive and negative regulators of immune responses as well as the key components in the integration of these reactions with other physiological systems such as the complement and hematopoietic systems. The capacity of cytokines to activate diverse cell types and to incite equally diverse responses underscores the pleiotropism of these inflammatory mediators. The bioactivities of different cytokines are significantly overlapping. According to their variations in effects *in vivo*, depending on time and location, there are basically three classes of cytokines: proinflammatory (T helper—Th1), anti-inflammatory (Th2), and Th17, different form both Th1 and Th2. Cytokines with proinflammatory effects are tumor necrosis factor- (TNF-) alpha, interleukin- (IL-) 1, IL-8, and HMGB-1; anti-inflammatory effects are present, for example, in IL-10 and IL-1 receptor antagonist (ra), and some cytokines possess both characteristics, like IL-6. The effects of cytokines are initiated by their binding to specific receptors at the membrane of target cell. This binding starts a cascade of events that leads to induction, enhancement, or inhibition of cytokine-regulated genes in the nucleus of the target cell, which results in modulation of cell immune activity [12–14].

Therefore, one of key events in bacterial sepsis is activation of immune cells, either by whole bacteria or with products derived from the bacteria, which both lead to local and systemic inflammation. Inflammatory response is not a uniform event: its characteristics differ from organ to organ, as well as from organ to peripheral blood. This finding leads to the concept of compartmentalization, with the most prominent difference between the blood compartment and tissues. During infection, immune cells interact not only with live and dead bacteria (killed by complement, defensins, antimicrobial peptides, or antibiotics) but also with bacterial products, either cell wall antigens of Gram-positive bacteria, such as peptidoglycans and lipoteichoic acid; endotoxin of Gram-negative bacteria or derived from inside cells like bacterial DNA and HSPs. Whole bacteria and PAMPs are potent activators of immune cells. They interact with specific sensors, like TLRs and nucleotide-binding oligomerization domain: NOD1 and NOD2 molecules, which induce the production of inflammatory cytokines. Final results are activation and overexpression of early response genes, which are mostly driven through the activation of nuclear factor κB (NF-κB). Anti-inflammatory mediators tend to predominate within the circulation in order to avoid initiation of new inflammatory foci, but their presence within tissues may not always be sufficient to prevent the onset of dangerous proinflammatory response in the different compartments. Contrary to normal conditions, in severe infection cytokines are produced in excess, so their presence in blood becomes detectable. However, the cytokines in circulation are merely the tip of the iceberg, and leukocyte-associated cytokines can be identified even when amounts in plasma are undetectable. Traditionally, sepsis has been represented by an excess production of proinflammatory mediators in blood, with the presence of cytokines within the blood compartment being a key factor in maintenance of proinflammatory process. But, the presence of circulating cytokines may also deactivate leukocytes from a further migration within tissues in response to local gradients of chemokines. Hence, both proinflammatory and anti-inflammatory responses are concomitantly present in sepsis [15].

Accurate cause of organ failure and death in majority of patients who died of sepsis remains unknown. The results of postmortem investigations have shown a relative paucity of cell death in most organs [16]. According to one theory, the organ dysfunction in sepsis may be the consequence of a so-called *cellular hibernation response* [17, 18]. In the most recent review, Hotchkiss with his coauthors delineated three potential inflammatory responses

in sepsis [19]. Major factors determining the immune responses in sepsis include pathogen virulence, size of bacterial inoculums, and comorbidities. In the first scenario, the initial phase in previously healthy patients with severe sepsis is characterized by an excess hyperinflammatory—proinflammatory response with fever, hyperdynamic circulation, and shock. These features are present despite the fact that both proinflammatory and anti-inflammatory responses begin rapidly and concomitantly after sepsis onset. In this early phase of sepsis, patients die due to cardiovascular collapse, metabolic derangements, and multiple organ dysfunctions. Although no particular anti-inflammatory therapies have improved survival in large phase 3 trials, short acting anti-inflammatory or anticytokine therapies offer a theoretical benefit. Hotchkiss proposed a second scenario, in which many septic patients are elderly, with numerous comorbidities impairing their immune response. In these patients, sepsis development is commonly characterized by blunted or absent hyperinflammatory response, with predomination of anti-inflammatory phase. In this setting, boosting immunity with an immunoadjuvant therapy seems promising. Finally, the third theory of immune response in sepsis is featured by cycling between hyperinflammatory and hypoinflammatory states. In this scenario, septic patients first experience an initial hyperinflammatory response, followed by hypoinflammatory state. With the development of a new secondary infection, patients experience a new onset of hyperinflammatory reaction and may either recover or reenter the hypoinflammatory phase. Death may occur in either state. The longer the sepsis continues chances for a patient to develop profound immunosuppression increase. Autopsy results show that most patients admitted to intensive care units (ICUs) for treatment of sepsis had unresolved septic foci at postmortem. These findings suggest that septic patients were unable to eradicate invading pathogens and were more susceptible to nosocomial infections or both. In order to investigate modulation of the immunosuppressive phase of sepsis, Coopersmith and Hotchkiss with coworkers performed very interesting animal study using clinically relevant two-hit model of sepsis, that is, cecal ligation and puncture (CLP), followed by the induction of *Pseudomonas aeruginosa* pneumonia in mice. They applied an agent that blocks IL-10, a key mediator of immunosuppression, to investigate its ability to reverse immunoparalysis and improve survival. The improved survival was associated with restoration of interferon- (INF-) gamma synthesis, increased production of proinflammatory cytokines, and decreased bacterial growth. These authors found that immunosuppression, which occurs after the initial septic insult, increases susceptibility to secondary infection. However, seven days after CLP procedure, the host's immune system recovers sufficiently to generate an effective immune response. Modulation of the immunosuppressive phase of sepsis may help develop the therapeutic strategies [20].

Critically ill patients suffer a high rate of nosocomial infection. In fact, the common cause of death in these patients is secondary sepsis. This high prevalence of secondary infections argues for the influence of an immune suppression that may, at first glance, appear paradoxical in light of the proinflammatory nature of many critical illnesses. In ICU patients requiring organ support, the prevalence of nosocomial infections increases to 25–40% [21]. Research performed in the last 10 years revealed that many interventions applied in ICUs, such as high-volume crystalloid resuscitation, early total parenteral nutrition, liberal blood transfusions, high tidal volume mechanical ventilation, and intermittent hemodialysis, were, in fact, facilitating nosocomial infections and late MODS. There is growing evidence of the role of proinflammatory mediators in developing immune dysfunction. This observation may contribute to explanation of apparent paradox of immune suppression present in a patient with manifested hyper-inflammation [22]. Clinically, many patients show signs of persisting inflammation and immune-mediated organ damage while simultaneously remaining highly susceptible to secondary infections, suggesting the term *complex immune dysfunction syndrome (CIDS)* [23]. The novel investigations of sepsis point out that virtually all immune cells (both innate immune type such as neutrophils, monocytes, tissue macrophages, and dendritic cells and adaptive immune type like T cells, B cells, and natural killer (NK cells)) demonstrate immune hypoactivity. For example, neutrophils display dual state by concomitant presence of activation and dysfunction features. In critically ill patients, dysfunction of organs is, to a considerable degree, driven by neutrophils, which are key immune cells [24]. They tend to express surface markers of activation (increased levels of CD11b and CD64), but simultaneously they display major impairment of phagocytic capacity and generation of reactive oxygen species (ROS). This apparently paradoxical superposition of both proinflammatory activation and failure of key antimicrobial functions within the same cell type was illuminated by the finding that dysfunction was driven by an excess of the proinflammatory complement split product, anaphylatoxin, and C5a [25, 26]. Key role of the systemic complement activation in acute organ dysfunction during sepsis has been revealed 20 years ago [27].

Most recently, Lyle Moldawer and Frederick More, with their coworkers, proposed that *"persistent inflammation-immunosuppression catabolism syndrome—PICS"* is the predominant phenotype that has replaced late occurring MODS in surgical ICU patients who fail to recover [28]. Key effector cells that remove pathogens and present antigens in innate immunity are terminally differentiated macrophages (Kupffer cells and splenic macrophages), blood monocytes, and dendritic cells. Macrophage dysfunction is a significant contributor to both innate immunosuppression and adaptive immunosuppression. The state of immune paralysis is characterized by decreased bacterial clearance, decreased capacity to present antigens and to release proinflammatory cytokines. The main features of sepsis-induced immunosuppression are presence of defective T cells, with apoptotic depletion, decreased proliferation, and Th-2 polarization. Clinical relevance of PICS was elucidated by Moldawer and More. Over the years, the management of SIRS in ICUs has become more and more successful. That means the more patients reside in ICUs for weeks, with clinical manifestations of moderate SIRS and/or secondary infection, requiring life support. They commonly develop progressive protein catabolism resulting

in substantial loss of lean body mass followed by additional weakening instead of regaining strength. Considering these facts, the main challenge for clinicians today is to manage simultaneous chronic inflammation and adaptive immunosuppression, as well as to provide the protection against secondary nosocomial infection and prevent severe protein catabolism.

Myeloid-derived suppressor cells (MDSCs) are other important regulators of the immune system, representing heterogeneous myeloid-originated population of cells that comprise myeloid progenitor cells, immature macrophages, immature granulocytes, and immature dendritic cells. When activated, they produce reactive oxygen and nitrogen species and arginase 1. They are also potent suppressors of various T-cell functions, predominantly antigen-specific CD8+ and CD4+ T-cell responses [29]. In his work, Moldawer, with his coworkers, was particularly interested in a paradoxical role of MDSCs in sepsis and trauma [30]. He reported that there is important role of MDSCs in inflammatory processes, both acute and chronic, and suggested that MDSC expansion is rather a programmed response to inflammation, regardless of its source, contrary to the previously established opinion that it is simply a pathologic response to a growing tumor. Mature myeloid cells are a relatively diverse population; half-life of blood neutrophils is few hours, while, in terminally differentiated macrophages and dendritic cells, half-life is up to months and even years. Nevertheless, during infection and inflammation, there is rapid increase in requirements for and the consumption of these cells, so the host responds to PAMPs, alarmins, and DAMPs with emergency increase in production of myeloid cells. This response is probably mediated by growth factor (e.g., granulocyte/granulocyte-macrophage colony stimulating factor (G/GM-CSF)) and cytokines (IL-6 and IL-17) produced during the early SIRS response. In emergency myelopoiesis, MDSCs are present in bone marrow, secondary lymphoid organs, and even organs of the reticuloendothelial system. Unlike terminally differentiated macrophages and monocytes, these cells produce large amounts of IL-10 and TNF-alpha after sepsis or trauma. They also consume large quantities of arginine, producing nitric oxide (NO), ROS, and peroxynitrites, acting both in proinflammatory and immunosuppressive manner. Patients with sepsis and burn injury, in which the expansion of the MDSC population is prevented, show decreased survival. MDSCs may be crucial for maintenance of innate immunity and inflammatory responses to secondary infection.

2. TNF-Alpha in Severe Acute Pancreatitis as a Clinical Example of Immune Response in Sepsis

As previously mentioned, sepsis is frequently characterized by elevated blood concentrations of both pro- and anti-inflammatory cytokines which may be associated with increased mortality. Cytokines activate multiple cellular processes and also activate other inflammatory mediators that contribute to organ dysfunction. Hence, patients with severe infection often develop MODS, which lead to further increase in morbidity and mortality. Actual underlying cause of severe sepsis may be different, for example, severe acute pancreatitis, secondary peritonitis, and trauma-induced sepsis, but they all may lead to systemic inflammation. Several clinical trials have been conducted in patients with severe sepsis, septic shock, and MODS in order to investigate the efficacy of biomodulators in blocking or inhibiting inflammation, but they all generally failed to improve the outcome. Recently, the trials have been performed to investigate the role of counter-inflammatory signaling and newer concept of the cholinergic anti-inflammatory pathways [31].

TNF is one of the best described proinflammatory cytokines. It not only is a potent stimulator of the activation of many cell types such as macrophages/monocytes and NK cells but also can induce cell survival or cell death by apoptosis. This cytokine is tightly related to regulation of host innate immunity, inflammation, and apoptosis. It is primarily produced as a 212-amino acid type 2 trimeric transmembrane protein. The release of soluble form is enabled by proteolytic cleavage, mediated by the metalloprotease TNF converting enzyme (TACE, also called ADAM17) [32]. There are numerous different physiological stressors which may stimulate the secretion of TNF, such as endotoxin (LPS), hypoxemia, ischemia/reperfusion, hemorrhage, and complement system. Once secreted, TNF has multiple effects on the host response like increasing synthesis of a potent vasodilator NO, activating the arachidonic acid pathway, and inducing activation of cyclooxygenase and lipoxygenase. These processes increase the production of thromboxane A2 and prostaglandin E2 and augment their physiological effects. TNF also induces the production of selectins, platelet activating factor, and intracellular adhesion molecules (ICAM), which mediate neutrophil migration into tissues. This indicates that TNF plays a major role in activation of both thrombotic and fibrinolytic pathways on endothelial and epithelial cells. Besides macrophages and monocytes, there are other cells capable of TNF production, such as T cells after activation. TNF is early proximal cytokine with a short half-life (less than 20 minutes). This short half-life is long enough for induction of synthesis of the variety of pro- and anti-inflammatory cytokines like IL-6, IL-8, IFN-gamma, and IL-10. The other effects of TNF include altering the levels of corticosteroids. Not only these effects are the consequence of systemic release but also local release of TNF may lead to organ failure, independent of its blood concentrations. TNF acts via its receptors TNFR1 and TNFR2, which belong to a still growing number of TNF receptors in the TNFR superfamily. TNF recognition can lead to divergent results, depending on the specific receptor and environmental factors. TNFR1 has a death domain at the cytoplasmic tail. By binding to this receptor, TNF-alpha induces the transcription factors like NF-κB and subsequent transcription of inflammatory genes, which seems to protect cell against programmed death. This binding can also induce the apoptosis by caspase cascade activation. Activation of TNFR1 only signals for cell death under distinct circumstances, for example, when the protein synthesis is blocked or when NF-κB activation is inhibited. Contrary to TNFR1, which is expressed in most tissues and can be activated by both membrane-bound and

soluble trimeric forms of TNF-alpha, TNFR2 is expressed in immune competent cells and on the endothelium and becomes activated by membrane-bound TNF-alpha. Also, this receptor does not contain a death domain. Binding to TNF initiates conformational changes in its receptors, thus inducing downstream signaling which activates at least three different pathways including NF-κB, mitogen-activated protein kinases (MAPK), and death signaling [33].

Contact of TNF and TNFR2 activates signal transduction pathways such as NF-κB and Jun N-terminal kinase and also induces activation and proliferation of immune cells (neutrophils, NK cells, B cells, and peripheral T cells). In patients with chronic inflammatory diseases, but not in patients with sepsis, there was a considerable success with the administration of anti-TNF antibodies or soluble TNF receptors in order to inhibit TNF activity.

Acute pancreatitis (AP) is a disease with incidence varying from 5 to over 100/100 000 people per year. Severity and mortality rates also vary. The most frequent is mild form of the disease, but severe acute pancreatitis (SAP), complicated with local lesions and/or organ failure, is developed in about 20% of the cases. Clinical course of SAP may be fulminant, and the releasing of inflammatory mediators into the bloodstream may affect distant organs. Hence, the major cause of death in these patients is MODS which occurs as a complication of SAP in 20–80% of the cases [34]. Recently, we performed a study regarding plasma levels of TN-alpha, as one of the most important cytokines in pathogenesis of acute pancreatitis, in patients with severe acute pancreatitis (SAP) on admission as predictors of severity and outcome of SAP. Blood samples were obtained from 100 patients with SAP. According to severity, patients were divided into two groups: 69 patients were in SAP group and 31 in SAP-induced MODS group. Fifty-three patients were alive 90 days after taking the blood sample for cytokine measurement and thus were recorded as survivors. When comparing SAP group with SAP-induced MODS group, we found that mean values of TNF-alpha on admission were 191.5-fold lower in group with SAP-induced MODS ($P < 0.01$). When comparing nonsurvivors with survivors, we found that mean values of TNF-alpha on admission were 63-fold higher in survivors ($P < 0.01$). At cut-off level of 7.95 pg/mL sensitivity was 83.9% and specificity was 72.5%. Patients with TNF-alpha level lower that 7.95 pg/mL had 3.2-fold higher probability to develop SAP with MODS. At cut-off level of 10.5 pg/mL, sensitivity was 83.0% and specificity was 77.4%. Patients with TNF-alpha level higher than 10.5 pg/mL had 4.8-fold higher probability to survive. We concluded that TNF-alpha is good predictor of severity and outcome. Low TNF-alpha concentration in patients with SAP predicts development of MODS and fatal outcome in our study [35]. Several studies have produced conflicting results regarding levels of cytokines in circulation and severity and outcome of systemic inflammation in critically ill patients. Contrary to some authors [36, 37] who found that high serum TNF-alpha levels correlate positively with the severity of disease and fatal outcome, we showed in our investigation that patient with SAP-induced MODS and fatal outcome had very low serum TNF-alpha levels. Florence Riche with coauthors found, as did we in our study, that in patients with abdominal septic shock high serum TNF levels were associated with increased survival [38]. The high serum level of TNF may reflect the efficacy of peritoneal inflammatory response against abdominal sepsis, and SAP belongs to that category. Ten years ago Dugernier and coauthors published the results of their interesting study regarding compartmentalization of the inflammatory response during acute pancreatitis. Their investigation was conducted in large cohort of 60 patients with SAP in whom they did peritoneal lavage and thoracic duct drainage at the onset of MODS [39]. In order to assess the pro- and anti-inflammatory responses, the site of mediator production, and their route of diffusion, they collected simultaneous samples of ascites, thoracic lymph, and blood at the onset of MODS and for the following 6 days. In less than 15% of blood and lymph samples they detected TNF-alpha and IL-1beta. Levels of secondary pro- and anti-inflammatory cytokines were elevated in all compartments from the beginning of investigation and throughout the entire sampling period. Cytokine concentrations were the highest in ascites and decreased from lymph to blood, suggesting a splanchnic origin. Although a net proinflammatory activity ascribed to IL-1beta was detected in ascites, a net anti-inflammatory activity was measured virtually in all lymph and blood samples. That indicates that the pancreas and the splanchnic area are sites of proinflammatory response while an early and sustained anti-inflammatory activity dominates in circulating compartments. This suggests that local proinflammatory stimuli induce rapid, robust, and dominant anti-inflammatory response in circulatory compartments, which may lead to an increased risk of developing secondary infection [40]. The timing of measurement of the proinflammatory cytokines from the onset of the disease is of great importance. In SAP, TNF-alpha is released in circulation in the first few hours and rapidly disappears after that. Because our patients presented to the ED at various stages of SAP, in some cases the first samples might be collected after this early TNF-alpha peak. In addition, the presence of soluble TNF receptors can interfere with the detection of unbound TNF. The production of cytokines at various tissue sites depends, in part, on the proximity of given site to the injurious stimulus. The magnitude of injury may also influence the increase in cytokine levels. However, it has been difficult to correlate plasma concentration of a particular proinflammatory cytokine with the overall extent of tissue damage in clinical trials. This is supported by the fact that cytokines are a component of a paracrine system that is involved in signaling the local presence of inflammation to adjacent somatic tissue. Also, cytokines may occur both as free secreted and cell-associate forms. TNF-alpha exists as a high-molecular weight, cell-associated membrane form in inflammatory cells. This form of TNF-alpha acts by direct cell-to-cell contact. Its dual nature also helps explain why systemic concentration of circulating TNF-alpha may not be reflective of the degree of local TNF-alpha activity.

