Thesis

Sensitivity and Specificity of Sepsis Markers derived from Circulating Nucleic Acids

submitted by

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Declaration and Disclosures

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz."

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All co-authors had explicitly agreed to the use of their data in this thesis (written statements were submitted together with the thesis).

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Graz, 16 October 2020 Signature eh

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Contents

Abbreviations

AUROC *area under receiver operating characteristic curve*

bp *basepairs*

cfDNA *cell-free deoxyribonucleic acid*

ctDNA *circulating tumor deoxyribonucleic acid*

CNA *circulating nucleic acid*

CNS *central nervous system*

Cq Cycle of quantification

CRP *C-reactive protein*

DNA *deoxyribonucleic acid*

dPCR *digital PCR*

EGDT *early goal-directed therapy*

ESICM *European Society of Intensive Care Medicine*

FiO_2 *Fraction of inspired oxygen*

GRCh38 *current human reference genome*

ICU *intensive care unit*

IL-6 *Interleukin-6*

NPV *negative predictive value*

NOBI(C)S *novel biomarker invasive (candiasis) and sepsis cohort*

$PaCO_2$ *Partial Pressure of Carbon Dioxide in Arterial Blood*

PaO2 *Partial Pressure of Oxygen in Arterial Blood*

PCR *polymerase chain reaction*

PCT *Procalcitonin*

PPV *positive predictive value*

qPCR *real time PCR*

qSOFA *quick sequential (sepsis related) organ failure assessment score*

RNA *ribonucleic acid*

SIRS *systemic inflammatory response syndrome*

SOFA *sequential (sepsis related) organ failure assessment score*

suPAR *soluble urokinase plasminogen activator receptor*

Tm *melting temperature*

Figures

Tables

Abstract

Background

Sepsis is a life-threatening host response to infection. We are investigating circulating nucleic acids (CNA) as possible early diagnostic markers (motifs). The diagnostic value of CNA has been suspected since the 1970s, but only next-generation sequencing has made it possible to characterize CNA in detail. We hypothesize that specific DNA motifs exist in the plasma of patients who are developing sepsis, which can be used as markers for predicting sepsis at an early stage of the disease. These markers can be identified via Real-time PCR (qPCR) assays.

Material and methods

In this study, CNA were isolated and amplified from plasma of patients with sepsis, drawn concomitantly to positive blood cultures and compared with samples from controls. Via high-throughput Illumina paired-end sequencing with subsequent bioinformatics analysis of the CNA molecules, differences in the sets of CNA patterns between healthy controls and patients were identified. Subsequently, determination of these motifs by qPCR has been established as a method to predict sepsis at an early onset.

Results

We identified 24 molecular markers, based on circulating nucleic acids (CNA) which in combination can be used in a qPCR assay to identify the presence of human sepsis. Sepsis group included 147 samples from 69 patients who met SEPSIS-3 criteria. Control cohort consisted of 71 samples from 58 people including healthy volunteers as well as influenza and lymphoma patients. Overall balanced accuracy of the qPCR was 89.6% with a sensitivity of 93.8 % and specificity of 85.4%.

Conclusion

As our markers are host-based, they can be used to capture bacterial as well as fungal sepsis, unlike the current PCR-based tests, which require species-specific primer sets for each organism causing human sepsis.

Zusammenfassung

Hintergrund

Sepsis ist eine lebensbedrohliche Wirts-Reaktion auf eine Infektion. In dieser Studie wurden zirkulierende Nukleinsäuren (CNA) als diagnostische Marker (Motive) zur frühen Sepsis-Erkennung identifiziert. Seit den 1970er Jahren werden CNA als mögliche Marker in der Diagnostik von Erkrankungen gehandelt. Erst durch die Etablierung neuer Techniken wie „Next-Generation Sequencing“ konnten sie genauer untersucht werden. Wir nehmen an, dass im Plasma von Patienten, die eine Sepsis entwickeln, spezifische DNA-Motive vorhanden sind, die als diagnostische Marker in sehr frühen Stadien der Krankheit verwendet werden können. Zur Identifizierung dieser Motive wurde eine real-time PCR (qPCR) entwickelt.

Material und Methoden

Plasmaproben wurden gleichzeitig mit Blutkulturen von Sepsis PatientInnen abgenommen und mit Plasmaproben der Kontrollgruppe verglichen. CNA wurden aus Plasma isoliert und amplifiziert und mittels Illumina-Paired-End Methode sequenziert. Durch bioinformatische Analyse wurden Unterschiede in den Mustern von Erkrankten und Kontrollen charakterisiert. Anschließend wurde zur raschen Identifizierung jener Marker eine qPCR entwickelt.

Ergebnisse

Wir konnten 24 Motive identifizieren, die auf zirkulierenden Nukleinsäuren (CNA) basieren und in Kombination mit einer qPCR das Vorhandensein einer Sepsis zu einem sehr frühen Zeitpunkt nachweisen. Die Sepsis-Gruppe umfasste 147 Proben von 69 Patienten, welche die SEPSIS-3-Kriterien erfüllten. Die Kontrollkohorte bestand aus 71 Proben von 58 Personen, darunter gesunde Freiwillige sowie Influenza- und LymphompatientInnen. Die Treffergenauigkeit (balanced accuracy) der qPCR erreichte 89,6% bei einer Sensitivität von 93,8% und einer Spezifität von 85,4%.

Fazit

Durch den Wirt-basierten Ansatz ist es möglich die Entstehung einer Sepsis unabhängig vom verursachenden Erreger (Bakterien, Pilze) zu einem sehr frühen Zeitpunkt zu erfassen.

