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Streamlining antimicrobial stewardship tools in critically ill patients

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Thesis submitted to fulfill the requirements for the degree of doctor in biomedical and pharmaceutical sciences 2022

ACKNOWLEDGEMENTS

"There is a crack in everything, that's how the light gets in".

Je remercie vivement ma promotrice, le Pr C. Oury ainsi que mon co-promoteur, le Pr A. Gothot, de m'avoir permis de réaliser une activité de recherche translationnelle parallèlement à mon activité de spécialiste en médecine intensive. Je vous suis reconnaissante pour vos conseils, votre exigence, votre rigueur et votre aide dans mon cheminement scientifique. J'ai été accueillie avec bienveillance dans les laboratoires qui se sont révélés être, pour moi, avant tout, une source de rencontres humaines enrichissantes.

Je suis reconnaissante vis-à-vis de l'ensemble des membres du jury, en particulier le Pr Moutschen, président du comité de thèse ainsi qu'au Pr Damas qui m'a accueillie au CHU de Liège et dans son service.

Je remercie chaleureusement le Pr De Waele, de l'Université de Gand ainsi que le Pr Cavaillon, de l'Institut Pasteur, membres extérieurs du jury, pour leur participation attentive.

Je remercie évidemment tous mes collègues du service des Soins Intensifs, qui ont permis et participé à la réalisation de ce travail. En particulier, je remercie le chef de service, le Pr Misset et mon plus proche collègue, le Pr Canivet, pour leur disponibilité et leurs conseils avisés et bienveillants. Je souhaite remercier tout particulièrement toutes les équipes infirmières et leurs chef(fe)s respectifs/ves avec qui nous venons de vivre une période particulièrement marquante de nos vies professionnelles et qui a mis en exergue, une fois encore, les fondements de notre fonctionnement : humanité, solidarité, team work.

Un merci tout particulier à Séverine Marck qui a participé à la réalisation pratique de ce manuscrit.

Je remercie également particulièrement les différentes équipes de recherche et de routine rencontrées au fil des ans, Céline, Christelle, Justine, Christian, Jacques et Mickaël, Raphaël et Virginie, le Pr Charlier, le Pr Hayette et le laboratoire de bactériologie. Pour certains, la collaboration perdure au-delà des projets scientifiques, au service quotidien du patient.

Merci au Centre B-STAT ainsi qu'au Pr Albert pour leurs précieux soutien et conseils en biostatistique.

Je tiens à remercier les patients qui ont participé aux différentes études exposées ci-après.

Je remercie le CHU de Liège, le fonds d'investissement de recherche scientifique du CHU de Liège ainsi que le Fonds National de Recherche Scientifique de m'avoir permis de mener à bien mes projets.

Enfin, mes remerciements et mon amour inconditionnels vont à mon époux, Eric, à mes enfants, Alexandre et Danaé et à mes parents, pour leur soutien indéfectible dans la réalisation de ce travail mais aussi et surtout, de celui accompli quotidiennement auprès des malades.

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LIST OF ABBREVIATIONS

%fT>MIC: fraction of time free compound of beta-lactam remains over MIC of bacteria

AMR: antimicrobial resistance

AMS: Antimicrobial stewardship

AMT: antimicrobial therapy

ARC: augmented renal clearance

AUC: area under the curve

BSAC: British Society for Antimicrobial Chemotherapy

BSI: blood stream infection

CA-SFM : Comité de l'Antibiogramme de la Société Française de Microbiologie

CAP: community-acquired pneumonia

CAZ-AVI: ceftazidime-avibactam

CI: confidence interval

CI: continuous infusion

CLABSI: central line associated blood stream infection

CRP: C-reactive protein

DAMPS: danger associated molecular patterns

DDD: daily defined dose (per 100 ICU days)

DIC: disseminated intravascular coagulation

ELF: epithelial lung fluid

ESBL: extended-spectrum β-lactamase

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FCM: flow cytometry

Fg: Fibrinogen

GM-CSF: granulocyte macrophage colony stimulating factor

HAI: hospital-acquired infections

HAP: hospital-acquired pneumonia

HBP: heparin-binding protein

HLA: human leucocyte antigen

ICU: intensive care unit

ID: infectious diseases

II: intermittent infusion

IPF: immature platelet fractions

IQR: interquartile range

LOS: length of stay

LRTI: lower respiratory tract infections

MFI: median fluorescence index or median of fluorescence intensity

MHC: major histocompatibility complex

MIC: minimal inhibitory concentration

MIPD: model-informed precision dosing

MV: mechanical ventilation

OR: odds ratio

PAMPS: pathogen associated molecular patterns

PCT: procalcitonin

PD: pharmacodynamics

PE: phycoerythrin-linked

PerCP: perinidin-chlorophyll protein-linked

PK: pharmacokinetics

PS: P-selectin

PSP: pancreatic stone protein

PTA: probability of target attainment

RCT: randomized controlled trial

ROC: receiver operating characteristic

RRT: renal replacement therapy

SAPS II: Simplified Acute Physiology Score II

SOFA: Sequential Organ Failure Assessment

SSC: Surviving Sepsis Campaign

SSTI: skin and soft tissue infection or surgical site and soft tissue infection

sTREM-1: soluble triggering receptor expressed on myeloid cells

SUPAR: soluble urokinase receptor

TDM: therapeutic drug monitoring

TLR: toll-like receptor

TNF-α: tumor necrosis factor α

UTI: urinary tract infections

VAP: ventilator-acquired pneumonia

VAT: ventilator-acquired tracheobronchitis

SUMMARY

Sepsis carries a high burden in intensive care units, not only in terms of morbidity and mortality in patients but also in terms of massive antimicrobial therapy consumption. The critically ill patient is the highest per-capita consumer of antimicrobials. While international guidelines advocate timely administration of broad-spectrum antimicrobial therapy to reduce sepsis mortality, it has also been shown that up to 50% of critically ill patients who receive antimicrobials have no definite diagnosis of infection. ICU physicians should strive to reduce pressure selection, drug side-effects and costs, through active implementation of antimicrobial stewardship (AMS) tools in therapeutic decision-making. Early and accurate diagnosis of sepsis, selection of narrow-spectrum antimicrobials with adequate knowledge of their PK/PD properties are some of the cornerstones of AMS programs in the critically ill patient.

In Chapter 2, we studied host response biomarkers, such as cell-surface markers and procalcitonin, for prediction and diagnosis of sepsis in a selected population of ICU patients. Indeed, it has previously been demonstrated that high-risk critically ill patients such as trauma, surgery and burn patients, experience susceptibility to sepsis due to innate and adaptive immune reprogramming secondary to the insult. Biomarkers such as cell-surface markers, present on leucocytes and platelets, could help identify those at risk of secondary sepsis. Concerning leucocytes, a combination of low levels of mHLA-DR and neutrophil CD88 along with elevated levels of T_{regs}, was recently shown to be associated with secondary sepsis occurrence in patients who were admitted in the ICU for trauma, sepsis or surgery. We studied 63 leucocyte surface markers in critically ill patients who had sustained such an injury (except for sepsis) in order to look for a change of phenotype that would be associated with secondary sepsis. We showed that patients exhibiting high monocytes counts and low expression of L-selectin on their monocytes upon ICU admission, and low levels of mHLA-DR 48-72h later, had higher risk of developing sepsis in the first week of ICU admission.

Concerning platelets, several preclinical models have shown that platelets influence innate and adaptive immune responses in infection. However, whether platelets contribute to

predisposition to sepsis is unknown. We studied two platelet activation markers, P-selectin (a marker of degranulation) and Fibrinogen-binding in the same cohort of critically ill injured patients. We found that when patients were stratified according to admission SOFA score (>8) and level of Fibrinogen-binding on platelets (>50%), the risk of secondary sepsis was 87% in the first week of ICU admission. We conclude that platelet activation secondary to injury could alter platelet ability to recognize bacterial components such as ligands of αIIbβ3 and, thus, affect recruitment of immune cells to the infectious site. Furthermore, we found no association between aspirin usage and protection from sepsis or levels of platelet activation. The latter suggests that platelet activation in the critically ill injured patient happens independently from TXA2 production.

Then, we studied procalcitonin (PCT) as a diagnostic tool for sepsis, in an attempt to initiate AMT only in those patients most likely to benefit. Procalcitonin is a prohormone that is ubiquitously secreted, in hyperinflammatory non-specific conditions, by non-neuroendocrine parenchymal cells throughout the body. In clinical practice, it has a more favorable kinetic profile, compared to CRP, making it a promising tool for AMS either for the decision to initiate antimicrobials or for stopping them once infection has been ruled out or circumscribed. Two large randomized trials showed that it could allow a reduction of AMT in pneumonia when used as a diagnostic tool with the help of an algorithm. Based on low, intermediate and high levels, the use of AMT was withheld or encouraged in suspected pneumonia and this strategy led to a 50% reduction in AMT prescribing.

We applied the same strategy in a randomized manner (PCT unveiled/PCT masked to the ICU treating physician) to a population of critically ill patients, in need of organ support, who were expected to stay for more than 48h. Patients who were suspected of having sepsis, either on admission or during ICU stay, had PCT sampled once. Moreover, the ICU physician had to classify sepsis diagnosis as sure, probable, possible or uncertain. The primary endpoint was the reduction of AMT use. We found no difference in the overall AMT use in the 2 groups despite a significantly higher number of antimicrobial treatments being withheld in the unveiled PCT group, in the group classified by the ICU physician as “possible sepsis”. Moreover, when looking at the decision to treat according to PCT levels in both groups, *a*

posteriori, no difference between groups could be observed. From this study, we concluded that PCT was a poor diagnostic marker of sepsis (AUC 0.69).

In Chapter 3, we focused our study on the therapeutic compound of AMS in the ICU by characterizing the population pharmacokinetics (PK) of two different modes of infusion of temocillin in severe pneumonia. Indeed, knowledge and integration of PK/PD properties of prescribed antimicrobials is a pragmatic objective of AMS. Use of temocillin, a revived narrow-spectrum beta-lactam, has been advocated in systemic infections such as severe pneumonia caused by Gram-negative pathogens. However, despite use of high doses in sicker patients with limited evidence for clinical efficacy, PKPD data are lacking and so are clinical breakpoints, aside for UTI. The lung penetration ratio of temocillin (infused either in CI or II) was determined at 73% via serial plasma and ELF samplings in mechanically ventilated patients. Probability of target attainment for a series of MICs were determined. We showed that even for the most minimal pharmacodynamic (PD) targets, breakpoints were much lower than expected for both modes of infusion, in plasma and ELF. The highest breakpoint of 8mg/L in ELF could only be reached in patients with moderate renal insufficiency thereby hindering wide use of this antimicrobial in severe pneumonia without further evidence of efficacy.

To summarize, we identified four biomarkers predictive of sepsis occurrence in a specific category of critically ill patients. These cell surface markers, among which three could be readily implemented in clinical practice, should be included in future prospective trials in the hope of a future pharmacological mitigation of secondary immune suppression leading to sepsis. We also contributed to PK characterization of a narrow-spectrum beta-lactam in severe pneumonia and proposed clinical breakpoints in this era of ever-growing antimicrobial resistance.

RESUME

Le sepsis est responsable d'une morbi-mortalité importante en soins intensifs, en ce compris celle qui est engendrée par une consommation importante d'antibiotiques. Le patient critique est celui qui génère le plus de prescriptions d'antimicrobiens. Alors que les recommandations internationales préconisent l'administration empirique d'une antibiothérapie de large spectre dans un délai de 1-3h maximum, afin de réduire la mortalité liée au sepsis, des études ont montré que jusqu'à 50% de patients séjournant en soins intensifs pour sepsis recevaient indûment des antibactériens, sans qu'aucune infection n'ait été confirmée. Les intensivistes doivent s'attacher à intégrer dans leur algorithme thérapeutique quotidien des outils de stewardship antibiotique afin de réduire la pression de sélection, les coûts et les effets secondaires liés aux médicaments, tous engendrés par la surconsommation antibiotique. Parmi ces outils, il y a la prédiction et le diagnostic précis du sepsis de manière à initier les antimicrobiens uniquement chez les patients les plus à même d'en bénéficier. D'autre part, au plan thérapeutique, il est recommandé d'avoir une connaissance adéquate des caractéristiques pharmacocinétiques des antibactériens utilisés afin d'adapter la posologie et le timing d'administration aux caractéristiques cliniques du patient, ce qui favorise à la fois l'efficacité clinique mais aussi l'écologie individuelle et collective.

Dans le chapitre 2, nous avons étudié des biomarqueurs de la réponse de l'hôte à l'infection (en ce qui concerne la procalcitonine, pour le volet diagnostique) et au mécanisme lésionnel (en ce qui concerne les marqueurs de surface leucocytaires et plaquettaires, pour le volet prédiction).

Il est établi que des mécanismes lésionnels tels que la brûlure, la chirurgie lourde et le traumatisme étendu peuvent entraîner un état d'immunosuppression prédisposant au sepsis. La majorité des études réalisées jusqu'ici, portant sur un nombre limité de marqueurs, a démontré un état de déficit fonctionnel des monocytes (mHLA-DR bas) ou une élévation des lymphocytes $T_{regs,}$ dans un délai variable après l'événement déclencheur. Nous avons étudié 63 marqueurs leucocytaires, ciblant 7 sous-types cellulaires, dans une démarche prospective de monitoring à J1 et J3 de l'admission en USI de patients lésés

(opérés du cœur, traumatisés, ayant subi un AVC) afin de déterminer si un changement de phénotype pouvait s'avérer prédictif de la survenue d'un sepsis. Nous avons montré que le fait d'avoir un haut taux de monocytes et une faible expression de L-selectin sur ceux-ci à l'admission ainsi qu'un m-HLA-DR bas 48h après l'admission, était associé à un plus haut risque de survenue de sepsis endéans la première semaine de séjour à l'USI.

En ce qui concerne les marqueurs plaquettaires, des études précliniques ont démontré l'influence des plaquettes dans la réponse innée et adaptative à l'infection. En ce qui concerne la prédisposition au sepsis, rien n'a été démontré. Nous avons étudié deux marqueurs d'activation plaquettaire, la P-selectin (dégranulation) et la liaison du fibrinogène (Fg-binding) dans la cohorte de patients critiques sus-jacente. Nous avons démontré que le niveau de Fg-binding (>50%) associé à un SOFA score élevé (>8) permettait d'identifier les patients présentant un risque de sepsis secondaire élevé (87%). Nous en concluons que l'activation plaquettaire secondaire à un mécanisme lésionnel pourrait altérer la capacité des plaquettes à identifier des composants bactériens tels que les. ligands d'αIIbβ3 et inhiber secondairement le recrutement de cellules immunitaires au site infectieux. D'autre part, nous n'avons pas trouvé d'association entre l'usage préalable d'aspirine par les patients et leur niveau d'activation plaquettaire ou un effet protecteur contre le sepsis. Ceci suggère que l'activation plaquettaire chez le patient avec un mécanisme lésionnel critique survient indépendamment de la production de TXA2.

Pour le volet diagnostique, nous avons étudié la procalcitonine (PCT) qui est une pré-hormone ubiquitaire qui est sécrétée de façon non spécifique, dans une série de maladies inflammatoires (cancer, pancréatite, brûlures..). En routine clinique, sa cinétique intéressante par rapport à la CRP a permis son implémentation comme biomarqueur dans le sepsis, entre autres pour conforter le diagnostic et limiter l'initiation de l'antibiothérapie aux patients qui sont les plus susceptibles d'en bénéficier. Nous avons utilisé un algorithme basé sur des seuils de PCT pour mener une étude randomisée en simple aveugle (1 groupe d'intensivistes aveugle au résultat/l'autre pas) visant à déterminer si une stratégie basée sur la PCT pouvait réduire l'initiation d'une antibiothérapie chez un patient suspect de présenter un sepsis en USI. Nous n'avons pas pu démontrer de différence de consommation antibiotique entre les deux groupes. L'AUC de la PCT en tant que marqueur diagnostique du sepsis s'est révélé médiocre (AUC 0.69), invalidant l'apport de ce marqueur en tant qu'outil

de stewardship dans une démarche d'initiation de traitement. Ces résultats ont été confortés par la littérature parue depuis lors, ainsi que par plusieurs recommandations récentes (sepsis et pneumonie communautaire) positionnant favorablement la PCT lorsqu'il s'agit d'arrêter un traitement (VPN>90%), même chez les patients à haut risque. Ces données ont fait l'objet d'un article de revue inclus dans le chapitre 2 en faisant le lien avec la littérature récente.

Dans le chapitre 3, nous avons étudié les propriétés pharmacocinétiques d'une béta-lactamine de spectre étroit, la témocilline, qui pourrait être un agent intéressant dans le traitement des pneumonies à bacilles gram négatifs (BGN), afin de limiter l'usage des carbapénèmes. En effet, cette molécule permet de traiter la plupart des BGN sécréteurs de beta-lactamases (à l'exception des non-fermentants, tels que *Pseudomonas Aeruginosa, Burkholderia, Acinetobacter*) en raison d'une conformation chimique particulière qui la rend résistante aux enzymes. La littérature sur les hautes posologies usuellement utilisées en USI (sans breakpoints disponibles) est assez disparate, notamment en terme de site infectieux et de sévérité d'infection. Nous avons déterminé que la pénétration au niveau de l'epithelial lung fluid de patients ventilés mécaniquement pour une pneumonie sévère était de 73%, ce qui est supérieur à de nombreuses beta-lactamines, à l'exception du cefepime. Néanmojns, les breakpoints suggérés par la modélisation Monte-Carlo se sont révélés plus bas qu'attendu, à l'exception des patients en insuffisance rénale modérée (Clcréat 30-60ml/min). L'usage de cette béta-lactamine à spectre étroit s'en trouve limité, même aux hautes doses pratiquées, en tout cas dans la pneumonie sévère et dans l'attente de données supplémentaires.

Pour conclure, nous avons identifié quatre biomarqueurs prédictifs de la survenue d'un sepsis chez des patients ayant subi une pathologie critique lésionnelle. Parmi ces marqueurs de surface, trois pourraient être implémentés en routine clinique immédiatement, à condition qu'ils fassent l'objet d'une validation. Nous avons également contribué à la caractérisation pharmacocinétique d'une beta-lactamine de spectre étroit dans la pneumonie sévère, en précisant l'usage qu'on pourrait en attendre en fonction des breakpoints proposés.

CHAPTER 1. INTRODUCTION

1.1. SEPSIS DEFINITION, EPIDEMIOLOGY AND BURDEN ON ICU PATIENTS

Sepsis is defined by the presence of infection and of organ dysfunction due to a dysregulated host response (1). It is a major health problem worldwide and the leading cause of morbidity and mortality in hospitalized patients (2, 3). The prevalence of severe infections in the hospital and particularly in the ICU has significantly risen in the last 30 years (4-6). The latest global, multinational, worldwide study estimating the incidence and mortality of hospital-treated sepsis suggested that there were 19.4 million cases of hospital-treated sepsis a year and 5.3 million deaths attributable to sepsis annually (7). The burden on morbidity and mortality in the ICU is significant. Two worldwide point-prevalence studies evaluating infection in critically ill patients, antibiotic exposure and all-cause in-hospital mortality have risen from 62% to 70% and from 17% to 30%, respectively, in 3 decades (8, 9). Predominant sites of infection diagnosed in critically ill patients, were the lung (60%) followed by the abdomen (18%) and the bloodstream (15%). Among 65% of patients who had at least one positive microbiological culture, Gram-negative pathogens were predominant (67%) followed by Gram-positives (37%) and fungal pathogens (16%). Among others, independent risk factors for higher mortality were found to be ICU-acquired infections (OR 1.32 [95%CI], 1.10-1.60) and infection with *Klebsiella sp*. resistant to β-lactamases (OR, 1.29[95%CI] 1.02-1.63) and carbapenem-resistant *Acinetobacter sp.* (OR, 1.40[95%CI]1.08-1.81). This highlights the burden of antimicrobial resistance on mortality in ICU patients and the necessity of a multi-faceted antimicrobial stewardship (AMS) intervention.

1.2. ANTIMICROBIAL STEWARDSHIP: DEFINITION AND STREAMLINED IMPLEMENTATION IN THE ICU

A very recent global survey, which was published in the Lancet, in January 2022, showed that 1.27 million deaths worldwide could be directly attributed to antimicrobial resistance (AMR) (10). Moreover, a United Nations Coordination Group on AMR estimate for 2050 is around 10 million deaths per year if no further action is taken.

Antimicrobial stewardship (AMS) is defined by "optimal selection, dosage and duration of antimicrobial treatment that results in the best clinical outcome for the treatment or prevention of infection with minimal toxicity to the patient and minimal impact on subsequent resistance" (11). AMS remains a significant challenge in the ICU environment for many reasons. The Surviving Sepsis Campaign (SSC) guidelines strongly recommend both prompt (<3h) and broad-spectrum administration of antimicrobial therapy (AMT) (12). Indeed, timely delivery has been shown to lower mortality and inadequate empiric choice of AMT is associated with higher mortality in the sickest patients (13, 14). On the other hand, a gold standard definition of sepsis is still lacking and clinicians are reluctant to miss the window of opportunity in such sick patients although as many as 40% of them are inappropriately exposed to AMT (15-17). As a consequence, the critically ill patient is the highest per-capita consumer of antimicrobials in a globalized world where antimicrobial resistance has become a public health threat calling for immediate action (18, 19).

Recently, the WHO issued a priority list of antibiotic-resistant bacteria to support research and development of effective drugs among which carbapenem-resistant *Acinetobacter Baumanii* and *Pseudomonas Aeruginosa* as well as carbapenem-resistant and 3rd-generation cephalosporin-resistant *Enterobacterales* were given critical priority (20). Antimicrobial stewardship comprises many tools designed to withhold or discontinue unnecessary and harmful AMT. It also encompasses the preferred use of narrow-spectrum antimicrobials (such as temocillin) in order to spare wide spectrum antimicrobials such as carbapenems which contribute to widespread antimicrobial resistance (21-23).

Table 5 Implementation and objectives of antibiotic stewardship programs in the ICU

Implementation of ASP	
Pre-requisites	Evidence-based, ideally bundled change package Dynamic data collection systems with feedback to prescribers Strategy for building capacity, including the appointment of an ICU staff member as ASP leader
Pre-implementation phase	Identification of determinants for antibiotic prescription and opportunities for improvement at the ICU level
Implementation phase	Building of a customized plan to solve quality gaps, based on educational and behavioral interventions Continuous collaboration between ICU staff members, microbiologists, pharmacists, and infection control units Clear definition of goals and indicators
Pragmatic objectives of ASP	
Stewardship of empirical antibiotic therapy	Distinction between bacterial infections, non-bacterial infections, and non-infectious inflammatory syndromes Early identification of sepsis (*antibiotic initiation might be delayed pending microbiological data in certain patients without new or worsening organ failure*) Consideration of local resistance patterns and patient's individual risk factors for MDRB for the choice of empirical drugs Efforts to obtain early microbiological documentation (including rapid diagnostic tools, conventional cultures, and source control when appropriate) Optimization of PK/PD Promotion of single-drug regimen whenever possible for patients without septic shock and/or risk factors for MDRB Restricted use of broad-spectrum, costly, and/or potentially toxic antibiotics
Stewardship of definite antibiotic therapy	Reappraisal of the diagnosis of bacterial infection at day 2–3 (microbiological and radiological data, clinical evolution) Early antibiotic cessation in patients without confirmed infection In patients with likely or confirmed infection: dosing adaptation when appropriate (e.g., if changes in Vd and/or renal clearance), routine discussion for de-escalation (e.g., spectrum narrowing and switching from combination to single-drug regimen) and shortening of treatment duration (e.g., PCT-based algorithms, adequate source control, favorable clinical evolution)
Overall objectives	Improvement in patient outcomes Reduction in ecological and non-ecological (e.g., toxicity or allergy) side effects of antibiotics Reduction of antibiotic- and resistance-related costs

From: Timsit *et al,* ICM 2019.

Antimicrobial stewardship tools can therefore be used for prediction, diagnosis and treatment of sepsis.

In a tentative to streamline AMS in the ICU, we first focused our studies on host-response biomarkers such as leucocyte and platelet surface markers and procalcitonin (PCT), in order to predict and diagnose sepsis earlier and better (24-27). This allows initiation of AMT exclusively in patients who are most likely to benefit. Then, we aimed to describe the PKPD characteristics of a revived narrow-spectrum beta-lactam, temocillin, which has a particular chemical conformation that renders it resistant to most beta-lactamases(28). Temocillin has been used in several severe infections in critically ill patients, while lacking extensive data on site penetration (29). Pharmacokinetic characterization was done in plasma and ELF of critically ill mechanically ventilated patients with pneumonia, in order to define clinical breakpoints (until then unavailable) for a drug that could contribute to a better individual and collective ecology.

We therefore aimed at contributing to better prediction, diagnosis and therapeutic management of sepsis, focusing on some cornerstones of AMS.

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CHAPTER 2. HOST RESPONSE BIOMARKERS FOR EARLY DIAGNOSIS OF SEPSIS

2.1. LEUCOCYTES AND PLATELET SURFACE MARKERS FOR EARLY DIAGNOSIS OF SEPSIS

Core elements of AMS include prescribing AMT when it is truly needed. This led us to determine whether we could identify patients admitted in the ICU for critical injury who would develop hospital-acquired infections (HAI) via a prospective immune monitoring of circulating leucocytes and platelets.

2.1.1. PROSPECTIVE FLOW CYTOMETRY ANALYSIS OF LEUCOCYTE SUBSETS IN CRITICALLY ILL PATIENTS WHO DEVELOP SEPSIS: A PILOT STUDY (SUBMITTED IN INFECTION)

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2.1.1.1. ABSTRACT

Purpose: Sepsis in critically ill patients with injury bears a high morbidity and mortality. Extensive phenotypic monitoring of leucocyte subsets in critically ill patients at ICU admission and during sepsis development is still scarce. The main objective of this study was to identify early changes in leukocyte phenotype which would correlate with later development of sepsis.

Methods: Patients who were admitted in a tertiary ICU for organ support after severe injury (elective cardiac surgery, trauma, necessity of prolonged ventilation or stroke) were sampled on admission (T1) and 48-72h later (T2) for phenotyping of leukocyte subsets by flow cytometry and cytokines measurements. Those who developed secondary sepsis or septic shock were sampled again on the day of sepsis diagnosis (Tx).

Results: Ninety-nine patients were included in the final analysis. Nineteen (19.2%) patients developed secondary sepsis or septic shock. They presented significantly higher absolute

monocyte counts and CRP at T1 compared to non-septic patients (1030/µl versus 55/µl, p=0.013 and 5.1mg/ml versus 2.5mg/ml, p=0.046, respectively). They also presented elevated levels of monocytes with low expression of L-selectin ($CD62L_{neg}$monocytes)(OR[95%CI]: 4.5 (1.4-14.5) p=0.01) and higher SOFA score (p<0.0001) at T1 and low mHLA-DR at T2 (OR[95%CI]: 0.003 (0.00-0.17) p=0.049). Stepwise logistic regression analysis showed that both monocyte markers and high SOFA score (>8) were independent predictors of nosocomial sepsis occurrence. No other leucocyte count or surface marker nor any cytokine measurement correlated with sepsis occurrence.

Conclusion: Monocyte counts and change of phenotype are predictive of secondary sepsis in critically ill patients with injury.

Keywords: injury; sepsis; flow cytometry; monocytes; HLA-DR; L-selectin.

2.1.1.2. BACKGROUND

It is estimated that 25-35% of critically ill patients develop sepsis which is associated with increased length-of-stay (LOS), morbidity and mortality (1-4). As in sepsis-induced immunosuppression, immune alterations affecting patients with critical injuries such as trauma, major surgery or burns, have been associated with increased susceptibility to secondary infections and mortality (5-8). The first reports of monocyte anergy and endotoxin tolerance date back to the 70's in major surgical and burn patients (9, 10). Since then, most studies relying on flow cytometric analysis of peripheral blood cells, have focused on single and restricted types of immune cells defects such as T-lymphocytes, monocytes and neutrophils (11-14). The most commonly studied parameter of immune dysfunction associated with injury is the low HLA-DR expression on monocytes (mHLA-DR), which induces an impaired functional state of these cells. The latter feature has been associated with secondary sepsis and sometimes outcome in severe trauma, burn and postoperative patients (15-21).Targeted treatment has been tempted in that context. Older studies have shown contrasted clinical outcomes after immunotherapy, based on GM-CSF or IFNγ administration, despite efficacious restoration of mHLA-DR and/or IFNγ endogenous

secretion (22-24). In a hypothesis-driven approach, other markers such as elevated levels of regulatory T-helper cells ($T_{regs)}$ were recently shown to be predictive of nosocomial sepsis in combination with low levels of mHLA-DR and neutrophil CD88 in an ICU patient population comprising but not restricted to trauma and postoperative patients (25). So far, only three studies relying on wide flow cytometry panels to predict secondary sepsis in critically ill patients have been conducted and the first two included only septic patients (26-28). These authors showed that clinical deterioration at 48h could be predicted in septic patients with circulating immature granulocytes which induced T-cell lymphopenia after enrichment. A very recent study focused on the overtime changes of the injury-induced immune profile in a large cohort of septic, trauma and surgical patients during the first week of ICU admission (28). The authors used a restricted number of immune markers determined by flow cytometry, combined with transcriptomic and functional tests to show that the initial adaptive immune response to injury, whatever the etiology, was not associated with a risk of secondary infections. Moreover, only a subset of patients exhibiting late combined immune alterations (such as low CD3D, CD74 messenger RNA and mHLA-DR and high S100A9 messenger RNA at days 5-7) developed secondary infections. Our study aimed at describing the temporal changes of various leucocyte surface markers, via flow cytometric analysis, in non-septic patients, after critical injury, in association with nosocomial sepsis occurrence. The studied panel included subsets of B and T lymphocytes, as well as monocyte and neutrophil characterization.

2.1.1.3. MATERIALS AND METHODS

Study patients

This single-center, prospective, observational study was conducted in 3 tertiary ICUs over a 7-month period at CHU de Liège. The institutional ethics committee approved the study (Belgian number: B707201111981) and written informed consent was obtained from the patient or his/her legal representative. Inclusion criteria included: age over 18 years, elective cardiac surgery (CABG or valve replacement), trauma, acute ischemic or hemorrhagic stroke

and invasive ventilation (>48h) for reasons other than infection. Exclusion criteria were: life expectancy of less than 48h, systemic or oral antibiotic therapy for active infection, active hematological or solid organ proliferative disease, HIV (+) status, chronic viral hepatitis B and C and use of any immunosuppressive therapy. Upon admission to ICU, the following demographic characteristics were recorded: gender, age, type of admission (surgical or medical) and treatment with vasopressors. The sequential organ failure assessment score (SOFA) score was calculated (29). For each patient, the following data were also collected: length of ICU and hospital stay (days), duration of ventilation (days), administration of vasopressors prior to and during ICU stay, antibiotic treatment, site of infection and microbiological documentation, necessity of hemofiltration or intermittent hemodialysis during and/or after ICU stay. All patients included were followed up until 1 year after inclusion in the study or death. In case of death, time was recorded.

Blood samples were collected within 24 h (T1) of admission, 48 h (T2) after admission and on the day of diagnosis of sepsis and/or septic shock (Tx). The Sepsis-3 definition (30) was used for this study. Definitions of infection were based on Center for Disease Control (CDC) criteria (31-33). Our institution does not recommend routine use of selective digestive tract decontamination. Patients were compared to an age-matched (>50 years) cohort of healthy controls (n=18).

Immunophenotyping

Automated blood counts were obtained using the Sysmex XS-800 hematology analyzer (Kobe, Japan) for quantification of the absolute cell counts. Immunophenotyping was performed by adding combinations of monoclonal antibodies to 100 µl of whole blood, incubated for 20 minutes at 4°c in the dark, after which red cell lysis was achieved by adding BD FACS Lysing Solution. Cells were centrifuged and resuspended in HBSS 1% formaldehyde. Flow cytometric data were acquired on a FACS Verse flow cytometer (BD Biosciences). The daily setup procedure involved a one-step performance check, using BD FACSuite™ CS&T Research Beads to adjust photomultiplier tube voltages. This ensured that the target MFI values were held constant from day to day.

The following combinations of monoclonal antibodies were used. For NK cells and T lymphocytes: anti-CD3-FITC, CD4-PerCP, CD8-APC-H7, CD14-V450, CD45-V500, CD56-PE-Cy7, CD69-APC and CD279 PE. For B and regulatory T lymphocytes: anti-CD3-FITC, CD4-PerCP, CD19-PE-Cy7, CD25-PE, CD45-V500 and CD127-AlexaFluor 647. For monocytes: anti-CD14-V450, CD16-AlexaFluor647, CD45-V500, CD64-PE-Cy7, CD279-PE, and HLA-DR-PerCP. For neutrophils: anti-CD11b-PE, CD11c-PE, CD16-PE, CD45-V500, CD62L APC and CD64-PE-Cy7. All antibodies were from BD Biosciences.

Cytokine measurements

Plasma was prepared from citrated whole blood samples to quantify plasma levels of TNFα, IL-10, IL-17A, IL6, IL-7 and IFNγ. Cytokine levels were measured using multiplex Cytometric Bead Arrays (BD Biosciences) on the FACSVerse System. Analysis was performed with the FCAP Array™ software (BD Biosciences).

Statistical analysis

Results were expressed as mean and standard deviation (SD) for quantitative data and as median and interquartile range (IQR) for durations. For categorical findings, frequency tables were used. Comparisons between septic and non- septic patients characteristics were done by the ANOVA or Kruskal-Wallis test for continuous variables and Chi-square or Fisher exact test for categorical variables. The predictive value of sepsis was assessed for each baseline variable by logistic regression analysis on log-transformed biological variables. The variables significant at $p<0.10$ were combined in a stepwise multivariate logistic regression analysis to identify independent baseline predictors of sepsis. The odds ratio (OR) with 95% confidence interval [95%CI] and ROC (receiving operating curve) curve analysis with area under the curve (AUC) were used to quantify the ability of the selected predictors to discern between septic and non-septic patients. The Youden method was applied to define an optimal cut-off point for those predictors. Data recorded on the same patients but at different time points were compared by the Wilcoxon signed rank test. Results were considered significant at the 5% critical level ($p<0.05$). All statistical calculations were performed with SAS (version 9.4)

and R (version 3.0.3).

2.1.1.4. RESULTS

Patients baseline characteristics

A total of 99 adult patients with complete data were included in the final analysis. The demographic and clinical characteristics at admission are presented in Table 1. There were predominantly male patients (60.6%) with a mean age of 64 ± 15 years. The type of admission was mainly surgical (86.9%) and cardiac surgery accounted for most patients (68.7%). Ten (10.1%) patients received vasopressors before admission, 67 (67.7%) received prophylactic antibiotics during surgery. The median admission SOFA score was 5 [IQR: 4-8].

Table 1. Demographic and clinical characteristics of the patients at ICU admission (N=99)

	Total N=99	Nonseptic N=80	Septic N=19	p-value
Age (years)	64 ± 15	65± 15	62 ± 15	0.46
Gender: male	60 (60.6)	48 (60.0)	12 (63.2)	0.80
Surgical admission	86 (86.9)	70 (87.5)	16 (84.2)	0.70
Reason for admission				0.0022
Cardiac surgery	68 (68.7)	61 (76.2)	7 (36.8)	
Acute brain injury	12 (12.1)	6 (7.5)	6 (31.6)	
Trauma	13 (13.1)	10 (12.5)	3 (15.8)	
Ventilation > 48h	6 (6.1)	3 (3.8)	3 (15.8)	
SOFA at ICU admission	5 (4 – 8)	4 (3 – 7)	10 (8 – 12)	<0.0001
Diabetes	17 (17.2)	13 (16.2)	4 (21.0)	0.74
Cardiovascular disease	79 (79.8)	68 (85.0)	11 (57.9)	0.021
Vasopressor before admission	10 (10.1)	6 (7.5)	4 (21.0)	0.096
Prophylactic antibiotics	67 (67.7)	61 (76.2)	6 (31.6)	0.0002
Total hospital LOS (days)	11 (9 – 19)	11 (9 – 16)	26 (16 – 71)	<0.0001
ICU LOS ((days)	3 (2 – 7)	3 (2 – 4)	15 (10 – 22)	<0.0001
28-days mortality	13 (13.1)	6 (7.5)	7 (36.8)	0.0028
90-days mortality (N=97)	14 (14.4)	7 (8.9)	7 (38.9)	0.0038

ICU : Intensive Care Unit, SOFA: Sequential Organ Failure Assessment, LOS : Length Of Stay

Results are expressed has mean± SD, median (IQR), or n(%) as appropriate and p-values from ANOVA, Kruskal-Wallis, Chi-square or Fischer exact tests respectively

Sepsis occurrence

Nineteen (19.2%) patients developed sepsis or septic shock during follow-up, after a median time of 5 [IQR: 3-7] days and 80 did not. As shown in Table 1, age, gender, category of

admission, history of diabetes and use of vasopressor prior to ICU admission were not associated with sepsis occurrence. By contrast, higher SOFA score, admission for brain injury and lack of prophylactic antibiotics were predominant in patients who developed sepsis. Moreover, septic patients displayed higher hospital and ICU length-of-stay compared to non-septic patients (26 days [16-71] versus 11 days [9-16], $p<0.0001$ and 15 days [10-22] versus 3 days [2-4], $p<0.0001$, respectively). Septic patients also displayed a higher 28-day and 90-day mortality compared to non-septic patients (36.8% versus 7.5%, $p=0.0028$ and 38.9% versus 8.9%, $p=0.0038$, respectively). Infections sites and microbiological documentation are shown in Table S1.

Table S1

Site of infection	N (frequency of infection)	Microbiological documentation
HAP-VAP	16	*MSSA, Serratia Marcescens, Morganella Morganii,Klebsiella Pneumoniae, Haemophilus Influenzae, Moraxella Catarrhalis, Proteus Vulgaris, Citrobacter Koseri, Enterococcus Faecalis, Escherischia Coli, Klebsiella Ornitholytica*
SSTI	3	*Staphylococcus Epidermidis, Enterobacter Cloacae Complex, Enteroccus Faecalis*
CLABSI	1	*Staphylococcus Epidermidis*
BSI	3	*Escherichia Coli, Citrobacter Koseri, Morganella Morganii, Serratia Marcescens*

Sites of infection and microbiological documentation

HAP-VAP: hospital-acquired pneumonia

VAP: ventilator-associated pneumonia

SSTI: surgical site and soft tissue infection

CLABSI: central line associated blood stream infection

BSI: primary blood stream infection

Some patients developed more than one infection and some infections were polymicrobial. Two episodes of VAP were clinically diagnosed and empirically treated although no organism grew in culture

Standard laboratory tests and cytokines

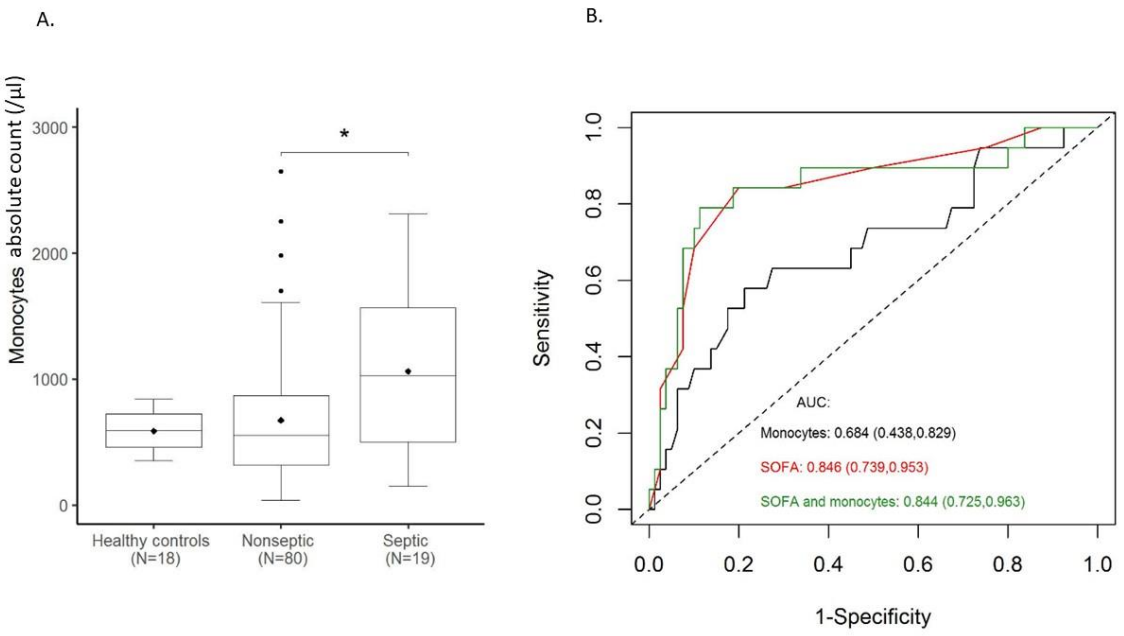
Comparison of standard laboratory tests and cytokine levels obtained within 24h after admission to the ICU is shown in Table 2. Absolute monocyte counts and CRP were significantly higher in patients who developed sepsis compared to non-septic patients (1030/µl versus 55/µl, p=0.013 and 5.1mg/ml versus 2.5mg/ml, p=0.046, respectively). Monocyte counts did not add to the performance of SOFA score alone (AUC 0.84 with a cut-off level >8) for prediction of secondary sepsis as shown in Fig S1.

Table 2. Comparison of biological parameter levels recorded upon admission to ICU according to later occurrence of sepsis (n = 99 patients)

	Non-septic n = 80	Septic n = 19	P-value
CRP (mg/ml)	2.5 (1.1-9.1)	5.1 (2.5-17.4)	0.046
Fibrinogen (g/l)	2.4 (2.0-3.0)	3.0 (2.0-3.7)	0.13
Platelet count (k/µl)	134 (105-166)	169 (117-213)	0.12
White blood cells count (K/µl)	9.0 (7.0-12.2)	9.8 (6.8-16.3)	0.47
TNFα(pg/ml)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.074
IL10 (pg/ml)	4.2 (0.0 - 11.8)	3.8 (0.0-10.1)	0.95
IL17A (pg/ml)	4.9 (0.76 - 11.6)	2.0 (0.0-7.8)	0.17
IL6 (pg/ml)	97.0 (34.8 - 189.2)	105.7 (39.3-240.3)	0.75
IL7 (pg/ml)	1.4 (0.17 - 4.3)	1.2 (0.21-1.5)	0.28
IFN?	0.0 (0.0 – 0.0)	0.0 (0.0-0.0)	0.66
Neutrophils (counts/µl)	7045 (5704 – 9344)	6405 (5919 – 7298)	0.62
Monocytes (counts/µl)	550 (320 – 873)	1030 (430 – 1600)	0.013
Lymphocytes (counts/µl)	1200 (810 – 1715)	1180 (990 – 1470)	0.97

Results are expressed as median and interquartile range (IQR). P-value of Kruskal-Wallis test; null values for TNFα and IFNγ correspond to values under the level of detection (3.8pg/ml); MFI, Median fluorescence intensity

Fig S1



Panel A: Measurements at ICU admission in nonseptic and septic patients and in healthy controls (> 50 years). (**: p<0.05)*

Panel B: Predictive value of monocyte absolute count (/µl) obtained at T1. ROC curve analysis of sepsis occurrence based on levels of monocytes and of SOFA is shown.

