NOVEL APPROACHES FOR DIAGNOSING SEPSIS IN THE CRITICALLY ILL

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Novel approaches for diagnosing sepsis in the critically ill.
PhD thesis, Utrecht University, the Netherlands.

ISBN: 978-94-6361-443-6
Layout and Printing: Optima Grafische Communicatie (www.ogc.nl)
Cover design: Optima Grafische communicatie.
Based on an Incan mask found on the archeological site 'La Tolita'. The design is typical of masks of the sungod Inti with zig-zag rays bursting from the head and ending in human faces or figures.

Financial support by the Julius Center for Health Sciences and Primary Care for the printing of this thesis is gratefully acknowledged.

Novel Approaches for Diagnosing Sepsis in the Critically Ill

Nieuwe benaderingen voor het diagnosticeren van sepsis
in kritiek zieke patiënten
(Met een samenvatting in het Nederlands)

P R O E F S C H R I F T

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 10 september 2020
des middags te 2:30 uur

door

Diana Milena Verboom
geboren op 9 maart 1990 te Dordrecht

Promotoren:
Prof. dr. M.J.M. Bonten
Prof. dr. O.L. Cremer

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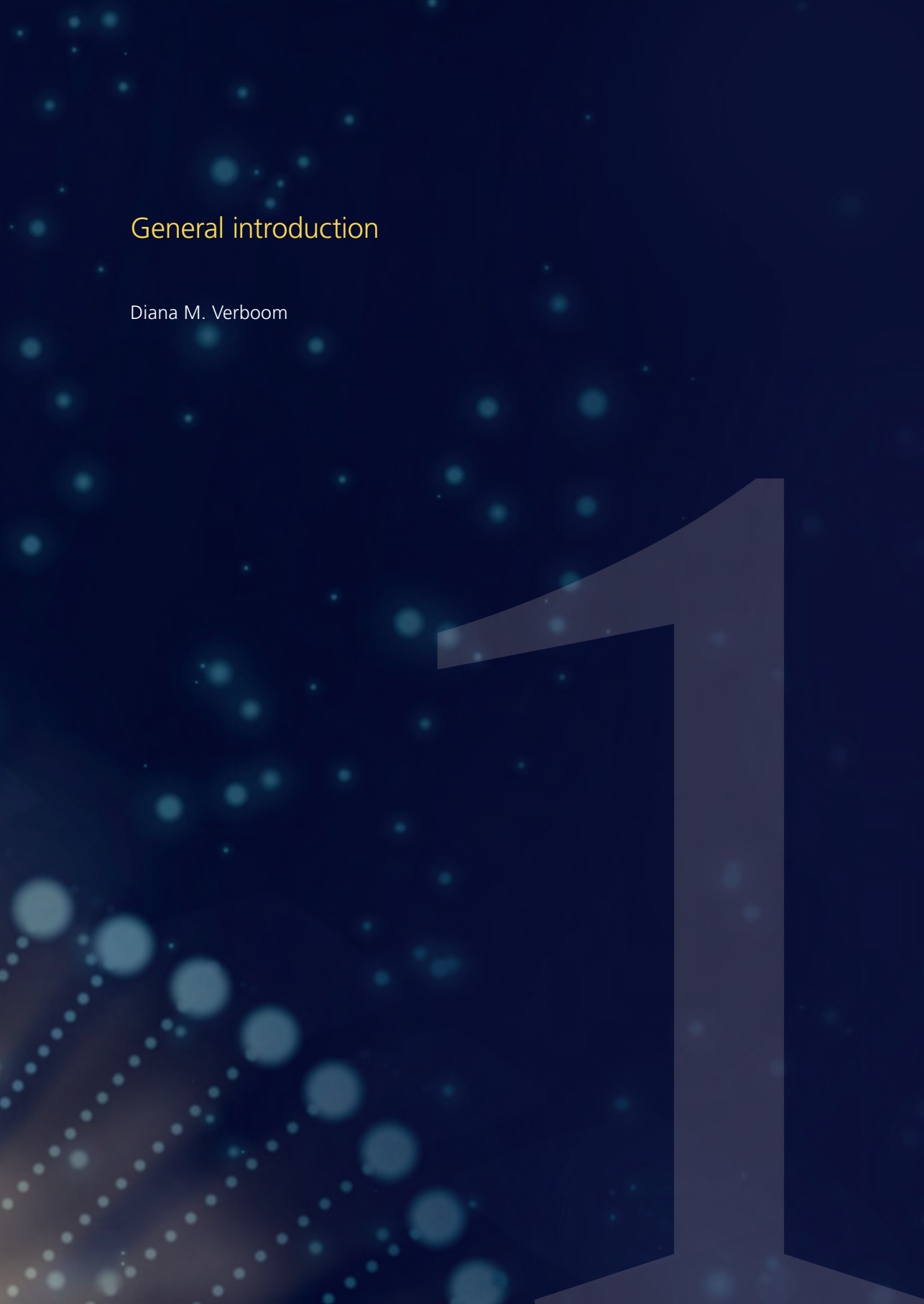
General introduction

Diana M. Verboom



General introduction

Sepsis results from the body's response to infection and is the leading cause of morbidity and mortality in hospitalized patients [1]. Worldwide, 21 million cases occur yearly, with mortality rates ranging between 6 and 34% [1]. Approximately half of these patients will need supportive care on the Intensive Care Unit (ICU). Conversely, about 18% of the ICU patients are admitted with a primary diagnosis of sepsis, and an additional 15% develop sepsis during ICU admission, meaning that almost a third of the ICU population will go through a septic episode during ICU admission [2,3]. When patients survive sepsis, they often suffer from cognitive, and functional disability [4,5]. Early recognition, and prevention of progressive organ failure is thought to improve short, and long-term outcomes in sepsis [4,6]. Hence early recognition of sepsis is important and there is a great need for accurate diagnostic tools that can be applied in an early phase. This thesis will focus on the classification of sepsis-related organ failure, diagnosis of sepsis, and bloodstream infections on the ICU.

Classifying organ dysfunction

Over time, sepsis definitions and diagnostic approaches have changed. The first consensus reports on what sepsis is and how it should be defined were published in the 1990s [7]. Ever since, efforts have been made to improve the broader consensus of the definition. Until 2015, the presence of systemic inflammatory response syndrome (SIRS) was included in the definition of sepsis [8]. However, a systemic response to invasive infection can be appropriate, enables the host to respond more effectively to the infection, and therefore does not help to distinguish an uncomplicated flu, from a severe pneumonia. Also, the clinical presentation of SIRS is similar between infectious and non-infectious entities, such as trauma or pancreatitis. In addition, patients with severe sepsis, do not necessarily present with SIRS [9,10]. As it lacked both specificity and sensitivity, SIRS was excluded from the sepsis definition, and instead, organ failure was proposed as the most important pathologic feature of the host response in the Third International Consensus Definitions as of 2016 (i.e. sepsis-3) [11].

As a result, sepsis is currently defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. These updated sepsis criteria aim to identify a subgroup with increased risk of adverse outcome among patients suspected of infection. Its predictive ability was confirmed multiple times among different cohorts [11–13]. However, sepsis remains a very heterogeneous syndrome which is reflected by the diversity of causative pathogens, sources of infection, patient characteristics, and host response. Each of these factors may influence sepsis-related outcomes and the response to specific therapies. A standardized and explicitly stated use of the definition is important to compare the epidemiology of sepsis between cohorts and over time. In **Chapter 2** we discuss the robustness of the current sepsis criteria and focus on the effect of differences in operationalization of sepsis-3, and its effect on the apparent incidence of sepsis and sepsis-related mortality.

Diagnosing infection

There are no unambiguous criteria for diagnosing infection and there is a large variety in signs and symptoms of sepsis depending on site of infection, severity of organ dysfunction and timing of presentation to the ICU [13–15]. Diagnostic uncertainty will lead to undetected cases of early sepsis, delaying initiation of adequate therapy and thus reducing the chances of survival [6,16,17]. On the other hand, initiating antimicrobial therapy in patients without an infection selects for antimicrobial resistance, causes side-effects, and may lead to delays in diagnosing (alternative) non-infectious conditions [18,19]. In some instances, single plasma biomarkers, such as PCT, can help in diagnosing sepsis [20].

However, single biomarkers are most likely not accurate enough to guide initiation of antibiotics, as they cannot accurately discriminate infection from non-infectious systemic inflammatory response syndrome (SIRS) [21–23].

There is a need for accurate biomarkers in the early phases of sepsis, and new approaches consist of molecular strategies based on transcriptomics, proteomics, or metabolomics [24]. The availability of these novel molecular techniques and automation of elaborate laboratory analyses have stimulated the development of more complex, multi-analyte indicators of the host response [25]. For example, transcriptomic profiling has shown largely different gene expression patterns between septic patients and other non-infectious entities, such as acute respiratory distress syndrome, burn injury and trauma [26,27]. These gene expression patterns can be converted into a simplified gene signature that keeps its discriminative ability [28]. In **Chapters 3, 4 and 5** we will discuss the clinical utility of SeptiCyte LAB, the first RNA signature that was cleared by the FDA in the United States as a test to aid in the diagnosis of infection in critically ill patients.

Documenting bloodstream infection

When bacteria or fungi invade the bloodstream, they may cause a bloodstream infection, sometimes triggering sepsis [29]. Bloodstream infection causes significant patient morbidity and mortality, especially in critically ill patients, in whom the case fatality rate is between 35 and 50% [30, 31]. Early appropriate antibiotic administration is associated with decreased sepsis-associated mortality, suggesting we should aim to initiate adequate therapy as soon as possible [32]. Detection of bloodstream infections is less unambiguous than infection without a positive blood culture, nevertheless this infectious entity has its own diagnostic challenges. The first challenge is to recognize a clear indication for taking a blood culture. The pre-test probability for a positive culture is very dependent on the clinical context and ranges significantly (between 2-69%) [33]. Clinical decision models for obtaining blood cultures can be helpful but are not sensitive enough. As a result, in some settings bloodstream infections might be missed [33].

The second challenge is to recognize false positive results. These are either due to contamination, or asymptomatic (and transient) bacteremia [34,35]. False positive results may result

in unnecessary antimicrobial treatment and increase costs and resource utilization through longer hospital stay and additional diagnostic analyses [36]. Therefore, clinical protocols usually suggest to only perform blood cultures when there is a clear indication [33,35]. In **Chapter 6** we describe the diagnostic yield of routine blood cultures in critically ill patients upon ICU admission as we explored the potential of such a strategy in the early detection of critical ICU admissions.

In **Chapter 7** we describe a cohort of patients with *Escherichia coli* bloodstream infection and risk factors for invasive *E. coli* disease. We performed O-serotyping of *E. coli* isolates to describe the O-antigens located on the bacterial surface of *E. coli*. Extra-intestinal pathogenic *E. coli* (ExPEC) is a Gram-negative rod and the most common identified causal pathogen in septic hospitalized patients, and the second most common pathogen causing bloodstream infections [1,40]. Antimicrobial resistance, particularly among Gram-negative bacteria, continues to increase [37,38]. At the same time, the development of new antibiotics has not kept up with the global increase in antimicrobial resistance, and alternative strategies are needed [39]. A glycoconjugate vaccine, targeting ten *E. coli* O-antigens, is in development and offers an alternative to antimicrobial therapy [41,42]. The chosen implementation strategy and effectiveness of such a strategy, will depend on target population characteristics, and potential vaccine coverage.

The thesis was written within the framework of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project. MARS was initiated in 2011 to aid diagnosis and prognostication of sepsis. The project was initiated to advance the diagnosis and prognostication of sepsis through development of new tools that provide rapid information on the host response in infection and the presence of causative pathogens. The MARS database contains daily data and biorepository material on from a large number of patients with a special interest for the detailed description of numerous infectious episodes occurring upon and during ICU admission. The central theme of this thesis is the complexity of diagnosing sepsis in an ICU population, and we discuss different diagnostic approaches for sepsis and bloodstream infections. We discuss sepsis-related organ failure, molecular and established diagnostic approaches. With this thesis we aim to improve diagnosis, and facilitate management of sepsis and bloodstream infections. C**hapter 8** provides a general discussion on these topics in light of previous literature, future perspectives and research directions.

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Robustness of sepsis-3 criteria in critically ill patients

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Published in Journal of Intensive Care (2019)

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Abstract

Background

Early recognition of sepsis is challenging, and diagnostic criteria have changed repeatedly. We assessed the robustness of sepsis-3 criteria in intensive care unit (ICU) patients.

Methods

We studied the apparent incidence and associated mortality of sepsis-3 among patients who were prospectively enrolled in the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) cohort in the Netherlands, and explored the effects of minor variations in the precise definition and timing of diagnostic criteria for organ failure.

Results

Among 1081 patients with suspected infection upon ICU admission, 648 (60%) were considered to have sepsis according to prospective adjudication in the MARS study, whereas 976 (90%) met sepsis-3 criteria, yielding only 64% agreement at the individual patient level. Among 501 subjects developing ICU-acquired infection, these rates were 270 (54%) and 260 (52%), respectively (yielding 58% agreement). Hospital mortality was 234 (36%) versus 277 (28%) for those meeting MARS-sepsis or sepsis-3 criteria upon presentation ($p<0.001$), and 121 (45%) versus 103 (40%) for those having sepsis onset in the ICU ($p<0.001$). Minor variations in timing and interpretation of organ failure criteria had considerable effect on the apparent prevalence of sepsis-3, which ranged from 68% to 96% among those with infection at admission, and from 22% to 99% among ICU-acquired cases.

Conclusion

The sepsis-3 definition lacks robustness as well as discriminatory ability, since nearly all patients presenting to ICU with suspected infection fulfill its criteria. These should therefore be specified in greater detail, and applied more consistently, during future sepsis studies.

Introduction

Sepsis is a life-threatening disease caused by a dysregulated host response to infection. Unfortunately, both early recognition and definitive confirmation of the diagnosis have proven to be difficult as sepsis is a very heterogeneous syndrome [1]. Since 1991, conceptual thinking about sepsis has focused on the presence of a systemic inflammatory response syndrome (SIRS). However, SIRS criteria are neither sensitive nor specific for infection, and do not necessarily indicate a dysregulated or life-threatening host response [2, 3]. Furthermore, sepsis definitions that relied on SIRS criteria were highly sensitive to minor variations in frequency and timing, thereby affecting reliability of the sepsis diagnosis [2].

Sepsis-3 definitions were developed to improve risk stratification among patients with a suspected infection, and their predictive validity regarding unfavorable clinical outcomes have been confirmed several times by now [4-12]. Rather than a systemic inflammatory response syndrome, these sepsis definitions require the development of organ failure during an infectious episode, which is operationalized by an increase in the Sequential Organ Failure Assessment (SOFA) score [13, 14]. Similarly, the septic shock-3 definition requires the presence of elevated serum lactate levels in addition to fluid resistant hypotension [15].

Sepsis-3 definitions were also established to increase uniformity among reported incidence and mortality rates [13-15]. A consistent diagnosis of sepsis and septic shock between centers is particularly important for research and benchmarking purposes. Clinical data can be sensitive to different coding approaches, complicating comparisons of sepsis epidemiology among different cohorts [16, 17]. However, as only little attention has been focused to the robustness of sepsis-3 criteria, we studied the effects of minor variations in the interpretation of the criteria on the incidence and related mortality of sepsis-3.

Methods

Study design and population

This study was embedded within the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) cohort [18]. Consecutive adult patients with newly suspected infection either upon presentation or during ICU stay were enrolled in two Dutch tertiary ICUs between June 2011 and April 2015 (University Medical Center Utrecht) or between June 2011 and January 2014 (Academic Medical Center Amsterdam).

Patients who had been admitted to another ICU for more than one day before transfer to one of the study centers were excluded, because information about possible previous infections and organ failures was not available. Patients who had been treated for an infection in the week prior to ICU admission and subsequently were admitted with a new infection were also excluded to avoid possible overlap between pre-existent and newly acquired organ

failures. The institutional review board approved an opt-out consent procedure (protocol number 10-056C).

Data and definitions

Trained researchers attended daily multidisciplinary rounds in the participating ICUs and prospectively recorded the presence of infection, SIRS, and organ failure [18, 19]. In this study we use the terms "MARS-sepsis" and "MARS-shock" to indicate severe sepsis and septic shock according to prospective assessment of the presence of SIRS and organ failure, based on the 1991 and 2001 definitions of sepsis [20, 21] (see table 1). The incidence and related mortality of MARS-sepsis are shown for illustrative purposes only and are not intended to provide a head to head comparison with sepsis-3 (which would have no clinical significance) nor to appraise the robustness of sepsis-3.

Table 1. Sepsis definitions

Old sepsis	
MARS-sepsis	Presence of ≥2 SIRS criteria and organ failure within a 4-day window around suspected infection [a, b]
MARS-septic shock	MARS-sepsis and use of vasopressor for hypotension within a 4-day window [a, c]
Sepsis-3	
Sepsis-3 (4-day window)	Suspected infection and an acute SOFA score increase of ≥2 points within a 4-day window [a]
Septic shock-3	Sepsis-3 and vasopressor-dependent hypotension (i.e. circulatory SOFA score ≥2) plus an increased serum lactate level of >2 mmol/L within a 4-day window [a, d]
Assessments of minor variations in diagnostic criteria	
Reduced observation window	Similar to sepsis-3, but with a 2-day window around suspected infection (i.e. an increase between the day before and the day of the onset of infection)
Absolute SOFA score	Suspected infection and an absolute SOFA score of ≥2 points at the day of onset of infection and within a 4-day window [a]
Septic shock-3 ignoring lactate	Similar to septic shock-3, but without the requirement of increased serum lactate levels if not measured

SIRS=Systemic Inflammatory Response Syndrome, SOFA=Sequential Organ Failure Assessment.

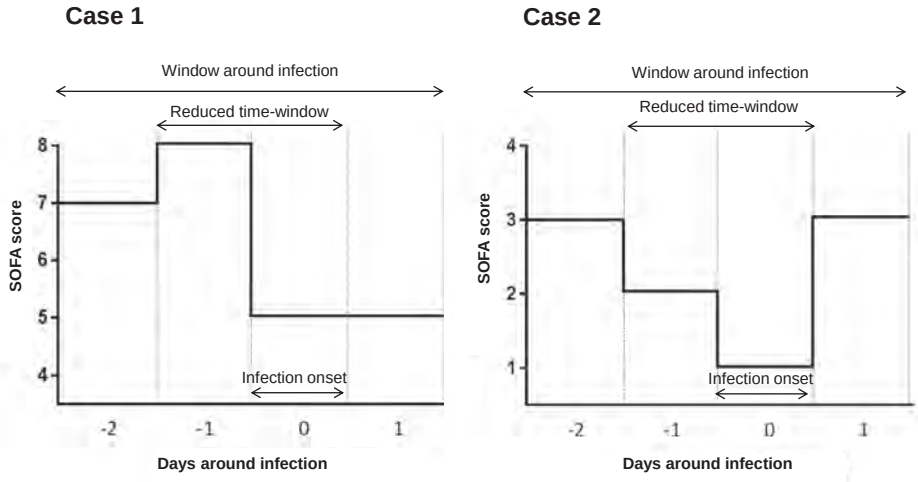
[a] 4-day window = an observation window ranging from 2 days before the initiation of empirical antibiotics (onset of infection) until 1 day after the onset of infection.

[b] Organ failure for MARS-sepsis was defined as the following signs of organ hypoperfusion or dysfunction: areas of mottled skin; capillary refilling requiring 3 seconds or longer; urine output <0.5 ml/kg for at least 6 hours, >1.5 fold elevated creatinine or renal replacement therapy; lactate >2 mmol/l; abrupt change in mental status; abnormal electroencephalographic findings consistent with septic encephalopathy; platelet count <100,000 platelets/ml or disseminated intravascular coagulation; acute respiratory distress syndrome and cardiac dysfunction, as defined by echocardiography or direct measurement of the cardiac index [25]".

[c] MARS-septic shock was defined as the use of norepinephrine in a dose of > 100 ng/kg/min for more than 50% of an observation day, dopamine >5 mcg/kg/ min or epinephrine for hypotension despite adequate fluid resuscitation (e.g. not including induced hypertension).

[d] Lactate was considered increased if it was increased once at any day during the 4-day time window

The terms "sepsis-3" and "septic shock-3" were used to indicate events meeting the updated definitions. Organ failure for sepsis-3 was defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [14]. We operationalized organ failure as an acute SOFA score increase of ≥2 points compared to pre-existing (acute or chronic) organ dysfunction before the onset of infection (table 1). The increase in SOFA score had to occur between 2 days before the onset of infection and 1 day after the onset of infection (i.e. a 4-day window, see figure 1). This window was used because organ dysfunction may occur prior to, near the moment, or after the infection is recognized [5]. An infection was registered when empirical antimicrobial therapy was started by attending clinicians irrespective of the presence of SIRS or organ failure, and this day was regarded as its onset. Subsequently, the likelihood of each infection was subsequently adjudicated as none, possible, probable or definite, using detailed definitions derived from Center of Disease Control and International Sepsis Forum Consensus Conference criteria [18, 22, 23]. Only first ICU infections occurring during a hospital admission were included for analysis. Infections present at admission (having onset between 1 day before and 2 days after ICU admittance)



	Sepsis-3 (core definition)	Reduced time window	Absolute SOFA score ≥2	Absolute SOFA score ≥2 (reduced time-window)
Case 1	No	No	Yes	Yes
Case 2	Yes	No	Yes	Yes

Figure 1. Hypothetical cases showing the influence of variations in organ failure definitions. SOFA=Sequential Organ Failure Assessment. The onset of infection (i.e. start of empirical antibiotic therapy) is day 0. Case 1 does not fulfill the sepsis-3 definition as there is no SOFA score increase of ≥2 points within the 4-day (or 2-day) time-window. However, case 1 fulfills the criteria if sepsis is defined by the presence of an absolute SOFA score of ≥2 (both in the 4-day and 2-day time-window). Case 2 fulfills the sepsis-3 criteria since there is an increase of ≥2 points between day 0 and day 1. In a reduced time-window there is no increase observed between the day before infection and day of the onset of infection, and sepsis-3 criteria are not met.

and ICU-acquired infections (having onset more than 2 days after ICU admittance) were analyzed separately since we hypothesized that the extent of new organ failure might vary between these types of infection.

To reconstruct baseline SOFA scores, raw pre-ICU clinical data were extracted from the hospital electronic health care record. All ICU data were collected prospectively [19]. In cases on dialysis dependency or having chronic renal insufficiency the renal SOFA was assumed to be 3.

To evaluate the robustness, we assessed the influence of minor variations in implementation of the sepsis-3 definitions (see Table 1). We based our variations on methodology that was used in previous studies [4, 6, 13, 15]. First, we shortened the time window of observation by only including the day of clinical diagnosis and one day before (2-day window). Second, we explored the effects of an absolute SOFA score at the time of recognition of infection. Third, to mimic settings in which lactate is not always available, only vasopressor-dependent hypotension was required to fulfill the septic shock definition in cases where lactate levels were missing (see table 1 and figure 1 for further explanations).

Statistical analyses

We calculated apparent incidences and related in-hospital mortality of sepsis-3 and MARS-sepsis. We calculated the percent agreement as the percentage of cases in which two sepsis definitions corresponded with each other. Sensitivity analysis was performed by excluding subjects with rejected infection (i.e. a post-hoc likelihood of none). All analyses were performed and reported separately for infections at admission and ICU-acquired infections. Missing data were handled as described in table S1 in the supplementary material. Differences at baseline and clinical characteristics between the subgroups were analyzed using a Mann-Whitney U test, Chi-square test, or McNemar test, as appropriate. Differences in mortality were calculated accounting for partially overlapping samples [24]. A p-value < 0.05 was considered statistically significant. All analyses were performed using SAS 9.2 (SAS Institute Inc.).

Results

Among 1743 patients treated for an infection in the ICU, 1081 with an infection at ICU admission and 501 with an ICU-acquired infection remained for analysis (figures 2 and 3). Patient and infection characteristics are presented in table 2.

Incidence and associated mortality

Table 3 shows the apparent incidences and related percent agreement of sepsis and septic shock according to the various definitions.

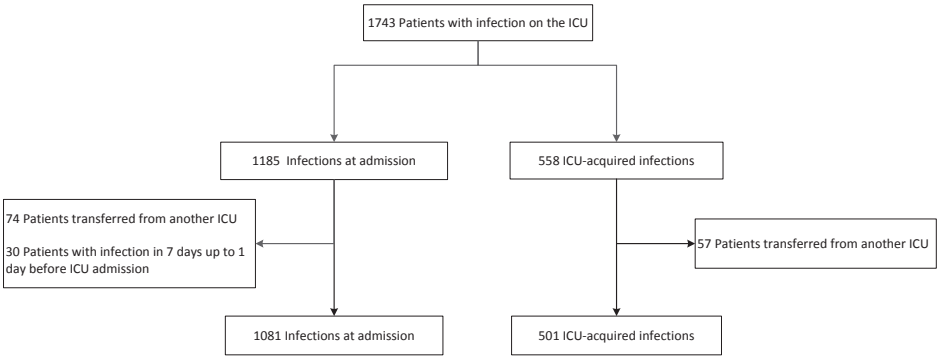


Figure 2. Flowchart. ICU=intensive care unit.

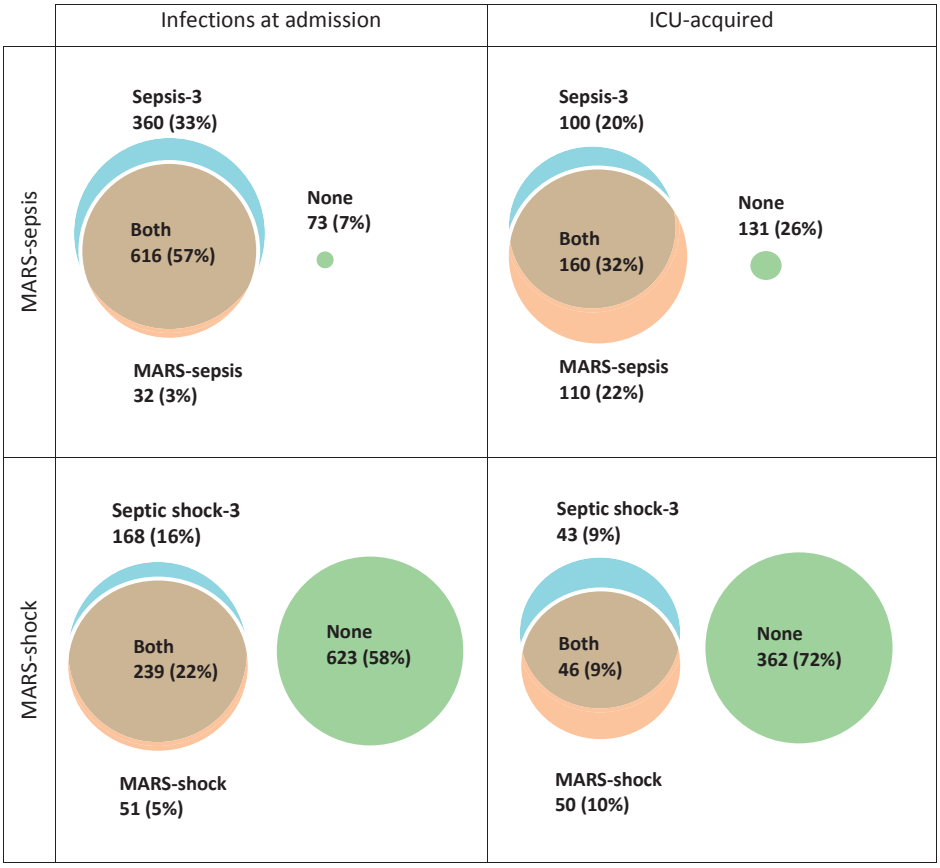


Figure 3. Venn diagram comparing MARS-sepsis and sepsis-3 definitions. ICU=intensive care unit. Presented as frequencies of patients (%).

Table 2. Characteristics of patients with infection on admission and with ICU-acquired infection and stratified by presence of sepsis-3 criteria

	Infection at admission (N=1081)			**ICU-acquired infection (N=501)**		
	No sepsis-3 (N=105)	Sepsis-3 (N=976)	P-Value	No sepsis-3 (N=241)	Sepsis-3 (N=260)	P-Value
Age (years)	61 (42, 69)	64 (53, 73)	0.005	62 (51, 71)	61 (50, 71)	0.64
Male	64 (61%)	621 (64%)	0.59	175 (73%)	177 (68%)	0.27
Charlson comorbidity index	0 (0, 2)	1 (0, 2)	0.002	0 (0, 2)	0 (0, 2)	0.33
Chronic renal insufficiency [a]	9 (9%)	114 (12%)	0.34	20 (8%)	25 (10%)	0.6
APACHE IV Score	69 (50, 89)	83 (66, 03)	0.001	76 (58, 95)	76 (62, 99)	0.24
Medical admission	69 (66%)	726 (74%)	0.06	102 (42%)	104 (40%)	0.60
At onset of infection						
Days from ICU admission	0 (0,2)	0 (0, 0)	<0.001	6 (4, 8)	6 (4, 9)	0.32
Hospital-acquired infection	65 (62%)	449 (46%)	0.002	100%	(100%)	-
Vasopressor use	36 (35%)	663 (68%)	<0.001	98 (41%)	141 (54%)	0.002
Mechanical ventilation	78 (74%)	664 (68%)	0.19	189 (78%)	240 (92%)	<0.001
Lactate measured	37 (35%)	676 (69%)	<0.001	72 (30%)	109 (42%)	0.005
Lactate	1.8 (1, 4)	3 (2, 5)	0.002	2 (1, 2)	2 (2, 4)	<0.001
≥ 2 SIRS criteria	88(83%)	900 (92%)	0.004	205 (85%)	226 (87%)	0.55
SOFA score	2 (1, 4)	6 (4, 9)	<0.001	6 (4, 8)	8 (5, 10)	<0.001
Source of infection			0.08			0.39
Pulmonary tract	70 (67%)	533 (55%)		138 (57%)	154 (59%)	
Abdominal tract	7 (7%)	156 (16%)		7 (3%)	12 (5%)	
Urinary tract	6 (6%)	57 (6%)		1 (0%)	1 (0%)	
CRBSI	1 (1%)	15 (2%)		36 (15%)	25 (10%)	
Other	21 (20%)	215 (22%)		59 (24%)	68 (26%)	
Infection likelihood			0.02			0.13
• None	11 (10%)	99 (10%)		85 (35%)	82 (32%)	
• Possible	46 (44%)	298 (31%)		109 (45%)	105 (40%)	
• Probable	30 (29%)	293 (30%)		31 (13%)	43 (17%)	
• Definite	18 (17%)	286 (29%)		16 (7%)	30 (12%)	
Outcome						
Length of ICU stay (days)	2 (1, 6)	4.0 (2, 10)	<.001	6 (3, 13)	7 (3, 15)	0.12
Length of hospital (days)	13 (5, 29)	15 (7, 31)	0.09	19 (9, 34)	22 (9, 38)	0.64
ICU mortality	8 (8%)	197 (20%)	0.002	52 (22%)	82 (32%)	0.01
Hospital mortality	12 (11%)	277 (28%)	0.001	72 (30%)	103 (40%)	0.05
90-day mortality	20 (19%)	328 (34%)	0.002	83 (35%)	114 (44%)	0.03

APACHE = Acute physiologic and chronic health evaluation, SIRS = Systemic inflammatory response syndrome, ICU = intensive care unit, CRBSI = Catheter-related bloodstream infection. Continuous data are presented as medians (IQR), dichotomous data are presented as frequencies (%).

[a] Creatinine >170 mmol/L or dialysis dependency

Table 3. Incidences of sepsis and related mortality according to core definitions.

		Sepsis-3		**MARS-sepsis**		
		Incidence % (95%CI) [a]	Mortality % (95%CI) [b]	Incidence % (95%CI) [a]	Mortality % (95%CI) [b]	Agreement (%)
	N					
Complete cohort	1582					
• Infection at admission	1081	90 (88-92)	28 (26-31)	60 (57-63)	36 (33-40)	64
• ICU-acquired	501	52 (48-56)	40 (34-46)	54 (50-58)	45 (39-51)	58
Probable infection cohort [c]	1304					
• Infection at admission [c]	971	90 (88-92)	29 (26-32)	61 (58-64)	37 (33-41)	65
• ICU-acquired infection [c]	334	53 (48-59)	44 (37-51)	56 (51-62)	51 (42-56)	59
		Septic shock-3		**MARS-shock**		
		Incidence % (95%CI) [a]	Mortality % (95%CI) [b]	Incidence % (95%CI) [a]	Mortality % (95%CI) [b]	Agreement (%)
	N					
Complete cohort	1582					
• Infection at admission	1081	38 (35-41)	41 (36-46)	27 (24-30)	50 (45-56)	80
• ICU-acquired infection	501	18 (15-21)	57 (47-67)	19 (16-23)	69 (59-78)	81
Probable infection cohort [c]	1304					
• Infection at admission [c]	971	39(36-42)	42(37-47)	28 (25-31)	51(46-57)	79
• ICU-acquired infection [c]	334	19 (15-23)	63 (50-74)	22 (18-27)	73 (62-82)	83

ICU=intensive care unit.

[a] Incidences are the apparent incidences of the various sepsis definitions.

[b] Mortality reflects in-hospital mortality. For all definitions, mortality of the sepsis-3 criteria was significantly lower than the MARS definitions ($p < 0.001$).