1. Introduction

1.1. Sepsis

1.1.1. Definition

Sepsis is a severe and life-threatening host-response to infection. For this reason, patient outcomes from sepsis are determined not only by the viability and virulence of the invading pathogen, but even more so by the host response, which may be excessive and result in organ and tissue damage. The pathobiology of sepsis is complex and heterogeneous. Until today, it is still not completely understood. The effort of defining this disease pattern implicates some problems. In 1991 initial definitions focused on the view that sepsis resulted from a host's systemic inflammatory response syndrome (SIRS) to infection (2). Temperature, heart rate, respiratory rate, $PaCO_2$ and the white blood cell count were the defining variables. But SIRS led to the following problems: The criteria were too sensitive – 93% of intensive care unit (ICU) patients had at least two SIRS criteria (3). Furthermore, SIRS could be caused by a variety of non-infectious clinical conditions, such as severe trauma, burns and ischaemic reperfusion events. A revision of the SIRS criteria at the International Sepsis Definitions Conference in 2001 failed. Despite the fact that SIRS criteria were overly sensitive and non-specific, the group of experts found – apart from expanding the list of signs and symptoms of sepsis – a lack of evidence for changing the definitions (4). In 2013 Vincent et al. appealed to a new definition of sepsis: *"Sepsis should be defined as a systemic response to infection with the presence of some degree of organ dysfunction"* (5). Finally, in 2016 "The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)" was published (6). According to the Sepsis-3 definitions, sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock should be defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a higher risk of mortality than with sepsis alone. Vasopressor requirement to maintain a mean arterial pressure of 65 mmHg or greater and serum lactate level greater than 2 mmol/L in the absence of hypovolemia are signs of septic shock. Assessing organ dysfunction, it is recommended to use the Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score. Organ dysfunction is defined by an increase of two points or more and associated with a higher in-hospital mortality. Outside the ICU, the simpler quick SOFA Score (qSOFA) provides easy-to-use bedside criteria to

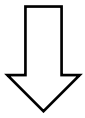
identify patients with suspected sepsis (due to infection) and probably poor outcome (Tab. 1.).

Tab. 1. Development of different diagnostic criteria and scores for sepsis

SIRS criteria, 1991, adapted from Bone et al. (2)

Two or more of:

- Temperature >38°C or <36°C
- Heart rate >90/min
- Respiratory rate >20/min or $PaCO_2$ <32mmHg
- White blood cell count >12 000/mm^3 or <4 000/mm^3 or >10% immature bands



Expanded diagnostic criteria for sepsis, 2001, adapted from Levy et al. (4)

Infection documented or suspected[a], and some of the following:

General variables

Fever (core temperature >38.3°C)

Hypothermia (core temperature <36°C)

Heart rate >90 min^{-1} or >2 SD above the normal value for age

Tachypnea

Altered mental status

Significant edema or positive fluid balance (>20 mL/kg over 24 hrs)

Hyperglycemia (plasma glucose >120 mg/dL or 7.7 mmol/L) in the absence of diabetes

Inflammatory variables

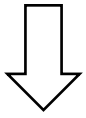
Leukocytosis (WBC count >12 000 μL^{-1})

Leukopenia (WBC count <4 000 μL^{-1})

Normal WBC count with >10% immature forms

Plasma C-reactive protein >2 SD above the normal value

Plasma procalcitonin >2 SD above the normal value



Expanded diagnostic criteria for sepsis, 2001, adapted from Levy et al. (4)

Hemodynamic variables

Arterial hypotension (SBP <90 mm Hg, MAP <70, or an SBP decrease

>40 mm Hg in adults or <2 SD below normal for age)

SvO_2 >70%

Cardiac index >3.5 $L*min^{-1}*M^{-23}$

Organ dysfunction variables

Arterial hypoxemia (PaO_2/FiO_2 <300)

Acute oliguria (urine output <0.5 $mL*kg^{-1}*hr^{-1}$ or 45 mmol/L for at least 2 hrs)

Creatinine increase >0.5 mg/dL

Coagulation abnormalities (INR >1.5 or aPTT >60 secs)

Ileus (absent bowel sounds)

Thrombocytopenia (platelet count <100 000 μL^{-1})

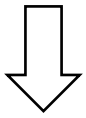
Hyperbilirubinemia (plasma total bilirubin >4 mg/dL or 70 mmol/L)

Tissue perfusion variables

Hyperlactatemia

Decreased capillary refill or mottling

WBC, white blood cell; SBP, systolic blood pressure; MAP, mean arterial blood pressure; SvO_2, mixed venous oxygen saturation; INR, international normalized ratio; aPTT, activated partial thromboplastin time. [a] Infection defined as a pathologic process induced by a microorganism



qSOFA and SOFA Score, 2016, adapted from Singer et al. (6)

qSOFA ≥2:

Respiratory rate ≥22/min

Altered mentation

Systolic blood pressure ≤100mmHg

qSOFA and SOFA Score, 2016, adapted from Singer et al. (6)

SOFA ≥2 (based on alterations of the following organ functions):

Respiration

PaO_2/FiO_2 ratio

Central nervous system

Glasgow Coma Scale Score

Cardiovascular

Mean arterial pressure

Administration of vasopressors with type and dose rate of infusion

Renal

Serum creatinine

Urine output

Liver

Bilirubin

Coagulation

Platelet count

1.1.2. Burden of disease

Sepsis is a major public health concern with an estimated number of 5.3 million deaths worldwide per annum (7). Although sepsis is the leading cause of death from infection, reliable epidemiological data is scarce, especially in Europe. A Norwegian survey found an annual population incidence of 140 patients per 100 000 inhabitants, ranging from 10 to 2 270 per 100 000 in different age groups during the years 2011 and 2012 (8). The predominance of men was statistically significant; this group reached an age-specific maximum annual incidence of 3 430 per 100 000 inhabitants. The overall hospital mortality rate for sepsis was 26.4%. Mortality increased with age and number of organ dysfunctions. A Spanish retrospective study describes increasing incidence rates of sepsis from 3.30 (between 2000 and 2004) to 4.28 (2005–2009) to 4.45 (2010–2013) per 1 000. The mortality rates increased from 6.34 to 7.89 per 10 000 over the three time periods. Average hospital cost per patient was calculated with € 9 090 during the whole study period (9). In 2014 approximately 10% of all US adult hospitalizations were due to suspected sepsis (10). Despite increased awareness and better identification of affected patients the mortality of

septic patients in ICUs has not changed noticeably in the last years. Tiru et al. estimate the average cost of care within a modern ICU setting to be $ 5 000 per day (11). One of the drivers of cost is of course the length of stay. In Europe the median length of a stay in an ICU is about 3.6 days longer than in the US (12). In summary, there is a huge economic and human burden of disease.

1.1.3. Management and Outcome

Early recognition and management of infection, hemodynamic issues and other organ dysfunctions are the most important measures in the management of these patients. To this effect, the international guidelines for management of severe sepsis and septic shock from 2012 (13) provide comprehensive recommendations. According to these guidelines, routine screening of potentially infected, seriously ill patients for severe sepsis is strongly recommended. There is a clear association between organ dysfunction and mortality (Fig. 1) (10). The early identification of affected patients and implementation of early evidence-based therapies improve outcomes and decrease sepsis-related mortality (Fig. 2) (14). The 2012 sepsis guidelines highly recommended protocolized resuscitation of sepsis-induced hypoperfusion within the first six hours with quantitative end points, a strategy called "*early goal-directed therapy (EGDT)*" (Tab. 2).