Leucocytes cell surface markers

When considering leucocytes subsets at T1 against healthy controls, elevated absolute counts of classical, intermediate and total monocytes, increased levels of $CD62L_{neg}$ monocytes and low expression of HLA-DR in total and intermediate monocytes were shown to be associated with further sepsis development in univariate analysis (Table 3). When all potential predictors of sepsis (p<0.10) recorded at ICU admission (T1) were combined into a stepwise logistic regression, only the absolute count of $CD62L_{neg}$ monocytes was independently associated with sepsis occurrence (OR[95%CI]: 4.5[1.4-14.5], p=0.011) (Fig.1A). By ROC curve analysis (Fig.1B), a cut-off value of 180/µl (AUC 0.69) was derived for $CD62L_{neg}$ monocytes at T1 to discriminate septic from non-septic patients. The $CD62L_{neg}$ monocytes count did not add to the performance of SOFA score alone for secondary sepsis

prediction, as seen in Fig.1B. In the 12 patients available for complete data at T1, T2 and Tx, there was no temporal change in the numbers of $CD62L_{neg}$ monocytes (Fig S2). When considering leucocyte subsets at T2, low expression of mHLA-DR by classical and intermediate monocytes and low levels of CD4+CD279+ lymphocytes were associated with sepsis development in univariate analysis (Table 4). When all potential predictors of sepsis ($p<0.10$) recorded at T2 were combined into a stepwise logistic regression, only low expression of HLA-DR by intermediate (CD14++CD16+) monocytes was independently associated with sepsis development (Fig.2A) (OR[95%CI]: 0.003[0-0.17], p=0.049). By ROC curve analysis (Fig.2B), a cut-off level of 1090 MFI (AUC 0.74) was derived for mHLA-DR to discriminate septic from non-septic patients. The level of m-HLA-DR did not add to the performance of SOFA score alone for secondary sepsis prediction, as seen in Fig.2B. In the 7 septic patients available for complete data at T1, T2 and Tx, there was no temporal change in the levels of the marker (Fig S3).

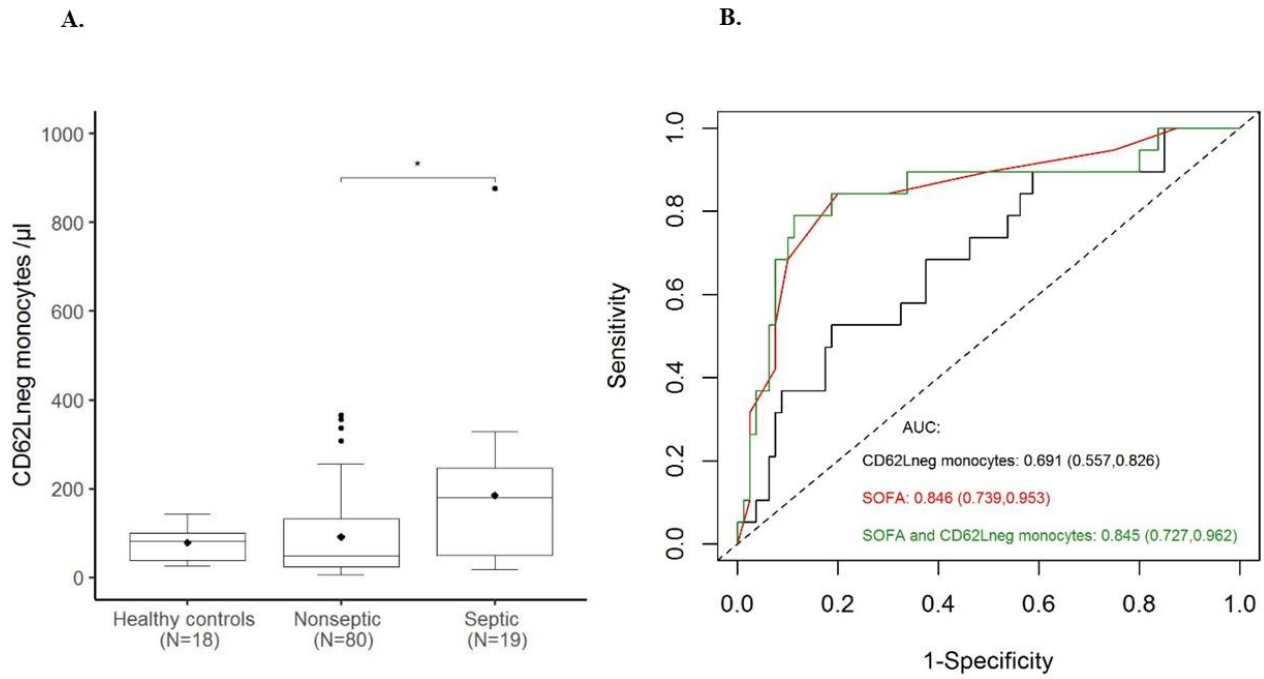
The temporal change (delta T2-T1) of the two monocyte markers, i.e. $CD62L_{neg}$ monocytes absolute count and HLA-DR expression by intermediate monocytes, was not predictive of sepsis occurrence (data not shown).

Table 3. Impact of parameters at ICU admission (T1) on the risk of sepsis.

	Nonseptic (N=80)			Septic (N=19)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1 ; Q3)	N	Mean ± SD	Median (Q1 ; Q3)	OR (95%CI)	p-value
HLA-DR MFI - total monocytes	80	1293 ± 632	1145 (805 ; 1682)	19	909 ± 477	776 (469 ; 1382)	0.030 (0.003 – 0.35)	0.0052
CD14 MFI - total monocytes	69	15709 ± 6886	13787 (11585 ; 19230)	12	15838 ± 7310	14613 (8432 ; 20030)	082 (0.022 – 31)	0.92
CD16 MFI - total monocytes	69	149 ± 167	111 (78 ; 171)	12	147 ± 70	150 (84 ; 190)	2.1 (0.28 – 15.2)	0.48
CD64 MFI - total monocytes	80	25273 ± 7449	23702 (19603 ; 29378)	19	25813 ± 5149	24679 (23119 ; 27938)	4.0 (0.052 – 301)	0.53
CD279 MFI - total monocytes	80	18 ± 100	-8.7 (-39 ; 43)	19	55 ± 127	33 (-24 ; 112)	2.9 (0.73 – 12)	0.13
Classical monocytes/µl	69	472 ± 324	419 (256 ; 598)	12	746 ± 433	742 (343 ; 1077)	11 (1.01 – 122)	0.049
Intermediate monocytes/µl	69	151 ± 171	82 (35 ; 221)	12	326 ± 221	392 (79 ; 502)	4.7 (1.2 – 19)	0.029
Non-classical monocytes/µl	57	22 ± 32	7.8 (3.3 ; 25)	10	36 ± 34	28 (5.7 ; 55)	2.7 (0.81 – 9.2)	0.11
CD279 MFI – classical monocytes	69	-16 ± 76	-23 (-58 ; 6.1)	12	3.8 ± 118	-20 (-58 ; 20)	1.1 (0.17 – 7.5)	0.90
HLA-DR MFI – classical monocytes	69	1126 ± 595	1030 (690 ; 1539)	12	756 ± 467	481 (373 ; 1155)	0.025 (0.001 – 0.47)	0.014
CD64 MFI – classical monocytes	69	25751 ± 7066	24756 (20707 ; 29028)	12	25712 ± 6122	24923 (22944 ; 27241)	1.3 (0.005 – 321)	0.93
CD279 MFI – intermediate monocytes	69	45 ± 109	11 (-7.4 ; 74)	12	103 ± 207	26 (-37 ; 164)	3.2 (0.60 – 17)	0.18
HLA-DR MFI – intermediate monocytes	69	1643 ± 791	1380 (1180 ; 2022)	12	1382 ± 756	1196 (647 ; 2053)	0.08 (0.003 – 2.1)	0.13
CD64 MFI - intermediate monocytes	69	25335 ± 7530	23912 (19508 ; 29242)	12	26032 ± 5305	25458 (22585 ; 29091)	4.4 (0.025 – 777)	0.58
CD279 MFI – non-classical monocytes	69	166 ± 133	142 (103 ; 211)	12	172 ± 85	192.3 (108 ; 221)	1.4 (0.21 – 8.9)	0.73
HLA-DR MFI – non-classical monocytes	69	6615 ± 4883	6328 (1962 ; 10108)	12	7973 ± 4160	6745 (4431 ; 11246)	4.0 (0.64 – 24.8)	0.14
CD64 MFI – non-classical monocytes	69	12141 ± 8841	8343 (5272 ;16776)	12	12653 ± 7537	10944 (6659 ; 16946)	1.9 (0.25 – 14)	0.55
CD62Lneg monocytes/µl	80	91 ± 94	48 (24 ; 131)	19	185 ± 196	179 (44 ; 247)	4.5 (1.4 – 14.5)	0.011
Total neutrophils/µl	80	7838 ± 3815	7045 (5365 ; 10160)	19	8601 ± 4456	7310 (4720 ; 12670)	2.1 (0.19 – 23)	0.55
CD62L MFI - neutrophils	80	7571 ± 2585	7677 (5704 ; 9344)	19	6658 ± 1751	6405 (5919 ; 7298)	0.21 (0.010 – 4.4)	0.32
CD16 MFI - neutrophils	69	1773 ± 654	1720 (1466 ; 2176)	12	1623 ± 395	1661 (1384 ; 1820)	0.49 (0.013 – 19)	0.70
CD64 MFI - neutrophils	80	1517 ± 1040	1293.5 (890 ; 1801)	19	1490 ± 876.1	1285 (699 ; 1849)	1.1 (0.20 – 6.4)	0.89
CD11b MFI - neutrophils	80	11569 ± 6583	9645 (7279 - 14752)	19	11000 ± 5935	9057 (6674 – 15051.)	0.59 (0.061 – 5.7)	0.65
CD11c MFI - neutrophils	80	723 ± 350	622.8 (522 ; 788)	19	853 ± 423	813 (476;- 982)	7.9 (0.47 – 131)	0.15
CD62Lneg neutrophils/µl	80	1067 ± 925	819.6 (245 ; 1613)	19	758 ± 698	487 (204 ; 1197)	0.54 (0.20 – 1.5)	0.24
Total lymphocytes/µl	80	1303 ± 688	1200 (810 ; 1715)	19	1261 ± 481	1180 (990 ; 1470)	1.2 (0.12 – 12)	0.87
CD4+ lymphocytes/µl	80	620 ± 342	610 (346.0 ; 829.1)	19	605 ± 232	612 (432 ; 779)	1.5 (0.21 – 11)	0.69
CD8+ lymphocytes/µl	80	281 ± 271	204 (143.7 ; 362.3)	19	261 ± 179	208 (150 ; 323)	0.88 (0.15 – 5.0)	0.88
CD4+CD69+ lymphocytes/µl	80	61 ± 62	45 (30.0 ; 67.0)	19	71 ± 46	54 (43 ; 97)	2.5 (0.52 – 12)	0.25
CD4+CD279+ lymphocytes/µl	80	168 ± 89	156 (101.8 ; 215.1)	19	189 ± 149	167 (113 ; 199)	1.5 (0.19 – 11)	0.71

CD8+CD69+ lymphocytes/µl	80	61 ± 102	33 (17.4 ; 53.7)	19	96 ± 125	51 (28 ; 137)	2.8 (0.93 – 8.2)	0.069
CD8+CD279+ lymphocytes/µl	80	89 ± 65	76 (46.8 ; 100.9)	19	103 ± 100	77 (47 ; 111)	1.5 (0.28 – 8.6)	0.62
CD69 MFI - CD4+CD69+ lymphocytes	80	369 ± 95	359 (320 ; 414)	19	343 ± 76	338 (280 ; 388)	0.042 (0.001 – 9.2)	0.25
CD69 MFI – CD8+CD69+ lymphocytes	80	683 ± 867	483 (397 ; 688)	19	1030 ± 1601	624 (504 ; 822)	4.4 (0.72 – 27)	0.11
CD279 MFI - CD4+CD279+ lymphocytes	80	232 ± 54	218 (193 ; 255)	19	236 ± 37	232 (206 ; 251)	4.3 (0.016 – 999)	0.61
CD279 MFI – CD8+CD279+ lymphocytes	80	269 ± 95	236 (201 ; 293)	19	310 ± 109	277 (247 ; 343)	26 (0.74 – 901)	0.073
B lymphocytes/µl	80	203 ± 242	149 (89 ; 233)	19	187 ± 166	147 (67 ; 225)	1.1 (0.28 – 4.0)	0.93
CD25+ B lymphocytes/µl	80	59 ± 218	16 (7.4 ; 37)	19	49 ± 91	19 (9.4 ; 40)	1.3 (0.51 – 3.0)	0.63
CD25 MFI - Tregs	80	3473 ± 691	3434 (2939 ; 3917)	19	3744 ± 980	3704 (291 ; 4682)	25 (0.096 – 999)	0.26
CD127 MFI - CD4+ lymphocytes	80	1378 ± 372	1313 (1111 ; 1660)	19	1420 ± 330	1496 (1095 ; 1671)	3.9 (0.05 – 304)	0.54
CD127 MFI - Tregs	80	209 ± 65	197 (162 ; 246)	19	211 ± 78	198 (161 ; 237)	0.89 (0.022 – 37)	0.95
Tregs/µl	80	59 ± 34	55 (32 ; 75)	19	56 ± 223	60 (39 ; 71)	1.0 (0.13 – 7.9)	0.99

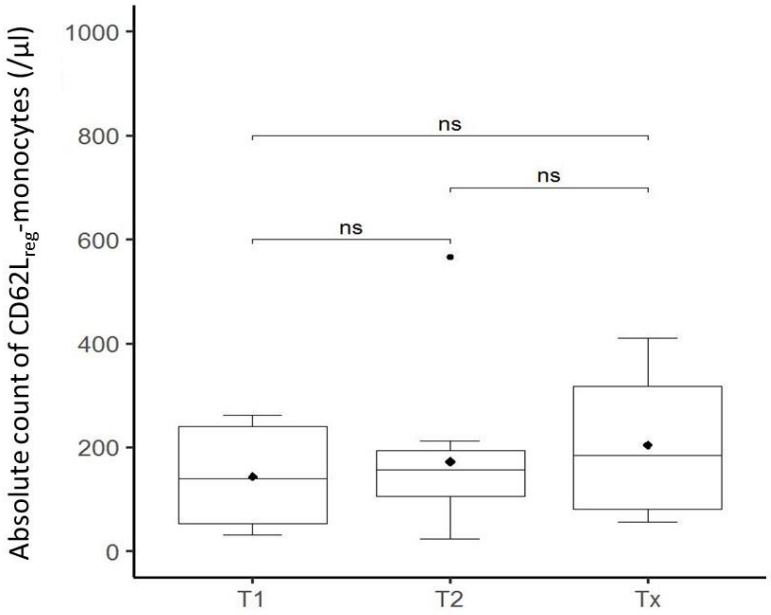
Fig 1



Panel A: Measurements at ICU admission in nonseptic and septic patients and in healthy controls (> 50 years). (**: p<0.05).*

Panel B: Predictive value of $CD62L_{neg}$ monocytes absolute count (/µl) obtained at T1. ROC curve analysis of sepsis occurrence based on levels of $CD62L_{neg}$ monocytes and SOFA is shown

Fig S2



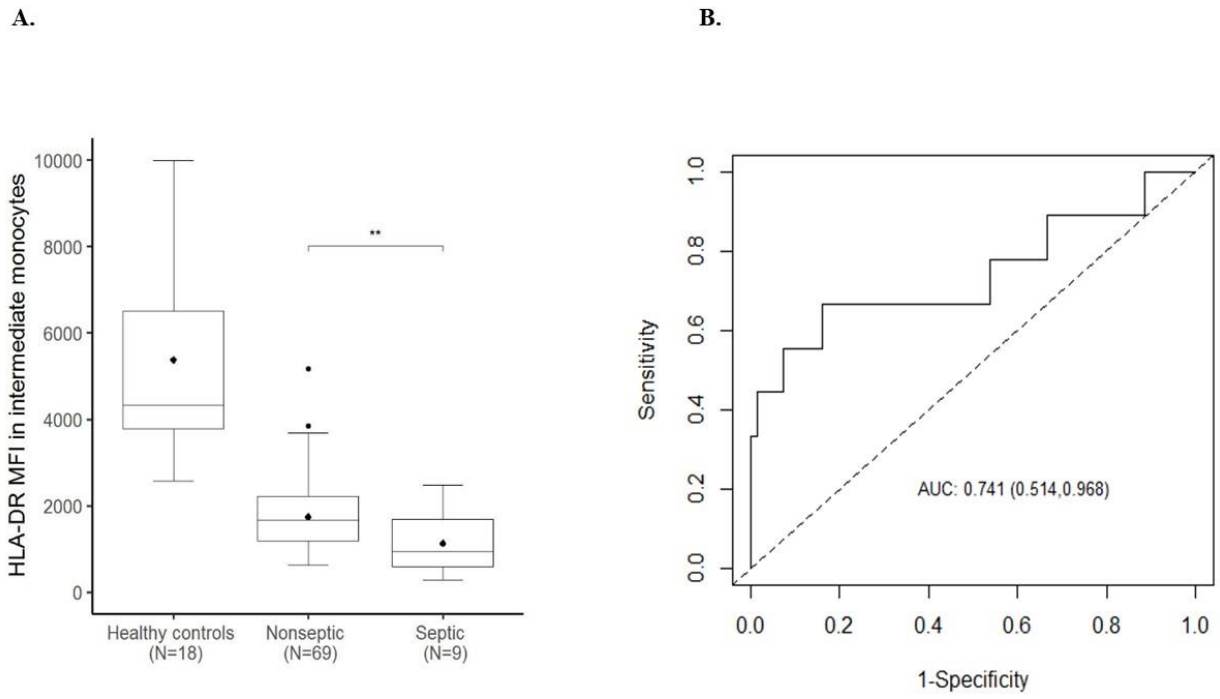
Absolute count of $CD62L_{neg}$ monocytes (/µl): evolution of septic patients (N=12 patients with measurement at ICU admission, 48 to 72h later and on the day of sepsis diagnosis). (ns: not statistically significant).

Table 4. Impact of parameters 48-72h after ICU admission (T2) on the risk of sepsis.

	Nonseptic (N=80)			Septic (N=19)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1 ; Q3)	N	Mean ± SD	Median (Q1 ; Q3)	OR (95%CI)	p-value
Total monocytes/µl	79	992 ± 452	950 (650 ; 1240)	15	1247 ± 997	980 (810 ; 1440)	4.5 (0.27 – 73)	0.30
HLA-DR MFI - total monocytes	79	1146 ± 556	972 (766 ; 1544)	15	690 ± 303	685 (435 ; 824)	0.004 (0.000 – 0.10)	0.0011
CD14 MFI - total monocytes	69	19581 ± 6805	18541 (15644 ; 23293)	9	19714 ± 5996	18758 (14917 ; 25446)	1.4 (0.011 – 169)	0.90
CD16 MFI - total monocytes	69	198 ± 127	171 (113 ; 240)	9	191 ± 88	174 (126 ; 198)	1.5 (0.077 – 28)	0.80
CD64 MFI - total monocytes	79	31472 ± 7345	32326 (26274 ; 36512)	15	28749 ± 7858	27011 (22435 ; 36294)	0.042 (0.000 – 4.7)	0.19
CD279 MFI - total monocytes	79	51 ± 160	24 (-21 ; 74)	15	100 ± 119	91 (20 ; 152)	2.9 (0.74 – 11)	0.13
Classical monocytes/µl	69	633 ± 328	589 (393 ; 828)	9	624 ± 245	562 (419 ; 747)	1.5 (0.054 – 43)	0.80
Intermediate monocytes/µl	69	271 ± 164	226 (143 ; 383)	9	283 ± 97	259 (195 ; 324)	3.3 (0.17 – 63)	0.43
Non-classical monocytes/µl	57	61 ± 47	50 (29 ; 75)	8	66 ± 31	68 (45 ; 83)	2.5 (0.25 – 26)	0.43
CD279 MFI – classical monocytes	69	6.6 ± 142	-19 (-39 ; 25)	9	62 ± 137	33 (-15 ; 43)	1.4 (0.32 – 5.8)	0.67
HLA-DR MFI – classical monocytes	69	976 ± 460	874 (642 ; 1296)	9	579 ± 228	560 (443 ; 761)	0.006 (0.000 – 0.26)	0.0081
CD64 MFI – classical monocytes	69	31749 ± 7633	32367 (26010 ; 36052)	9	29332 ± 7667	26825 (23234 ; 36640)	0.080 (0.000 – 26)	0.39
CD279 MFI – intermediate monocytes	69	89 ± 205	60 (-2.0 ; 115)	9	181 ± 191	154 (39 ; 178)	1.2 (0.35 – 4.0)	0.79
HLA-DR MFI – intermediate monocytes	69	1744 ± 769	1678 (1195 ; 2224)	9	1131 ± 733	941 (591 ; 1694)	0.003 (0.000 – 0.17)	0.0049
CD64 MFI - intermediate monocytes	69	33603 ± 7755	34545 (27854 ; 37880)	9	32401 ± 9496	30484 (24179 ; 38814)	0.20 (0.000 – 82)	0.60
CD279 MFI – non-classical monocytes	69	184 ± 124	160 (112 ; 232)	9	221 ± 97	206 (192 ; 241)	2.9 (0.29 – 30)	0.37
HLA-DR MFI – non-classical monocytes	69	8213 ± 3628	7898 (5738 ; 10756)	9	5929 ± 3234	5663 (3055 ; 7805)	0.074 (0.004 – 1.2)	0.070
CD64 MFI – non-classical monocytes	69	18781 ± 6999	18782 (14162 ; 23492)	9	17638 ± 6443	18175 (12364 ; 23814)	0.57 (0.018 – 19)	0.76
CD62Lneg monocytes/µl	79	158 ± 100	143 (82 ; 202)	15	170 ± 133	157 (74 ; 197)	0.94 (0.13 – 7.0)	0.95
Total neutrophils/µl	79	9057 ± 3048	8470 (7160 ; 10430)	15	8766 ± 3611	8040 (6780 ; 10560)	0.31 (0.007 – 13)	0.54
CD62L MFI - neutrophils	79	6618 ± 1519	6787 (5603 ; 7695)	15	6548 ± 2394	5984 (5057 ; 7099)	0.34 (0.002 – 50)	0.67
CD16 MFI - neutrophils	69	1956 ± 689	1872 (1442 ; 2318)	9	1863 ± 611	2027 (1233 ; 2495)	0.52 (0.007 – 40)	0.77
CD64 MFI - neutrophils	79	1901 ± 926	1619 (1263 ; 2305)	15	2281 ± 1689	1688 (1018;- 2787)	2.1 (0.16 – 27)	0.57
CD11b MFI - neutrophils	79	12027 ± 6866	9577 (7330 ; 16463)	15	13650 ± 7155	13145 (8932 ; 19791)	2.3 (0.23 – 23)	0.48
CD11c MFI - neutrophils	79	1220 ± 641	1056 (748 ; 1512)	15	1329 ± 687.0	1250.7 (666 ; 1926)	1.8 (0.13 – 26)	0.66
CD62Lneg neutrophils/µl	79	853 ± 849	512 (324;- 974)	15	827 ± 1153	551.3 (271 ; 893)	0.78 (0.19 – 3.2)	0.73
Total lymphocytes/µl	79	1220 ± 620	1140 (830 ; 1540)	15	1011 ± 336	1120 (760 ; 1210)	0.21 (0.013 – 3.5)	0.28
CD4+ lymphocytes/µl	79	524 ± 240.8	490 (356 ; 639)	15	434 ± 175	421 (291 ; 568)	0.15 (0.009 – 2.7)	0.20
CD8+ lymphocytes/µl	79	255 ± 172.9	206 (135 ; 344)	15	213 ± 131	183 (90 ; 308)	0.49 (0.084 – 2.9)	0.43
CD4+CD69+ lymphocytes/µl	79	60 ± 34.8	51 (33 ;- 75)	15	57 ± 30	61 (35 ; 70)	0.87 (0.10 – 27.6)	0.90
CD4+CD279+ lymphocytes/µl	79	179 ± 98	159 (116 ; 217)	15	121 ± 59	107 (97 ; 121)	0.044 (0.003 – 0.69)	0.026

CD8+CD69+ lymphocytes/µl	79	53 ± 89	27 (19 ;- 53)	15	71 ± 65	46 (24 ;- 97)	2.8 (0.75 – 11)	0.12
CD8+CD279+ lymphocytes/µl	79	95 ± 70	77 (50 ; 126)	15	80 ± 62.2	55 (31 ; 106)	0.56 (0.10 – 3.1)	0.50
CD69 MFI - CD4+CD69+ lymphocytes	79	329 ± 56	324 (289 ; 356)	15	343 ± 81	319 (287 ; 381)	11 (0.008 – 999)	0.51
CD69 MFI – CD8+CD69+ lymphocytes	79	771 ± 1162	527 (410 ; 731)	15	733 ± 441	605 (490 ; 725)	1.7 (0.19 – 16)	0.64
CD279 MFI - CD4+CD279+ lymphocytes	79	258 ± 58	247 (212 ; 283)	15	247 ± 24	253 (221 ; 257)	0.25 (0.001 – 226)	0.69
CD279 MFI – CD8+CD279+ lymphocytes	79	300 ± 91	289 (235 ; 346)	15	319 ± 79	311 (241 ; 385)	8.2 (0.092 – 724)	0.36
B lymphocytes/µl	79	216 ± 297	159 (108 ; 223)	15	191 ± 252	116 (56 ; 235)	0.50 (0.11 – 2.3)	0.38
CD25+ B lymphocytes/µl	79	66 ± 285	167 (7.5 ; 31)	15	55 ± 147	14 (7.4 ;- 35)	0.85 (0.29 – 2.5)	0.76
CD25 MFI - Tregs	79	3970 ± 1060	3818 (3319 ; 4600)	15	4150 ± 942	4135 (3593 - 4633)	6.2 (0.039 – 999)	0.48
CD127 MFI - CD4+ lymphocytes	79	1193 ± 373	1181 (878 ; 1493)	15	1230 ± 337	1195 (997 ; 1551)	2.9 (0.049 – 171)	0.61
CD127 MFI - Tregs	79	184 ± 69	176 (145 ;- 217)	15	197 ± 65	204 (133 - 237)	4.7 (0.12 – 180)	0.41
Tregs/µl	79	53 ± 27	46 (34 ; 72)	15	41 ± 16	37 (30 – 48)	0.12 (0.008 – 2.0)	0.14

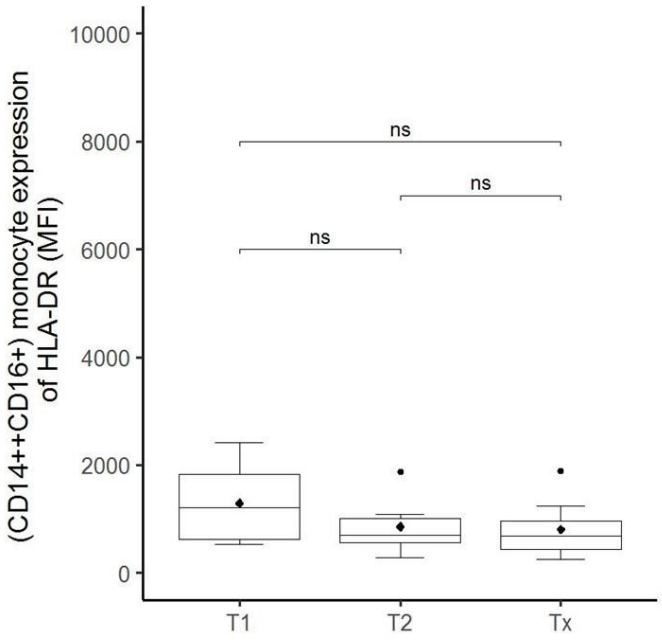
Fig 2



Panel A: Measurements at T2 in nonseptic and septic patients and in healthy controls (> 50 years). (**: p<0.001).

Panel B: Predictive value of intermediate (CD14++CD16+) monocyte expression of HLA-DR (MFI) obtained at T2

Fig S3



Intermediate monocytes (CD14++CD16+) median HLA-DR (MFI): evolution of septic patients (N=7 with measurement at ICU admission, 48 to 72h later and on the day of sepsis diagnosis). (ns: not statistically significant).

2.1.1.5. DISCUSSION

In this single-center study, we showed that, in critically ill injured adults, increased levels of absolute monocyte counts and of $CD62L_{neg}$ monocytes at ICU admission and reduced mHLA-DR in intermediate monocytes 48-72h later, were independently associated with later sepsis occurrence. To the best of our knowledge, such a wide leucocyte panel, including 63 markers, exploring innate and adaptive immunity by flow cytometry, has not been reported in critical injury (34). Concerning the absolute count of monocytes, although these cells exert a pivotal role in sepsis, the diagnostic and prognostic value of monocyte count is contrasted in the literature (35). Small observational trials including mainly trauma and sepsis patients have shown elevated or low monocyte counts to be associated with sepsis occurrence or outcome (36-39). A very recently published observational study including more than 300 severely injured patients (out of which a third were already septic patients) looked into 30 immune markers, among which 12 were determined by flow cytometry (28). The authors

showed that monocyte count was not associated with secondary infection acquisition.

Considering the downregulation of L-selectin, identified here as increased numbers of $CD62L_{neg}$ monocytes, little is known in terms of sepsis prediction apart from conflicting data in neonates (40-42). In a prospective older study including newborn infants with suspected bacterial infection, L-selectin expression was significantly reduced in both granulocytes and monocytes of infected newborns compared with controls (41). L-selectin is a leucocyte surface glycoprotein which mediates extravasation and recruitment of white blood cells to sites of inflammation. Its downregulation *in vitro* had been shown in murine and human neutrophils and this was the first report of *in vivo* downregulation of L-selectin on granulocytes and monocytes (43-46). Authors postulated that bacterial stimuli such as FMLP (N-formyl-methionyl-leucyl-phenylalanine)-related peptides or lipopolysaccharides or host-derived soluble mediators such as those released during acute systemic inflammatory response syndrome (cytokines, C5a, leukotriene B4) may have triggered L-selectin downregulation. Furthermore, a more recent study focusing on regional and systemic immune responses before, during and after major splanchnic surgery showed that intraoperative splanchnic hypoperfusion and mucosal acidosis led to monocyte deactivation (47). In that study, 20 patients who underwent resection for cancer of the esophagus, had no difference in monocyte marker expression in the preoperative period. They were categorized into 3 groups according to the nadir perioperative intestinal pH. Those who developed postoperative sepsis (5/20) had the lowest intestinal pH, a persistently lower postoperative expression of L-selectin and m-HLA-DR and a more acute phase response (higher CRP) compared to non-sepsis patients, similar to our findings. The authors concluded that severe mucosal acidosis, secondary to splanchnic hypoperfusion and increased intestinal permeability during major surgery, was associated with regional and systemic immune suppression predisposing to sepsis.

Our results are not in accordance with an observational study including 41 severely traumatized patients who underwent sampling and staining of 3 leucocyte subsets for CD62L, 1h and 20 hours after trauma(48). The authors found that monocytes, lymphocytes and neutrophils showed an early increase in CD62L cell surface expression and that this persisted in the later samples up to 20 hours. However, association with subsequent sepsis occurrence was not an endpoint in the latter study. In a more recent study aiming at guiding

the optimal timing of non-lifesaving orthopedic surgery for trauma, authors hypothesized that neutrophils and monocytes express activation markers prior to sepsis development(49). They found that in the perioperative period, elevated monocyte L-selectin (AUC 0.76 [95%CI 0.63-0.89] was a significant predictor of sepsis, thereby precluding urgent surgery. However, these patients were not critically ill.

Considering expression of mHLA-DR, our results confirm those of older single-center single-biomarker studies (20, 21) and of two more recent multi-center studies (25, 28). The first multi-center study validated a combined immune dysfunction score associated with sepsis development in a cohort of patients described as requiring organ support for more than 48h in the ICU (25). Trauma and surgery were among the inclusion criteria but sepsis patients were also included. The score encompassed low mHLA-DR (Youden index optimal cutoff <10000 molecules/cell), elevated T_{regs} and low neutrophil CD88. In our study, T_{regs} were not found to be predictive of sepsis probably because of earlier serial sampling and different case-mix. Indeed, elevation of T_{regs} was only seen 6-10 days after ICU admission in the aforementioned study and sepsis patients were included, contrary to our study. Elevated levels of these suppressor cells have frequently been reported in sepsis patients, reflecting severity of disease and predisposition to secondary infections, but very seldomly in injury, such as in our study, prior to the occurrence of a primary infection (50-52). The second recent large multicenter study explored mHLA-DR and *ex vivo* TNF-α release in sepsis, trauma and postoperative patients in association with adverse clinical outcome (death or secondary infection)(19, 28). It showed persistent decreases of both markers at days 5-7 post ICU admission to be associated with both outcomes, whatever the type of injury.

Furthermore, our results are partly corroborated by a recent monocentric study investigating the potential of HLA-DR expression by monocyte subsets in diagnosing sepsis in cardiac surgery patients (53). The authors showed that that there was a significant downregulation, in the postoperative period, of mHLA-DR on both intermediate (p=0.0477) and non-classical monocytes (p=0.033). However, in contrast to our findings, it is the combination of the reduced preoperative count and postoperative HLA-DR expression of the non-classical compound that was found to be associated with sepsis occurrence at 48h post cardiac surgery, with a 100% sensitivity and 69.2% specificity.

Another monocentric study, which took place in the emergency department and which included mostly patients with suspected infection among which very few Sepsis-3 patients (16/291) showed that in the latter cohort of patients, the combination of mHLA-DR on CD14+ monocytes, hyaluronidase and creatinine levels yielded an interesting AUC 0.92 [95%CI: 0.87-0.97] for prediction of sepsis(54). The authors argued that this study actually addressed mostly accurate detection of bacterial infection in non-severe patients (hence difficult to flag outside the ICU), with the combination of a limited number of biomarkers among which HLA-DR% on CD14%+ monocytes was consistently lower in infected patients.

Finally, our results are not in accordance with a recent multicenter study which aimed at discriminating SIRS (Systemic Inflammatory Response Syndrome) from sepsis in ICU patients(55). However, this study included patients according to the old Sepsis-2 definition of sepsis (56) but established sepsis diagnosis according to the Sepsis-3 criteria and hence, poor discriminative validity of biomarkers (among which mRNA HLA-DR) which was derived, might be misleading. Moreover, heterogeneous patients were sampled only once, upon AMT initiation, hence not in the period preceding sepsis and without serial kinetics.

Our study has several limitations among which, a single-center design and a small sample size. Furthermore, due to its exploratory nature, there was no *a priori* planned hierarchical clustering of surface markers, rendering consistency and fit-of-the model arguable. Validation of the two monocyte markers and of sampling times in a bigger cohort of patients could help to identify an optimal combination for sepsis prediction. Third, sampling times were limited and evolution of the biomarkers cannot be inferred past the third day of ICU admission. Furthermore, in patients who went on to develop sepsis, there are missing data in 7/19 for $CD62L_{neg}$ monocytes and 12/19 for mHLA-DR, respectively, thereby hindering interpretation of the biomarkers' levels time course. Fourth, potential confounders affecting the immune response to injury, such as blood transfusions and general anesthetics, were not taken into account at this stage(57). Fifth, sepsis occurrence was lower than expected (19% versus 25-35% in other studies) probably owing to the predominance of cardiac surgery patients who received prophylactic antibiotic therapy. Finally, we cannot exclude that some patients might have been in a pre-septic condition although high expression of neutrophil

CD64, which is a recognized marker of bacterial infection, was not found at ICU admission (58-61). Furthermore, CRP and fibrinogen levels were within normal ranges at ICU admission. It must be emphasized that procalcitonin was purposely not included in the design of the study because of known poor specificity as a diagnostic marker of sepsis in injured patients, as shown previously by our group (62).

In conclusion, this preliminary study showed that, in a selected population of critically injured patients, monocytes either in absolute count or via downregulation of specific surface markers, are predictive of subsequent sepsis development upon ICU admission and 48h later. Further validation in a bigger cohort of patients, perhaps in combination with recently published biomarkers, is warranted before envisaging a preventive immunomodulatory approach of sepsis in injured patients (63). In clinical practice, the latter approach could be feasible thanks to the readily available complete blood count and to a recent proof-of-concept study showing promising results for mHLA-DR bedside monitoring (64).

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2.1.2. SEPSIS PREDICTION IN CRITICALLY ILL PATIENTS BY PLATELET ACTIVATION MARKERS ON ICU ADMISSION: A PILOT STUDY.

Layios *et al. Intensive Care Medicine Experimental* (2017) 5:32
DOI 10.1186/s40635-017-0145-2

Intensive Care Medicine Experimental

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Sepsis prediction in critically ill patients by platelet activation markers on ICU admission: a prospective pilot study

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2.1.2.1. BACKGROUND

The Third International Consensus Task Force (Sepsis-3) defines sepsis as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (1). In this concept, growing experimental and preclinical evidence indicates that platelets could play an active role either in immune surveillance or in the response to infection. Indeed, in addition to their role in hemostasis and thrombosis, several studies in animal models suggest a contribution of platelets to infectious diseases due to their ability to influence innate and adaptive immune responses (2). First, platelets may act as sentinels of the immune system. They indeed express many major receptors of the innate immune system, including most Toll-like receptors (TLRs). Platelets are able to recognize molecular features of microbes and secrete many immunomodulatory mediators essential for alerting and recruiting cells of the immune system (3-7). Second, platelets may contain infection both directly and through functional interactions with immune cells (8). Platelets produce various antimicrobial molecules, including defensin, thrombocidins, and kinocidins, and they are able to interact with and kill bacteria directly (9-11). For instance, it has been shown that activated platelets facilitate the clearance of adherent Streptococci in experimental infective endocarditis (12); β-defensins released from platelets activated by the Staphylococcus aureus α-toxin impair bacterial

growth and induce neutrophil extracellular trap formation (4). Platelets also help trap blood pathogens on Kupffer cells in hepatic sinusoids, which limits systemic infection (13). Notably, platelets express CD40L, an essential player in host defense against infection that mediates interactions between platelets, antigen- presenting cells, and lymphocytes (14).

In overwhelming sepsis, platelets contribute to activation of the procoagulant cascade and ensuing complications linked to microvascular thrombosis and subsequent organ dysfunction(15). It has been demonstrated that critically ill injured adult patients, such as burn, trauma, or cardiac surgery patients, experience susceptibility to sepsis because of innate and adaptive immune reprogramming due to the insult (16, 17). However, whether platelets may participate in dysregulated host response to infection leading to sepsis remains unclear. One recent study showed that immature platelet fractions (IPF) could predict sepsis occurrence in critically ill subjects (18). Further, in severe trauma, platelet activation and leukocyte-platelet aggregate formation have been incriminated in the pathogenesis of tissue lesions leading to organ failure (19). The present prospective observational study hypothesized that platelet activation markers triggered by common injuries may help to predict occurrence of sepsis in specific ICU patient populations.

2.1.2.2. MATERIALS AND METHODS

Study patients

This was a single-center, prospective, observational, 7-month study based on a cohort of 99 consecutive adult patients, expected to stay for at least 48 h in tertiary ICU. Inclusion criteria included elective cardiac surgery (coronary artery bypass grafting or valve replacement), trauma, invasive ventilation >48 h for reasons other than sepsis, and acute brain injury (including subarachnoid, subdural, intra-parenchymal hemorrhage, and ischemic stroke). Patients were excluded from the study if they received oral or parenteral antibiotics other than for prophylaxis and if they were treated with any immunosuppressive agent except substitutive doses of corticosteroids, suffered from chronic hepatitis B or C, HIV, solid organ, or hematologic proliferative disease.

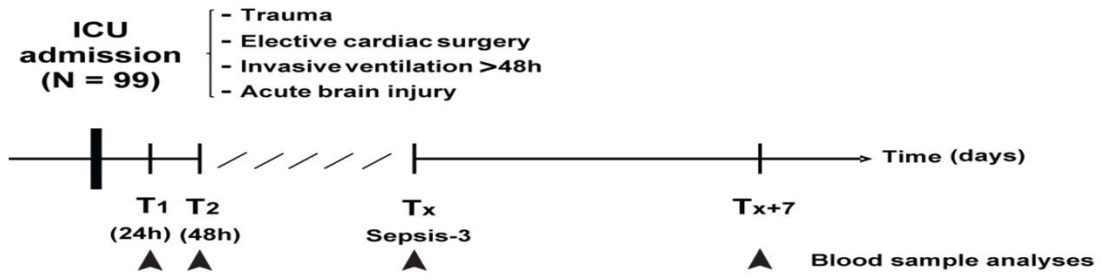
Characteristics at ICU admission

Upon admission to ICU, the following baseline characteristics were recorded: gender, age, type of admission (surgical or medical), history of diabetes and cardiovascular disease, previous treatment by vasopressor, prophylactic antibiotics, aspirin, and anticoagulants (anti-αIIbβ3). The sequential organ failure assessment (SOFA) score was computed. Blood samples were collected within 24 h (T1) for flow cytometry analyses (see the "Flow cytometry" section below). The following laboratory parameters were also assayed: C-reactive protein (CRP, mg/ml), fibrinogen (g/l), partial thromboplastin time (PTT, s), prothrombin time index (%), platelet count (k/µl), D-dimers (µg/l), and WBC count (K/µl). The ISTH scoring system for overt disseminated intravascular coagulation (DIC) was calculated based on Toh et al (20).

Follow-up and sepsis occurrence

Patients were sampled again 48 h (T2) after admission, on the day of diagnosis of sepsis (Tx), and 7 days later. All blood specimens were analyzed by flow cytometry as in T1 (Fig.S1).

Fig S1



Additional Figure 1. Follow-up and sepsis occurrence. Timeline of samplings.

Criteria for sepsis or septic shock are in agreement with the new definitions of sepsis (Sepsis-3) (1). For each study patient, the following data were also collected: length of ICU and of hospital stay (days), duration of ventilation (days) if required, administration of vasopressor during ICU admission, antibiotic treatment, use of curative antibiotics, red blood cell transfusion, plasma transfusion and platelet transfusion, and hemofiltration or intermit- tent hemodialysis during or after ICU stay. In case of death, time was also recorded. In case of discharge from the hospital, follow-up was at least 1 year.

Flow cytometry

Citrated whole blood was collected through an indwelling arterial catheter. Samples were processed within maximum 1 h following blood drawing. Platelet activation levels were assessed by measuring the expression of P-selectin (PS), a marker of degranulation, and fibrinogen (Fg) binding, as a result of integrin αIIbβ3 activation, on cell surface. Specifically, blood samples were fixed and incubated with peridinin-chlorophyll protein-linked (PerCP)-anti-CD61 antibodies (BD Biosciences), fluorescein isothiocyanate-linked (FITC)- anti-fibrinogen antibodies (Dako), and phycoerythrin-linked (PE)-anti-CD62P antibodies (BD Biosciences). Levels of platelet activation markers were determined by recording medians of FITC and PE fluorescence intensity (MFI) in platelets (CD61 positive cells) and percentages (%) of fibrinogen-positive (FITC) or CD62P-positive (PE) platelets on a FACS Verse flow cytometer (BD Biosciences). Data were analyzed using the BD FACSuite software. Platelets-monocytes and platelets-neutrophils aggregates were analyzed in citrated whole blood samples using an antibody panel, including anti-CD45-V500, anti-CD14- APC (monocytes), anti-CD15-PE (neutrophils), and anti-CD61-PerCP. Medians of CD61- PerCP fluorescence intensity in CD14-positive and CD15-positive cells, and percentages of cells double positive for CD61 and CD14, or CD61 and CD15 were recorded as above. In all cases, threshold of positivity was set by use of marker-specific antibodies or their corresponding IgG isotype controls in blood samples that were left unstimulated or activated with a supra-optimal dose of collagen-related peptide. Plasma was prepared from the citrated whole blood samples to quantify plasma levels of TNFα, IL10, sCD40L, IL17A, IL6, IL7, and IFNγ, all expressed in pg/ml. Cytokine levels were measured using customized multiplex BDTM Cytometric Bead Array on the FACSVerse System. Analysis was performed with the FCAP ArrayTM software.