[c] A subgroup of patients in whom the infection diagnosis was either possible, probable or definite based on microbiology, clinical symptoms, and radiology, as defined by post-hoc assessment.

Compared to prospectively recorded MARS-sepsis events, more patients fulfilled sepsis-3 and septic shock-3 criteria at ICU admission (60% vs 90%, and 27% vs 38%, respectively). Furthermore, agreement between the definitions was only 64% and 80%, respectively. For patients with ICU-acquired infections the overall incidences of sepsis (54% vs 52%) and septic shock (19% vs 18%) were similar, yet the MARS and sepsis-3 criteria selected different individuals (58% and 81% agreement for sepsis and septic shock, respectively) (Table 3).

Hospital mortality was lower for patients with sepsis-3 and septic shock-3 than for patients with MARS-sepsis and MARS-shock (table 3). Indeed, those patients who were exclusively identified by sepsis-3 at admission (33% of all patients) had a lower mortality rate than patients with organ failure according to both MARS-sepsis and sepsis-3 (37% vs 14%, respectively) (table S2). Nevertheless, mortality was >10% for all definitions (table 3, table S2 and table S3). There were 110 (10%) and 167 (33%) patients with a rejected infection (i.e. those with a post-hoc likelihood rated as none) at ICU admittance and during admission respectively. The exclusion of patients with rejected infection had negligible effect on apparent sepsis incidences, mortality and agreement (table 3).

Robustness of the sepsis-3 definitions

Table 4 shows the results of the analyses to assess the robustness of sepsis-3 criteria. Minor variations in the timing of observations and criteria for organ failure considerably affected the apparent incidence of sepsis-3 at admission, ranging from 68% to 96% for the most restrictive and the most liberal definition, respectively. Using the same criteria, the incidence of septic shock-3 varied from 30% to 42%. For ICU-acquired infections, the incidence of sepsis-3 and septic shock-3 ranged from 22% to 99% and from 7% to 28%, respectively. Whereas these minor variations did not affect hospital mortality rates for infections at admission, and only marginally for ICU-acquired sepsis (table 4).

Table 4. The influence of minor variations in diagnostic criteria on the apparent incidence and related mortality of sepsis

Minor variations			
	Incidence, % (95%CI)	Mortality, % (95%CI)	Agreement (%) [a]
Infection at admission			
Sepsis-3			
• SOFA increase ≥2	90 (88-92)	28 (26-31)	n/a
• Reduced time window	68 (66-71)	28 (25-31)	78
• SOFA ≥2 (4-day window)	96 (95-97)	27 (25-30)	94
• SOFA ≥2 at onset of infection	89 (87-91)	28 (25-31)	88
Septic shock-3			
• SOFA increase ≥2	38 (35-41)	41 (36-46)	n/a
• Reduced time window	30 (27-32)	41 (36-46)	92
• SOFA ≥2 (4-day window)	39 (36-42)	41 (36-45)	99
• SOFA ≥2 at onset of infection	37 (34-40)	41 (37-46)	97
• Shock-3 ignoring lactate	42 (40-45)	41 (36-45)	95
ICU-acquired infection			
Sepsis-3			
• SOFA increase ≥2	52 (48-56)	40 (34-46)	n/a
• Reduced time window	22 (19-26)	42 (33-51)	70
• SOFA ≥2 (4-day window)	99 (97-100)	35 (31-39)**	53
• SOFA ≥2 at onset of infection	96 (94-98)	35 (31-40)**	53
Septic-shock-3			
• SOFA increase ≥2	18 (15-21)	57 (47-67)	n/a
• Reduced time window	7 (5-9)	65 (48-79)*	89
• SOFA ≥2 (4-day window)	27 (23-31)	54 (45-62)	91
• SOFA ≥2 at onset of infection	26 (22-30)	53 (44-61)	90
• Shock-3 ignoring lactate	28 (24-32)	50 (42-58)*	90

SOFA=Sequential Organ Failure Assessment. Incidences are the apparent incidences of the various sepsis-3 variations.

[a] Percentage agreement indicates the agreement of the incidence with the incidence of the core definition (≥2 increase in SOFA score) of sepsis-3. * p-value <0.05 ** p-value <0.001.

Discussion

We assessed the incidence, mortality, and robustness of the sepsis-3 definitions in a large prospectively monitored cohort of ICU patients. We found that virtually all patients with a suspected infection met clinical criteria for organ failure and, as such, the sepsis-3 criteria did not have discriminative power in our setting. Furthermore, minor variations in the precise interpretation of the criteria required to meet the sepsis-3 definitions considerably impacted the apparent incidences of both sepsis and septic shock, while mortality remained comparable among the variations.

An anticipated advantage of the sepsis-3 definitions is that they may increase the comparability of sepsis incidence and related mortality among studies. Organ failure is explicitly defined by means of the SOFA score, possibly reducing subjective interpretation. Still, studies published to date have used many subtle variations on the original definition. For example, the original publication suggested to define organ failure as an acute change in the SOFA score of ≥2 points as a consequence of infection [14]. Subsequent validation studies, however, have largely disregarded this requirement of an acute SOFA increase. Instead they used an absolute SOFA score of ≥2 points, applied different time-windows, and used different ways of taking chronic comorbidities into account [4-10, 13]. By applying similar (minor) variations to our data we explored the robustness of the criteria and observed considerable variations in the apparent incidences of sepsis-3. Similar variations in incidence of sepsis-3 and septic shock-3 are likely to occur in other studies, hence affecting the comparability of study results. Standardization of the operationalization of sepsis-3 criteria is therefore paramount to improve generalizability of studies.

One of the most used and straightforward methods of defining organ failure for sepsis diagnosis is the use of an absolute SOFA score, thereby disregarding any pre-existent organ failure. And yet, several problems might arise using this approach. First, almost all ICU patients fulfill these criteria, indicating that the criteria have no discriminatory power in ICU settings. Second, an absolute SOFA score disregards the etiology of organ failure. Organ failure might have been present already before infection (e.g. due to non-infectious diseases or pre-existent co-morbidities) and is therefore not caused by the infection itself. To illustrate, in the current study up to 33% of the patients who developed sepsis-3 actually did not have an infection in a post-hoc adjudication. It therefore remains essential to differentiate between infectious and non-infectious causes for organ failure. We find that future efforts should also be directed to improve (risk) stratification of septic patients, and enrich classification by inclusion of additional variables, such as type of organ failure, number of different organ dysfunctions, site of infection and possibly biomarkers [26].

Our study has some limitations. First, organ failure data were often missing before ICU admission, which was also noticed in the original assessment of sepsis-3 [13]. Second, we based our severe sepsis and septic shock definitions on consensus literature. Nevertheless, the exact

application of the definitions in our study might be different from others. Of note, some of the described restraints of the sepsis-3 criteria also apply to previous sepsis definitions.

Conclusions

Virtually all patients who have suspected infection upon presentation to the ICU meet sepsis-3 criteria, making this definition less suitable for risk stratification in this setting. Furthermore, caution should be taken when using the sepsis-3 definitions to report incidences and related outcomes of sepsis, as they are very sensitive to minor variations in timing and interpretation of organ failure criteria. These criteria should therefore be specified in great detail, and applied very consistently, in all future publications on the topic.

Notes

Ethics approval

The institutional review board approved an opt-out consent procedure (protocol number 10-056C).

Funding

This work was supported by the Center for Translational Molecular Medicine (CTMM) [grant number 04I-201].

Potential conflicts of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest with the subject matter.

Acknowledgements

We thank the MARS consortium, including the participating ICUs and research physicians of the two medical centers for their help in data acquisition.

Members of the MARS Consortium also include the following persons: Academic Medical Center, University of Amsterdam (NL): Friso M. de Beer, MD; Lieuwe D. J. Bos, PhD; Gerie J. Glas, MD; Arie J. Hoogendijk, PhD; Roosmarijn T. M. van Hooijdonk, MD, PhD; Janneke Horn MD, PhD; Mischa A. Huson, MD, PhD; Laura R. A. Schouten, MD; Brendon Scicluna, PhD; Marcus J. Schultz, MD, PhD; Marleen Straat, MD; Lonneke A. van Vught, MD, PhD; Luuk Wieske, MD, PhD; Maryse A. Wiewel, MD, PhD; Esther Witteveen, MD. University Medical Center Utrecht (NL): Peter M.C. Klein Klouwenberg, MD, PharmD, PhD; Jos F. Frencken, MD; Maria E. Koster-Brouwer, MSc; Diana M. Verboom, MD; Meri R.J. Varkila MD.

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Supplementary material

Table S1. Missing data

Variable	Pre-ICU days	Days on ICU
Number of days	1100	4057
All SOFA components missing	2.5%	0.0%
5 SOFA components missing	14.3%	0.9%
2-4 SOFA components missing	71.6%	0.1%
1 SOFA component missing	10.3%	0.1%
No SOFA components missing	1.5%	98.8%
Missing data by SOFA score component[a]		
Central nervous system	81.4%	0.1%
Circulatory	13.4%	1.0%
Renal	24.8%	0.9%
Respiratory	58.6%	1.1%
Liver	77.6%	1.1%
Coagulation	47.2%	1.0%
Missing data of other variables		
Serum lactate	81.3%	45.6%

ICU=intensive care unit, SOFA=Sequential Organ Failure Assessment.

[a] SOFA components were assumed to be normal when missing, except for renal scores. The renal SOFA score was assumed to be 3 in case of dialysis dependency and chronic renal insufficiency (creatinine > 170 mmol/L).

Table S2. Incidence, organ failure, and mortality of sepsis-3 and MARS-sepsis

S2a. Incidence

Infection at admission

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	616 (57%)	360 (33%)
	No	32 (3%)	73 (7%)

ICU-acquired infection

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	160 (32%)	100 (20%)
	No	110 (22%)	131 (26%)

S2b. Median SOFA scores at onset of infection

Infection at admission

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	8 (5-10)[a]	4 (3-6)[a]
	No	4 (3-9)	1 (1-3)

ICU-acquired infection

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	9 (7-12)[a]	6 (4-7)[a]
	No	3 (2-8)	5 (3-7)

S2c. Hospital mortality

Infection at admission

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	228 (37%)[b]	49 (14%)[b]
	No	6 (19%)	6 (8%)

ICU-acquired infection

		MARS organ failure	
		Yes	No
Sepsis-3 organ failure	Yes	77 (48%)[b]	26 (26%)[b]
	No	44 (40%)	28 (22%)

ICU=intensive care unit, SOFA=Sequential Organ Failure Assessment Data are presented as frequencies (%) and SOFA score as median (IQR).

[a] Organ failure was significantly lower in patients with sepsis-3 than for patients with both types of organ failure for both infection at admission and ICU-acquired infection ($p<0.0001$).

[b] Hospital mortality of patients with organ failure according to sepsis-3 was significantly lower than for patients with both types of organ failure for both infection at admission and ICU-acquired infection ($p<0.0001$).

Table S3. Incidence, organ failure, and mortality of septic shock-3 and MARS-shock

S3a. Incidence

Infection at admission

		MARS-shock	
		Yes	No
Septic shock-3	Yes	239 (22%)	168 (16%)
	No	51 (5%)	623 (58%)

ICU-acquired infection

		MARS-shock	
		Yes	No
Septic shock-3	Yes	46 (9%)	43 (9%)
	No	50 (10%)	362 (72%)

S3b. Median SOFA scores at onset of infection

Infection at admission

		MARS-shock	
		Yes	No
Septic shock-3	Yes	10 (7-12)[a]	8 (5-10)[a]
	No	9 (6-11)	4 (2-6)

ICU-acquired infection

		MARS-shock	
		Yes	No
Septic shock-3	Yes	12 (9-15)[a]	9 (7-12)[a]
	No	11 (8-13)	6 (4-8)

S3c. Hospital mortality

Infection at admission

		MARS-shock	
		Yes	No
Septic shock-3	Yes	128 (54%)[b]	38 (23%)[b]
	No	18 (35%)	105 (17%)

ICU-acquired infection

		MARS-shock	
		Yes	No
Septic shock-3	Yes	33 (72%)[b]	18 (42%)[b]
	No	33 (66%)	91 (25%)

ICU=intensive care unit, SOFA=Sequential Organ Failure Assessment Data are presented as frequencies (%) and SOFA score as median (IQR).

[a] Organ failure was significantly lower in patients with septic shock-3 than for patients with both types of septic shock for both infection at admission and ICU-acquired infection ($p<0.01$).

[b] Hospital mortality of patients with septic shock according to sepsis-3 was significantly lower than for patients with both types of septic shock for both infection at admission and ICU-acquired infection ($p<0.01$).

Validation of a novel molecular host response assay to diagnose infection in hospitalized patients admitted to the ICU with acute respiratory failure

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Published in Critical Care Medicine (2018)

3

Abstract

Objective

Discrimination between infectious and non-infectious causes of acute respiratory failure (ARF) is difficult in patients admitted to the intensive care unit (ICU) after a period of hospitalization. Using a novel biomarker test (SeptiCyte LAB) we aimed to distinguish between infection and inflammation in this population.

Methods

This was a nested cohort study in two tertiary mixed ICUs in the Netherlands. We included hospitalized patients with ARF requiring mechanical ventilation upon ICU admission from 2011 to 2013. Patients having an established infection diagnosis or an evidently non-infectious reason for intubation were excluded. Blood samples were collected upon ICU admission. Test results were categorized into four probability bands (higher bands indicating higher infection probability) and compared with the infection plausibility as rated by post-hoc assessment using strict definitions. Of 467 included patients, 373 (80%) were treated for a suspected infection at admission. Infection plausibility was classified as ruled-out, undetermined, or confirmed in 135 (29%), 135 (29%), and 197 (42%) patients, respectively. Test results correlated with infection plausibility (Spearman's rho 0.332; $p < 0.001$). After exclusion of undetermined cases, positive predictive values were 29%, 54%, and 76% for probability bands 2, 3, and 4, respectively, whereas the negative predictive value for band 1 was 76%. Diagnostic discrimination of SeptiCyte LAB and CRP was similar ($p=0.919$).

Conclusion

Among hospitalized patients admitted to the ICU with clinical uncertainty regarding the etiology of ARF, the diagnostic value of SeptiCyte LAB was limited.

Introduction

Numerous biomarkers have been evaluated for diagnostic utility in distinguishing infection from sterile inflammation in critically ill patients, including C-reactive protein (CRP), procalcitonin, and several coagulation markers [1, 2]. Despite the clear association of these biomarkers with the presence of systemic inflammation, most did not diagnose or rule-out infection with sufficient rigor [1-4]. Distinct protein biomarkers likely provide an (over)simplified representation of the host immune response to infection [2, 5], which is very complex yet largely similar to that following major surgery, trauma, and various other diseases triggering systemic inflammation [6]. As a result, the use of single biomarkers may be predestined to yield only limited diagnostic value [2, 5].

Recently, a novel diagnostic test (SeptiCyte LAB, Immunexpress, Seatle, WA) was developed which aims to provide a probability of infection based on the expression of a specific genomic fingerprint consisting of CEACAM4 (carcinoembryonic antigen-related cell adhesion molecule 4), LAMP1 (lysosomal-associated membrane protein 1), PLA2G7 (phospholipase A2 group VII), and PLAC8 (placenta-specific 8-gene protein) [7]. The simultaneous analysis of RNA transcription by these four genes in peripheral blood potentially utilizes information that is contained in various unrelated pathways of the host response at the transcriptome level. This new technology was recently approved by the American Food and Drug Administration. In two technical validation studies SeptiCyte LAB was highly specific for infection in selected groups of both adult and pediatric patients, including some subjects for whom presence or absence of infection was already self-evident at the time of testing [7, 8]. As a result, the precise clinical utility of the test for discriminating infectious and non-infectious causes of inflammation in the ICU remains unknown.

Patients for whom a diagnostic biomarker for infection is particularly relevant are those admitted to the intensive care unit (ICU) with acute respiratory failure (ARF) after a previous stay in hospital wards. They frequently suffer from prolonged ICU stays and high mortality [9], yet dyspnea in these patients is a very non-specific symptom and its differential diagnosis is thus extensive, including congestive heart failure, pleural effusion, atelectasis, pulmonary embolus, acute respiratory distress syndrome, and —virtually always— infection. Early confirmation of infection allows timely initiation of antimicrobial therapy, whereas early rejection might prompt a comprehensive diagnostic work-up for non-infectious causes of respiratory distress. Therefore, we aimed to determine the diagnostic and prognostic value of SeptiCyte LAB in hospitalized patients admitted to the ICU with ARF.

Methods

Study design

For this nested cohort analysis, we selected patients who were enrolled in the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project, a prospective observational cohort study in the tertiary mixed ICUs of the University Medical Center Utrecht and the Academic Medical Center Amsterdam in the Netherlands [10]. Ethical approval for the study was provided by the Medical Ethics Committees of both hospitals, and an opt-out procedure to obtain consent from eligible patients was in place (protocol number 10-056).

Patients

Patients were included if they had been admitted to the ICU between January 2011 and December 2013 with ARF (evidenced by a need for mechanical ventilation within 24 hours of presentation) following prior hospital stay (on a general ward, coronary care unit, or medium care unit) of at least 48 hours. Furthermore, all patients had to have an early warning score >5 (a clinical screening tool based on 6 cardinal vital signs [11] and/or presence of ≥2 systemic inflammatory response syndrome criteria at ICU admission. Patients were excluded if they had another pertinent need for intubation and ARF was evidently not caused by an infection (including, but not limited to, chronic respiratory insufficiency, primary cardiac arrest, and airway obstruction) or if a diagnosis of infection already had been established at the time of ICU admission (i.e., confirmed infections for which antimicrobial therapy had been started >2 days prior to ICU admission) as the SeptiCyte LAB test was considered to offer little added value in clinical decision making in such patients.

Reference diagnosis

Infectious events were registered upon each occasion that antimicrobial therapy was initiated, and subsequently adjudicated using detailed definitions derived from Center of Disease Control (CDC) and International Sepsis Forum (ISF) Consensus Conference criteria [10, 12, 13]. To this end, dedicated physicians not involved in patient care categorized infection plausibility as none, possible, probable, or definite, based on a comprehensive post-hoc review of available clinical, microbiological, and radiological data. Daily discussions between observers and the attending team served to reach consensus in case of any uncertainties. For use as reference test in the current analysis, we reclassified all plausibility ratings into the following categories: infection ruled-out (patients with a post-hoc likelihood rated none, or patients who were not treated for infection), infection undetermined (patients with possible infection), or infection confirmed (patients with a post-hoc likelihood rated probable or definite).

SeptiCyte LAB

Blood specimens were collected within 24 hours of ICU admission in all patients using 2.5 mL PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Samples were kept for a period of 2 to 72 hours at room temperature, and subsequently stored at -20°C (for a maximum of 1 month) and finally stocked at -80°C until analysis. RNA was then isolated on a QIAcube workstation using a PAXgene blood miRNA kit (Qiagen, Venlo, the Netherlands). The concentration of total RNA per sample was assessed by Nanodrop spectrophotometry (Agilent, Amstelveen, the Netherlands) and had to be between 2 and 50 ng/uL to be eligible for further analysis.

SeptiCyte LAB tests were performed in 96-well microtiter amplification plates on an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific, Carlsbad, CA). During each amplification run, 3 control samples were included. PCR results were initially quantified using ABI Sequence Detection Software version 1.4. The SeptiCyte LAB score was then calculated from the threshold cycle numbers (Ct-values) measured per gene as follows: *SeptiCyte LAB score* = $(Ct_{PLA2G7} + Ct_{CEACAM4}) - (Ct_{PLAC8} + Ct_{LAMP1})$. The resulting score was finally classified into 4 probability bands reflecting an increasing sepsis likelihood according to the manufacturer's specification; scores ≤3.1 represented band 1 and were categorized as 'sepsis unlikely', whereas scores 3.1-4, 4-6, and >6 represented bands 2, 3, and 4, respectively, and were categorized as 'sepsis likely'.

Statistical analysis

We performed mainly descriptive analyses to determine the diagnostic value of SeptiCyte LAB as formal assessment of test characteristics was precluded due to the large proportion of patients in whom infection status remained inconclusive. However, some diagnostic measures were calculated, not taking into account these latter patients. Furthermore, we calculated the area under the receiver-operating curve (AUROC) to compare the performance of SeptiCyte LAB with CRP, a biomarker commonly used in clinical practice. CRP was not measured at ICU admission in 115 (25%) cases, these values were replaced with estimates derived from multiple imputation (details can be found in Appendix I) [14, 15].

To assess the potential utility of SeptiCyte LAB for risk stratification of patients upon ICU admission, we studied the relation of test results with case fatality (after correction for disease severity). We constructed two prognostic models, using the APACHE IV score either alone or combined with the SeptiCyte LAB score to predict 30-day mortality. We used generalized linear mixed models with a binomial distribution and logit link, and added a random intercept to accommodate possible outcome differences between participating hospitals. Model evaluation was based on Akaike's information criterion (AIC) and the AUROC.

Differences between subgroups of patients were tested using the Wilcoxon Rank-Sum test or the Chi-square test, as appropriate. To test differences in patient characteristics associated with increasing SeptiCyte LAB scores, p-values for trend were calculated using the Cochran-

Armitage trend test for dichotomous variables, or one-way ANOVA for continuous variables. If ANOVA suggested a significant association, linear regression with the SeptiCyte probability band as group determinant was performed. All analyses were performed in SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC) and R Studio (R Studio Team 2015, Boston, MA).

Results

Patients

Among 1399 hospitalized patients admitted to the ICU with ARF during the study period, 638 subjects were eligible for inclusion (Figure 1). Blood samples were unavailable in 157 of these, mostly because specimens had been used for prior studies within the MARS-project. Fourteen other patients could not be evaluated due to technical issues during sample preparation or processing, leaving 467 (73%) subjects for final analysis. Compared to included patients, patients without samples for analysis had less congestive heart failure, more chronic cardiovascular insufficiency, and higher APACHE IV scores and ICU mortality (Table S1).

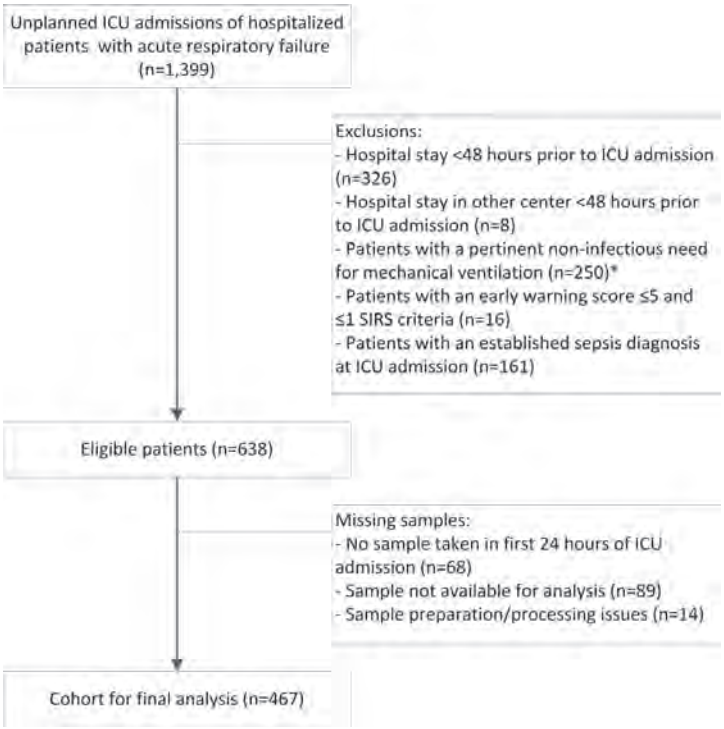

Figure 1. Flowchart of patient inclusions. ICU: intensive care unit. SIRS: systemic inflammatory response syndrome. *Including, but not limited to, patients with chronic respiratory insufficiency (n=107), in-hospital cardiac arrest (n=103), and airway obstruction (n=18).

Presence of infection

Because of presumed infection, 359 (77%) of 467 included patients received antimicrobial therapy on day 1 in the ICU, and another 14 subjects (3%) started treatment on day 2. Among these, the post-hoc plausibility of infection was rated none in 41 (11%) cases. An additional 94 patients did not receive antimicrobial therapy during the first 2 days in the ICU, yielding a total of 135 subjects in whom infection was considered ruled-out. The remaining 332 patients were classified as undetermined (n=135) or infection confirmed (n=197). Hence, in the total study population, the pre-test probability of infection was 197/467 (42%). Of the patients in whom infection was undetermined or confirmed, the most commonly suspected sites of infection were respiratory tract infections (n=228), abdominal infections (n=52), and bloodstream infections (n=36).

SeptiCyte LAB results

In patients in whom infection was ruled-out (n=135), undetermined (n=135), or confirmed (n=197), median (IQR) SeptiCyte LAB scores were 4.8 (3.7-6.1), 5.3 (3.9-6.4), and 6.5 (5.2-8.1), respectively (Figure 2). Formal analysis yielded a significant correlation between test scores and the probability of infection (Spearman's rho 0.320; p<0.001). However, Ct-values for all four individual genes were largely overlapping (Figure S1).

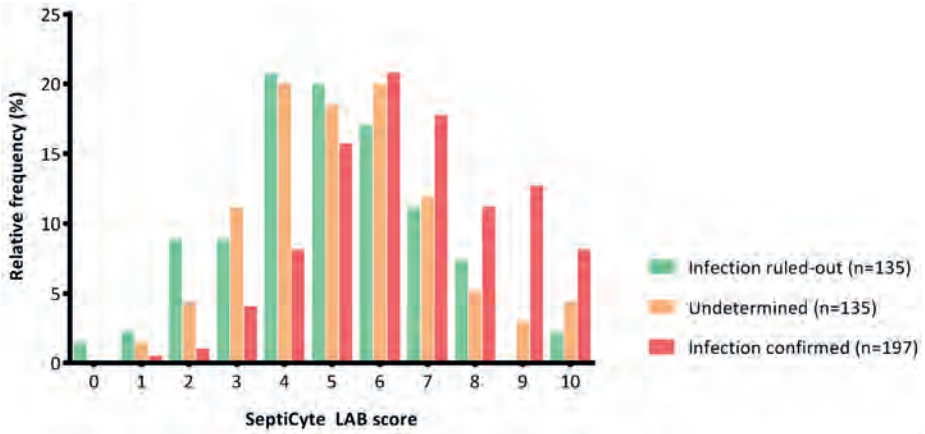

Figure 2. Distribution of SeptiCyte LAB scores by reference diagnosis. A higher SeptiCyte LAB score indicates a higher likelihood of sepsis. A score ≤3.1 should be interpreted as sepsis unlikely according to the manufacturer's specification.

Table 1 shows the probability bands for infection at admission according to the SeptiCyte LAB score in relation to the reference diagnosis. Dichotomizing test results at band ≥2 (as per manufacturer specification [7]), concordance was observed in 189 (96%) of 197 patients with confirmed infection, yet in only 25 (18%) of 135 patients in whom infection was eventually ruled-out. Using SeptiCyte LAB at this cut-off to select patients for antimicrobial treatment

would have led to inappropriate prescriptions in 110 cases (of which only 38 were currently treated). After exclusion of undetermined cases, the positive predictive values for probability bands 2, 3, and 4 were 29%, 54%, and 76%, respectively, and the negative predictive value for probability band 1 was 76%.

Table 1. SeptiCyte LAB result versus reference diagnosis

Probability band[a]	Infection ruled-out (n=135)	Undetermined (n=135)	Infection confirmed (n=197)
Band 1: Sepsis unlikely (n=52)	25 (19)	19 (14)	8 (4)
Band 2: Sepsis likely (n=39)	17 (13)	15 (11)	7 (4)
Band 3: Sepsis likely (n=181)	57 (42)	57 (42)	67 (34)
Band 4: Sepsis likely (n=195)	36 (27)	44 (33)	115 (58)

Data presented as n (%). All percentages are column percentages. [a] Higher SeptiCyte probability bands indicate increased likelihood of sepsis.

Table 2. Characteristics of patients according to test result (discrepancy analysis)

Patient and ICU characteristics	Infection ruled-out (n=135)		Infection confirmed (n=197)	
	True negative	False positive	True positive	False negative*
Patients, N	25	110	189	8
Gender, female	14 (56)	48 (44)	58 (31)	1 (13)
Age, years	60 (48-67)	64 (53-75)	65 (56-73)	72 (64-77)
Surgical reason for admission	1 (4)	9 (8)	9 (5)	1 (13)
Comorbidities				
- Congestive heart failure	3 (12)	10 (9)	10 (5)	0 (0)
- COPD	3 (12)	8 (7)	23 (12)	2 (25)
- Chronic cardiovascular insufficiency	3 (12)	1 (1)	6 (3)	1 (13)
- Diabetes mellitus	4 (16)	24 (22)	36 (19)	2 (25)
Immune deficiency	3 (12)	16 (15)	30 (16)	2 (25)
APACHE IV Score	73 (60-86)	81 (64-100)	84 (70-101)	64 (61-103)
Suspected site of infection				
- Respiratory tract	1 (4)	20 (18)	93 (49)	6 (75)
- Abdominal	0 (0)	3 (3)	40 (21)	1 (13)
- Cardiovascular	1 (4)	3 (3)	24 (13)	1 (13)
- Other/unknown	1 (4)	12 (11)	32 (17)	0 (0)
- No suspicion	22 (88)	72 (65)	0 (0)	0 (0)
Prior ICU admission during hospital stay	5 (20)	57 (52)	92 (49)	6 (75)
ICU length of stay, days	2 (1-8)	4 (2-8)	7 (3-12)	4 (2-8)
ICU mortality	2 (8)	18 (16)	36 (19)	0 (0)

COPD: chronic obstructive pulmonary disease. CHF: congestive heart failure. APAHCE: acute physiology and chronic health evaluation. ICU: intensive care unit. Continuous data are presented as medians (IQR), dichotomous data are presented as n (%). *Case descriptions for these patients are provided in Appendix III.

Discrepancy analysis

We observed 8 discordant cases where the test suggested that infection could be safely ruled-out, whereas infection was confirmed on post-hoc assessment. Review of these false negative cases revealed that these patients were older, had lower severity of illness upon presentation to the ICU, and more frequently had been previously admitted to the ICU than the 189 patients with true positive results (Table 2). Case descriptions for these patients are provided in Appendix II. In-depth analysis of the 110 patients with false positive results revealed that they had similar age, higher severity of illness, more previous ICU admissions, and were more likely to have been clinically suspected of infection than their 25 true negative counterparts. In addition, we compared individual RNA transcripts in discrepant cases. Ct-values differed significantly for the PLA2G7, CEACAM4 and (possibly) PLAC8 genes, but not for LAMP1, when comparing false positive to true negative results (Figure 3A). Similarly, there were significant differences in the median Ct-values of PLAC8 and PLA2G7 when comparing false negative and true positive test results, but not for LAMP1 and CEACAM4 (Figure 3B).

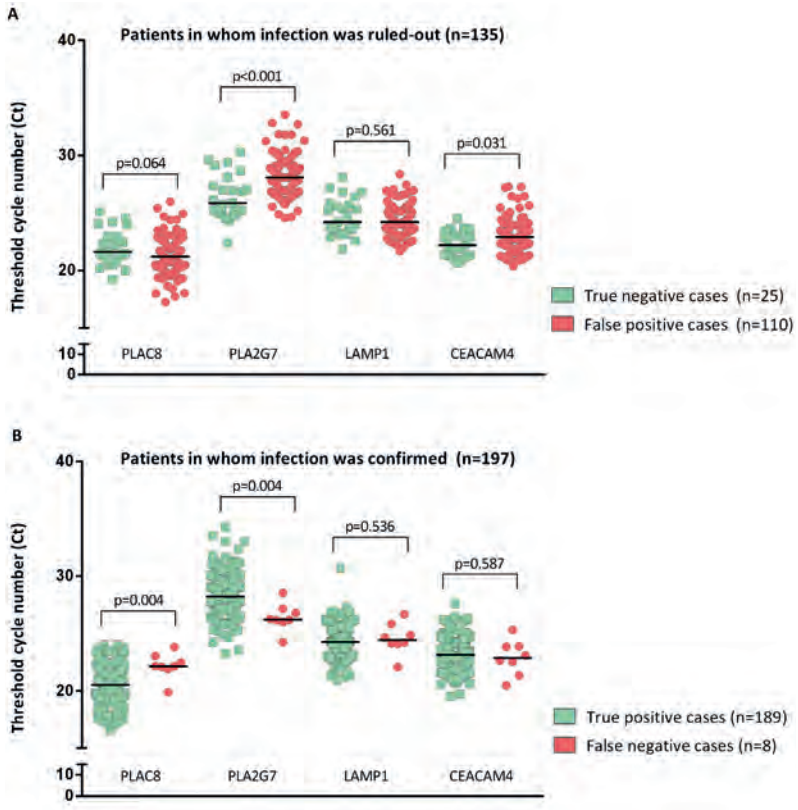

Figure 3. Median Ct-values per gene for non-infectious and infectious cases according to test result. For this analysis, cases in which infection was undetermined (n=135) were not taken into account as it was unknown whether the test classified them correctly.

Comparative diagnostic evaluation

To better assess the clinical utility of SeptiCyte LAB in the ARF population, we compared its diagnostic performance to CRP. In patients in whom infection was ruled-out, undetermined, or confirmed, median (IQR) plasma concentrations of CRP at ICU admission were 67 (22-152), 109 (63-207), and 166 (93-252) mmol/L, respectively. After exclusion of undetermined cases, ROC analysis yielded an AUC of 0.727 (95%CI 0.666-0.788) for CRP versus 0.731 (95%CI 0.677-0.786) for SeptiCyte LAB (mean difference 0.004, 95%CI –0.077-0.086; p=0.919).