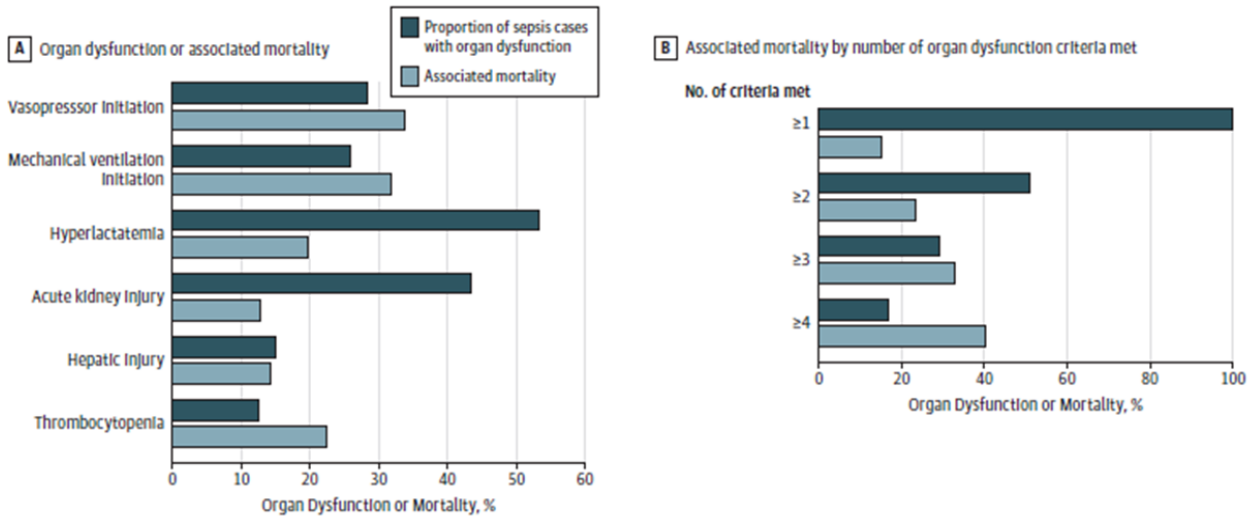


Fig. 1. Organ Dysfunction Distribution and Associated Mortality in Patients with Sepsis in 2014 and Associated Mortality by Number of Organ Dysfunction Criteria met. Reproduced from (10) with permission of American Medical Association.

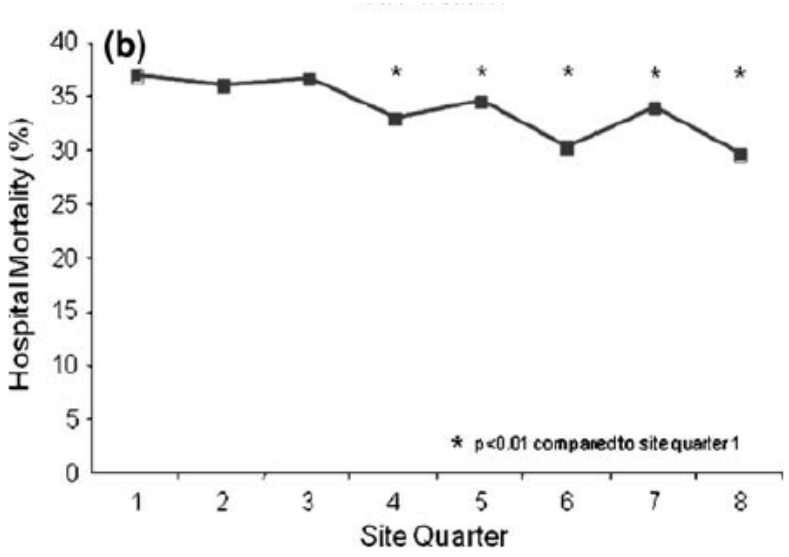


Fig. 2. Change in hospital mortality over time (*P<0.01 compared to first quarter) after implementation of a sepsis management bundle. Reproduced from (14) with permission of Wolters Kluwer Health, Inc.

Tab. 2. Goals of initial resuscitation of sepsis-induced hypoperfusion. Reproduced from (13) with permission Springer Nature.

(a) CVP 8–12 mmHg
(b) MAP ≥65 mmHg
(c) Urine output ≥0.5 mL kg h^{-1}
(d) Superior vena cava oxygenation saturation ($ScvO_2$) or mixed venous oxygen saturation (SvO_2) 70 or 65 %, respectively.
CVP, central venous pressure; MAP, mean arterial pressure;

Due the fact that three key randomized trials (PROCESS, PROMISE, ARISE) (15–17) suggest no difference in mortality between EGDT and usual care, the 2016 guidelines has removed these static EDGT targets. Frequent clinical reassessment and the use of dynamic measures predict fluid responsiveness better than static measures. Nevertheless, guidelines of both 2012 and 2016 still emphasize the importance of early administration of antibiotics. Sepsis is caused by infection, therefore managing infection is perhaps the most critical component of sepsis therapy. Administration of effective intravenous antimicrobial therapy should be initiated within an hour of recognition of severe sepsis and septic shock. Kumar et al. showed that each hour of delay after the first hour of documented sepsis-induced

hypotension was associated with a measurable increase in mortality (Fig. 3) (18). These findings were confirmed by a number of studies (19,20). Initial empiric anti-infective therapy should include one or more drugs that have activity against all likely pathogens (13). Under this point of view, the early diagnosis of sepsis is mandatory. We need reliable diagnostic tools with a high predictive value, because early detection of affected patients leads to rapid administration of therapy, which saves lives.