3. Immune Response in Severe Trauma with or without Secondary Sepsis

In 1536 the army of King Francis I of France fought at the city of Turin against the army of the Holy Roman Emperor Charles V. After the French army recaptured the city in 1537, their surgeon general, Amroise Pare, wrote a passage in which he reported inevitable consequence of nonfatal wound, dreaded by soldiers. We now entitle Amroise Pare to be the father of modern trauma surgery, and the described entity is now termed *posttraumatic sepsis* [41].

Worldwide, in the general population under the age of 45, trauma is one of the main causes of death. Mortality rate after major trauma is different regarding time period after injury. First, we recognize the immediate effects of trauma with death at the scene or within the first hour with mortality rate of 53–72%. These deaths are common consequences of massive head injury or bleeding. The second peak is somewhat smaller and occurs in the first 24 hours. The deaths are mainly due to hypoxia, hypovolemia, or traumatic brain injury. In survivors, we recognize the third pattern, characterized by high risk of developing immune dysfunction and subsequently sepsis, leading to MODS with high mortality rate. All severe posttraumatic complications (systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and MODS) are directly related to synthesis and release of inflammatory mediators into the bloodstream, which is in fact the *first hit*—representing the severity of injury itself, and then there is *second hit*, such as surgical intervention and/or bacterial infection [42–44]. Based on this concept, a new immunoinflammatory paradigm in critically injured patients is developed [45]. Current concept explains the complications of severe injury as the consequence of excessive proinflammatory response (SIRS), representing excessive innate immune response, then followed by compensatory anti-inflammatory response syndrome (CARS), representing suppressive adaptive immune response. A second-hit phenomenon results from sequential insults that lead to more severe, recurrent SIRS and organ dysfunction. The proposed new paradigm considers rapid and simultaneous induction of innate genes (both pro- and anti-inflammatory) and suppression of adaptive immunity genes. Recoveries from complications are delayed, and patients are captured in prolonged state of immune-inflammatory dysregulation. Patient is defending against the bacterial invasion by his/her first line of defense, epithelial barrier, but it is often disrupted in trauma, allowing the penetration of microorganisms. Other lines of defense include activation of immune cells and production of cytokines.

Six years ago we focused our research on immune cytokine response in very specific group of injured patients, namely, combat casualties, regarding secondary sepsis development [46]. Combat operations are becoming more frequent worldwide. Considering this, we wanted to evaluate the immune response in combat casualties who suffered from blast or explosive trauma, with or without secondary sepsis, and to assess the prognostic values of certain proinflammatory (TNF-alpha and IL-8) and anti-inflammatory (IL-4 and IL-10) cytokines, regarding severity and outcome. To our knowledge, until that time, next to nothing was done in research of cytokine response to combat trauma with or without sepsis in war time condition.

The study group consisted of 76 male combat casualties. The moment of sustaining injury was establish in 76% of the cases. In 61% of the cases time interval between sustaining injury and admission, when the first set of blood samples was taken, was 6 hours. In these patients, initial surgical treatment was administered in Military Medical Academy (MMA) in Belgrade. In the rest of the cases, time interval between injury and taking the first blood sample was 12 hours, for these patients were initially surgically treated in front line hospitals and then transferred to MMA. Group I consisted of 56 casualties with blast of explosive trauma who developed secondary sepsis (trauma + sepsis group). The criteria for establishing diagnosis of sepsis included positive blood culture. Group II consisted of 20 casualties, selected to match Group I. They all suffered from blast or explosive trauma equally severe as in Group I, but without sepsis (trauma group). Trauma severity was determined according to injury severity score (ISS). In Group I there were 15 and in Group II 5 true blast victims (without any evidence of being struck by any object) in which ISS was determined intraoperatively. There was no statistically significant difference in ISS between two groups (mean ± SD): 29±10.4 in Group I (trauma + sepsis) and 31.7± 12.5 in Group II (trauma), respectively. Also, there was no statistically significant difference in percentage of abdominal wounds between two groups (35.7% in Group I versus 40.0% in Group II). Severity of shock according to SOFA score was significantly higher in Group I (trauma + sepsis): 6.09 ± 3.73 versus 2.95 ± 3.87 (mean ± SD), $P < 0.01$.

According to severity of clinical status the patients were also divided into two groups, SIRS (less severe) and MODS (more severe) group. When compared trauma + sepsis group with trauma group, we found statistically highly significant difference ($P < 0.01$) in IL-8, IL-10 and statistically significant difference ($P < 0.05$) in TNF-alpha concentrations; mean values of IL-8 were 230-fold, IL-10 42-fold, and TNF-alpha 17-fold higher in trauma + sepsis group. When comparing MODS with SIRS group, we found statistically highly significant difference ($P < 0.01$) in IL-8, TNF-alpha, and IL-10 concentrations; mean values of IL-8 were 60-fold, TNF-alpha 43.5-fold, and IL-10 70-fold higher in MODS group. Concentrations of the same three cytokines were significantly different ($P < 0.01$) when we compared nonsurvivors with survivors; mean values of IL-8 were 2.3-fold and IL-10 1.4-fold higher in non-survivors, while mean values of TNF-alpha were 2.2-fold higher in survivors. Unexpectedly, concentrations of IL-4 were not statistically different between groups regarding secondary sepsis, severity, and outcome.

Meduri proposed interesting explanation for poor outcome in our patients with excessive proinflammatory and anti-inflammatory response. Since some studies have shown positive correlation between sustained and intense inflammatory responses and the incidence of bacterial infections, he hypothesized that cytokines secreted by the host during MODS may indeed favor the growth of bacteria and hence establish a relationship between exaggerated and protracted

systemic inflammation and the frequent development of infections [47].

Last year, Charles Wade and his group published comprehensive, systematic review of the literature regarding comparison of mortality associated with sepsis in the burn, trauma, and general intensive care unit patients [48]. Sepsis outcomes in these three distinct patient populations were compared for the first time. Conclusion of this review was that trauma patients showed relatively low mortality associated with sepsis, while in burn patients as well as in the older critical care patients the prevalence of sepsis was higher with worse outcomes. Out of 334 titles, 97 abstracts, and 65 full texts retrieved, 38 studies fulfilled strict criteria to be reviewed. Our study was included with the highest level of evidence rating (IV: evidence from well-designed studies) and quality (A grade). The same group of authors suggested that combining markers of inflammation and coagulation and standard clinical indices would improve early prediction of in-hospital mortality in burn and nonburn trauma (but not combat trauma) patients [49]. They concluded that, compared to previous methods, the proposed model improves prediction of in-hospital mortality. In spite of our best efforts, we could not find, in the literature available to us, studies regarding systemic effects of cytokines in combat casualties. What we did find were several articles regarding inflammatory biomarkers and combat wound dehiscence and healing. Most of them concluded that the cytokine and chemokine protein and gene transcript expression patterns demonstrate a condition of inflammatory dysregulation associated with war wound failure and that molecular biomarker panel may predict wound healing outcome and warrants prospective validation [50–53]. In one study, encouraged by the correlation between systemic and local inflammatory cytokines and microbial colonization assessed by quantitative cultures, authors proposed the concept of interplay between the systemic response to injury and local wound environment as a determinant of outcome [54]. The same authors also stated that this relationship remains poorly defined and requires further investigation in both clinical and preclinical studies; we completely agree with that statement.

Severe trauma, commonly followed by substantial blood loss, leads to decreased endothelially derived NO, which further leads to increased platelet aggregation, increased neutrophil infiltration, and deregulation of vasorelaxation. As a result, the increase of microvascular permeability, concomitantly with ultimate loss of endothelial integrity, simultaneously occurs. The first line of defense against invading microorganism is formed by the innate immune responses, which rapidly react to DAMPs. Adaptive immunity responds slower because antigen-specific reaction requires initial sensibilisation [55]. Antigen presentation after injury is the function of monocytes and macrophages as their mature phagocytotic phenotype. They recognize, uptake, and kill invading microorganisms, which initiate an adequate immune response. In cases when this monocyte function is impaired, antigen-presenting ability is decreased, together with disrupted monocyte—T cell interaction; that has been related to development of septic complications after severe trauma. Restitution of monocyte function is reflected by the significant increase of TNF-alpha, for example, after a temporary state of predominant anti-inflammatory production of IL-4 and IL-10 by Th2 cells. Endogenous inflammatory mediators such as TNF-alpha and NO activate premature apoptosis of immune effector cells, which may contribute to the sepsis-associated MODS after severe trauma. Neurohumoral signaling, via binding of glucocorticoids, catecholamines, or adrenergic agonists to the corresponding receptors on immune cells, can, for example, suppress cytokine production and thus impair a competent immune regulatory cell-cell interaction [56]. The concept of T-cell mediated immunosuppression is now somewhat improved by reports of the activity of Th17 cells. This lineage of inflammatory CD4+ T-cell subpopulation exhibits particular developmental and phenotypic characteristics different from both Th1 and Th2-types and is capable of IL-17 production [57]. Numerous authors use cytokines as prognostic markers regarding outcome of trauma in patients with SIRS, sepsis, or MODS. Some authors favor IL-6 in this regard and propose threshold level of 800 pg/mL on admission to be a good indicator for differentiating between patients with or without organ failure [58].

Besides its role in sepsis (as we elucidated in the first part of this review), complement also plays a significant role in activation of the innate immune system in contributing to the pathogenesis of trauma-induced sequelae and adverse outcome. Complement system takes part in the first line of defense, where it acts as a potent effector of innate immunity, which implicates this system in mediation of the early posttraumatic inflammatory response. Despite its generic beneficial functions, including pathogen elimination and immediate response to danger signals, complement activation may exert detrimental effects after trauma in terms of mounting an "innocent bystander" attack on host tissue. Ischemia-reperfusion injury after trauma is classical example of tissue damage mediated by complement activity. Complement activity may also exacerbate local and systemic inflammation and release of toxic mediators, thus adding to the "antigenic load." This activity may consequently sustain SIRS after major trauma, ultimately contributing to remote organ injury and death. This pathophysiological pattern represents the fundament of new therapeutic approach named *site-targeted complement inhibition* [59].

Severe trauma commonly leads to major impairment of the immune system. Hyperinflammation state after trauma mediates remote organ damage and may lead to MODS, while, on the other hand, immunosuppression enhances risk of developing acquired infectious complications. Pathophysiological substrates for these opposite consequences of trauma involve the role of endogenous danger signals, such as HMGB-1 and HSPs, generated in destroyed tissues, which mediate trauma-induced immune dysfunction [60]. The major danger signals that initiate immune response after trauma are dual-function alarmins HMGB-1, IL-1 alpha, and IL-33. They play the most important role in activation and propagation of the inflammatory response after disruption of cellular integrity. The common characteristics of these three alarmins are their activity as transcription factors and

extracellular mediators of inflammation; however, each dual-function protein exerts distinct functions. In addition, a new field for investigation of danger sensing and transmission is opened by the discovery of mitochondrial DAMPs, which activate immune response after cellular disruption by mimicking bacterial infection [61]. Mitochondria emerged as crucial mediators in the induction of apoptosis during traumatic shock [62]. Besides apoptosis, there is now evidence of presence of *necroptosis*, a form of organized cell necrosis. Necroptosis can be induced by TNFR and other so-called death receptors [63, 64]. The important role of mitochondria in activation of innate immunity is supported by the fact that they contain constituents of their bacterial ancestors which are potentially immunogenic [65]. Key receptor for danger signals is TLR-4. This receptor has been extensively studied, and ten TLR homologues have been identified in humans. In addition to its ability to recognize the bacterial LPS, it has been now revealed that TLR-4 can be activated by danger signal molecules released after cellular injury. Hemorrhagic shock and consequent resuscitation, that make common chain of events following severe trauma, may lead to global ischemia/reperfusion injury and MODS as a final result. The potential role of TLR-4 in this process is supported by its expression in liver, lungs, and myocardium during hemorrhagic shock and resuscitation [66]. The immune response may be influenced by the type of trauma. In the study of Mace and coauthors, burns were associated with a greater and more sustained immune-inflammatory response than nonburn trauma (evidenced by increased concentrations of IL-6 and IL-8 in plasma during the first week after trauma). They found no association between MODS and plasma cytokine concentrations [67].

There are several factors that contribute to the immune response and end organ damage after trauma. Some etiological factors are intrinsic, including genetic physiological status and predisposition, while others are extrinsic, such as type of injury ("trauma load" or "intervention load," meaning surgery). The only factor that can be altered by the attending physician is the intervention load. The damage caused by immune response to trauma hence may be attenuated by the adjustment of the therapeutic approach and surgical treatment strategy [68].

4. Is Immune Response in Sepsis and Trauma, at Least in Part, Genetically Determined?

The inflammatory response contributes significantly to the morbidity and mortality of critically ill patients and may extremely vary between individuals. In patients with similar infection, there is tremendous variability reported in the clinical profile and outcome. The risk of sepsis and its outcome are influenced by host predisposition [69, 70]. That predisposition may be explained by interindividual genetic variability, represented by genetic polymorphisms [71]. Genetic polymorphisms in the immune response to infection are associated with the susceptibility to certain infection and with clinical outcome. Understanding the biology of inflammation is significantly improved, but so far it is not followed by substantial improvements in clinical outcome; furthermore, the sporadic promising results have been related to supportive care efforts rather than to specific therapies. As a result, mortality and cost of treatment of patients suffering from severe infections remain high.

Twenty-five years ago, Sorensen and coauthors reported that in adult adoptees the risk of dying from infection has been 5.81-fold higher when one of their biologic parents died of infection before the age of 50. This risk exceeded the relative risk (RR) of dying of malignancy (1.19) and cerebral and/or cardiovascular causes (4.5). These findings suggest a significant genetic susceptibility to lethal infection and sepsis [72]. Studies performed in order to determine polymorphisms related to sepsis have been mainly focused on one or more polymorphisms for specific genes that generate proteins involved in immune response in sepsis such as pro- and anti-inflammatory cytokines and elements of innate immunity and coagulation/fibrinolysis pathways. Severe injury also activates the innate immune response as part of the inflammatory response, which in turn can lead to secondary MODS, so there may be genetic predisposition to adverse effects due to trauma [73]. The connection between phenotype and sepsis has been established by genetic mapping of the single nucleotide polymorphisms of IL-6, IL-18, TNF-alpha, IFN-gamma, and TLRs [74].

The most common form of stable genetic variation in the population is a *single nucleotide polymorphism* (SNP), which refers to single-base-pair positions in genomic DNA in which sequence alternatives exist with a frequency of more than 1%. SNPs are not the cause of disease itself, but they may alter the risk for disease development. They may also influence the outcome of a disease. Of all SNPs in the human genome, it has been estimated that 10% have the potential of modifying some biologic processes [75].

Three years ago we investigated whether distributions of TNF-alpha$_{308}$, IL-10$_{1082}$, CD14$_{159}$, and IL-1ra gene intron 2 genotypes in critically ill are associated with outcome, underlying cause of sepsis, type of microorganism. Blood samples from 106 critically ill Caucasian patients (severe acute pancreatitis, secondary peritonitis, and trauma with or without sepsis) were genotyped by methodology based on polymerase chain reaction (PCR) for TNF-alpha$_{308}$, IL-10$_{1082}$, cluster of differentiation, CD14$_{159}$, and IL-1 receptor antagonist gene intron 2. All patients with TNF-alpha$_{308}$AA genotype survived; RR of death in patients with AG was 3.250 and with GG 1.923 ($P < 0.01$). In patients with Gram-positive sepsis IL-10$_{1082}$AA and then AG genotypes were the most frequent ones (OR 18.67 and 7.20, resp., $P < 0.01$). When comparing IL-10$_{1082}$AA with AG, RR of pancreatitis being underlying cause of sepsis was 1.80; OR was 3.40. When AA and GG were compared, RR was 7.33; OR was 20.00. In patients with GG, RR of peritonitis being underlying was 4.07; OR was 5.88 ($P < 0.01$). In patients with Gram-positive sepsis CD14$_{159}$CT was the most frequent one with OR 5.25. Distribution of six IL-1ra gene intron 2 genotypes showed no significant association. We concluded that distribution of TNF-alpha$_{308}$ genotypes is associated with outcome, IL-10$_{1082}$ with type of microorganism and underlying cause of sepsis, and CD14$_{159}$ with type of microorganism [76]. We are aware that there are inconsistent findings in current studies

of genetic association in human trauma and/or sepsis (same as ours, opposite to ours, or with no association at all). Strict critics have focused on methodological and analytical problems (namely, underpowered studies), but they also state that, for example, it has been calculated, for a general ICU population with sepsis or septic shock, that a sample size of 2000 patients would be required to detect a mortality RR of 1.5 from any polymorphism to confidently exclude false negative associations. To our knowledge no genetic association study recruited this number of patients. Until then, relatively small population studies should be taken into account.

Identification of strong associations between certain genetic polymorphisms and increased mortality rate, underlying cause of sepsis or the type of infecting microorganism, is intriguing and requires further research. Despite previously mentioned limitations of the most studies of association between genetic polymorphisms and sepsis, this approach is promising. Genetic and molecular aspects of host immunoinflammatory response to various insults in critically ill patients are complex, and establishing a certain association does not mean revealing the causative relationship. Genetic studies might allow for earlier differentiation among patients with immunoinflammatory response to either infection or trauma, allowing for more focused and timely treatment. Molecular profiles might be established to distinguish a good versus a poor response to therapeutic intervention.

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Review Article

Immunomodulation in Sepsis: The Role of Endotoxin Removal by Polymyxin B-Immobilized Cartridge

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Severe sepsis results in high morbidity and mortality. Immunomodulation strategies could be an adjunctive therapy to treat sepsis. Endotoxin is a component of gram-negative bacteria and plays an important role in the pathogenesis of septic shock when it is recognized by immune cells. Removal of endotoxin could be an effective adjunctive approach to the management of sepsis. Devices to adsorb endotoxin or inflammatory cytokines have been designed as a strategy to treat severe sepsis, especially sepsis caused by gram-negative bacteria. Polymyxin B-immobilized cartridge has been successfully used to treat patients with sepsis of abdominal origin. Although this cartridge was conceived to adsorb endotoxin, several other immunological mechanisms have been elucidated, and this device has also yielded promising results in patients with nonseptic respiratory failure. In this paper, we summarize the immune modulation actions of Polymyxin B-immobilized cartridge to explore its potential usefulness beyond endotoxin elimination.