Statistics

Results were expressed as mean and standard deviation for quantitative data and as median and interquartile range (IQR) for durations. For categorical findings, frequency tables were used. The predictive value of sepsis was assessed for each baseline variable by logistic regression analysis. Then variables significant at $P < 0.10$ were combined in a stepwise logistic regression analysis to identify independent baseline predictors of sepsis. The odds ratio (OR) with 95% confidence interval (95%CI) and ROC curve analysis with AUC were used to quantify the ability of the selected predictors to discern patients who will later develop sepsis. The Youden method was applied to define an optimal cutoff point for platelet marker predictors and SOFA score. Comparisons of hospital data and outcomes between septic and non-septic patients were done by the Kruskal-Wallis test for continuous variables and the Fisher exact test for categorical variables. Data recorded on the same patients but at different time points were compared by the Wilcoxon signed rank test. Results were considered significant at the 5% critical level ($P < 0.05$). All statistical calculations were performed with SAS (version 9.4) and R (version 3.0.3).

2.1.2.3. RESULTS

Baseline characteristics of patients

The baseline ICU admission characteristics of the 99 study patients are displayed in additional Table 1.

Additional Table 1 - Baseline clinical characteristics of study patients (n=99)

Variable	Baseline[1]
Age (years)	64 ± 15
Gender (male)	60 (60.9)
Category of admission	
Surgical	86 (86.9)
Medical	13 (13.1)
Reason for admission	
Cardiac surgery	68 (68.7)
Acute brain injury	12 (12.1)
Trauma	13 (13.1)
Ventilation >48h	6 (6.1)
Score at admission	
SOFA	6.0 ± 3.3
Diabetes	17 (17.2)
Cardiovascular disease	79 (79.8)
Vasopressor before the admission	10 (10.1)
Prophylactic antibiotics	67 (67.7)
Aspirin	53 (53.5)
Anticoagulant	14 (14.1)

[1]Mean ± SD for quantitative variable and number (%) for qualitative parameters

There were 60 men and 39 women aged 64±15 years. The type of admission was surgical for 86 patients and the main reason was predominantly cardiac surgery (68.7%). Sixty-seven patients received prophylactic antibiotics for surgery, 53 were under aspirin, 3 took αIIbβ3 antagonists and 14 patients took prophylactic anticoagulants. The mean SOFA score was 6.0±3.3. Data of routine biological parameters and flow cytometry results upon admission and 48 h later are displayed in additional Table 2. No difference was evidenced between

aspirin (n = 53) or anticoagulant users (n = 14) and non-users in terms of their biological profile (data not shown).

Additional Table 2 - Baseline and 48-hour biological characteristics of study patients (n=99)

Variable	Baseline	48h
Routine		
CRP (mg/L)	17.1 ± 43.4	93.9 ± 182.6
Fibrinogen (g/L)	2.7 ± 1.3	NA
PTT (s)	14.4 ± 2.1	NA
Prothrombin Time Index (%)	66 ± 16.4	NA
Platelet count (10^3/□L)	126 ± 61	111 ± 58
D-dimers (□g/L)	2977 ± 6124	2100 ± 4111
DIC score	1.8 ± 1.3	NA
White blood cell count (10^3/□L)	10.2 ± 4.6	11.3 ± 3.5
Flow cytometry		
TNF-□□(pg/mL)	0.27 ± 1.0	0.17 ± 0.82
IL-10 (pg/mL)	17.4 ± 81.2	2.8 ± 7.2
sCD40L (pg/mL)	82 ± 77.1	89.9 ± 64.1
IL-17A (pg/mL)	8.9 ± 12.2	7.8 ± 11
IL-6 (pg/mL)	402 ± 2404	122 ± 266
IL-7 (pg/mL)	2.6 ± 3.5	2.7 ± 4.4
IFN-□ (pg/mL)	0.12 ± 0.83	0.04 ± 0.21
Platelet-Fg (%)	33.6 ± 30.5	70.2 ± 25.5
Platelet-Fg (MFI)	1960 ± 1335	3388 ± 1301
Platelet-PS (%)	3 ± 2.4	3.1 ± 2.0
Platelet-PS (MFI)	33.6 ± 30.5	70.2 ± 25.5
Platelets-neutrophils (%)	3.6 ± 5.2	3.3 ± 2.9
Platelets-neutrophils (CD61 MFI)	315 ± 128	302 ± 82
Platelets-monocytes (%)	20.3 ± 23.4	20.0 ± 16.5
Platelets-monocytes (CD61 MFI)	1443 ± 2597	1102 ± 1149

Results are expressed as means ± SD. Platelet-Fg, platelet-bound fibrinogen; platelet-PS, platelets expressing P-selectin

on their surface; null values for TNF-α and IFN-γ correspond to values under the level of detection (3.8pg/ml); MFI,

Median fluorescence intensity; %, percentage of positive cells for the indicated marker; NA, not available

Sepsis occurrence

Of the 99 study subjects, 19 (19.2%) developed sepsis after a median time of 5 [IQR 3–7] days and 80 did not. As seen in Table 1, age, gender, type of admission, history of diabetes, use of vasopressor, anti-platelet, or anticoagulation medication use were not associated with sepsis occurrence. By contrast, patients who later developed sepsis presented with higher SOFA score at admission. They were also predominantly admitted for acute brain surgery or prolonged ventilation and lacked prophylactic antibiotics prior to admission.

Table 1 Predictive value of patient demographic and baseline clinical data for sepsis development during ICU stay

Variable	Development of sepsis[a]		*P* value[b]
	No (*N* = 80)	Yes (*N* = 19)	
Age (years)	65 ± 15	62 ± 15	0.46
Gender			0.80
Male	48 (80)	12 (20)	
Female	32 (82.1)	7 (17.9)	
Category of admission			0.70
Surgical	70 (81.4)	16 (18.6)	
Medical	10 (76.9)	3 (23.1)	
Reason for admission			0.0052
Cardiac surgery	61 (89.7)	7 (10.3)	
Acute brain injury	6 (50)	6 (50)	
Trauma	10 (76.9)	3 (23.1)	
Ventilation >48 h	3 (50)	3 (50)	
Score at admission			
SOFA	5.2 ± 2.7	9.6 ± 3.1	<0.0001
Diabetes			0.62
Yes	13 (76.5)	4 (23.5)	
No	67 (81.7)	15 (18.3)	
Cardiovascular disease			0.012
Yes	68 (86.1)	11 (13.9)	
No	12 (60)	8 (40)	
Vasopressor before the admission			0.091
Yes	6 (60)	4 (40)	
No	74 (83.2)	15 (16.8)	
Prophylactic antibiotics			0.0005
Yes	61 (91)	6 (9)	
No	19 (59.4)	13 (40.6)	
Aspirin			0.93
Yes	43 (81.1)	10 (18.9)	
No	37 (80.4)	9 (19.6)	
Anticoagulant			0.24
Yes	13 (92.9)	1 (7.1)	
No	67 (78.8)	18 (21.1)	

[a]Means ± SD for quantitative variable and numbers (%) for qualitative parameters
[b]Logistic regression

Complementary results of septic compared to non-septic patients are shown in additional Table 3.

Additional Table 3 – Comparison of ICU- and hospital-related characteristics of patients with and without sepsis

Variable	Patients with sepsis[1]		P-value[2]
	No	Yes	
Duration of ICU stay (days)	3 (2-4)	15 (10-22)	<0.0001
Duration of hospital stay (days)	11 (9-16)	26 (16-71)	<0.0001
Ventilation	12 (15.0)	16 (88.9)	<0.0001
Duration of ventilation (days)	1 (1-1)	10 (6-15)	<0.0001
Vasopressor during the admission in ICU	14 (17.5)	12 (66.7)	<0.0001
Antibiotic treatment	62 (77.5)	12 (66.7)	0.24
Curative antibiotics	1 (1.3)	6 (33.3)	0.0001
Red blood cell transfusion	17 (21.3)	6 (33.3)	0.37
Plasma transfusion	9 (11.3)	2 (11.1)	0.99
Platelet transfusion	7 (8.9)	2 (11.1)	0.68
Hemofiltration or intermittent haemodialysis	0 (0)	6 (33.3)	<0.0001
28-day mortality	6 (7.5)	6 (33.3)	0.0055
90-day mortality	7 (8.9)	7 (38.9)	0.0026

[1]Medians and IQR for duration values and numbers (%) for qualitative parameters

[2]P-value of Kruskal-Wallis test or Fisher exact test

Moreover, when considering laboratory tests and flow cytometry parameters recorded within 24 h of admission to ICU, D-dimers and fibrinogen binding to platelets (platelet-Fg expressed as MFI or %) were markedly higher ($P < 0.001$) in patients who later developed sepsis (Table 2). To a lesser extent, ISTH DIC score ($P < 0.05$) also differed between septic and non-septic patients. Interestingly, levels of sCD40L, P-selectin on circulating platelets (MFI or %), platelets-monocytes, and platelets-neutrophils aggregates were not associated with sepsis occurrence.

Table 2 Predictive value of laboratory tests assessed at admission for sepsis development during ICU stay

Variable	Development of sepsis[a]		*P* value[b]
	No (*N* = 80)	Yes (*N* = 19)	
Routine			
CRP (mg/L)	14.1 ± 37.7	29.1 ± 61.7	0.053
Fibrinogen (g/L)	2.6 ± 0.99	3.3 ± 2.0	0.11
PTT (s)	14.4 ± 1.7	14.4 ± 3.3	0.78
Prothrombin Time Index (%)	66.3 ± 15.6	69.7 ± 19.4	0.61
Platelet count (10^3/μL)	124 ± 55	133 ± 84	0.59
D-dimers (μg/L)	2617 ± 6353	4456 ± 4957	0.0032
ISTH score	1.6 ± 1.3	2.6 ± 0.9	0.041
White blood cell count (10^3/μL)	10.0 ± 4.4	11.1 ± 5.2	0.42
Flow cytometry			
TNF-α (pg/mL)	0.17 ± 0.8	0.65 ± 1.5	0.091
IL-10 (pg/mL)	19.4 ± 90	9.4 ± 16.2	0.50
sCD40L (pg/mL)	88.8 ± 81.8	53.3 ± 43.6	0.89
IL-17A (pg/mL)	9.1 ± 12.2	7.6 ± 15.5	0.31
IL-6 (pg/mL)	459 ± 2673	162 ± 164	0.94
IL-7 (pg/mL)	2.9 ± 3.7	1.4 ± 1.7	0.65
IFN-γ (pg/mL)	0.14 ± 0.91	0	0.99
Platelet-Fg (%)	28.1 ± 27.8	56.5 ± 31.2	0.0054
Platelet-Fg (MFI)	1770 ± 1266	2752 ± 1359	0.0026
Platelet-PS (%)	2.9 ± 2.4	3.5 ± 2.7	0.61
Platelet-PS (MFI)	29.8 ± 15.1	37 ± 18.3	0.068
Platelets-neutrophils (%)	3.4 ± 5	4.4 ± 6.2	0.72
Platelets-neutrophils (CD61 MFI)	313 ± 127	323 ± 137	0.72
Platelets-monocytes (%)	19.8 ± 23.1	22.1 ± 25.5	0.73
Platelets-monocytes (CD61 MFI)	1413 ± 2462	1569 ± 3182	0.82

Null values for TNF-α and IFN-γ correspond to values under the level of detection (3.8 pg/ml)
Platelet-Fg platelet-bound fibrinogen, *platelet-PS*, platelets expressing P-selectin on their surface, *MFI* median fluorescence intensity, % percentage of positive cells for the indicated marker
[a]Results are expressed as means ± SD
[b]Logistic regression

Correlations

Platelet-Fg correlated weakly with platelet P-selectin (r = 0.32378, P = 0.0011, N = 98), and plasma levels of D-dimers (r = 0.35502, P = 0.0004, N=96) and fibrinogen (r=0.34592, P=0.0005, N=98). No significant correlation was found with platelet count (r = 0.071, P = 0.49, N = 98), sCD40L (r = −0.10377, P = 0.3222, N = 93), or cytokine levels.

Temporal changes

Flow cytometry parameters recorded 48 h after admission were not associated with sepsis occurrence, although a tendency (P<0.10) remained for platelet-Fg (data not shown). When looking at serial platelet-Fg levels in patients who developed sepsis, a significant increase was observed and a peak was reached on the day of sepsis (Fig. 1).

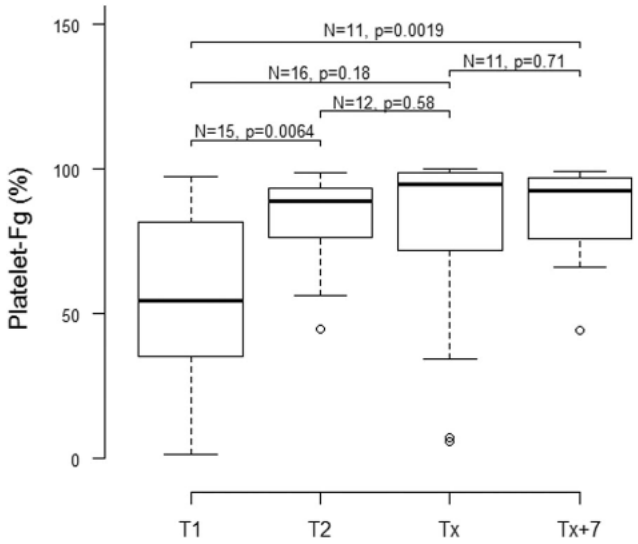
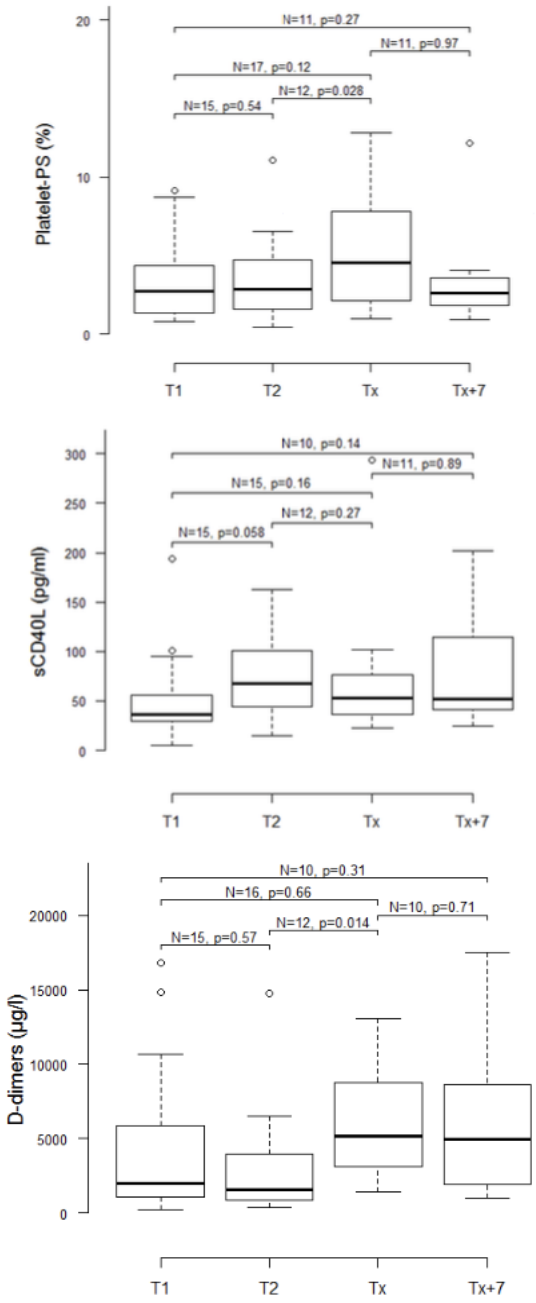


Fig. 1 Serial measurements of levels of platelet-bound fibrinogen (platelet-Fg) for patients who developed sepsis. Platelet-Fg levels were analyzed by flow cytometry on the day of ICU admission (*T1*), after 48 h (*T2*), at the time of sepsis diagnosis (*Tx*), and 7 days later (*Tx + 7*). Median values of percentages of Fg-positive platelets and IQR are shown (*P* value: Kruskal-Wallis test)

By contrast, sCD40L remained fairly stable as sepsis developed while D-dimers and platelet P-selectin levels increased significantly from T2 to the time of sepsis diagnosis (additional Fig 2). `



Additional Figure 2. Serial measurements of platelet markers and D-dimers for patients who developed sepsis. Percentages of platelets exposing P-selectin (Platelet-PS), levels of plasma sCD40L, and D-dimers were analyzed on day of ICU admission (T1), after 48h (T2), at the time of sepsis diagnosis (Tx) and 7 days later (Tx+7). Median values and IQR are shown (P-value: Kruskal-Wallis test).

Platelet markers at admission and sepsis prediction

All potential predictors of sepsis (P < 0.10) recorded at ICU admission (T1) were combined into a stepwise logistic regression analysis. As diagnosis of sepsis includes organ dysfunction, SOFA score was not included in our regression model. It turned out that platelet-Fg % levels at T1 (P = 0.0031) and admission for acute brain injury (P = 0.012) were the only independent predictors of sepsis occurrence. By ROC curve analysis (Fig. 2), an optimal cutoff point equal to 50% was derived for platelet-Fg % (AUC=0.75) to discern patients who will later develop sepsis from those who will not.

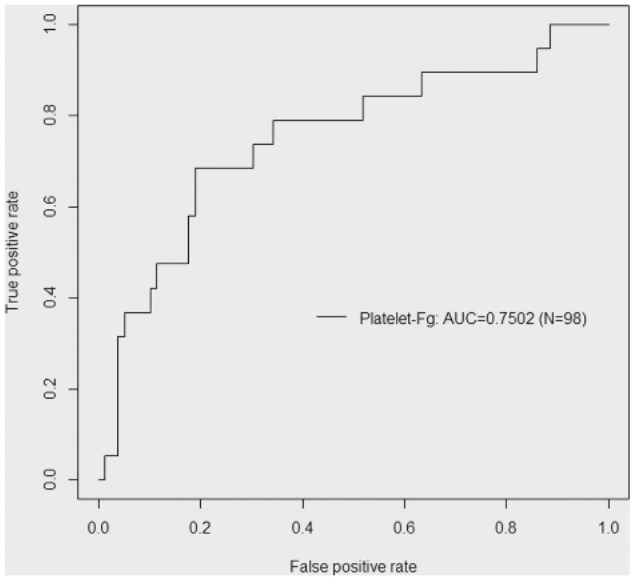


Fig. 2 Predictive value of platelet-Fg (%) obtained at admission. ROC curve analysis of sepsis prediction based on platelet-Fg is shown

The number of patients who developed sepsis was respectively equal to 13 (46.4%) for the 28 patients with platelet-Fg >50% and to 6 (8.6%) for the 70 patients with platelet-Fg <50% (data missing for one patient). As shown in Table 3, when accounted for SOFA score at admission (cutoff value of 8), in patients with elevated SOFA and platelet-Fg >50%, the risk of sepsis rose up to 85.7%. By contrast, in patients with low SOFA and platelet-Fg <50%, the occurrence of sepsis was negligible (3.8%).

Table 3 Risk stratification of patients according to sepsis development during ICU stay

	SOFA <8		SOFA ≥8	
	Platelet-Fg (%)		Platelet-Fg (%)	
Development of sepsis	<50	≥50	<50	≥50
Yes ($N = 19$)	2	1	4	12
No ($N = 79$)	51	13	13	2
Total	53	14	17	14
Risk of sepsis (%)	3.8	7.1	23.5	85.7

SOFA score and Platelet-Fg (%) plasma levels recorded on admission

Moreover, we found no association between aspirin therapy before admission and levels of platelet activation markers upon admission (T1) (TableS5).

Table S5. Effect of aspirin therapy before admission on platelet activation markers measured on patients upon admission to ICU (T1)

	Aspirin therapy before admission		
	No (n = 46)	Yes (n = 53)	P-value
Platelet-Fg (MFI)	1692 (1082 – 2304)	1506 (1080 – 2285)	0.77
Platelet-PS (MFI)	26.8 (20.2 – 34.4)	26.9 (20.4 – 33.4)	0.88
D-dimers (µg/l)	790 (338 – 5766)	506 (335 – 1315)	0.053

Results expressed as median and interquartile range (IQR) of median intensity of fluorescence (MFI) of indicated markers. P-value of Kruskal-Wallis test.

2.1.2.4. DISCUSSION

The major findings of this study concern the clear relationship between patient levels of Fg binding to circulating platelets (platelet-Fg) measured upon ICU admission and sepsis occurrence, regardless of the patient's baseline clinical characteristics. In particular, the

study demonstrated that for patients presenting a SOFA score ≥8, platelet-Fg % level above 50 predicted sepsis with a high accuracy. Importantly, neither platelet membrane-bound P-selectin expression plasma levels of sCD40L nor any other standard hemostasis parameter showed similar predictive value as platelet-Fg. The optimal timing of measurement was also determined since only levels obtained within 24 h after ICU admission and not 48 h later were associated with sepsis occurrence, thus saving blood sampling in future studies. Platelet-Fg levels can be obtained in 1 h by using whole blood flow cytometry in unstimulated samples. Thus, this work provides the clinician with a simple and practical tool to assess the risk of sepsis in critically ill patients admitted to the ICU.

To date, several clinical studies investigated platelet markers in various conditions of critical illness. However, none of them searched for a potential association of these platelet markers with a risk for sepsis. Most of these studies described altered platelet phenotype in injured patients, characterized by either differential expression of platelet activation markers or platelet dysfunction as compared to healthy controls (21-26). In ischemic stroke, two studies showed increased expression of platelet P-selectin and fibrinogen binding to platelets as compared to controls (21, 27). The latter finding is interesting in view of our results, in particular since predisposition to severe pneumonia is clinically well established in such patients (28, 29). Unfortunately, no association was searched between high levels of the biomarker and pneumonia. Several other clinical studies focusing on platelets as potential biomarkers for sepsis diagnosis and prognostication have been carried out but almost all concerned patients with sepsis as an inclusion criterion (30, 31).

Despite multiple experimental data demonstrating antimicrobial activity of platelets and a role for platelet aggregation in limiting pathogen growth and dissemination in the vasculature (2, 6), direct clinical evidence from human studies was lacking and there are no epidemiologic data showing that platelet function inhibition affects sepsis prediction or prognosis. The present observational prospective study provides the first clinical evidence that, in patients with critical illness and related organ dysfunction, platelets may intervene in the dysregulated host response to infection leading to sepsis. Although demonstration of a causal link requires further investigation, we speculate that injury-associated platelet activation and subsequent fibrinogen binding may alter platelet ability to recognize bacterial

components, some of which are ligands of αIIbβ3 (32, 33), and affect their ability to alert and recruit cells of the immune system(8). Our observation that platelet-Fg weakly correlates with D-dimer levels suggests that fibrinogen binding to platelets and the activation of coagulation could be driven by the same factors. In injured patients, plasma fibrinogen would both bind platelets and be actively converted into fibrin; fibrinolysis would then increase D-dimer levels.

Antiplatelet drugs have beneficial and detrimental effects in systemic inflammation and in organ dysfunction, as shown in preclinical models and in humans (15, 34, 35). Their usage has been variably associated with sepsis prognosis (36, 37). In this study, we found no protective effect of aspirin against sepsis 38). Our results are in line with a recent propensity-based analysis of 972 patients admitted for sepsis in which no association between aspirin therapy and sepsis prognosis could be evidenced (39). Our results however differ in that they encompassed the period before sepsis, a period during which the abovementioned authors could not assess the potential benefits of aspirin. In addition, we could not find any association between aspirin therapy and the levels of platelet activation, which suggests that platelet activation pathways independent of thromboxane A_2 production could be involved in the patient's platelet response to injury.

Limitations

The study has a number of limitations such as a small sample size, the predominance of postoperative patients and possible confounders such as immunomodulatory properties of anesthetic drugs. The findings of this pilot study call for a confirmatory prospective evaluation focusing on fibrinogen levels on platelets in a larger cohort. In our study, the platelet activation markers analyzed, namely levels of fibrinogen, platelet P-selectin expression, platelets-leucocytes aggregates and sCD40L, behaved differently in their ability to predict sepsis development, which might reflect differences in platelet activation mechanisms or sequences. It has indeed been proposed that platelet activation, in terms of P-selectin expression and fibrinogen binding, and release of immunological molecules (sCD40L, RANTES) result from independent signaling pathways(40). The utility of other

markers, such as platelet microparticles or soluble glycoprotein VI should be analyzed since the latter is shed from platelet surface and increases in patients with DIC (41).

2.1.2.5. CONCLUSION

In critically ill patients with comorbidities and post-trauma or post-surgical injury, platelet abnormalities are associated with altered host defense mechanisms. We found that admission levels of fibrinogen binding to platelets of ICU patients were associated with later sepsis occurrence. Combining it with stratification based on SOFA score at admission has a higher predictive ability. Hence, our observations could trigger non- specific preventive interventions such as better supportive care or prophylactic antibiotics as well as research aiming at developing a specific therapeutic tool. Also, the fact that the identified marker was independent of aspirin use might have important future therapeutic implications regarding its actual worldwide implementation of primary or secondary prophylaxis.

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2.1.3. CONCLUSION

From the above studies, we can conclude that host-response biomarkers such as platelet and leucocyte surface markers can be helpful AMS diagnostic tools for sepsis prediction. We showed that upon admission to the ICU for critical injury, patients who are at risk of developing sepsis have elevated monocyte counts, downregulation of L-selectin on monocytes and high levels of platelet-bound Fg. Interestingly, when the latter marker (>50%) is combined with a high (>8) admission SOFA score, the predictive value of secondary sepsis exceeds 85%. Moreover, 48-72h later, these patients show signs of monocyte deactivation

with low levels of mHLA-DR, thus confirming the robustness of this biomarker as a global immunosuppression surrogate marker in that patient population. All three markers, excluding L-selectin could be easily implemented in routine ICU sampling with a turn-around-time of approximately 1h.

2.2. PROCALCITONIN AS A DIAGNOSTIC ANTIMICROBIAL STEWARDSHIP TOOL FOR SEPSIS

2.2.1. INTRODUCTION

Core elements of AMS include the elaboration of more accurate diagnostic tools of bacterial sepsis as well as withholding or discontinuing antimicrobials in the absence of sepsis(1). The recently updated definition of sepsis includes the fact that the dysregulated host response in the infected patient, when associated with organ failure, plays an essential role in driving mortality (2). Specific immune and host-response markers such as presepsin, neutrophil CD64 and PCT have been proposed to improve early sepsis diagnosis and clinical management as well as prognosis (3). PCT, a precursor of the mature 32-amino acid hormone calcitonin is secreted ubiquitously by nonneuroendocrine parenchymal cells throughout the body (lung, liver, kidney, muscle, fat) in hyperinflammatory conditions of variable severity and origin. Indeed, its levels are raised in localized infection and sepsis but also burns, pancreatitis, extensive surgery, mesenteric infarction, etc.. (4, 5). In clinical practice, as an acute phase reactant, it has a more favorable kinetic profile than CRP, with levels increasing within 2-4h and peaking at 24h post infection (Fig.1 from Becker, K.L., CCM 2008).

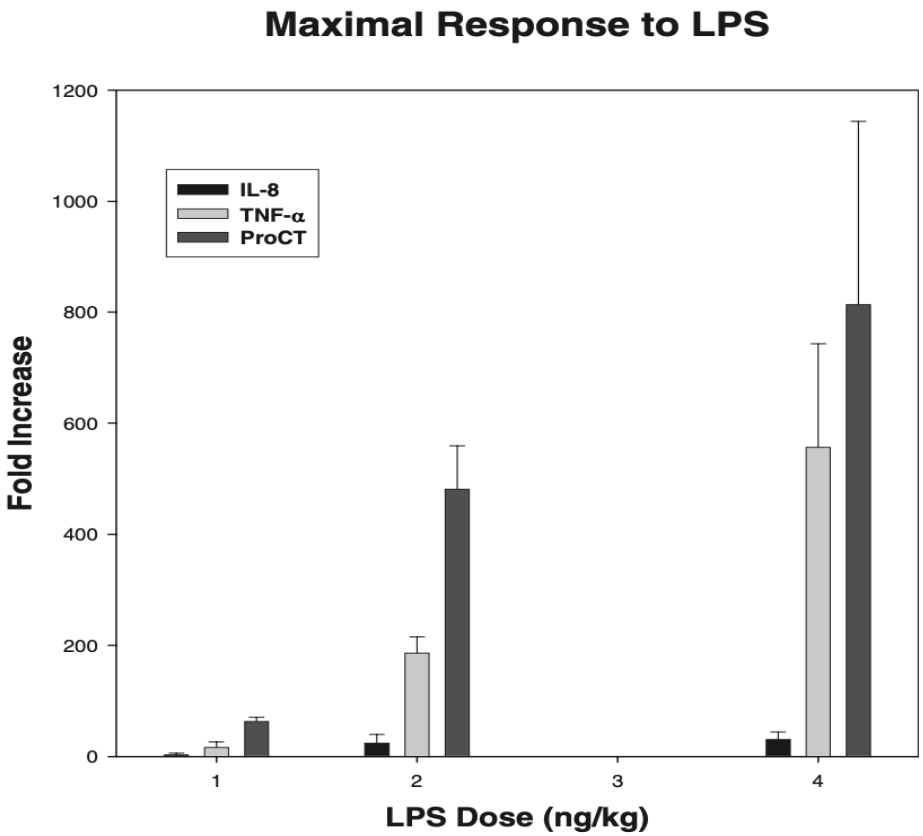
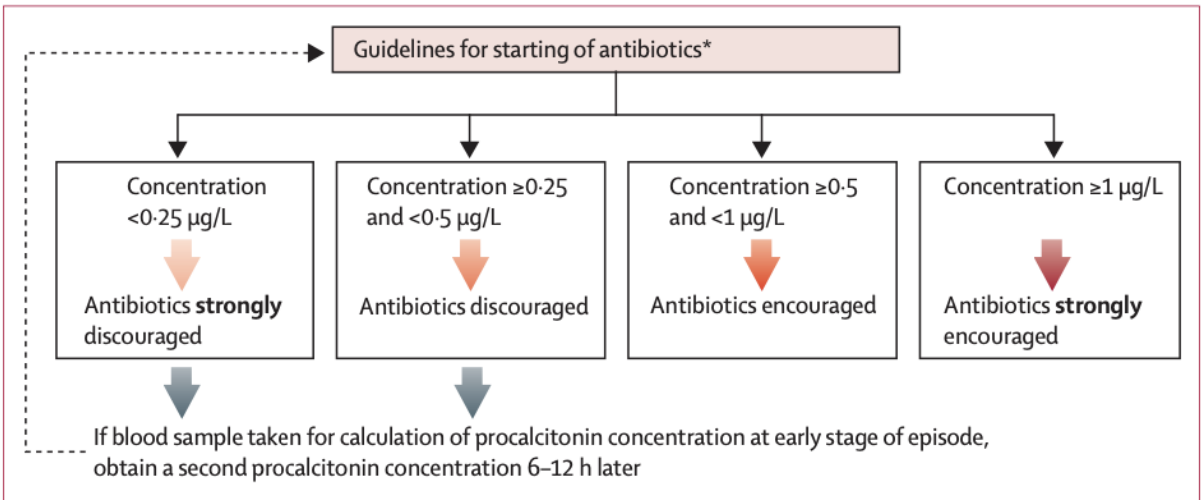


Figure 1. Peak (*fold*) increase of interleukin (*IL*)-8, tumor necrosis factor (*TNF*)-α, and procalcitonin (*ProCT*) in four healthy volunteers after increasing doses of endotoxin (lipopolysaccharide [*LPS*]; 1, 2, and 4 ng/kg). IL-8 reached peak levels at 4 hrs; TNF-α peaked at 1.5 hrs, and ProCT peaked at 24 hrs (unpublished data from Suffredini et al (164)).

Its levels remain high, as long as the inflammatory process is not controlled. In bacterial infections, as opposed to viral infections in which IFN-γ down-regulates PCT, serum levels of PCT raise in response to mediators released (TNF-α, Il-6) correlating with the extent and severity of infection(6, 7). Its levels fall by half daily when the infection is controlled (8, 9).

PCT was extensively studied between 1996 and 2011 and demonstrated a good discriminatory ability for sepsis (AUC of 0.85) with pooled sensitivity and specificity of 0.77 and 0.79, respectively (10). The use of a PCT-based algorithm helped to reduce AMT by almost 50% in suspected LRTI in two landmark studies, the ProCAP and ProRESP studies (11, 12). This was done by more or less withholding AMT upon PCT levels <0.1µg/L or <0.25µg/L or more or less encouraging AMT upon PCT levels ≥0.5µg/L or ≥0.25µg/L called the Muller classification (13) (Fig.1. from Bouadma, Lancet 2010). We applied the same algorithm in the following randomized controlled trial.


Guidelines for starting of antibiotics*
Concentration <0·25 µg/L
Antibiotics **strongly** discouraged
Concentration ≥0·25 and <0·5 µg/L
Antibiotics discouraged
Concentration ≥0·5 and <1 µg/L
Antibiotics encouraged
Concentration ≥1 µg/L
Antibiotics **strongly** encouraged
If blood sample taken for calculation of procalcitonin concentration at early stage of episode, obtain a second procalcitonin concentration 6–12 h later

2.2.2. PROCALCITONIN USEFULNESS FOR THE INITIATION OF ANTIBIOTIC TREATMENT IN INTENSIVE CARE UNIT PATIENTS

Clinical Investigations

Procalcitonin usefulness for the initiation of antibiotic treatment in intensive care unit patients*

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Crit Care Med 2012 Vol. 40, No. 8

DOI: 10.1097/CCM.0b013e318251517a

2.2.2.1. MATERIALS AND METHODS

Study patients

Procalcitonin serum level was obtained for all adult consecutive patients expected to stay more than 48h and suspected of developing infection either on admission or during ICU stay, over a 9-month period, in five tertiary single-center ICUs of University Hospital of Liège. The use of antibiotics was more or less strongly discouraged or recommended according to the Muller classification. Patients were randomized into two groups: one using the PCT results (PCT group) and one where the ICU physician was blinded to the PCT results (control group). PCT serum level was measured once, everytime an infection was suspected, using a time-resolved amplified cryptate emission technology assay (Kryptor® PCT; Pasteur Mérieux,

Paris, France) with a functional assay sensitivity of 0.06 µg/L. The primary endpoint was the reduction of antibiotic use expressed as a proportion of treatment days and of daily defined dose per 100 ICU days using a PCT-guided approach. Secondary endpoints included: *a posteriori* assessment of the accuracy of the infectious diagnosis done by the ICU physician in the PCT group, by review of the charts by a blinded to PCT result ID specialist and assessment of the diagnostic concordance between the ICU physician and the ID specialist. Admissions were classified into trauma, unscheduled surgery, elective surgery or medical. Patients readmitted to the ICU during the study period remained in the same group (PCT or control). For each patient, the total ICU stay was calculated, and all infectious episodes were recorded to account for the total antibiotic consumption throughout the study period.

Statistical Analysis

Continuous variables were reported as mean ± sd for normally distributed variables or as median and interquartile range (IQR) for variables with skewed distribution. Proportions were compared by the chi-square test while mean values were compared by one-way analysis of variance of the Kruskal-Wallis test. Interobserver agreement between clinicians and ID specialists was assessed by Cohen's kappa coefficient. Assuming a mean stay of 7 days with 50% anti- biotic exposure, a study sample of at least 250 patients in each group was deemed necessary to detect a 20% reduction in antibiotic consumption with 95% power at the 5% significance level.

2.2.2.2. RESULTS

During the study period, 1501 patients were admitted in the five ICUs (Fig 1. Trial profile).

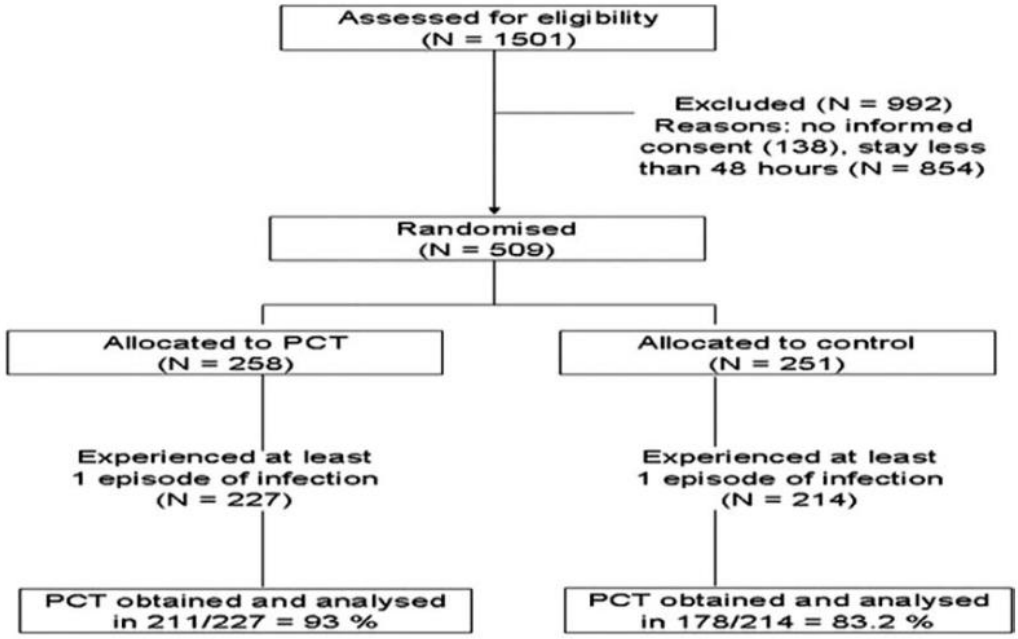

Figure 1. Trial profile.

Five hundred and nine patients were eligible for the study and were randomized to PCT group (n=258) and the control group (n=251). The baseline characteristics of patients at admission were comparable in both groups in terms of age, sex, type of admission, comorbidities and severity index score (SAPSII) (Table 1).

Table 1. Characteristics of patients at admission

Variable	Procalcitonin Group n = 258	Control Group n = 251	*p*
Age (yr) median (interquartile range)	66 (55–76)	65 (53–75)	.33
Sex, n (%)			
Male	154 (59.7)	153 (61.0)	.97
Female	104 (48.3)	98 (39.0)	
Underlying disease, n (%)			
Nonfatal	160 (62.0)	154 (61.4)	.66
Ultimately fatal	74 (28.7)	68 (27.1)	
Rapidly fatal	24 (9.3)	29 (11.6)	
Comorbidities, n (%)			
Coronary disease	29 (11.2)	21 (8.4)	.77
Chronic heart failure	36 (14.0)	34 (13.6)	
Cerebrovasc disease	12 (4.7)	16 (6.4)	
Renal dysfunction	30 (11.6)	36 (14.3)	
Liver disease	20 (7.8)	16 (6.4)	
Diabetes	45 (17.4)	38 (15.1)	
Chronic obstructive pulmonary disease, asthma	72 (27.9)	66 (26.3)	
Solid cancer	42 (16.3)	44 (17.5)	
Hematological cancer	17 (6.6)	15 (6.0)	
Transplant	8 (3.1)	8 (3.2)	
Type of admission, n (%)			
Medical	155 (60.1)	147 (58.6)	.97
Scheduled surgery	22 (8.5)	23 (9.2)	
Emergency surgery	56 (21.7)	53 (21.1)	
Trauma	25 (9.7)	28 (11.2)	
Simplified Acute Physiology Score II	39.3 ± 16.3	39 ± 16.7	.84
Readmission, n (%) 0	249 (96.5)	239 (95.2)	.51
1	6 (2.3)	11 (4.4)	
2	3 (1.2)	1 (0.4)	

2306

Among the 509 patients, 227 (88%) in the PCT group and 214 (85.3%) had at least one suspected episode of infection (Table 2). PCT results were available for 389 (88.2%) of the 441 patients. Together, these 441 patients presented 667 episodes of suspected infections (323 on admission to ICU and 344 during ICU stay). ICU LOS, SOFAmax, number of patients with RRT and MV, duration of MV and ICU mortality were similar between groups.

Table 2. Characteristics of intensive care unit stay

Variable	Procalcitonin Group n = 258	Control Group n = 251	*p*
Patients with suspected infections *n* (%)			
Yes	227 (88.0)	214 (85.3)	.43
No	31 (12.0)	37 (14.7)	
Number of episodes of infection/patients	1.4 ± 1.1	1.2 ± 1.0	.15
Procalcitonin assays *n* (%)			
Yes	211 (81.8)	178 (70.9)	.005
No	47 (18.2)	73 (29.1)	
Number of procalcitoninmeasurement/ patients	1.2 ± 1.0	0.9 ± 0.8	.003
Intensive care unit stay (days), median (interquartile range)	7 (4–16)	7 (4–18)	.38
SOFAmax			
Ventilation > 24 hrs, n (%)	9.3 ± 4.9	9.1 ± 5.4	.42
Yes	150 (58.1)	149 (59.4)	.79
No	108 (41.9)	102 (40.6)	
Duration of ventilation days			
Median (interquartile range)	3 (1–11)	3 (0–11)	.99
Renal-replacement therapy			
Yes	44 (17.1)	45 (17.9)	.81
No	214 (82.9)	206 (82.1)	
Intensive care unit mortality	56 (21.7)	53 (21.1)	.91
Antibiotic consumption: % of intensive care unit days	62.6 ± 34.4	57.7 ± 34.4	.11
Antibiotic consumption defined daily dose/100 intensive care unit days mean ± SD, median (interquartile range)	147.3 ± 206.0	141.1 ± 136.9	.96

SOFAmax, sum of all the dysfunction and failure occurring during the intensive care unit stay according to the Sequential Organ Failure Assessment score.

Antibiotic consumption did not differ between groups: the treatment days represented 62.6 ± 34.4% and 57.7 ± 34.4% of the ICU stays in the PCT and control groups, respectively (*p* = .11). Similarly, there was no difference in terms of DDD/100 ICU days between the two groups: a mean of 147.3 ± 206.00 DDD/100 ICU days in the PCT group vs. 141.1 ± 136.9 DDD/100 ICU days in the control group, or a median of 108.3 (IQR 47.7–200) DDD/100 ICU days in the PCT group vs. 108.7 (IQR 52.3– 180.7) DDD/100 ICU days in the control group (*p* = .96).

When looking at the number of withheld treatments in both groups according to the ICU clinician's confidence it is only in the episodes classified as possible that we were we able to show a significanly higher proportion of withheld treatments in the PCT group compared to the control group (50.5% vs. 34.2 %; p = .034)(Table 4). As expected, all episodes classified as certain were treated with antibiotics.