Prognostic evaluation

Higher SeptiCyte LAB scores were associated with both greater disease severity upon ICU admission and increased mortality (Table S2). However, a prognostic model that included both APACHE IV and SeptiCyte LAB scores was not superior in predicting 30-day mortality compared to a model using only the APACHE IV score (AUROC 0.737 versus 0.735, p=0.724; AIC 498 versus 497).

Discussion

In this study, we evaluated the clinical utility of SeptiCyte LAB to diagnose infection in patients admitted to the ICU because of ARF after a period of hospitalization. Infectious episodes were correctly identified by the test in 96% of the patients with confirmed infection. However, in patients in whom an infection was refuted, the test yielded a correct result in only 18% of patients. In fact, in this population the test did not offer better diagnostic discrimination than the more commonly used biomarker CRP. In addition, SeptiCyte LAB did not improve prognostication when added to the APACHE IV score.

Previous studies of SeptiCyte LAB reported very high discriminative power for infection (AUCs of 0.88 and 0.99) compared to what we observed [7, 8]. The major difference between those studies and ours concerns the study domain. Early validation studies have mostly used cohorts in which infectious and non-infectious patients could clearly be distinguished on clinical grounds. For instance, one study compared children after cardio-pulmonary bypass surgery to children with severe sepsis [8]. In another preliminary evaluation of SeptiCyte LAB (which included 23 subjects also enrolled in the current study), the test performed better in a cohort of highly selected patients (AUC 0.95; 95%CI 0.91-1.00) than in a cohort representing a more real-life setting (AUC 0.85; 95%CI 0.75-0.95) [7]. Furthermore, an assessment of its diagnostic performance across 39 publicly available datasets yielded highly variable findings, with reported AUCs ranging from 0.24 to 1 for individual datasets [16]. In search of a possible explanation for the lack of discriminative performance of SeptiCyte LAB in certain subgroups (some of which did not represent the intended use population of the test), it was noted that the expression of one of the four genes involved in calculating the SeptiCyte LAB

score (CEACAM4) was down regulated during sepsis in the discovery cohort, but not in other cohorts [16]. In our study, we observed only minimal differences in gene expression between infectious and non-infectious cases for all four genes, including CEACAM4 (Figure S1).

We deliberately focused on a target population in which it was difficult to diagnose infections with certainty. Many patients had significant (acute) comorbidities, had stayed in the hospital for prolonged periods of time prior to ICU admission, and had previously been exposed to antimicrobials for (presumed) infections. To avoid selection, we enrolled consecutive patients. However, 171 of 638 eligible patients were excluded from analysis, mostly because they had a clinically apparent sepsis syndrome (due to confirmed infection) and their samples had already been used for other studies within the MARS-project. These exclusions thus enriched our study cohort with infectious episodes that were more challenging to diagnose. Yet, we believe there is little value in using SeptiCyte LAB (or any other biomarker) in patients with clinically overt infection.

Although the probability of infection was prospectively adjudicated by trained observers based on available post-hoc clinical, radiological, and microbiological findings, some diagnostic misclassification will most likely have occurred [10]. For instance, infectious episodes for which treating physicians did not initiate antimicrobial therapy may have been erroneously classified as infection ruled-out. It is important to stress that, in the presence of an imperfect reference test, the maximal discriminative performance (in terms of AUROC) that can be attained by a diagnostic test will be necessarily lower than 1. Thus, diagnostic misclassification may have reduced the apparent diagnostic utility of SeptiCyte LAB in our cohort. This merely emphasizes the difficulty of performing diagnostic studies in patients with infection, where a gold standard simply does not exist. However, it is unlikely that the lack of a robust reference diagnosis explains the observed differences in discriminative power of the test between our study and previous validation cohorts, nor the equipoise between SeptiCyte LAB and a more common host-response marker such as CRP in classifying infections [7].

Conclusions

In our clinical evaluation of SeptiCyte LAB in patients presenting to the ICU with ARF after prior hospitalization for other acute diseases, the discriminative power of this new biomarker test was lower than previously reported in more selective validation cohorts. As SeptiCyte LAB scores are based on gene expression profiles, test results might vary between specific populations and/or settings. Therefore, more prospective studies are needed to determine the clinical utility of this novel test.

Notes

Funding

Supported, in part, by the Center for Translation Molecular Medicine (http://www.ctmm.nl), project MARS (grant 041-201). We received SeptiCyte LAB kits and technical assistance from Immunexpress. Neither the sponsor nor Immunexpress played a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the article; or the decision to submit the article for publication. The University Medical Center Utrecht received funding from Immunexpress. Furthermore, Immunexpress provided SeptiCyte LAB kits and technical assistance in kind. The Academic Medical Center Amsterdam and the University Medical Center Utrecht received funding from the Center for Translational and Molecular Medicine, project MARS.

Potential conflicts of interest

The authors have disclosed that they do not have any potential conflicts of interest.

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Supplementary material

Table S1. Characteristics of in- and excluded patients

Patient characteristics	Exclusions (patients without sample and/or test result, n=171)	Inclusions (patients with test result, n=467)	P-value
Gender, female	66 (39)	169 (36)	0.577
Age, years	65 (54-73)	65 (54-73)	0.870
Surgical reason for admission	6 (4)	25 (5)	0.337
Comorbidities			
- Congestive heart failure	4 (2)	33 (7)	0.024
- COPD	20 (12)	52 (11)	0.843
- Chronic cardiovascular insufficiency	13 (8)	17 (4)	0.036
- Diabetes mellitus	24 (14)	88 (19)	0.157
Immune deficiency	33 (19)	68 (15)	0.147
APACHE IV Score	87 (70-111)	81 (65-98)	0.010
Admission diagnosis (top 3 categories)			0.098
- Pneumonia	38 (22)	131 (28)	
- Congestive heart failure	13 (8)	34 (7)	
- Atelectasis	12 (7)	32 (7)	
Prior ICU admission during hospital stay	75 (44)	227 (49)	0.287
Intubation	137 (80)	394 (84)	0.203
ICU length of stay, days	4 (2-11)	5 (2-10)	0.104
ICU mortality	42 (25)	74 (16)	0.012

COPD: chronic obstructive pulmonary disease. CHF: congestive heart failure. APACHE: acute physiology and chronic health evaluation. ICU: intensive care unit. Continuous data are presented as medians (IQR), dichotomous data are presented as n (%).

Table S2. Patient characteristics by SeptiCyte LAB result

Patient characteristics	SeptiCyte probability band				P-value for trend
	Band 1 (n=52)	Band 2 (n=39)	Band 3 (n=181)	Band 4 (n=195)	
Gender, female	21 (40)	13 (33)	67 (37)	68 (35)	0.562
Age	67 (54-76)	65 (53-75)	63 (53-73)	65 (55-73)	0.710
Immune deficiency	7 (13)	5 (13)	21 (12)	35 (18)	0.253
APACHE IV score	74 (63-96)	69 (59-89)	80 (64-96)	84 (70-102)	<0.001
Prior ICU admission during hospital stay	17 (33)	22 (56)	97 (54)	91 (47)	0.352
ICU length of stay, days	5 (2-9)	4 (2-8)	6 (3-11)	6 (3-10)	0.306
ICU mortality	4 (8)	6 (15)	24 (13)	40 (21)	0.020
30-day mortality	7 (14)	11 (28)	37 (20)	46 (24)	0.271

Continuous data are presented as medians (IQR), dichotomous data are presented as n (%).

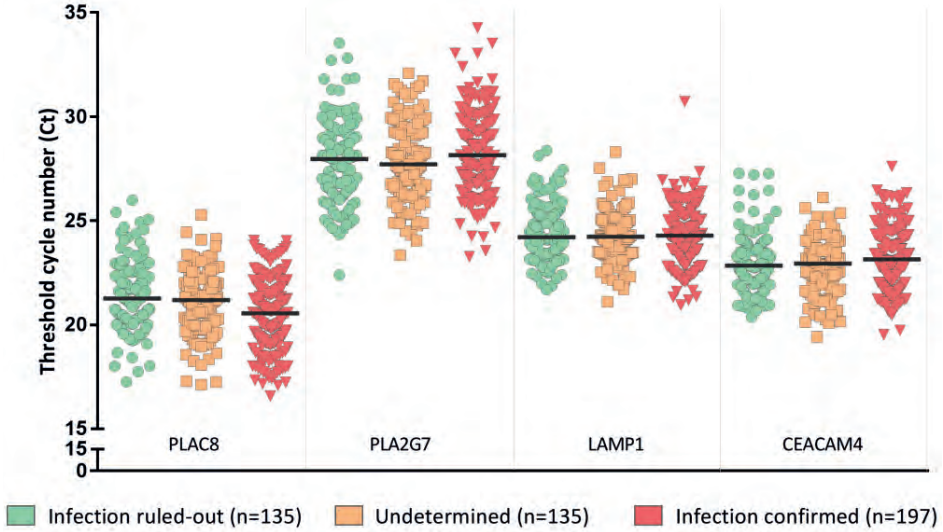

Figure S1. Ct-values per gene in the SeptiCyte LAB test per reference category. The vertical black lines indicate the median Ct-value for that reference category.

Appendix I: Multiple imputation model and handling of imputed data

Multiple imputation model

CRP was not measured at ICU admission in 115 (25%) of 467 cases. Since complete case analysis in this situation may lead to bias [1], we chose to perform multiple imputation. The use of multiple imputed datasets accounts for uncertainty introduced by imputation, providing larger standard errors when the number of missing values increases. To impute CRP values that were missing on the first day in the ICU we used an imputation method based on chained equations (R-package 'mice', version 2.25, 2015) [2-3]. In this we assumed data were missing at random (i.e., that any systematic differences between missing and observed values could be explained by other parameters in our data set) [4]. Variables that were used in the imputation model to predict missing CRP values included: gender, age, race, medical or surgical admission, immune deficiency, Charlson comorbidity index, APACHE IV score, SOFA score at ICU admission, presence of infection at admission, SIRS criteria (fever, tachycardia, tachypnea, and abnormal white blood cell count) at ICU admission, ICU length of stay, and the SeptiCyte LAB score.

If needed, rounding and boundaries of imputed values were used to assure that clinically possible values were replaced for missing values [5]. Considering that 25% of cases had missing values for CRP, we performed 25 imputations with 30 iterations per imputation. This resulted in stable imputations as evidenced by summary statistics and density plots [3, 6-8].

Handling of imputed data

We averaged AUROCs as estimated from the 25 separate datasets in order to arrive at a robust estimate for the discriminative power of CRP. Also, the difference in AUCs was calculated as the average difference between the AUROCs of SeptiCyte and CRP across all imputed datasets. Using Rubin's rules, we calculated the accompanying 95% confidence intervals and test-statistics for these estimations to arrive at correct effect estimates and standard errors [9-10].

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Appendix II: Case vignettes of false negative cases

Patient #1

Patient had been previously admitted to ICU because of bilateral pneumonia. After 2 weeks she was readmitted with respiratory insufficiency mainly due to fluid overload and atelectasis. However, the patient was also suspected of recurrent pneumonia and because of earlier growth of Enterobacter cloacae, she was empirically started on vancomycin and ciprofloxacin. Sputum cultures taken at admission subsequently grew Staphylococcus aureus, after which treatment was switched to flucloxacillin. Blood cultures remained negative.

Patient #2

Patient had been treated in the ICU for mediastinitis following a spontaneous retropharyngeal abscess for 6 weeks. Five days after discontinuation of antimicrobial therapy he was readmitted with tachypnea and fever. CRP 31/93. The differential diagnosis on admission included HAP, pulmonary embolism, recurrent abscess, and empyema. Empirical treatment with ceftriaxone plus metronidazole was initiated. CT-guided percutaneous drainage of evident mediastinal pus collections was performed two days after admission, cultures of which remained negative.

Patient #3

Patient had been admitted to ICU for 5 days following esophagectomy. After 3 months he was readmitted with dyspnea, fever and back pain. CRP 79/193. PCT 2/4. The differential diagnosis on admission included HAP, pulmonary embolism, and late anastomotic leakage. Chest X-ray showed both pulmonary infiltrates and spinal fractures (due to metastatic disease). Blood cultures taken at admission grew Klebsiella pneumonia and Klebsiella oxytoca. The patient was treated with ceftriaxone.

Patient #4

History of DM induced nephropathy, for which continuous ambulatory peritoneal dialysis. Patient had been treated for 3 months with intraperitoneal vancomycin for catheter peritonitis (due to corynebacterium) until 4 months earlier, and had undergone CABG 1 month before ICU admission. He was admitted with shock, respiratory insufficiency and left-sided pleural effusion. CRP 234. The differential diagnosis on admission included pneumosepsis, abdominal sepsis (due to recurring CAPD peritonitis), bowel ischemia, and hematothorax. An explorative laparotomy was performed, but was negative. Sputum cultures taken on the day of admission grew Klebsiealla pneumoniae and Serratia marcescens. The patient recovered following ciprofloxacin treatment.

Patient #5

Patient had been admitted to ICU for 5 days following pancreatectomy. After 3 weeks he was readmitted with hypothermia, lactic acidosis and hypercapnia. CRP 55. The differential diagnosis on admission included anastomotic leakage, abdominal abscess, pleural effusion, and exacerbation of COPD. Laparotomy showed small bowel perforation with an infected pocket in the right upper quadrant, pus cultured from the pocket grew a small amount of Candida albicans. Patient was empirically treated with ciprofloxacin, clindamycin, and anidulafungin.

Patient #6

History of laryngectomy. Patient underwent uncomplicated mandibular resection. Three days later he was admitted to the ICU with hypoxia and (some) fever (38.2). CRP 76/155. PCT 0/0. The diagnosis was evident: massive aspiration pneumonia due to malfunction of his pre-existing (one-way) tracheal-oesophageal speaking valve. He was empirically started on ceftriaxone and therapy was completed before the final results of the sputum cultures, taken on the day of admission, were known. These cultures grew Enterobacter cloacae and Pseudomonas aeruginosa.

Patient #7

Immunocompromised patient following allogenic stem cell transplant for CML. Medical history of recurrent infections, most currently cellulitis of the left lower leg for which he was still receiving flucloxacillin. Presentation to ICU with hematemesis (most likely due to GvHD) and a new pulmonary infiltrate. Patient was treated empirically with ceftazidime, clindamycin and voriconazole. Neither BAL nor blood cultures (which were both performed while patient was receiving antimicrobial treatment) yielded a probable causative pathogen.

Patient #8

Patient had been admitted to ICU for 4 days following acute subdural hematoma (while on anticoagulation) for which surgical decompression had been performed. Ten days later he was readmitted with fever, new-onset atrial fibrillation, renal insufficiency, and respiratory distress. CRP 112/185. The differential diagnosis on admission included aspiration pneumonia (due to difficulty swallowing), wound infection (he had a lesion on the back of his head), secondary meningitis, and decompensated heart failure. Patient was empirically treated with ceftriaxone (for possible pneumonia) and flucloxacillin (for presumed wound infection), but later switched to ciprofloxacin when the sputum and blood cultures taken at admission both grew Serratia marcescens.

A pilot study of a novel molecular host response assay to diagnose infection in patients after high-risk gastro-intestinal surgery

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Published in Journal of Critical Care (2019)

4

Abstract

Purpose

SeptiCyte LAB measures the expression of four host-response RNAs in peripheral blood to distinguish sepsis from sterile inflammation. This study evaluates whether sequential monitoring of this assay has diagnostic utility in patients after esophageal surgery.

Methods

Patients who developed a complication within 30 days following esophageal surgery and a random sample of 100 patients having an uncomplicated course. SeptiCyte LAB scores (ranging 0-10 reflecting increasing likelihood of infection) were compared to post-hoc physician adjudication of infection likelihood.

Results

Among 370 esophagectomy patients, 120 (32%) subjects developed a complication requiring ICU (re)admission, 63 (53%) of whom could be analyzed. Immediate postoperative SeptiCyte LAB scores were highly variable, yet similar for patients having a complicated and uncomplicated postoperative course (median score of 2.4 (IQR 1.6-3.3) versus 2.2 (IQR 1.3-3), respectively). In a direct comparison of patients developing a confirmed infectious (n=34) and non-infectious complication (n=12), addition of SeptiCyte LAB to CRP improved diagnostic discrimination of infectious complications (AUC 0.88 (95%CI 0.77-0.99)) compared to CRP alone (AUC 0.76 (95%CI 0.61-0.91); p=0.04).

Conclusions

Sequential measurement of SeptiCyte LAB may have diagnostic value in the monitoring of surgical patients at high risk of postoperative infection, but its clinical performance in this setting needs to be validated.

Introduction

Major gastro-intestinal surgery, such as esophageal, gastric, and pancreatic interventions, are associated with a high risk of developing postoperative complications [1,2]. Indeed, complication risks can be as high as 33.5%, and affected patients suffer from a significantly increased length of stay and excess mortality [2,3]. Most of the complications are of infectious origin (principally pneumonia and anastomotic leakage), which can readily lead to the development of sepsis in postoperative patients [1–3].

Timely recognition and treatment of and treatment of sepsis may improve outcome [4]. However, sepsis diagnosis is complicated by the almost universal presence of Systemic Inflammatory Response Syndrome (SIRS) in patients who just had gastrointestinal surgery [5]. Currently, sequential measurements of C-reactive protein (CRP) and procalcitonin (PCT) are most commonly used to monitor onset of postoperative infectious complications [6]. Although these biomarkers have adequate negative predictive values (ranging from 91% to 100%), their positive predictive values remain poor [7–11].A variety of alternative biomarkers have thus been proposed for the diagnosis of sepsis, however their diagnostic accuracy is variable across settings and their clinical utility not always evident [12,13].

Whole-blood transcriptomics-based technologies (i.e., measurement of RNA transcripts that are generated during gene expression in leukocytes) can detect rapid change when the host is exposed to infectious stress, and may therefore possibly yield an earlier diagnostic signal of sepsis than traditional protein biomarkers [14–17]. SeptiCyte™ LAB is the first RNA-based host response signature cleared by the US Food and Drug Administration for sepsis diagnosis[18]. It measures the expression of four genes (carcinoembryonic antigen-related cell adhesion molecule 4 (CEACAM4), lysosomal-associated membrane protein 1 (LAMP1), phospholipase A2 group VII (PLA2G7), and placenta-specific 8-gene protein (PLAC8)) in peripheral blood. In several evaluations SeptiCyte LAB discriminated better between critically ill patients with (overt) sepsis and a non-infectious SIRS than PCT [17,19,20]. Although the test performed less favorably in a recent cohort of difficult-to-diagnose cases of (nosocomial) sepsis after prolonged prior hospitalization [21].

We hypothesized that sequential measurement of SeptiCyte LAB could have superior diagnostic performance over established biomarkers in postoperative patients at high risk for infectious complications, as the test characterizes the host response to infection at a relatively early —and thus possibly more specific— stage [22]. To explore this idea further, we performed a pilot study in consecutive esophagectomy patients in order to 1) determine a normal range for SeptiCyte LAB measured directly following esophageal surgery, 2) evaluate temporal changes in SeptiCyte LAB scores as complications ensue in the postoperative setting, and 3) compare the ability of SeptiCyte LAB to discriminate infectious from non-infectious complications in postoperative patients to that of a more commonly used biomarker, CRP.

Methods

Study design

We performed a case-control analysis that was nested in the MARS (Molecular Diagnosis and Risk Stratification of Sepsis) cohort, which enrolled subjects in two Dutch university hospitals from 2011 to 2013. From this cohort we selected consecutive patients who had undergone elective esophageal resection. Ethical approval for the study was provided by the Medical Ethics Committees of both participating hospitals, and an opt-out procedure to obtain consent from patients was in place (protocol number 10-056). Blood samples for RNA analysis were collected within 24 hours of surgery and whenever a complication occurred in the ICU during the first 30 postoperative days. This complication could be either a (suspected) infection, acute kidney injury (AKI), acute respiratory distress syndrome (ARDS), acute myocardial infarction (AMI), or readmission to the ICU for any (other) reason. For the present study, we selected cases having ≥1 postoperative complication and for whom (at least) a single paired RNA sample was available. In addition, we randomly selected 100 control subjects having an uncomplicated postoperative course after esophagectomy in order to establish (a range of) normal SeptiCyte LAB scores following major surgery.

Samples were collected in 2.5 mL PAXgene blood RNA tubes and processed in accordance with predefined acceptance criteria as set by the manufacturer of the assay (Immunexpress, Seattle, WA) [18].Tests were performed in 96-well microtiter amplification plates on an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific, Carlsbad, CA), yielding a threshold cycle number (Ct-value) per individual gene. A score was then calculated as $(Ct_{PLA2G7} + Ct_{CEACAM4}) - (Ct_{PLAC8} + Ct_{LAMP1})$. This 'SeptiScore' ranges from 0 to 10, and may be categorized into 4 probability bands according to the manufacturer's specification [18]. SeptiScores ≤3.0 (band 1) indicate that sepsis is unlikely, whereas scores 3.1—4.4 (band 2), 4.5—5.9 (band 3), and >6 (band 4) represent increasing sepsis likelihoods.

Reference test for infection

Suspected infectious events were recorded prospectively upon each occasion that antimicrobial therapy was initiated by the clinician. All patients treated for a suspected infectious event in the ICU also met SIRS criteria and had a Sequential Organ Failure Assessment (SOFA) score ≥2, thus fulfilling current Sepsis-3 definitions [23,24]. The likelihood of infection was subsequently classified as none, possible, probable, or definite based on daily discussions with the attending team as well as a post-hoc review of all available clinical, microbiological, and radiological data collected during ICU stay by trained physicians according to predefined definitions [25]. This reference diagnosis was established without knowledge of SeptiCyte LAB results, yet observers were not blinded to CRP. However, CRP in and of itself could not lead to a diagnosis of infection in the absence of other clinical and inflammatory symptoms.

For use as a reference test in the current study, all observed complications were reclassified as infection ruled-out (patients with a post-hoc likelihood rated none, or patients who were never suspected of an infection), infection undetermined (patients with possible infection), or infection confirmed (patients with a post-hoc likelihood rated probable or definite). In case of multiple concurrent events, SeptiCyte LAB test results were related to the complication that occurred nearest in time to the moment the sample was taken.

Statistical analysis

Immediate postoperative SeptiScores were analyzed to determine a normal range following major surgery, both in patients who would later develop a complication as well as in the 100 control subjects. Subsequently, in esophagectomy patients having a complicated postoperative course only, we performed within-patient pairwise comparisons of scores measured in samples obtained directly after surgery and at complication onset. To assess the diagnostic potential of sequential SeptiCyte LAB measurement, we focused this analysis on differences between subjects having non-infectious, undetermined and confirmed infectious complications. In addition, we compared SeptiScores to CRP concentrations measured in plasma obtained at the same time point. To this end, we standardized differences between the post-operative moment and the moment of complication onset by calculating Z-scores. We compared discriminative ability of SeptiCyte LAB and CRP using receiver operating characteristic (ROC) curves. For this latter analysis we excluded subjects having an undetermined infectious state according to physician adjudication.

Differences in categorical and continuous variables between groups were assessed using Chi-square, Wilcoxon signed rank or Mann-Whitney U tests, as appropriate. All analyses were performed in SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC) and R Studio (R Studio Team (2015), Boston, MA).

Results

During the study period, a total of 370 patients were admitted to the ICU after elective esophagectomy, of whom 120 (34%) developed a complication resulting in prolonged ICU stay or readmission to the ICU within 30 days. Among these, 74 (62%) subjects had immediate postoperative PAXgene blood samples available for analysis, and 63 (53%) also had a sample taken at complication onset (figure 1). Patients without a postoperative sample (n=46) had a shorter ICU stay (9, IQR 2-16 versus 12, IQR 7-24, p=0.04), less ICU readmissions (54% versus 97%, p<0.001), and were treated for infection less frequently (70% versus 92%, p<0.05) than patients with an available sample. However, in-hospital mortality was similar between the groups (13% versus 16%, p=0.79).

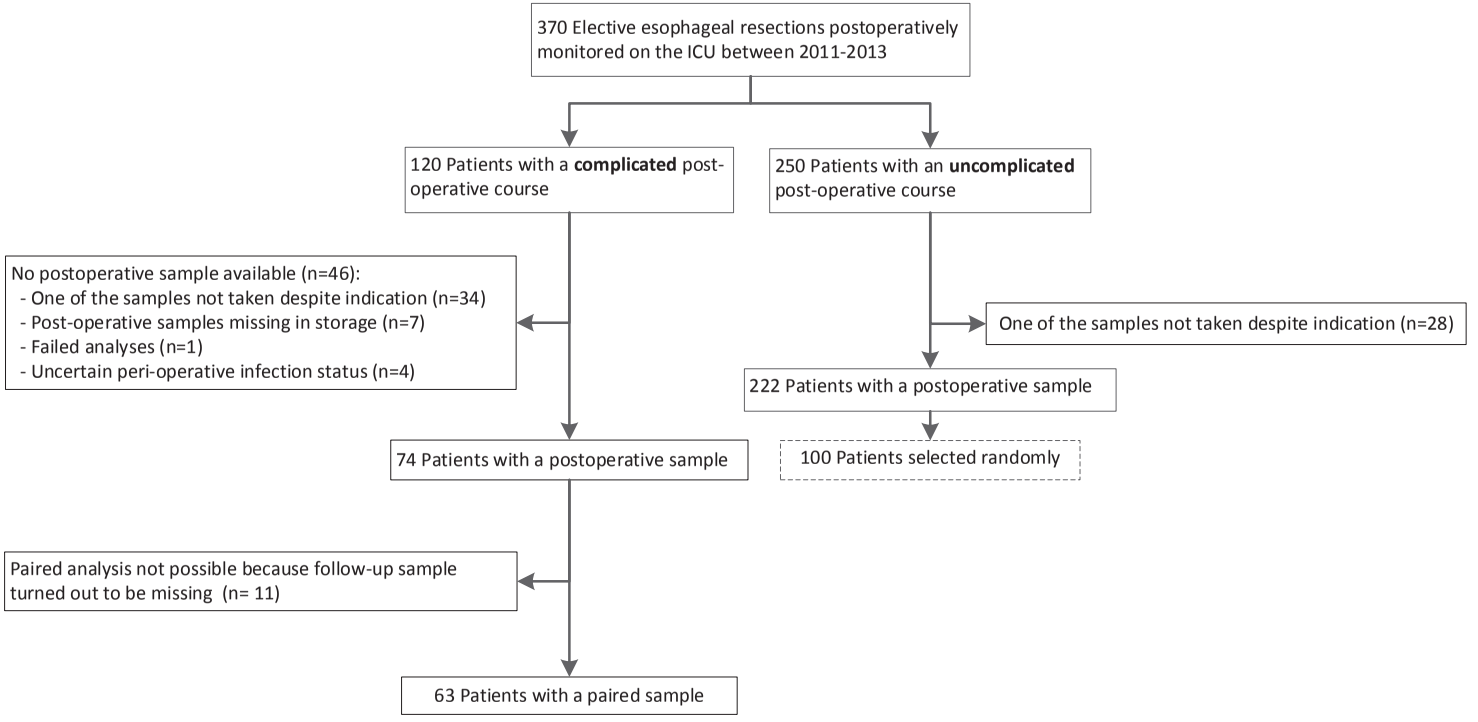

Figure 1. Flowchart of patient inclusion. *ICU;* Intensive Care Unit.

Immediate postoperative PAXgene samples were additionally analyzed in a random subsample (n=100) of the 250 remaining esophagectomy patients who had an uncomplicated postoperative course. These patients developed SIRS less frequently, required less vasopressors, and exhibited a trend towards lower APACHE scores and CRP levels compared to patients in the complicated cohort (Table 1).

Table 1. Characteristics of 174 esophagectomy patients stratified by their postoperative clinical course

Variable	No complication (N=100)	≥1 complication(s) (N=74)	P-value
Age	64 (57-71)	65 (59-70)	0.85
Male gender	77 (77%)	48 (65%)	0.08
Charlson Comorbidity Index	2 (2-2)	2 (2-2)	0.25
Malignancy	90 (90%)	69 (93%)	0.45
APACHE IV Score	45 (38-55)	47 (41-58)	0.14
≥ 2 SIRS criteria (postop day 1)	26 (26%)	40 (54%)	<0.001
Vasopressor use (postop day 1)	50 (50%)	47 (64%)	0.24
CRP (postop day 1)	46 (3-92)	70 (6-108)	0.050
Time to complication onset (days)	NA	3 (2-6)	NA
ICU stay (days)	0.9 (0.8-1.0)	6.6 (3.7-17.2)	<0.001
Hospital stay (days)	11 (8-14)	28 (20-53)	<.001
In-hospital mortality	0 (0%)	12 (16%)	<.001
1-year mortality	3 (3%)	15 (20%)	<.001

APACHE, Acute Physiology and Chronic Health Evaluation; *COPD*, Chronic Obstructive Pulmonary Disease; *CRP,* C-reactive protein; *ICU*, Intensive Care Unit; *SIRS,* Systemic Inflammatory Response Syndrome. Continuous data presented as median and IQR and dichotomous data as n (%).

Among the 63 patients with postoperative complications who were included in the pairwise analyses of SeptiCyte LAB, 34 (54%) subjects had a confirmed infection, 17 (27%) an undetermined infectious state, and 12 (19%) a non-infectious complication (i.e., 5 were empirically treated with antibiotics but classified as having no infection in retrospect, whereas 7 were never suspected of infection). Frequently observed infections included intrathoracic sources (34%; most commonly mediastinitis or pleural empyema due to anastomotic leakage) and pneumonia (24%) (Table 2). Of note, multiple complications could coexist.

SeptiScore distribution in the immediate postoperative setting

Among the total number of 174 analyzed patients, immediate postoperative SeptiScores were highly variable (range 0-10), with a median of 2.3 (IQR 1.4-3.1). Overall, 45 (26%) samples corresponded to probability band 2 or higher, which —in case sepsis were to be clinically suspected— would have incorrectly resulted in a "sepsis-likely" label according to the manufacturer's specification (Table S1). Median SeptiScores of patients having an uncomplicated postoperative course tended to be slightly lower than those of patients who would later develop a complication (2.2 (IQR 1.3-3) versus 2.4 (IQR 1.6-3. 3)), although this difference did not reach statistical significance (p= 0.14).

Table 2. Clinical events among 63 esophagectomy patients with ≥1 postoperative complication

Complications	Non-infectious (n=12)	Undetermined (n=17)	Confirmed infection (n=34)
Total number of events	18	39	97
(Presumed) infectious source(s)			
- intrathoracic[a]	0 (0%)	6 (15%)	24 (25%)
- pulmonary	4 (22%)[b]	10 (26%)	11 (11%)
- abdominal	1 (6%)[b]	2 (5%)	7 (7%)
- other	0 (0%)	0 (0%)	2 (2%)
Other (non-infectious) complication(s)			
- readmission to ICU	10 (56%)	16 (41%)	33 (34%)
- acute respiratory distress syndrome	1 (6%)	3 (8%)	13 (13%)
- acute kidney injury	2 (11%)	1 (3%)	7 (7%)
- acute myocardial infarction	0 (0%)	1 (3%)	0 (0%)

[a] Intrathoracic infections include mainly cases of lung emypema and mediastinitis

[b] Initially suspected focus of infection among patients in whom infection likelihood was later classified as 'none'

ICU, Intensive Care Unit. Multiple complications could occur at the same time.

Temporal changes in SeptiScore

Figure 2 shows SeptiScores as measured immediately after surgery and at the time of complication onset for the 63 patients in whom paired samples were available. Median lag time between surgery and the development of a complication was 5 (IQR 3-9) days. However, it should be noted that 8 (14%) of the repeat samples were already taken within 2 days of surgery. SeptiScores increased in all patients who developed a complicated disease course after surgery, but this rise was more pronounced in those developing infection versus another complication (median score differences 2.1 (IQR 0.4- 3.6), 4 (IQR 2.5- 5), and 4.7 (IQR 4.1- 5.8) for subjects having a non-infectious, undetermined, and confirmed infectious event, respectively; p<0.0001 (Table S2)).

Discriminative ability of SeptiCyte LAB

Standardized differences (expressed as Z-scores) between samples collected immediately after surgery and at complication onset revealed a greater increase in patients with confirmed infections than in other patients for both SeptiCyte LAB and CRP (figure 3). However, among the 34 patients with confirmed infection, the observed standardized increase in SeptiScore was more pronounced than that in CRP, although this difference did not reach statistical significance (median Z-score 0.28 versus 0.08, p=0.08).

In a direct comparison of patients developing a confirmed infectious (n=34) and definite non-infectious complication (n=12), ROC analysis yielded an AUC of 0.87 (95%CI 0.76-0.98) for SeptiCyte LAB, compared to an AUC of 0.76 (95%CI 0.61-0.91) for CRP (p=0.14). Adding SeptiCyte LAB to CRP resulted in improved diagnostic discrimination (AUC 0.88 (95%CI 0.77-0.99), p=0.04).