1. Introduction

Sepsis is a major healthcare problem. Despite advances in supportive care of critically ill patients, sepsis remains an important cause of death worldwide in adults and children [1–3]. The Surviving Sepsis Campaign (SSC), which standardized the approach to sepsis, was recently updated [4]. Several efforts have been made to improve adherence to SSC guidelines [5, 6]. Nevertheless, mortality and costs are still high [2, 7, 8]. Sepsis is characterized by a complex systemic inflammatory response to a microbial pathogen. First, the presence of microorganisms in the bloodstream causes an innate immune response characterized by the stimulation of monocytes and release of proinflammatory cytokines and the activation of a medley of different immune pathways. Toll-like receptors (TLRs) play a key role in this initial immune activation, acting as innate immune system sensors through the recognition of highly conserved components of a variety of microorganisms [9]. The activation of TLRs induces an inflammatory response to control the infection, which results in local vasodilatation, release of various cytotoxic chemicals, and, hopefully, destruction of the invading pathogen. Many of these same components of inflammation that are beneficial in host defenses against infection can, under some circumstances, be deleterious, causing cell and tissue damage and hence multiple organ failure. Endotoxin, also known as lipopolysaccharide, is a component of gram-negative bacteria and a strong activator of TLR4. The recognition of endotoxin by immune cells is important in the pathogenesis of septic shock [10, 11].

Conventional therapy such as antibiotics and surgical procedures to remove the source of infection is crucial for

treating sepsis, but these approaches cannot reverse the effects of the bacterial toxins already released into blood or of the endogenous mediators produced by the host in response to bacteria. Over recent years, numerous attempts have aimed to intervene in the inflammatory cascade. Attempts to stop the inflammatory cascade using antiendotoxin strategies such as monoclonal antibodies or vaccines have failed [12, 13]. Phospholipid emulsion to neutralize endotoxin does not improve outcome in septic patients [14]. A recent phase 2 trial found a nonsignificant trend toward better survival in patients with severe sepsis treated with eritoran tetrasodium, a TLR-4 antagonist [15].

Blood purification techniques including hemoperfusion, plasma exchange, and hemofiltration with hemoperfusion are associated with lower mortality in patients with sepsis as it has been demonstrated in a recent meta-analysis [16]. Removing endotoxin would be an effective adjunctive approach in the management of severe sepsis. Devices to remove endotoxin or inflammatory cytokines have been designed as a strategy to reduce the morbidity and mortality associated with sepsis, especially with sepsis due to gram-negative bacteria. These devices have also been successfully used in patients with sepsis due to gram-positive microorganisms and in patients with acute respiratory distress syndrome (ARDS), suggesting that they could have an immunomodulating action in addition to endotoxin elimination [17–21]. Indeed, many studies report additional beneficial immunological mechanisms for endotoxin removal devices. This review aims to summarize the immune modulation actions of Polymyxin B-immobilized cartridge to understand the potential usefulness of this device beyond endotoxin elimination.

2. Devices to Remove Endotoxin and Inflammatory Molecules

Over recent years, devices to eliminate endotoxin, inflammatory molecules such as cytokines and immune cells, have been designed to mitigate the deleterious effects of the inflammatory cascade. These extracorporeal devices act through convection or adsorption. A double lumen catheter for extracorporeal use is needed. Most of these devices are designed to combine the effect of molecules removal with renal replacement therapies (hemofiltration, dialysis, or hemodiafiltration). The biocompatibility of these devices is the main limitation for its use, thrombocytopenia and bleeding risk are the potential side effects [22]. Table 1 summarizes the mechanism of action and molecules removed by each membrane.

2.1. Polymyxin B-Immobilized Cartridge (Toraymyxin 20-R, Toray Industries, Japan). Polymyxin B is a cationic polypeptide antibiotic with activity against gram-negative bacteria and a high affinity to endotoxin, but its intravenous use has been limited due to nephrotoxicity and neurotoxicity [23]. Since 1994, Polymyxin B has been fixed and immobilized with polystyrene fiber in a hemoperfusion column Polymyxin B-immobilized cartridge (PMX) that allows endotoxin removal without the toxic effects of this antibiotic. This treatment has been widely used in Japan for septic shock due to gram-negative bacteria, and its use was authorized in Europe in 1998. Recent studies support the safety and efficacy of this treatment [16, 24–26].

2.2. LPS Adsorber (Alteco Medical AB, Sweden). This medical device designed for extracorporeal use contains a series of porous polyethylene plates coated with a peptide specific to endotoxin and has a high adsorption capacity. It has been used in patients with septic shock [27–29]. Yaroustovsky et al. [28] compared LPS adsorber and PMX hemoperfusion in a small sample of patients with gram-negative sepsis. The authors did not find differences in outcome. However, due to limitations of the study, the authors concluded that further studies were necessary to clarify the efficacy of LPS adsorber.

2.3. OXiris (Gambro-Hospal, France). This AN-69 (polysulfone and polyacrylonitrile) based membrane adsorbs a large spectrum of plasma inflammatory mediators such as endotoxin and cytokines [30, 31]. To date, clinical experience with this device is limited, but two trials are underway in septic patients [32]. The results of these two trials are crucial to determine its usefulness compared with the current standard of care.

2.4. MATISSE-Fresenius System (Fresenius SE, Germany). Based on the endotoxin-binding abilities of human albumin, this adsorber contains human serum albumin immobilized on polymethacrylate beads. Although in vitro experiments were promising, phase 2 study results have been disappointing [33].

2.5. Coupled Plasma Filtration Adsorption, CPFA Bellco, Italy. This extracorporeal treatment is based on nonspecific adsorption of cytokines and other proinflammatory mediators onto a specially designed resin cartridge, coupled with hemofiltration. This device does not adsorb endotoxin. Some studies have shown interesting results regarding hemodynamics and respiratory parameters [34–36]. This is a promising therapy, although further studies are necessary to determine its usefulness in septic patients. One clinical trial, COMPACT 2, is underway to clarify whether adding high doses of CPFA to current clinical practice can reduce hospital mortality in septic shock patients (ClinicalTrials.gov number NCT01639664) [37].

2.6. CytoSorb (Cytosorbents Inc., USA). This extracorporeal device removes cytokines through adsorption to a high-surface-area biocompatible porous polymer sorbent. This device does not target endotoxin, but it does rapidly eliminate several key cytokines by adsorption in both in vitro and in vivo experiments [38, 39]. This device is very promising, but more studies in septic patients are needed.

Due to the broad information existing about safety and efficacy of Polymyxin B-immobilized cartridge, we will review the immunological mechanisms described in this treatment.

Table 1: Devices designed to remove endotoxin and cytokines in patients with septic shock.

Device	Company	Composition	Mechanism	Substance eliminated
Toraymyxin 20R	Toray Industries, Japan	Polymyxin B covalently bound to polypropylene-polystyrene fibers fabric	Adsorption	Endotoxin
LPS adsorber	Alteco Medical, Sweden	Synthetic polypeptide bound to porous polyethylene discs	Adsorption	Endotoxin
oXiris	Gambro-Hospal, France	AN69-based membrane, surface treated with a polyethyleneimine (PEI) and grafted with heparin	Adsorption Convection	Endotoxin Cytokines
MATISSE	Fresenius SE, Germany	Human serum albumin immobilised on polymethacrylate beads	Adsorption	Endotoxin
CPFA	Bellco, Italy	Polyethersulfone Plasma filter with adsorption on an unselective hydrophobicresin cartridge, and a synthetic high-permeability polyethersulfone hemofilter for continuous hemofiltration	Adsorption Plasma filtration	Cytokines
Cytosorb	Cytosorbents, USA	Polystyrenedivinyl benzene copolymer beads with a biocompatible polyvinylpyrrolidone coating.	Adsorption Convection	Cytokines

3. Immunological Mechanisms Described for Polymyxin B-Immobilized Cartridge

Polymyxin B-immobilized cartridges (PMX) are designed to bind endotoxin. However, other mechanisms of immunomodulation have also been described. Whereas some of these mechanisms are derived from endotoxin elimination, others result from direct action on other inflammatory molecules and cells or from a combination of endotoxin elimination and direct action on these mediators. Table 2 summarizes these mechanisms.

3.1. Endotoxin Removal. Endotoxin is a major component of the outer membrane of gram-negative microorganisms [40]. Immune cells recognize endotoxin and other bacterial compounds through the TLR, a group of transmembrane proteins that play crucial roles in the host defense against invading pathogens [41]. During a gram-negative infection, TLR-4 recognizes endotoxin and originates a systemic inflammatory response in sepsis with potentially fatal effects in hosts. As a consequence, proinflammatory molecules such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) are released and generate other cell responses in the inflammatory cascade (Figure 1). This increase in cytokines is followed by a major expression of tissue factor, which activates coagulation, and by an increase in nitric oxide synthesis, which induces vasodilation [42]. Endotoxin levels are high in septic patients [43, 44], but they are also high in critical patients without sepsis, such as patients undergoing cardiopulmonary bypass and those with chronic heart failure, chronic kidney disease, and other medical conditions [44–47]. In critical patients without gram-negative infection, elevated endotoxin levels are related to translocation of gut bacterial antigens and endotoxin into the bloodstream due to gut barrier dysfunction [48–50].

Polymyxin B binds endotoxin through hydrophobic and ionic interactions. Polymyxin B's hydrophobic amino acids (Phe, Leu) form hydrophobic bonds with lipid A fatty acid in endotoxin, and the amino groups of Polymyxin B form ionic bonds with the negatively charged phosphate groups of lipid A [51]. This binding results in an antibiotic-endotoxin complex that is highly effective in neutralizing the deleterious effects of endotoxin.

Many studies have reported diverse benefits of PMX hemoperfusion in septic patients, including improved hemodynamics [24, 25, 52–60], increased ratio of partial pressure arterial oxygen and fraction of inspired oxygen (PaO_2/FiO_2) [24, 54, 55, 57, 58, 60, 61], decreased 28-day mortality [24, 52, 53, 59], and decreased endotoxin levels [52, 53, 55, 58, 59, 62–64]. In a multicenter study, Vincent et al. [25] found that PMX hemoperfusion was safe and improved cardiac and renal function due to sepsis or septic shock; however, they could not demonstrate a reduction in mortality or in endotoxin levels from baseline to the end of treatment. In a systematic review of 28 studies, Cruz et al. [65] concluded that PMX hemoperfusion was associated with lower mortality (RR 0.53, 95% CI: 0.43–0.65) and improvements in mean arterial pressure (MAP), use of inotropes, and PaO_2/FiO_2. In 17 of these studies in which endotoxin levels were measured, endotoxin levels decreased by 33% to 80% after PMX hemoperfusion [65]. More recently, the same authors published the EUPHAS study [24], a prospective multicenter randomized controlled trial that enrolled 64 patients with severe sepsis or septic shock who underwent emergency surgery for intra-abdominal infection. Patients were randomized to conventional therapy or to conventional therapy plus two sessions of PMX hemoperfusion. After the results of the scheduled interim, analysis revealed that PMX hemoperfusion significantly improved hemodynamics and organ dysfunction and reduced 28-day mortality; the study was discontinued because it was considered unethical to deprive high risk patients of a potentially beneficial therapy; however, early discontinuation resulted in a modest sample size. This study did not measure endotoxin. Two adequately powered prospective trials are underway, and the results of these trials should elucidate the benefit of endotoxin removal (ClinicalTrials.gov numbers NCT01046669 and NCT01222663) [66, 67].

Table 2: Summary of mechanisms described for polymyxin B-immobilized cartridge hemoperfusion.

Molecules	Description	Effect of PMX	Clinical features after PMX	References
Endotoxin	Component of the external membrane of gram-negative microorganisms, recognized by immune cells	↓	Interruption of inflammatory cascade	[52–55, 58, 59, 62–64]
IL-1; IL-6; IL-8; IL-9; IL-10; IL-12; IL-17; αTNF	Pro- and anti-inflammatory cytokines; their overproduction is deleterious in sepsis	↓	Decrease in the excessive systemic host inflammatory response to infection	[54, 61, 63, 68–70, 72, 88]
Plasminogen activator inhibitor (PAI-1)	Component of the coagulation system that downregulates fibrinolysis in the circulation, favoring coagulation	↓	Regulation of fibrinolysis and reversal of the occurrence of sepsis-associated thrombosis	[54, 55, 61]
Neutrophil elastase	Protease that hydrolyzes lung elastase and other proteins	↓	Reduction of pulmonary tissue destruction	[61, 71]
High mobility group box protein 1, HMGB-1; receptor for advanced glycation end-products (RAGE), S100A12	HMBG-1 is a cytokine to trigger inflammatory mediators; RAGE is a receptor involved in HMBG-1 signaling; S100A12 is a mediator involved in acute lung injury	↓	Decrease in the excessive systemic host inflammatory response to infection	[63, 74, 102]
Anandamide	Intrinsic cannabinoid that induces hypotension in septic shock	↓	Decrease in septic shock-associated hypotension	[78]
Nitric oxide	Produces vasodilatation and hypotension	↓	Decrease in septic shock-associated hypotension	[79]
Erythropoietin	Protein that controls red blood cells production, elevated in sepsis	↓	Prognostic biomarker in sepsis	[70]
Troponin T	Protein found in cardiac muscle	↓	Decrease in myocardial cell damage	[80]
Angiopoietin-1 and -2	Angiopoietin-1 reduces pulmonary inflammation and permeability. Angiopoietin-2 interferes with angiopoietin-1, resulting in pulmonary inflammation and increased permeability	Balance	Decrease in acute lung injury	[75]
Vascular endothelial growth factor (VEGF)	Growth factor involved in several acute and chronic lung diseases	↓	Improvement of lung function	[69]
Monocytes, neutrophils, and lymphocytes	Immune cells involved in inflammatory response	↓	Decrease in the interaction between monocytes and functionally associated cells, decreasing inflammatory response, and decrease in neutrophil and lymphocyte response	[82, 83]
Platelet activator factors (PAF) (P-selectin, β-Thromboglobulin, Platelet factor 4)	PAF stimulates platelets, increasing procoagulation status in sepsis	↓	Decrease inprothrombotic status	[52]
HLA-DR and CD16 expression monocytes on granulocytes	Surface antigen expressions HLA-DR and CD-16 are decreased in sepsis	↑	Increasing in surface antigen expression on immune cells helps the recovery from immunoparalysis in sepsis	[56]
CD4+CD25+Foxp3+ Treg	T-lymphocytes, responsible formaintaining immunological homeostasis and tolerance, are increased in sepsis	↓	Recovery from immunoparalysis in sepsis	[88]
Apoptotic factors (Fas- and caspase-mediated)	Factors that activate cell programmed death of tubular cells	↓	Improvement in renal function by reduction of proapoptotic factors	[94]
Metalloproteinase MMP9	Protease involved in degradation of the basement membrane associated with the alveolar epithelium	↓	Decrease in alveolar destruction and improvement in respiratory function	[62, 101]

IL: interleukin; PMX: polymyxin-B immobilized cartridge.

3.2. Elimination of Cytokines and Inflammatory Molecules. Several studies report a reduction in cytokines and inflammatory molecules in patients' plasma after PMX hemoperfusion [54, 56, 61, 68–70].

3.2.1. Cytokines and Inflammatory Proteins. In patients with severe sepsis, Tani et al. [54] found reductions in endotoxin, TNFα, IL-6, IL-10, and plasminogen activator inhibitor-1 (PAI-1) activities after PMX hemoperfusion. In patients with ARDS, Kushi et al. [61] found a reduction in blood levels of PAI-1, neutrophil elastase (NE), and IL-8 after PMX hemoperfusion. NE is a protease that hydrolyzes lung elastin. In these patients, PaO_2/FiO_2 increased significantly after the treatment, and the authors related this increase to the elimination of IL-8 and NE. In another study, the same group reported a decrease in NE in 20 septic patients treated with PMX hemoperfusion [71]. In 12 patients with septic shock receiving conventional treatment plus two sessions of PMX hemoperfusion, Zagli et al. [72] found a decrease in IL6, IL10, and TNFα in patients' serum after the treatment, especially in survivors. Most authors attribute the decrease in cytokines and inflammatory molecules to the removal of endotoxin and to the effect of this removal on the inflammatory cascade.

3.2.2. High Mobility Group Box-1 Protein. Patients with sepsis have increased high mobility group box-1 protein (HMGB1), a cytokine secreted by immune cells that triggers inflammatory mediators [73]. The receptor for advanced glycation end-products (RAGE) is involved in HMGB1 signaling. The inhibition of the HMGB-1-RAGE axis could be an effective therapeutic strategy for septic shock. Nakamura et al. [63] compared IL-6, HMGB1, and RAGE in serum between 15 patients with septic shock treated with PMX hemoperfusion and healthy volunteers. The levels of the three molecules decreased after PMX hemoperfusion and correlated with a decrease in endotoxin. Abe et al. [74] studied the effects of PMX hemoperfusion on HMGB1 in patients with acute exacerbation of idiopathic pulmonary fibrosis. PMX hemoperfusion both significantly decreased the serum HMGB-1 level and improved the PaO_2/FiO_2 ratio. Moreover, HMGB-1 was detected in washing medium from the PMX column, suggesting that the decrease in this molecule was not only secondary to endotoxin removal but also to direct removal by the device. A recent study exploring the meaning of HMGB-1 levels in 60 patients with septic shock treated with PMX hemoperfusion found a significant positive correlation between the Sequential Organ Failure Assessment (SOFA) score and HMGB-1 level ($P < 0.05$). The authors concluded that HMGB-1 is a useful prognostic biomarker in sepsis-induced organ failure in patients undergoing PMX hemoperfusion, but formal establishment of the utility of HMGB-1 as a prognostic biomarker still remains to be performed [68].

3.2.3. Vascular and Coagulation Proteins. PAI-1, a marker of vascular endothelial cell activation elevated by endotoxin and cytokines, is one of the fibrinolysis inhibitory factors. PAI-1 levels decrease after PMX hemoperfusion, decreasing the stimulation of vascular endothelial cells [54, 55, 61]. PMX hemoperfusion may have a role in modulating fibrinolysis and inhibiting the development of ischemic organ dysfunction in sepsis.

Angiopoietin-1 is a positive regulator of blood vessel development, remodeling, and maturation. Angiopoietin-2 is a competitive inhibitor of angiopoietin-1. Angiopoietin-1 and -2 play a contributory role in the pathogenesis of acute lung injury (ALI) in septic patients. Angiopoietin-1 reduces pulmonary inflammation and permeability. Angiopoietin-2 interferes with angiopoietin-1, resulting in pulmonary inflammation and increased permeability. Ebihara et al. [75] reported that PMX hemoperfusion could ameliorate the angiopoietin balance in septic patients with ALI.