Table 4. Number of withheld or withdrawn treatment according to the clinician confidence

Clinician Confidence	Total, n = 667 (%)	Procalcitonin Group, n = 353 (%)	Control Group, n = 314 (%)	*p*
Sure	0/192 (0)	0/101 (0)	0/91 (0)	.99
Probable	17/249 (6.8)	6/123 (4.9)	9/126 (7.1)	.60
Possible	78/179 (43.6)	52/103 (50.5)	26/76 (34.2)	.034
Uncertain	29/47 (61.7)	13/26 (50.0)	16/21 (76.2)	.080

When looking at the decision to treat according to PCT levels in both groups, *a posteriori*, no difference between groups could be observed (Table 5).

Table 5. Number of withheld or withdrawn treatment according to the procalcitonin levels in procalcitonin patients (n = 211) and in control patients (n =178)

Procalcitonin Levels	Total, n = 536 (%)	Procalcitonin Group, n = 306 (%)	Control Group, n = 230 (%)	*p*
>1 µg/L	31/259 (12.0)	16/140 (11.4)	15/119 (12.6)	.85
0.5–1 µg/L	13/67 (19.4)	9/39 (23.1)	4/28 (14.3)	.53
0.25– <0.5 µg/L	14/75 (18.7)	9/47 (19.1)	5/28 (17.9)	.99
<0.25 µg/L	55/135 (40.7)	37/80 (46.3)	18/55 (32.7)	.15

PCT levels were >1µg/L in 259 episodes (48.3% of 536 infectious episodes with PCT measurement) and <0.25µg/L in 135 episodes (25.2%).

A posteriori reviewing of charts by the ID specialist yielded a disappointing AUC of 0.69 for the ability of PCT levels to discriminate between certain/probable infection and possible/no infection upon initiation of AMT (Fig.3). The observed proportion of agreement between the ICU clinician and the ID specialist was 53% for the PCT group and 49% for the control group (not significant), yielding a kappa coefficient of 0.46 in both groups.

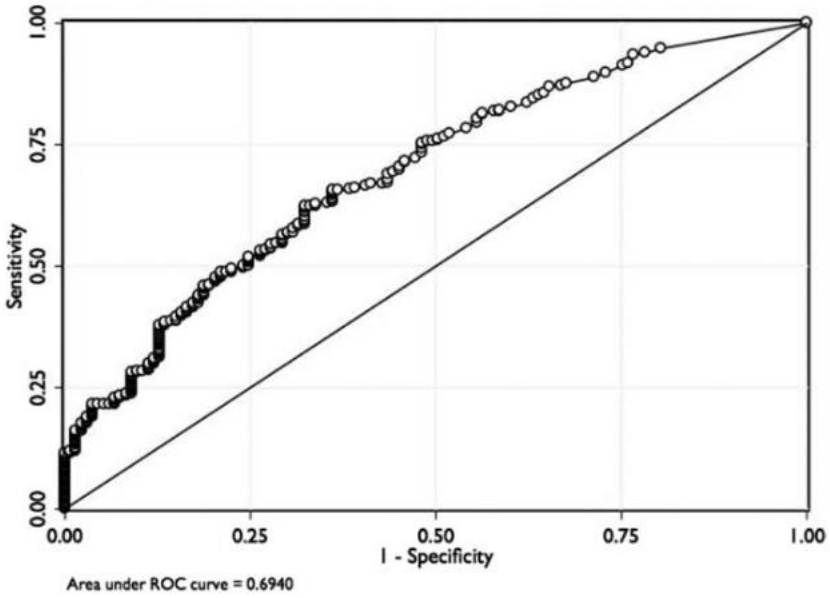

Figure 3. Discrimination power of procalcitonin (*PCT*) levels. The outcome was diagnosis of infection assessed by infectious-disease specialist as sure or probable. The area under the receiving operating curve was 0.69.

2.2.2.3. DISCUSSION

The present study failed to show a significant reduction in AMT consumption with PCT as a diagnostic tool for initiation of antimicrobials in critically ill patients suspected of having infection upon admission or during ICU stay. Although ICU clinicians could significantly decrease the number of treatments when infection was considered as possible and when PCT was available, the overall consumption was the same between the two groups. A reason for this failure may be that almost half of PCT serum samples were >1 µg/L thus encouraging the antibiotic treatment. Only 25% of the samples were below the lowest cutoff. A second reason might lie in the fact that clinical skills and judgment superseded PCT results and protocol recommendations since only 46.3% of the patients with a low level of PCT were not treated. Indeed, 43 patients had signs of severe sepsis and/or comorbidities that prompted physicians to treat them. It must be emphasized that the majority of these treatments (30 of 43, 69.8%) were *a posteriori* confirmed as correct by the ID specialist. A third reason could be that, in this study, the proportion of patient-days with antibiotic treatment in the control group was already low (57%) compared to other studies in which that proportion was >80% (13). Still, the main question raised by the present study is the accuracy of PCT as a marker of infection. Despite limitations (single-center design, open design, no serial measurements,

case-mix including 40.7% of trauma and surgical patients potentially inducing early false positives) we, as others, found a disappointingly low AUC of 0.69 for PCT as a diagnostic marker of infection in this cohort of patients among whom a proportion displayed severe sepsis according to the old definition (14, 15). PCT did not help to distinguish between probable, possible or no infection upon AMT initiation in critically ill patients displaying organ dysfunction.

2.2.2.4. CONCLUSION

PCT levels did not appear to be helpful in a strategy aiming at decreasing AMT consumption in suspected infection in critically ill patients in need of organ support, a proportion of whom displayed severe sepsis according to the old definition. PCT is a poor marker of severe infection when it comes to initiating antimicrobials in critically ill patients. Protocol violation is a common finding in trials investigating PCT as an AMS tool and a major hurdle to overcome.

2.2.3. A REVIEW ARTICLE ON PCT

Curr Infect Dis Rep (2013) 15:394–399
DOI 10.1007/s11908-013-0360-2

SEPSIS AND ICU (J RUSSELL, SECTION EDITOR)

Procalcitonin for Antibiotic Treatment in Intensive Care Unit Patients

Nathalie Layios · Bernard Lambermont

This review focused on the three main roles of PCT in guiding AMT use in critically ill patients: treatment initiation, prognosis and assessment of treatment duration allowing safe discontinuation of AMT in case of absence of sepsis or rapid control of the infectious process.

For **treatment initiation**, PCT was first thought to be a sensitive biomarker that would help to discriminate between severe infection and nonspecific hyperinflammatory states (16). However, unacceptably low sensitivity values in the setting of critically ill patients, ranging from 67 % to 80 % depending on the chosen cutoff (17-20), led to its being considered rather as a prognostic tool in terms of severity of illness and outcome. Moreover, PCT met the fate of other acute phase reactants that did not show satisfactory specificity. PCT is notoriously raised, in the absence of infection, in pancreatitis, ischemic bowel disease, cardiopulmonary bypass, and metastatic disease(21) and with the intake of some drugs (monoclonal antibodies, antithymocyte globulin, etc.) (22, 23). PCT does not rise in case of local bacterial, viral, parasitic, or fungal infection. Furthermore, evidence surrounding its role as a diagnostic marker of sepsis in immunocompromised patients is conflicting, owing to different patient populations and study quality (24-26).

Since 2013, two prospective, observational studies confirming the low diagnostic ability of PCT have been published (27, 28). The first study included hospitalized patients with community-acquired pneumonia (CAP) in whom no reliable cut-off value of PCT was found, highlighting the risk of not initiating AMT in CAP (27). The second study included

mechanically ventilated CU patients with VAP in whom PCT and CRP were measured once, in order to differentiate VAT from VAP. Although PCT and CRP presented lower values in VAT as compared to VAP, there was a marked overlap of both biomarkers' values in both conditions, not allowing adequate discrimination (28). Finally, a recent systematic review and meta-analysis found insufficient sensitivity and specificity to PCT levels (0.55 and 0.76, respectively) to distinguish viral from bacterial pneumonia in the setting of AMT initiation for CAP (29). Thus, the current evidence still does not support the use of PCT for AMT initiation in critically ill patients.

When considering PCT as a **prognostic tool**, ancillary studies had shown that a strategy combining PCT levels with other biomarkers (CRP, sTREM-1, SUPAR, TNF-α, IL-6, IL-8) or clinical scoring systems (SAPSII) or lactate levels, was better associated with outcome than using PCT alone (AUC 0.72-0.88). For example, higher values (1.5-over 5µg/L) in high-risk patients have been correlated with bacterial load, bacteremia and severity of organ failure (30-32). However, when considering mortality, although PCT levels seemed to correlate well with this outcome in older studies, the evidence is nowadays much more conflicting (3, 33, 34).

In fact, PCT proved to be a valuable asset as a tool to rule out bacterial infection, thanks to a high negative predictive value. The latter ranges from 92% to 98% depending on the cut-off level of the biomarker and the setting and design of the study (35, 36). The use of PCT seems to have a favorable impact on **AMT discontinuation,** thereby reducing cumulative antibiotic exposure, while proving safe in sepsis patients(37). A recent review comparing different PCT protocols found PCT to be most helpful when used for early stopping AMT, particularly in the setting of high-risk patients such as patients with positive blood cultures, using the 0.25 µg/l cut-off for the emergency room setting and 0.5 µg/l for the ICU setting(38). Thus, the current evidence supporting the use of PCT to discontinue AMT is stronger.

2.2.4. CONCLUSION

Despite being useful as a guide to discontinue AMT, significant limitations regarding the use of PCT as an AMS tool have to be taken into account. Low algorithm compliance, no report of AMS program implementation in the control groups, algorithms including other biomarkers (mainly CRP), longer and fixed duration of AMT in the control groups, constitute, among others, significant bias for the implementation of PCT to guide AMT use in sepsis. Moreover, high cost of the test has hindered serial testing in many studies, which is considered mandatory for proper and secure AMS in high-risk patients(39). The updated version of the Surviving Sepsis Campaign, published on Oct. 2nd, 2021, recommend against the use of PCT for AMT initiation (in addition to clinical evaluation) in sepsis and septic shock patients (40). Finally, published guidelines for the management of CAP recommend initiation of AMT regardless of PCT level (41).

2.2.5. REFERENCES

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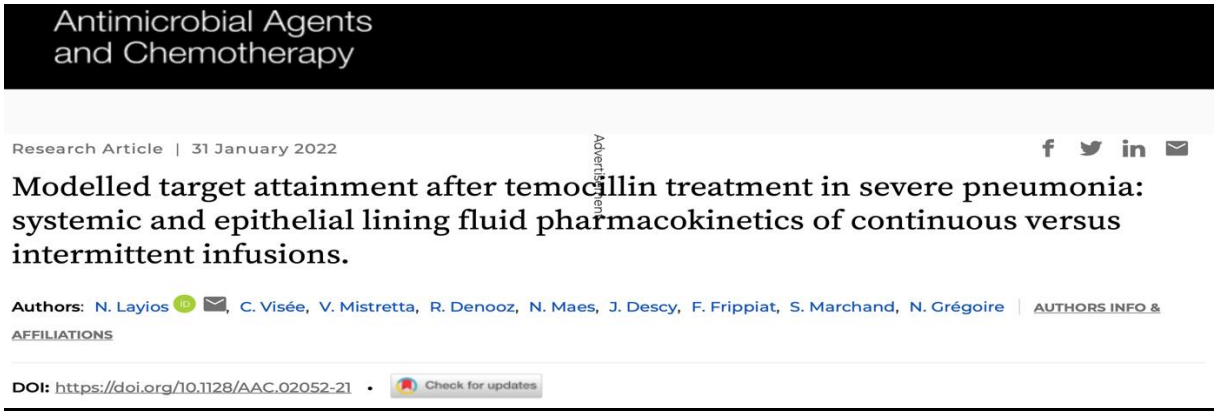
CHAPTER 3. PKPD CHARACTERISATION OF TEMOCILLIN IN PLASMA AND ELF OF MECHANICALLY VENTILATED PATIENTS WITH PNEUMONIA: A THERAPEUTIC ANTIMICROBIAL STEWARDSHIP TOOL

3.1. INTRODUCTION

Core elements of AMS include prescribing appropriate antibiotics with appropriate dosages, which includes considerations about the choice of the drug, its posology, mode of infusion and PKPD characteristics. This led us to determine whether a revived antibiotic such as temocillin, which bears a distinctive chemical conformation, demonstrated different PKPD properties according to the mode of infusion. The latter would enable us to use it to treat severe hospital-acquired pneumonia (HAP) caused by potentially resistant *Enterobacterales* (ESBL pathogens), in order to spare carbapenems.



3.2. BACKGROUND

Temocillin is an interestingly revived antibiotic, which is rendered resistant to most β-lactamases secreted by Gram-negative pathogens (excluding non-fermenters) thanks to its 6-α methoxy terminal structural modification (1). There is evidence for usage of higher

(6g/day) than manufacturer-approved dosages in severe infections, ideally in continuous infusion in order to optimize the %fT>MIC (fraction of time free compound of beta-lactam remains over MIC of bacteria), which is the cornerstone of beta-lactam efficacy (2-7). For temocillin, although no detailed analysis of its pharmacodynamics (PD) in vitro exists, it is assumed that a minimal bacteriostatic target of 40 to 50% should be considered by comparison with other penicillins (8, 9).

Furthermore, the above mentioned reports rely on determination of surrogate plasmatic concentrations of free drug, in small cohorts of patients, displaying varying degrees of severity and sites of infection (lung, intra-abdominal, urinary, blood). As stated in a recent position paper, more data on target-site tissue PK of antimicrobials are needed to evaluate the appropriateness of the currently used dosing regimens in critically ill patients. Indeed, they have notoriously modified PKs (augmented renal clearance, increased volume of distribution, extra-corporeal therapies…) hindering optimal antimicrobial therapy and therapeutic success (10). Furthermore, for clinical practice, only ancillary BSAC and CA-SFM breakpoints for systemic infections (susceptible [S] ≤ 8mg/L and resistant [R] >8mg/L) are available (11). Recently, European EUCAST clinical breakpoints were released, only for urinary tract infections (UTIs), owing to lack of PK/PD data for other sites (12). Hence, the primary objective of this study was to provide a characterization of temocillin PKPD breakpoints in ELF and plasma of critically ill patients treated for severe pneumonia. Two modes of infusion (continuous over 24h versus intermittent, 30 minutes bolus injection) of the same posology (6g/day) were compared.

3.3. MATERIALS AND METHODS

Study design and participants

This was a single-center, prospective, randomized study that was conducted in six ICUs at the Centre Hospitalier Universitaire du Sart-Tilman, Liège, during one year. Eligible adult patients had to meet the following inclusion criteria: diagnosis of HAP or VAP with a documented pathogen showing temocillin Vitek-2 *in vitro* sensitivity of ≤8 mg/L and

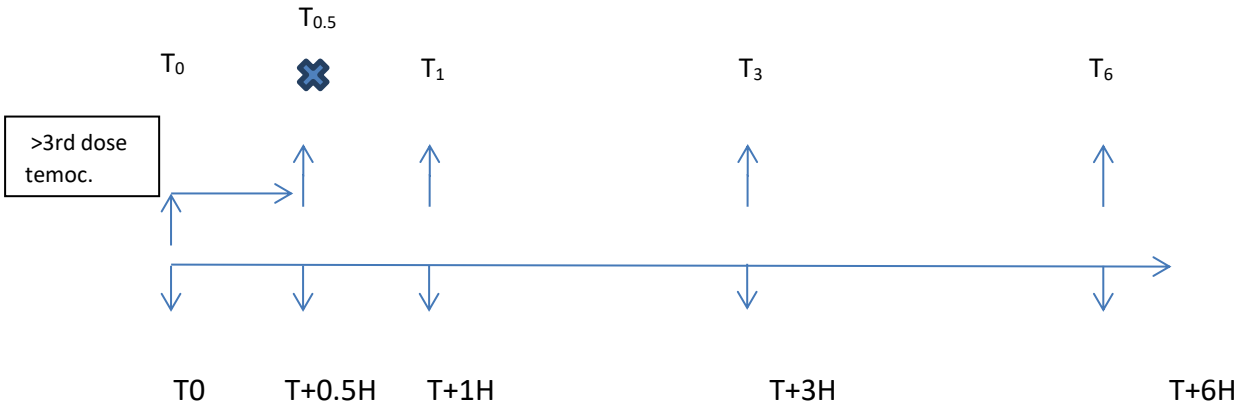
requiring mechanical ventilation; creatinine clearance based on 24-h urine output collection measured ≥30 mL/min/1.73 m2. The enrolled patients were prospectively randomized in a 2.5:1 ratio to either the intermittent infusion group (II; 2 g over 0.5 h every 8 h) or the continuous infusion group (CI; 6 g over 24 h after a loading dose of 2 g over 0.5 h). The a priori defined ratio was chosen to study the temocillin concentration at five time points, using only one ELF sample per patient (Fig.1 Trial profile). No power size calculation was deemed necessary for this descriptive study.

Data collection, study drug, and sampling

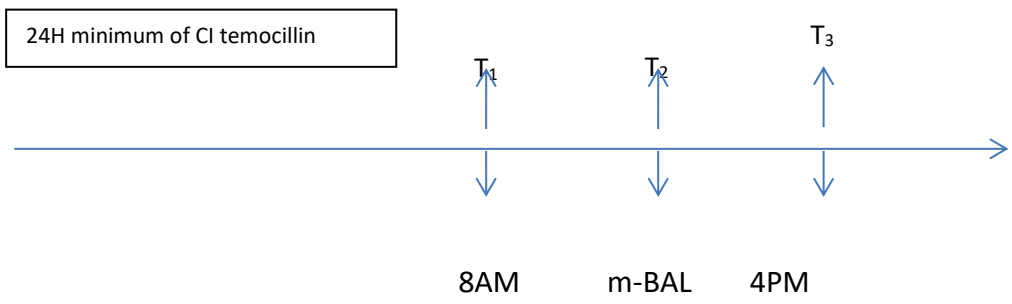
Demographic and clinical data were prospectively collected. Stability of temocillin continuous infusion through volumetric pump has been published elsewhere(12). All serum and mini-bronchoalveolar lavage (mini-BAL) samples were obtained within 15 min either side of the expected time of sampling after at least 24 h of infusion in the CI group and at least 3 doses in the II group (Fig.1 Trial profile). Serum samples (10 mL) were collected from indwelling arterial catheters at three predetermined time points for each patient in the CI group: i.e., 8am, time of the mini-BAL, and 4 pm. In the II group, blood samples were obtained at pre-dose and 0.5, 1, 3, and 6 h after the start of temocillin infusion. Mini-BAL samples (one per patient, evenly at the blood sampling times) were collected through a standardized mini-BAL procedure as follows: 2 x 40 mL of sterile 0.9% saline solution using a non-bronchoscopy catheter (Bal-Cath® system; Kimberly Clark, Zaventem, Belgium).

Fig. 1 Trial profile : plasma and ELF sampling times at steady-state

- **II** :
  - T0 (before the 4th temocillin dose)
  - T+ 0.5H, T+ 1H, T+ 3H and T+ 6H après le début de la perfusion de témocilline



- **CI** : at 3 different timings on the day of m-BAL (after at least 24hrs of CI of temocillin)
  - T1 at 8AM
  - T2 simultaneous with m-BAL
  - T3 at 4PM



In the II group (N=25): Plasma samples were done at 5 different timepoints. One plasma sample was drawn simultaneously with the m-BAL sampling, in each patient, in order to determine:

- Temocillin total and free concentrations in plasma
- Temocillin total and free concentrations in ELF
- Plasma urea and protein levels
- ELF urea concentration

Analytical methods

Blood and mini-BAL samples were immediately centrifuged at 3,000 rpm for 10 min and 10,900 rpm for 5 min, respectively; the supernatant was immediately separated and kept at 220°C until analysis, except for the BAL microbiological culture. For determination of total temocillin, 200 ml of BAL were spiked with ticarcillin (internal standard) and cleaned up by liquid-liquid extraction prior to chromatographic analysis. For determination of free temocillin concentration, 500 mL of serum or BAL was beforehand filtered by centrifugation using an Amikon 10-kDa ultrafiltration device (Millipore). Then, 300 mL of this ultrafiltered serum (or 200 mL of ultrafiltered BAL) were spiked with ticarcillin and were cleaned up by liquid-liquid extraction. The ultrafiltered serum/BAL was directly analyzed without extraction. All pretreated samples were analyzed using a validated method on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Acquity Waters-Quattro Premier Waters) equipped with a solvent quaternary pump, an injector, an Acquity HSS T3 column (100 x2.1 mm; 1,8 mm) thermostatized at 40°C, and MassLynx computer software (Waters Corporation).

Measure of urea and determination of ELF concentrations

The concentrations of urea in the se-rum and ELF were determined as described by Rennard et al. (28) with the urea nitrogen/1900 kit (Roche Professional Diagnostics, Mannheim, Germany). The concentration of temocillin in ELF was thereafter determined using urea as an endogenous marker according to the following formula (27, 28).

$$TEM_{ELF} = \frac{TEM_{BAL} \times urea_{PLA}}{urea_{BAL}}$$

MIC determinations

MICs were first determined using the automated system Vitek 2 (bioMérieux®) and subsequently by Etest (bioMérieux®).

PK analysis. A population PK model was developed. A nonlinear mixed effects modeling approach was performed with NONMEM version 7.4.0 (double precision; Icon Development Solutions, Ellicott City, MD, USA) and PsN-toolkit version 4.6.0 (13). The first-order conditional estimation method with interaction was used. One- and two-compartment structural models were fitted to free (Cu) and total (Ct) serum and total ELF (Celf) concentrations. The estimated pharmacokinetic parameters reflect the unbound concentrations of temocillin. The relationship between bound and unbound concentrations of temocillin was described by an E_{max}-type model of parameters Cb_{max}, the maximal concentration of temocillin that can be bound and BC_{50}, the concentration of unbound temocillin for which half of Cb_{max} is reached. The passage of unbound temocillin from plasma to ELF was modeled with an entry clearance into ELF (Q_{in}) and an exit clearance from ELF (Q_{out}). At steady state, the AUC ratio (R_{AUC}) between Cu and Celf corresponds to the Q_{out}/Q_{in} ratio. The interindividual variability in the PK parameters was estimated with the use of exponential models. The correlation between individual values of plasma clearance and central volume of distribution was estimated. Additive, proportional, and mixed error models were investigated to describe the residual variability. Weight, body surface area, and creatinine clearance were tested as covariates on volumes of distribution and/or clearance parameters. Power functions were used for this purpose. A decrease in objective function of .3.84 was used to consider a covariate as statistically significant with a 5% type I error. The correlation between unbound and total temocillin concentration measurements from the same sample was tested using the L2 function in NONMEM. Precision of the estimations was evaluated by using the sampling importance resampling (SIR) procedure, implemented with

PsN (14). An internal validation of the model was performed by visual inspection of goodness-of-fit (GOF) plots, based on model predictions and residuals, and visual predictive checks (VPCs).

Monte Carlo simulations. PK/PD analysis. Steady-state concentrations of temocillin in serum and ELF were generated for 32,000 virtual subjects by Monte Carlo simulations, with the same demographic characteristics as the 32 patients included in the study, for each of the two dosing scenarios. Subsequently, the %T>MIC were calculated as well as the probabilities of target attainment (PTA) for different PD targets based on Cu for plasma. The BSAC defined breakpoints for systemic infections caused by *Enterobacterales* were used (15).

3.4. RESULTS

Patient enrollment, exclusions, and adverse events.

Forty-four patients were enrolled in the study. Thirty-two patients were included in the final PK analyses, 23 in the II group and 9 in the CI group. Temocillin and mini-BAL were well tolerated without any significant adverse events.

Patient demographics. Patient demographics and clinical characteristics are described in Table 1. The two groups were clinically and demographically similar. Thirty-one percent of patients had augmented renal clearance (ARC) defined as >120 mL/min/1.73 m2 (7/23 in the II versus 3/9 in the CI group, respectively(16). The mean creatinine clearance was 107.2 ± 49.5 mL/min/1.73 m2.

TABLE 1 Demographic and clinical characteristics of patients

Demographic and clinical data	All ($n = 32$)	CI group ($n = 9$)	II group ($n = 23$)	P value
Age (yr)	64.9 ± 11.4	66.1 ± 7.0	64.4 ± 12.8	0.71
Male (no. [%])	24 (75.0)	8 (88.9)	16 (69.6)	0.39[a]
Wt (kg)	74.4 ± 13.7	75.6 ± 16.7	73.9 ± 12.8	0.76
BMI (kg/m^2)	25.1 ± 4.6	25.1 ± 4.5	25.1 ± 4.7	0.99
Hospital stay before onset of pneumonia (days)	15.6 ± 15.0	18.3 ± 13.9	14.5 ± 15.6	0.33[b]
ICU stay before onset of pneumonia (days)	10.3 ± 10.1	13.8 ± 12.9	9.0 ± 8.8	0.35[b]
Simplified CPIS	7.8 ± 1.0	8.1 ± 1.2	7.7 ± 0.93	0.25
SAPS III	72.2 ± 12.7	74.8 ± 11.9	71.3 ± 13.2	0.49
SOFA score	9.7 ± 3.3	10.0 ± 3.7	9.6 ± 3.2	0.77
APACHE II	28.6 ± 8.6	28.3 ± 9.6	28.7 ± 8.4	0.92
Septic shock (no. [%])	12 (37.5)	5 (55.6)	7 (30.4)	0.24
Concomitant bacteremia with the targeted bacteria (no. [%])	5 (15.6)	3 (33.3)	2 (8.7)	
Cl_{CR} (mean ± SD)[c]	115.6 ± 51.7	119.2 ± 33.2	114.2 ± 58.0	0.81
>120 mL/min/1.73 m^2	14 (43.7)	6 (66.7)	8 (35.8)	
90–119 mL/min/1.73 m^2	8 (25.0)	2 (22.2)	6 (26.1)	
60–89 mL/min/1.73 m^2	3 (9.4)	0 (0.0)	3 (13.0)	
30–59 mL/min/1.73 m^2	7 (21.9)	1 (11.1)	6 (26.1)	
CVVH	0 (0.0)	0 (0.0)	0 (0.0)	

[a]Fisher's exact test.
[b]Kruskal-Wallis test.
[c]Using urine output collection over 24 h.

CPIS, clinical pulmonary infectious score; MDRD, Modification of Diet in Renal Disease formula; CVVH, *Continuous Veno-Venous Hemofiltration;*

Clinical PK and microbiology. A high PK interindividual variability was observed in the serum and ELF concentrations in both groups.

Mean observed concentrations in plasma and ELF are displayed in Fig. 1.

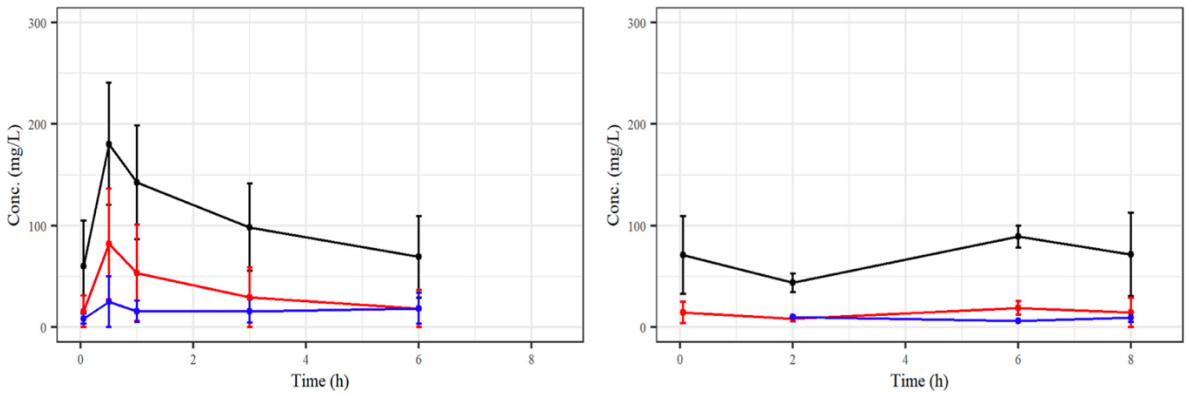


FIG 1 Mean (± standard deviation [SD]) observed free (red) and total (black) concentrations of temocillin in plasma and total concentration of temocillin in ELF (blue), after intermittent infusion, 2 g every 8 h over 0.5 h (n = 23) (left), and continuous infusion, 6 g over 24 h (n = 9) (right).

Forty-six pathogens were isolated from the 32 patients (11 in the CI group; 35 in the II group), among which were 33 nonfermenter *Enterobacterales* (data not shown). Based on Vitek 2, the majority (85%) of pathogens had an MIC of ≤4 mg/L for temocillin, and 15% had an MIC of 8 mg/L. Based on Etest, 10 (30.3%) pathogens had an MIC of ≤4 mg/L, 12 (36.4%) had an MIC of 4 to 8 mg/L, and 11 (33.3%) had an MIC of >8 mg/L, mainly *Escherichia coli* and *Serratia Marcescens*, corresponding to resistant strains according to BSAC recommendations. Four strains (12.1%) were ESBL producers, one in the II group and three in the CI group. None were carbapenemase producers. Based on Etest, the mean MIC was 9.94 mg/L (± 7.86 mg/L), and the median was 8 mg/L ([IQR], 4-13 mg/L).

Population PK model building and internal validation. A two-compartment model best fitted the plasma unbound concentrations, and an additional compartment was added to describe ELF concentrations. Creatinine clearance was retained as a significant covariate on

clearance (power relationship). According to the model, the plasma free fraction increases on average from 10% to about 75% when the total concentration increases from 10mg/L to 400 mg/L, with quite high inter-individual variability (CV=36%).

Total temocillin concentrations were related to unbound concentrations according to the equation: $Ct=Cu+\frac{Cb_{max}\times Cu}{BC_{50}+Cu}$. The ratio of AUC between Celf and Cu (Rauc) was estimated to be 0.73. Basic goodness-of-fit plots for total plasma, unbound plasma and total ELF concentrations showed adequate fitting performances of the model to the data (data not shown, available in the supplementary material) and visual predictive checks showed an acceptable agreement between the predicted and observed data over the dosing interval, for both free and total plasma and ELF total concentrations (Fig.2).

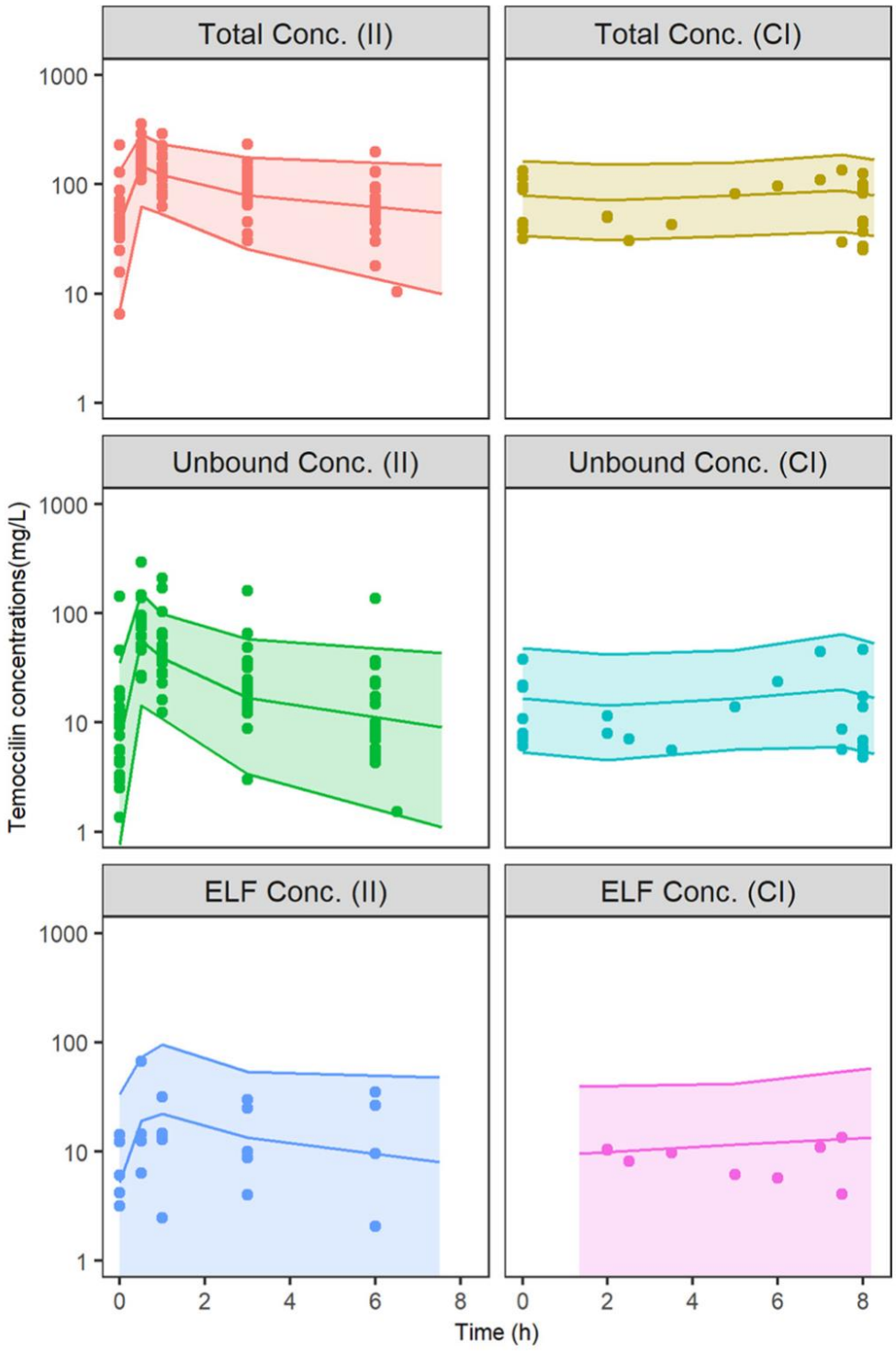


FIG 2 Visual predictive checks (simulations of the data set) of total and free plasma and total ELF concentrations after intermittent infusions (II) and continuous infusions (CI). Solid lines, medians and 90% prediction intervals; filled circles, observed concentrations.

PK/PD analysis. The probability of target attainment (PTA) was computed for two PK/PD targets (50% T>MIC for II and 100% T>MIC for both modes of infusion) against a range of MICs in plasma (free concentrations) and ELF (total concentrations) (Fig.3).

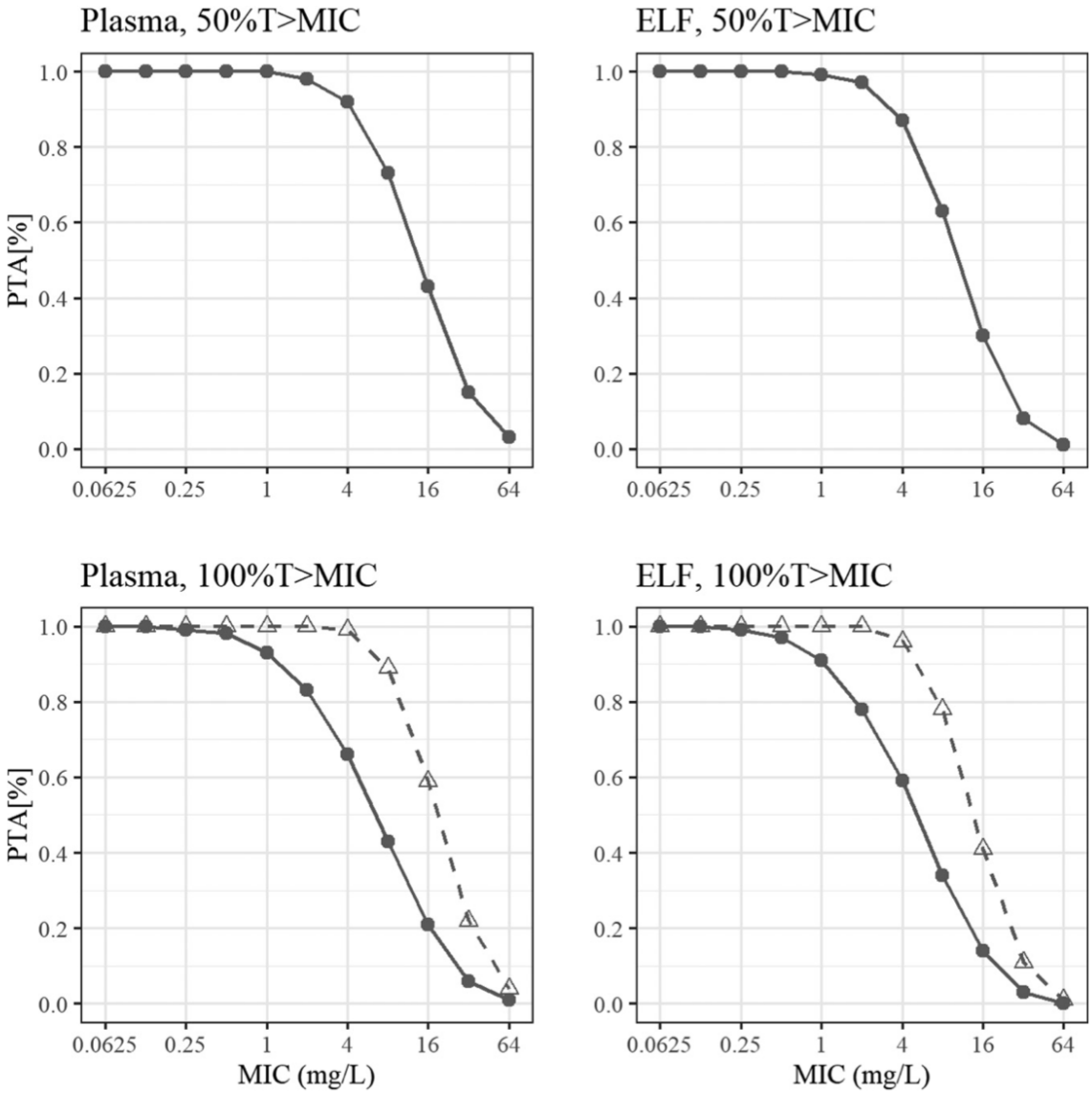


FIG 3 Probability of target attainment (obtained with the Monte Carlo simulations, n_{sim} = 32,000) for free plasma concentrations (left) and total ELF concentrations (right) for different dosing regimens and PK/PD targets (50% or 100% of time above the MIC). Continuous lines with filled circles, II (2 g/8 h over 0.5 h infusion); broken line with open triangles, CI (6 g/24 h).

The same targets were considered after dichotomization of creatinine clearance between ≥60ml/min and <60ml/min, respectively as well as in case of ARC (data not shown, available in the Supplementary material). Furthermore, PTA were performed for 60≤Clcr<90ml/min and 90≤Clcr<120ml/min (data not shown, available in the Supplementary material). The corresponding PK/PD breakpoints are determined using a probability of success of 90 % and are summarized in Table 2.

TABLE 2 PK/PD breakpoints in plasma (free concentrations) and ELF (total concentrations) for specific PD targets according to different modes of administration using a probability of success of 90%[a]

	Mode of administration			
	II		CI	
PD target	Plasma	ELF	Plasma	ELF
50% T > 1× MIC	4	2	NA	NA
Cl_{CR} < 60 mL/min/1.73 m²	8	8	NA	NA
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	8	4	NA	NA
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	4	4	NA	NA
Cl_{CR} ≥ 60 mL/min/1.73 m²	4	2	NA	NA
Cl_{CR} > 120 mL/min/1.73 m²[b]	2	2	NA	NA
50% T > 4× MIC	1	0.5	NA	NA
Cl_{CR} < 60 mL/min/1.73 m²	2	2	NA	NA
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	2	1	NA	NA
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	1	1	NA	NA
Cl_{CR} ≥ 60 mL/min/1.73 m²	1	0.5	NA	NA
Cl_{CR} > 120 mL/min/1.73 m²	0.5	0.5	NA	NA
100% T > 1× MIC	1	1	4	4
Cl_{CR} < 60 mL/min/1.73 m²	4	4	8	8
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	2	2	8	8
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	2	1	8	4
Cl_{CR} ≥ 60 mL/min/1.73 m²	1	0.5	4	4
Cl_{CR} > 120 mL/min/1.73 m²	0.5	0.5	4	4
100% T > 4× MIC	0.25	0.25	1	1
Cl_{CR} < 60 mL/min/1.73 m²	1	1	4	2
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	0.5	0.5	2	2
90 ≤ Cl_{CR}<120 mL/min/1.73 m²	0.5	0.25	2	1
Cl_{CR} ≥ 60 mL/min/1.73 m²	0.25	0.125	1	1
Cl_{CR} > 120 mL/min/1.73 m²	0.125	0.125	1	0.5

[a]NA, not applicable; II, intermittent infusion; CI, continuous infusion; Cl_{Cr}, creatinine clearance.
[b]These patients are included in the group of patients with Cl_{CR} ≥ 60 mL/min.

3.5. DISCUSSION

To the best of our knowledge, this is the first report of temocillin PK in the ELF of critically ill patients with pneumonia. Ratios of AUCs show that penetration ratio is higher than previously published for most other β-lactams, except cefepime, for both modes of infusion (17-21). As illustrated in Table 2, CI offers better PK/PD indexes than II in all scenarios considered: for the less stringent PD targets (50% T>1xMIC in II and 100% T>1xMIC in CI), the

breakpoints in plasma and ELF were found to be 4 mg/L and 2 mg/L in II, respectively, versus 4 mg/L in CI. For the most stringent PD target (100% T>4x MIC for both II and CI), the breakpoints in plasma and ELF were 0.25 mg/L in the II versus 1 mg/L in the CI, respectively. Nonetheless, these values remain well below the only available to date BSAC breakpoints (≤8 mg/L) that recommend the usage of temocillin in systemic infections and, moreover, below the mean MIC of 9.94 mg/L (based on Etest) of the pathogens isolated in this cohort of patients. At best, an MIC of 8 mg/L was achieved for the less stringent PD targets in ELF for both II and CI in patients with moderate renal impairment (30 to 60 mL/min/1.73 m^2). However, as already pointed out, as many as 33.3% of the pathogens in this cohort had an MIC>8 mg/L to temocillin by Etest, thereby precluding its usage even in the less stringent scenario.

The renal function was found to be a clinically relevant covariate on the drug clearance in the population pharmacokinetics (popPK) analysis, which is consistent with temocillin's renal elimination(22). This is also in line with PKPD findings for other renally excreted beta-lactams (23-25). The incidence of ARC in our study is also in line with current reports in critically ill patients (26).

Two previous PK studies have been undertaken with temocillin in critically ill patients; however, they were not focused on severe pneumonia (2, 3). With the same dose given by CI, Laterre *et al*. (5) reported higher average concentrations of free temocillin in plasma (mean, 37 mg/L; n = 11) than that observed in our study (13.7 ± 11.8 mg/L). This difference can probably be explained by differences in the distribution of the creatinine clearance, 56 ±34 mL/min/1.73 m^2 in the study by Laterre *et al.* versus 119.2 ±33.2 mL/

min/1.73 m^2 in the present study. Moreover, in the De Jongh *et al.* (4) study, temocillin was given at a lower dose of 4 g/day via CI in 6 patients who displayed a higher mean free plasma concentration of 21.5 mg/L and a higher plasma breakpoint of 16 mg/L in parallel with a lower CL_{CR} (102 ±18 mL/min/1.73 m^2) than in our study, thereby also possibly explaining the discrepancies observed with our results.