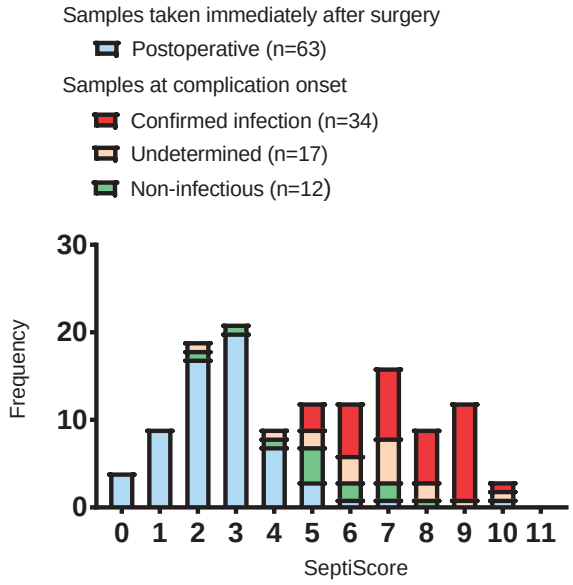

Figure 2. SeptiScores among 63 esophagectomy patients with ≥1 postoperative complication. Frequencies represent the number of patients.

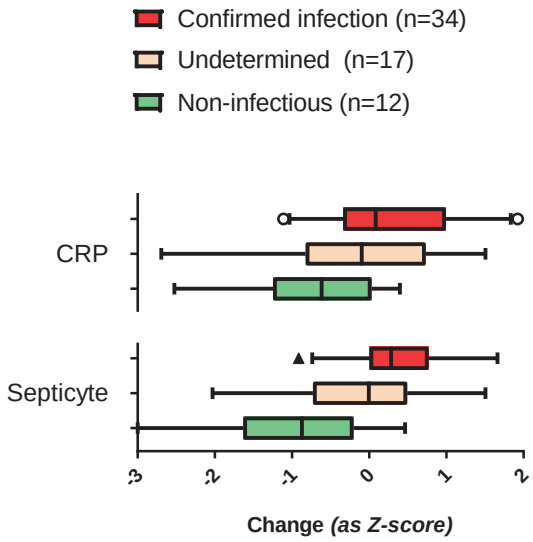

Figure 3. Temporal changes in SeptiScores and CRP levels among 63 esophagectomy patients developing a postoperative complication. *CRP,* C-reactive protein. CRP concentration and SeptiScores were measured in the immediate postoperative sample and the sample taken at complication onset. Subsequently, observed differences between both time points were transformed into a standardized z-score (z=x-**μ/σ**), having a mean value of 0 and standard deviation of 1. Boxes show median standardized differences with IQR, whiskers show 5-95th percentiles.

Discussion

This pilot study explored whether temporal changes in SeptiCyte LAB could be used to help diagnose infectious complications after esophageal surgery. Although SeptiScores varied widely between individuals, median scores immediately after surgery were comparable between subjects who went on to have either an eventful or uncomplicated subsequent postoperative course. However, the increase of SeptiScore over time was greater in patients developing postoperative infections than in those with other complications. Furthermore, this appeared to be more pronounced than the simultaneous rise in CRP observed in these patients.

SeptiCyte LAB was originally developed to help diagnose infection in critically ill patients presenting to an ICU with SIRS. Previous studies evaluating its diagnostic performance in this setting have reported variable discriminative ability for SeptiCyte LAB, with AUC's ranging from 0.73 (0.68-0.79) to 0.99 (95%CI 0.96-1.00) in different cohorts [17,19–21,26,27]. In particular, specificity was lower in patients presenting with suspected pneumonia as well as in those who had already been subjected to a prolonged clinical course in hospital prior to ICU admission [21,26]. In the current study we observed favorable discrimination (AUC of 0.87 (95%CI 0.76-0.98), albeit after exclusion of patients having an uncertain infectious state.

Observed variations in diagnostic performance are most likely explained by differences in study size, clinical setting, and distribution of underlying infectious etiologies. The optimal intended use scenario for SeptiCyte LAB therefore requires further exploration. Our data suggest that adding SeptiCyte LAB to CRP may improve diagnostic discrimination in patients following major surgery [7–9]. However, any possible use of SeptiCyte LAB for routine screening of postoperative patients will require careful evaluation before it can be considered. As physicians base their probabilistic decision-making mainly on clinical information (rather than biomarkers alone), it is unlikely that SeptiCyte LAB will be able to significantly decrease antimicrobial drug use when the pre-test probability for infection is high. The test will also be much more expensive than routinely available alternative biomarkers, and a stepped diagnostic approach might thus be appropriate. Clearly, the clinical utility and cost-effectiveness of such strategies can only be evaluated in a randomized controlled diagnostic trial.

Our pilot study has several limitations related to its relatively small sample size, which limits statistical power, as well as the unavailability of paired PAXgene samples in almost half the target population. Also, not all study patients presented to the ICU having a true diagnostic dilemma regarding the presence or absence of infection, which precludes final conclusions regarding diagnostic accuracy. In addition, we did not collect SeptiCyte LAB follow-up samples in patients without a postoperative complication, hindering the comparison of temporal changes in SeptiScores between patients having infectious complications, non-infectious complications, and uneventful postoperative courses. Furthermore, although

our pilot series aimed to explore the potential clinical utility of successive SeptiCyte LAB measurements in patients following major surgery, we evaluated score changes between two timepoints only. Thus, our findings cannot be directly extrapolated to a setting of sequential daily monitoring of this biomarker in postoperative patients. Also, for the prompt initiation of PAXgene specimen collection researchers were dependent on the clinical recognition of complications by attending physicians, which may have led to between-patient variability in timing of samples. Finally, even though the post-hoc likelihood of all suspected infections was carefully adjudicated by trained physician-observers according to standardized definitions, diagnostic misclassification cannot be ruled out [25]. However, patients with the greatest uncertainty regarding their reference diagnosis (i.e., those with an undetermined infectious status), were excluded from some comparative analyses, as has been done before in similar studies [17,21,28].

Conclusions

Repeated measurement of SeptiCyte LAB may have diagnostic value in the monitoring of surgical patients at high risk for postoperative infection. However, this has to be further evaluated in prospective studies that enroll both patients who are merely at risk for developing postoperative infections, as well as in those with suspected sepsis after major surgery.

Notes

Funding

D. Verboom and M. Bonten were funded by the Innovative Medicine Initiative (IMI), European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in-kind contribution (COMBACTE-CARE, grant agreement 115 620).

Potential conflicts of interest

The University Medical Center Utrecht received funding from the Center for Translation Molecular Medicine (http://www.ctmm.nl), project MARS (grant 041-201). Additional support (both in cash and in kind) was provided by Immunexpress (Seattle, WA).

None of the sponsors, in particular Immunexpress, played a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review of the article; or the decision to submit the article for publication.

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Supplementary material

Table S1. Number of samples by SeptiCyte LAB probability band and reference test

SeptiCyte LAB probability band[a]	Interpretation	Postoperative (n=174)	Reference test		
			Non-infectious complication (n= 12)	Undetermined (n=17)	Confirmed infection (n=34)
Band 1 (SeptiScore 0-3)	Sepsis unlikely	129 (74)	1 (8)	1 (6)	0 (0)
Band 2 (SeptiScore 3.1-4.4)	Sepsis likely	28 (16)	2 (17)	1 (6)	0 (0)
Band 3 (SeptiScore 4.5-5.9)	Sepsis likely	14 (8)	6 (50)	4 (24)	7 (21)
Band 4 (SeptiScore 6-10)	Sepsis likely	3 (17)	3 (25)	11 (65)	27 (79)

Presented as n (%). Percentages are column percentages. Reference test presented by post-hoc likelihood [a]Higher SeptiCyte LAB probability bands indicate increased likelihood of sepsis

Table S2. SeptiScore and CRP in infectious complications and non-infectious complications in 63 patients

	Non-infectious (n=12) Median (IQR)	Undetermined (n=17) Median (IQR)	Confirmed infection (n=34) Median (IQR)	p-value[a]
SeptiScore				
- immediate postop sample	2.7 (1.4-4.6)	2.7 (2.2-2.9)	2.5 (1.6-3.4)	-
- complication onset sample	5.3 (4.2-6.1)	6.8 (5.5-7.4)	7.6 (6.3-8.7)	0.0002
- delta score	2.1 (0.4-3.6)[*]	4.0 (2.5-5)[*]	4.7 (4.1-5.8)[*]	<0.0001
CRP concentration (mmol/L)				
- immediate postop sample	87 (46-132)	70 (4-108)	60 (9-102)	-
- complication onset sample	184 (128-275)	257 (181-320)	315(194-386)	0.0083
- delta concentration	124 (53-197)[*]	185 (107-279)[*]	206 (162-312)[*]	0.0043

CRP, C-reactive protein. Delta is the difference in SeptiScore and CRP between the postoperative moment and the moment when a complication occurred.
[a]Mann whitney U Test comparing non-infectious complication to confirmed infection.
[*]Wilcoxon signed rank test for the delta $p<0.05$, meaning that the delta was significantly different from 0.

Profile of the SeptiCyte™ LAB gene expression assay to diagnose infection in critically ill patients

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Published in Expert Review of *Molecular Diagnostics (2019)*

Abstract

Sepsis is a severe and frequently occurring clinical syndrome, caused by the inflammatory response to infections. Recent studies on the human transcriptome during sepsis have yielded several gene-expression assays that might assist physicians during clinical assessment of patients suspected of sepsis. SeptiCyte™ LAB (Immunexpress, Seattle, WA) is the first gene expression assay that was cleared by the FDA in the United States to distinguish infectious from non-infectious causes of systemic inflammation in critically ill patients. The test consists of the simultaneous amplification of four RNA transcripts (CEACAM4, LAMP1, PLAC8, and PLA2G7) in whole blood using a quantitative real-time PCR reaction. This review provides an overview of the challenges in the diagnosis of sepsis, the development of gene expression signatures, and a detailed description of available clinical performance studies evaluating SeptiCyte™ LAB.

1. Introduction

Sepsis is defined as life-threatening organ failure caused by a dysregulated host response to infection [1]. With approximately 1.7 million hospitalized adults and 270 000 deaths in the US each year, sepsis represents a major healthcare problem [2, 3]. Early detection and initiation of treatment improve outcomes in sepsis [4-6]. Current guidelines recommend the use of clinical scores, such as the SOFA (sequential organ failure assessment) and qSOFA (a simplified version based on the presence of impaired consciousness, hypotension and tachypnea) for early risk stratification of patients suspected of infection [1, 7]. However, sepsis and infection-negative systemic inflammatory syndromes often present as clinically similar entities, and both are frequently complicated by organ failure [8]. Identification of sepsis, therefore, remains somewhat subjective as there are no unambiguous criteria for diagnosing infection in such cases [8-10].

The difficulty to distinguish sepsis from non-infectious systemic inflammation is illustrated by the fact that up to 40% of patients receiving antimicrobial treatment in the ICU have no microbiologically confirmed infection [11]. If a sepsis syndrome is not caused by a bacterial infection, antimicrobial treatment is not necessary and may actually lead to adverse drug reactions, delays in the diagnosis and treatment of the true underlying cause of inflammation, and disturbances of the microbiome. The latter may result in selection of microorganisms not susceptible to the antibiotics used [12-14]. A diagnostic test that can reliably discriminate between infectious and non-infectious inflammation might therefore improve appropriate use of antibiotics, reduce the occurrence of complications, shorten length of hospital stay, and lower the costs of sepsis care.

Biomarkers reflecting the host immune response have long been proposed as useful tools for diagnosing infection. Traditionally the search has focused on single (plasma) biomarkers, including procalcitonin (PCT), C-reactive protein (CRP), soluble triggering receptor expressed on myeloid cell-1 (sTREM1), neutrophil expression of the high-affinity immunoglobulin-Fc fragment receptor I (cluster of differentiation 64, CD64), interleukin-6 (IL-6), IL-1 receptor antagonist (IL-1ra), pro-vasopressin (or copeptin), and pro-adrenomedullin (pro-ADM) [15, 16]. Although CRP and PCT may help to decrease antibiotic use, most external validation studies have shown only limited accuracy in discriminating infection from a non-infectious systemic inflammatory response syndrome (SIRS) [17, 18]. To improve diagnostic discrimination, several combinations of biomarkers have been proposed [19, 20]. The availability of novel molecular techniques and automation of elaborate laboratory analyses have stimulated the development of more complex, multi-analyte indicators of the host response [21, 22].

The analysis of host gene expression through RNA transcripts - coined "transcriptomics"- offers one such approach. Host gene expression differs greatly between septic patients and healthy individuals [23, 24], and while genetic expression between septic and non-infectious inflammation overlap to a large extent, a number of distinct differences observed in the

sepsis pathway may be exploited to distinguish infection from other causes of inflammation [23].

The SeptiCyte™ LAB transcription assay was developed to diagnose infection in critically ill patients presenting to an ICU with suspected sepsis [25]. It is the first host response assay based on quantitative reverse transcriptase PCR (qRT-PCR) methods that was cleared by the Food and Drug Administration (FDA) for this purpose. In this review we will discuss the available clinical data regarding the diagnostic accuracy of this new test and compare its performance to other RNA transcription assays that have been developed for a similar purpose.

2. Biomarker discovery using a transcriptomics approach

Biomarker research can be divided into quests for specific marker molecules (with known biological action) and systems biology-based approaches [26]. Single biomarkers (or combinations thereof) are likely to provide only a simplified representation of the host immune response to infection, whereas the rapid development of omics technologies now allows us to integrate data from many different biological systems, identify interactions between such systems, and decipher how these interactions relate to structure, function, and temporal dynamics. In the context of sepsis research this entails the study of complex interactions between host signaling and response pathways by genomics, epigenetics, transcriptomics, proteomics, and metabolomics methodologies. Proteomics and metabolomics refer to the study of proteins and metabolite profiles. Genomics and epigenetics refer to characterization of the genetic code and methylation status of DNA molecules, as well as the measurement of single nucleotide polymorphisms, whereas transcriptomics refers to the study of RNA expression [26].

Non-infectious triggers and infectious triggers can cause similar clinical responses. However, the broad approach offered by transcriptomics technologies could be helpful to understand differences in the underlying molecular pathways causing disease, and detect molecular evidence of pathophysiological derangement even before sepsis-related organ failure becomes clinically apparent (figure 1). These techniques may then be translated into tests that answer clinical questions regarding the (type of) trigger that caused the observed host response, the severity of inflammation, and the probability of adverse outcomes.

Whereas DNA maintains the genetic information of an organism, RNA transcripts provide information about the expression of genetic material at the molecular level in living cells. Messenger RNA (mRNA) is an intermediary molecule in the process of protein synthesis, while noncoding RNA (such as micro RNA) has regulatory functions in this transcription process. The 'transcriptome' is defined as the sum of all transcripts in a biological sample and reflects the transcriptional activity at that particular moment [27]. These processes occur early during sepsis (typically within 2-6 hours of the initial trigger), as demonstrated by an experimental study of endotoxin-induced inflammation in healthy individuals [28]. Transcriptional changes can be studied either in whole blood or in a subfraction of peripheral blood mononuclear cells

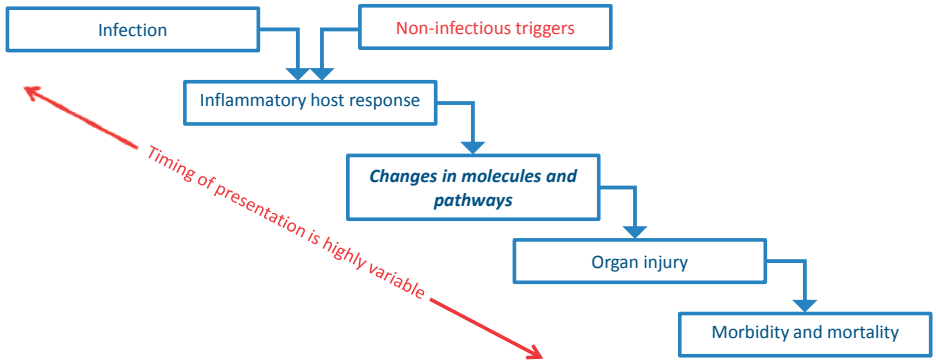

Figure 1. Challenges in the diagnosis of sepsis. Transcriptomics-based biomarker discovery may focus on changes in molecules and pathways that are specific for different triggers, the type and severity of underlying pathophysiological derangements, or clinical outcomes. Development of biomarkers may be complicated by similar clinical responses to infectious and non-infectious triggers, such as cell damage caused by trauma, ischemia, or surgery, and a highly variable timing of clinical presentation. Discriminating gene signatures could thus be used for diagnostic and prognostic purposes, as well as guidance for therapeutic decision making.

(PBMCs) specifically [29]. As gene expression is cell-type specific, expression profiles derived from whole blood represent a weighted sum of total gene expression in all leucocyte subpopulations [29, 30]. However, transcriptional changes will be largely driven by neutrophils since these make up 70% of white blood cells during inflammation [30, 31]. For practical reasons, therefore, whole blood specimens are typically used to derive a transcriptome. This allows for a less complicated sample preprocessing, and reduces susceptibility to artifacts that may arise from incomplete separation of leucocyte subfractions [29, 30].

The transcriptome can be measured using either DNA microarrays or next-generation RNA sequencing. Both techniques yield large amounts of information, and complex mathematical techniques are thus needed to reduce the raw data into a signature based on a manageable number of genes. This so-called dimensionality reduction is mostly performed by unsupervised (i.e., completely data-driven) variable selection techniques (such as principal component analysis) with the aim to find meaningful aggregates of data [32]. The next step in the data analysis process involves the selection of a simplified gene signature that provides the highest possible discriminative ability. In this way, expression profiles incorporating a large number of genes are scaled down to a final signature containing only a few genes, while preserving diagnostic value [33, 34]. Such signature may then be implemented on automated, accessible RT-PCR platforms to yield a final test having relatively fast turnaround time at an acceptable cost when compared to traditional biomarker panels [22].

Without appropriate statistical measures to correct for multiple testing, almost any analysis will 'discover' genes that significantly differ between subjects with sepsis and those with sterile inflammation, including many false positive findings. Several methods, such as the Bonferroni or Benjamini-Hochberg's correction, have been proposed to overcome this prob-

lem [35]. However, even though statistical techniques will reduce the rate of false discoveries, empirical testing of the proposed signature in a representative and clinically relevant target population remains a crucial step in order to uncover the true diagnostic potential as a new biomarker [36]. Rigorous clinical validation of the signature is therefore typically required.

An important role of the transcriptomics technologies might lie in their potential use to accomplish a more accurate and faster diagnosis of infection [37]. Several RNA transcription assays have shown promise as early markers of inflammation, and some studies suggest they yield a higher accuracy in the diagnosis of infectious etiologies than most clinical variables or biochemical markers [23, 31, 38-41]. Furthermore, transcription assays could help classify septic patients into distinct clinical phenotypes according to the dominant underlying pathophysiological aberrations. For instance, patients may represent subgroups that could benefit from targeted interventions, if they have increased expression of genes associated with the adaptive and innate immune system, or alternatively, higher expression in specific coagulopathic pathways [42-46]. Other applications for host response transcription assays could be the identification of causative pathogens or prognostication of sepsis [42, 47-52]. However, in the present review we will focus exclusively on the diagnosis of infection in critically ill patients.

3. Market profile of transcriptomics-based diagnostic sepsis markers

Several RNA signatures have been proposed to distinguish critically ill patients with sepsis from those with non-infectious inflammation, including the FAIM3:PLAC8 ratio [23], sNIP score [39], Sepsis Meta Score [31], and Bauer's Gene Expression Score [40] (table 1).

Below, we will discuss these markers considering the framework of a clinical performance study, since all studies reported diagnostic discrimination of a particular target gene signature for a specific disease (i.e., sepsis) and population (i.e., critically ill patients). Although clinical performance studies can have mixed characteristics, we classified each as either explanatory or pragmatic [56, 57]. An explanatory study design is predominantly used to evaluate the performance of a diagnostic test in a well-defined and controlled setting in which the target condition is established using strict definitions, patient inclusion is informative (i.e., subjects with clear presence or absence of disease), sample size is generally small, multiple cohorts are frequently used in parallel, and technical failures of the test are typically not taken into account during analysis. In contrast, a pragmatic approach aims to evalute clinical performance of a diagnostic test (and its implications) in routine practice. Consequently, target conditions are defined according to usual clinical practice, patient inclusion reflects the intended-use population, sample size is relatively large (with few exclusion criteria being applied), and all failures of the test are ideally taken into account [56, 57]. Table 1 shows a summary of findings yielded by the clinical performance studies that have been performed to date using these four sepsis gene signatures.

Table 1. Transcriptomics-based host response signatures to distinguish infectious from non-infectious systemic inflammation in critically ill patients

Signature	Genes	Cases	Controls	Study type	AUC	Sensitivity	Specificity
FAIM3/ PLAC8 ratio [23]*	FAIM3, PLAC8	Patients with probable/ definite CAP	Patients suspected of CAP, having their diagnosis refuted	Explanatory	0.78(23) (23)(23) (23)	97%	28%
sNIP score [39]	NLRP1, IDNK, PLAC8	Patients with abdominal sepsis	Postoperative GI surgery patients	Explanatory	0.86-0.98	93-95%	79-86%
Bauer Gene expression score [40]	TLR5,CD59,CLU, FGL2, IL7R, HLA-DPA1,CPVL	Patients with sepsis	Patients with SIRS	Explanatory	0.81	80%	59%
Sepsis Meta Score [31, 39, 53-55]	CAECAM1, ZDHHC19, C9orf95, GNA15, BATF, C3AR1, KIAA1370, TGFBI, MTCH1, RPGRIP1, HLA-DPB1	Adults with sepsis, Neonates with sepsis	Adults with SIRS, COPD patients, Patients after trauma Neonates with SIRS	Explanatory	0.83-0.92	95%	53-60%

CAP Community-acquired pneumonia, *SIRS* Systemic Inflammatory Response Syndrome, *COPD Chronic Obstructive Pulmonary Disease, GI gastrointestinal*. Shown test characteristics refer to (a range of) estimates as reported in validation (rather than discovery) cohorts. Sensitivity and specificity are threshold dependent but were always pre-specified by the investigators, except for reference [54].
*Some additional validation cohorts are not reported as they did not reflect the intended use population of this test.

Using a genome-wide whole blood transcription assay, Scicluna et al. found some significant differences in the host leucocyte response when comparing 101 subjects with probable or definite severe community-acquired pneumonia (CAP) according to *post hoc* physician assessment to 33 subjects in whom an (initially suspected) diagnosis of pneumonia was refuted [23]. Gene expression patterns that differed between CAP and noninfectious patients were associated with pathways related to EIF2 signaling (protein translation), T-cell receptor signaling, and mTOR signaling. These differences were the foundation for a FAIM3:PLAC8 marker showing reasonable discriminative ability (AUC of 0.78 (95% CI 0.67-0.89) in a validation sample containing 70 cases and 30 control subjects [23]. Both the derivation and validation cohorts consisted of patients having a true diagnostic dilemma, which is an important quality criterion for diagnostic accuracy studies. Yet, as no patients were studied in whom CAP could neither be confirmed or refuted with certainty (i.e., all 'undetermined' cases were excluded), this study may have resulted in over-optimistic estimation of diagnostic performance measures for the FAIM3:PLAC8 marker.

The sepsis NLRP1, IDNK, and PLAC8 gene expression score (sNIP score), incorporating three genes, was developed in a discovery cohort consisting of 60 patients with confirmed

abdominal sepsis and 42 patients who were enrolled after uncomplicated major gastrointestinal surgery [39]. The investigators found 1 196 genes to be overexpressed and 686 to be underexpressed in abdominal sepsis cases when compared to control subjects after gastrointestinal surgery. Subsequently, these differences were reduced to a 3-gene signature, which was then tested in an independent validation cohort. In this cohort, which included 46 sepsis cases and 27 control patients, the observed AUC was 0.91 (95% CI 0.84-0.97), yielding a sensitivity and specificity of 95% and 79%, respectively [39]. Additionally, the investigators evaluated sNIP score performance in publicly available gene expression data from external cohorts including a total of 53 septic patients and 33 controls. In these cohorts, discriminative ability remained good with AUC estimates varying between 0.86 (95% CI 0.75-0.95) and 0.98 (95% CI 0.92-1).

The Sepsis Meta Score (SMS) is an 11-gene expression signature that was derived using publicly available sepsis gene expression data sets obtained from 22 independent cohorts, including a total of 823 children and adults with community- and hospital-acquired sepsis and 747 controls without infection [31]. Interestingly, diagnostic performance was not only estimated in cases and controls at the time of ICU admission, but also in subjects who developed infection during their ICU stay and their time-matched controls. In one validation cohort, which included patients admitted after trauma (181 controls and 37 cases who later developed sepsis), the mean AUC for infection increased from 0.73 on day 3 to 0.89 on day 10 after ICU admission. In another validation sample, 23 trauma patients who had developed an infection were matched to non-infectious controls from other data sets, and yielded an AUC that increased over time from 0.68 to 0.84 [31]. This suggests that diagnosis of infection became more accurate once the traumatic injury began to recover.

The SMS has been validated in two additional studies in critically ill patients. One study assessed its diagnostic performance across 16 publicly available microarray datasets and yielded an overall AUC of 0.82 (range 0.73-0.89) [54]. Another study included patients admitted to the ICU with and without infection and yielded a comparable AUC of 0.80 (95% CI 0.67-0.92) [55].

The diagnostic performance studies for both SMS and sNIP scores share some limitations, the most important being that diagnostic performance characteristics were estimated based on highly selected patient populations in which not all patients were actually suspected of infection (thus lacking a clinical need for a diagnostic test). This negatively impacts generalizability of the study findings and makes the susceptible to bias.

The Bauer Gene Expression Score is a 7-gene signature which was validated in a cohort consisting of 56 patients with SIRS (i.e., definite absence of infection) and 190 patients having various degrees of infection plausibility (i.e., including possible, probable or definite infections) [40]. The biomarker had an estimated AUC of 0.81 (95% CI 0.76-0.87), resulting in a sensitivity of 80% and specificity of 59% at the chosen threshold [40]. Importantly, patients were consecutively enrolled and subjects with an uncertain diagnosis of infection

remained included in the analysis, which reflects a pragmatic approach and enhances generalizability of the results. However, the control group partially consisted of patients without clinically suspected infection, and the cohort was thus not fully representative of patients that will need the test in practice.

3.1 Unmet needs in the market

Although diagnostic accuracy measures of the gene expression assays discussed in the previous section were promising overall, most studies were explanatory by nature and based on a retrospective case-control design, which is known to positively bias discriminatory performance estimates [58, 59]. Further studies in consecutive patient series across various settings, therefore, need to be conducted to produce more robust assessments of clinical performance. Furthermore, some studies focused on specific subgroups such as CAP, abdominal sepsis, or trauma patients. While developing separate biomarkers for specific infectious etiologies might be an intentional strategy [23, 39], this limits the applicability of these signatures to the general ICU population.

Despite considerable efforts and progress in the development of transcriptomic biomarkers, several issues still remained to be addressed. First, there is a need for a test that can be used in a general ICU population, irrespective of clinical domain. Second, there is a lack of approved tests. None of the previously described tests has been cleared by the FDA or by the European In Vitro Diagnostic Directive[1] for use during the diagnosis of sepsis.

1. in May 2018 the In Vitro Diagnostic Regulation (EU) 2017/746 was published and will become applicable in 2022. This regulation will replace the Directive and aims to strengthen the current approval system. It includes changed risk classification rules, and requirements for conformity assessment. New in vitro diagnostic categorized with a higher risk profile will have to deliver a performance evaluation, based on scientific validity, analytical and clinical performance data providing sufficient clinical evidence.

Third, as treatment needs to start as early as possible in sepsis patients [60], there is a need for tests providing rapid turnaround times while reducing hands-on labor. Cartridge-based implementation on automated analysis platforms will provide a likely solution for this, but has not been accomplished so far. As the main objective of this paper, we will focus on SeptiCyte™ LAB and discuss to which extent development and validation of this test has fulfilled any of the current market needs.

4. How SeptiCyte™ LAB works

SeptiCyte™ LAB serves as an indicator of the host response to infection by measuring the expression of specific genes involved in immune function and inflammatory signaling in whole blood. The test consists of an assay that simultaneous amplifies and measures 4 RNA transcripts (i.e., carcinoembryonic antigen-related cell adhesion molecule 4 (CEACAM4), lysosomal-associated membrane protein 1 (LAMP1), placenta-specific 8 (PLAC8), and phospholipase A2 group VII (PLA2G7)) using a quantitative real-time PCR reaction. The gene transcripts and their (presumed) biological actions are listed in table 2. Subsequently, a risk score (SeptiScore™) is calculated (ranging 0-10) from their relative expression levels as quantified by the number of PCR cycle times, estimating the probability that SIRS observed in critically ill patients is due to infection [25]. The SeptiCyte™ LAB kit as used in the studies described in this review will not become commercially available, but has been used as a proof-of-concept device to demonstrate performance to US regulatory standards.

Table 2. The four transcripts measured by SeptiCyte™ LAB*

Gene	Locus and function	Description
CEACAM4	chromosome 19 q13.2 encodes carcinoembryonic antigen (CEA) -related cell adhesion molecule 4	An oncofetal cell surface glycoprotein. It has been widely used as a human tumor marker because its expressed highly in tumors and secreted in serum. It belongs to the CEA –related cell adhesion molecule (CEACAM) family, which is a immunoglobulin superfamily. It is also involved in phagocytosis by human granulocytes [61].
LAMP1	chromosome 13 q34 encodes Lysosomal-associated membrane protein 1	A glycoprotein from the family of lysosome-associated membrane glycoproteins. It is a marker of degranulation on lymphocytes and works synergistically with LAMP2 in phagosome maturation [62, 63].
PLAC8	chromosome 4q21.22. encodes Placenta-specific 8 gene protein	An upstream regulator of brown fat differentiation and function. This protein product could act as a transporter for divalent cations. Homozygous deleted mice for PLAC8 display impaired host defense involving a decreased ability of phagocytes to kill bacteria [64].
PLA2G7	chromosome 6 p21.2-p12 encodes Phospholipase A2, group VII (platelet-activating factor acetylhydrolase)	Catalyzator of the degradation of platelet-activating factor (PAF). Defects of PAF-acetylhydrolase deficiency are associated with asthma and atopy. Human pre-term infants with necrotizing enterocolitis (NEC) have increased circulating and luminal levels of PAF and decreased levels of the PLA2G7-encoded PAF-acetylhydrolase. Homozygous deletion in mice for PLA2G7 leads to enhanced susceptibility to NEC after birth [65].

*Table adapted from McHugh et al. [41]

The test has been developed by Immunexpress (Seattle, WA) and is validated for use on the Applied Biosystems® 7500 Fast Dx Real-Time PCR System (Applied Biosystems®, Foster City, CA). The format of the test is a boxed kit with turnaround time (from sample draw to report) of approximately 6 hours. Each SeptiCyte™ LAB kit includes reagents sufficient for up to 12

patient samples. The specimen used for the SeptiCyte™ LAB is a 2.5 mL sample of whole blood collected directly from the patient, using the PAXgene™ collection tubes within the PAXgene™ Blood RNA System (Qiagen). A white blood cell count (WBC) of 2.7 X 10^5 WBC/mL or greater must be verified prior to testing patients.

Total RNA isolation is performed using the procedures specified in the PAXgene™ Blood RNA kit (a component of the PAXgene™ Blood RNA System). Purification can be carried out manually, using a microcentrifuge, or semi-automated on the QIAcube (Qiagen). The purified total RNA must be evaluated for concentration (A260 indicating a concentration of ≥2 ng/μL) and purity (as estimated by A260/A280 ratio ≥ 1.6). RNA specimens may need to be adjusted in concentration to facilitate a constant input volume of 10 μL and a total input into the RT reaction between 20 and 500 ng. Within these validated input ranges no further normalization of RNA expression data is needed. Purified total RNA should be tested immediately after extraction or stored frozen in single-use portions at or below -70°C until ready for testing.

Subsequently, the transcription and amplification steps take place. Extracted RNA is converted to cDNA. The cDNA is immediately run in the qPCR portion of the test. Transcripts CEACAM4, LAMP1, and PLAC8 are amplified, detected, and quantified in a multiplex reaction. There is a separate reaction (singleplex) well for PLA2G7. During cycles of PCR amplification there is a real-time generation of fluorescence from hydrolysis of dye-quencher hydrolysis probes. Each kit includes a high positive control, low positive control and negative control for each in vitro transcripts (IVT) (LAMP1, CEACAM4, PLA2G7, PLAC8), designed to produce high, medium or low SeptiScore™ values. If all controls are valid, then the batch run is valid.

The SeptiScore™ score is calculated from the cycle threshold numbers. The results of four RNA transcripts are translated into a single numerical result with the following formula: $(Ct_{PLA2G7} + Ct_{CEACAM4}) - (Ct_{PLAC8} + Ct_{LAMP1})$. Analysis software which accompanies the SeptiCyte™ LAB is designed to analyze.sds run files from the ABI 7500 Fast Dx instrument. The higher the SeptiScore™, the higher the likelihood of sepsis. Although a dichotomization threshold value of 3.1 has been used to separate likely from unlikely sepsis cases during early test validation, the manufacturer has subsequently suggested the use of four continuous SeptiScore™ interpretation bands. These classification bands and their associated sepsis likelihood ratios originate from a large validation study and are provided in the FDA decision summary [25].

SeptiCyte™ LAB is currently (2018) developed into a product to be used on the Biocartis Idylla™ platform for further commercialization as molecular diagnostic test [66]. When released, it will be a self-contained and fully automated cartridge, providing a test result directly from whole blood in approximately 75 minutes, with a hands-on time of less than 2 minutes. The cartridge is expected to become available for commercial use in Europe in late 2019. Technical details of the Idylla system can be found elsewhere [67].