Vascular endothelial growth factor (VEGF) is a pluripotent growth and permeability factor that has a broad impact on endothelial cell function. VEGF also plays a role in several acute and chronic lung diseases [76]. Oishi et al. [69] recently studied nine patients with acute exacerbation of idiopathic pulmonary fibrosis treated with conventional therapy and PMX hemoperfusion 6 hours/day on two successive days. They found a high concentration of cytokines and VEGF in the eluate from used PMX cartridge fibers, and the clinical improvement in these patients correlated with the amount of VEGF in the eluate. This is the first study to demonstrate that cytokines and VEGF can be directly adsorbed by PMX hemoperfusion independently from endotoxin removal. The authors suggest that cytokines can bind to PMX hemoperfusion fibers directly through ionic/hydrophobic interactions like endotoxin or indirectly via heparin coated in the fibers.

3.2.4. Other Molecules. Anandamide is an intrinsic cannabinoid that has been related with hypotension in septic shock, although currently its direct link to sepsis is only established in a small patient population. Polymyxin-B directly binds anandamide in vitro [77]. One study in 24 patients with septic shock treated with PMX hemoperfusion found that anandamide levels decreased after PMX hemoperfusion in the nine patients who survived; the authors conclude that removal of anandamide by PMX hemoperfusion, whether directly or as a result of endotoxin elimination, could be key to successful septic shock treatment [78]. Further studies are necessary to elucidate the effect of PMX hemoperfusion on anandamide, in order to establish it as a useful treatment for hypotension.

Elevation of nitric oxide (NO) plays an important role in septic patients, producing vasodilatation and hypotension. Nakamura et al. [79] compared NO breakdown products in urine in 20 patients with PMX hemoperfusion, 15 patients with conventional therapy, and 20 healthy controls. They found that septic patients increased NO production and that PMX hemoperfusion reduced NO levels and thus increased blood pressure.

Troponin is a biomarker that may be elevated in septic patients as a result of subclinical myocardial cell damage. Nakamura et al. [80] found increased troponin T in septic patients compared to nonseptic patients and age-matched healthy controls; interestingly, troponin T decreased after PMX hemoperfusion $P < 0.05$.

Erythropoietin levels may be higher in patients with sepsis; erythropoietin levels decrease after PMX hemoperfusion and could be a prognostic indicator in patients with septic shock [70].

3.3. Removal of Cells and Phenotype Change. During sepsis, different populations of leukocytes are activated and change their adhesive phenotype. The capture of leukocytes through extracorporeal blood purification could alter the immune response to sepsis [81]. After ex vivo perfusion of heparinized blood from patients with sepsis and septic shock through PMX hemoperfusion in a laboratory circuit, Kumagai et al. [82] found significant decreases in neutrophils (78%), monocytes (70%), and lymphocytes (10%). This marked reduction in white blood cells should be attributed mostly to the reduction in the circulation of proinflammatory cytokines that induce cell activation and proliferation, as opposed to a direct effect of removal of these cells by the cartridge. Nishibori et al. [83] examined the PMX hemoperfusion filters after treating 4 patients with sepsis; PMX hemoperfusion bound monocytes from the peripheral blood leucocytes. PMX hemoperfusion could produce a beneficial effect by reducing the interaction between monocytes and functionally associated cells, including endothelial cells.

The inflammatory response in sepsis involves activation of platelets. High levels of platelet activator factor (PAF) have been observed in sepsis. Nakamura et al. [52] studied the effect of PMX hemoperfusion on platelet activation, comparing 30 patients treated with conventional therapy plus PMX hemoperfusion and 20 patients with conventional therapy alone. Survival was 60% in the group that received PMX hemoperfusion and 30% in the group that received only conventional treatment. Septic patients had increased PAF (P-selectin, platelet factor 4, and β-thromboglobulin), and PMX hemoperfusion reduced the levels of PAF.

3.4. Effect on Immunoparalysis. The human body undergoes a biphasic immunological reaction in sepsis. A proinflammatory reaction takes place, marked by the release of proinflammatory cytokines like TNFα, as a reaction to the bacterial toxins. On the other hand, a counter regulatory anti-inflammatory reaction arises. This phase acts as negative feedback on the inflammation by inhibiting the proinflammatory cytokines. The persistence of a marked compensatory anti-inflammatory response is called "immunoparalysis" (Figure 1). This pronounced immunosuppressive state adversely affects immune function, making the patient vulnerable to opportunistic infections [84]. These two phases of sepsis may occur simultaneously with a lasting anti-inflammatory response in later phases [85]. Most septic patients survive the initial proinflammatory phase, but they die during this second stage. Strategies to stimulate this immunoparalysis phase of sepsis as IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been developed in animals, but extensive clinical studies are needed to test their safety and efficacy [86].

Recently, Ono et al. [56] showed that the expression of the surface antigens, HLA-DR on monocytes and CD16 on granulocytes, is extremely decreased in patients with septic shock and that PMX hemoperfusion beneficially increases this expression on these leukocytes. Thus, PMX hemoperfusion might help septic patients recover from immunoparalysis.

The regulatory T cells (T_{reg}) that express CD4, CD25, and Foxp3 comprise a small percentage of the T-lymphocyte population in the immune system, but they are central to the maintenance of immunological homeostasis and tolerance. In septic patients, the percentage of T_{reg} is increased, and this presumably contributes to sepsis-induced immunosuppression. Polymyxin-B induces T_{reg} cell death in mice through the modulation of the purinergic P2X7 receptor [87]. Ono et al. [88] studied the effect of PMX hemoperfusion on the recovery from the immunosuppression owing to septic shock. T_{reg}, IL-6, and IL-10 were higher in patients with septic shock than in patients with sepsis. After PMX hemoperfusion, T_{reg} cells, Il-6, and IL-10 decreased. In survivors, the decrease in T_{reg} cells was accompanied by an increase in CD4+ cells. Although further studies are necessary to confirm a causative relationship between T_{reg} depletion and PMX hemoperfusion in septic patients, this mechanism could explain why PMX hemoperfusion can be useful in patients without endotoxemia or in those with gram-positive sepsis [89, 90]. The authors also suggest that the second PMX hemoperfusion treatment might provide additional benefits for recovery from immunoparalysis. This study sheds new light on the benefits of treating septic patients with PMX hemoperfusion beyond endotoxin removal.

Apoptosis, programmed cell death, is an energy-dependent process [91]. Endotoxin may cause an inappropriate activation of proapoptotic pathways in immune cells during sepsis, and this may contribute to the impaired immune response that characterizes sepsis [92]. Endotoxin can also cause apoptosis of renal tubular cells through Fas-mediated and caspase-mediated pathways [93]. Cantaluppi et al. [94] tested the hypothesis that PMX hemoperfusion might prevent gram-negative sepsis-induced acute renal failure by reducing the activity of proapoptotic circulating factors. They randomized 16 patients with gram-negative sepsis to receive standard care or standard care plus PMX hemoperfusion. Proapoptotic activity was significantly reduced in the plasma of the PMX hemoperfusion group, with decreases in Fas upregulation and caspase activity, and these patients also had improved renal function.

4. Usefulness in Acute Respiratory Failure

Several studies have found that PMX hemoperfusion has beneficial effects on oxygenation in patients with sepsis [24, 61, 65]. Moreover, PMX hemoperfusion has been successful in patients with influenza A infection [89, 95], ARDS in drug-induced injury [96, 97], interstitial pneumonia [19, 20, 98], and idiopathic fibrosis [18, 69, 74, 99]. Mechanisms other than endotoxin removal could explain the beneficial effects of PMX hemoperfusion in patients with respiratory failure.

Chemical mediators have an important role in the pathogenesis of ARDS, and decreasing them through direct or indirect removal could be beneficial in ARDS patients. Kushi et al. [61] found decreases in PAI1, neutrophil elastase (NE), and IL-8 in ARDS after PMX hemoperfusion. Abe et al. [74]

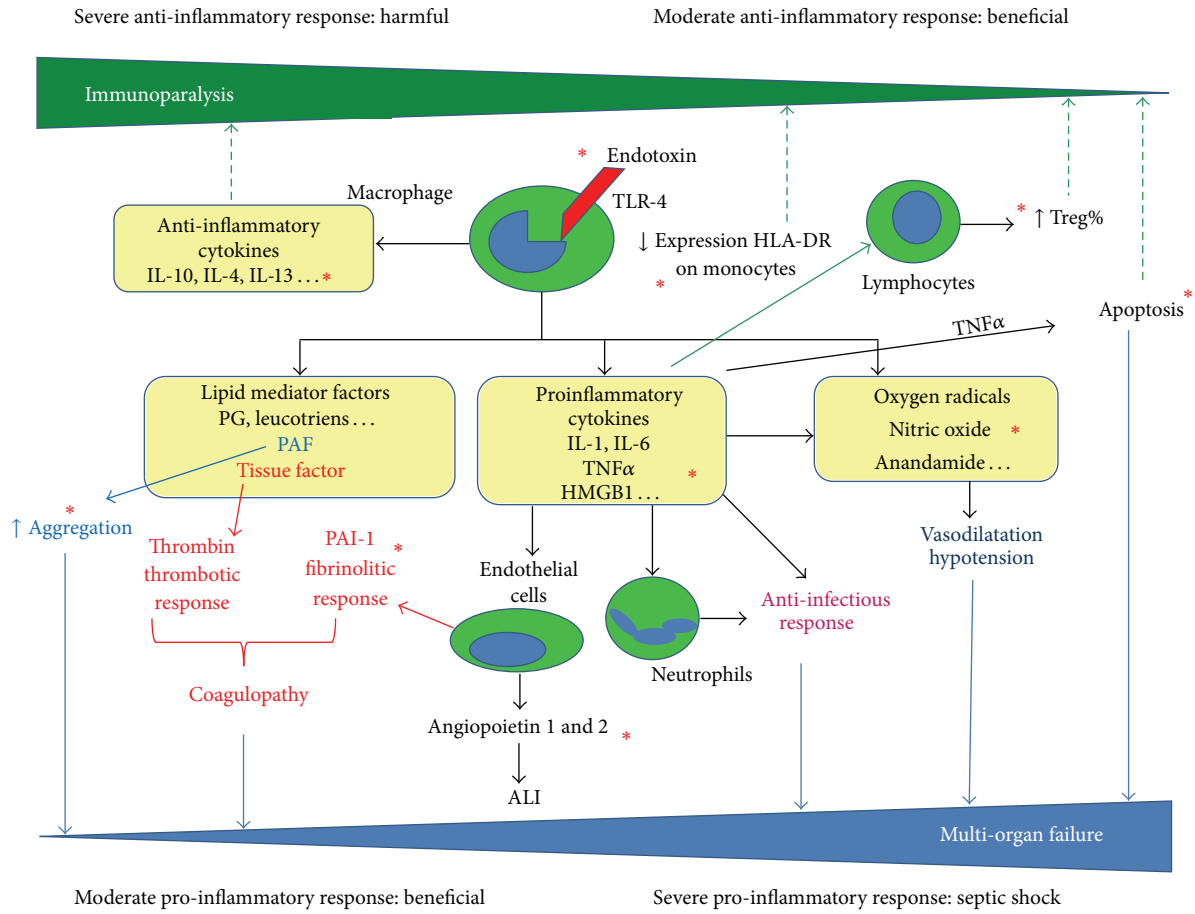

Figure 1: Proinflammatory and anti-inflammatory responses to endotoxin. Red asterisk: immunological mechanisms described for Polymyxin-B hemoperfusion in this review; TLR: toll-like receptor; IL: interleukin; T_{reg}: T regulatory lymphocytes; TNFα: tumor necrosis factor alpha; HMGB1: high mobility group box protein 1; PAF: platelet activator factors; PAI: plasminogen activator inhibitor; ALI: acute lung injury.

studied the role of decreases in HMGB1 after PMX hemoperfusion in patients with acute exacerbation of idiopathic fibrosis. When the same authors investigated the effects of PMX hemoperfusion in a retrospective multicenter study of 160 patients with acute exacerbation of idiopathic pulmonary fibrosis or interstitial pneumonia, they found that the PaO_2/FiO_2 ratio significantly increased after PMX hemoperfusion [18]. They concluded that PMX hemoperfusion might be an effective adjunctive therapy for these patients, although the mechanisms underlying the benefits of the treatment are uncertain. Likewise, Hara et al. [20] reported that PMX hemoperfusion resulted in improved PaO_2/FiO_2 ratio 72 hours and 1 week after treatment in 33 patients with acute exacerbation of interstitial pneumonia. Tsushima et al. [100] treated 20 patients with ARDS with PMX hemoperfusion and compared the outcomes with a historical control group. They found improved PaO_2/FiO_2 ratio and survival; however, the methodology of the study limits its power to draw conclusions.

Collectively, the matrix metalloproteinases (MMP) are capable of degrading all kinds of extracellular matrix proteins. MMP-9 is a protease involved in the degradation of the basement membrane, a part of the extracellular matrix associated with the alveolar epithelium and vascular endothelium. MMP-9 is essential for the remodeling of basement membranes in various inflammatory lung diseases, including ARDS. Increased amounts of MMP-9 in the vasculature are likely to enhance vascular permeability and to facilitate cell homing and inflammatory remodeling. Nakamura et al. [62] studied the effect of PMX hemoperfusion on MMP levels in ARDS patients by treating 12 ARDS patients with two sessions of PMX hemoperfusion and comparing their laboratory data with those of healthy controls. After treatment, the PaO_2/FiO_2 ratio and hemodynamic parameters clearly improved. Patients with ARDS had significantly higher levels of MMP-9 than controls. After PMX hemoperfusion, MMP-9 decreased and chest X-ray findings improved. However, the precise mechanism is still unclear. The authors suggest the need for more studies to elucidate the beneficial effect of PMX hemoperfusion in ARDS. In a pilot study of 16 patients, Abe et al. [101] studied the effects of PMX hemoperfusion for acute exacerbation of interstitial pneumonia and demonstrated neutrophil adsorption and a decrease in MMP-9.

In recent years, the mediators S100A12 and RAGE have drawn attention as specific markers of ALI [102]. The expression of S100A12 in neutrophils increases in the presence

of endotoxin. Takahashi et al. [103] studied the changes in serum S100A12 and RAGE after PMX hemoperfusion in postoperative septic shock. They found a significant decrease in S100A12 in serum after PMX hemoperfusion and an improvement in PaO_2/FiO_2 ratio but no decrease in RAGE. The authors attributed the decrease in S100A12 to the concomitant decrease in endotoxin.

As mentioned above, Oishi et al. [69] found cytokines and VEGF in the eluate from PMX hemoperfusion cartridges used to treat patients with acute exacerbation of pulmonary fibrosis, suggesting a new explanation for the improvement in oxygenation in nonseptic patients treated with PMX hemoperfusion.

5. Conclusions

In recent years, many studies have shown that PMX hemoperfusion is a promising strategy for immunomodulation in septic shock, and two ongoing clinical trials will be key in determining its usefulness.

Although most studies have focused on the removal of endotoxin as the principal mechanism through which PMX hemoperfusion improves outcome in sepsis, other studies have revealed mechanisms involving diverse immunological pathways through which PMX hemoperfusion could improve outcome not only in sepsis but also in non-septic respiratory failure. However, these studies are limited by their small samples, their observational and in some cases retrospective design, and the lack of control groups in many cases. Well-designed clinical investigations with larger samples are needed to confirm these findings.

It is interesting to note that the elimination of endotoxin brings about a reduction in many inflammatory molecules and cells involved in the inflammatory cascade. Endotoxin removal devices act at the onset of this complex cascade, and their benefits in terms of immunomodulation are encouraging. Only a part of the consequences of endotoxin elimination has been studied and summarized in this review. Future studies might reveal other mediators and cells involved in sepsis that might be altered after endotoxin removal.

Some of the studies reviewed here found mediators and cells in the eluate from PMX hemoperfusion cartridges or by direct examination of the filter. Further studies are necessary to elucidate how PMX hemoperfusion eliminates these molecules, whether through ionic/hydrophobic interactions like in endotoxin removal or indirectly via heparin that coats the fibers.

Additional mechanisms could potentially explain why PMX hemoperfusion can be beneficial in gram-positive sepsis or non-septic respiratory failure. Endotoxin can be elevated in other medical conditions apart from gram-negative infections, and its removal could also partially explain this benefit. The immunomodulating effects of PMX hemoperfusion in patients with interstitial pneumonia and acute exacerbations of pulmonary fibrosis are especially interesting, given the high mortality associated with these conditions. However, well-designed clinical trials are needed to assess the efficacy of PMX hemoperfusion in these medical conditions.

Future potential directions such as combination of different hemoperfusion devices to treat septic patients, in order to alter the host inflammatory response in more than one step, are currently speculative. Technically, it could be viable, but no experience has been reported to date.

In summary, antimicrobial therapy, surgical treatment of the focus of infection, and hemodynamic stabilization are crucial in the treatment of severe sepsis. PMX hemoperfusion is an effective adjunctive treatment in septic shock. It seems that PMX hemoperfusion might have other beneficial immunological mechanisms in addition to endotoxin removal; however, the limited evidence suggests that we must be cautious with other indications for PMX hemoperfusion, and future studies are necessary.

Disclosure

Esteban and Ferrer have received honorariums as scientific advisor to Ferrer Farma. Artigas has been invited and received honorariums to present conferences in symposiums organized by Ferrer Farma, Toray Co. and Thermo Fisher. Artigas has received honorariums as scientific advisor to Ferrer Farma, Almirall and Gambro Co.

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Review Article

The Role of Mannose-Binding Lectin in Severe Sepsis and Septic Shock

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Severe sepsis and septic shock are a primary cause of death in patients in intensive care unit (ICU). Investigations upon genetic susceptibility profile to systemic complications during severe infections are a field of increasing scientific interest. Particularly when adaptive immune system is compromised or immature, innate immunity plays a key role in the immediate defense against invasive pathogens. Mannose-binding lectin (MBL) is a serum protein that recognizes a wide range of pathogenic microorganisms and activates complement cascade via the antibody-independent pathway. More than 30% of humans harbor mutations in MBL gene (MBL2) resulting in reduced plasmatic levels and activity. Increased risk of infection acquisition has been largely documented in MBL-deficient patients, but the real impact of this form of innate immunosuppression upon clinical outcome is not clear. In critically ill patients higher incidence and worse prognosis of severe sepsis/septic shock appear to be associated with low-producers haplotypes. However an excess of MBL activation might be also harmful due to the possibility of an unbalanced proinflammatory response and an additional host injury. Strategies of replacement therapies in critically ill patients with severe infections are under investigation but still far to be applied in clinical practice.