This study's limitations include its single-center design, the fact that it was not designed to test clinical efficacy of temocillin, and a relatively low number of patients, some of which

were severely ill with late-stage ARC (26). Furthermore, the choice of microbiological diagnostic techniques such as Vitek 2 and Etest, which was anterior to EUCAST guidelines, might have underestimated or overestimated sensitivity to temocillin in comparison to disk diffusion and broth microdilution tests, which are now recommended (24). Moreover, this study included mainly normal weight patients; therefore, no conclusions may be drawn as to PTA of temocillin in obese (BMI >30) critically ill patients (8). Finally, MIC distributions of various ESBL-producing organisms are largely unknown at this stage, making it difficult to generate recommendations for temocillin usage solely based on PTA analysis.

3.6. CONCLUSION

Penetration ratios that were estimated by MC simulations at 73% were higher than previously demonstrated for other β-lactams, except cefepime. However, the current BSAC breakpoint of 8 mg/L was achieved for II and CI only in patients with creatinine clearance of <60 mL/min/1.73 m^2 and with the least stringent PD target both in plasma and ELF. While it has not been demonstrated that efficacy of a β-lactam in severe pneumonia is entirely dependent on its ELF concentration levels, our results suggest that temocillin should not be recommended in severe nosocomial pneumonia without further clinical data in accordance with recent EUCAST clinical breakpoints.

CHAPTER 4. CONCLUSIONS AND PERSPECTIVES

Streamlining AMS tools in critically ill patients was done by using **host-response biomarkers** in order to initiate AMT only in those patients who are most likely to benefit and by **characterizing the PKPD principles** of a revived antibiotic in order to spare the use of extended spectrum antibiotics.

In the first part of this thesis, we looked for surface markers on leucocytes and platelets, which could be associated with secondary sepsis occurrence in critically ill injured patients.

First, we identified high monocytes counts and low expression of CD62L on monocytes, sampled at admission, to be independently associated with sepsis occurrence in patients admitted in the ICU for cardiac surgery, brain injury, trauma and mechanical ventilation (expected to last>48h). The same was shown for low mHLA-DR levels sampled 48-72h following admission. These findings emphasize the role of monocytes as key signaling cells in peri-operative immunology, as already shown by a genome-wide expression study investigating patterns of blood leucocytes in trauma and burn patients (1). This study showed that more than 80% of the leucocyte transcriptome was altered secondary to severe trauma, during the first 28 days after injury and that there was early concomitant activation and repression of innate and adaptive immune responses, respectively. Concerning monocyte counts, most studies have evaluated them during sepsis, with controversy regarding impact on organ dysfunction and mortality and different settings of evaluation (2-5). One recent retrospective study, which included more than 2000 patients, showed that low levels (<250/mm3) were associated with mortality, organ dysfunction and bacteremia in septic shock non-survivors (6). Interestingly, in the subset of patients in whom premorbid cell counts were available, monocyte counts increased in survivors between the premorbid period and full-blown sepsis and decreased in non-survivors, thereby conferring a prognostic significance to the biomarker. However, sample size does not allow to draw the same conclusion in our study. Concerning L-selectin findings on monocytes, data are scarce and conflicting in preclinical and observational studies, as already mentioned. Indeed,

impairment of monocyte trafficking at sites of inflammation has been associated with improved and worse outcomes in humans and mice, in trauma and sepsis (7-9). Interestingly, although we could not confirm this finding in our study, down-regulation of L-selectin on neutrophils has been associated with occurrence of nosocomial infections in blunt trauma patients (10). This has been attributed to immunosuppressive activity on T-cells in a Mac-1 fashion. An old randomized controlled trial aiming at preventing resuscitation injury caused by neutrophils, failed to show a reduction of mortality or infection rate in patients treated with aselizumab (anti L-selectin monoclonal antibody) versus placebo (11).

Concerning mHLA-DR, we confirmed the robustness of this marker in its ability to reflect post-injury immunosuppression that leads to secondary sepsis. Potential therapeutic modulation can be exerted on monocytes by administration of several agents such as IFN-γ and GM-CSF (12-14). Interestingly, in the early 90's, in 2 RCTs including severe trauma patients, use of therapeutic fixed dose and timing of IFN-γ (100µg/day, 10-21days) did not result in substantial reduction of serious infections (15, 16). The authors of those trials raised a number of questions among which, the timing of administration of immunotherapy and the lack of stratification of patients on mHLA-DR admission levels. Then, to validate the latter hypothesis, Docke *et al* nicely showed that sepsis could be cleared in 8/9 septic patients who received IFN -γ based on levels of mHLA-DR<30% (17). This was also confirmed in 2 patients who were sampled serially until mHLA-DR normalized after IFN-γ administration (18). Unfortunately, after the relative failure of the randomized ancillary studies, interest in IFNγ-mediated reversal of monocyte deactivation wore out although timing and stratification issues remain unanswered.

Concerning treatment with granulocyte macrophage colony stimulating factor (GM-CSF) for monocyte deactivation reversal, findings from two older studies had shown the efficacy of immunotherapy in patients exhibiting immunoparesis secondary to sepsis and multiple organ dysfunction syndrome (19, 20). When considering injured patients, only one recent RCT reported the benefit of GM-CSF administration based on low pre and post-operative levels of mHLA-DR (<10000mAB/C) in major abdominal surgery(21). The treatment was safe and restored monocyte competence while reducing the duration (but not the rate) of infections in immune suppressed patients undergoing esophageal or pancreatic resection. A

more recent observational trial included 119 patients undergoing elective major abdominal surgery, 44 (37%) of whom developed a nosocomial infection at a median of 9 days post-operatively (22). Investigators determined IL-10 protein levels by ELISA and the expression of selected genes pre and postoperatively by qRT-PCR. Perioperative cell surface mHLA-DR was determined using flow cytometry. Investigators demonstrated that mHLA-DR cell surface expression levels decreased by a factor of three from the pre-operative time point to 24 hours post-operatively (P<0.001) and were then unchanged between 24 and 48 hours post-operatively. No difference was detected between patients who did and did not subsequently develop an infection. By contrast, higher IL-10 mRNA (p=0.007) and protein levels (p=0.001) were associated with increased risk of infection. Reduced production, rather than intracellular sequestration which had been previously hypothesized, accounted for the postoperative decline in mHLA-DR expression and this was mediated by IL-10 dependent pathways. *In vitro* addition of GM-CSF and IFN-γ restored mHLA-DR levels, each via a distinct pathway. The findings of this recent study are in line with those of authors arguing for the benefit of immunotherapy in sepsis-induced immunosuppression (23). Hence, biomarker-guided immunotherapy that is administered to patients at the proper phase of injury-induced immune suppression might still hold its promises.

Second, immune monitoring on platelets led to the finding that elevated D-dimers and platelet-bound fibrinogen levels within 24 h of ICU admission help identifying critically ill patients at risk of developing sepsis. However, we did not observe thrombocytopenia nor disseminated intravascular coagulation nor leucocyte-platelet aggregates, the first of which has been associated with impaired host defence in animal models (24).

Hence, we observed partial features of immunothrombosis, such as recently described in the early stages of sepsis, which is characterized by early activation and reprogramming of the innate immune system and platelets (25). From a mechanistic point of view, our findings across the two studies are coherent with the common physiopathological pathway between sepsis and injury (26, 27). From the preventive point of view, apart from immunotherapy in selected patients discussed above, use of aspirin was shown to be associated with protection against sepsis in patients without established cardiovascular disease in a recently published

observational study (28). However, no randomized trial has yet investigated the effect of antiplatelet agents before or during sepsis.

In the second part of this thesis, we, as others, found that **PCT** was a poor marker of severe infection. PCT bears an AUC of 0.69 for its ability to discriminate between certain/probable infection and possible/no infection when initiating AMT for sepsis. Using PCT as a stewardship tool using that strategy did not contribute to lessen AMT use (29, 30). Instead, PCT has been suggested as a safe de-escalation tool in high-risk patients thanks to its high negative predictive value (31-33). Various algorithms are available based on regression of cut-off levels (PCT kinetics>80% or 90% depending on the initial cut-off level, <0.25µg/L or ≤0.5µg/L, respectively) and even on the site of infection (34, 35). However, despite the number of publications over the past three decades, there are significant hurdles to overcome in order to implement PCT as an effective AMS tool in daily clinical practice. First, it has been proven that continuous education about its use as a de-escalation tool is essential to guarantee better protocol adherence (36). Moreover, the impact of such a strategy on mortality in the sickest patients is still controversial (37) because improved survival and decreased duration of AMT were mainly observed in studies with low protocol adherence (less than 50% in two main trials) and use of concomitant biomarkers such as CRP (38-40). Finally, PCT is of uncertain use in immunocompromised patients and its actual cost of a single test (10-30€) seriously hinders serial samplings that could allow its wide implementation as an AMS tool (41, 42). These are the hurdles to overcome that explain the current suggestion of SSC regarding PCT and AMT discontinuation: adding PCT to clinical evaluation is weakly recommended because of low quality of evidence (43).

In the third part of this thesis, we characterized the PKPD properties of a narrow-spectrum beta-lactam in critically ill patients who were treated for HAP caused by Gram-negative pathogens. It is increasingly admitted that personalized treatment with adequate knowledge of patient characteristics and PKPD properties of the used antimicrobial, combined with TDM, should ameliorate outcome and improve overall AMS (44, 45). Pharmacokinetics of temocillin, particularly in critically ill patients with unpredictable and sudden PK variations, are largely unknown. Yet, temocillin use is licensed for systemic infections, including

pneumonia. We showed that although the penetration ratio was surprisingly high (73%) only a minority of critically ill patients with moderate renal impairment would reach potential target of success in pneumonia, provided that temocillin be used at 6g/day through a continuous infusion. This was somehow expected: it is in line with numerous publications advocating higher than usual posologies for beta-lactams to treat serious infections (46, 47). The main driver for underdosing was high renal clearance which was found to be present in 44% of patients included in this pragmatic trial. Future areas of research should focus on obese patients who exhibit distinct risk factors for underdosing of beta-lactams and determination of MIC distributions of various ESBL organisms (48).

Finally, our findings indirectly support the use of TDM, given the risk of underdosing and therapeutic failure (44). The first hurdle to routine implementation of TDM is lack of standardization and hence, high costs destined to development of the technique and realization of the test (not reimbursed in Belgium). Furthermore, the high turn-around time hinders timely adaptation of posology. An easier and faster enzymatic colorimetric technique has been described and could enable a point-of-care testing in patients(49). This could ensure day-to-day adaptation of AB, limited drug toxicity and inherent costs. Moreover, lack of standardization does not allow for initial dosing regimen suggestion, especially in patients with high PK variability. This is one of the concerns that was raised recently after the completion of the TARGET trial which showed lack of evidence-based clinical benefit following traditional and cumbersome TDM optimization of piperacillin-tazobactam levels in septic patients (50, 51). An interesting alternative are model-informed precision dosing (MIPD) softwares, which propose *a priori* and *a posteriori* dosing regimens and related probability of target attainment, based on patients' covariates or integration of TDM results, respectively (52).

In conclusion, we provided a contribution to translational research focusing on streamlining AMS tools in critically ill patients, which will necessitate external validity through further studies. The use of biomarkers for diagnosis and prognostication of sepsis as well as for antimicrobial stewardship has already come a long way. A recent reappraisal review identified 80 new biomarkers in sepsis research almost a decade after reporting on 178

previously identified ones, among which proteins, cytokines and soluble receptors(53). The authors stressed that little real progress has been made in identifying biomarkers with clinical significance, mainly due to a lack of improved methodological approaches for such a complex pathophysiological syndrome, an argument which is shared by other experts (54, 55). Nevertheless, apart from proteomics and plasma protein biomarkers such as PSP (pancreatic stone protein) and HBP (heparin-binding protein), promising molecular (genomic, transcriptomic) diagnostic biomarkers aimed at assessing host-pathogen interactions are under development.

PSP is a protein that is released during splanchnic hypoperfusion and a recent multicenter prospective study showed that serial measurements, through a rapid point-of care test, helped to detect sub-clinical sepsis in ICU patients earlier than PCT and CRP(56). Interestingly, prediction of sepsis was shown by the same group in severely burned patients, irrespective of trauma severity (57). Moreover, besides already evaluated HBP levels and the direct measurement of endothelial cells in blood, which both indicate the disruption of the endothelial barrier, some experts advocate an entire plasma proteomic analysis in order to gain insights into theranostics (58-60).

Advances in genomics offer supplementary opportunities for translational research in host-pathogen interactions (61). After identification of myriads of SNP (single nucleotide polymorphisms) in host genome that are associated with susceptibility and type of response to sepsis, experts now advise to conduct large genome-wide association studies (62-64). These approaches should avoid poor reproducibility which affected candidate gene approaches studies. Interestingly, very recently, a combined approach using host and pathogen metagenomic profiles in a cohort of hospitalized and critically ill patients helped to distinguish infectious from non-infectious conditions with excellent sensitivity (97-100%) (65). However, the diagnostic model, which combined patients' whole blood and plasma nucleic acid mNGS analysis and machine-learning modelling, suffered from poor specificity (78%) thereby hindering its use in antibiotic stewardship.

Advances in transcriptomics have allowed to move from the vast initial heterogeneity observed between individual septic patients' transcription gene profiles to the ability, nowadays, to discriminate between septic and non-septic inflammatory states, through the identification of common gene clusters (66-68). From the antimicrobial stewardship point of view, these are important findings since they allow early avoidance of AMT in non-septic

patients. Concerning the source, available concordant evidence leans towards a large overlap in blood leukocytes transcriptome profiles in abdominal and pulmonary infections, some of which were associated with a worse prognosis (69, 70). Moreover, notable differences between infectious sources were found in hemostasis, cytokine signalling, innate and adaptive immune as well as metabolic transcriptional pathways thereby hindering the "one size fits all" immune modulatory drug approach. Ultimately, cluster analysis of transcriptional patterns should enable stratification of patients for personalized future therapeutic interventions (71, 72).

Finally, machine-learning derived risk prediction modelling is in development for early recognition of sepsis (73, 74). It is however based on large databases fed by millions of electronic medical records of patients encompassing mainly physiologic data and very few biologic findings and the evidence towards its performance ability, compared to clinical diagnosis, is conflicting (75, 76). Enriching these databases with biomarkers could prove to be useful in a precision medicine approach of sepsis (77).

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ANNEXES

Articles

Prospective flow cytometry analysis of leucocyte subsets in critically ill patients who develop sepsis: a pilot study (submitted)

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Acknowledgements

We are grateful for the insightful revision of Profs B. Misset and JM Cavaillon.

We are thankful to BD Sciences for the generous gift of monoclonal antibodies.

Abstract:

Purpose: Sepsis in critically ill patients with injury bears a high morbidity and mortality. Extensive phenotypic monitoring of leucocyte subsets in critically ill patients at ICU admission and during sepsis development is still scarce. The main objective of this study was to identify early changes in leukocyte phenotype which would correlate with later development of sepsis.

Methods: Patients who were admitted in a tertiary ICU for organ support after severe injury (elective cardiac surgery, trauma, necessity of prolonged ventilation or stroke) were sampled on admission (T1) and 48-72h later (T2) for phenotyping of leukocyte subsets by flow cytometry and cytokines measurements. Those who developed secondary sepsis or septic shock were sampled again on the day of sepsis diagnosis (Tx).

Results: Ninety-nine patients were included in the final analysis. Nineteen (19.2%) patients developed secondary sepsis or septic shock. They presented significantly higher absolute monocyte counts and CRP at T1 compared to non-septic patients (1030/µl versus 55/µl, p=0.013 and 5.1mg/ml versus 2.5mg/ml, p=0.046, respectively). They also presented elevated levels of monocytes with low expression of L-selectin ($CD62L_{neg}$monocytes)(OR[95%CI]: 4.5 (1.4-14.5) p=0.01) and higher SOFA score (p<0.0001) at T1 and low mHLA-DR at T2 (OR[95%CI]: 0.003 (0.00-0.17) p=0.049). Stepwise logistic regression analysis showed that both monocyte markers and high SOFA score (>8) were independent predictors of nosocomial sepsis occurrence. No other leucocyte count or surface marker nor any cytokine measurement correlated with sepsis occurrence.

Conclusion: Monocyte counts and change of phenotype are predictive of secondary sepsis in critically ill patients with injury.

Keywords: injury; sepsis; flow cytometry; monocytes; HLA-DR; L-selectin.

Statements and Declarations

Ethics approval and consent to participate

The study was appointed the Belgian number B707201111981 by the local ethics committee of University Hospital of Liège (number 707) and written informed consent was obtained from the patient or his/her legal representative.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed (beyond those included in the supplementary files) during the current study are available from the corresponding author on reasonable request.

Competing interests

Not applicable.

Funding

No funds or grants, was received.

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

NL, PD, CO and AG designed the study; NL, CG, CD, CL, AH and AG did the experiments; NL, CG, NM and AG analysed the data; NL wrote the manuscript, CG and AG revised the manuscript.

Background:

It is estimated that 25-35% of critically ill patients develop sepsis which is associated with increased length-of-stay (LOS), morbidity and mortality[1-4]. As in sepsis-induced immunosuppression, immune alterations affecting patients with critical injuries such as trauma, major surgery or burns, have been associated with increased susceptibility to secondary infections and mortality[5-8]. The first reports of monocyte anergy and endotoxin tolerance date back to the 70's in major surgical and burn patients[9, 10]. Since then, most studies relying on flow cytometric analysis of peripheral blood cells, have focused on single and restricted types of immune cells defects such as T-lymphocytes, monocytes and neutrophils[11-14]. The most commonly studied parameter of immune dysfunction associated with injury is the low HLA-DR expression on monocytes (mHLA-DR), which induces an impaired functional state of these cells. The latter feature has been associated with secondary sepsis and sometimes outcome in severe trauma, burn and postoperative patients[15-21].Targeted treatment has been tempted in that context. Older studies have shown contrasted clinical outcomes after immunotherapy, based on GM-CSF or IFNγ administration, despite efficacious restoration of mHLA-DR and/or IFNγ endogenous secretion[22-24]. In a hypothesis-driven approach, other markers such as elevated levels of regulatory T-helper cells (T_{regs}) were recently shown to be predictive of nosocomial sepsis in combination with low levels of mHLA-DR and neutrophil CD88 in an ICU patient population comprising but not restricted to trauma and postoperative patients[25]. So far, only three studies relying on wide flow cytometry panels to predict secondary sepsis in critically ill patients have been conducted and the first two included only

septic patients [26-28]. These authors showed that clinical deterioration at 48h could be predicted in septic patients with circulating immature granulocytes which induced T-cell lymphopenia after enrichment. A very recent study focused on the overtime changes of the injury-induced immune profile in a large cohort of septic, trauma and surgical patients during the first week of ICU admission[28]. The authors used a restricted number of immune markers determined by flow cytometry, combined with transcriptomic and functional tests to show that the initial adaptive immune response to injury, whatever the etiology, was not associated with a risk of secondary infections. Moreover, only a subset of patients exhibiting late combined immune alterations (such as low CD3D, CD74 messenger RNA and mHLA-DR and high S100A9 messenger RNA at days 5-7) developed secondary infections. Our study aimed at describing the temporal changes of various leucocyte surface markers, via flow cytometric analysis, in non-septic patients, after critical injury, in association with nosocomial sepsis occurrence. The studied panel included subsets of B and T lymphocytes, as well as monocyte and neutrophil characterization.

Materials and methods:

Study patients

This single-center, prospective, observational study was conducted in 3 tertiary ICUs over a 7-month period at CHU de Liège. The institutional ethics committee approved the study (Belgian number: B707201111981) and written informed consent was obtained from the patient or his/her legal representative. Inclusion criteria included: age over 18 years, elective cardiac surgery (CABG or valve replacement), trauma, acute ischemic or hemorrhagic stroke and invasive ventilation (>48h) for reasons other than infection. Exclusion criteria were: life expectancy of less than 48h, systemic or oral antibiotic therapy for active infection, active hematological or solid organ proliferative disease, HIV (+) status, chronic viral hepatitis B and

C and use of any immunosuppressive therapy. Upon admission to ICU, the following demographic characteristics were recorded: gender, age, type of admission (surgical or medical) and treatment with vasopressors. The sequential organ failure assessment score (SOFA) score was calculated[29]. For each patient, the following data were also collected: length of ICU and hospital stay (days), duration of ventilation (days), administration of vasopressors prior to and during ICU stay, antibiotic treatment, site of infection and microbiological documentation, necessity of hemofiltration or intermittent hemodialysis during and/or after ICU stay. All patients included were followed up until 1 year after inclusion in the study or death. In case of death, time was recorded.

Blood samples were collected within 24 h (T1) of admission, 48 h (T2) after admission and on the day of diagnosis of sepsis and/or septic shock (Tx). The Sepsis-3 definition[30] was used for this study. Definitions of infection were based on Center for Disease Control (CDC) criteria[31-33]. Our institution does not recommend routine use of selective digestive tract decontamination. Patients were compared to an age-matched (>50 years) cohort of healthy controls (n=18).

Immunophenotyping

Automated blood counts were obtained using the Sysmex XS-800 hematology analyzer (Kobe, Japan) for quantification of the absolute cell counts. Immunophenotyping was performed by adding combinations of monoclonal antibodies to 100 µl of whole blood, incubated for 20 minutes at 4°c in the dark, after which red cell lysis was achieved by adding BD FACS Lysing Solution. Cells were centrifuged and resuspended in HBSS 1% formaldehyde. Flow cytometric data were acquired on a FACS Verse flow cytometer (BD Biosciences). The daily setup procedure involved a one-step performance check, using BD FACSuite™ CS&T Research

Beads to adjust photomultiplier tube voltages. This ensured that the target MFI values were held constant from day to day.

The following combinations of monoclonal antibodies were used. For NK cells and T lymphocytes: anti-CD3-FITC, CD4-PerCP, CD8-APC-H7, CD14-V450, CD45-V500, CD56-PE-Cy7, CD69-APC and CD279 PE. For B and regulatory T lymphocytes: anti-CD3-FITC, CD4-PerCP, CD19-PE-Cy7, CD25-PE, CD45-V500 and CD127-AlexaFluor 647. For monocytes: anti-CD14-V450, CD16-AlexaFluor647, CD45-V500, CD64-PE-Cy7, CD279-PE, and HLA-DR-PerCP. For neutrophils: anti-CD11b-PE, CD11c-PE, CD16-PE, CD45-V500, CD62L APC and CD64-PE-Cy7. All antibodies were from BD Biosciences.

Cytokine measurements

Plasma was prepared from citrated whole blood samples to quantify plasma levels of TNFα, IL-10, IL-17A, IL6, IL-7 and IFNγ. Cytokine levels were measured using multiplex Cytometric Bead Arrays (BD Biosciences) on the FACSVerse System. Analysis was performed with the FCAP Array^{TM} software (BD Biosciences).

Statistical analysis:

Results were expressed as mean and standard deviation (SD) for quantitative data and as median and interquartile range (IQR) for durations. For categorical findings, frequency tables were used. Comparisons between septic and non- septic patients characteristics were done by the ANOVA or Kruskal-Wallis test for continuous variables and Chi-square or Fisher exact test for categorical variables. The predictive value of sepsis was assessed for each baseline variable by logistic regression analysis on log-transformed biological variables. The variables significant

at $p<0.10$ were combined in a stepwise multivariate logistic regression analysis to identify independent baseline predictors of sepsis. The odds ratio (OR) with 95% confidence interval [95%CI] and ROC (receiving operating curve) curve analysis with area under the curve (AUC) were used to quantify the ability of the selected predictors to discern between septic and non-septic patients. The Youden method was applied to define an optimal cut-off point for those predictors. Data recorded on the same patients but at different time points were compared by the Wilcoxon signed rank test. Results were considered significant at the 5% critical level ($p<0.05$). All statistical calculations were performed with SAS (version 9.4) and R (version 3.0.3).

Results:

Patients baseline characteristics:

A total of 99 adult patients with complete data were included in the final analysis. The demographic and clinical characteristics at admission are presented in Table 1. There were predominantly male patients (60.6%) with a mean age of 64 ± 15 years. The type of admission was mainly surgical (86.9%) and cardiac surgery accounted for most patients (68.7%). Ten (10.1%) patients received vasopressors before admission, 67 (67.7%) received prophylactic antibiotics during surgery. The median admission SOFA score was 5 [IQR: 4-8].

Table 1. Demographic and clinical characteristics of the patients at ICU admission (N=99)

	Total N=99	Nonseptic N=80	Septic N=19	p-value
Age (years)	64 ± 15	65± 15	62 ± 15	0.46
Gender: male	60 (60.6)	48 (60.0)	12 (63.2)	0.80
Surgical admission	86 (86.9)	70 (87.5)	16 (84.2)	0.70
Reason for admission				0.0022
Cardiac surgery	68 (68.7)	61 (76.2)	7 (36.8)	
Acute brain injury	12 (12.1)	6 (7.5)	6 (31.6)	
Trauma	13 (13.1)	10 (12.5)	3 (15.8)	
Ventilation > 48h	6 (6.1)	3 (3.8)	3 (15.8)	
SOFA at ICU admission	5 (4 – 8)	4 (3 – 7)	10 (8 – 12)	<0.0001
Diabetes	17 (17.2)	13 (16.2)	4 (21.0)	0.74
Cardiovascular disease	79 (79.8)	68 (85.0)	11 (57.9)	0.021
Vasopressor before admission	10 (10.1)	6 (7.5)	4 (21.0)	0.096
Prophylactic antibiotics	67 (67.7)	61 (76.2)	6 (31.6)	0.0002
Total hospital LOS (days)	11 (9 – 19)	11 (9 – 16)	26 (16 – 71)	<0.0001
ICU LOS ((days)	3 (2 – 7)	3 (2 – 4)	15 (10 – 22)	<0.0001
28-days mortality	13 (13.1)	6 (7.5)	7 (36.8)	0.0028
90-days mortality (N=97)	14 (14.4)	7 (8.9)	7 (38.9)	0.0038

ICU : Intensive Care Unit, SOFA: Sequential Organ Failure Assessment, LOS : Length Of Stay
Results are expressed has mean± SD, median (IQR), or n(%) as appropriate and p-values from ANOVA, Kruskal-Wallis, Chi-square or Fischer exact tests respectively

Sepsis occurrence

Nineteen (19.2%) patients developed sepsis or septic shock during follow-up, after a median time of 5 [IQR: 3-7] days and 80 did not. As shown in Table 1, age, gender, category of admission, history of diabetes and use of vasopressor prior to ICU admission were not associated with sepsis occurrence. By contrast, higher SOFA score, admission for brain injury and lack of prophylactic antibiotics were predominant in patients who developed sepsis.

Moreover, septic patients displayed higher hospital and ICU length-of-stay compared to non-septic patients (26 days [16-71] versus 11 days [9-16] , p<0.0001 and 15 days [10-22] versus 3 days [2-4], p<0.0001, respectively). Septic patients also displayed a higher 28-day and 90-day mortality compared to non-septic patients (36.8% versus 7.5%, p=0.0028 and 38.9% versus 8.9%, p=0.0038, respectively). Infections sites and microbiological documentation are shown in Table S1.

Table S1

Site of infection	N (frequency of infection)	Microbiological documentation
HAP-VAP	16	*MSSA, Serratia Marcescens, Morganella Morganii,Klebsiella Pneumoniae, Haemophilus Influenzae, Moraxella Catarrhalis, Proteus Vulgaris, Citrobacter Koseri, Enterococcus Faecalis, Escherischia Coli, Klebsiella Ornitholytica*
SSTI	3	*Staphylococcus Epidermidis, Enterobacter Cloacae Complex, Enteroccus Faecalis*
CLABSI	1	*Staphylococcus Epidermidis*
BSI	3	*Escherichia Coli, Citrobacter Koseri, Morganella Morganii, Serratia Marcescens*

Sites of infection and microbiological documentation

Legend:

HAP-VAP: hospital-acquired pneumonia

VAP: ventilator-associated pneumonia

SSTI: surgical site and soft tissue infection

CLABSI: central line associated blood stream infection

BSI: primary blood stream infection

Some patients developed more than one infection and some infections were polymicrobial. Two episodes of VAP were clinically diagnosed and empirically treated although no organism grew in culture.

Standard laboratory tests and cytokines

Comparison of standard laboratory tests and cytokine levels obtained within 24h after admission to the ICU is shown in Table 2. Absolute monocyte counts and CRP were significantly higher in patients who developed sepsis compared to non-septic patients (1030/µl versus 55/µl, p=0.013 and 5.1mg/ml versus 2.5mg/ml, p=0.046, respectively). Monocyte counts did not add to the performance of SOFA score alone (AUC 0.84 with a cut-off level >8) for prediction of secondary sepsis as shown in Fig S1.

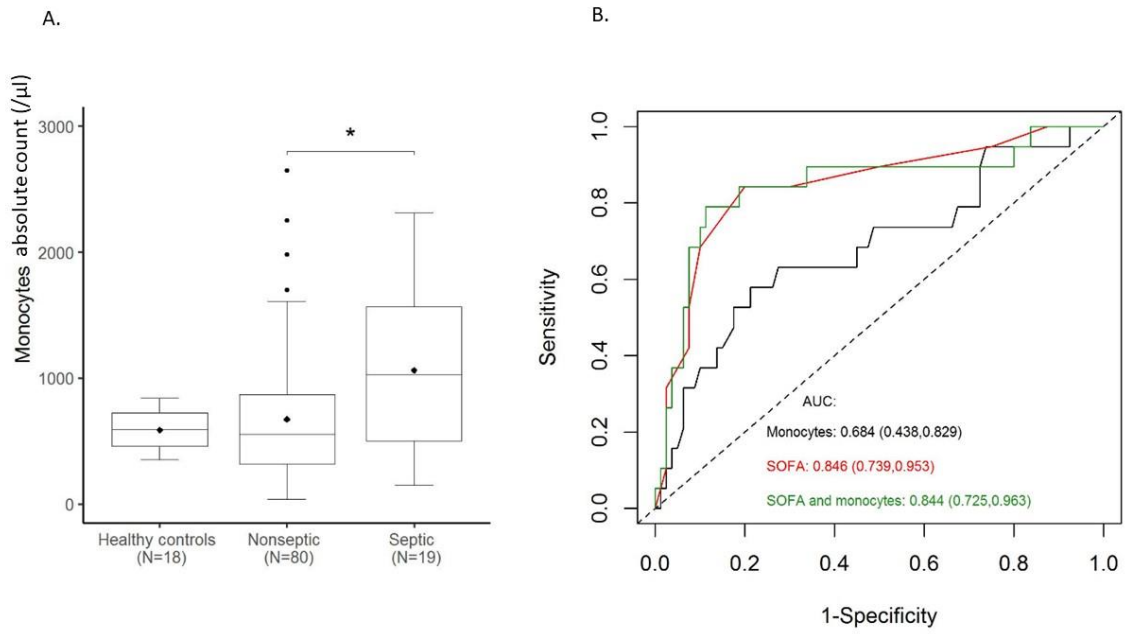
Table 2. **Comparison of biological parameter levels recorded upon admission to ICU according to later occurrence of sepsis** (n = 99 patients)

	Non-septic n = 80	Septic n = 19	P-value
CRP (mg/ml)	2.5 (1.1-9.1)	5.1 (2.5-17.4)	0.046
Fibrinogen (g/l)	2.4 (2.0-3.0)	3.0 (2.0-3.7)	0.13
Platelet count (k/µl)	134 (105-166)	169 (117-213)	0.12
White blood cells count (K/µl)	9.0 (7.0-12.2)	9.8 (6.8-16.3)	0.47
TNF (pg/ml)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.074
IL10 (pg/ml)	4.2 (0.0 - 11.8)	3.8 (0.0-10.1)	0.95

IL17A (pg/ml)	4.9 (0.76 - 11.6)	2.0 (0.0-7.8)	0.17
IL6 (pg/ml)	97.0 (34.8 - 189.2)	105.7 (39.3-240.3)	0.75
IL7 (pg/ml)	1.4 (0.17 - 4.3)	1.2 (0.21-1.5)	0.28
IFN□	0.0 (0.0 – 0.0)	0.0 (0.0-0.0)	0.66
Neutrophils (counts/µl)	7045 (5704 – 9344)	6405 (5919 – 7298)	0.62
Monocytes (counts/µl)	55 (320 – 873)	1030 (430 – 1600)	0.013
Lymphocytes (counts/µl)	1200 (810 – 1715)	1180 (990 – 1470)	0.97

Results are expressed as median and interquartile range (IQR). P-value of Kruskal-Wallis test; null values for TNFα and IFNγ correspond to values under the level of detection (3.8pg/ml); MFI, Median fluorescence intensity

Fig S1



Panel A: Measurements at ICU admission in nonseptic and septic patients and in healthy controls (> 50 years). (**: p<0.05)*

Panel B: Predictive value of monocyte absolute count (/µl) obtained at T1. ROC curve analysis of sepsis occurrence based on levels of monocytes and of SOFA is shown.

Leucocytes cell surface markers

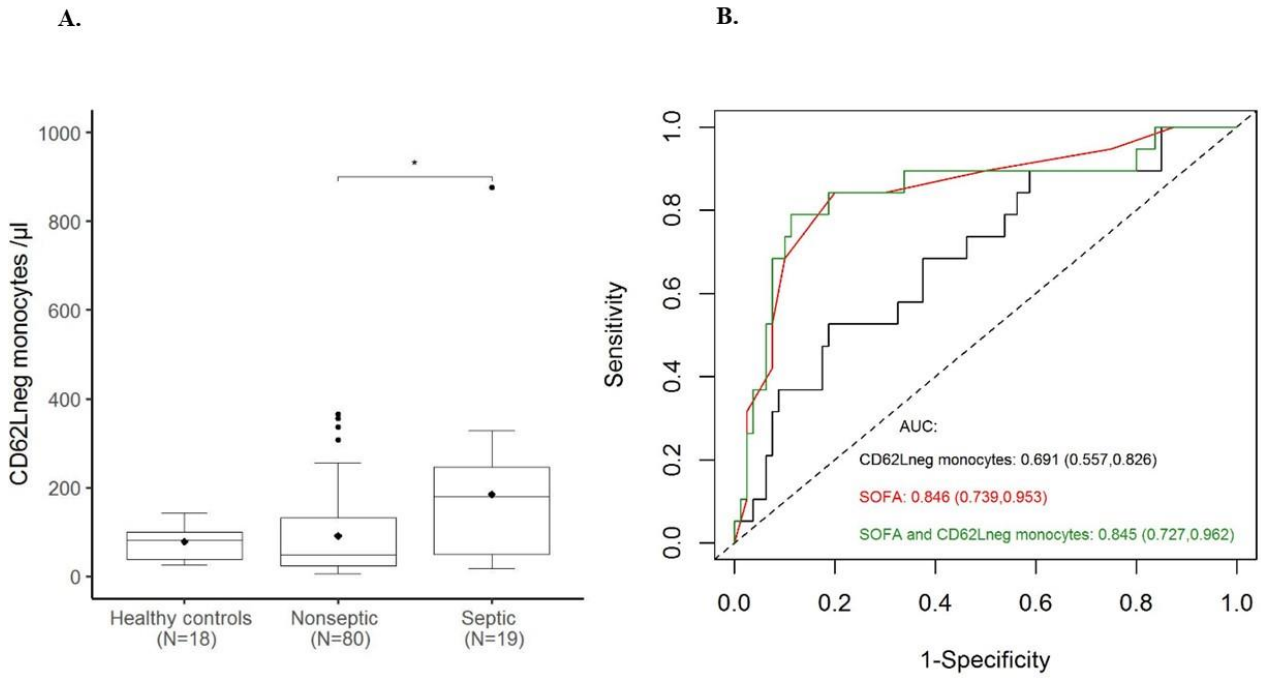
When considering leucocytes subsets at T1 against healthy controls, elevated absolute counts of classical, intermediate and total monocytes, increased levels of $CD62L_{neg}$ monocytes and low expression of HLA-DR in total and intermediate monocytes were shown to be associated with further sepsis development in univariate analysis (Table 3). When all potential predictors of sepsis ($p<0.10$) recorded at ICU admission (T1) were combined into a stepwise logistic regression, only the absolute count of $CD62L_{neg}$ monocytes was independently associated with sepsis occurrence (OR[95%CI]: 4.5[1.4-14.5], p=0.011)(Fig.1A). By ROC curve analysis (Fig.1B), a cut-off value of 180/µl (AUC 0.69) was derived for $CD62L_{neg}$ monocytes at T1 to discriminate septic from non-septic patients. The $CD62L_{neg}$ monocytes count did not add to the performance of SOFA score alone for secondary sepsis prediction, as seen in Fig.1B. In the 12 patients available for complete data at T1, T2 and Tx, there was no temporal change in the numbers of $CD62L_{neg}$ monocytes (Fig S2). When considering leucocyte subsets at T2, low expression of mHLA-DR by classical and intermediate monocytes and low levels of CD4+CD279+ lymphocytes were associated with sepsis development in univariate analysis (Table 4). When all potential predictors of sepsis ($p<0.10$) recorded at T2 were combined into a stepwise logistic regression, only low expression of HLA-DR by intermediate (CD14++CD16+) monocytes was independently associated with sepsis development (Fig.2A) (OR[95%CI]: 0.003[0-0.17], p=0.049). By ROC curve analysis (Fig.2B), a cut-off level of 1090 MFI (AUC 0.74) was derived for mHLA-DR to discriminate septic from non-septic patients. The level of m-HLA-DR did not add to the performance of SOFA score alone for secondary sepsis prediction, as seen in Fig.2B. In the 7 septic patients available for complete data at T1, T2 and Tx, there was no temporal change in the levels of the marker (Fig S3).

The temporal change (delta T2-T1) of the two monocyte markers, i.e. $CD62L_{neg}$ monocytes absolute count and HLA-DR expression by intermediate monocytes, was not predictive of sepsis occurrence (data not shown).

Table 3. Impact of parameters at ICU admission (T1) on the risk of sepsis.