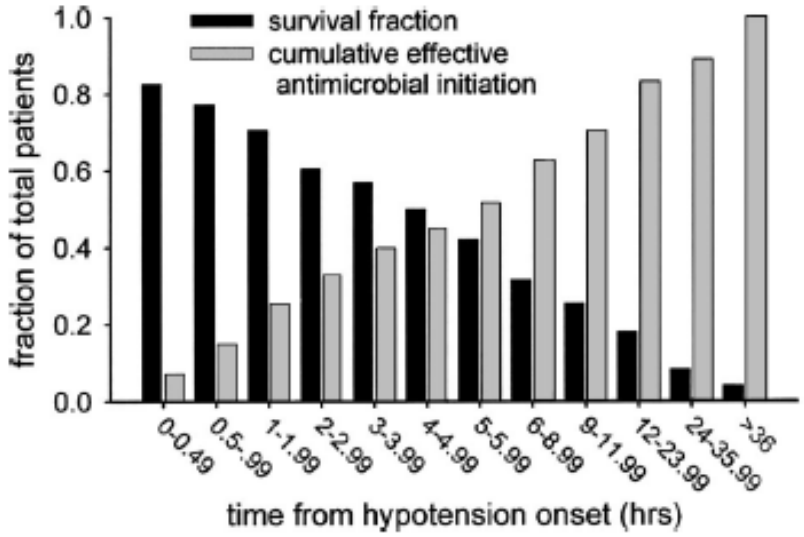


Fig. 3. Cumulative effective antimicrobial initiation following onset of septic shock-associated hypotension and associated survival. Reproduced from (18) with permission of Wolters Kluwer Health, Inc. The x-axis represents time (hrs) following first documentation of septic shock-associated hypotension. Black bars represent the fraction of patients surviving to hospital discharge for effective therapy initiated within the given time interval. The grey bars represent the cumulative fraction of patients having received effective antimicrobials at any given time point.

1.2. Diagnostic Markers and Scores

1.2.1. Diagnostic Scores

Until today, diagnosis of sepsis remains challenging. There is no reliable predictive marker or score, which detects affected patients with a high degree of certainty or at an early point in time. A lot of different scores and biomarkers are in use, but there is no single biomarker which is sensitive or specific enough to be relied on completely (21).

1.2.1.1. SOFA (Sepsis-related Organ Failure Assessment) score and qSOFA (quick SOFA) score

Already in the nineties of the last century the need for simple but objective criteria to define the degree of organ dysfunction has been recognized. In addition, time has been considered as a fundamental factor. The so-called sepsis-related organ failure assessment (SOFA) score was created by the European Society of Intensive Care Medicine (ESICM) in October 1994 (Tab. 3) (22). More than twenty years later The Third International Consensus Conference on the Definition of Sepsis and Septic Shock (Sepsis-3) simplified and established more feasible diagnostic criteria in form of a modified simple SOFA Score, called quick SOFA score (Tab. 4) (5). The Sepsis-3 guideline assumed that a SOFA or qSOFA score ≥2 points reflects an increased mortality risk of approximately 10%.

Tab. 3. SOFA Score. Reproduced from (22) with permission of Springer Nature

SOFA score	1	2	3	4
Respiration PaO_2/FiO_2, mmHg	<400	<300	<200	<100
			——— with respiratory support ———	
Coagulation Platelets $\times 10^3/mm^3$	<150	<100	<50	<20
Liver Bilirubin, mg/dl (μmol/l)	1.2–1.9 (20–32)	2.0–5.9 (33–101)	6.0–11.9 (102–204)	>12.0 (<204)
Cardiovascular Hypotension	MAP < 70 mmHg	Dopamine ≤5 or dobutamine (any dose)[a]	Dopamine >5 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central nervous system Glasgow Coma Score	13–14	10–12	6–9	<6
Renal Creatinine, mg/dl (μmol/l) or urine output	1.2–1.9 (110–170)	2.0–3.4 (171–299)	3.5–4.9 (300–440) or <500 ml/day	>5.0 (>440) or <200 ml/day

[a] Adrenergic agents administered for at least 1 h (doses given are in μg/kg·min)

Tab. 4. qSOFA Score

qSOFA Score, adapted from Singer et al., 2016 (6)

qSOFA ≥2:

- Respiratory rate ≥22/min
- Altered mentation
- Systolic blood pressure ≤100mmHg

Assessment of these clinical scores leads to the following findings: Among ICU patients with suspected infection, the predictive validity for in-hospital mortality of SOFA was statistically higher than SIRS criteria and qSOFA score, supporting its use in clinical criteria for sepsis. The score should be calculated at admission and every 24 hours until discharge, using the worst parameters measured during the prior 24 hours. Among patients with suspected infection outside of the ICU, the predictive validity for in-hospital mortality of qSOFA was statistically higher than SOFA and SIRS, supporting its use as a bedside test to consider possible sepsis. A retrospective analysis of 184 875 ICU patients reported that an increase of 2 or more points in SOFA score predicted a significantly higher in-hospital mortality than qSOFA or SIRS (AUROC, 0.75 vs. 0.60 vs. 0.59) (Tab. 5) (23,24). A meta-analysis from 2018 including 38 studies showed that qSOFA score had a higher specificity for predicting mortality from sepsis but a lower sensitivity compared to SIRS criteria. The sensitivity of qSOFA was higher in ICU populations than in non-ICU populations (87.2% vs. 51.2%); in contrast, specificity decreased in ICU populations (33.3% vs. 79.6%) (25). The value of the qSOFA score is still debatable. It is more a predictive tool that calculates the risk of death from sepsis than a diagnostic tool.

Tab. 5. Area under the receiver operating characteristic curve (AUROC) SIRS vs. qSOFA vs. SOFA. Reproduced from (23) with permission of American Medical Association.

				Between-Group Difference		*P*
	SIRS	qSOFA	SOFA	SOFA vs SIRS	SOFA vs qSOFA	Value
In-Hospital Mortality (Primary Outcome)						
Crude AUROC (99% CI)	0.589 (0.585-0.593)	0.607 (0.603-0.611)	0.753 (0.750-0.757)	0.164 (0.159-0.169)	0.146 (0.142-0.151)	<.001
In-Hospital Mortality or ICU Stay ≥3 Days (Secondary Outcome)						
Crude AUROC (99% CI)	0.609 (0.606-0.612)	0.606 (0.602-0.609)	0.736 (0.733-0.739)	0.127 (0.123-0.131)	0.131 (0.127-0.134)	<.001

1.2.2. Biomarkers and pathogen diagnostics

Sepsis is caused by the host's response to an infection. Due to the complex pathophysiology of sepsis, which involves almost all cell types, tissues, and organ systems, there are numbers of potential biomarkers and molecules under research (Fig. 4) (26). A few of them are used in clinical routine. Unfortunately, at present there is no "gold standard biomarker" to diagnose sepsis and no reliable way to assign risk profiles or predict the patient's outcome. The ideal biomarker can be obtained easily and determined in daily-routine laboratories. It

has a short turn-around time, a high sensitivity and specificity and is low-priced. It can be used to distinguish sepsis from non-infectious illness, to predict patient outcome and to determine causative pathogens to initiate the best possible treatment (Fig. 5) (27).