1. Introduction

Despite the diffusion of effective care bundles and the implementation of new technologies able to support organ function, severe sepsis and septic shock still represent a leading cause of intensive care unit (ICU) admission with a case fatality rate of 30–40% [1, 2]. Systemic inflammation, surrounding multiorgan failure and septic shock, results from a maladaptive unbalance between early antimicrobial immune reactions and uncontrolled local infection and inflammation. Innate immune system is the primitive first-line organism's response to invasive pathogens, and it interacts with other homeostatic patterns, including inflammation and coagulation. Early activation of immune response is mediated by soluble pattern recognition molecules that, in addition to complement proteins, cytokines, and coagulation factors, activate humoral and cellular effectors, identifying and neutralizing the invasive pathogen [3]. Mannose-binding lectin (MBL) is a soluble pattern recognition molecule which activates the lectin pathway of the complement system and the subsequent inflammatory mechanisms [4, 5]. Low MBL plasmatic levels, mainly due to genetic influences, have been largely described to be associated with susceptibility to invasive infections and poor outcome [6]. On the other hand, the excessive activation of this ancient protective system may be responsible for a detrimental unbalanced inflammatory and coagulation response, as observed in inflammatory diseases, transplant rejections, and diabetic nephropathy [7]. Many authors have investigated whether MBL may influence the susceptibility to common pathogens and the development of severe infections, but, still, there is no consensus about the clinical relevance of its deficiency or the indications for replacement therapies.

The purpose of this review is to summarize the results of relevant recent studies where the role of MBL in severe sepsis and septic shock has been investigated.

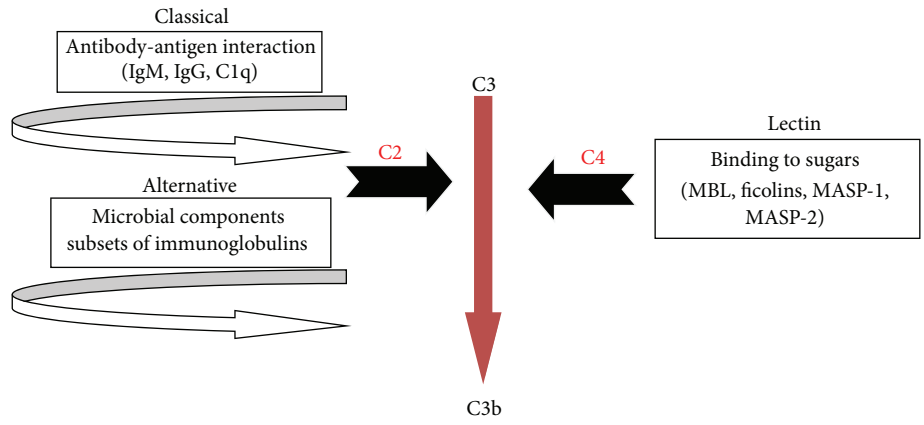

Figure 1: Complement activation pathways.

2. The MBL Protein and Its Deficiency

Mannose-binding lectin is a serum calcium-dependent protein, synthesized by the liver and is detectable in the sites of inflammation, particularly in epithelial-lining fluid [5]. Small amounts of this protein are also produced in other organs (kidney, thymus, tonsil, small intestine, and vagina). MBL is a collectin (collagen-like lectin) and is characterized by a high-ordered oligomeric structure that is essential for its function and interaction with MBL-associated serine proteases (MASPs) [8, 9]. MBL harbors a carbohydrate recognition domain (CRD) through which it binds to specific carbohydrates (i.e., mannose or N-acetylglucosamine) exposed on pathogenic agents surface, and it is therefore called a "pattern-recognition molecule" [4]. Subsequently MASPs (mainly MASP-2) are able to trigger the lectin complement pathway cleaving C4 and C2 to form C3 convertase. Complement system may be activated by three pathways: the classic and the alternative ones are antibody dependent and belong to the adaptive immune response; the lectin one is antibody independent and, as part of the innate immune system, comes into play within the first 12 hours from microorganisms' contact [10] (Figure 1). MBL plays a central role as a first-line defense against invading pathogens by triggering complement system, directly mediating opsonophagocytosis, and possibly functioning as a toll-like receptor coreceptor [11]. In humans there are two genes that might code for MBL, but only MBL2 gene is functional, located on the long arm of chromosome 10 [12]. MBL deficiency may be due to the presence of single nucleotide polymorphisms (SNP) either in the gene-coding or in the promoter regions. The wild-type gene is called "A" (homozygous haplotype, A/A); instead the variants alleles are widely classified as "0" (0/A and 0/0). Three points mutations involve the exon 1, identifying allele B (Codon 54), C (Codon 57), and D (Codon 52). Additionally, many SNPs may affect the promoter region and determine low MBL protein serum levels and activity (variants H/L, Y/X, and P/Q). Even though all these mutations could be combined with exon 1 alleles, seven haplotypes are typically found in humans genome [13, 14].

These polymorphisms determine the production of unstable proteins, with shorter half-life, mainly due to the absence of the high-ordered oligomeric structure. These variants are not able to efficiently activate the complement pathway [15].

Healthy individuals (genotype A/A) generally present MBL levels above 1000 ng/mL. In the newborn this protein is detectable at concentrations of two-thirds of their mothers. Normal levels are reached within a month [16]. MBL levels are not influenced by age, circadian cycle, and physical exercise and, during inflammation, do not increase over 3-4 folds than baseline level [17]. MBL deficiency is generally defined by plasmatic protein levels below 500 ng/mL or by an MBL function lower than 0.2 U/μL C4 deposition [18]. The detection of pulmonary MBL concentration is quite difficult. Some authors have reported bronchoalveolar lavage (BAL) levels ranging between 20 and 80 ng/mL, but these results were not corrected for dilution factors (i.e., urea or other lung proteins with known lung concentration) that could explain the large distance from the minimum concentration needed to activate complement proteins (300-400 ng/mL) [19]. Plasmatic levels ranging between 500 and 1000 ng/mL are generally detected in heterozygous patients (A/O genotype); instead homozygous variant MBL2 alleles usually present very low concentrations (<50 ng/mL). Similarly the haplotypes that include mutations in promoter regions are associated with significant reduction of MBL protein production and activity. However, even though the degree of MBL deficiency is strictly dependent upon patients' genotypes, in some cases low MBL plasmatic levels have been also associated with wild-type genes [13]. This gene was already present in early invertebrates more than fifty million years ago and has been highly conserved throughout animal and human evolution. This would suggest that the correct function of MBL protein is crucial for the survival of living animal species. It is of interest to note that there is geographic distribution of different alleles: the B variant is predominant in Eurasian populations, the C variant mainly among Asians and America Indians, and the D haplotype seems to be frequently expressed in Caucasian region. This particular distribution might be linked to the initial human migrations

Table 1: MBL deficiency and susceptibility to diseases (human and animal studies).

Bacterial infections	Viral infections	Parasitic infections	Fungal infections	Miscellaneous
(i) *Streptococcus pneumoniae*	(i) CMV	(i) *Plasmodium falciparum*	(i) *Candida spp*	(i) Recurrent respiratory infections
(ii) *Neisseria meningitidis*	(ii) Coronavirus	(ii) *Cryptosporidium parvum*	(ii) *Aspergillus spp.*	(ii) Chemotherapy-induced febrile neutropenia
(iii) *Staphylococcus aureus*	(iii) HIV			(iii) Systemic lupus erythematosus
(iv) *Pseudomonas aeruginosa*	(iv) HCV			(iv) Chronic renal failure
(v) *Escherichia coli*	(v) HBV			(v) Cystic fibrosis
(vi) *Legionella spp.*	(vi) HSV			

out of Africa and induced by some specific advantages due to MBL deficiency. For example, high MBL production has been observed to be associated with higher incidence of preterm births; instead moderately low levels could protect the organism from mycobacteria systemic infection and from the complement induced inflammatory-mediated damage of some diseases (i.e., meningococcemia and rheumatoid arthritis) [20, 21]. Additionally sporadic reports have not found a clear association between MBL deficiency and increased rate of infectious episodes [22–24]. The wide range of clinical effects linked to MBL haplotypes has also been attributed to the role of associated mutations in other genes encoding proteins with similar functions (i.e., L-ficolin, MASP2, and surfactant proteins) [25, 26]. However, to date, there are few data upon the clinical role of these combined deficiencies.

3. MBL and Severe Infections

The prevalence of mutations in one or both MBL2 gene alleles is relevant, ranging between 30% and 40% in analyzed populations [13, 27]. During the last twenty years an increasing body of evidence has indicated that MBL deficit, due to specific haplotypes, generally increases frequency and severity of infectious episodes [28, 29] (Table 1). However the structure of our immune system is redundant, and this may explain why in many cases polymorphisms of MBL2 gene were not observed to influence susceptibility to infections [24]. The role of this lectin is particularly relevant when adaptive immune system is immature or compromised [30]. In a case-control study upon 47 infants, lower MBL cord blood concentrations were associated with a higher incidence of Gram-negative sepsis ($P = 0.036$) [31], and an observational cohort study upon 100 pediatric ICU patients identified MBL2 gene exon 1 polymorphisms as a main determinant of progression from sepsis to septic shock [32]. Additionally, the incidence and outcome of severe infections appear to be influenced by the levels and activity of mannose-binding lectin. In a cohort of leukemic patients undergoing chemotherapy, severe infections (bacteremia, pneumonia or both) occurred more frequently in those individuals with lower MBL concentrations ($P < 0.001$) [33]. In an ethnically homogeneous English population, homozygotes for MBL codon variant alleles showed a significantly higher risk of invasive infections due to *Streptococcus pneumoniae*, "the captain of men of death" [34]. Similarly allelic variants of this gene seem to be associated with increased susceptibility to meningococcal disease [35]. Among respiratory tract infections, independently from the causal pathogen, MBL insufficiency has been observed to predispose to higher severity and poor outcome [36]. Even though *Legionella spp.* act as an intracellular pathogen, MBL function was lower in infected cases during an Australian *Legionnaires' disease* outbreak [37]. Increased susceptibility and worse outcome in 212 Caucasian patients with acute respiratory distress syndrome (ARDS) were also observed in presence of MBL2 gene polymorphisms [38]. Regarding viruses, in Chinese population, the presence of MBL2 gene B variant was associated with increased risk of *Coronavirus* infection [39, 40]; instead normal MBL function seems to worsen pandemic H1N1 and avian H9N2 infections by potentially upregulating inflammatory response [41].

Furthermore, in a recent large retrospective study involving 102 donor-recipient orthotopic liver transplantation pairs, patients who received MBL-deficient livers showed a three-fold increased risk of clinically significant infections including *Cytomegalovirus*-related diseases [42].

Few authors have studied the role of MBL in severe fungal infections. Polymorphisms of this gene were observed in seven of ten white patients with chronic necrotizing aspergillosis compared with 25% of controls [43]. In addition variations of MBL plasmatic levels seem to correlate with the occurrence of invasive candidiasis [44].

MBL genetic, plasmatic, and functional profiles were investigated in numerous clinical settings obtaining different results. The critically ill patient, affected by severe infections with severe sepsis and septic shock, might be a field of particular interest for a better knowledge of their clinical relevance and the possible development of novel therapeutic strategies.

4. MBL and Severe Sepsis/Septic Shock

Mannose-binding lectin is not only part of the innate recognition system of invasive pathogens but effectively modulates the cytokines' production by macrophages during phagocytosis. This effect, upon interleukin (IL)-6, IL-1β, and tumor necrosis factor-α, was clearly shown in an "*ex vivo*" model of immediate immunity response to *Neisseria Meningitidis* infection [45]. MBL deficiency may be associated with unbalanced proinflammatory responses to infective and

noninfective triggers. In a cohort of critically ill pediatric patients, *Fidler and coworkers* observed that MBL levels less than 1000 ng/mL, consistent with MBL-2 gene exon 1 polymorphisms, significantly increased the risk of developing systemic inflammatory response syndrome (SIRS) and progression to severe sepsis/septic shock [32]. Additionally in patients with SIRS, MBL insufficiency degree was observed to correlate with severity of systemic infection, according to the genetic profile [46]. The association between the deficiency of this protein and worse outcome during severe systemic infections (i.e., evolution to refractory septic shock) may be also related to the significant interaction between complement activation, inflammatory cytokines' "storm", and coagulation cascade. The influence of complement activation upon septic shock development was largely investigated. Many studies have shown how the classical and alternative pathways are activated during septic shock and are involved in mechanisms aimed to clear endotoxin. This role has been more recently studied also for lectin complement activation due to MBL [47, 48]. Disseminated intravascular coagulation (DIC) may worsen the course of septic shock but the occurrence of this severe complication is unpredictable. However recent data suggest that MBL deficit may be a significant risk factor for the early development of DIC and organ failure during severe infections [9]. Conversely, excessive MBL expression might be harmful, since this molecule may contribute to the pathogenesis of inflammatory induced vascular damage and organ failure, as observed in patients undergoing solid organ transplantation [49]. Hence MBL, due to its pivotal role in the crosstalking among complement activation, coagulation, and systemic inflammation, may represent a key point for the understanding of the development of systemic severe infections, as interestingly investigated in animal models and clinical studies involving patients with severe sepsis/septic shock.

4.1. Animal Models. Even though many differences between animal models and humans limit the "translationalability" of preclinical data, several mouse experiments support the role of MBL deficiency in severe infections, especially after bacteria inoculation. Two functional MBL genes exist in the mouse, and the generation of double knockout gene-deficient mice has increased the investigations in this field.

After inoculation of 5×10^7 CFU *Staphylococcus Aureus*, MBL-null mice showed at 48 hours 100% mortality compared with wild-type (WT) mice which survived in a percentage of 55%. Additionally, pretreatment of MBL-null mice with rhMBL increased their survival rate of about 50% [50]. In another model of MBL and/or MASP 1/3 deficient mice *Takahashi and coworkers* observed that this deficiency was associated with early occurrence of DIC and liver injury after *S. aureus* inoculation, suggesting the role of this protein in the development of organ failure and systemic coagulation activation during severe infections [9].

Another study demonstrated that MBL is able to strongly bind to O-antigen region of LPS, contributing to mice platelets activation and rapid occurrence of septic shock [48].

Susceptibility of MBL null mice to *Pseudomonas aeruginosa* postburn infection was also investigated [51]. All MBL-null mice, after burn and bacterial inoculation, early developed septic shock and died; instead the majority of WT animals (two-thirds) survived. These observations underline the relevance of innate immunity and mannose-binding lectin in the susceptibility and outcome of severe bacterial infections occurring in this population.

Regarding fungal diseases, the protective role of this lectin was also observed in murine models of invasive pulmonary aspergillosis after *ex vivo* MBL administration [52].

However lectin pathway activation does not only depend on MBL function. Some authors have observed how deficient mice models, without the capability to activate MBL-independent lectin cascade (i.e., ficolins and other collectins), are more susceptible to develop severe systemic pneumococcal infections [53].

Although most of literature evidence obtained by animal studies supports the importance of MBL in the acquisition and outcome of severe infections, these observations, due to unresolved several limits of animal studies, may not be considered conclusive and strongly need clinical human studies to definitely identify its clinical relevance.

4.2. Human Pediatric and Adult Studies. The MBL key role as part of innate immunity is the reason why haplotypes associated with its deficiency mainly influence infectious episodes involving neonates and children or immunosuppressed adults. In a population-based prospective study performed in Greenland upon almost 300 Eskimo children, both heterozygous and homozygous subjects, aged 6 to 17 months, for variant alleles presented a twofold increased risk of acute respiratory infections, including pneumonia [30]. Additionally, *Capoluongo and colleagues*, analyzing 75 preterm newborns, identified two MBL2 gene variants as independent risk factors associated with unfavorable outcome, including higher bronchopulmonary dysplasia prevalence [54]. Turkish authors have investigated the possible relationship between cord blood MBL levels and neonatal sepsis. The results indicated that lower MBL levels during fetal inflammatory response syndrome (FIRS) were associated with higher risk of sepsis development independently from gestational age and birth weight [55]. Another prospective study conducted on 62 neonates (27 of them were preterm) showed how lowest MBL levels were detected in infants with septic shock, especially in case of fatal outcome ($P < 0.05$). Relevant sensitivity, specificity, positive, and negative predictive values for detecting sepsis episodes were also documented [56].

In a recent Swiss investigation, MBL levels were detected in cord blood of 141 newborns. Forty-seven developed sepsis (28% within the first 72 hours of life) and 13% required catecholamines because of septic shock. After excluding those infants who underwent surgery, low MBL concentrations resulted independently being associated with increased risk of early-onset Gram-negative sepsis [31].

In pediatric oncological patients, MBL deficiency was associated with susceptibility, poor outcome, and duration of febrile neutropenic episodes [57]. In a prospective study MBL deficit was observed to increase the severity of disease during pediatric ICU admission after febrile neutropenia [58]. Additionally also MBL-related proteins deficit was

investigated in this setting. In a cohort of 94 children treated with chemotherapy for cancer, MASP-2 deficit (<200 ng/mL) significantly increased the risk of febrile neutropenia and bacteraemia development and prolonged cumulative duration of hospitalization and antimicrobial treatment [59].

The importance of MBL function during the first months of life, when the efficacy of innate immunity is crucial, has induced some authors to propose its dosing as part of a biomarkers panel for the early detection of severe neonatal infections in low-resource settings [60].

Impaired innate immune mechanisms may also increase the risk of nosocomial infections in critically ill patients as observed by *Sutherland and colleagues*. In a genetic association study, the authors identified the relationship between SNP in CD 14, MBL and Toll-like receptor-2 with increased prevalence of positive cultures and sepsis [61]. In a cohort of 195 adult septic patients, MBL deficiency resulted also independently being associated with higher sequential organ failure assessment (SOFA) score at day 3, suggesting its role as a risk factor for the development of severe sepsis and septic shock [62]. Additionally in a multicenter prospective study involving eight adults ICUs in U. K., the association between MBL-2 exon and promoter polymorphisms with the outcome of 174 patients affected by severe sepsis and septic shock was studied [63]. Compared with healthy subjects, MBL deficient patients were at increased risk of sepsis, with a significant higher mortality rate in presence of levels below 1000 ng/mL (47.2% versus 22.2%, $P = 0.05$).

During severe sepsis and septic shock, the increase of MBL plasmatic levels, as acute phase response molecule, may be different. In a report of 128 adult critically ill patients, *Dean and colleagues* observed that regardless of MBL-2 genotype those patients who were MBL deficient at study entry were not able to reach normal plasmatic levels during severe sepsis and septic shock [17]. Furthermore, a well-conducted prospective study, performed in Denmark, investigated the MBL genetic and plasmatic profile in a population of 272 critically ill ICU patients with documented SIRS [46]. Among enrolled patients 172 met the criteria for severe sepsis and 70 for septic shock. Compared with noninfectious SIRS, these patients shared the carriage of MBL variants alleles and low serum levels according to the severity of disease ($P = 0.03$).