	Nonseptic (N=80)			Septic (N=19)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1 ; Q3)	N	Mean ± SD	Median (Q1 ; Q3)	OR (95%CI)	p-value
HLA-DR MFI - total monocytes	80	1293 ± 632	1145 (805 ; 1682)	19	909 ± 477	776 (469 ; 1382)	0.030 (0.003 – 0.35)	0.0052
CD14 MFI - total monocytes	69	15709 ± 6886	13787 (11585 ; 19230)	12	15838 ± 7310	14613 (8432 ; 20030)	082 (0.022 – 31)	0.92
CD16 MFI - total monocytes	69	149 ± 167	111 (78 ; 171)	12	147 ± 70	150 (84 ; 190)	2.1 (0.28 – 15.2)	0.48
CD64 MFI - total monocytes	80	25273 ± 7449	23702 (19603 ; 29378)	19	25813 ± 5149	24679 (23119 ; 27938)	4.0 (0.052 – 301)	0.53
CD279 MFI - total monocytes	80	18 ± 100	-8.7 (-39 ; 43)	19	55 ± 127	33 (-24 ; 112)	2.9 (0.73 – 12)	0.13
Classical monocytes/µl	69	472 ± 324	419 (256 ; 598)	12	746 ± 433	742 (343 ; 1077)	11 (1.01 – 122)	0.049
Intermediate monocytes/µl	69	151 ± 171	82 (35 ; 221)	12	326 ± 221	392 (79 ; 502)	4.7 (1.2 – 19)	0.029
Non-classical monocytes/µl	57	22 ± 32	7.8 (3.3 ; 25)	10	36 ± 34	28 (5.7 ; 55)	2.7 (0.81 – 9.2)	0.11
CD279 MFI – classical monocytes	69	-16 ± 76	-23 (-58 ; 6.1)	12	3.8 ± 118	-20 (-58 ; 20)	1.1 (0.17 – 7.5)	0.90
HLA-DR MFI – classical monocytes	69	1126 ± 595	1030 (690 ; 1539)	12	756 ± 467	481 (373 ; 1155)	0.025 (0.001 – 0.47)	0.014
CD64 MFI – classical monocytes	69	25751 ± 7066	24756 (20707 ; 29028)	12	25712 ± 6122	24923 (22944 ; 27241)	1.3 (0.005 – 321)	0.93
CD279 MFI – intermediate monocytes	69	45 ± 109	11 (-7.4 ; 74)	12	103 ± 207	26 (-37 ; 164)	3.2 (0.60 – 17)	0.18
HLA-DR MFI – intermediate monocytes	69	1643 ± 791	1380 (1180 ; 2022)	12	1382 ± 756	1196 (647 ; 2053)	0.08 (0.003 – 2.1)	0.13
CD64 MFI - intermediate monocytes	69	25335 ± 7530	23912 (19508 ; 29242)	12	26032 ± 5305	25458 (22585 ; 29091)	4.4 (0.025 – 777)	0.58
CD279 MFI – non-classical monocytes	69	166 ± 133	142 (103 ; 211)	12	172 ± 85	192.3 (108 ; 221)	1.4 (0.21 – 8.9)	0.73
HLA-DR MFI – non-classical monocytes	69	6615 ± 4883	6328 (1962 ; 10108)	12	7973 ± 4160	6745 (4431 ; 11246)	4.0 (0.64 – 24.8)	0.14
CD64 MFI – non-classical monocytes	69	12141 ± 8841	8343 (5272 ;16776)	12	12653 ± 7537	10944 (6659 ; 16946)	1.9 (0.25 – 14)	0.55
CD62Lneg monocytes/µl	80	91 ± 94	48 (24 ; 131)	19	185 ± 196	179 (44 ; 247)	4.5 (1.4 – 14.5)	0.011
Total neutrophils/µl	80	7838 ± 3815	7045 (5365 ; 10160)	19	8601 ± 4456	7310 (4720 ; 12670)	2.1 (0.19 – 23)	0.55
CD62L MFI - neutrophils	80	7571 ± 2585	7677 (5704 ; 9344)	19	6658 ± 1751	6405 (5919 ; 7298)	0.21 (0.010 – 4.4)	0.32
CD16 MFI - neutrophils	69	1773 ± 654	1720 (1466 ; 2176)	12	1623 ± 395	1661 (1384 ; 1820)	0.49 (0.013 – 19)	0.70
CD64 MFI - neutrophils	80	1517 ± 1040	1293.5 (890 ; 1801)	19	1490 ± 876.1	1285 (699 ; 1849)	1.1 (0.20 – 6.4)	0.89
CD11b MFI - neutrophils	80	11569 ± 6583	9645 (7279 - 14752)	19	11000 ± 5935	9057 (6674 – 15051.)	0.59 (0.061 – 5.7)	0.65
CD11c MFI - neutrophils	80	723 ± 350	622.8 (522 ; 788)	19	853 ± 423	813 (476;- 982)	7.9 (0.47 – 131)	0.15
CD62Lneg neutrophils/µl	80	1067 ± 925	819.6 (245 ; 1613)	19	758 ± 698	487 (204 ; 1197)	0.54 (0.20 – 1.5)	0.24
Total lymphocytes/µl	80	1303 ± 688	1200 (810 ; 1715)	19	1261 ± 481	1180 (990 ; 1470)	1.2 (0.12 – 12)	0.87
CD4+ lymphocytes/µl	80	620 ± 342	610 (346.0 ; 829.1)	19	605 ± 232	612 (432 ; 779)	1.5 (0.21 – 11)	0.69
CD8+ lymphocytes/µl	80	281 ± 271	204 (143.7 ; 362.3)	19	261 ± 179	208 (150 ; 323)	0.88 (0.15 – 5.0)	0.88
CD4+CD69+ lymphocytes/µl	80	61 ± 62	45 (30.0 ; 67.0)	19	71 ± 46	54 (43 ; 97)	2.5 (0.52 – 12)	0.25
CD4+CD279+ lymphocytes/µl	80	168 ± 89	156 (101.8 ; 215.1)	19	189 ± 149	167 (113 ; 199)	1.5 (0.19 – 11)	0.71
CD8+CD69+ lymphocytes/µl	80	61 ± 102	33 (17.4 ; 53.7)	19	96 ± 125	51 (28 ; 137)	2.8 (0.93 – 8.2)	0.069
CD8+CD279+ lymphocytes/µl	80	89 ± 65	76 (46.8 ; 100.9)	19	103 ± 100	77 (47 ; 111)	1.5 (0.28 – 8.6)	0.62
CD69 MFI - CD4+CD69+ lymphocytes	80	369 ± 95	359 (320 ; 414)	19	343 ± 76	338 (280 ; 388)	0.042 (0.001 – 9.2)	0.25
CD69 MFI – CD8+CD69+ lymphocytes	80	683 ± 867	483 (397 ; 688)	19	1030 ± 1601	624 (504 ; 822)	4.4 (0.72 – 27)	0.11
CD279 MFI - CD4+CD279+ lymphocytes	80	232 ± 54	218 (193 ; 255)	19	236 ± 37	232 (206 ; 251)	4.3 (0.016 – 999)	0.61
CD279 MFI – CD8+CD279+ lymphocytes	80	269 ± 95	236 (201 ; 293)	19	310 ± 109	277 (247 ; 343)	26 (0.74 – 901)	0.073
B lymphocytes/µl	80	203 ± 242	149 (89 ; 233)	19	187 ± 166	147 (67 ; 225)	1.1 (0.28 – 4.0)	0.93
CD25+ B lymphocytes/µl	80	59 ± 218	16 (7.4 ; 37)	19	49 ± 91	19 (9.4 ; 40)	1.3 (0.51 – 3.0)	0.63
CD25 MFI - Tregs	80	3473 ± 691	3434 (2939 ; 3917)	19	3744 ± 980	3704 (291 ; 4682)	25 (0.096 – 999)	0.26
CD127 MFI - CD4+ lymphocytes	80	1378 ± 372	1313 (1111 ; 1660)	19	1420 ± 330	1496 (1095 ; 1671)	3.9 (0.05 – 304)	0.54
CD127 MFI - Tregs	80	209 ± 65	197 (162 ; 246)	19	211 ± 78	198 (161 ; 237)	0.89 (0.022 – 37)	0.95
Tregs/µl	80	59 ± 34	55 (32 ; 75)	19	56 ± 223	60 (39 ; 71)	1.0 (0.13 – 7.9)	0.99

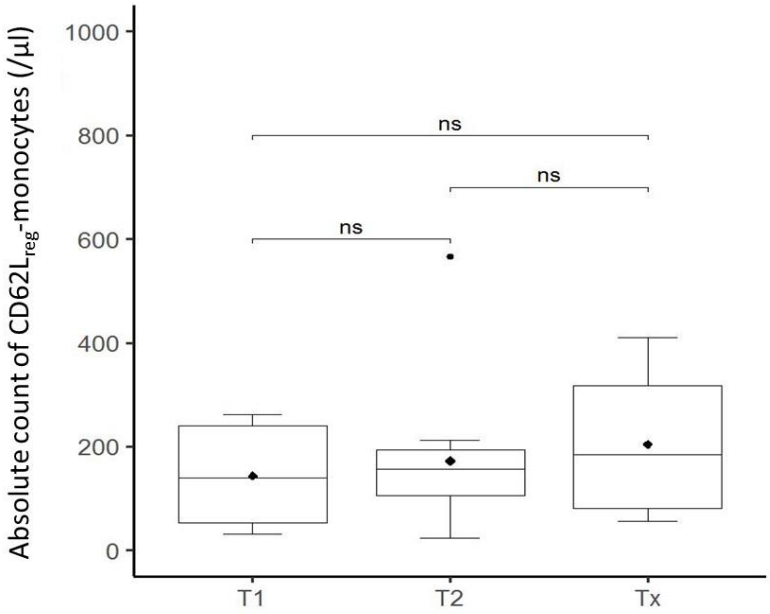
Fig1



Panel A: Measurements at ICU admission in nonseptic and septic patients and in healthy controls (> 50 years). (*: *p<0.05).*

Panel B: Predictive value of $CD62L_{neg}$ monocytes absolute count (/µl) obtained at T1. ROC curve analysis of sepsis occurrence based on levels of $CD62L_{neg}$ monocytes and SOFA is shown

Fig S2

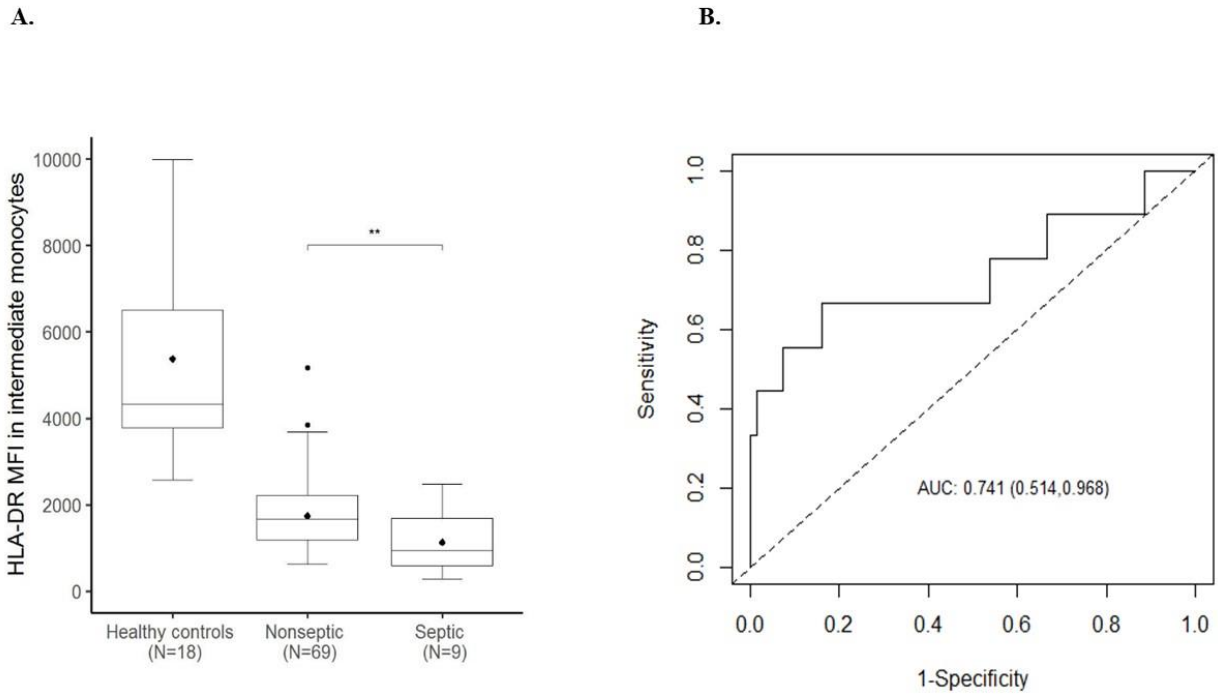


Absolute count of $CD62L_{neg}$ monocytes (/µl): evolution of septic patients (N=12 patients with measurement at ICU admission, 48 to 72h later and on the day of sepsis diagnosis). (ns: not statistically significant).

Table 4. Impact of parameters 48-72h after ICU admission (T2) on the risk of sepsis.

	Nonseptic (N=80)			Septic (N=19)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1 ; Q3)	N	Mean ± SD	Median (Q1 ; Q3)	OR (95%CI)	p-value
Total monocytes/µl	79	992 ± 452	950 (650 ; 1240)	15	1247 ± 997	980 (810 ; 1440)	4.5 (0.27 – 73)	0.30
HLA-DR MFI - total monocytes	79	1146 ± 556	972 (766 ; 1544)	15	690 ± 303	685 (435 ; 824)	0.004 (0.000 – 0.10)	0.0011
CD14 MFI - total monocytes	69	19581 ± 6805	18541 (15644 ; 23293)	9	19714 ± 5996	18758 (14917 ; 25446)	1.4 (0.011 – 169)	0.90
CD16 MFI - total monocytes	69	198 ± 127	171 (113 ; 240)	9	191 ± 88	174 (126 ; 198)	1.5 (0.077 – 28)	0.80
CD64 MFI - total monocytes	79	31472 ± 7345	32326 (26274 ; 36512)	15	28749 ± 7858	27011 (22435 ; 36294)	0.042 (0.000 – 4.7)	0.19
CD279 MFI - total monocytes	79	51 ± 160	24 (-21 ; 74)	15	100 ± 119	91 (20 ; 152)	2.9 (0.74 – 11)	0.13
Classical monocytes/µl	69	633 ± 328	589 (393 ; 828)	9	624 ± 245	562 (419 ; 747)	1.5 (0.054 – 43)	0.80
Intermediate monocytes/µl	69	271 ± 164	226 (143 ; 383)	9	283 ± 97	259 (195 ; 324)	3.3 (0.17 – 63)	0.43
Non-classical monocytes/µl	57	61 ± 47	50 (29 ; 75)	8	66 ± 31	68 (45 ; 83)	2.5 (0.25 – 26)	0.43
CD279 MFI – classical monocytes	69	6.6 ± 142	-19 (-39 ; 25)	9	62 ± 137	33 (-15 ; 43)	1.4 (0.32 – 5.8)	0.67
HLA-DR MFI – classical monocytes	69	976 ± 460	874 (642 ; 1296)	9	579 ± 228	560 (443 ; 761)	0.006 (0.000 – 0.26)	0.0081
CD64 MFI – classical monocytes	69	31749 ± 7633	32367 (26010 ; 36052)	9	29332 ± 7667	26825 (23234 ; 36640)	0.080 (0.000 – 26)	0.39
CD279 MFI – intermediate monocytes	69	89 ± 205	60 (-2.0 ; 115)	9	181 ± 191	154 (39 ; 178)	1.2 (0.35 – 4.0)	0.79
HLA-DR MFI – intermediate monocytes	69	1744 ± 769	1678 (1195 ; 2224)	9	1131 ± 733	941 (591 ; 1694)	0.003 (0.000 – 0.17)	0.0049
CD64 MFI - intermediate monocytes	69	33603 ± 7755	34545 (27854 ; 37880)	9	32401 ± 9496	30484 (24179 ; 38814)	0.20 (0.000 – 82)	0.60
CD279 MFI – non-classical monocytes	69	184 ± 124	160 (112 ; 232)	9	221 ± 97	206 (192 ; 241)	2.9 (0.29 – 30)	0.37
HLA-DR MFI – non-classical monocytes	69	8213 ± 3628	7898 (5738 ; 10756)	9	5929 ± 3234	5663 (3055 ; 7805)	0.074 (0.004 – 1.2)	0.070
CD64 MFI – non-classical monocytes	69	18781 ± 6999	18782 (14162 ; 23492)	9	17638 ± 6443	18175 (12364 ; 23814)	0.57 (0.018 – 19)	0.76
CD62Lneg monocytes/µl	79	158 ± 100	143 (82 ; 202)	15	170 ± 133	157 (74 ; 197)	0.94 (0.13 – 7.0)	0.95
Total neutrophils/µl	79	9057 ± 3048	8470 (7160 ; 10430)	15	8766 ± 3611	8040 (6780 ; 10560)	0.31 (0.007 – 13)	0.54
CD62L MFI - neutrophils	79	6618 ± 1519	6787 (5603 ; 7695)	15	6548 ± 2394	5984 (5057 ; 7099)	0.34 (0.002 – 50)	0.67
CD16 MFI - neutrophils	69	1956 ± 689	1872 (1442 ; 2318)	9	1863 ± 611	2027 (1233 ; 2495)	0.52 (0.007 – 40)	0.77
CD64 MFI - neutrophils	79	1901 ± 926	1619 (1263 ; 2305)	15	2281 ± 1689	1688 (1018;- 2787)	2.1 (0.16 – 27)	0.57
CD11b MFI - neutrophils	79	12027 ± 6866	9577 (7330 ; 16463)	15	13650 ± 7155	13145 (8932 ; 19791)	2.3 (0.23 – 23)	0.48
CD11c MFI - neutrophils	79	1220 ± 641	1056 (748 ; 1512)	15	1329 ± 687.0	1250.7 (666 ; 1926)	1.8 (0.13 – 26)	0.66
CD62Lneg neutrophils/µl	79	853 ± 849	512 (324;- 974)	15	827 ± 1153	551.3 (271 ; 893)	0.78 (0.19 – 3.2)	0.73
Total lymphocytes/µl	79	1220 ± 620	1140 (830 ; 1540)	15	1011 ± 336	1120 (760 ; 1210)	0.21 (0.013 – 3.5)	0.28
CD4+ lymphocytes/µl	79	524 ± 240.8	490 (356 ; 639)	15	434 ± 175	421 (291 ; 568)	0.15 (0.009 – 2.7)	0.20
CD8+ lymphocytes/µl	79	255 ± 172.9	206 (135 ; 344)	15	213 ± 131	183 (90 ; 308)	0.49 (0.084 – 2.9)	0.43
CD4+CD69+ lymphocytes/µl	79	60 ± 34.8	51 (33 ;- 75)	15	57 ± 30	61 (35 ; 70)	0.87 (0.10 – 27.6)	0.90
CD4+CD279+ lymphocytes/µl	79	179 ± 98	159 (116 ; 217)	15	121 ± 59	107 (97 ; 121)	0.044 (0.003 – 0.69)	0.026
CD8+CD69+ lymphocytes/µl	79	53 ± 89	27 (19 ;- 53)	15	71 ± 65	46 (24 ;- 97)	2.8 (0.75 – 11)	0.12
CD8+CD279+ lymphocytes/µl	79	95 ± 70	77 (50 ; 126)	15	80 ± 62.2	55 (31 ; 106)	0.56 (0.10 – 3.1)	0.50
CD69 MFI - CD4+CD69+ lymphocytes	79	329 ± 56	324 (289 ; 356)	15	343 ± 81	319 (287 ; 381)	11 (0.008 – 999)	0.51
CD69 MFI – CD8+CD69+ lymphocytes	79	771 ± 1162	527 (410 ; 731)	15	733 ± 441	605 (490 ; 725)	1.7 (0.19 – 16)	0.64
CD279 MFI - CD4+CD279+ lymphocytes	79	258 ± 58	247 (212 ; 283)	15	247 ± 24	253 (221 ; 257)	0.25 (0.001 – 226)	0.69
CD279 MFI – CD8+CD279+ lymphocytes	79	300 ± 91	289 (235 ; 346)	15	319 ± 79	311 (241 ; 385)	8.2 (0.092 – 724)	0.36
B lymphocytes/µl	79	216 ± 297	159 (108 ; 223)	15	191 ± 252	116 (56 ; 235)	0.50 (0.11 – 2.3)	0.38
CD25+ B lymphocytes/µl	79	66 ± 285	167 (7.5 ; 31)	15	55 ± 147	14 (7.4 ;- 35)	0.85 (0.29 – 2.5)	0.76
CD25 MFI - Tregs	79	3970 ± 1060	3818 (3319 ; 4600)	15	4150 ± 942	4135 (3593 - 4633)	6.2 (0.039 – 999)	0.48
CD127 MFI - CD4+ lymphocytes	79	1193 ± 373	1181 (878 ; 1493)	15	1230 ± 337	1195 (997 ; 1551)	2.9 (0.049 – 171)	0.61
CD127 MFI - Tregs	79	184 ± 69	176 (145 ;- 217)	15	197 ± 65	204 (133 - 237)	4.7 (0.12 – 180)	0.41
Tregs/µl	79	53 ± 27	46 (34 ; 72)	15	41 ± 16	37 (30 – 48)	0.12 (0.008 – 2.0)	0.14

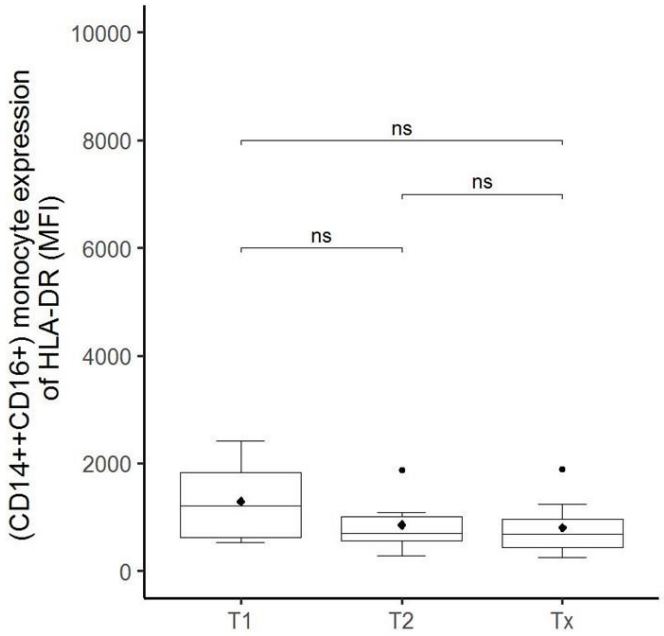
Fig 2



Panel A: Measurements at T2 in nonseptic and septic patients and in healthy controls (> 50 years). (**: $p<0.001$).

Panel B: Predictive value of intermediate (CD14++CD16+) monocyte expression of HLA-DR (MFI) obtained at T2

Fig S3



Intermediate monocytes (CD14++CD16+) median HLA-DR (MFI): evolution of septic patients (N=7 with measurement at ICU admission, 48 to 72h later and on the day of sepsis diagnosis). (ns: not statistically significant).

Discussion

In this single-center study, we showed that, in critically ill injured adults, increased levels of absolute monocyte counts and of $CD62L_{neg}$ monocytes at ICU admission and reduced mHLA-DR in intermediate monocytes 48-72h later, were independently associated with later sepsis occurrence. To the best of our knowledge, such a wide leucocyte panel, including 63 markers, exploring innate and adaptive immunity by flow cytometry, has not been reported in critical injury[34]. Concerning the absolute count of monocytes, although these cells exert a pivotal role in sepsis, the diagnostic and prognostic value of monocyte count is contrasted in the literature[35]. Small observational trials including mainly trauma and sepsis patients have

shown elevated or low monocyte counts to be associated with sepsis occurrence or outcome[36-39]. A very recently published observational study including more than 300 severely injured patients (out of which a third were already septic patients) looked into 30 immune markers, among which 12 were determined by flow cytometry[28]. The authors showed that monocyte count was not associated with secondary infection acquisition.

Considering the downregulation of L-selectin, identified here as increased numbers of $CD62L_{neg}$ monocytes, little is known in terms of sepsis prediction apart from conflicting data in neonates[40-42]. In a prospective older study including newborn infants with suspected bacterial infection, L-selectin expression was significantly reduced in both granulocytes and monocytes of infected newborns compared with controls[41]. L-selectin is a leucocyte surface glycoprotein which mediates extravasation and recruitment of white blood cells to sites of inflammation. Its downregulation *in vitro* had been shown in murine and human neutrophils and this was the first report of *in vivo* downregulation of L-selectin on granulocytes and monocytes[43-46]. Authors postulated that bacterial stimuli such as FMLP (N-formyl-methionyl-leucyl-phenylalanine)-related peptides or lipopolysaccharides or host-derived soluble mediators such as those released during acute systemic inflammatory response syndrome (cytokines, C5a, leukotriene B4) may have triggered L-selectin downregulation. Furthermore, a more recent study focusing on regional and systemic immune responses before, during and after major splanchnic surgery showed that intraoperative splanchnic hypoperfusion and mucosal acidosis led to monocyte deactivation[47]. In that study, 20 patients who underwent resection for cancer of the esophagus, had no difference in monocyte marker expression in the preoperative period. They were categorized into 3 groups according to the nadir perioperative intestinal pH. Those who developed postoperative sepsis (5/20) had the lowest intestinal pH, a persistently lower postoperative expression of L-selectin and m-HLA-DR and a more acute phase response (higher CRP) compared to non-sepsis patients, similar to

our findings. The authors concluded that severe mucosal acidosis, secondary to splanchnic hypoperfusion and increased intestinal permeability during major surgery, was associated with regional and systemic immune suppression predisposing to sepsis.

Our results are not in accordance with an observational study including 41 severely traumatized patients who underwent sampling and staining of 3 leucocyte subsets for CD62L, 1h and 20 hours after trauma[48]. The authors found that monocytes, lymphocytes and neutrophils showed an early increase in CD62L cell surface expression and that this persisted in the later samples up to 20 hours. However, association with subsequent sepsis occurrence was not an endpoint in the latter study. In a more recent study aiming at guiding the optimal timing of non-lifesaving orthopedic surgery for trauma, authors hypothesized that neutrophils and monocytes express activation markers prior to sepsis development[49]. They found that in the perioperative period, elevated monocyte L-selectin (AUC 0.76 [95%CI 0.63-0.89] was a significant predictor of sepsis, thereby precluding urgent surgery. However, these patients were not critically ill.

Considering expression of mHLA-DR, our results confirm those of older single-center single-biomarker studies[20, 21] and of two more recent multi-center studies[25, 28]. The first multi-center study validated a combined immune dysfunction score associated with sepsis development in a cohort of patients described as requiring organ support for more than 48h in the ICU[25]. Trauma and surgery were among the inclusion criteria but sepsis patients were also included. The score encompassed low mHLA-DR (Youden index optimal cutoff <10000 molecules/cell), elevated T_{regs} and low neutrophil CD88. In our study, T_{regs} were not found to be predictive of sepsis probably because of earlier serial sampling and different case-mix. Indeed, elevation of T_{regs} was only seen 6-10 days after ICU admission in the aforementioned study and sepsis patients were included, contrary to our study. Elevated levels of these suppressor cells have frequently been reported in sepsis patients, reflecting severity of disease

and predisposition to secondary infections, but very seldomly in injury, such as in our study, prior to the occurrence of a primary infection[50-52]. The second recent large multicenter study explored mHLA-DR and *ex vivo* TNF-α release in sepsis, trauma and postoperative patients in association with adverse clinical outcome (death or secondary infection)[19, 28]. It showed persistent decreases of both markers at days 5-7 post ICU admission to be associated with both outcomes, whatever the type of injury.

Finally, our results are partly corroborated by a recent study investigating the potential of HLA-DR expression by monocyte subsets in diagnosing sepsis in cardiac surgery patients[53]. The authors showed that that there was a significant downregulation, in the postoperative period, of mHLA-DR on both intermediate (p=0.0477) and non-classical monocytes (p=0.033). However, in contrast to our findings, it is the combination of the reduced preoperative count and postoperative HLA-DR expression of the non-classical compound that was found to be associated with sepsis occurrence at 48h post cardiac surgery, with a 100% sensitivity and 69.2% specificity.

Our study has several limitations among which, a single-center design and a small sample size. Furthermore, due to its exploratory nature, there was no *a priori* planned hierarchical clustering of surface markers, rendering consistency and fit-of-the model arguable. Validation of the two monocyte markers and of sampling times in a bigger cohort of patients could help to identify an optimal combination for sepsis prediction. Third, sampling times were limited and evolution of the biomarkers cannot be inferred past the third day of ICU admission. Furthermore, in patients who went on to develop sepsis, there are missing data in 7/19 for $CD62L_{neg}$ monocytes and 12/19 for mHLA-DR, respectively, thereby hindering interpretation of the biomarkers' levels time course. Fourth, potential confounders affecting the immune response to injury, such as blood transfusions and general anesthetics, were not taken into account at this stage[54]. Fifth, sepsis occurrence was lower than expected (19% versus 25-35% in other studies)

probably owing to the predominance of cardiac surgery patients who received prophylactic antibiotic therapy. Finally, we cannot exclude that some patients might have been in a pre-septic condition although high expression of neutrophil CD64, which is a recognized marker of bacterial infection, was not found at ICU admission[55-58]. Furthermore, CRP and fibrinogen levels were within normal ranges at ICU admission. It must be emphasized that procalcitonin was purposely not included in the design of the study because of known poor specificity as a diagnostic marker of sepsis in injured patients, as shown previously by our group[59].

In conclusion, this preliminary study showed that, in a selected population of critically injured patients, monocytes either in absolute count or via downregulation of specific surface markers, are predictive of subsequent sepsis development upon ICU admission and 48h later. Further validation in a bigger cohort of patients, perhaps in combination with recently published biomarkers, is warranted before envisaging a preventive immunomodulatory approach of sepsis in injured patients[60]. In clinical practice, the latter approach could be feasible thanks to the readily available complete blood count and to a recent proof-of-concept study showing promising results for mHLA-DR bedside monitoring[61].

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Abbreviations list

AUC: area under the curve

BSI: blood stream infection

CI: confidence interval

CLABSI: central line associated blood stream infection

CRP: C-reactive protein

HAP: hospital-acquired pneumonia

HLA-DR : human leucocyte antigen

ICU : intensive care unit

IQR: interquartile range

LOS : length of stay

MFI: median fluorescence index or median of fluorescence intensity

OR: odds ratio

PE: phycoerythrin-linked

PerCP: perinidin-chlorophyll protein-linked

ROC: receiver operating characteristic

SOFA: Sequential Organ Failure Assessment

SSTI: surgical site and soft tissue infection

TNF-α: tumor necrosis factor α

VAP: ventilator-associated pneumonia

Layios et al. Intensive Care Medicine Experimental (2017) 5:32
DOI 10.1186/s40635-017-0145-2

Intensive Care Medicine Experimental

RESEARCH Open Access

Sepsis prediction in critically ill patients by platelet activation markers on ICU admission: a prospective pilot study

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Abstract

Background: Platelets have been involved in both immune surveillance and host defense against severe infection. To date, whether platelet phenotype or other hemostasis components could be associated with predisposition to sepsis in critical illness remains unknown. The aim of this work was to identify platelet markers that could predict sepsis occurrence in critically ill injured patients.

Methods: This single-center, prospective, observational, 7-month study was based on a cohort of 99 non-infected adult patients admitted to ICUs for elective cardiac surgery, trauma, acute brain injury, and post-operative prolonged ventilation and followed up during ICU stay. Clinical characteristics and severity score (SOFA) were recorded on admission. Platelet activation markers, including fibrinogen binding to platelets, platelet membrane P-selectin expression, plasma soluble CD40L, and platelet-leukocytes aggregates were assayed by flow cytometry at admission and 48 h later, and then at the time of sepsis diagnosis (Sepsis-3 criteria) and 7 days later for sepsis patients. Hospitalization data and outcomes were also recorded.

Methods: Of the 99 patients, 19 developed sepsis after a median time of 5 days. These patients had a higher SOFA score at admission; levels of fibrinogen binding to platelets (platelet-Fg) and of D-dimers were also significantly increased compared to the other patients. Levels 48 h after ICU admission no longer differed between the two patient groups. Platelet-Fg % was an independent predictor of sepsis ($P = 0.0031$). By ROC curve analysis, cutoff point for Platelet-Fg (AUC = 0.75) was 50%. In patients with a SOFA cutoff of 8, the risk of sepsis reached 87% when Platelet-Fg levels were above 50%. Patients with sepsis had longer ICU and hospital stays and higher death rate.

Conclusions: Platelet-bound fibrinogen levels assayed by flow cytometry within 24 h of ICU admission help identifying critically ill patients at risk of developing sepsis.

Keywords: Sepsis, Prediction, Flow cytometry, Platelet markers, Fibrinogen, SOFA, Biomarker

Background

Despite sustained research on the immune pathophysiology of sepsis, sepsis occurrence remains the leading cause of mortality (20–50%) in the intensive care unit (ICU) [1, 2].

Therefore, the identification of predictive biomarkers of sepsis is instrumental to improve ICU patients' outcome. The Third International Consensus Task Force (Sepsis-3) defines sepsis as a "life-threatening organ dysfunction caused by a dysregulated host response to infection". In this concept, growing experimental and preclinical evidence indicates that platelets could play an active role either in immune surveillance or in the response to infection. Indeed, in addition to their role in hemostasis and thrombosis, several studies in animal models suggest a contribution of platelets to infectious diseases due to their ability to influence innate and adaptive immune responses [3]. First, platelets may act as sentinels of the immune system. They indeed express many major receptors of the innate immune system, including most Toll-like receptors. Platelets are able to recognize molecular features of microbes and secrete many immunomodulatory mediators essential for alerting and recruiting cells of the immune system [4–7]. Second, platelets may contain infection both directly and through functional interactions with immune cells [8]. Platelets produce various antimicrobial molecules, including defensins [9], thrombocidins [10], and kinocidins, and they are able to interact with and kill bacteria directly [11]. For instance, it has been shown that activated platelets facilitate the clearance of adherent *Streptococci* in experimental infective endocarditis [12]; β-defensins released from platelets activated by the *Staphylococcus aureus* α-toxin impair bacterial growth and induce neutrophil extracellular trap formation [13]. Platelets also help trap blood pathogens on Kupffer cells in hepatic sinusoids, which limits systemic infection [14]. Notably, platelets express CD40L, an essential player in host defense against infection that mediates interactions between platelets, antigen-presenting cells, and lymphocytes [15].

In overwhelming sepsis, platelets contribute to activation of the procoagulant cascade and ensuing complications linked to microvascular thrombosis and subsequent organ dysfunction [16]. It has been demonstrated that critically ill injured adult patients, such as burn, trauma, or cardiac surgery patients, experience susceptibility to sepsis because of innate and adaptive immune reprogramming due to the insult [17, 18]. However, whether platelets may participate in dysregulated host response to infection leading to sepsis remains unclear. One recent study showed that immature platelet fractions (IPF) could predict sepsis occurrence in critically ill subjects [19]. Further, in severe trauma, platelet activation and leukocyte-platelet aggregate formation have been incriminated in the pathogenesis of tissue lesions leading to organ failure [20]. The present prospective observational study hypothesized that platelet activation markers triggered by common injuries may help predicting occurrence of sepsis in specific ICU patient populations.

Methods

Study patients

This was a single-center, prospective, observational, 7-month study based on a cohort of 99 consecutive adult patients, expected to stay for at least 48 h in tertiary ICU. Inclusion criteria included elective cardiac surgery (coronary artery bypass grafting or valve replacement), trauma, invasive ventilation >48 h for reasons other than sepsis, and acute brain injury (including subarachnoid, subdural, intra-parenchymal hemorrhage, and ischemic stroke). Patients were excluded from the study if they

received oral or parenteral antibiotics other than for prophylaxis and if they were treated with any immunosuppressive agent except substitutive doses of corticosteroids, suffered from chronic hepatitis B or C, HIV, solid organ, or hematologic proliferative disease.

Characteristics at ICU admission

Upon admission to ICU, the following baseline characteristics were recorded: gender, age, type of admission (surgical or medical), history of diabetes and cardiovascular disease, previous treatment by vasopressor, prophylactic antibiotics, aspirin, and anticoagulants (anti-αIIbβ3). The sequential organ failure assessment (SOFA) score was computed. Blood samples were collected within 24 h (T1) for flow cytometry analyses (see the "Flow cytometry" section below). The following laboratory parameters were also assayed: C-reactive protein (CRP, mg/ml), fibrinogen (g/l), partial thromboplastin time (PTT, s), prothrombin time index (%), platelet count (k/μl), D-dimers (μg/l), and WBC count (K/μl). The ISTH scoring system for overt disseminated intravascular coagulation (DIC) was calculated based on Toh et al. [21].

Follow-up and sepsis occurrence

Patients were sampled again 48 h (T2) after admission, on the day of diagnosis of sepsis (Tx), and 7 days later. All blood specimens were analyzed by flow cytometry as in T1. A time line diagram is provided as Additional file 1: Figure S1. Criteria for severe sepsis or septic shock are in agreement with the new definitions of sepsis (Sepsis-3) [22]. For each study patient, the following data were also collected: length of ICU and of hospital stay (days), duration of ventilation (days) if required, administration of vasopressor during ICU admission, antibiotic treatment, use of curative antibiotics, red blood cell transfusion, plasma transfusion and platelet transfusion, and hemofiltration or intermittent hemodialysis during or after ICU stay. In case of death, time was also recorded. In case of discharge from the hospital, follow-up was at least 1 year.

Flow cytometry

Citrated whole blood was collected through an indwelling arterial catheter. Samples were processed within maximum 1 h following blood drawing. Platelet activation levels were assessed by measuring the expression of P-selectin (PS), a marker of degranulation, and fibrinogen (Fg) binding, as a result of integrin αIIbβ3 activation, on cell surface. Specifically, blood samples were fixed and incubated with peridinin-chlorophyll protein-linked (PerCP)-anti-CD61 antibodies (BD Biosciences), fluorescein isothiocyanate-linked (FITC)-anti-fibrinogen antibodies (Dako), and phycoerythrin-linked (PE)-anti-CD62P antibodies (BD Biosciences). Levels of platelet activation markers were determined by recording medians of FITC and PE fluorescence intensity (MFI) in platelets (CD61 positive cells) and percentages (%) of fibrinogen-positive (FITC) or CD62P-positive (PE) platelets on a FACS Verse flow cytometer (BD Biosciences). Data were analyzed using the BD FACSuite software. Platelets-monocytes and platelets-neutrophils aggregates were analyzed in citrated whole blood samples using an antibody panel, including anti-CD45-V500, anti-CD14-APC (monocytes), anti-CD15-PE (neutrophils), and anti-CD61-PerCP. Medians of CD61-PerCP fluorescence intensity in CD14-positive and CD15-positive cells, and percentages

of cells double positive for CD61 and CD14, or CD61 and CD15 were recorded as above. In all cases, threshold of positivity was set by use of marker-specific antibodies or their corresponding IgG isotype controls in blood samples that were left unstimulated or activated with a supra-optimal dose of collagen-related peptide. Plasma was prepared from the citrated whole blood samples to quantify plasma levels of TNFα, IL10, sCD40L, IL17A, IL6, IL7, and IFNγ, all expressed in pg/ml. Cytokine levels were measured using customized multiplex BD™ Cytometric Bead Array on the FACSVerse System. Analysis was performed with the FCAP Array™ software.

Statistics

Results were expressed as mean and standard deviation for quantitative data and as median and interquartile range (IQR) for durations. For categorical findings, frequency tables were used. The predictive value of sepsis was assessed for each baseline variable by logistic regression analysis. Then variables significant at $P < 0.10$ were combined in a stepwise logistic regression analysis to identify independent baseline predictors of sepsis. The odds ratio (OR) with 95% confidence interval (95%CI) and ROC curve analysis with area under the curve (AUC) were used to quantify the ability of the selected predictors to discern patients who will later develop sepsis. The Youden method was applied to define an optimal cutoff point for platelet marker predictors and SOFA score. Comparisons of hospital data and outcomes between septic and non-septic patients were done by the Kruskal-Wallis test for continuous variables and the Fisher exact test for categorical variables. Data recorded on the same patients but at different time points were compared by the Wilcoxon signed rank test. Results were considered significant at the 5% critical level ($P < 0.05$). All statistical calculations were performed with SAS (version 9.4) and R (version 3.0.3).

Results

Baseline characteristics of patients

The baseline ICU admission characteristics of the 99 study patients are displayed in Additional file 2: Table S1. There were 60 men and 39 women aged 64 ± 15 years. The type of admission was surgical for 86 patients, and the main reason was predominantly cardiac surgery (68.7%). Seventeen patients had a history of diabetes and 79 of cardiovascular disease. Ten patients received vasopressor before admission, 67 received prophylactic antibiotics during surgery, 53 were under aspirin, 3 were under αIIbβ3 antagonist, and 14 patients were taking anticoagulant (only prophylactic doses of low molecular weight heparin). The mean SOFA score was 6.0 ± 3.3. Data of routine biological parameters and flow cytometry results upon admission and 48 h later are displayed in Additional file 1: Table S2. No difference was evidenced between aspirin ($n = 53$) or anticoagulant users ($n = 14$) and non-users in terms of their biological profile (data not shown).

Sepsis occurrence

Of the 99 study subjects, 19 (19.2%) developed sepsis after a median time of 5 [IQR 3–7] days and 80 did not. As seen in Table 1, age, gender, type of admission, history of diabetes, use of vasopressor, anti-platelet, or anticoagulation medication use were not associated

Table 1 Predictive value of patient demographic and baseline clinical data for sepsis development during ICU stay

Variable	Development of sepsis[a]		*P* value[b]
	No (*N* = 80)	Yes (*N* = 19)	
Age (years)	65 ± 15	62 ± 15	0.46
Gender			0.80
Male	48 (80)	12 (20)	
Female	32 (82.1)	7 (17.9)	
Category of admission			0.70
Surgical	70 (81.4)	16 (18.6)	
Medical	10 (76.9)	3 (23.1)	
Reason for admission			0.0052
Cardiac surgery	61 (89.7)	7 (10.3)	
Acute brain injury	6 (50)	6 (50)	
Trauma	10 (76.9)	3 (23.1)	
Ventilation >48 h	3 (50)	3 (50)	
Score at admission			
SOFA	5.2 ± 2.7	9.6 ± 3.1	<0.0001
Diabetes			0.62
Yes	13 (76.5)	4 (23.5)	
No	67 (81.7)	15 (18.3)	
Cardiovascular disease			0.012
Yes	68 (86.1)	11 (13.9)	
No	12 (60)	8 (40)	
Vasopressor before the admission			0.091
Yes	6 (60)	4 (40)	
No	74 (83.2)	15 (16.8)	
Prophylactic antibiotics			0.0005
Yes	61 (91)	6 (9)	
No	19 (59.4)	13 (40.6)	
Aspirin			0.93
Yes	43 (81.1)	10 (18.9)	
No	37 (80.4)	9 (19.6)	
Anticoagulant			0.24
Yes	13 (92.9)	1 (7.1)	
No	67 (78.8)	18 (21.1)	

[a]Means ± SD for quantitative variable and numbers (%) for qualitative parameters
[b]Logistic regression

with sepsis development. By contrast, patients who later developed sepsis presented with higher SOFA score at admission. They were also predominantly admitted for acute brain surgery or prolonged ventilation and lacked prophylactic antibiotics prior to admission. Complementary results of septic compared to non-septic patients are shown in Additional file 2: Table S3.

When considering laboratory tests and flow cytometry parameters recorded within 24 h of admission to ICU (Table 2), D-dimers and fibrinogen binding to platelets (platelet-Fg expressed as MFI or %) were markedly higher ($P < 0.001$) in patients who later

Table 2 Predictive value of laboratory tests assessed at admission for sepsis development during ICU stay

Variable	Development of sepsis[a]		*P* value[b]
	No (*N* = 80)	Yes (*N* = 19)	
Routine			
CRP (mg/L)	14.1 ± 37.7	29.1 ± 61.7	0.053
Fibrinogen (g/L)	2.6 ± 0.99	3.3 ± 2.0	0.11
PTT (s)	14.4 ± 1.7	14.4 ± 3.3	0.78
Prothrombin Time Index (%)	66.3 ± 15.6	69.7 ± 19.4	0.61
Platelet count (10^3/μL)	124 ± 55	133 ± 84	0.59
D-dimers (μg/L)	2617 ± 6353	4456 ± 4957	0.0032
ISTH score	1.6 ± 1.3	2.6 ± 0.9	0.041
White blood cell count (10^3/μL)	10.0 ± 4.4	11.1 ± 5.2	0.42
Flow cytometry			
TNF-α (pg/mL)	0.17 ± 0.8	0.65 ± 1.5	0.091
IL-10 (pg/mL)	19.4 ± 90	9.4 ± 16.2	0.50
sCD40L (pg/mL)	88.8 ± 81.8	53.3 ± 43.6	0.89
IL-17A (pg/mL)	9.1 ± 12.2	7.6 ± 15.5	0.31
IL-6 (pg/mL)	459 ± 2673	162 ± 164	0.94
IL-7 (pg/mL)	2.9 ± 3.7	1.4 ± 1.7	0.65
IFN-γ (pg/mL)	0.14 ± 0.91	0	0.99
Platelet-Fg (%)	28.1 ± 27.8	56.5 ± 31.2	0.0054
Platelet-Fg (MFI)	1770 ± 1266	2752 ± 1359	0.0026
Platelet-PS (%)	2.9 ± 2.4	3.5 ± 2.7	0.61
Platelet-PS (MFI)	29.8 ± 15.1	37 ± 18.3	0.068
Platelets-neutrophils (%)	3.4 ± 5	4.4 ± 6.2	0.72
Platelets-neutrophils (CD61 MFI)	313 ± 127	323 ± 137	0.72
Platelets-monocytes (%)	19.8 ± 23.1	22.1 ± 25.5	0.73
Platelets-monocytes (CD61 MFI)	1413 ± 2462	1569 ± 3182	0.82

Null values for TNF-α and IFN-γ correspond to values under the level of detection (3.8 pg/ml)
Platelet-Fg platelet-bound fibrinogen, *platelet-PS*, platelets expressing P-selectin on their surface, *MFI* median fluorescence intensity, % percentage of positive cells for the indicated marker
[a]Results are expressed as means ± SD
[b]Logistic regression

developed sepsis. To a lesser extent, ISTH DIC score ($P < 0.05$) also differed between septic and non-septic patients. Interestingly, levels of sCD40L, P-selectin on circulating platelets (MFI or %), platelets-monocytes, and platelets-neutrophils aggregates were not associated with sepsis occurrence. Platelet-Fg correlated weakly with platelet P-selectin ($r = 0.32378$, $P = 0.0011$, $N = 98$), and plasma levels of D-dimers ($r = 0.35502$, $P = 0.0004$, $N = 96$) and fibrinogen ($r = 0.34592$, $P = 0.0005$, $N = 98$). No significant correlation was found with platelet count ($r = 0.071$, $P = 0.49$, $N = 98$), sCD40L ($r = -0.10377$, $P = 0.3222$, $N = 93$), or cytokine levels. Flow cytometry parameters recorded 48 h after admission were not associated with sepsis occurrence, although a tendency ($P < 0.10$) remained for platelet-Fg (data not shown). When looking at serial platelet-Fg levels in patients who developed sepsis, a significant increase was observed and a peak was reached on the day of sepsis (Fig. 1). By contrast, sCD40L remained fairly stable as sepsis developed (Additional file 1: Figure S2). D-dimers and platelet P-selectin levels

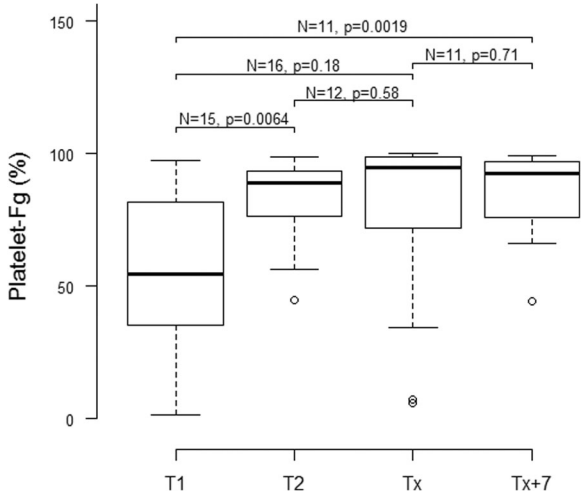

Fig. 1 Serial measurements of levels of platelet-bound fibrinogen (platelet-Fg) for patients who developed sepsis. Platelet-Fg levels were analyzed by flow cytometry on the day of ICU admission (*T1*), after 48 h (*T2*), at the time of sepsis diagnosis (*Tx*), and 7 days later (*Tx + 7*). Median values of percentages of Fg-positive platelets and IQR are shown (*P* value: Kruskal-Wallis test)

increased significantly from T2 to the time of sepsis diagnosis (Additional file 1: Figure S2).