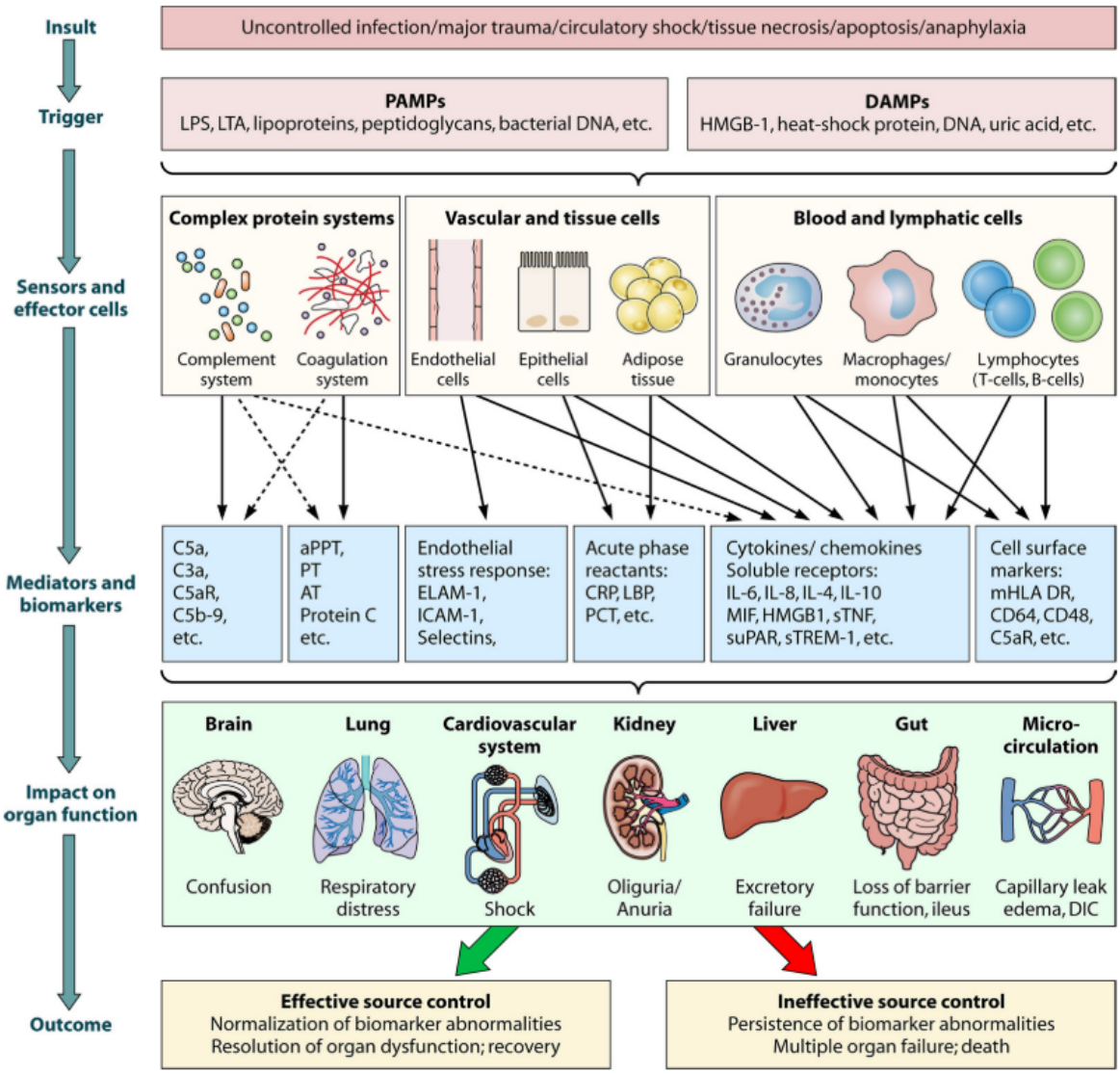


Fig. 4. The inflammatory response. This simplified overview shows the course of the inflammatory response. Reproduced from (26) with permission of American Society of Microbiology.

An insult triggers the release of PAMPs (pathogen-associated molecular patterns) and/or DAMPs (danger-associated molecular patterns), which are sensed by pattern recognition mechanisms such as receptors (pattern recognition receptors – PRRs) on the cell surface or within the cytosol or nucleus of sensor cells as well as by pattern-recognizing complex systems such as the complement system and others. Therefore, sensors can be different types of cells, tissues/organs, or proteins/other molecules, which themselves may function as effectors to modulate the immune response through various different pro- or anti-inflammatory mediators or biomarkers. As a result, the underlying insult can be cleared or not, and organ function may be temporarily or permanently impaired. LPS, lipopolysaccharide (part of the membrane of Gram-negative bacteria); LTA, lipoteichoic acid

(part of the cell wall of Gram-positive bacteria); HMGB1, high-mobility-group protein B1; C5a and C3a, complement components 5a and 3a; C5aR, C5a receptor protein; C5b-9, terminal complement complex; aPPT, activated partial thromboplastin time; PT, prothrombin time; AT, antithrombin; ELAM-1, endothelial leukocyte adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; CRP, C-reactive protein; LBP, LPS-binding protein; PCT, procalcitonin; IL-6, interleukin-6; MIF, macrophage migration inhibitory factor; sTNF, soluble tumor necrosis factor; suPAR, soluble urokinase- type plasminogen activator receptor; sTREM-1, soluble triggering receptor expressed on myeloid cells 1; mHLA-DR, monocytic human leukocyte antigen DR; CD64 and CD48, integral membrane glycoproteins; DIC, disseminated intravascular coagulation