Another recent Korean study in ICU patients investigated whether MBL2 gene polymorphisms and serum levels might influence severity and prognosis of sepsis [64].

The authors compared 26 septic patients with 398 healthy controls, analyzing three SNP and dosing MBL serum levels on day one. Among sepsis group, homozygosis for the polymorphism at codon 54 (A/A) resulted in a significant risk factor for severe sepsis development ($P = 0.001$). MBL serum levels ≥ 1.3 mcg/mL were associated with a lower 28-day mortality rate in the septic shock group ($P = 0.02$).

The role of MBL deficiency in critically ill patients with severe pneumonia, a still leading cause of death due to an infectious disease, has been investigated by many authors. In a large case-control study, 848 patients affected by community-acquired pneumonia (CAP) were compared with 1447 healthy control subjects and 519 patients without relevant infectious diseases. MBL2 and MASP2 haplotypes were equally distributed among those subjects. In the multivariate analysis, MBL deficiency was associated with poor outcome measures (i.e., severe sepsis, acute respiratory failure, multiorgan dysfunction syndrome, and death) [36].

Eisen and colleagues have reanalyzed data from six studies involving 675 patients affected by severe infections [18]. First, the authors defined a MBL cutoff value of 0.5 mcg/mL as a reliable predictor of low producing status (negative predictive value 98%). They confirmed that MBL deficiency significantly increased the risk of death due to severe infection, also in ICU setting, especially when *Streptococcus pneumoniae* was the invasive causative agent (odds ratio 5.6, 95% confidence interval, 1.27–24.3). The association between MBL deficiency and *S. pneumoniae* invasive infection outcome has been recently investigated in a Spanish prospective cohort study [65]. During the study period 117 patients with invasive pneumococcal infection were enrolled: the rate of allelic variants was 32%. SNP MBL2 (AO/OO) and septic shock were the factors independently associated with in-hospital mortality. Otherwise early adequate antibiotic dose ≤ 4 hours resulted in a significant protective determinant.

MBL deficiency role was also studied in some other systemic infections due to specific organisms. *Resman and colleagues* recently described the case of a necrotizing myositis and septic shock due to *Haemophilus Influenzae* in a patient where IgG3 and MBL deficiency were diagnosed [66]. In a well-conducted prospective study, the correlation between MBL2 gene polymorphisms and the outcome of *Escherichia coli* pyelonephritis was investigated [67]. Although no association was found with the incidence of *E. coli* infections and the presence of bacteremia, those patients who shared low-expression MBL2 genotypes showed a significant higher risk of septic shock development (odd ratio: 9.1, 95% confidence interval: 1.23–65.9; $P = 0.03$). Finally, in nonbacterial severe systemic infections, invasive candidiasis (IC), especially candidemia, still remains a leading cause of death due to infections in critically ill patients. Serum MBL levels were measured in 68 patients with proven IC, 82 hospitalized not infected patients, and 70 healthy subjects [44]. Even though MBL concentration was significantly higher in IC patients than controls, the authors identified a marked decrease in its plasmatic levels during the first days of infection in association with mannans increase. These observations, although limited, suggest a crucial role of MBL also in the early phase of candidiasis.

4.3. MBL Replacement Therapy. MBL substitution therapy in patients with recognized lectin deficit has been proposed. Apart from genetic analyses, antigenic measurement is widely diffused as diagnostic test. Even though MBL serum levels < 500 ng/mL or MBL activity < 200 U/mL may be considered a significant deficiency, there are not standard guidelines aimed to define which patient categories need to be tested (i.e., in presence of severe recurrent respiratory infections or acquired immunesuppression). Recombinant human MBL use, to supplement MBL deficiency status, has been investigated in animal and phase I/II human studies [68, 69]. Although its clinical efficacy has not been clearly established, still now no adverse effects were observed. Sixty-five MBL

infusions were given to 12 MBL deficient chemotherapy-induced neutropenic children. The observed postadministration level was 1.06 mcg/mL (range: 0.66–2.05) which may be considered protective [70]. A similar pharmacokinetic profile was observed in 20 healthy MBL-deficient volunteers and two patients with *Staphylococcus Aureus* septicemia [71]. However, beyond these preliminary observations, MBL replacement needs to be further investigated in deficient patients affected by acute severe infections, especially in presence of multiple-level immune system impairment.

5. Conclusions

An increasing body of data support the role of MBL as central player of innate immunity. Several gene polymorphisms have been identified in association with decreased serum levels and activity. Many authors have showed the association of this molecule deficit with recurrent severe infections, particularly involving the respiratory tract and encapsulated bacteria. Additionally growing evidence suggests its importance during systemic severe infections as severe sepsis and septic shock. This correlation might derive from the cross-talking among complement system, coagulation patterns, and proinflammatory cytokines. Even though many patients with systemic infections, who present MBL serum levels below the functional threshold, are at higher risk to develop severe complications and poor outcomes (i.e., septic shock, multiple organ failure), in some cases low levels have appeared to be protective, probably reducing the inflammatory cytokines' storm. Moreover not all published studies have identified a clear association between deficiency and increased risk of infections. Replacement therapy with recombinant human protein during severe sepsis and septic shock affecting deficient patients has been proposed but it still remains an experimental treatment. Hence, until new promising and robust data will be available, the strict adherence to current standard recommendations still remains the mainstay of severe sepsis/septic shock management.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Eosinophil as a Protective Cell in *S. aureus* Ventilator-Associated Pneumonia

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Cell counts of leukocytes subpopulations are demonstrating to have an important value in predicting outcome in severe infections. We evaluated here the render of leukogram counts to predict outcome in patients with ventilator-associated pneumonia (VAP) caused by *Staphylococcus aureus*. Data from patients admitted to the ICU of Hospital Clínico Universitario de Valladolid from 2006 to 2011 with diagnosis of VAP caused by *S. aureus* were retrospectively collected for the study ($n = 44$). Leukocyte counts were collected at ICU admission and also at VAP diagnosis. Our results showed that nonsurvivors had significant lower eosinophil counts at VAP diagnosis. Multivariate Cox regression analysis performed by the Wald test for forward selection showed that eosinophil increments from ICU admission to VAP diagnosis and total eosinophil counts at VAP diagnosis were protective factors against mortality in the first 28 days following diagnosis: (HR [CI 95%], *P*): (0.996 [0.993–0.999], 0.010); (0.370 [0.180–0.750], 0.006). Patients with eosinophil counts <30 cells/mm^3 at diagnosis died earlier. Eosinophil counts identified survivors: (AUROC [CI 95%], *P*): (0.701 [0.519–0.882], 0.042). Eosinophil behaves as a protective cell in patients with VAP caused by *S. aureus*.

1. Introduction

Staphylococcus aureus has persisted as an important public health problem, mostly due to the emergence of strains that were resistant to methicillin and oxacillin (MRSA) in the 1960s [1]. Ventilator-associated pneumonia (VAP) is the most frequent infection among patients hospitalized in intensive care units (ICU), with *S. aureus* infection being a leading cause of VAP [2]. VAP maintains high morbidity and mortality. Because of that, a number of inflammatory biomarkers are under evaluation to guide duration of antibiotic therapy and to predict disease outcome in VAP, with heterogeneous results [3–5]. Cell counts of leukocytes subpopulations are demonstrating to have an important value in predicting outcome in severe infections [6–8]. Counting leukocytes is a routine and inexpensive test in ICU settings. We designed a retrospective study aimed to evaluate the influence of leukocyte subpopulations counts on the probability of death in patients with VAP caused by *S. aureus*.

2. Methods

Study Design and Subjects. Data from patients admitted to the ICU of Hospital Clínico Universitario de Valladolid from 2006 to 2011 with diagnosis of VAP caused by *S. aureus* were retrospectively collected for the study ($n = 44$). Patients who were intubated and ventilated in the moment of ICU

hospitalization and developed VAP were eligible. Patients who had been treated with corticosteroids or immunosuppressive drugs and immunocompromised patients were not eligible and were excluded from the present study. VAP was defined as the pneumonia arising more than 72 h after endotracheal intubation characterized by the presence of new or progressive radiographic infiltrate associated with two or more of the following criteria: (a) temperature of greater than 38.5°C or less than 36.5°C, (b) leukocyte count of greater than 12,000/μL or less than 4,500/μL, (c) purulent endotracheal aspirate, and (d) positive ($\geq 10^6$ cfu/mL) endotracheal aspirate. Microbiological diagnosis was performed by quantitative cultures of lower respiratory tract samples (endotracheal aspirate (BAS) or bronchoscopic alveolar lavage (BAL)), following the standard protocols for diagnosis of respiratory bacteria employed in our center. Patients with positive microbiological identification before 72 first hours following mechanical ventilation instauration were excluded because they did not satisfy VAP definition. A standard survey was employed to collect clinical data and leukogram counts from the patients. Patient identification remained anonymous and informed consent was waived due to the observational nature of the study. Approval of the study protocol in both the scientific and the ethical aspects was obtained from the Scientific Committee for Clinical Research of our hospital.

Statistical Analysis. For the demographic and clinical characteristics of the patients, differences between groups were assessed using the Chi-squared test for categorical variables and the Mann-Whitney *U* test for continuous variables when appropriate. We determined the hazard ratio (HR) and 95% confidence interval by Cox regression analysis, which was used to assess the impact of eosinophil increments and counts on mortality over time. Multivariate Cox regression analysis was performed by using the Wald test for forward selection. Statistical analysis was performed by using IBM-SPSS Statistics 20.0.

3. Results

The vast majority of our patients were elderly males, being hypertension, cardiovascular disease, and smoker habit the most frequent comorbidities. Both survivors and nonsurvivors spent four days under mechanical ventilation and presented an APACHE-II score of 18 on average. They were comparable in terms of age, sex, and accompanying comorbidities. We defined coinfection as those other bacteria infecting any localization of our patients with clinical significance. Bacterial coinfection was principally of the following foci: respiratory, urine, and blood. The principles and most frequent pathogens isolated were Gram negative rods from Enterobacteriaceae family (43.3%), *Acinetobacter baumannii* (26.7%), and coagulase negative staphylococci (23.3%).

The comparison of cell counts revealed significant lower eosinophil counts at VAP diagnosis in nonsurvivors (see Table 1). When cell increments ([counts at VAP diagnosis]–[counts at ICU admission]) were evaluated in survivors and nonsurvivors, nonsurvivors showed significant lower increments of eosinophil counts ($P = 0.010$) (see Figure 1(a)). Potential confounding variables introduced in the multivariate Cox regression analysis were age, sex, APACHE-II score, VAP caused by *Staphylococcus aureus* methicillin resistance/*Staphylococcus aureus* methicillin susceptible (MRSA/MSSA), bacterial coinfection, and days in mechanical ventilation. For eosinophil increments, the Wald test selected the APACHE-II score and the eosinophil increments as the variables associated with mortality: (HR [CI 95%], *P*): APACHE-II score: (1.073 [1.001–1.151], 0.050); (eosinophil increments): (0.996 [0.993–0.999], 0.010). For total eosinophil counts at VAP diagnosis (log values), the Wald test selected total eosinophil counts at VAP diagnosis (log values) as the only variable associated with prognosis: (HR [CI 95%], *P*): (0.370 [0.180–0.750], 0.006). Therefore, multivariate Cox regression analysis showed that eosinophil increments as well as total eosinophil counts at VAP diagnosis (log values) were protective factors against mortality in the first 28 days following diagnosis of VAP. Kaplan Meier analysis showed that patients with eosinophil counts less than 30 cells/mm^3 died earlier (see Figure 1(b)). Area under the receiver operating characteristic curve (AUROC) analysis confirmed eosinophil counts at VAP diagnosis as a good test to diagnose survival (see Figure 1(c)): (area [CI 95%], *P*): (0.701 [0.519–0.882], 0.042).

4. Discussion

Regression studies and AUROC analysis supported the protective role of eosinophils in VAP caused by *S. aureus*. Eosinophils are granulocytes that develop in the bone marrow from pluripotent progenitors. They are released into the peripheral blood in a phenotypically mature state, and they are capable of being activated and recruited into tissues in response to appropriate stimuli, most notably the cytokine interleukin-5 (IL-5) and the eotaxin chemokines [9]. Eosinophils are recruited to and activated in lung tissue as part of the pathophysiology of asthma, but recent findings confirm antimicrobial activities of eosinophils [9]. Catapult-like release of structures resembling neutrophil extracellular traps (NETs) from eosinophils has been documented [10]. Our results could in fact support the existence of an antibacterial activity of the eosinophil in the severe infection caused by *S. aureus*. In our patients, fail in expanding eosinophil counts was translated into a poorer outcome.

There is increasing evidence on eosinophils as a protective cell in critically ill patients. Abidi et al. described eosinopenia as a marker of sepsis on admission to ICU [11]. Recently, these authors have described eosinopenia as an early marker of increased mortality in critically ill medical patients [12]. Merino et al. have reported lower eosinophil counts in patients who died of sepsis than in those who survived [6]. Prince et al. have demonstrated that *S. aureus* α-hemolysin induces cell death in eosinophils [13], which could represent a microbial mechanism of evasion from host immune response.

Table 1: Clinical characteristics of survivors and nonsurvivors.

	Survivors ($n = 26$)	Nonsurvivors ($n = 18$)	P
Age (years)	60.0 (35.0)	63.5 (26.0)	n.s
Sex (male)	23 (88.5)	7 (38.9)	n.s
APACHE-II score	17.0 (11.0)	22.0 (12.0)	n.s
Days under mechanical ventilation until VAP diagnosis	4.0 (4.0)	4.5 (4.0)	n.s
MRSA/MSSA	8 (30.8)	4 (22.2)	n.s
Bacterial coinfection (Y/N)	20 (76.9)	10 (55.6)	n.s
Diabetes (type I or II) (Y/N)	4 (15.4)	2 (11.1)	n.s
Cardiovascular disease (Y/N)	6 (23.1)	5 (27.8)	n.s
Chronic renal disease (Y/N)	2 (7.7)	1 (5.6)	n.s
Chronic respiratory disease (Y/N)	6 (23.1)	0 (0.0)	n.s
Cerebrovascular disease (Y/N)	6 (23.1)	0 (0.0)	n.s
Smoker (ever) (Y/N)	8 (30.8)	1 (5.6)	n.s
Neurological disease (Y/N)	4 (15.4)	0 (0.0)	n.s
Hypertension (Y/N)	12 (46.2)	8 (44.4)	0.084
Hematologic malignancy (ever) (Y/N)	1 (3.8)	0 (0.0)	n.s
Cirrhosis of the liver (Y/N)	2 (7.7)	0 (0.0)	n.s
Metastatic solid cancer (ever) (Y/N)	0 (0.0)	2 (11.1)	0.018
Gastrointestinal disease (Y/N)	9 (34.6)	1 (5.6)	n.s
Chemotherapy (ever) (Y/N)	1 (3.8)	2 (11.1)	n.s
Alchohol abuse (Y/N)	4 (15.4)	0 (0.0)	n.s
Intravenous drug abuse (Y/N)	2 (7.7)	0 (0.0)	n.s
Obesity (Y/N)	3 (11.5)	1 (5.6)	n.s
Dyslipidemia (Y/N)	4 (15.4)	2 (11.1)	n.s
Lymphocytes at admision to ICU (cells/mm^3)	960.9 (1017.3)	812.3 (1336.9)	n.s
Monocytes at admision to ICU (cells/mm^3)	480.3 (442.9)	465.8 (436.6)	n.s
Neutrophils at admision to ICU (cells/mm^3)	7801.9 (7053.9)	9504.0 (9101.8)	n.s
Basophils at admision to ICU (cells/mm^3)	11.5 (22.0)	10.5 (28.0)	n.s
Eosinophils at admision to ICU (cells/mm^3)	14.9 (119.0)	20.3 (195.0)	n.s
Lymphocytes at diagnosis (cells/mm^3)	983.0 (734.4)	919.6 (973.1)	n.s
Monocytes at diagnosis (cells/mm^3)	501.3 (238.2)	707.1 (473.8)	n.s
Neutrophils at diagnosis (cells/mm^3)	8261.4 (5372.3)	12182.5 (15051.7)	n.s
Basophils at diagnosis (cells/mm^3)	13.3 (32.3)	14.2 (16.5)	n.s
Eosinophils at diagnosis (cells/mm^3)	112.2 (231.0)	51.5 (118.8)	0.043
Ratio N/L at admission to ICU	7.2 (9.2)	11.7 (20.1)	n.s
Ratio N/L at diagnosis	7.3 (7.6)	10.4 (13.5)	0.059

Survival time was censored at day 28. Continuous variables are expressed as median (interquartile rank). Categorical variables are expressed as n (% over column). N.s: not significant.

Terradas et al. observed that both sustained eosinopenia and a high neutrophil to lymphocyte count ratio were independent markers of mortality in patients with bacteremia [8]. Based upon these results, we evaluated the neutrophil to lymphocyte count ratio in survivors and nonsurvivors. No differences were found between groups for this ratio although there was a trend to exhibit a higher ratio in nonsurvivors (see Table 1).

A limitation of our study was that only two samples were collected (at admission and at VAP diagnosis). In further studies, it will be interesting to assess eosinophil counts in other extra time points during the disease course.

5. Conclusion

We document here for the first time a protective effect of eosinophils in patients suffering from VAP caused by *S. aureus*. Eosinophil counting is an inexpensive biomarker easy to implement in clinical practice.