5. Clinical profile of SeptiCyte™ LAB

Discovery and preliminary validation of the 4-gene SeptiCyte™ LAB classifier occurred through mathematical analysis of an Australian cohort consisting of both sepsis patients and surgical patients having postoperative systemic inflammation [41]. The 4-gene signature was converted from microarray to reverse-transcription quantitative polymerase chain reaction (RTqPCR) format, which was then further evaluated in several clinical performance studies. A SeptiScore™ threshold ≥3.1 favored a high sensitivity in the discovery cohort and was initially chosen as the cut-off level for dichotomization during further test evaluation. Below, we will summarize the diagnostic accuracy studies that have been published to date (November 2018), and assess their risk of bias based on Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) guidelines (tables 3 and 4) [68]. Of note, the questions listed in table 3 were adapted from the original QUADAS-2 publication in order to better reflect the purpose of the current diagnostic profile.

Table 3. Domains of bias in risk of bias assessment of diagnostic test accuracy studies*

Domain	Questions	Risk of bias assessment (high=H/low=L/unclear= ?)
A. Patient selection	Was a consecutive or random sample of patients enrolled? Was a case-control design avoided? Did the study avoid inappropriate exclusions? Was the spectrum of patients representative of the patients who will receive the test in practice?	Could the selection of patients have introduced bias?
B. Index test	Were the methods to obtain results technically reliable? If a threshold was used, was it pre-specified?	Could the conduct or interpretation of the index test have introduced bias?
C. Reference test	*Is the reference standard likely to correctly classify the target condition? Were the reference standard results interpreted without knowledge of the results of the index test? Was the reference standard applied in a way it can be reproduced?*	Could the reference standard, its conduct, or its interpretation have introduced bias?
D. Flow and timing	Was there an appropriate interval between index test(s) and reference standard? Did all patients receive a reference standard? Did all patients receive the same reference standard? Were all patients included in the analysis?	Could the patient flow have introduced bias?

*Table was adapted from Whiting et al. [68]

The first clinical performance study of SeptiCyte™ LAB analyzed 345 patients who were enrolled as part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) cohort in two tertiary ICU's in The Netherlands between 2011 and 2013 [41]. Patients were categorized as having either infection negative systemic inflammation or sepsis based on a post-hoc physician assessment of available clinical, radiological and microbiological evidence [69]. Across five separate validation cohorts, estimates for AUC (0.77 to 0.99), sensitivity (79% to 100%), and specificity (33% to 91%) varied widely, depending on the prevalence of sepsis, the level of confidence regarding the reference diagnosis, and whether patient enrollment was consecutive.

Among a post-hoc subgroup of 157 patients with complete data, SeptiCyte™ LAB outperformed PCT (AUC 0.88 (95% CI 0.81-0.93) versus AUC 0.84 (95% CI 0.76-0.92), respectively, p<0.001). However, the best discriminative ability was achieved by a combination of both biomarkers (AUC 0.89, 95% CI 0.82-0.95, p<0.01). In contrast, combining five commonly available clinical variables (i.e., PaO2/FIO2 ratio, bilirubin, urine output, blood glucose and maximum heart rate on the first day of ICU admission) with SeptiCyte™ LAB, did not improve diagnostic performance (data not reported).

A small pediatric study compared 40 children (aged 0 to 18 years) who presented with microbiologically confirmed or highly probable community-acquired bacterial sepsis to 30 children who had undergone uncomplicated cardio-pulmonary bypass surgery (i.e., without infection) [70]. In this study the reported AUC for SeptiCyte™ LAB in diagnosing sepsis was 0.99 (95% CI 0.96-1.00), and did not seem to be correlated with disease severity, immune status, or positivity of culture results. Although transcriptomic responses to sepsis are known to evolve as children mature [71], this study did not suggest an altered performance of the SeptiScore™ in children compared to adults.

Another study, explored the potential utility of longitudinal monitoring of SeptiScores™ in 67 patients who suffered from complications after elective esophagectomy [72]. SeptiCyte™ LAB results were analyzed pair-wise, comparing samples drawn immediately after surgery and at onset of a postoperative complication, which was then categorized as being of infectious, undetermined, or non-infectious origin [69]. SeptiCyte™ LAB results were highly variable immediately after surgery, yet scores increased significantly as complications evolved (median 2.7 (IQR 1.6-3.4) versus 7.3 (5.7-8.7); p<0.001). Importantly, samples taken during infection yielded higher SeptiScores™ than specimens taken at onset of a non-infectious complication (median 7.3 (IQR 5.7-8.7) versus 5.2 (IQR 4-6.3); p<0.001). An analysis of the scores obtained at the time that a complication had become evident yielded an AUC of 0.87 (95%CI 0.76-0.98) for SeptiCyte LAB, compared to an AUC of 0.76 (95%CI 0.61-0.91) for CRP (p=0.14). Adding SeptiCyte LAB to CRP resulted in improved diagnostic discrimination (AUC 0.88 (95%CI 0.77-0.99), p=0.04). However, these performance estimates may suffer from bias as the study included cases with unambiguous non-infectious and infectious samples only.

In addition to the above-mentioned clinical performance studies, which were specifically designed to yield estimates of diagnostic accuracy, three other studies aimed to compare SeptiCyte™ LAB to other RNA signatures. The first study included 60 patients with (suspected) abdominal sepsis and 42 controls after uncomplicated gastro-intestinal surgery who had been enrolled in the MARS cohort [39]. Discriminative ability of SeptiCyte™ LAB was generally adequate (AUC 0.89, 95% CI 0.83-0.95), but lower than observed for the sNIP score (0.97 (95% CI 0.94-0.99); p<0.01). Yet, the sNIP score was derived in (and optimized for) this particular cohort of abdominal sepsis patients, possibly leading to overestimation of its performance compared to SeptiCyte™ LAB.

Table 4. Overview of clinical performance studies of SeptiCyte™ LAB

Explanatory studies											
			Patient classification		**Risk of bias**						
Reference	**Source**	**Sepsis cases**	**Undetermined cases**	**Infection-negative controls**	***A***	***B***	***C***	***D***	**AUC (95%CI)**	**Sensitivity (%)**	**Specificity (%)**
McHugh et al. [41]											
1.High confidence cohort	MARS	24 probable and definite sepsis cases	-	35 SIRS controls	H	L	L	L	0.95 (0.91-1)	79 (58-93)	91(77-98)
2. Randomly picked including possibles	MARS	3 probable and definite sepsis cases	6 possible cases (results reported)	27 SIRS controls (2 were treated for suspected sepsis)	H	L	L	L	0.77 (0.59-0.94)	100(29-100)	67 (46-84)
3.Randomly drawn after excluding possibles	MARS	29 probable and definite sepsis cases	-	77 SIRS controls (1 was treated for suspected sepsis)	H	L	L	L	0.93 (0.88-0.97)	97(82-100)	68 (56-78)
4. Sequential cohort	MARS	18 probable and definite sepsis cases	20 possible cases (results reported)	49 SIRS controls (11 were treated for suspected sepsis)	H	L	L	L	0.85 (0.75-0.95)	100(82-100)	33 (20-48)
5. Adding racial diversity	MARS	21 probable and definite sepsis cases	11 possible cases (results reported)	25 SIRS controls (2 were treated for suspected sepsis)	H	L	L	L	0.92 (0.85-1)	100(84-100)	52 (31-72)
Zimmerman et al. [70]	PICU Seattle	35 cases with overt sepsis at admission	-	29 SIRS & cardiopulmonary bypass surgery controls	H	L	L	H	0.99 (0.96-1)	-	-
Verboom et al. [72]	MARS	34 cases with infectious complications	21 possible cases (results reported)	12 controls with non-infectious complications	H	L	L	H	0.87 (0.76-0.98)	100	8
Scicluna et al. [39]	MARS	60 abdominal sepsis cases	-	42 SIRS & abdominal surgery controls	H	L	L	H	0.89 (0.83-0.95)	-	-
Sweeney et al. [54]	Public data*	532 cases from mixed cohorts	-	585 controls from mixed cohorts	H	H	?	H	0.75 (± 0.15)	~95	38

Table 4. Overview of clinical performance studies of SeptiCyte™ LAB

Pragmatic studies										
			Patient classification		**Risk of bias**					
Reference	**Source**	**Sepsis cases**	**Undetermined cases**	**Infection-negative controls**	**A**	**B**	**C**	**D**	**AUC (95%CI)**	**Sensitivity (%)**	**Specificity (%)**
Koster-Brouwer et al. [73]	MARS	197 probable and definite sepsis cases	135 possible cases (results reported)	135 controls with acute respiratory failure (41 were treated for suspected infection)	?	L	L	L	0.73 (0.67–0.79)	96	19
Miller III et al. [74]											
Unanimous	MARS/ VENUS	121 sepsis cases according to 3 panelists	157 without unanimous diagnosis (not reported)	171 SIRS controls according to 3 panelists	H	L	L	H	0.89 (0.85-0.93)	97	34
Consensus	MARS/ VENUS	180 sepsis cases according to ≥ 2 panelists	37 without consensus diagnosis (results reported)	230 SIRS controls according ≥2 panelists	H	L	L	L	0.85 (0.81-0.89)	94	35
Forced	MARS/ VENUS	202 sepsis cases by forced decision	0 cases (as panel was forced to make a decision)	245 SIRS controls by forced decision	H	L	H	L	0.82 (0.78-0.86)	92	65
Maslove et al. [55]	PREVAIL	14 probable and definite sepsis cases	9 possible cases (included as confirmed sepsis in the primary analysis)	38 ICU controls	H	L	L	L	0.68 (0.53-0.83	-	-

AUC Area Under the Curve. *MARS* Molecular diagnosis And Risk Stratification of Sepsis, *PICU* Pediatric Intensive Care Unit, *VENUS* Validation of septic gene ExpressioN Using SeptiCyte. *SOFA* Sequential Organ Failure Assessment. Sources of bias are expressed for various domains (A= Patient selection, B= Index test, C=Reference test, D= Flow and timing). Risk of bias per domain are expressed as high=H, low=L, or unclear= ?.

* Discriminative ability was evaluated across 16 separate cohorts derived from publicly available datasets (individual analyses can be found in reference [54]).

The second study compared SeptiCyte™ LAB to the SMS and FAIM3/PLAC8 signatures using various publicly available microarray datasets [54]. Array data were based on gene expressions measured either in whole blood, neutrophils or PBMCs only. Overall, 16 cohorts containing patients who were classified as having either SIRS or sepsis were included. Across these data sets, SeptiCyte™ LAB yielded a mean AUC of 0.75 (range 0.44-0.99) [54], which compared unfavorably to some of the other signatures tested, yet without reaching statistical significance. However, this microarray study had several limitations. First, most original cohorts had highly selective case-control designs. Second, normalization of raw data – needed to correctly compare the microarray datasets – was not always possible. Last, the reference tests used across the different cohorts varied widely.

The third comparative study evaluated SeptiCyte™ LAB, the SMS and the FAIM3/PLAC8 ratio in a cohort that was recruited completely independently from their discovery cohorts [55]. The study prospectively enrolled 23 ICU patients with and 38 without sepsis. The reference diagnosis was based on a post-hoc assessment of available clinical and microbiological evidence, and cases were considered to have sepsis if the plausibility of infection was adjudicated to be either possible, probable or definite, in a setting of organ dysfunction. Discrimination was highest for the SMS, although the performance difference with SeptiCyte**™** LAB did not reach statistical significance (AUC 0.80 (95% CI 0.67-0.92) versus 0.68 (95% CI 0.53-0.83), respectively). When uncertain (i.e. possible) infections were excluded from analysis, discriminative ability of all gene expression signatures increased, but their relative performances remained unchanged.

SeptiCyte™ LAB was also investigated in two larger studies that included consecutive patients, and therefore were considered to be predominantly pragmatic by design (table 4). The first study analyzed 467 patients presenting to the ICU with acute respiratory failure following a period of (prolonged) hospitalization, in whom the possibility of nosocomial infection was considered during diagnostic work up [73]. Of note, patients in whom an infectious diagnosis was already self-evident upon ICU admission were excluded, as these cases had no clinical indication to be tested. A post-hoc reference diagnosis was assigned by trained research physicians following a comprehensive review of available clinical, radiological and microbiological evidence [69]. Based on this classification, patients were categorized as having no infection, definite infection, or an undetermined infectious state. SeptiScores™ correlated with the probability of infection (Spearman's rho 0.320; $p < 0.001$), yet Ct values for the four individual genes were largely overlapping between patients with and without infection. Furthermore, the discriminatory ability of SeptiCyte™ LAB was comparable to CRP in a direct comparison that excluded all cases with an undetermined infection status (AUC 0.73 (95% CI 0.68-0.79) and 0.73 (95% CI 0.67-0.79), respectively). Of note, using the recommended SeptiCyte™ LAB cut-off of ≥3.1 to select patients for antimicrobial treatment would have led to inappropriate prescriptions in 110 cases (of whom 72 did not actually receive antibiotic treatment during the study).

A second pragmatic study combined 249 patients from the VENUS cohort (enrolled in seven ICU's in the USA between 2014 and 2016) and 198 patients from the MARS cohort (enrolled in the Netherlands between 2011 and 2013) [74]. SeptiCyte™ LAB was measured upon ICU admission in all patients meeting SIRS criteria. Subsequently, patients received a reference diagnosis based on retrospective adjudication by three expert panelists using case record forms containing clinical information about the first 24 hours of ICU admission (retrospective physician diagnosis, RPD) [74]. Three different methods were then used to construct reference tests reflecting various levels of diagnostic robustness (i.e. based on forced, consensus, and unanimous panelists' judgments). Test characteristics were determined for each, thereby acknowledging different levels of diagnostic uncertainty in the reference diagnosis. The resulting AUC estimates for SeptiCyte™ LAB ranged from 0.82 (when using the forced RPD) to 0.89 (when using the consensus RPD) (table 4). For comparison, estimated AUC of PCT for consensus RPD in this study was 0.80 (95% CI 0.75-0.85). Addition of clinical and laboratory variables (including PCT) to SeptiCyte™ LAB did not improve diagnostic discrimination.

5.1 Remarks on clinical performance

The aforementioned clinical performance data suggest that SeptiCyte™ LAB has a sensitivity above 90%, which is high compared to an overall sensitivity of only 60% for culture-based methods in ICU patients with sepsis [75]. Yet specificity is highly variable and discriminative ability (as expressed by AUCs) for distinguishing sepsis from non-infectious causes of systemic inflammation, is at least equivalent to PCT and CRP (table 4) [41, 72, 73, 74]. Possible explanations for these variable findings might be related to the fact that CEACAM4 (one of the four genes that make up the SeptiCyte™ LAB test) was not down-regulated (as much as observed in the original discovery cohort) in at least two studies [54, 73]. This may have led to inaccurately high SeptiScores™ and thus false positive findings. Secondly, it is not clear to what extent SeptiCyte™ LAB results are affected by clinical disease severity. In one study there seemed to be a trend towards increased SeptiScores™ in patients having higher APACHE IV scores and mortality [73], yet two other studies observed robust performance when evaluating different strata of disease severity in either adult or pediatric cases [41, 70]. Thirdly, in several studies the test performed considerably worse in subjects with pneumonia compared to other sepsis etiologies [73, 74]. This finding could be, in part, spurious and related to greater uncertainty in the reference standard used to diagnose lower respiratory tract infections. Indeed, inter-observer agreement for making a diagnosis of ventilator-associated pneumonia is poor, with reported Cohen's kappa varying between 0.2 and 0.4 [69, 74]. Still, as pneumonia is one of the most frequently occurring infections in both the ICU and hospital at large, a reduced diagnostic accuracy in these patients should be accounted for when implementing the test in clinical practice. Fifth, discriminative ability of SeptiCyte™ LAB in some studies was poor when sepsis patients were compared to healthy subjects [54, 55]. Although healthy subjects are not representative of a population

that will need a diagnostic test in practice, this observation suggests that the test might not function optimally in patients who are less severely ill. Furthermore, in some studies it was also noted that SeptiScores™ were slightly higher for African Americans compared to other racial groups [25, 74]. However, due to possible confounding by lifestyle factors, it remains unclear whether this would result in higher rates of false positive findings in these patients.

5.2 Assessment of possible bias

We used the QUADAS-2 tool as a framework to assess sources of bias in the studies discussed above (table 3). This tool uses four key domains, including A) patient selection, B) index test, C) reference test, and D) flow and timing (i.e., clinical flow of patients throughout the study and timing of both the index and the reference tests). In studies reporting multiple analyses, qualitative assessment of study methodology was focused on the estimation of discriminative ability of SeptiCyte™ LAB against a reference test for sepsis, and not on comparisons of SeptiCyte™ LAB with clinical scores and/or other biomarkers (such as PCT or CRP).

The risk of bias in the domain of patient selection was high for most studies (table 4). In some studies, patients were initially enrolled based on the presence of SIRS criteria or an increased SOFA score [41, 54, 55, 70, 74]. However, as SIRS and organ failure are virtually omnipresent and rather non-specific in ICU patients [7, 10], these cohorts may not be fully representative of patients having a true diagnostic dilemma who would receive the test in clinical practice. Equally important, most studies used a case-control design and thus included only highly selected patients [39, 41, 54, 70, 72]. This results in a study population that is enriched for obvious diagnoses, which may lead to overestimation of diagnostic performance [58]. A single study included patients admitted with acute respiratory failure after prolonged previous hospitalization, thus better reflecting subjects posing a true diagnostic dilemma [73]. However, this study also enrolled a considerable proportion of patients in whom no blood sample for SeptiCyte™ LAB analysis was available. As these missings were most likely not at random, bias cannot be excluded.

With regard to interpretation of the index test, information bias is expected to be low as thresholds for analysis were generally pre-specified [41] and there is virtually no ambiguity in the SeptiScores™. However, uniformity of the SeptiCyte™ assay might have been problematic as various normalization techniques were used across different datasets [54].

All studies incorporated a variety of data sources (e.g. clinical symptoms, radiology findings, and microbiological cultures) to construct a reference standard for diagnosis. This is a generally accepted method and acknowledged by several guidelines [76-78]. Four studies [39, 41, 72, 73] used definitions as proposed by the MARS consortium, which have demonstrated good concordance among observers for most types of infection [69]. Other studies used a similar approach [55], single expert-opinion [70], or an expert panel [74] to adjudicate patients. One study used aggregated data derived from a wide variety of studies, each using different reference standards (not all of which have been properly reported, which

precluded a formal assessment of possible bias) [54]. Except for this latter study, the risk of bias introduced by the use of these reference standards was generally considered to be low. Nonetheless, when studying a syndrome with a somewhat subjective definition such as sepsis, the reference test will always be prone to misclassification. As the direction and rate of misclassification is unknown, its impact on apparent diagnostic test performance cannot be estimated in a straightforward way. However, there are some methods that will decrease or increase misclassification. For instance, enforcing a diagnosis in highly uncertain cases will increase the overall misclassification rate [74].

As the patient flow might be an important source of selection bias it is important to fully report this in diagnostic studies, which was not always done, or showed a large proportion (~50%) of unavailable samples that was not corrected for [39, 54, 72]. Finally, the maximum time interval between performance of the index test (SeptiCyte™ LAB) and the reference standard (physician diagnosis) was explicitly stated to be between 24 and 72 hours in three studies [41, 70, 74]. This implies that all measurements, cultures and clinical assessments used for constructing the post-hoc reference standard needed to be performed within this timeframe. In contrast, this period was much longer in the remaining studies [39, 54, 72, 73]. Whether a time window of 24-72 hours is sufficient to collect all the evidence required for a robust reference standard is debatable, as later clinical findings, microbiology results, and even post-mortem findings could also contribute to a sepsis diagnosis. However, in general we considered the reported timeframes as acceptable.

6. Expert commentary

Data indicate that SeptiCyte™ LAB has favorable sensitivity, but highly variable specificity, for diagnosing sepsis in critically ill patients when used at a cut-off threshold of ≥3.1. Therefore, this assay should not be used primarily as a rule-in test, especially not in a setting of very severely ill patients suspected of nosocomial infection, where immediate treatment is needed. However, due to its high sensitivity, SeptiCyte™ LAB shows promise as a test to rule-out infectious causes of systemic inflammation in patients clinically presenting with sepsis-like symptoms. However, evaluation of diagnostic performance in populations posing a true diagnostic dilemma is still limited and has been subject to methodological shortcomings, precluding a definite assessment of clinical utility. In addition, not all the previously listed unmet marked needs have been completely fulfilled by SeptiCyte™ LAB at this time.

The first remaining challenge relates to the fact that diagnostic performance measures seem to vary widely across different patient populations. This concerns sensitivity and specificity, as well as likelihood ratios and predictive values. For example, specificity may be influenced by a high disease prevalence, in either a positive or negative direction [79]. Also, variances in patient selection can explain differences of up to 40% in sensitivity or specificity estimates

[58, 59, 79]. Our QUADAS-2 analysis revealed several risks of bias in all currently published clinical performance studies that could lead to both overestimation and underestimation of the diagnostic performance.

The second challenge refers to the lack of a perfect gold standard. Every sepsis biomarker will face the problem of an imperfect reference diagnosis, which will bias any estimate of diagnostic performance [80]. A frequently used approach to overcome this problem is to exclude patients with an uncertain (or undetermined) infection status, as was done in several studies evaluating SeptiCyte™ LAB [41, 74]. However, excluding "difficult-to-diagnose" patients might result in overestimation of diagnostic accuracy [58, 59]. Although certain techniques such as latent class modeling or use of composite reference standards can correct for this uncertainty, these are infrequently used as the assumptions underpinning these methods are not always met (such as conditional independence of the reference and novel test) [81-83]. One study evaluating SeptiCyte™ LAB nicely illustrates how apparent diagnostic performance changes with various degrees of diagnostic uncertainty [74].

Moreover, most of the clinical performance studies included patients presenting without a true diagnostic dilemma. This renders the estimated clinical utility of the test prone to overestimation [58]. In fact, inclusion of either apparent sepsis cases or clearly non-infectious controls precludes unbiased estimation of clinical utility, since neither reflect patients in whom the clinician would actually perform the test (or would base treatment decisions on test results).

Changing focus from mere diagnostic accuracy to clinical utility is an indispensable step for any new biomarker before implementation in clinical practice [84]. Clinical utility goes beyond the quality of a diagnostic test, because it implies that using the test improves outcomes in relevant patient populations, enhances healthcare quality, efficiency, and cost-effectiveness, or both. In the absence of a gold standard (i.e., confident reference diagnosis), a randomized controlled trial – comparing patient management with and without information provided by the new test – is the best study design to demonstrate clinical utility. To the best of our knowledge such a trial is neither ongoing, nor planned.

Without proof of clinical utility, we consider it unlikely that physicians will base their therapeutic decision-making primarily on SeptiCyte™ LAB (or any other biomarker for that matter). However, it is fair to say that critically ill patients suspected of sepsis represent a very challenging intended use population for any new test as the consequences of unjustly withholding antimicrobial treatment in sepsis patients based on a false negative test result can be disastrous. However, available clinical performance data suggest that SeptiScore™ values <3.1 (the likelihood threshold suggested by the manufacturer) are associated with a probability ranging between 10% and 15% of true infection in the populations studied to date. Provided that the patient is clinically stable, based on such values, antimicrobial therapy may be withheld or postponed on a case-by-case basis.

Conversely, imperfect specificity renders it implausible that SeptiCyte™ LAB results will prevent spurious diagnosis of infection, and introduction of this test into clinical practice will thus most likely not reduce unnecessary antibiotic use on a large scale. However, even modest benefits over currently used diagnostic markers of infection could improve daily care. Along the same line of reasoning, any probabilistic information that results from SeptiCyte™ LAB testing might expedite the diagnostic work-up of a critically ill patient suspected of infection in such a way that infection can be refuted sooner and the search for alternative diagnoses pursued. Whether such (perceived) benefits warrant large-scale use of the test should be evaluated in further clinical performance studies that are focused on specific clinical applications, such as guiding early discontinuation of antibiotic therapy or monitoring of patients at high risk of acquiring nosocomial infection.

7. Five-year view

Further development of PCR techniques and their implementation on automated analysis platforms will enable SeptiCyte™ LAB, and its competing gene signatures, to be implemented as efficient bedside clinical tools with fast turnaround times. The recent partnership between Immunexpress and Biocartis is expected to result in SeptiCyte™ LAB becoming available for use on the Idylla™ platform by late 2019.

The road from initial discovery to establishment as a clinically accepted biomarker is often painstakingly long and winding. For instance, the possible use of PCT as a marker of infection was already described in the early 1990s [85], but it was not until 2017 that PCT was granted FDA approval for predicting progression from severe sepsis to septic shock, for predicting 28-day mortality, and for managing antibiotic de-escalation. Even so, the clinical utility of PCT for diagnosing bacterial infections across a broad range of clinical settings remains being debated. In fact, measurement of PCT is currently not recommended to inform decisions about initiation of antibiotic therapy in ICU patients [86]. Similarly, a recent pragmatic study failed to find significant added value of PCT-guided antibiotic initiation in patients with suspected respiratory tract infections presenting to the emergency department [87]. This illustrates that clinical utility is not only dependent on the diagnostic accuracy of a test, but also depends on the intended use population. We therefore expect future biomarker studies to focus explicitly on cohorts where the largest potential for improvement of the diagnostic process exists (e.g., patient groups having a low pre-test probability of infection, or presenting with possibilities for early discontinuation of antimicrobial treatment).

A first hurdle that novel diagnostic RNA signatures need to overcome is to provide more robust evidence of clinical performance, as measures of diagnostic accuracy alone will never be convincing enough to implement any new biomarker into daily practice. Rather, extensive evidence of both efficacy and efficiency should be sought by performing intervention trials

in which SeptiCyte™ LAB is added to the standard care of critically ill patients presenting with a true diagnostic dilemma. Predictive values and likelihood ratios derived from this type of pragmatic research better reflect true diagnostic utility and will eventually be required to implement any new test.

Key issues

- Transcriptomics technologies are increasingly being used for biomarker discovery in sepsis, resulting in several gene expression signatures that have recently been proposed for use as a potential diagnostic tool.
- SeptiCyte™ LAB is the first gene expression assay available in the United States that was cleared by the FDA to distinguish sepsis from non-infectious causes of systemic inflammation in critically ill patients presenting to an ICU.
- The test consists of the simultaneous amplification of four RNA transcripts (CEACAM4, LAMP1, PLAC8, and PLA2G7) in whole blood using a quantitative real-time PCR reaction, resulting in a SeptiScore™ ranging from 0 to 10, with higher scores indicating a higher likelihood of infection.
- Although SeptiCyte™ LAB is more extensively validated than most competing gene expression assays, clinical performance studies available to date are subject to bias and have mostly focused on patient populations that (at least partially) consisted of subjects presenting without a true diagnostic dilemma, precluding a definite assessment of clinical utility
- The manufacturer's recommended threshold value of ≥3.1 for the SeptiScore™ has high sensitivity, yet limited specificity for diagnosing infections in critically ill patients, making the test primarily useful for ruling-out sepsis.
- The SeptiCyte™ LAB assay is currently being implemented onto the Biocartis Idylla™ platform and is expected to be marketed as a fully automated bedside molecular diagnostics system in Europe and elsewhere within the near future.

Notes

Funding

D. Verboom and M. Bonten were funded by the Innovative Medicine Initiative (IMI), European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in-kind contribution (COMBACTE-CARE, grant agreement 115 620).

Declaration of interest

The UMC Utrecht received a grant from CTMM (project MARS, grant 04I-201) in which Immunexpress was included as a providing party and received funding from Immunexpress for further research. Except for a factual accuracy check there was no scientific involvement by Immunexpress in preparing this review. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

Acknowledgements

We thank Immunexpress for reviewing technical information for accuracy.

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The diagnostic yield of routine admission blood cultures in critically ill patients

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Submitted

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Abstract

Objective

Although the Surviving Sepsis Campaign bundle recommends obtaining blood cultures within one hour of sepsis recognition, adherence is suboptimal in many settings. We, therefore, implemented routine blood culture collection for all non-elective ICU admissions (regardless of infection suspicion) and evaluated its diagnostic yield.

Methods

This was a before-after analysis in a mixed-ICU of a tertiary care hospital in the Netherlands. We included patients acutely admitted to the ICU between January 2015 and December 2018. Automatic orders for collecting a single set of blood cultures immediately upon ICU admission were implemented on January 1, 2017. Blood culture results and the impact of contaminated blood cultures were compared for 2015-2016 (*before period)* and 2017-2018 (*after period*). Positive blood cultures were categorized as bloodstream infection (BSI) or contamination. Blood cultures were obtained in 573 (32.3%) of 1 775 and in 1 582 (84.5%) of 1 871 patients in the before and after period, respectively (p<0.0001), and BSI was diagnosed in 95 (5.4%) and 154 (8.2%) patients in both study periods (RR 1.5, 95%CI 1.2-2.0; p=0.0006). The estimated number needed to culture for one additional patient with BSI was 17. Blood culture contamination occurred in 40 (2.3%) and 180 (9.6%) patients in the before period and after period, respectively (RR 4.3, 95%CI 3.0-6.0; p<0.0001). Incidence of vancomycin use, or presumed episodes of catheter-related BSIs treated with antibiotics did not differ between both study periods, however more blood cultures were taken in the days following admission (127 (12.3%) to 172 (15.8%), p=0.02).

Conclusion

Implementation of routine blood cultures was associated with a 1.5-fold increase of detected BSI. The 4.3-fold increase in contaminated blood cultures did not result in an increase of vancomycin use on the ICU.

Introduction

The current Hour-1 Bundle of the Surviving Sepsis Campaign guidelines recommends collection of two sets of blood cultures (2 aerobic vials and 2 anaerobic vials) before initiation of antimicrobial treatment in patients with suspected sepsis [1]. These cultures may guide streamlining of antimicrobial therapy in the intensive care unit (ICU), and narrow down the differential diagnosis that commonly includes both infectious and non-infectious diseases in critically ill patients. However, clinical detection of sepsis can be difficult during the early stages of the disease, and many episodes in patients admitted through emergency wards remain initially undetected [2,3]. Furthermore, complex logistics and errors of communication in the setting of an acute admission may negatively affect adherence to blood culture recommendations [4]. As a result the pursued clinical practice of obtaining blood cultures in all patients with suspected sepsis is suboptimal in many ICUs.

To optimize identification of bloodstream infections (BSI) in our setting, a clinical policy change was introduced that included an automatic order for a single set of blood culture collection in every critically ill patient immediately upon arrival in our ICU, regardless of a clinical suspicion of infection. Because of standardized prospective data collection and infection registration of all patients in the unit as part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study [5], we could evaluate the effect of this intervention on the proportion of patients with BSI. Naturally, an increase in contaminated blood cultures was expected and we, therefore, also determined the effects of the intervention on antibiotic use and repeated blood cultures among patients with contaminated blood cultures.

Methods

Patients and data collection

The Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study (NCT01905033) was initiated in 2011 and prospectively collects comprehensive clinical data, daily biological specimens (mainly plasma), and detailed descriptions of (presumed) infectious episodes in all patients having an expected ICU length-of-stay >24 hours [5].The current study was a before-after analysis of an intervention occurring in the 32-bed mixed-ICU of the UMC Utrecht, a tertiary care hospital in the Netherlands, which was a recruiting center for the MARS study.

Ethical approval for data and sample collection was provided by the Medical Ethics Committee of the University Medical Center Utrecht, including an opt-out consent method (IRB No. 10-056C).

We consecutively included adults (>18 years) who had been acutely admitted to our ICU (thus excluding all elective medical and surgical admissions). We used the protocolized prospective data collection within the MARS study to define a "clinical suspicion of infection

present in the first two days of ICU admission", which was based on the prescription of therapeutic antimicrobial therapy in these days [5].

Routine blood cultures were implemented as a quality improvement intervention on January 1st 2017 for all acute ICU admissions. To this end, an automatic order was implemented in the patient data management system, requesting the collection of a single blood culture set immediately upon presentation, regardless of whether blood cultures had been obtained at the Emergency Room or hospital ward before transfer to ICU. There were no other interventions implemented to optimize compliance of blood cultures obtainment, prior to this study. Decisions on antibiotic treatment (and thus of a patient being categorized as having a clinical suspicion of infection in our analysis) were at the physicians' discretion, based on the presence of a clinical suspicion of infection, and were not influenced by the changed policy. The culture policy was communicated to all attending physicians and clinical microbiologists. Subsequently, we compared blood culture results between January 2015 through December 2016 (*before period*) and January 2017 through December 2018 (*after period*).

Standard operating procedure of blood culture collection

All blood cultures consisted of one aerobic and one anaerobic vial (2 times 10 mL) and were processed following routine clinical protocols in our local laboratory. Bottles were incubated in a BD BACTEC™ blood culture system with automatic signaling of microbial growth, after which MALDI-ToF MS (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry, Bruker BV) and gram staining was used for pathogen identification. Until June 2016 blood cultures obtained after office hours were kept at room temperature and incubated the next morning. From June 2016 onwards blood cultures were incubated directly in a BD BACTEC™ blood culture system. Positive culture results were immediately communicated with clinicians through telephone consultation and made available via the patient data management system. Clinical microbiologists attended daily multidisciplinary meetings in the ICU.