Platelet markers at admission and sepsis prediction

All potential predictors of sepsis ($P < 0.10$) recorded at ICU admission (T1) were combined into a stepwise logistic regression analysis. As diagnosis of sepsis includes organ dysfunction, SOFA score was not included in our regression model. It turned out that platelet-Fg % levels at T1 ($P = 0.0031$) and admission for acute brain injury ($P = 0.012$) were the only independent predictors of sepsis occurrence. By ROC curve analysis (Fig. 2), an optimal cutoff point equal to 50% was derived for platelet-Fg % (AUC = 0.75) to discern patients who will later develop sepsis from those who will not. The number of patients who developed sepsis was respectively equal to 13 (46.4%) for the 28 patients with platelet-Fg >50% and to 6 (8.6%) for the 70 patients with platelet-Fg <50% (data missing for one patient). As shown in Table 3, when accounted for SOFA score at admission (cutoff value of 8), in patients with elevated SOFA and platelet-Fg >50%, the risk of sepsis rose up to 85.7%. By contrast, in patients with low SOFA and platelet-Fg <50%, the occurrence of sepsis was almost negligible (3.8%).

Discussion

The major findings of this study concern the clear relationship between patient levels of fibrinogen binding to circulating platelets (platelet-Fg) measured upon ICU admission and sepsis occurrence, regardless of the patient's baseline clinical characteristics. In particular, the study demonstrated that for patients presenting a SOFA score ≥8, platelet-Fg % level above 50 predicted sepsis with a high accuracy. Importantly, neither platelet membrane-bound P-selectin expression plasma levels of sCD40L nor any other standard hemostasis parameter showed similar

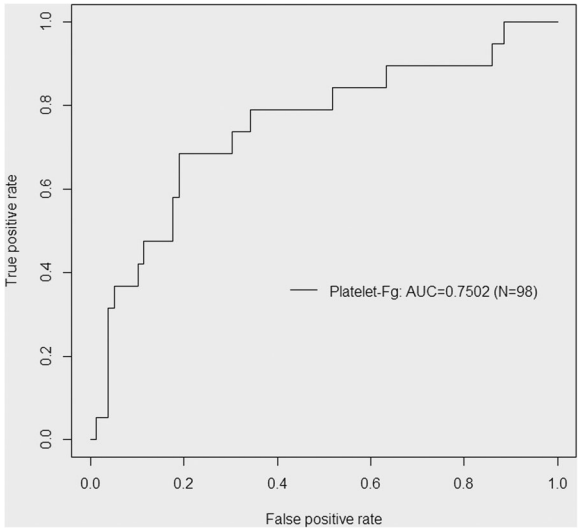

Fig. 2 Predictive value of platelet-Fg (%) obtained at admission. ROC curve analysis of sepsis prediction based on platelet-Fg is shown

predictive value as platelet-Fg. The optimal timing of measurement was also determined since only levels obtained within 24 h after ICU admission and not 48 h later were associated with sepsis occurrence, thus saving blood sampling in future studies. Platelet-Fg levels can be obtained in 1 h by using whole blood flow cytometry in unstimulated samples. Thus, this work provides the clinician with a simple and practical tool to assess the risk of sepsis in critically ill patients admitted to the ICU.

To date, several clinical studies investigated platelet markers in various conditions of critical illness. However, none of them searched for a potential association of these platelet markers with a risk for sepsis. Most of these studies described altered platelet phenotype in injured patients, characterized by either differential expression of platelet activation markers or platelet dysfunction as compared to healthy controls [23–28]. In ischemic stroke, two studies showed increased expression of platelet P-selectin and fibrinogen binding to platelets as compared to controls [25, 29]. The latter finding is interesting in view of our results, in particular since predisposition to severe pneumonia is clinically well established in such patients [30, 31]. Unfortunately, no association was searched between high levels of the biomarker and pneumonia. Several other clinical studies focusing on platelets as potential biomarkers for

Table 3 Risk stratification of patients according to sepsis development during ICU stay

	SOFA <8		SOFA ≥8	
	Platelet-Fg (%)		Platelet-Fg (%)	
Development of sepsis	<50	≥50	<50	≥50
Yes ($N = 19$)	2	1	4	12
No ($N = 79$)	51	13	13	2
Total	53	14	17	14
Risk of sepsis (%)	3.8	7.1	23.5	85.7

SOFA score and Platelet-Fg (%) plasma levels recorded on admission

sepsis diagnosis and prognostication have been carried out but almost all concerned patients with sepsis as an inclusion criterion [32, 33].

Despite multiple experimental data demonstrating antimicrobial activity of platelets and a role for platelet aggregation in limiting pathogen growth and dissemination in the vasculature [3, 6], direct clinical evidence from human studies was lacking and there are no epidemiologic data showing that platelet function inhibition affects sepsis prediction or prognosis. The present observational prospective study provides the first clinical evidence that, in patients with critical illness and related organ dysfunction, platelets may intervene in the dysregulated host response to infection leading to sepsis. Although demonstration of a causal link requires further investigation, we speculate that injury-associated platelet activation and subsequent fibrinogen binding may alter platelet ability to recognize bacterial components, some of which are ligands of αIIbβ3 [34, 35], and affect their ability to alert and recruit cells of the immune system [8]. Our observation that platelet-Fg weakly correlates with D-dimer levels suggests that fibrinogen binding to platelets and the activation of coagulation could be driven by the same factors. In injured patients, plasma fibrinogen would both bind platelets and be actively converted into fibrin; fibrinolysis would then increase D-dimer levels.

Antiplatelet drugs have beneficial and detrimental effects in systemic inflammation and in organ dysfunction, as shown in preclinical models and in humans [36–38]. Their usage has been variably associated with sepsis prognosis [39, 40]. In this study, we found no protective effect of aspirin against sepsis [41]. Our results are in line with a recent propensity-based analysis of 972 patients admitted for sepsis in which no association between aspirin therapy and sepsis prognosis could be evidenced [42]. Our results however differ in that they encompassed the period before sepsis, a period during which the abovementioned authors could not assess the potential benefits of aspirin. In addition, we could not find any association between aspirin therapy and the levels of platelet activation, which suggests that platelet activation pathways independent of thromboxane A_2 production could be involved in the patient's platelet response to injury.

Limitations

The study has a number of limitations. The small sample size and the predominance of elective surgical patients call for caution when interpreting results. Also, possible confounders such as immunomodulatory properties of anesthetic drugs were not taken into account at this stage. The findings of this pilot study call for a confirmatory prospective evaluation focusing on fibrinogen levels on platelets in a larger cohort. In our study, the platelet activation markers analyzed, namely levels of fibrinogen, platelet P-selectin expression, platelets-leukocytes aggregates, and sCD40L, behaved differently in their ability to predict sepsis development, which might reflect differences in platelet activation mechanisms or sequences. It has indeed been proposed that platelet activation, in terms of P-selectin expression and fibrinogen binding, and release of immunological molecules (sCD40L, RANTES) result from independent signaling pathways [43]. The utility of other markers, such as platelet microparticles or soluble glycoprotein VI, should be

analyzed, since the latter is shed from platelet surface and increases in patients with DIC [44].

Conclusions

In critically ill patients with comorbidities and post-trauma or post-surgical injury, platelet abnormalities are associated with altered host defense mechanisms. We actually found that admission levels of fibrinogen binding to platelets of ICU patients were predictive of later sepsis acquisition. Combining it with stratification based on SOFA score at admission has a higher predictive ability. Hence, our observations could trigger non-specific preventive interventions such as better supportive care or prophylactic antibiotics as well as research aiming at developing a specific therapeutic tool. Also, the fact that the identified marker was independent of aspirin use might have important future therapeutic implications regarding its actual worldwide implementation of primary or secondary prophylaxis.

Additional files

Additional file 1: Figure S1. Follow-up and sepsis occurrence. Timeline of samplings. **Figure S2.** Serial measurements of platelet markers and D-dimers for patients who developed sepsis. (DOCX 49 kb)

Additional file 2: Table S1. Baseline clinical characteristics of study patients (*n*=99). **Table S2.** Baseline and 48-h biological characteristics of study patients (*n*=99). **Table S3.** Comparison of ICU- and hospital-related characteristics of patients with and without sepsis. (PDF 157 kb)

Abbreviations

AUC: Area under curve; CI: Confidence interval; CRP: C-reactive protein; Fg: Fibrinogen; MFI: Median of fluorescence intensity; OR: Odds ratio; PS: P-selectin; ROC: Receiver operating characteristic; SOFA: Sequential organ failure assessment

Acknowledgements

The authors wish to thank the GIGA core facilities (Imaging and flow cytometry) for logistic and technical support.

Funding

The project was supported by grants from the FNRS (Fonds National pour la Recherche Scientifique FNRS-FRSM 3.4611.11, CDR J.0043.13) and by the French Community of Belgium (FSRC-12/13, ARC-SF 12/14-05). C.L. was a postdoctoral researcher at the FNRS. C.D. was supported by a "Fond pour la recherche industrielle et agricole" fellowship. C.O. is a senior research associate at FNRS. The authors thank BD Biosciences, Europe, for their support in providing the reagents for this study.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files.

Authors' contributions

CD, AH, CL, CG, AJ, PG, JH, NL, NM, and CO performed the experiments and analyzed the data. NL, CO, AG, CD, PL, NM, AA, and PD contributed to the experimental design, analysis, and interpretation of data. NL recruited the patients. NL, CO, and CD wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the ethics committee of the University of Liège Academic Hospital (CHU) (reference number B707201111981). Written informed consent was obtained from each participant or next of kin prior to sampling in agreement with the recommendations of the Declaration of Helsinki for investigations involving human subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 13 January 2017 Accepted: 3 July 2017
Published online: 12 July 2017

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Procalcitonin usefulness for the initiation of antibiotic treatment in intensive care unit patients*

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Objectives: **To test the usefulness of procalcitonin serum level for the reduction of antibiotic consumption in intensive care unit patients.**

Design: **Single-center, prospective, randomized controlled study.**

Setting: **Five intensive care units from a tertiary teaching hospital.**

Patients: **All consecutive adult patients hospitalized for > 48 hrs in the intensive care unit during a 9-month period.**

Interventions: **Procalcitonin serum level was obtained for all consecutive patients suspected of developing infection either on admission or during intensive care unit stay. The use of antibiotics was more or less strongly discouraged or recommended according to the Muller classification. Patients were randomized into two groups: one using the procalcitonin results (procalcitonin group) and one being blinded to the procalcitonin results (control group). The primary end point was the reduction of antibiotic use expressed as a proportion of treatment days and of daily defined dose per 100 intensive care unit days using a procalcitonin-guided approach. Secondary end points included: *a posteriori* assessment of the accuracy of the infectious diagnosis when using procalcitonin in the intensive care unit and of the diagnostic concordance between the intensive care unit physician and the infectious-disease specialist.**

Measurements and Main Results: **There were 258 patients in the procalcitonin group and 251 patients in the control group. A significantly higher amount of withheld treatment was observed in the procalcitonin group of patients classified by the intensive care unit clinicians as having possible infection. This, however, did not result in a reduction of antibiotic consumption. The treatment days represented 62.6 ± 34.4% and 57.7 ± 34.4% of the intensive care unit stays in the procalcitonin and control groups, respectively (p = .11). According to the infectious-disease specialist, 33.8% of the cases in which no infection was confirmed, had a procalcitonin value >1µg/L and 14.9% of the cases with confirmed infection had procalcitonin levels <0.25 µg/L. The ability of procalcitonin to differentiate between certain or probable infection and possible or no infection, upon initiation of antibiotic treatment was low, as confirmed by the receiving operating curve analysis (area under the curve = 0.69). Finally, procalcitonin did not help improve concordance between the diagnostic confidence of the infectious-disease specialist and the ICU physician.**

Conclusions: **Procalcitonin measuring for the initiation of antimicrobials did not appear to be helpful in a strategy aiming at decreasing the antibiotic consumption in intensive care unit patients. (Crit Care Med 2012; 40: 2304–2309)**

Key Words: antibiotic consumption; infection; intensive care unit patients; procalcitonin

Antibiotics are known to carry a high burden in the intensive care unit (ICU) setting, leading to an increased risk of bacterial resistance and higher treatment-related costs (1). ICU caregivers are daily challenged by the necessity of a prompt initiation of antibiotics in the setting of sepsis (2, 3) while avoiding misuse in the absence of infection (4, 5). A potentially discriminant biomarker could therefore prove to be helpful (6). Recently, it was shown that procalcitonin (PCT) guidance of antibiotic therapy reduced antibiotic consumption by almost 50% in patients suspected of having either a community-acquired pneumonia or an exacerbation of chronic obstructive pulmonary disease (7–9). The same strategy was recommended for ICU patients suspected of developing an infectious process (10).

In this context, the present study was designed to test the hypothesis that antibiotic consumption differs between patients with PCT guidance and those without PCT guidance for the decision to treat. Secondary objectives included the assessment of the quality of infectious-disease

*See also p. 2499.

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The authors have not disclosed any potential conflicts of interest.

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DOI: 10.1097/CCM.0b013e318251517a

(ID) diagnosis by the ICU physician and determination of its concordance with the ID specialist's diagnosis. The latter was blinded to PCT results.

MATERIALS AND METHODS

From April 2008 to December 2008, patients older than 18 yrs of age and hospitalized for >2 days in one of the five ICUs of the University Hospital of Liege (Liège, Belgium) were prospectively randomized to either a PCT-guided approach to antibiotic therapy (PCT group) or to a standard approach (Control group) in which the physician was blinded to PCT result, PCT levels being obtained in all suspected episodes of infection. The study was approved by the institutional ethics comity, and written or oral consent was obtained from all patients or their next of kin.

Age, sex, Simplified Acute Physiology Score II calculated during the first 24 hrs after admission to the ICU, as well as underlying diseases according to the McCabe classification were recorded. Admissions were classified into trauma, unscheduled surgery, scheduled surgery, or medical. Patients readmitted to the ICU during the study period remained in the same group (PCT or Control). For each patient, the total ICU stay was calculated, and all infectious episodes were recorded to account for the total antibiotic consumption throughout the study period. The patient's course of illness was characterized by the Sequential Organ Failure Assessment score measured daily and the Sequential Organ Failure Assessmentmax calculated for the first ICU stay as proposed by Moreno et al (11).

As soon as patients were suspected of developing an infection, a serum PCT was ordered and its level was revealed to physicians of the PCT group, while it was blinded to those of the Control group. According to the proposal by Müller et al, for patients in the PCT group, the use of antibiotics was more or less strongly discouraged if PCT level was <0.25 μg/L or 0.50 μg/L, respectively, and more or less recommended if PCT level was above 1 μg/L or 0.50 μg/L, respectively (10). This strategy was applied to all infectious episodes encountered during their ICU stay. Infections were defined on the basis of clinical history, symptoms, physical examination, and laboratory findings. They were also characterized according to mode of acquisition (community, hospital, or ICU-acquired), source, and microbiological documentation. The severity of infection was assessed according to the American College of Chest Physicians/Society of Critical Care Medicine Conference Guidelines on the grade of sepsis (12). Clinicians involved in the decision to treat were also asked to rate their diagnosis as certain, probable, possible, or unlikely. At the end of ICU stay, patients' charts were reviewed by ID specialists blinded to PCT results, who classified them as confirmed, probable, possible, or no infection using all the clinical data and biological results including microbiological cultures and results from investigational procedures.

PCT results in the control group were eventually unblinded for the statistical analysis.

PCT serum level was measured using a time-resolved amplified cryptate emission technology assay (Kryptor PCT; Pasteur Mérieux, Paris, France) with a functional assay sensitivity of 0.06 μg/L. Antibiotic consumption was evaluated by counting the days of therapy expressed as treatment days and by the amounts of antibiotics expressed as daily defined dose (DDD) (13).

The primary end point was the difference of antibiotic consumption between the PCT group and the control group. Secondary end points included: usefulness of PCT levels in the ICU diagnostic algorithm in deciding whether to initiate antibiotics or not whenever infection was suspected, and determination of concordance of the infection's diagnostic ratings by the ICU physician and the ID specialist, bearing in mind that the latter was blinded to PCT results in all of the cases.

Statistical Analysis. Continuous variables were reported as mean ± SD for normally distributed variables or as median and interquartile range (IQR) for variables with skewed distribution. Proportions were compared by the chi-square test while mean values were compared by one-way analysis of variance of the Kruskal-Wallis test. Interobserver agreement between clinicians and ID specialists was assessed by Cohen's kappa coefficient. Assuming a mean stay of 7 days with 50% antibiotic exposure, a study sample of at least 250 patients in each group was deemed necessary to detect a 20% reduction in antibiotic consumption with 95% power at the 5% significance level.

RESULTS

During the study period, 1501 patients were admitted to the five ICUs (see Fig. 1). Among them, 854 were expected to stay for ≤48 hrs, and 138 did not give informed consent. Five hundred and nine

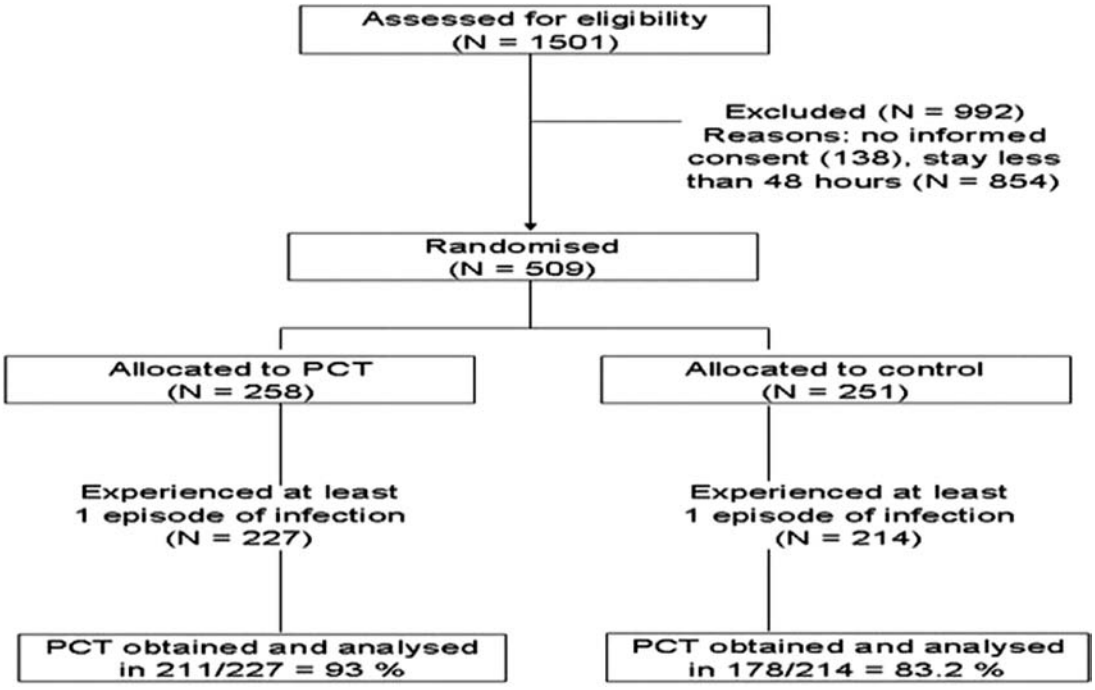

Figure 1. Trial profile.

patients were eligible for the study and randomized into the PCT group (n = 258) and the control group (n = 251). The baseline characteristics of patients at admission were comparable in both groups in terms of age, sex, type of admission, comorbidities, and Simplified Acute Physiology Score II score (Table 1).

The characteristics of the ICU stay are given in Table 2: among the 509 study patients, 227 in the PCT group (88%), and 214 in the control group (85.3%) had at least one suspected episode of infection. Together, these 441 patients presented 667 episodes of suspected infections (323 on admission to ICU and 344 during ICU stay) yielding a number of infectious episodes of 1.4 ± 1.1 episodes per patient in the PCT group and 1.2 ± 1 episodes per patient in the control group (p = .15). PCT results were available for 389 (88.2%) of the 441 patients. There were more patients with PCT results in the PCT group than in the control group (81.8% vs. 70.9%, p = .005), and so was the number of PCT results per patient (1.2 ± 1.0 vs. 0.8 ± 0.8; p = .003). The length of stay, Sequential Organ Failure Assessmentmax, number of patients with renal-replacement therapy and ventilation, duration of ventilation, and ICU mortality were similar in both groups.

Antibiotic consumption did not differ between groups: the treatment days represented 62.6 ± 34.4% and 57.7 ± 34.4% of the ICU stays in the PCT and control groups, respectively (p = .11). Similarly, there was no difference in terms of DDD/100 ICU days between the two groups: a mean of 147.3 ± 206.00 DDD/100 ICU days in the PCT group vs. 141.1 ± 136.9 DDD/100 ICU days in the control group, or a median of 108.3 (IQR 47.7–200) DDD/100 ICU days in the PCT group vs. 108.7 (IQR 52.3–180.7) DDD/100 ICU days in the control group (p = .96).

The characteristics of the 667 infectious episodes observed in the two groups are displayed in Table 3. The source of infection was predominantly respiratory (62.8%). Severe sepsis or septic shock were encountered in almost half of the infectious episodes (47.5%). Infection was bacteriologically documented in 61.5% of the cases, and ID specialists confirmed the diagnosis as certain or probable in 61.7% of the cases. No differences were noted between infectious episodes in the two groups. PCT levels were compared according to the bacteriological results of episodes considered at least as possible by ID specialists: PCT levels (median and IQR) were 1.02 µg/L (0.34–4.12) in infectious episodes due to *Staphylococcus aureus* (n = 43), 2.6 µg/L (0.78–10.5) in *Streptococcus pneumoniae* infections (n = 23), 2.0 µg/L (0.35–8.32) in other gram-positive *cocci* infections (n = 26), 1.20 µg/L (0.37–4.13) in *enterobacteriaceae* infections (n = 124); 2.6 µg/L (0.78–10.5) in nonfermenting gram-negative *bacilli* infections (n = 55), 1.27 µg/L (0.56–10.4) in infections caused by other microorganisms including *fungi* (n = 39), and 1.8 µg/L (0.38–8.0) in nonbacteriologically documented infections (n = 30). The differences were not significant (p = .84).

Table 4 displays the number of withheld treatments in both groups according to the clinician's confidence; all episodes classified as certain were treated with antibiotics. For the episodes classified as possible, the proportion of withheld treatments was significantly higher in the PCT group as compared to those in the control group (50.5% vs. 34.2 %; p = .034).

When looking at the decision to treat according to PCT level in both groups, no difference between PCT and control groups could be observed (Table 5). PCT levels were >1 µg/L in 259 episodes (48.3% of 536 cases with PCT measurement) and <0.25 µg/L in 135 episodes (25.2%). There was a trend toward a higher proportion of withheld treatment in suspected sepsis with PCT level < 0.25 µg/L in the PCT group (37 of 80, 46%) vs. the control group (18 of 55, 32.7%, p = .15). Forty-three episodes with PCT levels <0.25 µg/L in the PCT group were nevertheless treated by antibiotics: these were lower-tract respiratory infections (n = 27), intraabdominal infections (n = 2), soft-tissue infections (n = 4), sepsis (n = 9), and cryptococcosis (n = 1). Fifteen of these episodes of infection (34.9%) presented as severe sepsis or septic shock, 10 (23%) were accompanied by bloodstream infections (three *S. aureus*, one coagulase negative *Staphylococcus*, two *Streptococcus faecalis*, and four *Enterobacteriaceae*). At the end of the ICU stay, ID specialists classified these 43 episodes as certain (n = 18, 41.8%), probable (n = 12, 27.9%), possible (n = 4, 9.3%), or absence of infection (n = 9, 20.9%). By contrast, 16 episodes in the PCT group with PCT levels >1 µg/L were not treated. ID specialist *a posteriori* confirmed the absence of infection in 10 of them; only one should have been treated for an empyema which was, in fact, diagnosed 1 day later. The 15 other episodes turned out to be acute pulmonary edema (n = 4), pneumothorax at day 5 after cardiac surgery (n = 1), pericardial effusion with tamponade at day 5 after cardiac surgery (n = 1), hemorrhagic shock (n = 1), subdural hematoma (n = 1), ruptured aortic abdominal aneurysm (n = 1), toxic hepatitis (n = 1), lactic acidosis with acute renal failure secondary to drug abuse (n = 1), diabetic ketoacidosis (n = 1), acute pancreatitis (n = 1), acute exacerbation of chronic obstructive pulmonary disease (n = 1), and microbiologically nonconfirmed catheter-related infection (n = 1). Considering the 259 suspected infectious episodes with a PCT level >1 µg/L, 46 (17.8%) were not confirmed by the ID specialist.

Table 1. Characteristics of patients at admission

Variable	Procalcitonin Group n = 258	Control Group n = 251	p
Age (yr) median (interquartile range)	66 (55–76)	65 (53–75)	.33
Sex, n (%)			
Male	154 (59.7)	153 (61.0)	.97
Female	104 (48.3)	98 (39.0)	
Underlying disease, n (%)			
Nonfatal	160 (62.0)	154 (61.4)	.66
Ultimately fatal	74 (28.7)	68 (27.1)	
Rapidly fatal	24 (9.3)	29 (11.6)	
Comorbidities, n (%)			
Coronary disease	29 (11.2)	21 (8.4)	.77
Chronic heart failure	36 (14.0)	34 (13.6)	
Cerebrovasc disease	12 (4.7)	16 (6.4)	
Renal dysfunction	30 (11.6)	36 (14.3)	
Liver disease	20 (7.8)	16 (6.4)	
Diabetes	45 (17.4)	38 (15.1)	
Chronic obstructive pulmonary disease, asthma	72 (27.9)	66 (26.3)	
Solid cancer	42 (16.3)	44 (17.5)	
Hematological cancer	17 (6.6)	15 (6.0)	
Transplant	8 (3.1)	8 (3.2)	
Type of admission, n (%)			
Medical	155 (60.1)	147 (58.6)	.97
Scheduled surgery	22 (8.5)	23 (9.2)	
Emergency surgery	56 (21.7)	53 (21.1)	
Trauma	25 (9.7)	28 (11.2)	
Simplified Acute Physiology Score II	39.3 ± 16.3	39 ± 16.7	.84
Readmission, n (%) 0	249 (96.5)	239 (95.2)	.51
1	6 (2.3)	11 (4.4)	
2	3 (1.2)	1 (0.4)	

Table 2. Characteristics of intensive care unit stay

Variable	Procalcitonin Group n = 258	Control Group n = 251	*p*
Patients with suspected infections *n* (%)			
Yes	227 (88.0)	214 (85.3)	.43
No	31 (12.0)	37 (14.7)	
Number of episodes of infection/patients	1.4 ± 1.1	1.2 ± 1.0	.15
Procalcitonin assays *n* (%)			
Yes	211 (81.8)	178 (70.9)	.005
No	47 (18.2)	73 (29.1)	
Number of procalcitoninmeasurement/ patients	1.2 ± 1.0	0.9 ± 0.8	.003
Intensive care unit stay (days), median (interquartile range)	7 (4–16)	7 (4–18)	.38
SOFAmax			
Ventilation > 24 hrs, n (%)	9.3 ± 4.9	9.1 ± 5.4	.42
Yes	150 (58.1)	149 (59.4)	.79
No	108 (41.9)	102 (40.6)	
Duration of ventilation days			
Median (interquartile range)	3 (1–11)	3 (0–11)	.99
Renal-replacement therapy			
Yes	44 (17.1)	45 (17.9)	.81
No	214 (82.9)	206 (82.1)	
Intensive care unit mortality	56 (21.7)	53 (21.1)	.91
Antibiotic consumption: % of intensive care unit days	62.6 ± 34.4	57.7 ± 34.4	.11
Antibiotic consumption defined daily dose/100 intensive care unit days mean ± SD, median (interquartile range)	147.3 ± 206.0	141.1 ± 136.9	.96

SOFAmax, sum of all the dysfunction and failure occurring during the intensive care unit stay according to the Sequential Organ Failure Assessment score.

Table 3. Characteristics of infectious episodes in PCT patients (n = 227) or control patients (n = 214)

Variable	Procalcitonin Group n = 353	Control Group n = 314	*p*
Occurrence			
Upon admission	176 (49.9)	147 (46.8)	.64
During intensive care unit stay	177 (50.1)	167 (53.2)	
Type of infection			
Respiratory	229 (64.9)	190 (60.5)	.51
Intraabdominal	33 (9.3)	30 (9.6)	
Catheter related	7 (2.0)	3 (1.0)	
Soft Tissue	14 (4.0)	15 (4.8)	
Urine	10 (2.8)	7 (2.2)	
Miscellaneous	60 (17.0)	69 (22.0)	
Severity			
Sepsis	186 (52.7)	164 (52.2)	.50
Severe sepsis	54 (15.3)	58 (18.5)	
Septic shock	113 (32)	92 (29.3)	
Clinician confidence			
Sure	101 (29.6)	91 (29.0)	.39
Probable	123 (34.8)	126 (40.1)	
Possible	103 (29.2)	76 (24.2)	
Uncertain	26 (7.4)	21 (6.7)	
Bacteriologically documented			
Yes	205 (58.1)	205 (65.3)	.06
No	148 (41.9)	109 (34.7)	
Confirmed by infectious disease specialist			
Yes	218 (61.8)	204 (65.0)	.42
No	135 (38.2)	110 (35.0)	
Procalcitonin measurement			
Yes	306 (86.7)	230 (73.2)	<.0001
No	47 (13.7)	84 (26.8)	
C-reactive protein levels mg/L mean ± SD	156 ± 108	166 ± 112	.25

Figure 2 displays the distribution of PCT levels according to the ID specialist's diagnostic confidence upon reviewal of the charts. A statistically significant difference was found between the four PCT distributions ($p < .0001$) but also a large overlap between the results. In fact, 33.8% of the cases in which no infection was confirmed had a PCT value > 1 µg/L, and 14.9% with confirmed infection had PCT levels < 0.25 µg/L. The ability of PCT levels to differentiate between certain or probable infection and possible or no infection, upon initiation of antibiotic treatment, as assessed by ID specialists, was low as confirmed by ROC curve analysis (AUC = 0.69) (Fig. 3). The observed proportion of agreement between the ICU clinician and the ID specialist was 53% for the PCT group and 49% for the control group (not significant), yielding a kappa coefficient of 0.46 in both groups.

Lastly, the ICU length of stay and mortality were the same in both groups of patients.

DISCUSSION

In this study, we failed to show a significant reduction in antibiotic consumption when considering PCT as a diagnostic tool for the initiation of antibiotics for critically ill patients in whom sepsis is suspected. Although ICU clinicians could significantly decrease the number of treatments when infection was considered as possible and when PCT was available (Table 4), the overall consumption was the same between the two groups. A reason for this failure may be that almost half of PCT serum samples were >1 µg/L thus encouraging the antibiotic treatment. Only 25% of the samples were below the lowest cutoff. A second reason might lie in the fact that clinical skills and judgment superseded PCT results and protocol recommendations since only 46.3% of the patients with a low level of PCT were not treated. Indeed, 43 patients had signs of severe sepsis and/or comorbidities that prompted physicians to treat them. It must be emphasized that the majority of these treatments (30 of 43, 69.8%) were *a posteriori* confirmed as correct by the ID specialist. A third reason could be that in this study the proportion of patient-days with antibiotic treatment in the control group was already low (57%) compared to other studies (14) in which that proportion was >80%.

However, the main question raised by our study is the accuracy of PCT as a marker of infection. This is a question that has been already raised by others (15–17). In our study, 33.8% of episodes with no confirmed infection had a PCT value >1 µg/L, potentially leading to

Table 4. Number of withheld or withdrawn treatment according to the clinician confidence

Clinician Confidence	Total, n = 667 (%)	Procalcitonin Group, n = 353 (%)	Control Group, n = 314 (%)	*p*
Sure	0/192 (0)	0/101 (0)	0/91 (0)	.99
Probable	17/249 (6.8)	6/123 (4.9)	9/126 (7.1)	.60
Possible	78/179 (43.6)	52/103 (50.5)	26/76 (34.2)	.034
Uncertain	29/47 (61.7)	13/26 (50.0)	16/21 (76.2)	.080

Table 5. Number of withheld or withdrawn treatment according to the procalcitonin levels in procalcitonin patients (n = 211) and in control patients (n =178)

Procalcitonin Levels	Total, n = 536 (%)	Procalcitonin Group, n = 306 (%)	Control Group, n = 230 (%)	*p*
>1 µg/L	31/259 (12.0)	16/140 (11.4)	15/119 (12.6)	.85
0.5–1 µg/L	13/67 (19.4)	9/39 (23.1)	4/28 (14.3)	.53
0.25– <0.5 µg/L	14/75 (18.7)	9/47 (19.1)	5/28 (17.9)	.99
<0.25 µg/L	55/135 (40.7)	37/80 (46.3)	18/55 (32.7)	.15

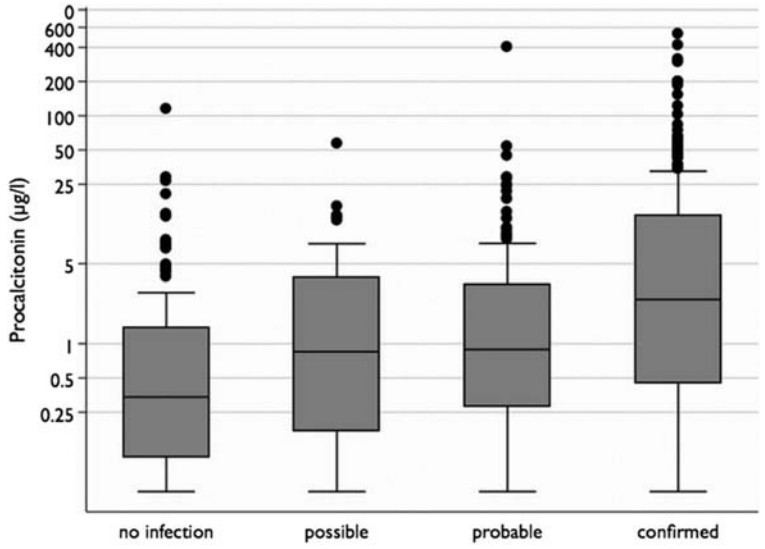

Figure 2. Procalcitonin (*PCT*) serum levels according to the diagnosis confidence of infectious-disease specialists.

antibiotic misuse, and 14.9% episodes with confirmed infection had PCT levels < 0.25 µg/L. Thus the area under the receiving operating curve was disappointingly 0.69, which is too low to propose PCT as a marker of infection in ICU patients, at least in the setting of deciding whether to initiate or withhold antibiotics.

Our study did not confirm the positive results of two recently published studies investigating the impact of PCT levels on the reduction of antibiotic consumption in critically ill patients (14, 18). The impact of serial PCT measurements as studied by Bouadma et al and Nobre et al ultimately determined treatment duration, which was not our primary objective (14, 18). The present study was designed to analyze the impact of PCT in a diagnostic strategy of infection and also to verify the accuracy of PCT as a marker of infection. To the best of our knowledge, the *a posteriori* reviewal of the charts and confirmation or rejection of infection by a blinded to PCT ID specialist has not been described in the ICU setting yet. Rather than the potential recurrent infection or excess mortality possibly caused by an inappropriate antimicrobial treatment driven by a PCT-guided strategy, the validation of true infection by an ID specialist in possession of all the clinical and final microbiological data appears to be more appropriate in recommending a particular strategy. It has been validated in febrile patients presenting to the emergency department (19).

The present study has weaknesses: First, its single-center design may be criticized. Second, the high number of surgery and trauma patients (40.7%) in our population could also be outlined in terms of case mix, since it is known that these conditions can induce early increase in PCT levels, causing false positives. However, infection occurring after trauma or surgery usually appears after several days, which allows PCT to come back to baseline value. Furthermore, surgical or trauma patients represent a substantial part of the critically ill patients hospitalized in the ICU. It was important to consider them in a systematical approach designed to reduce antibiotic consumption and misuse. Third, for obvious reasons, it was not possible to design a blind study in this setting. Some results of our study emphasize the bias provoked by the open design: clinicians aware of PCT availability for patients belonging to the PCT group, tended to suspect occurrence of sepsis more often in this group. The number of suspected episodes was slightly higher in PCT group (Table 2), the least confident classes (uncertain and possible) were also a little bit higher in this group and the proportion of bacteriologically documented infection was lower in the PCT group with a trend toward significance ($p = .06$, Table 3). Nevertheless this bias per se did not modify the decision to treat algorithms which followed classical recommendations (10). Fourth, the fact that no serial determinations of PCT throughout the antibiotic course were programmed upfront in the design of the study, for reasons of cost, is probably partly responsible for the negative finding considering reduction of antibiotic consumption, although some recent data suggest the opposite (20).

In conclusion, considering PCT levels for initiating antibiotic treatment did not appear to be helpful in a strategy aimed at decreasing the antibiotic consumption in critically ill patients. In addition, PCT proved to be a poor marker of infection when considering the accuracy of infection diagnosis made by the ICU clinician and the *a posteriori* reviewal by an ID specialist blinded to PCT result. These are the reasons why we cannot, at this point, recommend a PCT-guided strategy for

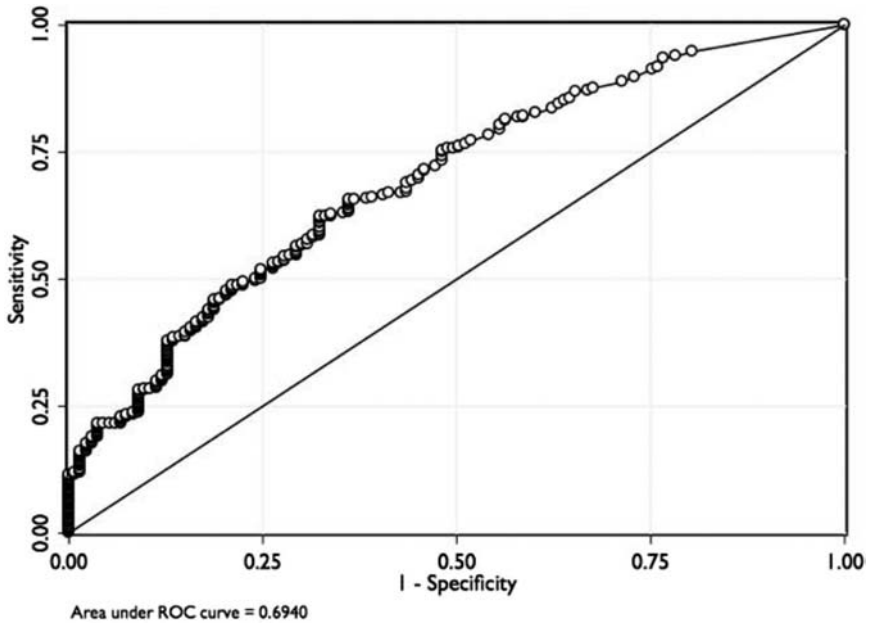

Figure 3. Discrimination power of procalcitonin (*PCT*) levels. The outcome was diagnosis of infection assessed by infectious-disease specialist as sure or probable. The area under the receiving operating curve was 0.69.

the initiation of antibiotics in critically ill patients.

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Curr Infect Dis Rep (2013) 15:394–399
DOI 10.1007/s11908-013-0360-2

SEPSIS AND ICU (J RUSSELL, SECTION EDITOR)

Procalcitonin for Antibiotic Treatment in Intensive Care Unit Patients

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Published online: 10 August 2013

Abstract Procalcitonin (PCT), a 116-aminoacids prohormone, has been substantially studied over the last 2 decades in the field of sepsis. Disappointingly low sensitivity values led to the abandonment of the concept of it as a diagnostic tool and then to its being considered more as a prognostic marker with a good correlation with severe infection. Later on, growing concerns about multidrug-resistant bacteria in the ICU environment and about the cost and side effects of antibiotics suggested that PCT might prove to be a valuable asset in stewardship programs. Numerous but hardly comparable randomized controlled trials assessing either initiation or deescalation in ICU patients have been published. Stewardship encompassing PCT should focus on the latter, because of the high negative predictive value of this biomarker. However, there still would be safety concerns if a systematic implementation of PCT were to be considered in daily stewardship programs in the ICU, especially in extra-thoracic sepsis.

Keywords Procalcitonin · Diagnosis · Prognosis · Initiation · De-escalation · Antibiotic Stewardship · Intensive care unit · Cost-effectiveness · Safety

Introduction

Since its first description in children and burned patients 2 decades ago [1], procalcitonin (PCT) has traveled a long way from diagnosis of infectious chronic obstructive pulmonary disease (COPD) exacerbation in the emergency department to its current concept as a possible tool for antibiotic stewardship in our busier intensive care units (ICUs) with worsening ecological environments [2, 3] (Fig. 1). Indeed, it was first thought to be helpful in the discrimination between severe infection and nonspecific hyperinflammatory states [4•]. Intensive care physicians are daily challenged with the risk of initiating useless and potentially toxic (for the patient and the environment) treatments in the absence of specific clinical signs and of a gold-standard biomarker in the field of sepsis. PCT was historically studied in various settings and clinical conditions, including emergency departments, neonatal sepsis, and ICU patients [5, 6]. However, unacceptably low sensitivity values in the setting of critically ill patients, ranging from 67 % to 80 % depending on the chosen cutoff [7, 8, 9•, 10•], led to its being considered rather as a prognostic tool in terms of severity of illness and outcome. In that sense, PCT met the fate of other acute phase reactants that did not show satisfactory specificity. PCT is notoriously raised, in the absence of infection, in pancreatitis, ischemic bowel disease, cardiopulmonary bypass, and metastatic disease [11] and with the intake of some drugs (monoclonal antibodies, antithymocyte globulin, etc.) [12••, 13]. PCT does not rise incases of local bacterial, viral, parasitic, or fungal infection. Between 2006 and 2008, numerous studies involving very different types of patients (medical vs. surgical, immunocompetent vs. immunocompromised) and indications (severe community-acquired pneumonia, sepsis) [14, 15] were undertaken. Some studies focused strictly on prognostic significance, whereas others combined the diagnostic and prognostic abilities of the test. Interestingly, PCT was combined with others biomarkers (CRP, sTREM-1, SUPAR, TNF-alpha, IL-6, IL-8), clinical scoring systems (SAPSII), and biological markers of sepsis such as lactate [16–18]. The results were better in the multimodal approach, as opposed to the use of PCT alone, for determination of outcome (AUC 0.72–0.88). It is now commonly admitted that higher values (1.5–over 5 μg/l) in high-risk patients are correlated with bacterial load and

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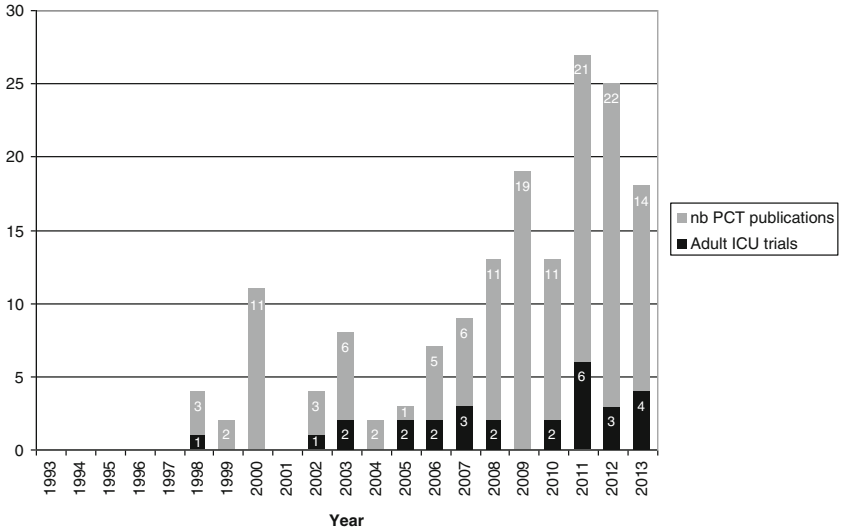

Fig. 1 Number of procalcitonin publications including adult ICU trials

bacteremia [19], severity of organ failure [20, 21], and, in some studies, mortality [6, 22].