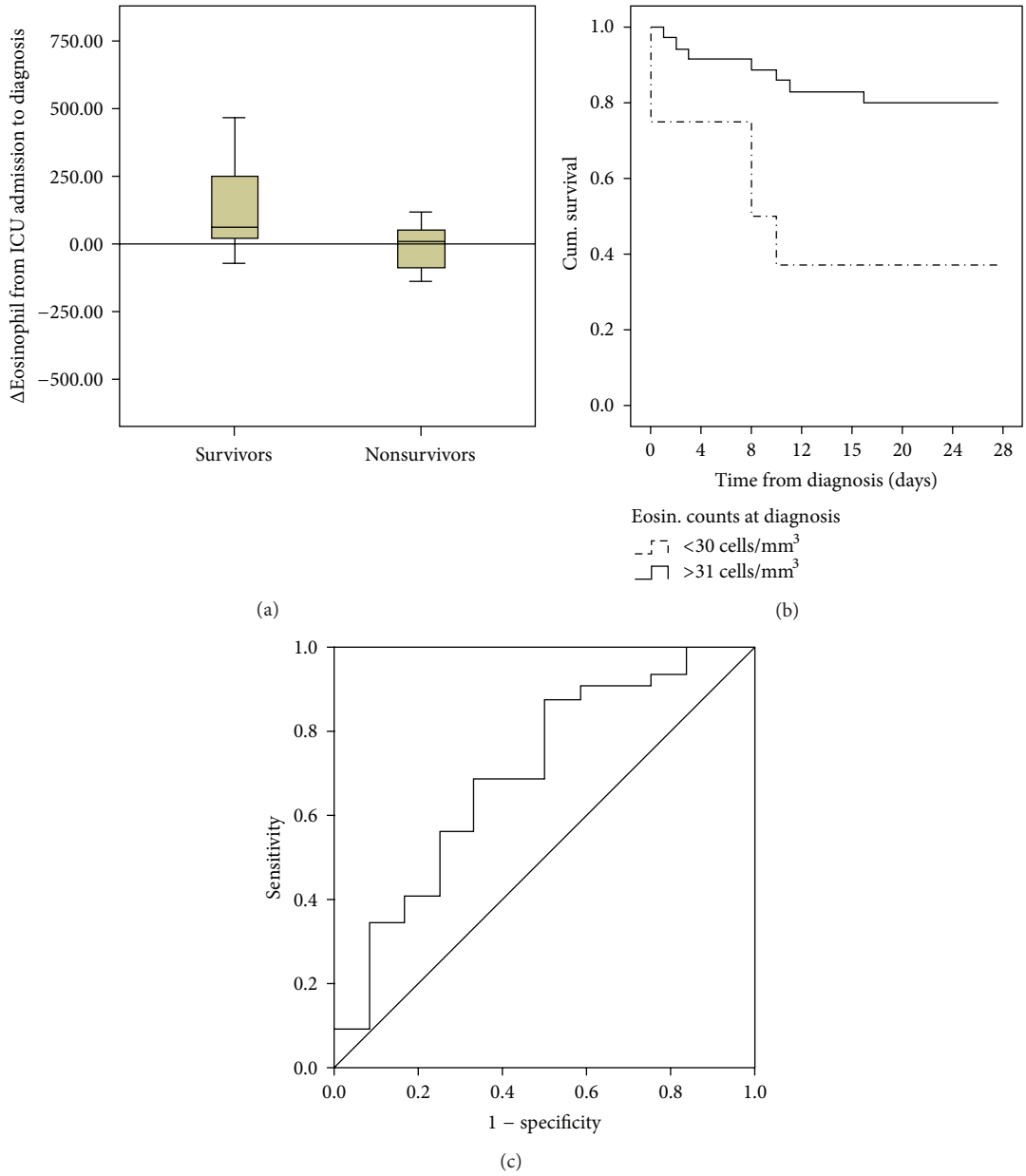

Figure 1: (a) Box plots showing increments of eosinophil counts: [counts at VAP diagnosis]–[counts at ICU admission] ($P = 0.016$). (b) Kaplan Meier curves for survival: deciles from percentile 10 to percentile 90 of eosinophil counts were calculated and used to compare survival times in those patients with low or high counts. The first decile showing significant differences between groups based upon the log-rank test was used as the cutoff (percentile 20). Time was censored at 28 days following VAP diagnosis. Cum. survival: cumulative survival. (c) AUROC analysis: the accuracy and the predictive values of eosinophil counts for detecting survivors in the first 28 days following VAP diagnosis were assessed calculating the AUROC.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Regulation and Prognostic Relevance of Symmetric Dimethylarginine Serum Concentrations in Critical Illness and Sepsis

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In systemic inflammation and sepsis, endothelial activation and microvascular dysfunction are characteristic features that promote multiorgan failure. As symmetric dimethylarginine (SDMA) impacts vascular tension and integrity via modulating nitric oxide (NO) pathways, we investigated circulating SDMA in critical illness and sepsis. 247 critically ill patients (160 with sepsis, 87 without sepsis) were studied prospectively upon admission to the medical intensive care unit (ICU) and on day 7, in comparison to 84 healthy controls. SDMA serum levels were significantly elevated in critically ill patients at admission to ICU compared to controls and remained stably elevated during the first week of ICU treatment. The highest SDMA levels were found in patients with sepsis. SDMA levels closely correlated with disease severity scores, biomarkers of inflammation, and organ failure (renal, hepatic, and circulatory). We identified SDMA serum concentrations at admission as an independent prognostic biomarker in critically ill patients not only for short-term mortality at the ICU but also for unfavourable long-term survival. Thus, the significant increase of circulating SDMA in critically ill patients indicates a potential pathogenic involvement in endothelial dysfunction during sepsis and may be useful for mortality risk stratification at the ICU.

1. Introduction

Alterations in microvascular perfusion are common characteristics of patients with systemic inflammation and sepsis and substantially contribute to the development of organ failure [1, 2]. Microcirculatory defects in critically ill patients such as capillary leakage and disturbed capillary perfusion are not necessarily reflected by macrohemodynamic parameters (e.g., mean arterial blood pressure, cardiac index, and central venous oxygen saturation) that are commonly assessed at the intensive care unit (ICU) [3]. In fact, a recent study revealed that although global hemodynamic variables were relatively preserved in patients with severe sepsis, their microvascular perfusion as assessed by complex invasive flow imaging techniques was severely altered, predicted the progression of organ failure and the overall mortality risk [2]. The underlying mechanisms of microvascular dysfunction in sepsis result from different factors such as endothelial dysfunction, leukocyte-endothelium interactions, coagulation and inflammatory disorders, hemorheologic abnormalities, and functional shunting [4].

The activation of the endothelium, as reflected by increased levels of circulating biomarkers, has been suggested as a main promoter in the pathogenesis of disturbed microcirculation [5]. Based on the potent vasodilative effects of nitric oxide (NO), the arginine-NO pathway might be substantially involved in inflammation, infection, and organ injury [6]. The natural inhibitor of NO synthase, asymmetric dimethylarginine (ADMA), has been found elevated in patients with sepsis and related to mortality risk [7–11]. ADMA is assumed to exert detrimental effects on endothelial function, cardiovascular homeostasis, and cardiovascular outcomes. In

contrast, relatively little is known about the other methylated form of L-arginine, symmetric dimethylarginine (SDMA) [12].

SDMA is generated as the isomer form of ADMA by protein hydrolysis [13]. Unlike ADMA, SDMA is not a direct inhibitor of NO synthase [14]. Thus, SDMA has long been regarded as an inert, functionally inactive molecule. However, using highly specific *in vitro* models with primary endothelial cells, SDMA was found to reduce endothelial NO synthesis via competition with arginine at the cellular transporter and increased intracellular reactive oxygen species in a dose-dependent manner, already at very low, "physiological" concentrations [15]. Circulating levels of SDMA in serum have been consecutively investigated in several cohorts of patients with cardiovascular and renal diseases, demonstrating an association of SDMA with glomerular filtration rate and extent of coronary artery disease and atherosclerosis [12].

We hypothesized that SDMA might be involved in endothelial dysfunction during critical illness and sepsis, resulting in organ failure. Therefore, we investigated SDMA serum levels in a large cohort of 247 consecutively enrolled critically ill patients in order to identify associations between SDMA and organ dysfunction, metabolism and disease severity as well as to assess the prognostic value of SDMA for ICU and long-term mortality.

2. Material and Methods

2.1. Study Design and Patient Characteristics. All patients that were admitted to the medical ICU were consecutively enrolled, except for patients who were expected to have a short-term (<72 h) intensive care treatment due to postinterventional observation or acute intoxication [16]. Patient data and blood samples were collected prospectively. Patients who met the criteria proposed by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference Committee for severe sepsis and septic shock were categorized as sepsis patients and the others as nonsepsis patients [17]. After discharge from our hospital, the outcome was assessed during a follow-up period by directly contacting the patients, their relatives, or primary care physician. The study protocol was approved by the local ethics committee (EK 150/06). Written informed consent was obtained from the patient, his or her spouse, or the appointed legal guardian.

As a control population, we analyzed 84 healthy blood donors (57 male, 27 female) with normal values for blood counts, C-reactive protein, and liver enzymes.

2.2. SDMA Measurements. Blood samples were collected upon admission to the ICU (prior to therapeutic interventions) as well as in the morning of day 7 after admission. After centrifugation at 4°C for 10 minutes, serum and plasma aliquots of 1 mL were frozen immediately at −80°C. SDMA serum concentrations were analysed using a commercial enzyme immunoassay (Immundiagnostik, Bensheim, Germany). The scientist performing experimental measurements was fully blinded to any clinical or other laboratory data of the patients or controls. Due to limited technical resources and changes in the original patient cohort due to discharges from the ICU and deaths, follow-up SDMA measurements were only performed in 42 patients.

2.3. Statistical Analysis. Due to the skewed distribution of most of the parameters, data are presented as median and range. Differences between two groups were assessed by Mann-Whitney *U* test, and multiple comparisons between more than two groups have been conducted by Kruskal-Wallis ANOVA and Mann-Whitney *U* test for post hoc analysis. Box plot graphics illustrate comparisons between subgroups, and they display a statistical summary of the median, quartiles, range, and extreme values. The whiskers extend from the minimum to the maximum values excluding outside and far out values which are displayed as separate points. An outside value (indicated by an open circle) was defined as a value that is smaller than the lower quartile minus 1.5-times interquartile range or larger than the upper quartile plus 1.5 times the interquartile range. A far out value was defined as a value that is smaller than the lower quartile minus three times interquartile range or larger than the upper quartile plus three times the interquartile range [18]. All values, including "outliers," have been included for statistical analyses.

Correlations between variables have been analysed using the Spearman correlation tests. Single parameters that correlated significantly with SDMA levels at admission were included in a multivariate linear regression analysis with SDMA as the dependent variable to identify independent predictors of elevated SDMA. The prognostic value of the variables was tested by univariate and multivariate analyses in the Cox regression model. Kaplan-Meier curves were plotted to display the impact on survival [19]. P values below 0.05 were considered statistically significant. Statistical analyses were performed with SPSS (SPSS, Chicago, IL, USA).

3. Results

3.1. SDMA Serum Levels Are Significantly Elevated in Critically Ill Patients, Especially in Conditions of Sepsis. In order to investigate SDMA in critical illness, we measured SDMA serum concentrations in a large cohort of medical ICU patients at admission (= before therapeutic intervention) and on day 7 (Table 1). SDMA serum levels were significantly higher in ICU patients ($n = 247$, median 0.84 μmol/L, and range 0.15–4.0) as compared with healthy controls ($n = 84$, median 0.38 μmol/L, range 0.20–1.06, and $P < 0.001$; Figure 1(a)). No associations between SDMA levels and sex or age were observed in controls (data not shown).

About two thirds ($n = 160$) of the ICU patients consecutively enrolled into our study presented with either sepsis or septic shock upon ICU admission (Table 2). Importantly, patients with sepsis ($n = 160$, median 0.89 μmol/L, and range 0.19–4.0) had significantly higher SDMA serum concentrations at ICU admission compared to patients with non-septic origin of critical illness ($n = 87$, median 0.67 μmol/L, range 0.15–3.86, Figure 1(b)). The site of infection (Table 2) was

Table 1: Baseline patient characteristics and SDMA serum measurements.

Parameter	All patients	Sepsis	Nonsepsis	*P* value
Number	247	160	87	—
Sex (male/female)	145/102	94/66	51/36	—
Age median (range) [years]	63 (18–90)	64 (20–90)	60 (18–85)	n.s.
APACHE-II score median (range)	17 (2–43)	19 (3–43)	15 (2–33)	0.002
SOFA score median (range)	9 (0–19)	11 (3–19)	7 (0–16)	<0.001
ICU days median (range)	8 (1–137)	10 (1–137)	6 (1–45)	<0.001
Death during ICU *n* (%)	60 (24.3)	45 (28.1)	15 (17.2)	—
Death during follow-up *n* (%)	115 (47.3)	85 (54.1)	30 (34.9)	—
Mechanical ventilation *n* (%)	171 (71.5)	117 (75.5)	54 (64.3)	—
Ventilation time median (range) [h]	126 (0–2966)	181 (0–2966)	63 (0–986)	0.019
Preexisting diabetes *n* (%)	72 (29.1)	45 (28.1)	27 (31.0)	—
BMI median (range) [m^2/kg]	25.8 (15.9–86.5)	25.8 (17.1–86.5)	25.4 (15.9–53.3)	n.s.
WBC median (range) [$\times 10^3/\mu$L]	12.7 (0.1–149)	13.8 (0.1–149)	11.4 (1.8–29.6)	0.010
CRP median (range) [mg/dL]	103 (5–230)	162 (5–230)	17 (5–230)	<0.001
Procalcitonin median (range) [μg/L]	1.0 (0.05–248)	3.2 (0.1–248)	0.24 (0.05–100)	<0.001
Creatinine median (range) [mg/dL]	1.3 (0.1–21.6)	1.6 (0.1–21.6)	1.0 (0.2–11.5)	n.s.
GFR Cystatin median (range) [mL/min]	33 (3–379)	27 (3–379)	58 (5–379)	0.013
INR median (range)	1.17 (0.9–4.64)	1.18 (0.92–4.64)	1.16 (0.9–4.32)	n.s.
SDMA day 1 median (range) [μmol/L]	0.84 (0.15–4.0)	0.89 (0.19–4.0)	0.67 (0.15–3.86)	0.018
SDMA day 7 median (range) [μmol/L]	0.82 (0.18–3.34)	0.85 (0.18–3.34)	0.81 (0.23–1.50)	n.s.

For quantitative variables, median and range (in parenthesis) are given. Differences between sepsis and nonsepsis patients were tested for significance (*P* values [*U* test] are given in the table). APACHE: Acute Physiology and Chronic Health Evaluation; BMI: body mass index; CRP: C-reactive protein; ICU: intensive care unit; INR: international normalized ratio; SDMA: symmetric dimethylarginine; SOFA: sequential organ failure assessment; WBC: white blood cell count.

not associated with SDMA levels (detailed data not shown). However, SDMA levels were related to disease severity, as patients with APACHE-II score values greater than 10 displayed significantly elevated SDMA serum concentrations (Figure 1(c)).

Elevated SDMA levels had been observed in patients with metabolic and cardiovascular disorders [12]. In our cohort of critically ill patients, SDMA levels did not differ between patients with or without type 2 diabetes or obesity, defined as a body mass index >30 kg/m^2 (detailed data not shown).

In 42 patients, paired blood samples were available for SDMA measurements at ICU admission and at day 7 of ICU treatment. Individual SDMA levels remained stable during the first week of ICU therapy (Table 1, Figure 1(d), not significant by paired Wilcoxon test).

3.2. SDMA Serum Concentrations at Admission to the ICU Are Closely Correlated to Organ Function, Inflammation, Metabolism, and Disease Severity. In order to understand possible mechanisms underlying elevated serum SDMA levels in critically ill patients, we performed extensive correlation analyses with various laboratory parameters. At admission to the ICU, serum SDMA concentrations were closely correlated to biomarkers displaying organ dysfunction. In detail, SDMA was found to correlate significantly with markers reflecting renal failure such as creatinine ($r = 0.687$, $P < 0.001$), cystatin C ($r = 0.714$, $P < 0.001$) or inversely with their glomerular filtration rates (Figure 2(a)). Moreover, SDMA levels correlated with clinically used biomarkers of hepatic dysfunction like reduced protein ($r = -0.172$, $P = 0.013$), pseudocholinesterase ($r = -0.292$, $P < 0.001$), or bilirubin excretion (Figure 2(b)).

In line with the elevated SDMA concentrations observed in patients with sepsis, various biomarkers indicating systemic inflammation were associated with circulating SDMA. In fact, SDMA levels correlated with white blood cell counts (0.190, $P = 0.003$), C-reactive protein ($r = 0.261$, $P < 0.001$), procalcitonin ($r = 0.407$, $P < 0.001$), tumor necrosis factor ($r = 0.324$, $P = 0.004$), and soluble urokinase plasminogen activator receptor (suPAR, $r = 0.494$, $P < 0.001$), a prognostic biomarker in sepsis [20]. SDMA levels correlated with ADMA ($r = 0.384$, $P < 0.001$) as well [11].

When selected parameters that were correlated with SDMA serum levels by univariate analysis (i.e., creatinine, pseudocholinesterase, bilirubin, suPAR, ADMA, C-reactive protein, and procalcitonin) were included in a multivariate regression analysis, only creatinine ($P < 0.001$) and procalcitonin ($P = 0.023$), but not liver function markers, ADMA, or suPAR, remained independent predictors of SDMA concentrations (Table 3).