Classification of blood culture results

All blood cultures obtained in the time window ranging from two days before until two days after ICU admission were evaluated (i.e. <48 hours). Positive results were classified as (presumed) pathogens or contaminants according to the flow chart shown in Supplementary Fig. S2. This differentiation between a (presumed) pathogen and a contaminant was based on an algorithm applied to potential contaminants and included culture characteristics associated with contamination; i.e. number of positive blood cultures with the organism of interest, number of positive vials, incubation time (as a proxy for time to positivity), and polymicrobial growth (Supplementary Fig. S2) [6,7]. The following (skin) commensals were considered potential contaminants: coagulase-negative staphylococci (CoNS), *Bacillus* spp., *viridans group Streptococci*, *Corynebacterium* spp., *Proprionibacterium* spp., *Aerococcus* spp., and *Micrococcus* spp.

Patients with BSI but without a clinically suspected infection (i.e., that were not treated with antibiotics immediately after or at the time that the blood culture was obtained) were adjudicated upon clinical chart review as contamination when patients had good clinical response without antimicrobial therapy (Supplementary Fig. S3). Blood culture results of patients with only skin contaminants results were considered contamination. Patients with both BSI and contamination were classified as having BSI.

Statistical analysis

To evaluate the diagnostic yield of routine blood culturing we calculated the relative risk (RR) of detecting BSI or contamination during the two study periods. Subsequently, we used a modified Poisson regression to adjust the relative risk estimate for BSI [8,9]. In this multivariable analysis we included the following potential confounders; age (>65), gender, Charlson comorbidity index, chronic immunodeficiency, APACHE-IV score, SIRS criteria, presence of shock, clinically suspected infection, previous antimicrobial treatment (>2 days before ICU admission) and hospital length of stay <48 hours prior to ICU admission, These covariables were selected based on their previously reported associations with BSI [10]. We estimated the number of patients needed to culture for one additional BSI by dividing the total number of additionally cultured patients by the total number of additional BSIs detected. Furthermore, we quantified potential adverse effects of contamination by comparing vancomycin use, the number of antibiotically treated catheter-related bloodstream infections and the number of patients with additional blood cultures obtained (on day 3-5) in patients without initial clinically suspected infection. We chose this time window for the repeat blood cultures in order to identify those episodes most likely resulting from initially obtained blood cultures.

Differences in admission and disease characteristics during both periods were analyzed using Wilcoxon rank sum, Chi-square, or Fischer exact tests, as appropriate. A Pearson correlation coefficient was calculated for the correlation between time and contamination rates. A p-value < 0.05 was considered statistically significant. All analyses were performed using SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC) and figures were made using GraphPad Prism version 7.04 (GraphPad Software, La Jolla, CA, USA).

Results

Patient characteristics

There were 1 775 (48.7%) and 1 871 (51.3%) patients acutely admitted to the ICU in the before and the after periods, respectively (Supplementary Fig. S1). In the after period fewer patients were immunocompromised, patients were less often previously admitted to the ICU, were more often transferred to other hospitals, and patients had a shorter length of hospital stay (11 versus 10 days; p=0.006; Table 1).

Table 1. Baseline characteristics of patients

Variables	Before period (n=1 775)	After period (n=1 871)	*p*-value
Age (years)	61 (50, 71)	61 (49, 70)	0.55
Sex (male)	1089 (61.4)	1183 (63.2)	0.24
APACHE–IV score	82 (63, 102)	81 (60, 103)	0.41
Charlson comorbidity index	0 (0, 2)	0 (0, 2)	0.37
Immune deficiency	324 (18.3)	255 (13.6)	<.001
Surgical reason for admission	533 (30)	514 (27.5)	0.09
Previous ICU admission	206 (11.6)	177 (9.5)	0.03
Hospital stay prior to ICU <48h	1263 (71.2)	1419(75.8)	0.001
Antibiotically treated for infection >2 days prior to ICU admission	210 (11.8)	237 (12.7)	0.44
Clinically suspected infection	744 (41.9)	779 (41.6)	0.86
SOFA at admission ≥ 2 or SIRS	1775 (100)	1775 (100)	1.00
Number of blood cultures collected:			<0.001
• 0	1202 (67.7)	289 (15.5)	
• 1	326 (18.4)	979 (52.3)	
• 2 or more	247 (13.9)	603 (32.2)	
Transfer to another hospital	218 (12.3)	306 (16.4)	<0.001
Length of ICU stay (days)	2.6 (0.8, 7.4)	2.0 (0.7, 5.7)	<0.001
Length of hospital stay (days)	11 (3, 24)	10 (2, 21)	0.006
ICU Mortality	333 (18.8)	305 (16.3)	0.05
30-day Mortality	516 (29.1)	523 (28.0)	0.45

AB: antimicrobial therapy. *APACHE*: Acute Physiology and Chronic Health Evaluation. *BC:* blood culture(s). *ICU*: intensive care unit. Continuous data presented as median (Q1, Q3) and dichotomous data as *n* (%).

Blood culture findings

One or more blood cultures were collected in 573 (32.3%) patients in the before and in 1 582 (84.5%) in the after period (p<0.0001). Overall, 998 and 2 646 blood cultures were obtained around ICU admission during both periods, reflecting 0.5 and 1.4 cultures per individual, respectively. We identified 95 (5.4%) patients with BSI in the before period and 154 (8.2%) in the after period (p=0.0006), yielding a crude RR of documented BSI in the after period of 1.5 (95%CI 1.2-2.0; p=0.0006) and an absolute risk difference of 2.9% (95% CI 1.3-4.5) (Table 2). The RR with adjustment for potential confounding was 1.6 (95%CI 1.2- 2.0; p<0.0001). Based on the average number of blood cultures obtained per patient we estimate that around 1 009 additional cultures were obtained in the after period, which yielded 59 BSIs (from 94 before to 154 after the intervention) corresponding to a number needed to culture of 17 to detect one additional patient with BSI. Among patients in whom blood cultures were obtained, the pre-test probability for BSI decreased from 16.6% (95%CI 13.5-19.6) in the before to 9.7% (95%CI 8.3-11.2) in the after period (p<0.0001). In the subgroup of patients suspected of infection the pre-test probability remained the same (19.3% (95%CI 15.7-22.8) vs. 19.0% (16.2-21.9 95%CI); p=0.93).

Table 2. Bloodstream infections and contaminated blood cultures

	Before period		After period		Relative risk	
	n	**% (95% CI)**	**n**	**% (95% CI)**	**(95% CI)**	***p*-value**
All patients	**1 775**		**1 871**			
Any blood culture obtained	573	32.3 (30.1-34.5)	1582	84.6 (82.9-86.2)	2.6 (2.4-2.8)	<0.0001
Detected bloodstream infections	95	5.4 (4.3-6.4)	154	8.2 (7.0-9.5)	1.5 (1.2-2.0)	0.0006
Only contaminated blood cultures [a]	40	2.3 (1.6-2.9)	180	9.6 (8.3-11.0)	4.3 (3.0-6.0)	<0.0001
Patients with a suspected infection	**744**		**779**			
Any blood culture obtained	483	64.9 (61.5-68.4)	746	95.8 (94.4-97.2)	1.5 (1.4-1.6)	<0.0001
Detected bloodstream infections	93	12.5 (10.1-14.9)	142	18.2 (15.5-20.9)	1.5 (1.1-1.9)	0.0020
Only contaminated blood cultures [a]	27	3.6 (2.3-5.0)	83	10.7 (8.5-12.8)	2.9 (1.9-4.5)	<0.0001
Patients without a suspected infection	**1031**		**1092**			
Any blood culture obtained	90	8.7 (7.0-10.5)	836	76.6 (74.0-79.1)	8.8 (7.2-10.7)	<0.0001
Detected bloodstream infections	2	0.2 (0-0.5)	12	1.1 (0.5-1.7)	5.6 (1.3-25.1)	0.0130
Only contaminated blood cultures [a]	13	1.3 (0.6-1.9)	97	8.9 (7.2-10.6)	7.4 (4.2-13.1)	<0.0001

This table reports the proportion of patients with blood culture results aggregated at patient-level within the defined time window. Number of patients were divided by the total number of patients admitted during the periods. Clinically suspected infection was evaluated on the first 2 days of ICU admission and defined upon the administration of antibiotics for presumed clinical infection. [a]Aggregated blood cultures yielding mere contaminants. Patients with both BSI and contaminated blood cultures (n=27) were classified as having BSI.

The proportion of patients with contaminated blood cultures increased from 2.3% (n=40) in the before period to 9.6% (n=180) in the after period (p<0.0001), corresponding to a crude RR of 4.4 (95%CI 3.1-6.1; Table 2). The monthly rates of contamination increased in the first year after the intervention (ρ=0.52; p<0.0001) and then declined in the second year (ρ=-0.42; p<0.0001; Fig. 1). The distribution of the different pathogen types did not differ between the before and after period (Table 3). Classification of the cultured pathogens can be found in Supplementary Table S1.

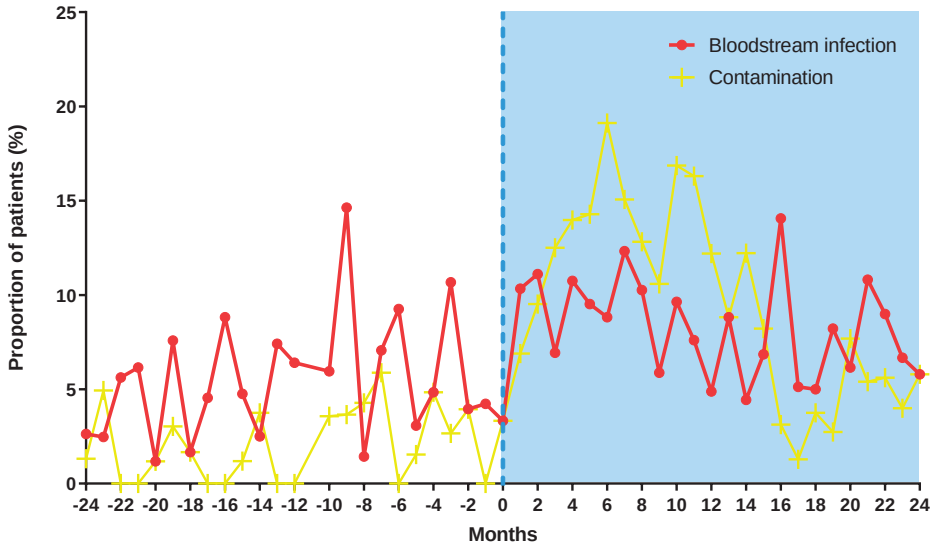

Figure 1. Proportion of patients with a bloodstream infection and contamination over time. *BSI:* bloodstream infection. The blue line indicates the start of the intervention.

Consequences of blood culture positivity

There were 1 031 and 1 092 patients admitted without clinically suspected infection during both study periods; i.e.,that they were not treated with antibiotics immediately after or at the time that the blood culture was obtained. Among these, 13 (1.3%) and 97 (8.9%) were considered to have contaminated blood cultures (RR 7.4 95%CI 4.2-13.1; <0.0001), for which three of 1 031 (0,3%) and seven of 1 092 (0,6%) received vancomycin (p=0.24). Overall, prophylactic or therapeutic vancomycin use, or presumed episodes of catheter-related BSIs treated with antibiotics were comparable between both study periods (Table 4). The number of cultures taken on day 3 to 5, suggestive for repeat blood cultures, increased from 127 (12.3) to 172(15.8; p=0.02) in the after period, however of those only 4 (0.4) and 19 (1.7; p=0.003) were associated with a previous positive culture (Table 4).

Table 3. Blood culture results

Blood culture results	Before period (n=998)	After period (n=2 646)	*p*-value
No growth	807 (80.9)	2157 (82.5)	0.65
Growth	191 (19.1)	489 (18.5)	0.65
Identified microorganisms by type (>1 possible per blood culture)			
Enterobacterales	33 (3.3)	70 (2.7)	0.29
Other Gram-negatives	17 (1.7)	40 (1.5)	0.68
Staphylococci	42 (4.2)	85 (3.2)	0.14
- *Coagulase negative staphylococci*	- 27 (2.7)	- 41 (1.5)	
- *Staphylococcus aureus*	- 15 (1.5)	- 45 (1.7)	
Streptococci	22 (2.2)	35 (1.3)	0.06
Enterococci	20 (2.0)	46 (1.7)	0.59
Other Gram-positives	9 (0.9)	17 (0.6)	0.41
Yeast	9 (0.9)	16 (0.6)	0.33
Contamination [a]	56 (5.6)	228 (8.6)	0.01

Values are presented as n (%) and proportion of total cultures. Cultures were counted multiple times when results contained more than two types of micro-organisms. This was the case for 54 (7.9%) of 680 positive cultures. See Supplemental Table 1 for results per micro-organism. [a] Including 18 initially presumed pathogens (in 17 patients), adjudicated as contamination according to Supplementary Fig. S3.

Table 4. Repeat blood cultures, vancomycin use and CRBSIs in patients without a clinically suspected infection on day 3 to 5.

	Before period (n=1 031)	After period (n=1 092)	*p*-value
One or more blood cultures	127 (12.3)	172 (15.8)	0.02
≥ 1 repeat blood cultures (any previous culture taken) [a]	19 (1.8)	147 (13.5)	<0.001
≥ 1 blood cultures in patients with a previous positive culture	4 (0.4)	19 (1.7)	0.003
≥ 1 blood cultures in patients with a previous contaminated culture	3 (0.3)	18 (1.7)	0.002
Prophylactic vancomycin	22 (1.0)	17 (0.8)	0.32
Therapeutic vancomycin	9 (0.4)	9 (0.4)	0.90
Vancomycin in patients with a contaminated culture [b]	3 (0.1)	7 (0.3)	0.24
Patients treated with antibiotics for infection (on day 3-5)			
• Suspected CRBSI	8 (10.8)	4 (6.0)	0.20
• Other infection	66 (7.2)	63 (6.1)	0.54

BC: blood culture(s). CRBSI: catheter-related bloodstream infection. Continuous data presented as median (Q1, Q3) and dichotomous data as *n* (%). [a] Previous cultures were taken in the first 2 days of admission (i.e. <48 hours). [b] Including prophylactic and therapeutic indications.

Patients with clinical suspicion of infection

Almost all detected BSIs (n=235 (94.4%)) were obtained from patients with clinically suspected infection during the first two days in ICU (i.e., those treated with antibiotics immediately after or at the time that the blood culture was obtained) (Supplementary Fig. S3). In this subgroup, one or more blood cultures were collected in 483 of 744 (64.9%) and in 746 of 779 (95.8%) patients in the before and after period, respectively (p<0.0001), and these yielded 93 (12.5% of 744) and 142 (18.2% of 779) patients with BSI in the before and after period, respectively (RR 1.5, 95%CI 1.1-1.9; p=0.002; Table 2). Comparing the incidence of obtaining blood cultures among patients categorized as having a clinical suspicion of infection we estimated that blood cultures were obtained in an additional 243 patients in the after period and that this yielded 49 additional BSIs (from 93 before to 142 after the intervention) corresponding to a number needed to culture of 5 to detect one additional patient with BSI.

Discussion

In this before-after analysis the implementation of routine blood cultures for all admitted patients to the ICU of a tertiary care hospital was associated with a 1.5 fold increase in patients with a blood culture collected (from 32.3% to 84.5%), a 1.5 fold increase in patients with documented BSI and a 4.3 fold increase in patients with contaminated blood cultures. In patients that were treated with antibiotics for a clinical suspicion of infection within two days of admission, the proportion with blood cultures obtained increased from 64.9% to 95.8%, considerably improving compliance to the Surviving Sepsis Campaign culture guidelines.

Not unexpectedly, the intervention was associated with an increase in blood culture contamination. The observed 8.6% contamination rate exceeds the 3% set as benchmark by the American Society of Microbiology [11]. Reported contamination rates in other cohorts ranged from 4 to 23% [12–14]. Of note, approximately 70% of the patients had been hospitalized less than 2 days when admitted to ICU, and we, therefore, expect that the observed contamination rate was minimally influenced by catheter colonization. We did not implement a wash-out, or run-in period as the intervention implied an automatic pop-up which we did not expect to require learning time after implementation. Yet, after the start of intervention an increase in contaminated blood cultures was noted, which initiated education on contamination and prevention measures for nursing teams from April 2017 onwards. The rate of contamination sharply declined in the second year of intervention, as has been observed before after educating staff [14–16].

False positivity of blood cultures is an undesirable outcome and has been associated repeatedly with additional costs and unnecessary antimicrobial use in other settings [17,18]. Yet, in our setting the increase in patients with contaminated blood cultures was not associated

with initiation of vancomycin treatment or diagnosed CRBSI events among patients without initially suspected infection. It did however result in a higher number of cultures taken on the days following admission. Of importance, in our academic hospital all culture results and antimicrobial therapy are daily discussed in multidisciplinary meetings, which limits generalizability of these results to ICU-settings where this is not standard of care. Without such antimicrobial stewardship measurements there might be a risk of overtreatment.

The decision to implement a policy of obtaining blood cultures in all patients regardless of a clinical suspicion of infection was based on several incidents where blood cultures had not been obtained in patients with obvious infections. This new policy increased the number of patients with detected BSI and the crude "numbers needed to culture for one additional episode of BSI" was five among patients with a clinical suspicion of infection, 17 for the total ICU population and 75 for patients without a clinical suspicion. In our hospital the average internal cost price per blood culture is around 25€.

During our study blood culture obtainment in patients with suspected infection increased substantially, at the cost of many negative and false-positive cultures. Obviously, a 100% adherence of obtaining blood cultures, restricted to patients with an infection would be the most cost-effective approach. Previously, compliance with sepsis care bundles, including timely blood culture obtainment, has been successfully improved by educational interventions, and (computerized) early screening interventions, resulting in blood culture adherence ranging from 78 to 98% [19–24]. Nevertheless, the effect of sepsis care bundles stands or falls with the completeness of screening. To overcome complex logistics and errors of communication during transfers, automated screening should overarch all hospital units of care. Our study results suggest that routine blood cultures may provide good value for health on the ICU.

Due to the observational design of our analysis, the a priori likelihood of culture positivity may have been slightly different between both periods. Yet, most patient characteristics were comparable between both periods and adjustment for potential confounders did not change relative risks of culture positivity.

Our results suggest that BSI diagnoses were missed during the before period and that a higher rate of contaminated blood cultures is the trade-off for not missing BSI. Research has focused on risk stratification in order to select high risk patients in whom blood cultures have a higher diagnostic value. However, to our knowledge, no clinical prediction rule has been able to identify patients with sufficient sensitivity and specificity for BSI [18,26,27]. Our intervention was motivated by the recognition that different clinical and logistical challenges negatively affect the pursued practice of obtaining blood cultures at an early stage of sepsis. Improving BSI detection aids in establishing an accurate diagnosis of infection and rapid adjustment of empiric antimicrobial therapy if needed, which may improve clinical outcomes and may help reducing unnecessary exposure to broad-spectrum antibiotic coverage [28,29]. Our findings illustrate the potential effect of obtaining blood cultures in every critically ill

patient, even when a clinical suspicion of infection is not (yet) obvious, instead of a strategy to obtain blood cultures only in patients with a clinical suspicion of infection. That aside, not all detected BSI episodes in the ICU will have clinical significance as the source and pathogen of infection might already be known from previous cultures or procedures, and therapeutic decision making is not always guided by the results of blood cultures alone.

Naturally, this analysis has limitations due to its uncontrolled design. Deliberately, we did not attribute differences in patient related outcomes between study periods to the implemented policy change. The validity of such analysis would be hampered by the used study-design (a before-after comparison), since differences in the population, and logistics (i.e., more transfers to other hospitals) are likely to develop over time in an uncontrolled study design. Also, we could not evaluate outcomes that reflect clinical utility, such as an expedited microbiological diagnosis, or antimicrobial change as a result of culture results. Furthermore, adjudication of blood culture results as BSI or contaminant was mostly based on culture characteristics, and misclassification in some cases cannot be excluded. Another limitation is that evaluation of vancomycin use and occurrence of CRBSI was restricted to the ICU stay, with no information being collected after ICU discharge. Importantly, the higher BSI detection was caused by low adherence to sepsis guidelines in the first place, therefore results may not be generalizable to settings with better adherence. Finally, incubation time of cultures obtained after office hours changed in the after period. Although this may have reduced time till positivity, there is no evidence that this could have led to an increase in the number of samples with bacterial growth [30].

Conclusion

In conclusion, in a response to suboptimal practice of obtaining blood cultures in patients with clinically suspected infection, a strategy of obtaining a single set of blood cultures in all acutely admitted ICU patients was associated with 1.5 fold increase in the detection of BSI, at the cost of a 4.3-fold increase in the proportion of patients with contaminated blood cultures, and more blood cultures in the days following admission. Yet, there was no evidence that this resulted in more vancomycin use on the ICU.

Notes

Potential conflicts of interest

The authors declare that they have no potential conflicts of interest in relation to the subject of the present manuscript.

Funding

D. Verboom and M. Bonten were funded by the Innovative Medicine Initiative (IMI), European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in-kind contribution (COMBACTE-CARE, grant agreement 115 620).

Acknowledgments

We thank the research physicians and research nurses of the UMC Utrecht for their help in data acquisition.

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Supplementary material

Table S1. Classification of pathogenic micro-organisms after adjudication

	Control period		**Intervention period**	
	N cultures	**N patients**	**N cultures**	**N patients**
Enterobacterales	**33**	**27**	**70**	**49**
• *Escherichia coli*	26	21	35	25
• *Klebsiella oxytoca*	-	-	7	4
• *Klebsiella pneumoniae*	1	1	5	4
• *Proteus mirabilis*	2	2	8	5
• *Raoultella species*	2	1	-	-
• *Salmonella enterica*	-	-	2	2
- Amp C producing				
• *Citrobacter freundii*	1	1	4	1
• *Enterobacter cloacae complex*	1	1	3	3
• *Morganella morganii*	-	-	1	1
• *Pantoea agglomerans*	-	-	1	1
• *Serratia marcescens*	1	1	4	3
	N cultures	**N patients**	**N cultures**	**N patients**
Other Gram-negatives	**17**	**15**	**40**	**24**
- Gram-negative anaerobes				
• *Bacteroides spp.*	3	3	4	3
• *Fusobacterium necrophorum*	-	-	1	1
• *Prevotella nigrescens*	-	-	1	1
• *Gram negative anaerobe (not further specified)*	**-**	**-**	1	1
- Other				
• *Aeromonas veronii*	**-**	**-**	2	1
• *Capnocytophaga species*	-	-	1	1
• *Haemophilus influenzae*	1	1	-	-
• *Moraxella catarrhalis*	-	-	2	1
• *Neisseria meningitidis*	1	1	2	1
• *Oligella urethralis*	-	-	1	1
• *Pseudomonas aeruginosa*	9	8	24	12
• *Pseudomonas putida*	-	-	1	1
• *Sphingomonas species*	2	1	-	-
• *Vibrio parahaemolyticus*	1	1	-	-
	N cultures	**N patients**	**N cultures**	**N patients**
Staphylococci	**42**	**19**	**85**	**34**
• *Coagulase negative staphylococci*	27	11	41	17
• *Staphylococcus aureus*	15	8	45	18

Streptococci	**N cultures**	**N patients**	**N cultures**	**N patients**
	22	**19**	**35**	**24**
- Pyogenic streptococci				
• *Streptococcus agalactiae*	2	2	-	-
• *Streptococcus dysgalactiae*	3	2	6	4
• *Streptococcus pyogenes*	3	3	2	2
- Non-pyogenic streptococci				
• *Streptococcus gallolyticus*	-	-	2	1
• *Streptococcus pneumoniae*	9	9	14	9
• *Viridans streptococci*	4	2	3	2
- Other streptococci				
• *Aerococcus species*	-	-	3	1
• *Gemella morbillorum*	-	-	1	1
• *Granulicatella adiacens*	-	-	2	2
	N cultures	**N patients**	**N cultures**	**N patients**
Enterococci	**20**	**13**	**46**	**25**
• *Enterococcus faecalis*	5	4	13	8
• *Enterococcus faecium*	16	10	33	17
Other Gram-positives	**9**	**5**	**17**	**13**
- Gram-positive anaerobes				
• *Actinomyces odontolyticus*			**3**	**3**
• *Clostridium innocuum*			**1**	**1**
• *Clostridium perfringens*			**3**	**2**
• *Clostridium symbiosum*	1	**1**		
• *Lactobacillus sakei*			**1**	**1**
• *Lactococcus lactis*	1	**1**		
• *Parvimonas micra*			**1**	**1**
• *Peptostreptococcus species*			**1**	**1**
- Other				
• *Bacillus species*	-	**-**	**1**	**1**
• *Kocuria species*	1	1	1	1
• *Listeria monocytogenes*	-	-	4	1
• *Pseudoclavibacter species*	2	1		
• *Rothia mucilaginosa*	4	1	1	1
	N cultures	**N patients**	**N cultures**	**N patients**
Yeast	**9**	**6**	**16**	**6**
• *Candida albicans*	2	2	6	3
• *Candida glabrata*	7	4	2	2
• *Candida krusei*	-	-	8	1
Total	**145**	**95**	**282**	**154**

This table reports the number of unique cultures and unique patients with a positive result, therefore numbers of cultures and patients do not necessarily add up per type of micro-organism. Eighteen presumed pathogens were adjudicated as contamination according to Supplementary Figure 2 and are reported under 'Contamination'.

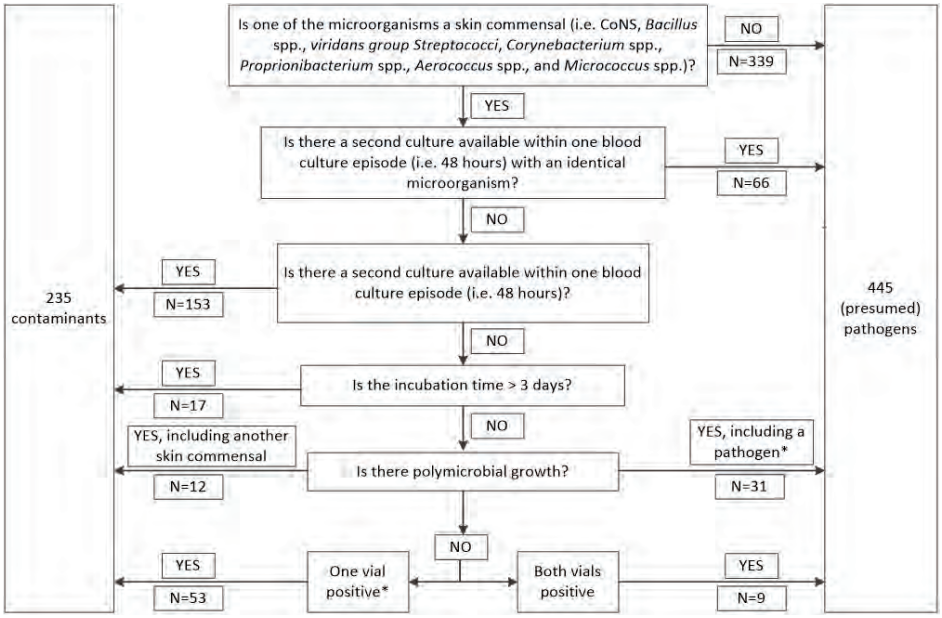


Figure S1. Classification of blood culture results. *Culture results with an obligate aerobe (*Micrococcus spp.*, n=5), were all classified as contaminants. Thirty-one cultures (in 27 patients) contained both a contaminant and a (presumed) pathogen according to this algorithm, and were classified as (presumed) pathogen in this figure.

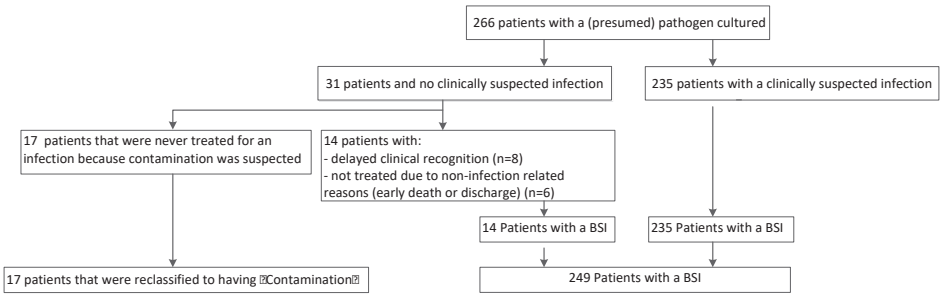


Figure S2. Adjudication of bloodstream infections and contamination in patients after aggregating blood culture results. *ICU:* intensive care unit. *BSI:* Bloodstream infection. Presence of a (clinically suspected) infection was based on the prospective data collection within the MARS study [1], and was based on the presence of sepsis or use of therapeutic antimicrobial therapy within the first two days of ICU admission.

[1] Klein Klouwenberg PM, Ong DS, Bos LD, et al. Interobserver agreement of Centers for Disease Control and Prevention criteria for classifying infections in critically ill patients. *Crit Care Med.* 2013; 41:2373–8.

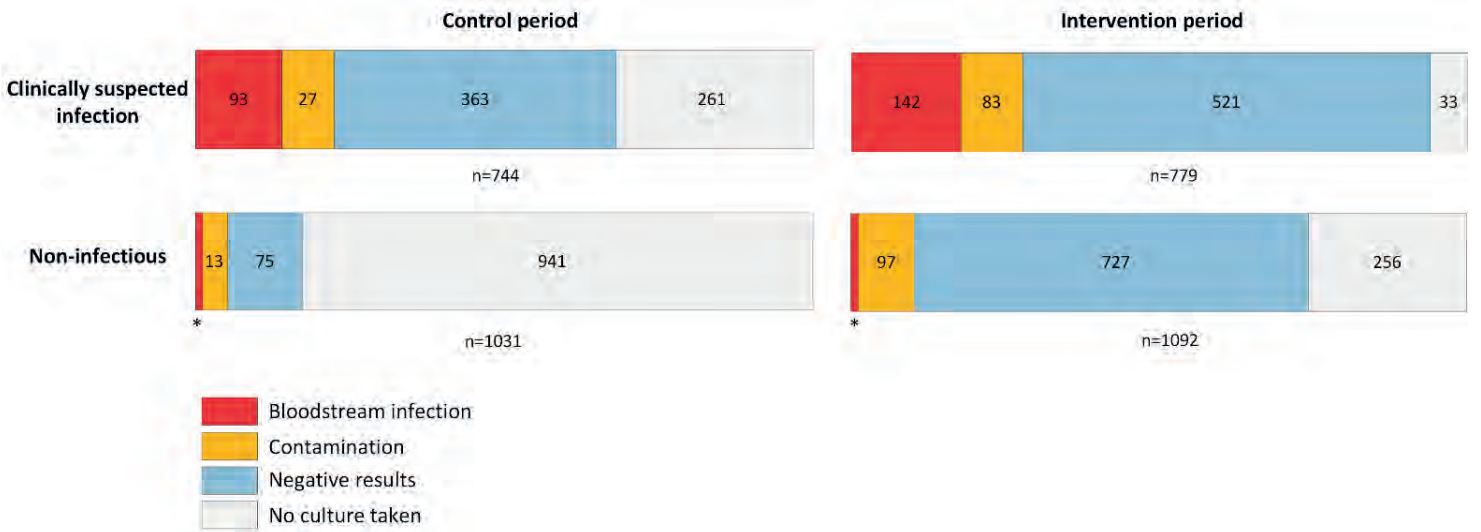
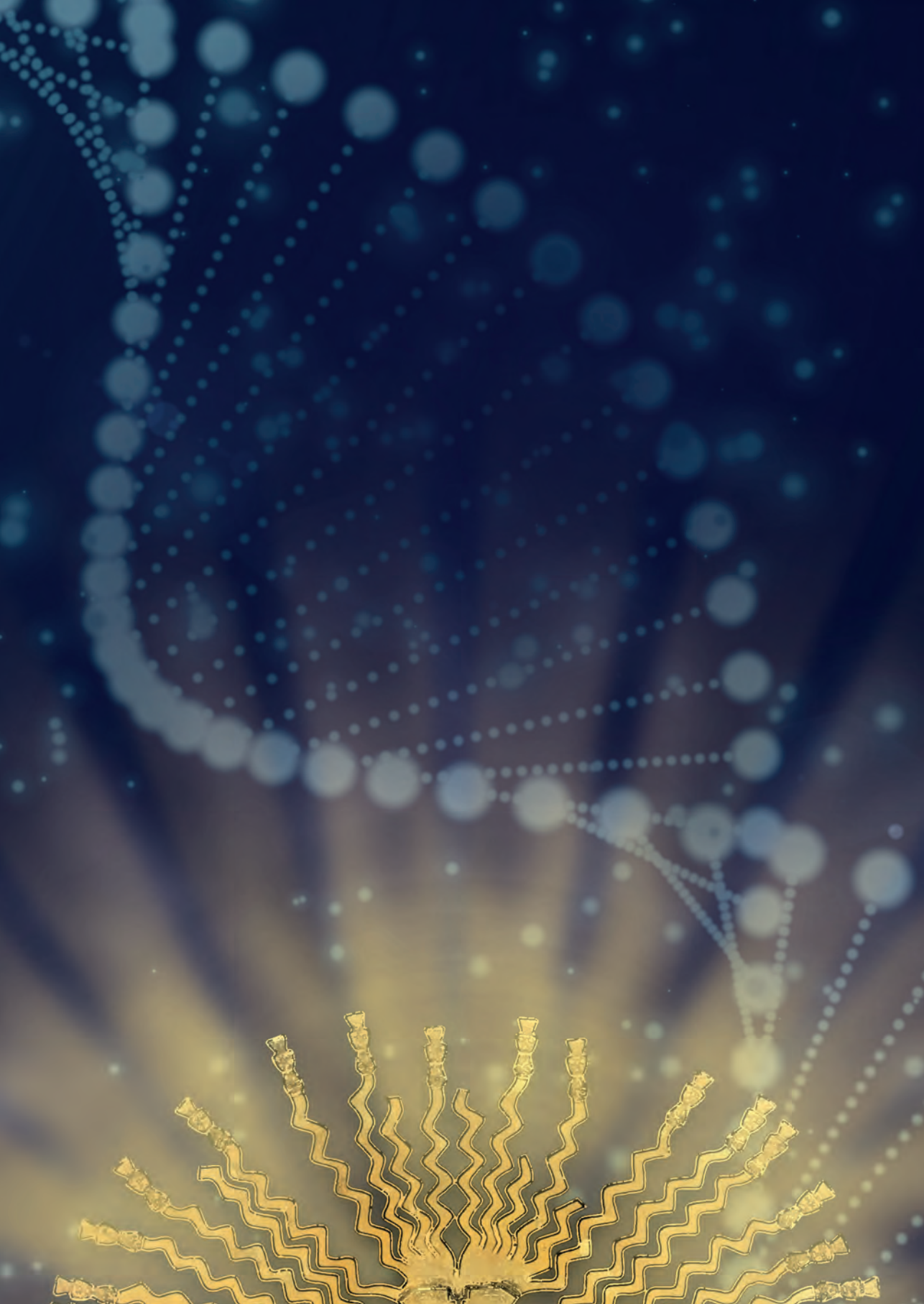


Figure S3. Included patients in the before and after period by clinically suspected infection. The box size represents the relative proportion of number of subjects within the particular subgroup. Clinically suspected infections include all infections in the first two days of ICU admission. *2 patients without clinical suspicion of infection had a BSI in the before period, compared to 12 in the after period.