Core Text

After an initial encouraging report on the usefulness of PCT for safe reduction of antibiotic therapy in lower respiratory tract infections (RTIs) in 2004 [23, 24], it was not until 2008 that the concept of PCT as a possible antibiotic stewardship tool emerged in clinical studies involving ICU patients [25••] (Table 1). The growing pressure of multidrug-resistant bacteria in the ICU environment, combined with considerations regarding cost and limitation of drug toxicity and interactions [26, 27], offered the perfect opportunity for a new appraisal of this biomarker, which had somehow failed to fulfill its promises. It is considered to be useful because of favorable kinetics [28] and a high negative predictive value [17, 29–31].

Antibiotic stewardship encompassing PCT can be regarded as a lack of initiation of antibiotics in the absence of bacterial infection, thus calling into question the sensitivity of the biomarker, versus rapid stopping of them, in cases of a decrease of PCT, on the basis of a daily check, either because clinical cure is achieved or because infection has been safely ruled out.

Now, the first strategy encounters two hurdles. First, it obviously does not fit into clinical practice dealing with seriously ill patients, since protocol-overruling reports range from 20 % to 65 % [30, 32•, 33, 34]. In the Layios study, in 43/80 patients (belonging to the PCT arm, which comprised 258 patients) who had a PCT <0.25 μg/l, the diagnosis was overruled by the treating physician, and they received antibiotics. Of note, 69.8 % of these treatments (30/43) were a posteriori confirmed by the infectious diseases specialist to have been appropriately initiated. Second, poor diagnostic sensitivity was once again confirmed recently (AUC 0.69), and two recent studies showed that the strategy is a failure in an escalating or initiation process [10•, 30].

The second strategy, however, is supported by the physiological decline within 48 h in noninfected patients [35, 36] and has recently been shown to be cost effective, thanks to a 2-day decrease in antibiotic consumption, although not altogether convincingly safe [32•, 37]. The same degree of concern about a possible excess of mortality had been raised in the PRORATA study [33], and the debate is ongoing. Prior to 2010, five randomized controlled trials (RCTs) evaluating deescalation in critically ill patients had shown reduction in antibiotic consumption, without excess of morbidity or mortality [38]. Interestingly, the same authors put into perspective the fact that in nondocumented sepsis, the optimal duration of antibacterial therapy is not known. Several other studies have been published since then, reporting the same proportion of safe antibiotic-free days (2–4 days) [31, 39•, 40], but only two focused merely on severe extra-thoracic sepsis. The ESICM meta-analysis reviewed seven RCTs in critically ill patients and confirmed the safety of shortening antibiotic administration by just over 3 days, in terms of a similar rate of superinfections and recurrence of infection in the PCT-guided arm. A consistent reduction of antibiotic therapy was also reported in the review published by Schuetz et al. [41••], mainly owing to shorter courses of antibiotic therapy (and not withholding of initiation) among moderate- and high-acuity care patients. The Schuetz study mixed lower RTIs and severe sepsis and septic shock without further definition. The proposed PCT cutoff values in the deescalating strategy in the ICU were roughly the same throughout all the recently published trials and meta-analyses, meaning a drop of 80 %–90 % from the peak value or a return to a level less than 0.25–1 μg/l in patients showing clinical signs of recovery. Mortality has not been significantly affected by that strategy in any of the trials published so far. Importantly, PCT was extensively studied in the setting of

Table 1 Publication of randomized controlled trials (RCTs) of procalcitonin (PCT) guided antibiotic (AB) treatment in critically ill adults

Author	Study Design	Diagnosis	*n*	Initiation/ esc	Deescalation	Major Endpoint	AB Reduction in the PCT Arm	Mortality	Main Results
Nobré et al. [25••], 2008	prospective RCT	severe sepsis/septic shock	79	no	yes	AB duration	−3.5 days	not affected	Safety of deescalation reduction of AB duration and ICU LOS
Schroeder et al, [48], 2009	prospective RCT	severe sepsis/septic shock	27	no	yes	AB use	−1.7 days	not affected	
Hochreiter et al. [49], 2009	prospective RCT	postoperative sepsis	110	no	yes	AB use	−2 days	not affected	
Stolz et al. [34], 2009	prospective RCT	VAP	101	no	yes	AB-free days alive	+3.5 days	not affected	
Bouadma et al. [33], 2010	prospective RCT	sepsis	621	yes	yes	AB-free days	+2.7 days	not affected	
Jensen et al. [9•], 2011	prospective RCT	sepsis	1,200	yes	no	death from any cause at day 28 AB use=second end-point	+2 days in the PCT arm	ARR=0.6 %	longer ICU LOS 1d, p=.004; more organ harm; possible 10 % risk increase in 60-day mortality
Agarwal et al. [50], 2011	review 5 RCTs+1 abstract	sepsis, VAP, septic shock	1,476	yes	yes	AB duration	−23 % to −37 %		
Schuetz et al. [41••], 2011	review 6 RCTs	VAP, sepsis/septic shock, postoperative sepsis	1,010	yes	yes	AB duration and AB-free days alive	in 5 studies, −20 % to −33 %	not increased in 2/5 studies	
Heyland et al. [32•], 2011	review 5 RCTs	VAP, sepsis/septic shock, postoperative sepsis		yes	yes	patient safety and cost analysis	−2 days		overall costs may be reduced under circumstances; 7 % risk increase in hospital mortality could not be ruled out
Matthaiou et al. [39•], 2012	meta-analysis 7 RCTs	VAP, sepsis/septic shock, postoperative sepsis	2,199 (1,098 PCT, 1,101 control)	yes	yes	AB duration for the first infectious episode and 28-day mortality	−3.5 days	not affected	
Layios et al. [30], 2012	prospective RCT	VAP, sepsis/septic shock, postoperative sepsis	509	yes	no	AB use	none	not affected	low sensitivity, useless for decision of initiation
Hohn et al. [51], 2013	retrospective 2005–2009	severe sepsis/septic shock	141	no	yes	AB days on ICU, ICU reinfection rate, 28-day mortality rate, LOS in ICU, mean AB costs (per patient) and ventilation hours. Data	−1 day/year over a 4-year period	not affected	

Note. LOS, length of stay;.VAP, ventilator-acquired pneumonia; ARR, adjusted risk ratio

lower RTIs namely, ventilator-acquired pneumonia (VAP) in immunocompetent adults—while severe sepsis (i.e., a syndrome defined as the host's systemic inflammatory response syndrome [SIRS] to infection) was the second cause of inclusion of patients. However, the source and/or the microbiological proof of infection have seldom been reported. This is very intriguing after almost 2 decades of striving—and with, sometimes, rigorous research—to establish the utility of a biomarker in less rigorous conditions. It is also, in our view, the biggest difficulty to overcome, since modern intensive care is becoming, alas, more and more syndromic. Of interest, the recently published and prematurely stopped study of Annane et al. [42] failed to include patients because 80.6 % of the eligible patients had a documented source of infection within 48 h of recognition of SIRS (before randomization), 77.6 % of whom had a documented pathogen. Now this was considered to be a major design flaw, but one definitely has to put into perspective the utility of a biomarker when modern pathogen identification techniques and experienced clinical judgment are combined. This point is very interestingly raised by Póvoa et al. [43], who reminded us of two studies, going 10 years back, that had shown the effectiveness of a shorter (6–8 days) duration of antibiotic therapy to be equal to that of a long-term course (10–21 days) in VAP, but without the use of any biomarker. Hence, the decision to recommend PCT's usage in the recent guidelines for deescalation in lower RTIs even in the case of septic shock [44] leaves us skeptical. Proposals and recommendations for the use of PCT in a strategy aiming at antibiotic stewardship were issued by Schuetz et al. [41••] and Foushee et al. in 2012 [12••], each in distinct environments (Europe vs. Northern America, primary setting vs. low-, intermediate-, and high-risk patients), recommending caution regarding their implementation in immunocompromised and unstable patients. This is in line with most studies focusing on the need for supplementary data in favor of safe antibiotic stewardship, always encompassing PCT in a multimodal approach. A prospective upcoming and well-enrolled study (the SAPS study), the largest to be conducted so far in ICU patients, will perhaps be able to answer questions about the cost, safety, and effectiveness of such a strategy [45].

However, although convincing from the physiopathological and, sometimes, evidence-based point of view, PCT's systematical implementation as a prognostic tool or, for therapeutic monitoring, as a clinical algorithm for ICU patients has not been widely encouraged so far.

Conclusion

PCT as an antibiotic stewardship tool aiming at appropriately initiating antibiotics—that is, only in the setting of severe infection—has recently once again proven to be futile, if not detrimental. The 2013 surviving sepsis campaign (SSC) guidelines propose PCT as a diagnostic aid, in conjunction with the usual clinical signs, provided its value is superior to 2 standard deviations above the normal value. This is, in our opinion, a surrogate marker for poor sensitivity, and it would have been more prudent not to include it at all in the diagnostic strategy. Now, interest in PCT's ability to contribute to infected critically ill patients' diagnosis and prognosis has not worn out, as large-scale ongoing clinical studies attest (accessed on clinicaltrials.gov on May 13, 2013), but we are doubtful about their ultimate daily clinical implementation, given the amount of literature already available and the understandable reluctance of the intensive care physician, facing the possibility of uncontrolled sepsis, not to initiate antibiotics.

Hence, PCT as a therapeutic monitoring tool has looked like an attractive alternative in view of its high negative predictive value, but conclusive data concerning safety of this strategy are still lacking, at least in extra-thoracic severe sepsis. Concerning VAP, past studies have shown efficacious and safe shorter duration of antibiotic therapy without the need for a biomarker. Low adherence to protocol, even in the setting of controlled infection, is another hurdle to its implementation in daily clinical practice, since reports of overruling range from 16 % to 65 % [32•, 43]. Only a grade 2C level of recommendation was attributed to PCT in the recently updated SSC guidelines when deescalation was considered. Rather, narrowing of the spectrum of antibiotics or stopping is left to "clinical judgment and information."

On the other hand, high-throughput molecular techniques such as multiplexed PCR and mass spectrometry allow more rapid and less empiric pathogen identification nowadays [46]. These techniques should be evaluated in terms of cost effectiveness, sensitivity, and specificity as part of a multimodal stewardship program in the ICU that could encompass bioscores such as the one recently described by Gibot et al. [47•]. This approach would thus imply a patient-tailored treatment based on individual phenotypic characteristics, combined with a biomarker allowing prompt stopping of antibiotics in the absence of infection or, even better, consensus on the optimal duration of therapy in sepsis without bacterial documentation.

Compliance with Ethics Guidelines

Conflict of Interest Nathalie Layios and Bernard Lambermont declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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PHARMACOLOGY

Modelled Target Attainment after Temocillin Treatment in Severe Pneumonia: Systemic and Epithelial Lining Fluid Pharmacokinetics of Continuous versus Intermittent Infusions

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ABSTRACT The objective of this article is to describe the population pharmacokinetics (PK) of temocillin administered via continuous infusion (CI) versus intermittent infusion (II) in critically ill patients with pneumonia. Secondary objectives included characterization of epithelial lining fluid (ELF)/plasma penetration ratios and determination of the probability of target attainment (PTA) for a range of MICs. Thirty-two mechanically ventilated patients who were treated for pneumonia with 6 g of temocillin daily for *in vitro* sensitive pathogens were assigned to either the II (2 g every 8 h over 0.5 h) or the CI (6 g over 24 h after a loading dose of 2 g) group. A population pharmacokinetic model was developed using unbound plasma, and total ELF concentrations of temocillin and related Monte Carlo simulations were performed to assess PTAs. The area under the concentration-time curve from 0 to 24 h ($AUC_{0–24}$) ELF/plasma penetration ratio was 0.73, at steady state, for both modes of infusion and whatever the level of creatinine clearance. Monte Carlo simulations showed that for the minimal pharmacodynamic (PD) targets of 50% $T > 1\times$ MIC (II group) and 100% $T > 1\times$ MIC (CI group), PK/PD breakpoints were 4 mg/L in plasma and 2 mg/L in ELF and 4 mg/L in plasma and ELF, respectively. The breakpoint was 8 mg/L in ELF for both modes of infusion in patients with creatinine clearance (CL_{CR}) < 60 mL/min/1.73 m^2. While CI provides better PKPD indexes, the latter remain below available recommendations for systemic infections, except in the case of moderate renal impairment, thereby warranting future clinical studies in order to determine the efficacy of temocillin in severe pneumonia.

KEYWORDS temocillin, epithelial lining fluid, PTA, critically ill patients, nosocomial pneumonia, Monte Carlo simulation, critical illness, pharmacokinetics

Temocillin is a derivative of ticarcillin, which, owing to its 6-α methoxy terminal structural modification, is resistant to most β-lactamases produced by extended-spectrum β-lactamases (ESBLs), excluding nonfermenters such as *Pseudomonas Aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* sp., and some carbapenemases (1). This feature makes it an interesting alternative in an era of highly resistant *Enterobacterales* infections in view of sparing carbapenems, supported by some retrospective study results (2, 3).

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The authors declare no conflict of interest.

Received 21 October 2021
Returned for modification 16 November 2021
Accepted 20 January 2022
Accepted manuscript posted online 31 January 2022
Published 15 March 2022

Temocillin is licensed in the United Kingdom, Belgium, France, and Germany for use in urinary tract infections (UTI), bloodstream infections, and lower respiratory tract infections (LRTI) at a posology of 2 g twice daily (4). However, studies have demonstrated higher efficacy with 6 g daily in continuous infusion in critically ill patients in various infectious settings (5). Extended or continuous infusions of β-lactams are being used in daily clinical practice in order to maximize the time that the antimicrobial free concentration remains above the MIC (%fT $>$ MIC), which is the cornerstone of β-lactam therapy efficacy (6–8). For temocillin, although no detailed analysis of its pharmacodynamics (PD) *in vitro* exists, it is assumed that a minimal bacteriostatic target of 40 to 50% should be considered by comparison with other penicillins (9, 10). Furthermore, in patients with severe pneumonia, data about epithelial lining fluid (ELF) pharmacokinetics (PK) and penetration ratio of temocillin are lacking. Currently, only the British Society for Antimicrobial Chemotherapy (BSAC) and the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) have defined temocillin clinical breakpoints (susceptible [S] $\leq$ 8 and resistant [R] $>$ 8 mg/L) for systemic infections (11). Very recently, EUCAST issued clinical breakpoints for urinary tract infections only (S $\leq$ 0.001 and R $>$ 16 mg/L), thereby excluding other sites of infection from its recommendations due to the lack of clinical and PK data (12). The aim of this study is to provide an insight to establishing temocillin PK/PD breakpoints in pneumonia via ELF and plasma samples.

RESULTS

Patient enrollment, exclusions, and adverse events. Forty-four patients were enrolled in the study. Thirty-five patients were treated either in the continuous infusion (CI) group (n = 10) or in the intermittent infusion (II) group (n = 25). Twelve were excluded from the PK and PK/PD analyses for the following reasons. Three patients were under continuous veno-venous hemofiltration (CVVH), and this limited number did not permit their PK characterization with acceptable robustness. Furthermore, in the II group, four patients were extubated before bronchoalveolar lavage (BAL), one patient had undetectable urea-BAL, and one patient died before BAL. In the CI group, one patient had undetectable cells in BAL, another patient was shifted from CVVH to intermittent hemodialysis, and one patient was deemed too unstable by the attending physician to undergo BAL.

Consequently, 32 patients were included in the final PK analyses, 23 in the II group and 9 in the CI group. Temocillin and mini-BAL were well tolerated without any significant adverse events.

Patient demographics. Patient demographics and clinical characteristics are described in Table 1. The two groups were clinically and demographically similar. Thirty-one percent of patients had augmented renal clearance (ARC) defined as $>$120 mL/min/1.73 m^2 (7/23 in the II versus 3/9 in the CI group, respectively) (13). The mean creatinine clearance was 107.2 $\pm$ 49.5 mL/min/1.73 m^2.

Clinical PK and microbiology. A high PK interindividual variability was observed in the serum and ELF concentrations in both groups as illustrated in Fig. S1 in the supplemental material. Mean observed concentrations in plasma and ELF are displayed in Fig. 1.

Forty-six pathogens were isolated from the 32 patients (11 in the CI group; 35 in the II group), among which were 33 nonfermenter *Enterobacterales* (data not shown). Based on Vitek 2, the majority (85%) of pathogens had an MIC of $\leq$4 mg/L for temocillin, and 15% had an MIC of 8 mg/L. Based on Etest, 10 (30.3%) pathogens had an MIC of $\leq$4 mg/L, 12 (36.4%) had an MIC of 4 to 8 mg/L, and 11 (33.3%) had an MIC of $>$8 mg/L, mainly *Escherichia coli* and *Serratia Marcescens*, corresponding to resistant strains according to BSAC recommendations. Four strains (12.1%) were extended-spectrum β-lactamase (ESBL) producers, one in the II group and three in the CI group. None were carbapenemase producers. Based on Etest, the mean MIC was 9.94 mg/L ($\pm$ 7.86 mg/L), and the median was 8 mg/L (interquartile range [IQR], 4 to 13 mg/L).

Population PK model building and internal validation. A two-compartment model best fitted the plasma unbound concentrations, and an additional compartment was added to describe ELF concentrations. Creatinine clearance was retained as a

TABLE 1 Demographic and clinical characteristics of patients

Demographic and clinical data	All (n = 32)	CI group (n = 9)	II group (n = 23)	P value
Age (yr)	64.9 ± 11.4	66.1 ± 7.0	64.4 ± 12.8	0.71
Male (no. [%])	24 (75.0)	8 (88.9)	16 (69.6)	0.39[a]
Wt (kg)	74.4 ± 13.7	75.6 ± 16.7	73.9 ± 12.8	0.76
BMI (kg/m^2)	25.1 ± 4.6	25.1 ± 4.5	25.1 ± 4.7	0.99
Hospital stay before onset of pneumonia (days)	15.6 ± 15.0	18.3 ± 13.9	14.5 ± 15.6	0.33[b]
ICU stay before onset of pneumonia (days)	10.3 ± 10.1	13.8 ± 12.9	9.0 ± 8.8	0.35[b]
Simplified CPIS	7.8 ± 1.0	8.1 ± 1.2	7.7 ± 0.93	0.25
SAPS III	72.2 ± 12.7	74.8 ± 11.9	71.3 ± 13.2	0.49
SOFA score	9.7 ± 3.3	10.0 ± 3.7	9.6 ± 3.2	0.77
APACHE II	28.6 ± 8.6	28.3 ± 9.6	28.7 ± 8.4	0.92
Septic shock (no. [%])	12 (37.5)	5 (55.6)	7 (30.4)	0.24
Concomitant bacteremia with the targeted bacteria (no. [%])	5 (15.6)	3 (33.3)	2 (8.7)	
Cl_{CR} (mean ± SD)[c]	115.6 ± 51.7	119.2 ± 33.2	114.2 ± 58.0	0.81
>120 mL/min/1.73 m^2	14 (43.7)	6 (66.7)	8 (35.8)	
90–119 mL/min/1.73 m^2	8 (25.0)	2 (22.2)	6 (26.1)	
60–89 mL/min/1.73 m^2	3 (9.4)	0 (0.0)	3 (13.0)	
30–59 mL/min/1.73 m^2	7 (21.9)	1 (11.1)	6 (26.1)	
CVVH	0 (0.0)	0 (0.0)	0 (0.0)	

[a]Fisher's exact test.
[b]Kruskal-Wallis test.
[c]Using urine output collection over 24 h.

significant covariate on clearance (power relationship). According to the model, the plasma free fraction increases on average from 10% to about 75% when the total concentration increases from 10 mg/L to 400 mg/L, with quite high interindividual variability (coefficient of variation [CV] = 36%).

Total temocillin concentrations were related to unbound concentrations according to the following equation: $Ct = Cu + \frac{Cb_{max} \times Cu}{BC_{50} + Cu}$. Parameter estimates and related covariate are summarized in Table S1 in the supplemental material. The ratio of area under the concentration-time curve (AUC) between concentration of total temocillin in ELF (Celf) and plasma concentration of unbound (free) temocillin (Cu) (R_{AUC}) was estimated to be 0.73. Basic goodness-of-fit plots for total plasma, unbound plasma, and total ELF concentrations are displayed in Fig. S2 to S4 in the supplemental material. They indicate adequate fitting performances of the model to the data. Visual predictive checks are presented on Fig. 2 and show an acceptable agreement between the predicted and observed data over the dosing interval for both free and total plasma and ELF total concentrations.

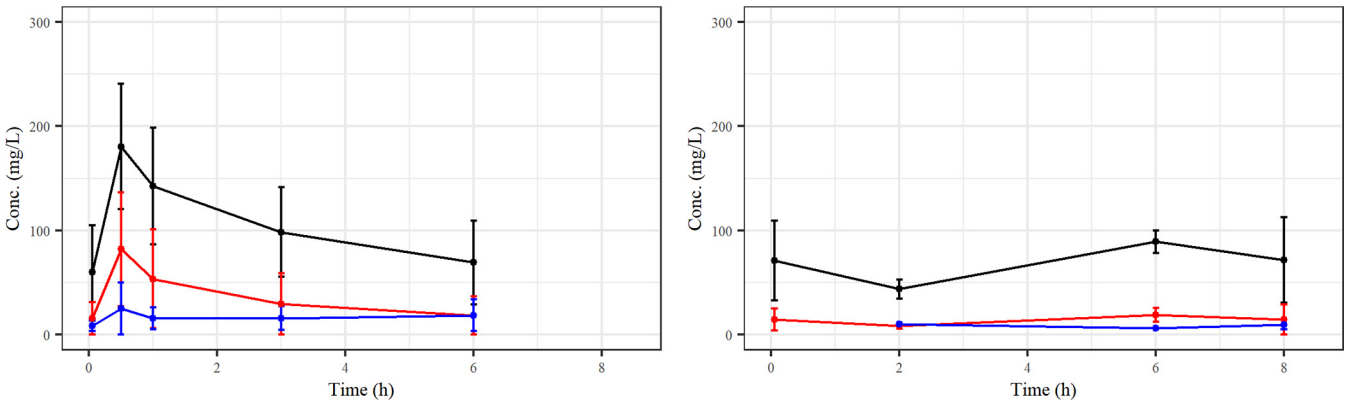

FIG 1 Mean (± standard deviation [SD]) observed free (red) and total (black) concentrations of temocillin in plasma and total concentration of temocillin in ELF (blue), after intermittent infusion, 2 g every 8 h over 0.5 h (n = 23) (left), and continuous infusion, 6 g over 24 h (n = 9) (right).

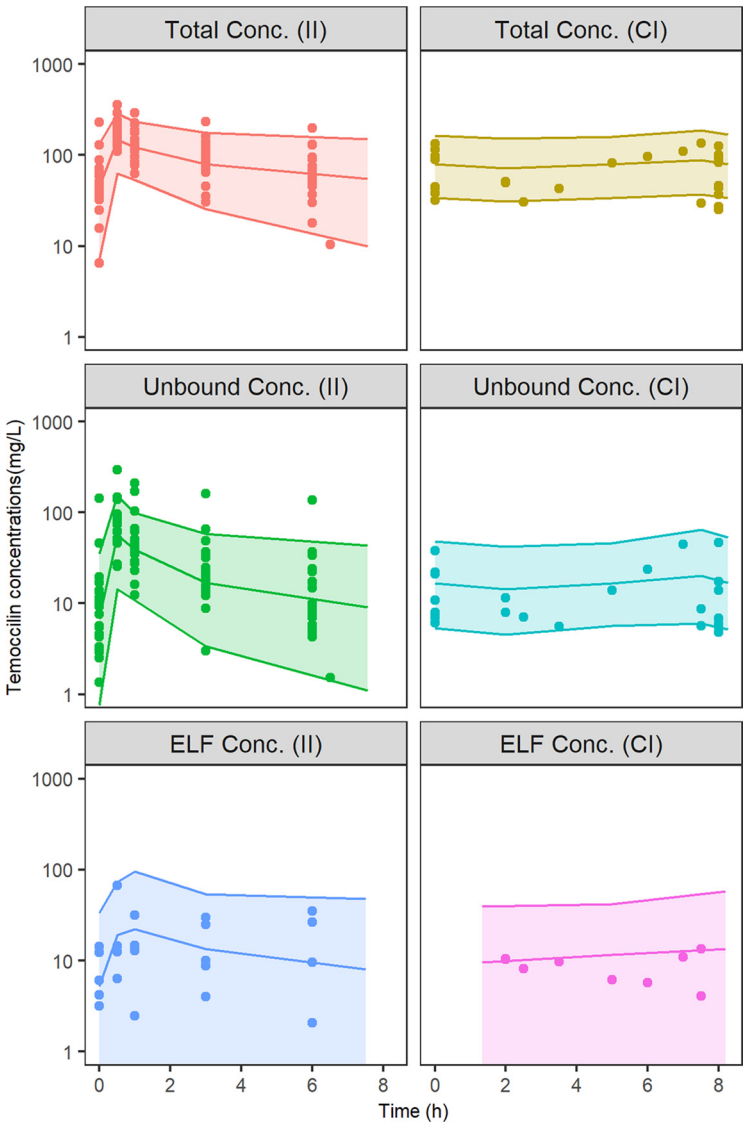

FIG 2 Visual predictive checks (simulations of the data set) of total and free plasma and total ELF concentrations after intermittent infusions (II) and continuous infusions (CI). Solid lines, medians and 90% prediction intervals; filled circles, observed concentrations.

PK/PD analysis. The probability of target attainment (PTA) was computed for two PK/PD targets (50% T $>$ MIC for II and 100% T $>$ MIC for both modes of infusion) against a range of MICs in plasma (free concentrations) and ELF (total concentrations) (Fig. 3). The same targets were considered after dichotomization of creatinine clearance between $\geq$60 mL/min/1.73 m^2 and $<$60 mL/min/1.73 m^2, respectively, as shown in Fig. S5 in the supplemental material, as well as in the case of ARC, as shown in Fig. S6 in the supplemental material. Furthermore, PTA was performed for 60 $\leq$ creatinine clearance (Cl_{CR}) $<$ 90 mL/min/1.73 m^2 and 90 $\leq$ Cl_{CR} $<$ 120 mL/min/1.73 m^2 as shown in Fig. S7 in the supplemental material. The corresponding PK/PD breakpoints are determined using a probability of success of 90% and are summarized in Table 2.

DISCUSSION

To the best of our knowledge, this is the first report of temocillin PK in the ELF of critically ill patients with pneumonia. Ratios of AUCs show that penetration ratio is higher than previously published for most other β-lactams, except cefepime, for both modes of infusion (14–18). As illustrated in Table 2, CI offers better PK/PD indexes than II in all scenarios considered: for the less stringent PD targets (50% T $>$ 1$\times$ MIC in II

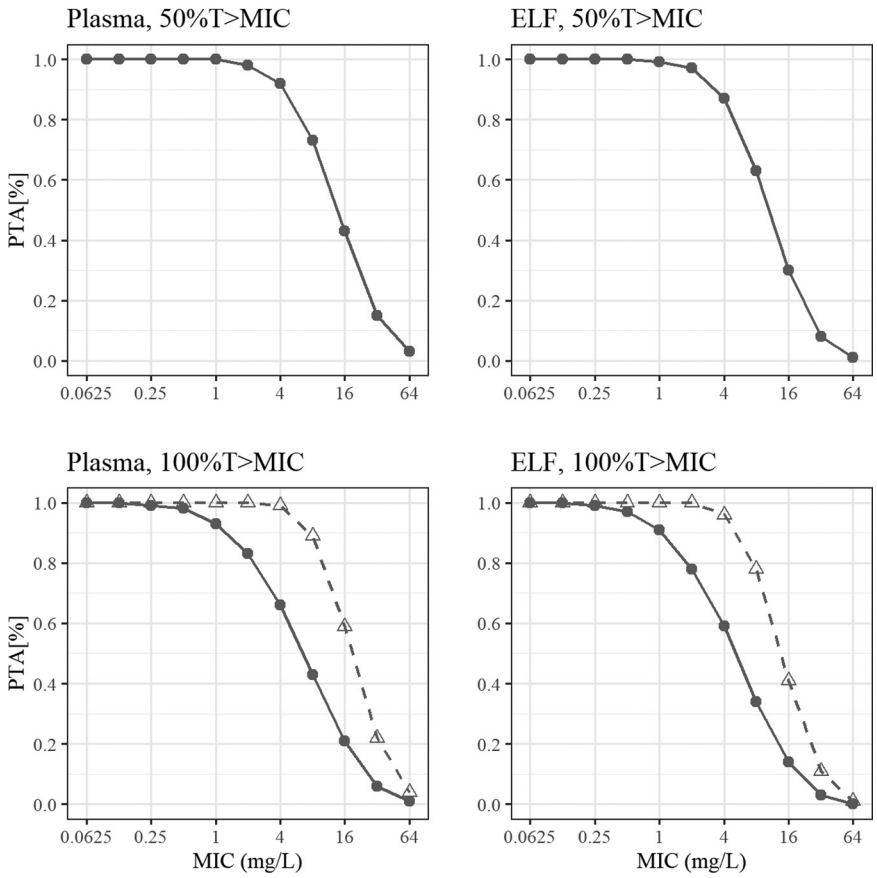

FIG 3 Probability of target attainment (obtained with the Monte Carlo simulations, n_{sim} = 32,000) for free plasma concentrations (left) and total ELF concentrations (right) for different dosing regimens and PK/PD targets (50% or 100% of time above the MIC). Continuous lines with filled circles, II (2 g/8 h over 0.5 h infusion); broken line with open triangles, CI (6 g/24 h).

and 100% T $>$ 1$\times$ MIC in CI), the breakpoints in plasma and ELF were found to be 4 mg/L and 2 mg/L in II, respectively, versus 4 mg/L in CI. For the most stringent PD target (100% T $>$ 4$\times$ MIC for both II and CI), the breakpoints in plasma and ELF were 0.25 mg/L in the II versus 1 mg/L in the CI, respectively. Nonetheless, these values remain well below the only available to date BSAC breakpoints ($\leq$8 mg/L) that recommend the usage of temocillin in systemic infections and, moreover, below the mean MIC of 9.94 mg/L (based on Etest) of the pathogens isolated in this cohort of patients. At best, an MIC of 8 mg/L was achieved for the less stringent PD targets in ELF for both II and CI in patients with moderate renal impairment (30 to 60 mL/min/1.73 m^2). However, as already pointed out, as many as 33.3% of the pathogens in this cohort had an MIC $>$ 8 mg/L to temocillin by Etest, thereby precluding its usage even in the less stringent scenario.

The renal function was found to be a clinically relevant covariate on the drug clearance in the population pharmacokinetics (popPK) analysis, which is consistent with temocillin's renal elimination (19). This is also in line with PK/PD findings for other renally excreted beta-lactams (20–22). The incidence of ARC in our study is also in line with current reports in critically ill patients (23).

Two previous PK studies have been undertaken with temocillin in critically ill patients; however, they were not focused on severe pneumonia (4, 5). With the same dose given by CI, Laterre et al. (5) reported higher average concentrations of free temocillin in plasma (mean, 37 mg/L; n = 11) than that observed in our study (13.7 $\pm$ 11.8 mg/L). This difference can probably be explained by differences in the distribution of the creatinine clearance, 56 $\pm$ 34 mL/min/1.73 m^2 in the study by Laterre et al. (5) versus 119.2 $\pm$ 33.2 mL/

TABLE 2 PK/PD breakpoints in plasma (free concentrations) and ELF (total concentrations) for specific PD targets according to different modes of administration using a probability of success of 90%[a]

	Mode of administration			
	II		CI	
PD target	Plasma	ELF	Plasma	ELF
50% T > 1× MIC	4	2	NA	NA
Cl_{CR} < 60 mL/min/1.73 m²	8	8	NA	NA
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	8	4	NA	NA
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	4	4	NA	NA
Cl_{CR} ≥ 60 mL/min/1.73 m²	4	2	NA	NA
Cl_{CR} > 120 mL/min/1.73 m²[b]	2	2	NA	NA
50% T > 4× MIC	1	0.5	NA	NA
Cl_{CR} < 60 mL/min/1.73 m²	2	2	NA	NA
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	2	1	NA	NA
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	1	1	NA	NA
Cl_{CR} ≥ 60 mL/min/1.73 m²	1	0.5	NA	NA
Cl_{CR} > 120 mL/min/1.73 m²	0.5	0.5	NA	NA
100% T > 1× MIC	1	1	4	4
Cl_{CR} < 60 mL/min/1.73 m²	4	4	8	8
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	2	2	8	8
90 ≤ Cl_{CR} < 120 mL/min/1.73 m²	2	1	8	4
Cl_{CR} ≥ 60 mL/min/1.73 m²	1	0.5	4	4
Cl_{CR} > 120 mL/min/1.73 m²	0.5	0.5	4	4
100% T > 4× MIC	0.25	0.25	1	1
Cl_{CR} < 60 mL/min/1.73 m²	1	1	4	2
60 ≤ Cl_{CR} < 90 mL/min/1.73 m²	0.5	0.5	2	2
90 ≤ Cl_{CR}<120 mL/min/1.73 m²	0.5	0.25	2	1
Cl_{CR} ≥ 60 mL/min/1.73 m²	0.25	0.125	1	1
Cl_{CR} > 120 mL/min/1.73 m²	0.125	0.125	1	0.5

[a]NA, not applicable; II, intermittent infusion; CI, continuous infusion; Cl_{Cr}, creatinine clearance.
[b]These patients are included in the group of patients with Cl_{CR} ≥ 60 mL/min.

min/1.73 m² in the present study. Moreover, in the De Jongh et al. (4) study, temocillin was given at a lower dose of 4 g/day via CI in 6 patients who displayed a higher mean free plasma concentration of 21.5 mg/L and a higher plasma breakpoint of 16 mg/L in parallel with a lower CL_{CR} (102 ± 18 mL/min/1.73 m²) than in our study, thereby also possibly explaining the discrepancies observed with our results.

This study's limitations include its single-center design, the fact that it was not designed to test clinical efficacy of temocillin, and a relatively low number of patients, some of which were severely ill with late-stage ARC (23). Furthermore, the choice of microbiological diagnostic techniques such as Vitek 2 and Etest, which was anterior to EUCAST guidelines, might have underestimated or overestimated sensitivity to temocillin in comparison to disk diffusion and broth microdilution tests, which are now recommended (24). Moreover, this study included mainly normal weight patients; therefore, no conclusions may be drawn as to PTA of temocillin in obese (BMI > 30) critically ill patients (25). Finally, MIC distributions of various ESBL-producing organisms are largely unknown at this stage, making it difficult to generate recommendations for temocillin usage solely based on PTA analysis.

In conclusion, penetration ratios that were estimated by MC simulations at 73% were higher than previously demonstrated for other β-lactams, except cefepime. However, the current BSAC breakpoint of 8 mg/L was achieved for II and CI only in patients with creatinine clearance of <60 mL/min/1.73 m² and with the least stringent PD target both in plasma and ELF. While it has not been demonstrated that efficacy of a β-lactam in severe pneumonia is entirely dependent on its ELF concentration levels, our results suggest that temocillin should not be recommended in severe nosocomial

pneumonia without further clinical data in accordance with recent EUCAST clinical breakpoints.

MATERIALS AND METHODS

Study design and participants. This was a single-center, prospective, randomized study that was conducted in six intensive care units (ICUs), with a total of 53 medical and surgical beds, at the Centre Hospitalier Universitaire du Sart-Tilman, Liège, Belgium, between March 2016 and February 2017. The study was approved by the local ethics committee (EudraCT number 2015-004591-30), and informed consent was obtained from relatives because all patients were ventilated at the time of inclusion.

Eligible patients had to meet the following inclusion criteria: age $>$ 18; diagnosis of ventilator-associated pneumonia (VAP) or hospital-acquired pneumonia (HAP) requiring mechanical ventilation with a documented pathogen showing temocillin Vitek 2 *in vitro* sensitivity of $\leq$8 mg/L; and creatinine clearance based on 24-h urine output collection and measurement $\geq$30 mL/min/1.73 m^2.

The enrolled patients were prospectively randomized in a 2.5:1 ratio to either the intermittent infusion group (II; 2 g over 0.5 h every 8 h) or the continuous infusion group (CI; 6 g over 24 h after a loading dose of 2 g over 0.5 h). The *a priori* defined ratio was chosen to study the temocillin concentration at five time points, using only one ELF sample per patient. No power size calculation was deemed necessary for this descriptive study.

Data collection, study drug, and sampling. Demographic and clinical data were prospectively collected including age, sex, weight, admission diagnosis, duration of ICU stay before temocillin treatment, clinical pulmonary infectious score (CPIS), simplified acute physiology score (SAPS) 3, sequential organ failure assessment (SOFA), and acute physiology and chronic health evaluation (APACHE) scores, presence of septic shock, and in-hospital and ICU mortality.

Temocillin (Négaban; Eumedica, Belgium) was dissolved in 50 mL of NaCl 0.9% saline solution and injected into a central venous catheter via a volumetric pump with an infusion dead space of less than 2 mL. Stability of the infusion has been published elsewhere (26).

All serum and mini-bronchoalveolar lavage (mini-BAL) samples were obtained within 15 min either side of the expected time of sampling after at least 24 h of infusion in the CI group and at least 3 doses in the II group. Serum samples (10 mL) were collected from indwelling arterial catheters at three predetermined time points for each patient in the CI group: i.e., 8am, time of the mini-BAL, and 4 pm. In the II group, blood samples were obtained at predose and 0.5, 1, 3, and 6 h after the start of temocillin infusion.

Mini-BAL samples (one per patient, evenly at the blood sampling times) were collected through a standardized mini-BAL procedure as follows: 2 $\times$ 40 mL of sterile 0.9% saline solution using a non-bronchoscopy catheter (Bal-Cath system; Kimberly Clark, Zaventem, Belgium).

Analytical methods. Blood and mini-BAL samples were immediately centrifuged at 3,000 rpm for 10 min and 10,900 rpm for 5 min, respectively; the supernatant was immediately separated and kept at $-$20°C until analysis, except for the BAL microbiological culture. For determination of total temocillin, 200 μl of BAL were spiked with ticarcillin (internal standard) and cleaned up by liquid-liquid extraction prior to chromatographic analysis. For determination of free temocillin concentration, 500 μL of serum or BAL was beforehand filtered by centrifugation using an Amikon 10-kDa ultrafiltration device (Millipore). Then, 300 μL of this ultrafiltered serum (or 200 μL of ultrafiltered BAL) were spiked with ticarcillin and were cleaned up by liquid-liquid extraction. The ultrafiltered serum/BAL was directly analyzed without extraction.

All pretreated samples were analyzed using a validated method on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Acquity Waters-Quattro Premier Waters) equipped with a solvent quaternary pump, an injector, an Acquity HSS T3 column (100 $\times$ 2.1 mm; 1,8 μm) thermostatized at 40°C, and MassLynx computer software (Waters Corporation).

Measure of urea and determination of ELF concentrations. The concentrations of urea in the serum and ELF were determined as described by Rennard et al. (27) with the urea nitrogen/1900 kit (Roche Professional Diagnostics, Mannheim, Germany). The concentration of temocillin in ELF was thereafter determined using urea as an endogenous marker according to the following formula (27, 28).

$$\mathrm{TEM_{ELF}} = \frac{\mathrm{TEM_{BAL}} \times \mathrm{urea_{PLA}}}{\mathrm{urea_{BAL}}}$$

where calculated $\mathrm{TEM_{ELF}}$ is the concentration of temocillin in ELF, $\mathrm{TEM_{BAL}}$ is the concentration of temocillin in the mini-BAL fluid, $\mathrm{urea_{PLA}}$ is the concentration of urea in serum (collected concomitantly with bronchoscopy), and $\mathrm{urea_{BAL}}$ is the concentration of urea in the mini-BAL fluid.

MIC determinations. MICs were first determined using the automated system Vitek 2 (bioMérieux) and subsequently by Etest (bioMérieux).

PK analysis. A population PK model was developed. A nonlinear mixed effects modeling approach was performed with NONMEM version 7.4.0 (double precision; Icon Development Solutions, Ellicott City, MD, USA) and PsN-toolkit version 4.6.0 (29). The first-order conditional estimation method with interaction was used. One- and two-compartment structural models were fitted to free (Cu) and total (Ct) serum and total ELF (Celf) concentrations. The estimated pharmacokinetic parameters reflect the unbound concentrations of temocillin. The relationship between bound and unbound concentrations of temocillin was described by an E_{max}-type model of parameters Cb_{max}, the maximal concentration of temocillin that can be bound and BC_{50}, the concentration of unbound temocillin for which half of Cb_{max} is reached. The

passage of unbound temocillin from plasma to ELF was modeled with an entry clearance into ELF (Q_{in}) and an exit clearance from ELF (Q_{out}). At steady state, the AUC ratio (R_{AUC}) between Cu and Celf corresponds to the Q_{out}/Q_{in} ratio. The interindividual variability in the PK parameters was estimated with the use of exponential models. The correlation between individual values of plasma clearance and central volume of distribution was estimated. Additive, proportional, and mixed error models were investigated to describe the residual variability. Weight, body surface area, and creatinine clearance were tested as covariates on volumes of distribution and/or clearance parameters. Power functions were used for this purpose. A decrease in objective function of >3.84 was used to consider a covariate as statistically significant with a 5% type I error. The correlation between unbound and total temocillin concentration measurements from the same sample was tested using the L2 function in NONMEM. Precision of the estimations was evaluated by using the sampling importance resampling (SIR) procedure, implemented with PsN (30). An internal validation of the model was performed by visual inspection of goodness-of-fit (GOF) plots, based on model predictions and residuals, and visual predictive checks (VPCs).

Monte Carlo simulations. (i) PK/PD analysis. Steady-state concentrations of temocillin in serum and ELF were generated for 32,000 virtual subjects by Monte Carlo simulations, with the same demographic characteristics as the 32 patients included in the study, for each of the two dosing scenarios. Subsequently, the %T > MIC were calculated as well as the probabilities of target attainment (PTA) for different PD targets based on Cu for plasma. The BSAC defined breakpoints for systemic infections caused by *Enterobacterales* were used (11).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.3 MB.

AKNOWLEDGMENTS

This work was partly funded by a FIRS (Fonds d'Investissement de Recherche Scientifique 981480500) at CHU de Liège.

N.L. and F.F. designed the study. N.L. obtained the funds, C.V. obtained the data. N.M., J.D., V.M., R.D., and N.G. did the analyses. N.L., F.F., C.V., and N.G. interpreted the data. N.L. and N.G. wrote the manuscript, which was reviewed by S.M.

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