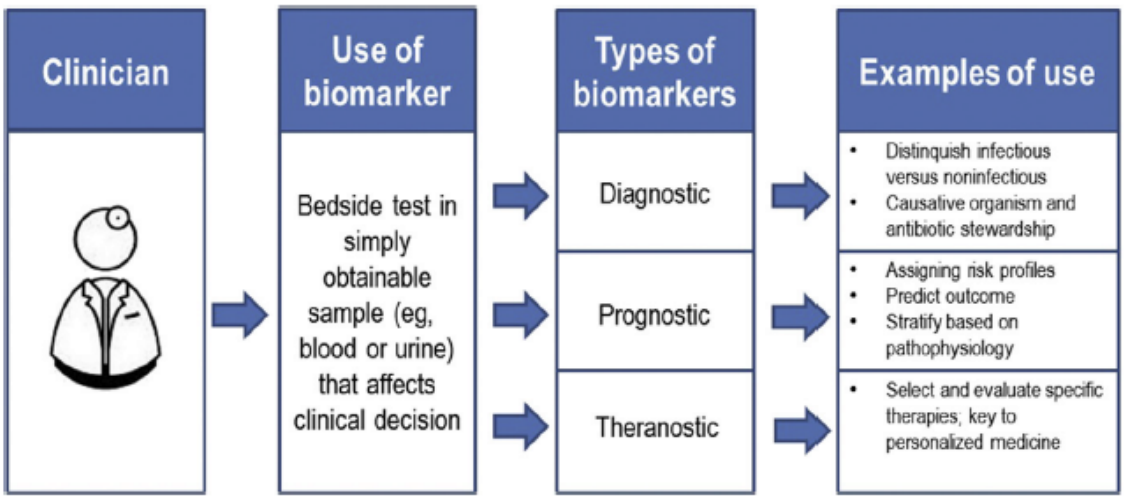


Fig. 5. The ideal biomarker. Reproduced from (27) with permission of Elsevier

What is a biomarker? Biomarkers are host characteristics, such as molecules or genes, by which particular physiologic or pathologic processes can be identified. When developing and validating novel biomarkers their potential clinical use is of utmost importance. Biomarkers can be used to distinguish sepsis from non-infectious critical illness or to determine causative pathogens to initiate the best possible treatment, thereby contributing to antibiotic stewardship. Furthermore, biomarkers can help to stratify patients based on risk profiles, and predict outcome or identify pathophysiologic pathways that can be the target for personalized therapy. Biomarker tests that select and monitor specific therapies are known as theranostics and are seen as a future aid for a targeted personalized approach in patients with sepsis.

1.2.2.1. Traditional (protein) biomarkers

Lactate

The German physician-chemist Johann Joseph Scherer (1841–1869) pioneered the first analysis of lactic acid in human blood (28). Already in 1927 Meakins et al. described the relationship between increased blood lactate levels and the presence of tissue hypoxia in

patients with circulatory shock (29). The accelerated anaerobic glycolysis and reduced mitochondrial metabolism occurring during shock led to increased lactate production. Many experimental studies have confirmed the relationship between tissue hypoxia and the elevation of serum lactate. On the other hand, Grocott et al. published their findings on the relationship between hypoxemia and lactate levels in climbers on Mount Everest. Despite chronic hypoxemia, none of the subjects in this study had clinically significant hyperlactatemia (30). So maybe lactate is not only related to poor tissue oxygenation. Nonetheless, lactate can be a helpful tool in predicting the development of multi-organ-failure in patients with septic shock (31). A cut-off value of serum lactate levels higher than 2 mmol/L seems to be adequate (32,33).

Lactate Clearance

Lactate clearance demonstrates the reduction of lactate concentration in blood, expressed as the removal from lactate in mL (volume) over time (minutes). It is calculated by the following formula: lactate clearance = $(\text{lactate}_{\text{initial}} - \text{lactate}_{\text{delayed}}) / \text{lactate}_{\text{initial}} \times 100$ (expressed as percentage) (34). A positive value indicates a decrease, a negative value an increase in lactate rate. Decreased lactate clearance is associated with increased mortality in patients with sepsis and septic shock. A study from Nguyen et al. showed an approximately 11% decrease likelihood of mortality for each 10% increase in lactate clearance (35). Authors conclude that lactate clearance obtained in the first 24 hours after ICU admission can be used as prognostic marker regarding mortality and outcome. Jansen et al. showed in their study better survival rates for patients who received a lactate guided therapy as for the control group (33.9% vs. 43.5%, $p= 0.067$). When adjusted for predefined risk factors, hospital mortality was even significantly lower in the lactate group ($p= 0.006$) (36). On the other hand, lactate metabolism is reduced in patients with severe acute or chronic liver disease and the value of lactate clearance is limited. Furthermore, lactate measurements can be confounded by the use of Ringer's lactate as a resuscitative fluid or exogenous causes of lactate production such as the use of metformin or large-volume packed red blood cell transfusion (37).

C-reactive protein (CRP)

C-reactive protein is an acute phase protein found in 1930 by Tillett and Francis in patients with pneumococcal pneumonia. It is produced by the liver with a maximum production 24–38 hours after the onset of inflammation. CRP binds to Gram-positive and Gram-negative

bacteria and stimulates their adhesion and complement dependent phagocytosis by leukocytes. It is a systemic marker of inflammation and tissue damage, but many different diseases and conditions can cause an elevation of CRP (Tab. 6) (38).

Tab. 6. CRP responses in disease. Reproduced from (38) with permission of American Society for Clinical Investigation.

Major CRP elevation in acute-phase response	
Infections	Bacterial Fungal Mycobacterial Viral
Complications of infection	Rheumatic fever Erythema nodosum
Inflammatory disease	Rheumatoid arthritis Juvenile chronic arthritis Ankylosing spondylitis Psoriatic arthritis Systemic vasculitis Polymyalgia rheumatica Reiter's disease Crohn's disease Familial Mediterranean fever
Necrosis	Myocardial infarction Tumor embolization Acute pancreatitis
Trauma	Surgery Burns Fractures
Malignancy	Lymphoma

	Carcinoma Sarcoma
Modest or absent CRP elevation in acute-phase response	
	Systemic lupus erythematosus Scleroderma Dermatomyositis Ulcerative colitis Leukemia Graft-versus-host disease

CRP has been considered a useful marker to distinguish bacterial from viral infection (39). The median concentration of CRP in healthy young adults is around 0.8 mg/L. But with certain stimuli (e.g., bacterial infection) values may increase more than 10 000-fold (40). For differentiating patients with bacteremia from patients without, the AUC for CRP was only 0.601 (Fig.7) (41). Due to the delayed release of CRP, a plethora of differential diagnosis and causes for an increase in CRP, it is not an ideal biomarker for the (early) diagnosis of sepsis. On the other hand, CRP increase is lower in patients with cirrhosis hepatis or other diseases causing liver dysfunction (42). Thus, CRP is not a reliable marker for infection in these patients.