O-serotype distribution of *Escherichia coli* bloodstream infection isolates in critically ill patients in The Netherlands

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Abstract

Objectives

Invasive infections by extra-intestinal pathogenic *Escherichia coli* (ExPEC) strains are increasing. We determined O-serotypes of *E. coli* isolates from ICU patients having bloodstream infections (BSI) and the potential coverage of a 10-valent O-polysaccharide conjugate vaccine currently in development for the prevention of invasive ExPEC disease.

Methods

We studied *E. coli* BSI among patients admitted to a tertiary ICU in the Netherlands between April 2011 and November 2016. O-serotypes were determined *in vitro* by agglutination and whole genome sequencing.

Results

Among 714 ICU patients having BSI, 70 (10%) had an *E. coli* BSI. Among 68 (97%) isolates serotyped, the most common serotypes were O25 (n=11; 16%), O8 (n=5; 7%), O2 (n=4; 6%), O6 (n=4; 6%), and O15 (n=4; 6%). The theoretical coverage of a 10-valent ExPEC vaccine was 54% (n=37).

Conclusions

A 10-valent ExPEC O-polysaccharide conjugate vaccine in development could potentially aid in the prevention of *E. coli* BSI in Dutch ICU patients.

Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a common pathogen causing bloodstream infections (BSI), frequently associated with antimicrobial resistance. Its incidence in hospitals across Europe is increasing, which is largely driven by an aging population [1,2]. Furthermore, *E. coli* is among the most frequently isolated pathogens among sepsis patients in the intensive care unit (ICU) [3].

As the development of new antibiotics has not kept up with the global increase in antimicrobial resistance, preventive strategies, such as vaccines, are needed. A multivalent ExPEC glycoconjugate vaccine, targeting 10 specific O-antigens located on the distal end of the lipopolysaccharide (LPS) of *E. coli* is currently under development. Its 4-valent predecessor was demonstrated to be both safe and *immunogenic in subjects* with recurrent urinary tract infections, as well as healthy adults [4,5].

Although more than 180 different O-serotypes have been described in *E. coli,* most ExPEC infections can be attributed to a smaller subset of O-serotypes. Previously, serotypes O2, O6, and O25 were reported to be the most common among invasive *E. coli* isolates obtained from urine and blood in the UK [6]. However, O-serotype distributions among invasive isolates may change over time and may differ according to age, source of infection, and geographical and clinical setting [6–8]. Furthermore, little is known about the O-serotype distribution among invasive *E. coli* isolates in ICU patients. In this report we describe patient and disease characteristics and O-serotype distribution in ICU patients with *E. coli* BSI in the Netherlands.

Methods

We identified all first occurrences of BSI caused by *E. coli* in a cohort of critically ill patients. Patients had been consecutively admitted to the mixed-ICU of a tertiary care hospital in the Netherlands, between April 2011 and November 2016. Data collection was part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study for which ethical approval was provided by the Medical Ethics Committee of the University Medical Center Utrecht, including an opt-out consent method (IRB No. 10-056C) [9]. Most likely sources of *E. coli* BSI had been recorded prospectively by researchers for each new antimicrobial therapy prescription. Previously reported risk-factors for *E. coli* BSI were retrieved, including the presence of solid tumors or hematological malignancies, chronic dialysis, chronic renal failure, underlying urological pathophysiology (including calculi, obstruction, and retention), recurrent urinary tract infections (UTI) (defined as the occurrence of 3 or more UTIs in the previous year), presence of a urinary catheter device (in the past two weeks for more than 48 hours), a previous solid organ transplant, and recent abdominal surgery (<30 days) [10,11]. Sepsis was defined as a sequential organ failure assessment (SOFA) score of 2 or more and septic shock was defined as the presence of sepsis and the need for vasopressors and a lactate >2 mmol/L [12]. BSI events were categorized as community-acquired if they occurred within 48 hours of

hospital admission, otherwise they were considered to have nosocomial onset. ICU-acquired BSI had an onset of 72 hours after ICU admission. We report recurrence of *E. coli* BSI until two years following the primary event.

A single clinical *E. coli* isolate was analyzed for each patient. BD BACTEC ™ (Becton and Dickinson Microbiology System, Sparks, MD, USA) blood culture bottles were incubated using a *BD BACTEC*™ blood culture system with automatic microbial growth signaling under 35 °C. Subsequently MALDI-ToF MS was used for pathogen identification. *E. coli* isolates were subsequently stored at -80 °C for later serotyping. Cultures yielding multiple species were considered polymicrobial (except for contamination caused by coagulase-negative staphylococci). Antimicrobial susceptibility testing was performed using phenotypic methodology, and the reference minimum inhibitory concentrations (MIC) provided by EUCAST. Individual *E. coli* isolates were categorized as susceptible or non-susceptible, which included isolates with intermediate susceptibility.

O-serotyping of *E. coli* isolates was conducted at the Pennsylvania State University (University Park, PA, USA) and Janssen Research and Development (Raritan, NJ, USA) by agglutination using O-antisera [13]. Due to an incomplete or absent LPS structure, *E. coli* strains can either respond to two or more antisera (i.e. multiple positive result), or not respond at all (i.e. negative result) in agglutination assays. *E. coli* isolates not typeable by agglutination were subjected to whole-genome sequencing (WGS) to allow for O-serotyping at the genetic level. The prediction of O-serotype from WGS was performed using O-serotyper v0.1, developed by Janssen Vaccines and Prevention. This tool uses the EcOH database to screen assembled contigs for allelic variants in O-antigen *rfb* cluster to infer *E. coli* O-serotypes using the *wzy*/*wzx* genes of published genomes with known *rfb* clusters [14–16]. Among the serotyped isolates vaccine coverage was estimated by calculating the percentage of *E. coli* isolates with an O-serotype included in the 10-valent O-polysaccharide conjugate vaccine that is currently in development. This vaccine includes the following serotypes: O25, O6, O2, O1, O75, O8, O15, O18, O16, and O4 [17]. Differences between community-acquired and nosocomial BSI were analyzed using the Wilcoxon rank sum, Chi-square or Fisher's exact test as appropriate. A p-value <0.05 was considered statistically significant.

Results

Of 9,660 admitted patients in the MARS cohort, 714 had a positive culture (excluding results suggesting contamination), and 70 (10%) had *E. coli* BSI of whom 34 (49%) were community-acquired, 36 (51%) were nosocomial, and 11 were ICU-acquired (Table 1). Patients with nosocomial *E. coli* BSI more frequently had indwelling urinary catheters and recent abdominal surgery, but other known risk factors for *E. coli* BSI were evenly distributed among patients with community-acquired and nosocomial infections. Median SOFA score was 10 (IQR 7, 13) at the day of *E. coli* BSI, and 24 of 70 patients (34%) died within 30 days after BSI.

Table 1. Characteristics of 70 patients and concordant strains with an *E. coli* bloodstream infection

Variable	Community-acquired (n=34)	Nosocomial (n=36)	P-value
Patient and disease characteristics			
Age (median IQR)	64 (56, 73)	65 (53, 71)	0.66
Male (n %)	18 (52.9)	24 (66.7)	0.24
APACHE IV score at admission (median, IQR)	102 (74, 132)	95 (80, 124)	0.36
Charlson comorbidity score (median, IQR)	2 (0, 3)	2 (0, 3)	0.51
SOFA score on the day of the BSI (median, IQR)	10 (8, 14)	9 (6, 13)	0.41
Septic shock on the day of the BSI (n, %)	24 (71)	24 (67)	0.72
Polymicrobial BSI (n, %)	8 (24)	6 (17)	0.47
Risk factors for E. coli BSI			
Solid tumor disease (n, %)	11 (32)	12 (33)	0.93
Hematologic Malignancy (n, %)	5 (15)	4 (11)	0.65
Chronic dialysis (n, %)	1 (3)	2 (6)	0.59
Chronic renal failure (n, %)	6 (18)	8 (22)	0.63
Underlying urological pathophysiology (including calculi, obstruction, and retention) (n, %)	5 (15)	2 (6)	0.20
Recurrent UTI's (3 UTIs in the last year) (n, %)	1 (3)	0 (0.0)	0.30
Urinary catheter device (in the past two weeks, >48hrs) (n, %)	5 (15)	30 (83)	<0.0001
Chronic catheter	5 (100)	1 (3)	
Solid organ transplant (n, %)	1 (3)	4 (11)	0.18
Recent abdominal surgery (<30 days) (n, %)	1 (3)	14 (39)	<0.0001
1 or more risk factors present (n, %)	19 (56)	26 (72)	0.46
Source of infection			0.61
- Intra-abdominal infection (n, %)	11 (32.4)	36)	
- Secondary peritonitis	3 (27)	10 (78)	
- Biliary tract	7 (64)	1 (8)	
- Other (translocation, primary peritonitis)	1 (9)	2 (15)	
- Urinary tract infection (n, %)	12 (35)	7 (19)	
- Pneumonia (n, %)	2 (6)	2 (6)	
- Skin or wound infection (n, %)	3 (9)	6 (17)	
- Other (n, %)	6 (18)	8 (22)	
Outcomes			
Recurrent *E. coli* BSI short term (<1 month) (n, %)	1 (3)	1 (3)	0.97
Recurrent *E. coli* BSI long term (<2 year) (n, %)	5 (15)	1 (3)	0.07
Total ICU length of Stay (median IQR)	3 (1, 12)	9 (3, 24)	0.02
30-day mortality (n, %)	10 (29)	14 (39)	0.40
1-year mortality (n %)	17 (50)	21 (58)	0.48
Strain characteristics			
Antimicrobial non-susceptibility (n, %)			
Gentamycin and/or tobramycin	4 (12)	6 (17)	0.56
Ceftriaxone	2 (6)	10 (28)	0.02

Table 1. Characteristics of 70 patients and concordant strains with an *E. coli* bloodstream infection (continued)

Variable	Community-acquired (n=34)	Nosocomial (n=36)	P-value
ESBL-production	2 (6)	9 (25)	0.03
Ciprofloxacin	5 (15)	9 (25)	0.28
Trimethoprim/sulfamethoxazole	16 (47)	14 (39)	0.49
Meropenem	0 (0)	0 (0)	NA
Colistin	0 (0)	1 (3)	0.33
O-serotypes (n, %) [a]			0.75
25	6 (18)	5 (15)	
15	3 (9)	1 (3)	
2	2 (6)	2 (6)	
6	2 (6)	2 (6)	
16	1 (3)	2 (6)	
8	3 (9)	2(6)	
75	1 (3)	2 (6)	
1	0 (0)	1 (3)	
18	0 (0)	1 (3)	
4	1 (3)	0 (0)	
17	2 (6)	1 (3)	
101	0 (0)	2 (6)	
78	2 (6)	0 (0)	
Non -typeable	1 (3)	2 (6)	
Other serotypes[b]	10 (29)	11 (32)	
10-valent vaccine coverage	19 (56)	18 (53)	0.81

APACHE=Acute Physiology and Chronic Health Evaluation, ICU=Intensive Care Unit, ESBL=Extended spectrum beta-lactamase, SOFA=Sequential Organ Failure Assessment. WGS=Whole-genome sequencing. Serotypes included in the 10-valent vaccine that is in development are shown in bold.

[a] Serotype percentages are based on the available strains (n=68).

[b] Other serotypes include: 162, 153, 117, 111, 107, 86, 77, 73, 68, 58, 45, 44, 23, 21, 13, 9 and 3. Non-typeable strains were either negative or multiple positive.

Nosocomial *E. coli* BSI were most frequently attributed to an intra-abdominal infection (n=13; 36%), in particular secondary peritonitis (n=10), whereas UTI was the most common source for community-acquired *E. coli* BSI (n=12; 35%). Recurrence of *E. coli* BSI within one month occurred in two patients (one with community-acquired and one with nosocomial BSI). An additional five patients with community-acquired *E. coli* BSI (15%) and one patient with nosocomial *E. coli* BSI (3%) had a recurrent BSI within two years (Table 1; p=0.07). Overall, antibiotic non-susceptibility was more prevalent among nosocomial BSI strains (Table 1). Of all strains, 12 (17% (95% CI 8%–26%) were non-susceptible to ceftriaxone and in all but one production of extended-spectrum beta-lactamases was demonstrated. All strains were susceptible to meropenem and one was non-susceptible to colistin.

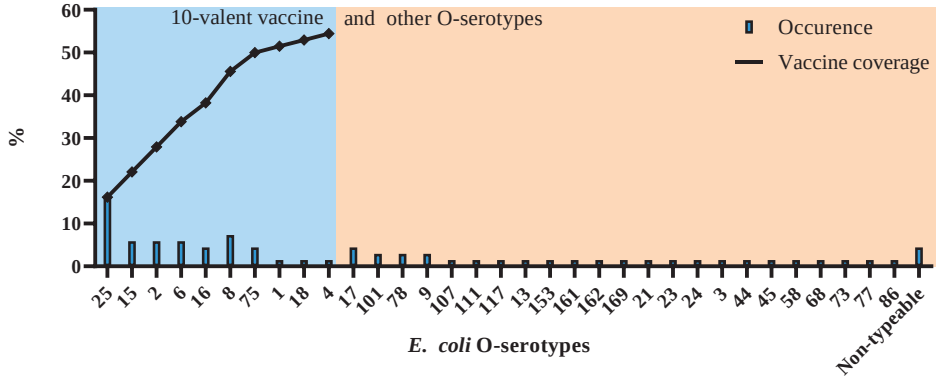

Figure 1. O-serotypes of the *E. coli* bloodstream isolates (n=68). Based on agglutination and whole-genome sequencing vaccine coverage was 54%.

Serotypes could be determined from 68 of 70 *E. coli* isolates (97%); agglutination yielded 58 O-serotypes, and 10 non-typeable strains. Of these 10 non-typeable strains, 9 were available for WGS, which yielded 7 O-serotypes, and 2 remained non-typeable. Therefore, in all there were 65 known O-serotypes (96%), and 3 non-typeable strains (4%) (Table 1). Overall, serotype O25 was most prevalent (11 isolates, 16%), followed by O8 (5 isolates, 7%), O2 (4 isolates, 6%), O6 (4 isolates, 6%), and O15 (4 isolates, 6%) (Table 1). In this study the theoretical coverage of the 10-valent vaccine that is in development was 54% overall; 56% for community-acquired and 53% for nosocomial *E. coli* BSI, (p=0.81, Fig. 1).

Discussion

In this cohort of *E. coli* isolates associated with BSI in 70 critically ill patients in a Dutch ICU the theoretical O-serotype coverage of a 10-valent *E. coli* vaccine that is in development was 54%. The coverage was similar for both community-acquired and nosocomial infections. In this population, O25 was the most prevalent serotype, which confirms findings in other disease settings [6–8].

The overall prevalence of third generation cephalosporin non-susceptibility (i.e. ceftriaxone) was 17% (95% CI 8%–26%) in this cohort, which is comparable to European surveillance data on blood and spinal fluid isolates (12–13.1%) [18]. The observed prevalence is higher than the average prevalence among *E. coli* blood culture isolates in the Netherlands [19], but reflects the prevalence in a critically ill patient population in an ICU of a tertiary care hospital that has been exposed to multiple antibiotics.

The rising incidence of invasive *E. coli* infections has prompted policy makers to target this infection for prevention. For instance, the UK's current national action plan aims to reduce gram-negative BSI (including *E. coli* BSI) by 50% in the next 5 years. As a considerable

proportion of invasive *E. coli* infections is community-acquired and predominantly occurring in older adults, generalized senior vaccination could be an effective measure [20]. To optimize vaccine strategies, additional risk-based approaches need to be explored. In the current study, 72.2% of patients with nosocomial infection and 55.9% of patients with community-acquired infections had at least one identifiable risk factor. Therefore, identifying patients with relevant comorbidities could be a starting point in selecting risk populations for the evaluation of prevention strategies.

This study has several limitations. Three strains could not be retrieved for serotyping by agglutination assay or WGS and calculations were based on the serotyped strains. Also, we did not examine sequence types (e.g. ST131) and O-antigen subtypes (e.g. O25AB). Furthermore, our results are not generalizable beyond the Dutch ICU population. Finally, our limited sample size precludes robust conclusions on risk factors for invasive ExPEC disease or O-serotype epidemiology. Further research is therefore needed for risk stratification of patients at risk for *E. coli* bloodstream infection.

In conclusion, the 10-valent ExPEC O-polysaccharide vaccine that is currently in development had a theoretical O-serotype coverage of 54% for *E. coli* isolates associated with BSI in Dutch ICU patients. Vaccine strategies to prevent *E. coli* BSI in critically ill patients should be further explored.

Notes

Ethics approval and consent to participate

The institutional review board approved an opt-out consent procedure (protocol number 10-056C).

Funding

D. Verboom and M. Bonten were funded by the Innovative Medicine Initiative (IMI), European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in-kind contribution (COMBACTE-CARE, grant agreement 115 620).

Potential conflicts of interest

TD, BM, are employees of Janssen Research & Development L.L.C. PIP and JP are employees of Janssen Vaccines & Prevention. PH was an employee of Janssen Vaccines & Prevention from 2012 to 2019. MB has received consultation and research fees from Janssen Pharmaceuticals, ED, ER, DV, MV, and OC declare that they have no conflicts of interest.

Acknowledgements

We thank the members of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) consortium for their contribution to this research.

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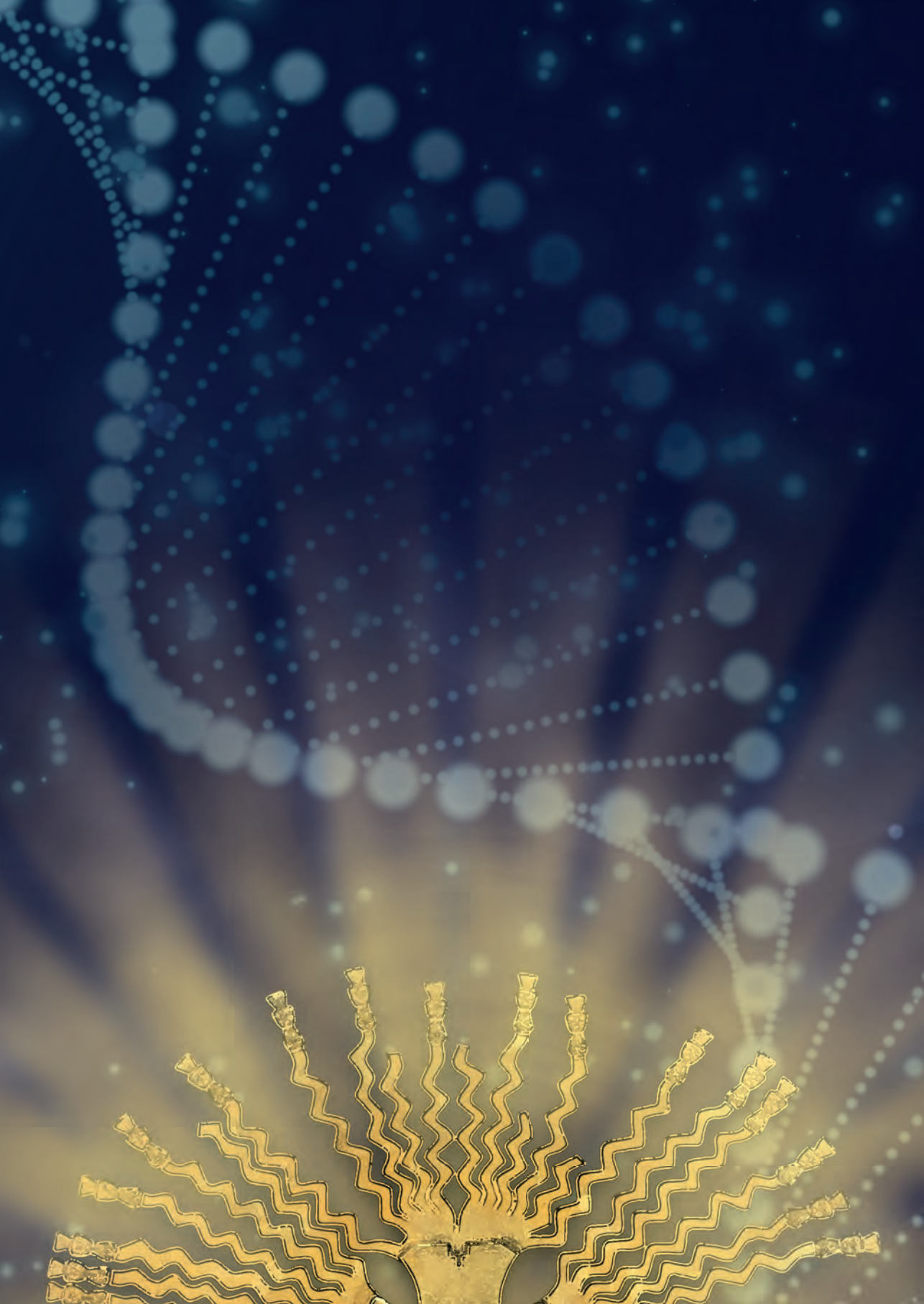

Summary and general discussion

Diana M. Verboom

Summary and general discussion

Sepsis is a complex syndrome, that involves physiologic, biologic, and biochemical responses to invasive infection. It has been difficult to capture the syndrome in definitions based on clinical symptoms. Also, the presence of infection is not always self-evident. Perhaps biochemical molecular methods will lead to a more consistent, accurate and faster diagnosis of sepsis [1]. The Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project was established to improve diagnosis and prognostication of sepsis, through development of molecular biomarkers. Many aspects that could influence the host response, such as gender, comorbidity and age have been studied within the project, which has attributed to our understanding of sepsis [2–6]. Furthermore, the MARS project has established four different sepsis endotypes. These were associated with traits, such as infection source and presence of septic shock, and clinical outcomes [7]. This type of endotyping might guide personalized patient management and assist patient selection for trials in the near future. Still, everyday clinical work is challenged by the early recognition of infection among already critically ill patients, impeding early and directed therapy. Previous work within the MARS project has focused on new strategies for fast pathogen detection in whole blood samples [8]. This thesis was written within the framework of this observational cohort aiming to evaluate tools to be used in the early diagnosis of sepsis. The purpose of this thesis was to evaluate a new host-response assay developed to diagnose infections in critically ill patients and to improve the understanding of bloodstream infections. This final chapter discusses our main findings, considerations and future perspectives of this thesis.

Towards more robust diagnostic criteria for sepsis

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. It has many clinical presentations and can evolve from a simple infection to a catastrophic disease. But the definition of sepsis is not unambiguous, as it cannot always classify patients as either a case or non-case. Presenting a case of a patient with purpura, hypotension and unconsciousness with a urine culture positive for *E. coli* to a group of clinicians would probably lead to an unonymous judgement of sepsis. However, in a rather confused patient that just suffered from a subarachnoid bleeding, moderate thrombopenia and a urine culture positive for *E. coli* the likelihood of having a sepsis would probably be deemed lower, as the symptoms could also be attributed to the subarachnoidal bleeding. Both, however, would fulfill the diagnostic criteria for sepsis.

SIRS was originally included in the sepsis definition, but appeared neither specific nor sensitive to predict poor outcome or infection [9,10]. SIRS was, therefore, subsequently excluded from the sepsis-3 definition in the 2016 consensus and replaced by organ failure, as operationalized by a SOFA (Sequential Organ Failure Assessment) score increase of 2 or more [11]. Although organ failure is also not pathognomic for the presence of infection, the

sepsis-3 criteria had predictive validity for unfavorable outcomes. They have shown to be able to select a group of patients at high risk of dying (mortality >10%) and with a prolonged ICU stay [12–16].

There is no diagnostic gold-standard test for sepsis, and this leaves room for different approaches in classifying cases in epidemiological studies which may result in inconsistent estimates of occurrence rates [17]. For example, we know that coding practices are generally more prone to changes in behavior and health policies, than extraction of clinical data from electronic health records [18,19]. Previously, diagnostic criteria for sepsis associated with organ dysfunction, hypoperfusion abnormality, or sepsis-induced hypotension consisted of a list of clinical signs and symptoms [20]. For sepsis-3 there is one clinical criterion for organ failure, which is the SOFA score. This score consists of clinical endpoints in 6 organ systems that together reflect the severity of organ failure. The SOFA score is a universally used, and widely validated prognostic score, and when calculated consistently, the score might promote consistent reporting of sepsis cases [21].

Previously, it was demonstrated that SIRS was susceptible to small changes in the definition [9]. In chapter 2 we assessed the robustness of the sepsis-3 criteria in an observational cohort study. This study included 1582 patients admitted to the ICU that were clinically suspected of infection. In this chapter we discuss how minor variations in the precise interpretation of the sepsis-3 criteria considerably impacted the apparent incidences of both sepsis and septic shock. These variations had been used at the time in several publications (all were consistent with the sepsis-3 consensus definition) and reflected different choices regarding the timing of organ failure assessment in relation to the onset of the clinically suspected infection. We therefore advocate a standardized use of the definitions, that is reproducible, and coding that is explicitly stated. We also feel that future guidelines should recommend a consistent time-window for identifying organ failure around suspected infection.

A limitation of our study was its restriction to an ICU population, and the presence of organ failure (i.e. SOFA scores) may be different in a pre-ICU setting. Also, we did not provide a "better" option or alternative. It was merely a demonstration of an effect that was to be expected. However, when not pointed out, in our opinion the lack of robustness of a definition is generally overlooked, and this can have large implications for future clinical practice and health policies. The way the definition of sepsis is used could cause a shift towards the inclusion of more patients that are less severely ill. This impedes a reliable sepsis surveillance and may affect implemented prevention strategies and nationwide sepsis quality measures [18].

We observed that virtually all patients with a suspected infection in the ICU met clinical criteria for organ failure, being non-discriminant in this setting. Nevertheless, the sepsis population is very heterogeneous when it comes to infection characteristics (i.e. pathogen, source), patient characteristics, and the incited host-response [7,17,22–24]. Also, infectious disease is difficult to distinguish from non-infectious systemic inflammatory syndromes, and

up to 33% of the patients with sepsis-3 that were treated with antimicrobial therapy did not have an infection in hindsight, as registered by the MARS researchers (chapter 2). To identify different subgroups within a sepsis cohort, and to achieve a more accurate infection diagnosis, we will need novel diagnostic methods. Omics technologies are potential sources of a huge variety of candidate biomarkers. Their value in clinical decision making is yet to be determined. In the next paragraph we will discuss how transcriptomics, or gene expression analyses, can be used for diagnosing sepsis.

Gene-expression to diagnose infection

There are two problems with the currently available tests for diagnosing sepsis. They are 1) not accurate enough and 2) not fast enough. C-reactive protein and PCT are the two most commonly used biomarkers in sepsis. Nevertheless, they have limited ability to differentiate between infectious and non-infectious causes of inflammation [25]. Many more single biomarkers (approximately 180) have been evaluated as diagnostic and prognostic markers of sepsis, yet none of these outperformed CRP or PCT, which led to a general belief that broader panels including multiple biomarkers might improve diagnostic accuracy [25–27]. With the development of the so-called 'Omics' technologies, it became possible to visualize an organism's physiological state by high-volume throughput data on transcription, protein synthesis and metabolites. Transcriptomics provide information on all present transcriptomes in a sample, or in other words, the RNA molecules in the cell that are ready to be transcribed into functional units, such as other RNA molecules or proteins [28]. The majority of the transcriptomic host response in sepsis is independent of the source and causative pathogen of the underlying infection and could be helpful to improve understanding of the underlying molecular pathways occurring in sepsis patients [6,29] Also, as transcriptomic changes occur very early in the inflammatory process, it might be possible to detect molecular evidence of pathophysiological derangement even before sepsis-related organ failure becomes clinically apparent [30,31]. If a test could rapidly and reliably discriminate between infectious and non-infectious inflammation, this would improve appropriate use of antibiotics, reduce the occurrence of complications, shorten length of hospital stay, and lower the costs of sepsis care [32].

Clinical utility of gene-expression assays for diagnosing infection

Once a transcriptomic profile is translated into an assay, or RNA-signature, it can be transformed into a test that can be validated for clinical use. In order to be useful in a clinical setting, it is important that such test has a fast turnaround time at an acceptable cost when compared to traditional biomarker panels. Several gene expression signatures have recently been introduced as a potential diagnostic tool [33–36], yet SeptiCyte™ LAB is the first gene expression assay available in the United States that was cleared by the FDA to distinguish sepsis from non-infectious causes of systemic inflammation in critically ill patients [37]. The

test consists of the simultaneous amplification of four RNA transcripts (CEACAM4, LAMP1, PLAC8, and PLA2G7) using a quantitative real-time PCR reaction on whole blood, with the technical potential to yield a result within 75 minutes. This assay provides a SeptiScore ranging from 0 to 10, with higher scores indicating a higher likelihood of infection. We performed two studies in which we compared the SeptiCyte LAB results to the clinical infection diagnosis, as established within the MARS cohort, to evaluate the potential diagnostic value that this test may have in critically ill patients. The clinical infection diagnosis was based on prospective adjudication by trained researchers using all patient, clinical, radiological, and microbiological data that became available during ICU stay. The likelihood of infection was adjudicated as being as none, possible, or confirmed, and this adjudication served as the reference standard to which SeptiCyte LAB results were compared.

In chapter 3 we report results from our clinical evaluation of SeptiCyte LAB in 467 patients admitted to the ICU with acute respiratory failure, that had been residing for more than 48 hours in the hospital. This cohort was chosen, as they reflect a population in whom infection is always part of the differential diagnosis, and would therefore qualify for a diagnostic test, such as SeptiCyte LAB. We found that SeptiScores were correlated to the likelihood of infection, and (when excluding cases with an uncertain infection diagnosis) we found a high sensitivity (96%). However, due to a large amount of false positive results we found a low specificity of 18%, and discriminative ability for infection did not outperform CRP. These results demonstrate that SeptiCyte LAB will not have added diagnostic value in patients that are admitted with acute respiratory failure after prolonged hospital admission.

Next, in chapter 4 we describe a pilot study that explored the ability of SeptiCyte LAB to diagnose infectious complications after esophageal surgery. We included 63 patients that underwent an esophagectomy and had a post-operative complication, and 100 patients that underwent esophagectomy without complications. Although SeptiScores varied widely between individuals, median scores immediately after surgery were comparable between subjects with and without a postoperative complication in their postoperative course. However, the increase of SeptiScore over time was greater in patients developing postoperative infections than in those with other complications. Our results suggest that SeptiCyte together with CRP could improve diagnostic performance in this setting. We recommend that future studies will further assess temporal change of clinical symptoms and biomarkers such as, but not limited to, SeptiCyte LAB (i.e. PCT, other potential RNA signatures), by daily monitoring and daily sampling of patients after high-risk surgery.

Chapter 5 is a review on the available gene-expression assays for sepsis, and an assessment of the studies performed on SeptiCyte LAB (including chapter 3 and 4). Overall, a review of the available studies suggests that SeptiCyte LAB has a consistent sensitivity of approximately 90%. Yet specificity is highly variable, depending on both the clinical setting and the criteria used to select patients for testing. Also, we observed that discriminative ability (as expressed by AUCs) is at least equivalent to PCT and CRP. Some studies showed a statistically improved

discriminative ability when SeptiCyte was used as an add-on diagnostic marker, together with CRP or PCT. Obviously, the cost-benefit ratio of such a strategy will be largely dependent on the additional costs of the novel biomarker. We also concluded that the available clinical performance studies are subject to bias and did not provide patient-based outcomes, precluding a definite assessment of clinical utility of the SeptiCyte LAB test. No real comparisons could be made to other RNA signatures currently available, as evidence on their clinical utility was even more limited and also biased. Furthermore, diagnostic performance of the current RNA signatures does not convincingly outperform established diagnostic markers for sepsis. Future efforts to evaluate the SeptiCyte LAB test, and other RNA signatures, should have a more pragmatic design. Pragmatic means that the study mimics the clinical conditions in which a test will function in patients with a real diagnostic dilemma, for example by consecutively enrolling patients within a certain domain, minimizing selection bias and overestimation of its diagnostic performance measures [38].