3.3. SDMA Serum Levels Are an Independent Prognostic Biomarker for ICU and Overall Long-Term Mortality in Critically Ill Patients. Based on the close correlation between SDMA levels at admission and disease severity scores, we hypothesized that circulating SDMA might be capable of identifying patients at high risk of mortality. Indeed, patients that died during the course of ICU treatment (about one

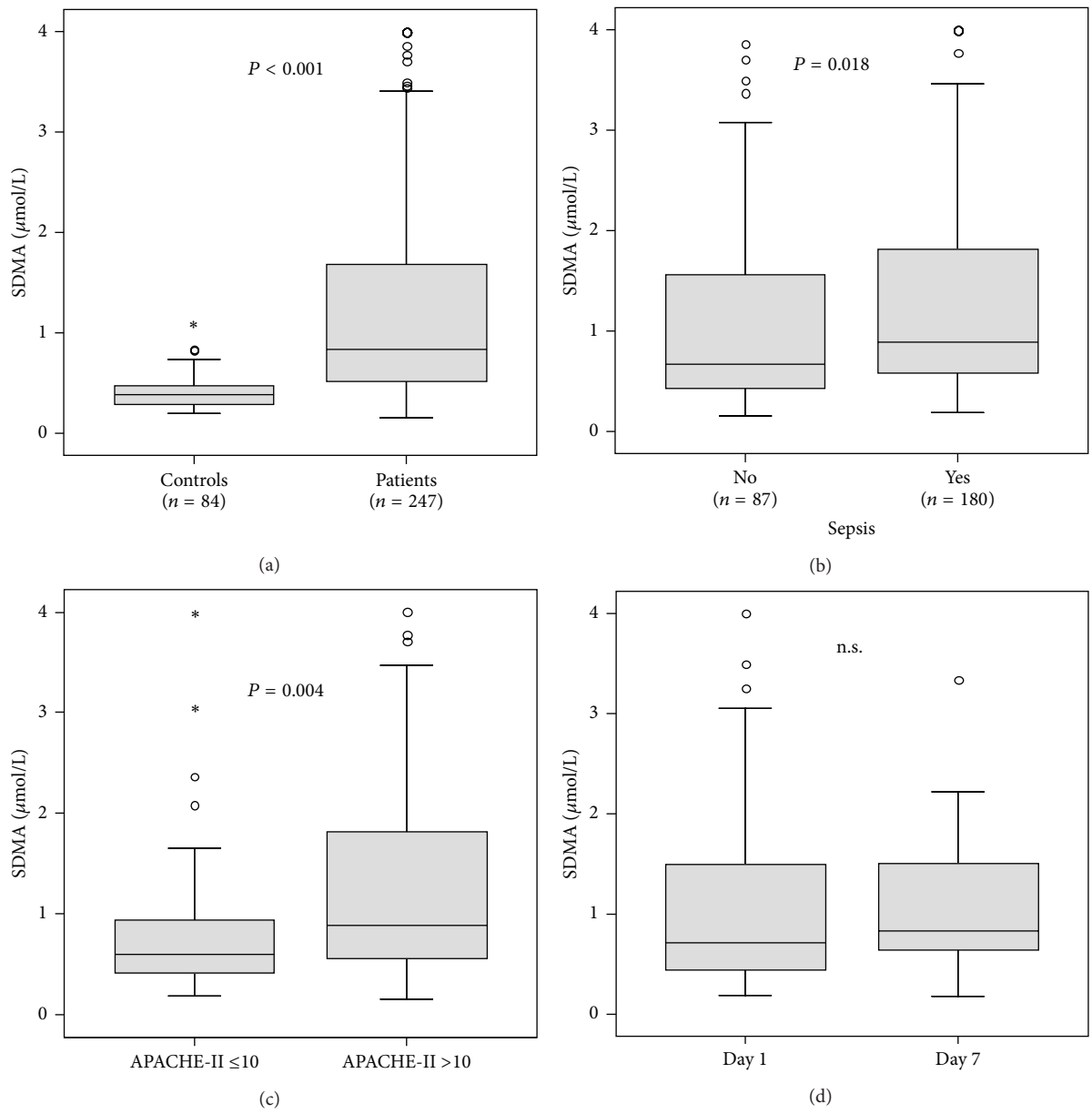

Figure 1: Serum SDMA concentrations in critically ill patients. (a) At admission to the medical ICU, serum SDMA levels were significantly ($P < 0.001$, U test) elevated in critically ill patients ($n = 247$) as compared to healthy controls ($n = 84$). (b) SDMA serum levels at ICU admission were significantly increased in ICU patients with sepsis ($n = 180$) compared to patients without sepsis ($n = 87$). (c) SDMA serum levels at ICU admission were significantly increased in ICU patients with higher degree of disease severity, as displayed by the APACHE-II score. (d) In 42 patients, SDMA levels were measured at admission (day 1) and at 1 week (day 7) of ICU therapy. SDMA levels remained stable during the first week of ICU treatment (paired Wilcoxon test).

quarter of the total cohort) had significantly higher serum SDMA levels at admission compared with the ICU survivors (median 1.33 versus 0.74 μmol/L, $P = 0.001$). We thus performed Cox regression analyses and Kaplan-Meier curves to assess the impact of the initial SDMA serum concentrations on ICU mortality among critically ill patients. Low SDMA levels upon admission to the ICU were a strong prognostic predictor for ICU survival ($P = 0.021$, Cox regression analyses). In multivariate Cox regression analyses, including markers of inflammation/infection (CRP, WBC), circulatory (lactate), hepatic (bilirubin, protein, and INR), and renal (creatinine) deterioration at admission, SDMA remained an independent significant prognostic parameter (hazard ratios and P values are presented in Table 4). Kaplan-Meier curves displayed that patients with SDMA levels of the upper quartile (>1.7 μmol/L) had the highest mortality (log rank 8.14, $P = 0.0171$, Figure 3(a)). We found the best cutoff value to discriminate survivors from non-ICU survivors for serum SDMA of 1.2 μmol/L (log rank 15.15, $P = 0.0001$, Figure 3(b)).

Table 2: Disease etiology of the study population.

	Sepsis $n = 160$	Nonsepsis $n = 87$
Etiology of sepsis critical illness n (%)		
Site of infection		
Pulmonary	91 (56.9)	
Abdominal	29 (18.1)	
Urogenital	14 (8.8)	
Other	26 (16.2)	
Etiology of nonsepsis critical illness n (%)		
Cardiopulmonary disorder		35 (40.2)
Acute pancreatitis		11 (12.6)
Decompensated liver cirrhosis		16 (18.4)
Severe gastrointestinal hemorrhage		7 (8.0)
Non-sepsis other		18 (20.7)

Table 3: Multivariate regression analysis of parameters determining SDMA levels.

Parameter	Standardized coefficient beta	t-value	P value
Creatinine	0.502	7.108	<0.001
Procalcitonin	0.164	2.311	0.023
suPAR	0.151	1.587	NS
C-reactive protein	0.019	0.258	NS
Pseudocholinesterase	−0.089	−1.112	NS
Bilirubin	0.003	0.042	NS
ADMA	0.109	1.378	NS

ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine; suPAR: soluble urokinase plasminogen activator receptor.

During the follow-up observation period of approximately three years, the overall case fatality rate increased to 47.3% of the study cohort (Table 1). SDMA serum concentrations at admission to the ICU were significantly higher in patients with unfavourable outcome (median 1.09 versus 0.67 μmol/L, $P < 0.001$). By Cox regression analysis, initial serum SDMA levels significantly predicted long-term prognosis ($P = 0.010$). The prognostic value remained significant also by multivariate analysis (Table 5). Kaplan-Meier curves proved that SDMA levels of the highest quartile (>1.7 μmol/L) were strongly associated with fatal outcome (log rank test 13.49, $P = 0.0012$, Figure 3(c)). SDMA levels of 0.75 μmol/L discriminated the long-term prognosis of critically ill patients (log rank test 14.15, $P = 0.0002$, Figure 3(d)). Interestingly, when SDMA levels were adjusted to renal function by calculating the SDMA/creatinine ratio, patients that died during the observation period still displayed significantly elevated SDMA/creatinine values ($P = 0.012$, detailed data not shown), confirming that the association of SDMA with long-term mortality was independent of renal function.

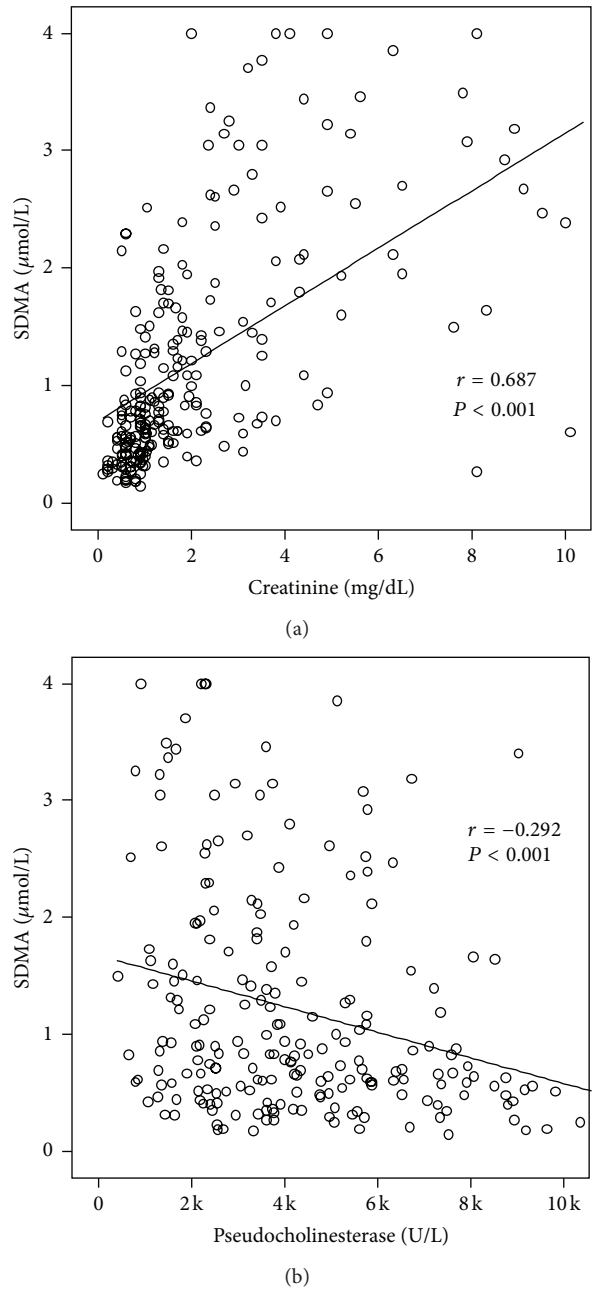

Figure 2: Serum SDMA concentrations in critically ill patients are correlated with renal and hepatic organ failure. Serum ADMA levels were measured in $n = 247$ critically ill patients at admission to the ICU. Serum SDMA correlated significantly with renal failure (creatinine, (a)) or hepatic failure (pseudocholinesterase, (b)). Spearman rank correlation test, r and P values are given in the figure.

4. Discussion

The excessive endothelial activation in systemic inflammation and sepsis affects hemostasis, leukocyte trafficking, vascular permeability, and the extent of disturbed microcirculation [5]. There is experimental and clinical evidence that dysregulation of the arginine-NO pathway critically contributes to this process [21]. It had been previously

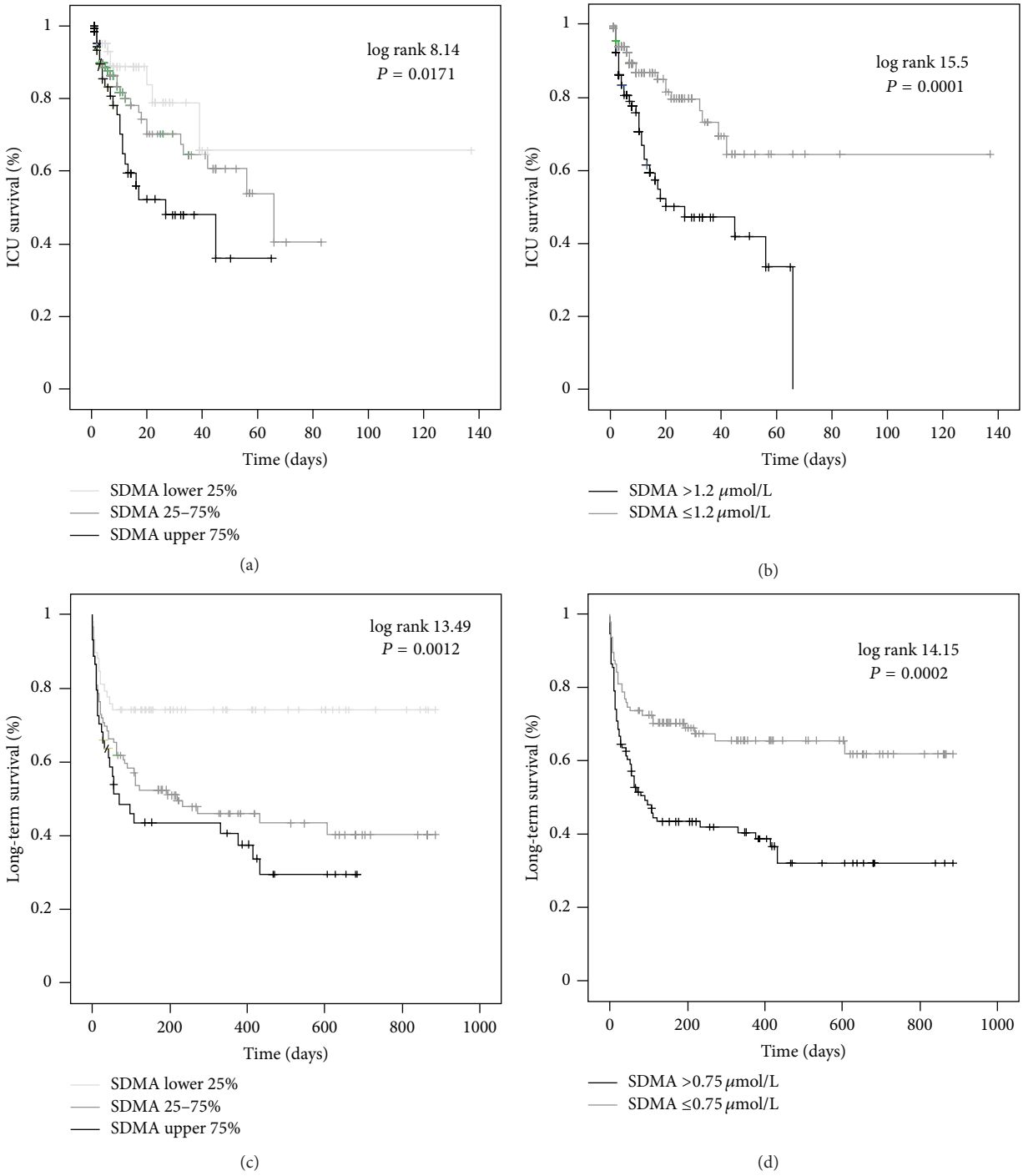

Figure 3: Prediction of mortality by SDMA serum concentrations. ((a)-(b)) Kaplan-Meier survival curves of ICU patients are displayed, showing that patients with SDMA levels of upper quartile (on admission >1.7 μmol/L; black (a)) had an increased short-term mortality at the ICU as compared to patients with ADMA serum concentrations of lower quartile (on admission <0.51 μmol/L; light grey) or middle 50% (grey). Best discrimination between ICU survivors and nonsurvivors was achieved with an SDMA cutoff value of 1.2 μmol/L (b). Log rank and P values are given in the figure. ((c)-(d)) Kaplan-Meier survival curves of ICU patients are displayed, showing that patients with SDMA levels of upper quartile (on admission >1.7 μmol/L; black (c)) had an increased long-term mortality at the ICU as compared to patients with SDMA serum concentrations of lower quartile (on admission <0.51 μmol/L; light grey) or middle 50% (grey). Best discrimination between overall survivors and nonsurvivors was achieved with an ADMA cutoff value of 0.75 μmol/L (d).

Table 4: Uni- and multivariate Cox regression analyses for SDMA levels at admission to predict ICU mortality.

Parameter	Unadjusted HR (95%-CI)	*P* value	Adjusted HR (95%-CI)	*P* value
SDMA	1.349 (1.059–1.719)	0.015	1.379 (1.012–1.879)	0.042
Protein	0.961 (0.939–0.984)	0.001	0.966 (0.940–0.993)	0.012
INR	1.890 (1.370–2.607)	<0.001	1.537 (1.004–2.354)	0.048
Lactate	1.169 (1.085–1.260)	<0.001	1.142 (1.058–1.231)	0.001
Creatinine	1.026 (0.937–1.123)	NS	—	NS
White blood cell count	0.986 (0.955–1.018)	NS	—	NS
C-reactive protein	1.0 (0.997–1.003)	NS	—	NS

Table 5: Uni- and multivariate Cox regression analyses for SDMA levels at admission to predict overall mortality.

Parameter	Unadjusted HR (95%-CI)	*P* value	Adjusted HR (95%-CI)	*P* value
SDMA	1.275 (1.067–1.524)	0.007	1.357 (1.088–1.692)	0.007
Protein	0.965 (0.948–0.982)	<0.001	0.973 (0.954–0.992)	0.006
INR	1.630 (1.226–2.168)	0.001	1.410 (0.997–1.994)	0.05
Lactate	1.133 (1.054–1.217)	0.001	1.122 (1.042–1.207)	0.002
Creatinine	1.009 (0.944–1.079)	NS	—	NS
White blood cell count	0.989 (0.967–1.012)	NS	—	NS
C-reactive protein	1.001 (0.999–1.004)	NS	—	NS

demonstrated that ADMA as an endogenous NO synthase inhibitor is a promoter of vascular dysfunction in patients with sepsis [7–10]. Our study now shows that also SDMA, another methylated form of L-arginine, is also significantly upregulated in critically ill patients, especially in conditions of sepsis, associated with inflammation and organ failure as well as a yet unrecognized indicator for mortality risk in medical ICU patients.

A prominent finding in our heterogeneous cohort of critically ill medical patients was the independent association of SDMA serum levels with biomarkers reflecting renal dysfunction and systemic inflammation by multivariate analyses. The fact that renal function was an important independent determinant of circulating SDMA levels was not surprising, because SDMA is excreted via the urine, and SDMA has been found elevated in studies of patients with end-stage renal disease [12, 22]. There is also experimental evidence that dimethylarginines can be metabolized by the liver as well [23], which would well explain its increase in ICU patients with hepatic dysfunction. Furthermore, the close correlation between SDMA levels and inflammatory biomarkers such as procalcitonin or tumor necrosis factor may indicate that protein catabolism induced by systemic inflammation might contribute to elevated systemic SDMA levels in critically ill patients. Due to our study design, which focussed on regulation of SDMA in critically ill patients at admission to the ICU, we were unable to further analyse whether the persistence of elevated SDMA, as observed in patients with available longitudinal SDMA measurements, reflects persistent systemic inflammation or is rather an epiphenomenon of multiorgan failure in these patients.

Our study identified SDMA as a prognostic marker in patients with critical illness, both for ICU and long-term mortality. Importantly, SDMA remained independently associated with mortality in multivariate regression analyses, corroborating that SDMA is not only an epiphenomenon of acute organ dysfunction. These data strongly indicate that elevated SDMA levels in ICU patients reflect prognostically relevant pathomechanisms such as microcirculatory dysfunction due to endothelial activation. The accumulation of SDMA might reduce endothelial NO synthesis, as it competes with arginine for cellular transport across the y^+ transporter and might promote endothelial stress, as it has been showed to increase intracellular reactive oxygen species in human endothelium [12, 15]. Similar cause-effect relationships have been proposed for chronic, "low-grade inflammatory" processes such as atherosclerosis [24]. One might speculate whether therapeutic interventions intended to increase vascular tension during the hyperdynamic state of sepsis via modulating arginine-NO interactions could be beneficial in critically ill patients [25].

Despite its potential pathogenic implications, SDMA serum levels were closely associated with ICU as well as long-term mortality risk in our cohort of critically ill medical patients. Our study now identified possible cutoff values of circulating SDMA as indicators for increased mortality risk. This raises the possibility that implementing SDMA in risk stratification algorithms might further increase the prognostic accuracy of current clinical scoring system at the ICU. Future studies should therefore not only aim at exploring the pathogenic role of SDMA in sepsis and concomitant endothelial dysfunction but also evaluate the clinical applicability of SDMA measurements as a prognostic biomarker in critical illness.

5. Conclusions

Our study demonstrates significantly upregulated serum levels of SDMA in critically ill patients, especially in patients with sepsis. The potential value of SDMA as an indicator

of endothelial dysfunction in medical ICU patients and its correlations to biomarkers of renal, liver, and circulatory failure function should be confirmed in experimental models of systemic inflammation and in different clinical settings. The clear association of circulating SDMA levels with clinically relevant endpoints such as ICU or long-term mortality gives rise to the expectation that integrating SDMA into current tools of risk assessment in critically ill patients might improve their prognostic accuracy.

Acknowledgments

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