Procalcitonin (PCT)

A promising biomarker in diagnosing bacterial infection and sepsis is procalcitonin. It is a protein consisting of 116 amino acids and a prohormone of calcitonin. It is secreted by C cells of the thyroid gland and can be identified in plasma within 2–3 h. Its concentration increases up to a maximum, typically after 12–48 h (43). Many studies and meta-analyses have assessed PCT as a helpful biomarker in early diagnosis of sepsis, especially in critically ill patients. Lower sensitivity and specificity was found in immunocompromised or neutropenic patients (44,45). In a study from Hönigl et al. PCT failed to predict bloodstream infections even at the lowest cut- off evaluated (i.e. 0.1 ng/mL) in 7% of patients (46). According to Tromp et al., 11% of bacteremic patients had a negative PCT (47). On the other hand, PCT can be elevated in different non-infectious conditions, such as in severe trauma,

surgical invasive procedures, and critical burn injury. Thus, false positive results are possible (typically after severe trauma or surgery) (48). In conclusion neither procalcitonin nor CRP fulfil the role of an ideal biomarker in the diagnosis of sepsis.

Interleukin-6 (IL-6)

IL-6 is an important cytokine during the acute phase reaction in response to inflammation and sepsis. It is particularly frequently used in paediatric wards. IL-6 is produced by different cells, like macrophages, monocytes, T-lymphocytes, epithelial cells, keratinocytes and fibroblasts. IL-6 acts as a differentiating factor for B-lymphocytes and as an activating factor for T-lymphocytes. The normal serum concentration of IL-6 is less than 5 pg/mL. Serum levels of IL-6 rise within 1 hour and peak within 2 hours after the infectious stimulus (Fig. 6) (21). A meta-analysis, including 2680 patients, provides evidence that the IL-6 test has only moderate diagnostic performance in differentiating sepsis from non-infectious SIRS in adults (49).

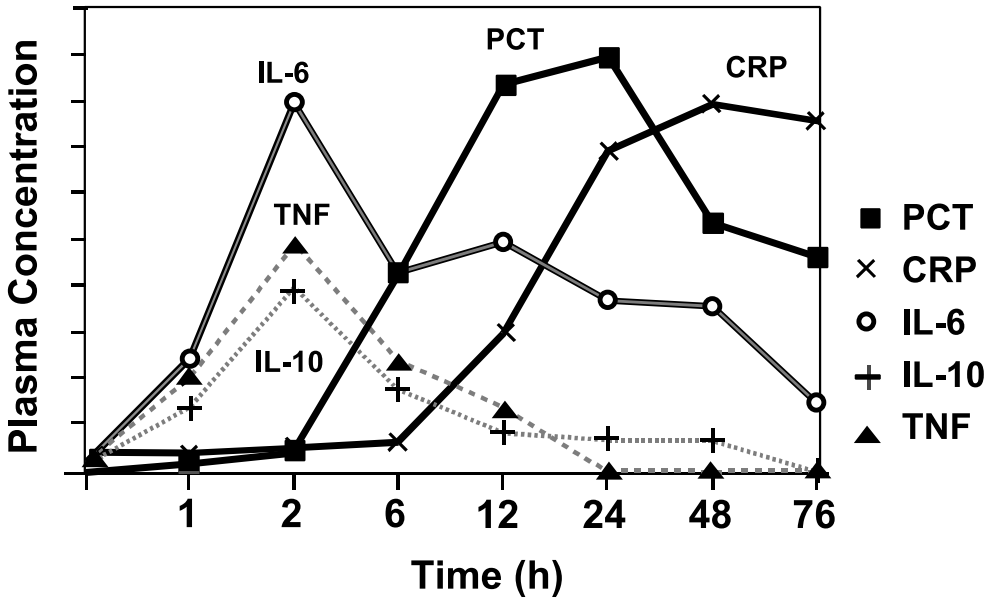


Fig. 6. Time course of induction of various parameters of the systemic inflammatory system after stimulus (thoracic surgery). Reproduced from (21) with permission of Elsevier.

Soluble urokinase plasminogen activator receptor (*suPAR)*

SuPAR is the soluble form of the urokinase plasminogen activator receptor (uPAR). The receptor is present on a variety of immunologically active cells, like monocytes, activated

T-lymphocytes and macrophages, but also on several other body cells. SuPAR can be measured in plasma, urine, blood, serum and cerebrospinal fluid and reflects “activation” of the immune system. SuPAR serum levels are increased due to a number of pathological conditions, like bacteremia, SIRS, sepsis, malaria, CNS infection and different forms of cancer (41,50). Furthermore, studies indicate that high levels of suPAR are predictive for mortality (51,52). For differentiating patients with bacteremia from patients without suPAR, PCT and IL-6 were nearly equal (AUC 0.726, 0.744 and 0.735, respectively); the AUC for CRP was 0.601 (Fig.7) (41).

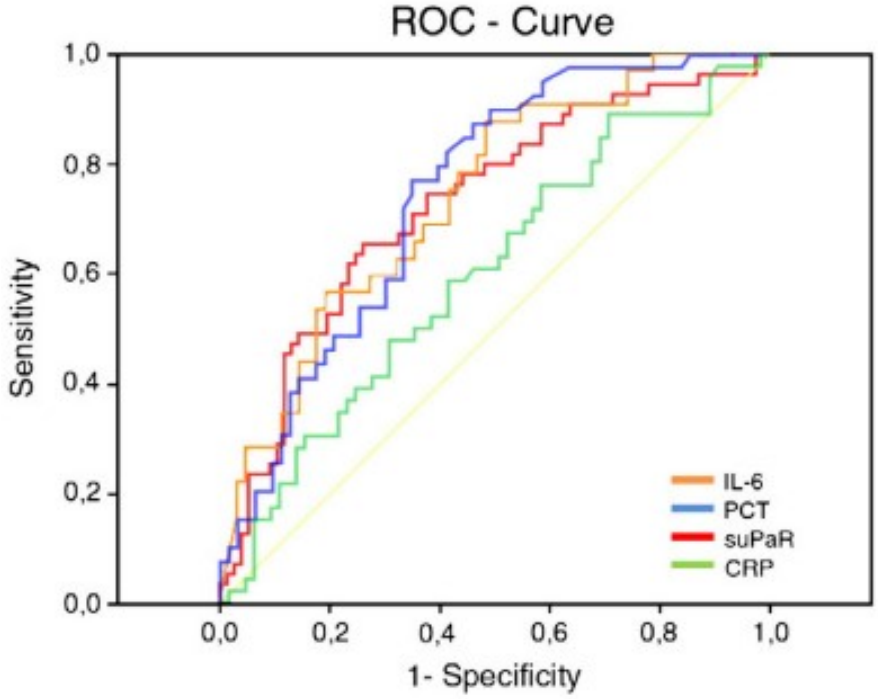


Fig. 7. ROC curve analysis: suPAR, PCT, IL-6 and CRP for differentiation between positive and negative blood cultures in SIRS patients. Reproduced from (41) with permission of Elsevier.