Gene-expression is a highly dynamic process, and we must consider that even gene-expression assays are but a simplified representation of the highly complex sepsis-associated host immune response. Further research on optimal applications of these biomarkers is thus warranted. The sepsis-associated host-response is characterized by a variety of pathophysiological pathways, with excessive inflammatory, catabolic, metabolic and immune-suppressive features [39]. Also changing gene-expression patterns in the same patient over time, besides being affected by secondary complications, are largely a result of recovery [35]. The sepsis-associated host response often results in sustained excessive inflammation and immune suppression. Some of these changes that occur after sepsis, are similar to changes in the host response after severe non-infectious injuries [39]. As gene-expression patterns in septic patients share similarity with those in patients with severe non-infectious injury, we should take into account that the diagnostic performance of a gene-expression assay when it comes to distinguish infection, from non-infectious inflammation, is time-dependent [30,33,35].

In our studies, patients were selected in a rather acute phase of sepsis (namely, the ICU admission, or when a complication became apparent). Indeed, this moment of clinical deterioration, is the most obvious onset of the "dysregulation" of the host response. Yet it would be valuable to collect expression patterns in infected patients, and non-infected patients, before dysregulation occurs. Future biomarker research should be designed with multiple samples taken over a longer period of time to enable appropriate analysis of early and time-dependent changes. These studies should transcend ward borders, and sampling should be performed in all settings from emergency departments, nursing wards and intensive care units. Patients should be included when they are at high-risk of developing an infectious complication, and biochemical processes of patients that develop an infection, could be compared to those with non-infectious inflammation.

To withheld antimicrobial therapy in critically ill patients suspected of sepsis, physicians need tests with high negative predictive value, to rule out sepsis. However, to be able to

further minimize antibiotic consumption, we will need tests with a higher specificity too, as the combination of high sensitivity and low specificity results in more false-positive results, and thus potentially more antibiotic usage [25]. The available evidence on the specificity of the SeptiCyte LAB test is not convincing. The test may be helpful as part of a combination of biomarkers, and in patients without a prolonged infectious course of disease before presentation (i.e. community-acquired and post-operative patients at high risk for infection). This should however be validated in prospective and pragmatic studies, assessing clinical outcomes such as earlier detection and treatment, shorter length of hospital stay, and reduced costs.

Bloodstream infections in the critically ill: exploring novel strategies for detection and prevention

Sepsis syndrome has many different clinical presentations that need specific management. In this thesis we dedicated two chapters to the detection and epidemiology of bloodstream infections. Although frequently used synonymously with bacteremia, sepsis is a different concept and will not always co-occur with a bloodstream infection. In fact, only 38–69% of septic patients have bacterial bloodstream infections, and not all bacteremic patients will have sepsis (i.e. a life-threatening organ dysfunction caused by a dysregulated host response to infection) [11,40] Approximately 5% of the patients will acquire a bloodstream infection during their ICU stay, and overall mortality in this group is between 35-50% [41,42]. Bad outcomes for patients with bloodstream infections are directly associated with delayed treatment and clinicians therefore aim for prompt initiation of treatment. When a patient is suspected of infection, several variables may be predictive for blood culture positivity, namely fever, chills, leukocytosis, suspected sepsis, or suspected endocarditis [43]. In part, some decision rules, seem to be able to select a population at high risk for true bacteremia and reduce negative cultures, however most of them have not been validated in an ICU setting, and are most likely not able to rule-out all BSI beforehand [40,44–47]. If we want to restrict the number of patients in whom a culture is taken, there always exist a risk that bloodstream infections will be missed.

Blood cultures should be taken in patients that are suspected of sepsis [48]. However, due to complex logistics and errors of communication in an acute setting, it can happen that a blood culture indication is missed within the first hours of presentation, also symptoms may develop over the course of time [49–52]. We encountered suboptimal culturing practices in our own center in patients acutely admitted to the ICU, and in response the department introduced a single blood culture for all patients, irrespective of the presence of suspected sepsis. In chapter 6 we describe a before-after analysis on the implementation of routine blood cultures for all admitted patients to the ICU of a tertiary care hospital. We observed that it was associated with an increase in the proportion of patients with a blood culture collected (from 32.3% to 84.5%) and a similar 1.5-fold increase in patients with a documented

bloodstream infection. Yet, routine blood cultures came at the expense of a 4.3-fold increase in patients with contaminated blood cultures and an increased number of blood cultures in the days following admission.

These results suggest that routine blood cultures improve BSI detection, at the cost of false-positive results. Of course, false-positive blood culture results are known to be associated with higher costs by increasing hospital length of stay, laboratory and pharmacy costs [53–55]. Yet, almost all these studies were performed before 2010, in settings without antibiotic stewardship, and they do not allow us to decide whether the potential negative effects are sufficiently balanced by the benefits of such an approach. As a higher rate of contaminated blood cultures is the trade-off for not missing BSI, we hypothesize that a more liberal culture strategy is cost-effective in an ICU where antimicrobial stewardship is implemented on a daily basis.

The results evaluating the effect of routine blood cultures upon ICU admission, should be interpreted in the light of its limitations. First, they might be not generalizable to settings without antimicrobial stewardship. Second, because of efficiency the implemented strategy consisted of the collection of a single blood culture set. This is a point for improvement as guidelines recommend two blood culture sets to achieve an adequate sensitivity [43]. Third, we did not evaluate whether the blood culture results changed the treatment decisions. As a result of these issues, a follow-up study is necessary to study the cost-effectiveness of such a practice and to evaluate whether routine blood cultures besides improved documentation, would expedite initiation of adequate therapy, potentially improving clinical outcomes.

Patients are worldwide becoming more susceptible to bacteremia due to increasing age and immunosuppression [57]. Also, antimicrobial resistance is increasing, and alternative strategies from antimicrobial treatment might be necessary, such as vaccination [58,59]. An O-polysaccharide vaccine against *E. coli* is currently under development. *E. coli* infections contribute to approximately 13% of the infections on the ICU, and about a third is resistant to one or more antimicrobial agents [60][61]**.** Therefore, in Chapter 7 we describe a cohort of patients with *E. coli* bacteremia on the ICU, and wanted to explore the potential vaccine coverage of this vaccine, by determining the O-serotype distribution in ICU patients with *E. coli* bacteremia. We found a theoretical serotype coverage of 54% for *E. coli* isolates associated with bloodstream infection in Dutch ICU patients; 56% for community-acquired and 53% for nosocomial *E. coli* BSI, (p=0.81). This estimate was based on combined results from agglutination assays and whole-genome sequencing (WGS). Results of agglutination assays resulted in a serotype distribution that would result in a potential vaccine coverage of 50%.

Our study describing an *E. Coli* cohort on the ICU, had a very limited scope, and potential vaccination strategies will have to be effectuated in a broader population, for example in patients with recurrent urinary tract infections or after gastro-intestinal surgery. In order to optimize vaccination strategies, additional risk-based approaches need to be explored. Only 73% of patients with nosocomial infection and 56% of patients with community-acquired

infections had at least one identifiable risk factor in our study. Identifying the right target population for preventive strategies might be complicated. We will need population-based studies, that include data that can be used for estimates of cost-effectiveness of different vaccine strategies.

Final considerations and future perspectives

This thesis discusses different diagnostic approaches for diagnosing sepsis. At this stage, we do not think there exists a one-size-fits all sepsis biomarker anymore. A broad range of advanced molecular diagnostics will aid clinical decision making in the future, and will improve our understanding of the different endo- and phenotypes in sepsis. Nevertheless, before biochemical profiling can be clinically implemented, further research is needed. I will now list some final considerations that relate to improving future diagnostics and management of sepsis and bloodstream infections.

- Diagnostic studies need to report diagnostic test characteristics in comparison (and in addition) to other more commonly available diagnostic tests and clinical data.
- To assess clinical validity of new diagnostic biomarkers we need pragmatic studies in a population with a true diagnostic dilemma [62].
- Future research is needed to evaluate clinical utility of novel diagnostic biomarkers by assessing cost-effectiveness, and clinical outcomes such as expedited diagnosis, adequate therapy, and hospital length of stay.
- As the performance of diagnostic biomarkers (and combinations) change over the course of sepsis, we also need studies with a longitudinal design and multiple sampling to evaluate performance over time [35,63].
- Advanced statistical approaches to develop decision-making models will be needed to integrate combinations of biomarkers and large volumes of data.
- We need high-throughput, high-quality and observational data. International multidisciplinary efforts are therefore necessary, and to facilitate this, policy makers should critically weigh the risk for individual privacy against expected benefits for public and individual health [64].
- Routine blood cultures on the ICU might improve BSI detection, and cost-effectiveness is likely to increase by the use contamination reducing strategies.
- The differences between the sepsis-associated host response and the host response after non-infectious injuries form a foundation for diagnostic biomarker discovery. The similarities, on the other hand, might offer possibilities for therapeutic interventions based on underlying pathophysiology, irrespective of the presence of infection [39].

In this thesis we demonstrate that novel molecular host-response assays could be of use in combination with other biomarkers in sepsis diagnosis. However, there is much to be done before clinical implementation is possible. The development and validation are hampered by

an imperfect reference standard, heterogeneity, and a fast and dynamic disease evolution. Future research efforts should be directed towards combinations of different diagnostic approaches, such as improved pathogen detection, clinical risk scores and daily monitoring of biomarkers. We will have to continue putting these novel methods to the test in large pragmatic studies, and assess whether they are indeed able to improve clinical outcomes.

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Appendix I
Nederlandse samenvatting

Nederlandse samenvatting

Sepsis is een disproportionele afweerreactie van het lichaam op een infectie. Het is een syndroom dat wordt gekarakteriseerd door orgaanschade. Het is de belangrijkste doodsoorzaak in ziekenhuizen. Wereldwijd maken ongeveer 21 miljoen patiënten sepsis door en afhankelijk van patiëntgebonden karakteristieken kan de mortaliteit oplopen tot 34%. Ongeveer de helft van de patiënt zal vanwege door sepsis uitgelokt orgaanfalen opgenomen moeten worden op een intensive care (IC) om de vitale functies te ondersteunen. Als patiënten een sepsis episode overleven, is er een aanzienlijk deel dat last krijgt van cognitieve en fysieke restverschijnselen. Om de ziektelast te verlagen is het belangrijk zo snel mogelijk te starten met de juiste behandeling. De behandeling bestaat uit antibiotische therapie, het wegnemen van een eventueel infectieus anatomisch substraat (e.g. een abces) en het ondersteunen van de vitale functies. Het is daarom erg belangrijk om sepsis in een vroeg stadium te herkennen en diagnostische tests te ontwikkelen die in een vroeg stadium kunnen vertellen of een patiënt septisch is.

Dit proefschrift zal zich richten op de classificatie van orgaanfalen bij sepsis, diagnostiek naar sepsis en de epidemiologie van bloedbaaninfecties in patiënten op de IC. In dit proefschrift wordt gebruikt gemaakt van data verzameld voor de MARS (Moleculaire diAgnose en Risicostratificatie van Sepsis) biobank. Deze biobank werd in 2011 gestart op twee IC's (het Universitair Medisch Centrum in Utrecht en het Academisch Medisch Centrum in Amsterdam) om het begrip van de gastheer-respons te vergroten en snellere en accuratere diagnostische instrumenten voor sepsis te ontwikkelen.

Classificatie van orgaanfalen

SIRS ("systemic inflammatory response syndrome") is een inflammatoire respons veroorzaakt door het immuunsysteem van de gastheer. SIRS wordt gekenmerkt door een afwijkende lichaamstemperatuur, een verhoogd of juist verlaagd aantal witte bloedcellen, een verhoogde hartslag en een snellere ademhalingsfrequentie. Omdat bij een invasieve infectie vaak SIRS optreedt en een invasieve infectie kan leiden tot sepsis, werd SIRS voorheen gebruikt in de definitie van sepsis. In 2016 werd er een nieuwe definitie van sepsis geïntroduceerd. In deze sepsis-3 definitie wordt sepsis gedefinieerd als een levensbedreigend orgaanfalen veroorzaakt door de immunologische respons op een infectie. Zowel SIRS als orgaanfalen komen ook veelvuldig voor in patiënten met weefselschade van niet-infectieuze origine, bijvoorbeeld bij postoperatieve weefselschade of brandwonden. Het is daarom moeilijk om in een vroeg stadium het onderscheid te maken tussen een infectieuze en een non-infectieuze oorzaak van orgaanfalen.

Er is geen gouden standaard voor het diagnosticeren van sepsis. Het kan hierdoor voorkomen dat de diagnostische criteria op verschillende manieren worden gehanteerd. Dit leidt ertoe dat epidemiologische studies verschillende resultaten opleveren, die puur worden

gedreven door verschillende manieren van het definiëren van sepsis en geen reflectie zijn van een verandering in de incidentie of verbeterde behandeling van sepsis. De sepsis-3 definitie maakt gebruik van de zogenaamde SOFA ("Sequential Organ Failure Assessment") score, waarbij wordt voldaan aan orgaanfalen als een patiënt een score heeft van twee of meer. Deze score evalueert orgaanfalen in zes verschillende orgaansystemen, waarin elk orgaansysteem wordt geëvalueerd met een score van nul tot en met vier.

In hoofdstuk twee beschrijven we een observationele studie naar de robuustheid van sepsis-3 in 1582 IC-patiënten met een verdenking op een infectie. We bespreken in dit hoofdstuk hoe het gebruik van kleine variaties in de definitie grote verschillen oplevert in de incidentie van sepsis-3. Daarentegen bleek de mortaliteit redelijk gelijk te blijven als er een andere groep patiënten werd geselecteerd. Het is daarom belangrijk dat het gebruik van de definitie expliciet wordt beschreven en dat elke onderzoeker de definitie en de data die daarvoor wordt gebruikt, op dezelfde manier hanteert. We zagen ook dat in deze studie vrijwel alle patiënten voldeden aan de sepsis-3 criteria voor orgaanfalen. Dit betekent dat deze criteria in deze populatie weinig bijdragen aan risicostratificatie. Sepsispatiënten zijn een heterogene groep. Deze heterogeniteit wordt veroorzaakt door grote verschillen in patiënt- en infectiekarakteristieken, maar ook door variatie in de immunologische respons op de infectie. Er zijn dus nieuwe diagnostische instrumenten nodig voor nauwkeurigere risicostratificatie en clustering van homogenere subgroepen van sepsispatiënten. Daarnaast blijkt een derde van de patiënten die worden behandeld voor een infectie achteraf geen infectie te hebben. Om onnodige antibiotische therapie te voorkomen, maar ook om infectieuze diagnoses niet te missen, zijn er nieuwe diagnostische tests nodig.

Het verbeteren van de diagnostiek naar sepsis

Met het ontstaan van nieuwe moleculaire technieken is het mogelijk geworden om een volledig beeld te krijgen van de moleculaire processen die in gang worden gezet bij ziekte. Nog nooit kon er zoveel informatie worden verkregen van de genetische expressie, de eiwitsynthese en het metabolisme in een septische patiënt. Door de analyses te automatiseren is het ook mogelijk om testen te ontwikkelen die bruikbaar zijn in een klinische setting. *Transcriptie* is een proces waarin genetische informatie van DNA wordt omgeschreven in RNA, voornamelijk ten behoeve van eiwitsynthese. Door de samenstelling van het transcriptoom te bepalen (d.w.z. een optelsom van alle transcriptiefactoren in een biologisch monster, als een reflectie van de transcriptie-activiteit) kunnen de verschillen op transcriptieniveau van twee patiënten met SIRS - met en zonder infectie - worden geïdentificeerd. Deze verschillen, veelal bestaand uit honderden genen, kunnen met behulp van statistische technieken worden omgezet in zogenaamde RNA-handtekeningen die bestaan uit een handzaam aantal RNA-genen, of transcriptiefactoren, die met behulp van PCR-technieken kunnen worden bepaald in de meeste gangbare ziekenhuislaboratoria. Een voorbeeld van een dergelijke test is de SeptiCyte LAB-test. Dit was de eerste RNA-handtekening die in de Verenigde

Staten werd geregistreerd door de FDA (Food and Drug Administration). De test meet de expressie van vier transcriptiefactoren (CEACAM4, LAMP1, PLAC8 en PLA2G7). Deze test wordt uitgevoerd op perifeer bloed en levert een score van 0 tot 10 op (i.e. de SeptiScore), die hoger wordt naarmate de diagnose sepsis waarschijnlijker is. In hoofdstuk drie, vier en vijf bespreken we de diagnostische waarde van deze biomarker in het vaststellen van een infectie in patiënten met SIRS.

In hoofdstuk drie beschrijven we de resultaten van een klinische evaluatie van SeptiCyte LAB in 467 patiënten die werden opgenomen op de IC na minstens 48 uur in het ziekenhuis te hebben verbleven en werden verdacht van infectie. De SeptiScores werden afgezet tegen de waarschijnlijkheid van infectie zoals deze werd vastgelegd in de MARS-database. De waarschijnlijkheid van een infectie werd uitgedrukt in vier categorieën oplopend in waarschijnlijkheid van onwaarschijnlijk tot een zekere infectie. De waarschijnlijkheid werd bepaald in alle gevallen dat er therapeutische antibiotica werd gestart door een arts bij een verdenking op een infectie. Getrainde onderzoekers werkten volgens vastgelegde richtlijnen en baseerden hun oordeel op klinische symptomen, medisch microbiologische bewijs en beeldvorming, die dagelijks werden besproken bij een multidisciplinair overleg gehouden op de Intensive Care. SeptiScores waren hoger als sepsis waarschijnlijker was volgens de MARS-database. De test bleek in bijna alle gevallen (96%) in staat om sepsis te diagnosticeren. Aan de andere kant waren er ook veel vals-positieve resultaten en was de specificiteit 18%. Ook bleek de test vergelijkbaar met CRP (een veelgebruikte en goedkopere bloedtest voor inflammatie) als het ging om het onderscheiden van patiënten met en zonder infectie.

In hoofdstuk vier onderzochten we of SeptiCyte LAB gebruikt kon worden om infectieuze complicaties te onderscheiden van non-infectieuze complicaties na slokdarmchirurgie. We analyseerden samples na slokdarmchirurgie in 63 patiënten die later een complicatie zouden ontwikkelen op de IC en 100 patiënten die geen complicatie zouden ontwikkelen. De SeptiCyte scores in de postoperatieve setting waren gelijk tussen de twee groepen. In de groep patiënten die een complicatie ontwikkelden, bleek de SeptiCyte meer te stijgen in patiënten met een infectieuze complicatie, dan in patiënten zonder infectie. Daarnaast nam het onderscheidende vermogen voor infectie toe als SeptiCyte werd toegevoegd aan CRP. Dit suggereert dat SeptiCyte gebruikt zou kunnen worden in een postoperatieve setting. Toekomstig onderzoek zou zich kunnen richten op een longitudinale monitoring van biomarkers in deze hoog-risicopatiënten.

Hoofstuk vijf is een overzicht van de beschikbare genexpressietesten ontwikkeld voor sepsis en een kwalitatieve evaluatie van de beschikbare studies van SeptiCyte LAB. SeptiCyte blijkt erg sensitief voor sepsis, maar het ontbreekt de test aan robuuste specificiteit. Anders gezegd, het gebruiken van de test zou leiden tot veel vals-positieve resultaten. Het onderscheidende vermogen lijkt tenminste zo goed als PCT en CRP, de huidige gebruikte testen in de klinische praktijk. Helaas is het nog niet gelukt om studies te doen in een setting die een goede afspiegeling is van de klinische praktijk. Er zijn ook nog geen klinische uitkomsten

geëvalueerd en er is dus nog geen basis voor het gebruik van de SeptiCyte LAB. Verder onderzoek zou zich moeten richten op klinisch bruikbare uitkomsten, zoals kosteneffectiviteit, antibioticagebruik en tijd tot de juiste diagnose.

Epidemiologie van bacteriëmieën

Sepsis is een syndroom met veel verschillende klinische presentaties, die elk specifiek management vereisen. In dit proefschrift worden er twee hoofdstukken gewijd aan detectie en epidemiologie van bacteriëmieën. Een bacteriëmie wordt gedefinieerd als de aanwezigheid van een bacterie in de bloedbaan. Sepsis wordt vaak in één adem genoemd met bacteriemie, echter zijn dit twee verschillende syndromen in hetzelfde infectieuze spectrum. Slechts 38-69% van de patiënten met sepsis heeft ook een aantoonbaar micro-organisme in het bloed. Daarnaast zullen niet alle patiënten met een positieve bloedkweek ook levensbedreigend orgaanfalen hebben ontwikkeld en dus septisch zijn. Vijf procent van de IC-patiënten zal een bloedbaaninfectie hebben bij opname, of ontwikkelen gedurende de IC-opname. Dit is tevens een groep met een zeer hoge mortaliteit, tussen de 35 en 50%. Snel starten met gerichte antibiotische therapie is essentieel voor goede klinische uitkomsten. Als een infectie bij een patiënt wordt vermoed, zijn er bepaalde variabelen die de kans vergroten op een positieve bloedkweek Voorbeelden zijn koorts, koude rillingen, een verhoogd aantal witte bloedcellen, aanwijzingen voor sepsis of endocarditis. Beslismodellen die deze variabelen gebruiken om te bepalen of er een bloedkweek moet worden afgenomen in patiënten, zijn niet in staat om een bloedbaaninfectie volledig uit te sluiten. Als men niet alle patiënten aan de poort wil kweken, zullen er dus altijd positieve bloedkweken worden gemist.

Als patiënten in een acute setting worden opgenomen, kan het voorkomen dat bloedkweken niet worden afgenomen in de hectiek van de complexe logistiek rondom overname. Daarnaast wordt een indicatie voor bloedkweekafname niet altijd direct herkend, of ontwikkelen symptomen zich pas in de loop van de opname. Op de IC in het UMC Utrecht, zagen we dat bloedkweken niet altijd waren afgenomen op het moment dat er een klinische verdenking was op een infectie en wilden we onderzoeken wat het zou opleveren om in alle patiënten die in een acute setting werden opgenomen standaard een bloedkweek af te nemen. In hoofdstuk zes beschrijven we de resultaten van deze analyse. We zagen een 50% toename van bloedkweken met pathogene verwekkers. Hiernaast zagen we ook viermaal zoveel gecontamineerde bloedkweken. We zagen hierbij geen toename van vancomycinegebruik op de IC of opnameduur. Deze resultaten suggereren dat het mogelijk loont om een standaard bloedkweek af te nemen in alle patiënten. Ook als er bij opname niet direct aan infectie wordt gedacht.

De incidentie van bacteriëmieën neemt wereldwijd toe, deels te wijten aan een toegenomen kwetsbaarheid van een ouder wordende populatie. Daarnaast neemt antimicrobiële resistentie onder pathogenen toe om de ziektelast in de toekomst te verlagen en zullen er alternatieve therapieën nodig zijn naast antimicrobiële therapie. Eén zo een alternatief dat

momenteel wordt ontwikkeld is een O-polysaccharide vaccin dat zich richt tegen tien veelvoorkomende *E. Coli* O-serotypes. De *E. coli* is een gramnegatieve bacterie die in de darmen voorkomt en één van de meest voorkomende veroorzakers is van infecties op de IC. In hoofdstuk zeven beschrijven we een cohort van *E. coli* bacteriëmieën op de IC. We beschrijven de potentiële dekking van dit vaccin door de O-serotypes te bepalen in een IC-populatie. Deze dekking bleek 54% te zijn en niet verschillend onder bacteriëmieën die waren opgelopen buiten (56%) en in het ziekenhuis (53%, p=0.81). Slechts 73% van de patiënten een infectie opgelopen in het ziekenhuis en 56% van de patiënten met een infectie opgelopen buiten het ziekenhuis, bleken één of meer risicofactoren te hebben. Dit zal toekomstige identificatie van de juiste populatie voor het vaccin hoogstwaarschijnlijk bemoeilijken. Om de juiste doelgroep te selecteren zal er in de toekomst data moeten worden verzameld over de kosteneffectiviteit van verschillende vaccinatiestrategieën.

Conclusie

Dit proefschrift laat zien dat moleculaire diagnostische tests gebaseerd op de gastheer response, zoals de SeptiCyte LAB-test, een rol kunnen gaan spelen in de diagnostiek van sepsis. Wel zal het nog enkele jaren vergen voordat deze testen toegepast kunnen worden in de dagelijkse praktijk, met name omdat er nog weinig patiënt-gerelateerde uitkomsten zijn geëvalueerd en de diagnostische waarde in de klinische praktijk nog niet bekend is. Daarnaast heeft dit proefschrift bijgedragen aan het begrijpen van het diagnostische proces van sepsis en de epidemiologie van bloedbaaninfecties op de IC. Toekomstig onderzoek zal zich moeten richten op het integreren van verschillende diagnostische benaderingen, zoals verbeterde pathogeen detectie, klinische risico-scores en dagelijkse monitoring van moleculaire processen in de septische patiënt.

Appendix II
Dankwoord

Dankwoord

" De omgeving van de mens is de medemens. "

J.A. Deelder

Een cliché op zijn tijd is nooit weg, soms ontzettend waar en eigenlijk best lekker. Zo schreef ik dit proefschrift niet alleen en werd ik omringd door mensen die hebben bijgedragen aan een waardevolle en bijzondere promotietijd. In dit hoofdstuk wil ik iedereen bedanken en sommigen in het bijzonder.

Marc, Professor Bonten. Met de welbekende "So what !?" vraag werd menig onderzoeksvoorstel terug naar de tekentafel gestuurd. Toch voelde ik me altijd gesteund en heb ik veel energie en enthousiasme gehaald uit de mogelijkheden die jij mij hebt geboden. Bedankt voor het vertrouwen en de kans om dit traject te volbrengen onder jouw toeziend oog.

Olaf, Professor Cremer. Je bent een betrokken (co-)promotor die mij nauw heeft begeleid en wiens lessen ik niet snel zal vergeten. Hout bewerken is jou niet vreemd en ook manuscripten worden door jou doortastend en nauwkeurig gesculpteerd tot het gewenste resultaat. Bedankt voor de tijd en aandacht die je hebt gestoken in mijn ontwikkeling. Bedankt dat jij met mij dit proefschrift tot stand wilde brengen en bleef bijschaven waar nodig.

Geachte leden van de beoordelingscommissie, prof. dr. D. van Dijk, prof. dr. N.P. Juffermans, prof. dr. H.A.H. Kaasjager, prof. dr. R.J.L. Willems en dr. M. van Smeden. Dank voor de tijd die u heeft genomen voor het beoordelen van mijn proefschrift.

Trialbureau, Ada, Joanne, José, Gea, Sandra. Zonder jullie hulp bij de dataverzameling was ik waarschijnlijk al vroeg in het proces verzopen. Het was me een waar genoegen om af toe te mogen binnenvallen voor een kletspraatje en een kop koffie. Het waren van die dingen, die mij zo thuis deden voelen op de kantoorgang.

David en Peter. Samen waren jullie de grondleggers van MARS. Wij konden bij jullie jaren later nog terecht voor vragen over de databases, scripts en voor adviezen. Peter, bedankt voor de begeleiding tijdens het schrijven van ons stuk. David, ook na je vertrek bleef je een graag geziene gast op F6. Je stond altijd voor de nieuwe MARS-ers klaar, ook als je niet direct meeschreef op onze stukken. Ik hoop jou dan ook nog vaak te mogen tegenkomen in het Franciscus Gasthuis.

VF-onderzoekers, Wietze, Ilse, Simone, Lisette, Jacqueline, Martine, Lynn en Tessa. Bedankt voor jullie nuttige bijdragen en gezelligheid. De ideale vergadertijd hebben we nooit gevonden, maar ik kijk met plezier terug op elk geslaagd vergadermoment.

Jos. De nestor, het geweten van MARS. Consciëntieus en kalm. Bedankt voor jouw oprechte interesse en de SAS-trucjes.

Ivo. Ik kan niet anders dan met een glimlach terugdenken aan al jouw kibbelmomentjes met Marlies en onze gesprekken op de fiets naar Lombok. Met jou hoefde ik me niet te vervelen want je had altijd wat te vertellen (en anders ik wel). Bedankt voor de gezelligheid.

Marlies. Jij had een doel voor ogen en was niet te stoppen. Ook niet tijdens de hormonale stormen omstreeks de zomer van 2017. Ik waardeer jouw betrokkenheid en kundigheid tijdens onze projecten. Ik heb geleerd van jouw scope die verder reikt dan de randstad, maar misschien belangrijker nog, dan werk.

Meri. Aan jouw kritische blik en relativerende droge humor heb ik veel gehad. Zeker toen ik al met één been in Zuid-Amerika stond, maar ook nog een proefschrift had om af te ronden.

Nikki en Emma. Bedankt voor jullie frisse blikken. Het afscheid van F6 was bitterzoet, maar met zulke gedreven en competente opvolgers heb ik alle vertrouwen in een mooie voortzetting van MARS en de geboorte van veel nieuwe onderzoeksprojecten.

Tessa en Denise. Het dynamische duo. Naast werk wisselden wij vooral van gedachten over sport, muziek of broeierige dichtkunst. Dit hielp mij om telkens weer vol nieuwe inspiratie en energie te beginnen aan mijn onderzoeksprojecten.

De WMM-groep. Samen deelden we koffie in het MiCafé, ECCMID, Madrid, colleges, (kerst)borrels en promovenski. Menige woensdagochtend werden hersenen gekraakt en werd er kritisch gespard. Wat heb ik gelachen en genoten van deze groep enthousiaste en ambitieuze collega's.

Elk sample voor MARS of ASPIRE werd ooit ontvangen en verwerkt in de illustere MMB-gangen. Dit ging niet altijd vlekkeloos, maar problemen werden gelukkig snel en effectief opgelost. Ik wil daarom de *MMB-analisten*, waaronder Daniel Kuis en Judith Vlooswijk, bedanken voor hun onmisbare hulp.

Kirsten. Jij was mijn kameraad in Utrecht. Met plezier denk ik terug aan de logeerpartijtjes, etentjes en borrels. Enige tijd tackelden wij samen de MARS en ASPIRE-obstakels en dat schept een band. Aan een half woord heb jij genoeg om te begrijpen waar ik vastloop in mijn denkproces. Bedankt voor jouw gezelligheid en steun de afgelopen jaren.

Marissa. Jouw huis en hart stonden altijd voor mij open. Als er stoom moest worden afgeblazen stond jij klaar met een flink bord eten en een glaasje wijn. Het waren constructieve jaren. Samen bouwden we Ikea-kasten, tuinhuisjes en feestjes. Bedankt voor jouw jarenlange vriendschap.

Misha. Vaak halen onze avonturen mijn rust-reinheid-regelmaat voornemens volledig overhoop. Het plezier ging soms ten koste van mijn schrijfuurtjes, maar heeft mij tevens ook door de moeilijke laatste momentjes geloodst. Zodoende, is jouw bijdrage aan dit proefschrift netto nul. Ik wil jou heel erg bedanken voor jouw aandeel in mijn leven.

Evelien. Van beugelbekkies tot cocktails nippen in Rotterdam. Bedankt voor jouw aanstekelijke enthousiasme en al die keren dat je voor me klaar stond.

Judith en Tessa. Toen spijbelen nog stoer was, en ik duidelijk niet, werden wij dikke vriendinnen. Jullie houden me met beide benen op de grond en het is altijd een feestje als we vrijmiborrelen, op weekend weggaan, of gewoon even een bakkie doen.

Pap en mam. Jullie houden van mij om wie ik ben en hebben mij altijd gesteund in mijn ambities. Toch leggen jullie nooit druk op mij. Ik dank jullie voor het warme nest dat jullie mij schonken. Ik dank jullie voor de vrijheid in denken, doen en laten die ik ervaar als jullie dochter.

Victor en Patricia. Mijn lieve broer en zus, ik ben trots op jullie. Bedankt voor jullie liefde, steun en aanwezigheid in mijn leven.

Erik. Mijn team- en reisgenoot. Met jou in mijn team, voel ik me lichter en lach ik meer. Ik wens nog veel meer jaren waarin wij genieten van elkaar en het leven waar we elke dag samen aan bouwen.

Appendix III
About the author

Curriculum Vitae

Diana Verboom (1990) was born in Dordrecht, the Netherlands. After finishing her secondary school at the Johan de Witt Gymnasium in 2008, she followed her medical training at the Erasmus University in Rotterdam. She wrote her research thesis on Tumor Necrosis Factor inhibitors in Behçet's disease with dr. Jan van Laar. Besides her medical training she obtained a Master's degree in Latin American studies at Leiden University. Her Master's thesis was on "Domestic Violence and structural support systems in Lima, Peru".

She developed a special interest for research, and wanted to broaden her scientific knowledge. Therefore she initiated her doctoral research within the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project in May 2015. She worked under supervision of Prof. dr. Marc Bonten and Prof. dr. Olaf Cremer, at the Julius Center of UMC Utrecht. Her research focuses on various novel diagnostic approaches for sepsis, including (but not limited to) the validation of a host RNA expression signature. Besides her work as a PhD candidate she assisted in teaching epidemiology and intensive care topics to medical students. Finally she obtained her Master's degree in Clinical epidemiology at Utrecht University in 2019. Afterwards, she travelled through South America for six months to indulge in the natural and cultural richness of this part of the world. In February 2020 Diana started to work at the Internal Medicine department of the Franciscus Gasthuis & Vlietland hospital where she is further developing her clinical skills.

List of publications

This thesis

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