Presepsin (soluble CD14 subtype)

Presepsin is the generic name of the soluble CD14 subtype. CD14 is a receptor for lipopolysaccharide-binding protein complexes and is expressed on the myelomonocytic cell surface. It activates a series of signal transduction pathways and inflammatory cascades and it promotes the response to bacterial infections (53). The concentration of presepsin in plasma increases within 6 hours after bacterial infection, peaks after 3 hours, and declines

after 4–8 hours (54,55). Presepsin values are much higher in patients with sepsis and severe sepsis than in healthy comparators (Fig. 8) (53,56). An important limitation of the study of Shozushima et al. is the low number of participants (normal, 22; local infection, 28; SIRS, 41; sepsis, 87; and severe sepsis, 14, referring to Fig. 8). Presepsin could be a helpful marker in early risk stratification. A study from Masson et al. indicates higher levels of presepsin on day one in sepsis-non-survivors than in survivors (57) compared to PCT.

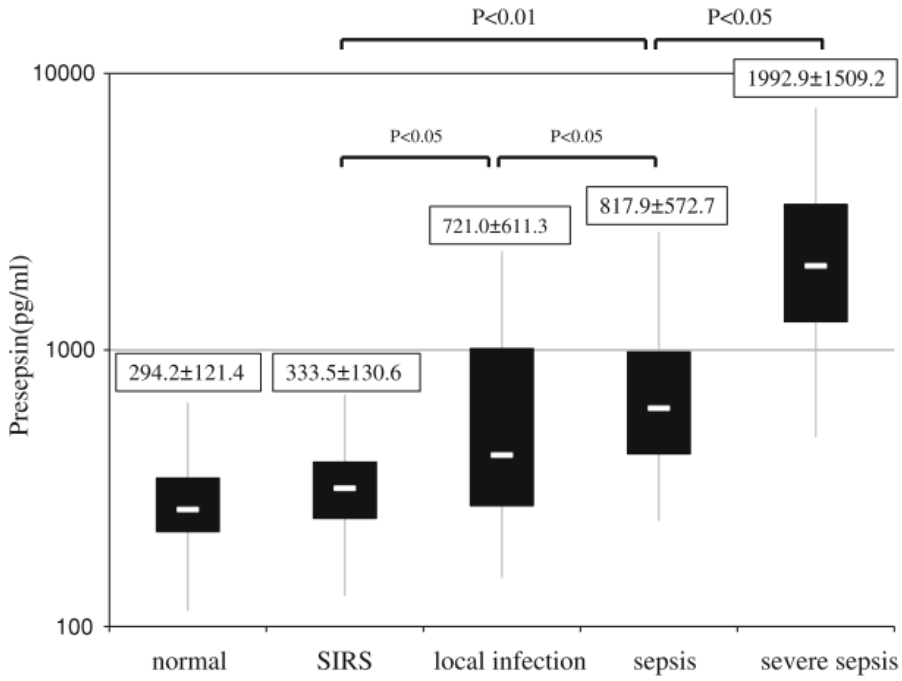


Fig. 8. Comparison of presepsin value in different pathological conditions. Reproduced from (53) with permission of Elsevier.

1.2.2.2. Blood cultures

A blood culture is defined as a specimen of blood obtained from a single venipuncture or intravenous access device. Blood cultures are frequently used as the "gold standard" diagnostic method for bacteremia and fungemia. Both are within the most common causes of sepsis. Culture of blood is usually the most common method for detection of bacteremia or fungemia and subsequent antimicrobial susceptibility testing. Due to increasing drug resistance problems, antibiotic stewardship efforts and, for a targeted antimicrobial therapy, knowledge of the causing pathogen and its resistance pattern is mandatory. For any patient, who is suspicious for bacteremia or fungemia, blood cultures are indicated and should be obtained. Microbiological pathogen detection is significantly higher if blood cultures are

taken prior to antimicrobial therapy (58). However, it usually takes two to seven days until blood cultures turn positive. Most clinically significant bacteremia and fungemia cases are detected within 48 hours with modern automated blood culture detection systems. Both, common and fastidious pathogens are usually detected within five days; only anaerobes sometimes benefit from an extended incubation period (59,60). A significant number of patients with sepsis never develop positive cultures. Positive blood cultures can be found for only approximately 30% of septic patients (61). Due to various reasons, blood cultures frequently yield low positive results. Besides the fact that some pathogens cannot be cultured in blood cultures (e.g. *Bartonella sp.*, *Coxiella sp*., *Bordetella sp*., *Legionella sp*., *Mycoplasma sp*., etc.), drawing an adequate volume of blood (40-60 ml) seems to be crucial for pathogen detection (62,63). Table 7 lists causes affecting the yield of microbiological cultures and leading to possibly false negative results (64). Furthermore, detection of fungi and anaerobic bacteria can be more challenging regarding time and sensitivity. In addition, even in patients whose cultures will finally be positive, there is a time lag until the results are available.

Tab. 7. Factors affecting yield of microbiological cultures. Reproduced from (64) with permission of Springer Japan KK.

Intermittent bacteremia
Small number of detectable colony-forming units
Bactericidal properties of blood components
Volume of blood drawn (optimum 20 ml)
Concomitant antibiotic use
Number of blood samples cultured
Timing of blood cultures
Length of incubation time
Culture media and collecting system use

1.2.2.3. Molecular and mass spectrometric approaches

In the last few years the search for biomarkers has shifted its focus from traditional protein and cytokine markers to molecular approaches. A number of molecular approaches and methods to improve conventional culture-based identification, including PCR, have been investigated and already partially established. One approach is to shorten the time between positive blood culture and identification of the pathogen. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS) decreases the time to result to 3 hours once the blood culture has become positive (65). The application of MALDI-TOF MS directly to positive blood cultures is still in the experimental phase but has the potential to identify a broad range of organisms. In their Lancet paper, Tissari et al. describe the extraction and amplification of microbial nucleic acids from positive blood cultures and subsequent hybridization on a microarray platform to detect genes of the 50 most common Gram-negative and Gram-positive bacterial species. Additionally, the identification of methicillin resistance was also possible. This method identified bacterial species about 18 hours before conventional culture methods did (66). There are several multiplex PCR platforms available detecting microorganisms directly from positive blood cultures within one to two hours. One example is a multiplex PCR-based platform, the BioFire® filmarray system (bioMerieux, Marcy-l'Étoile, France) uses nested multiplex PCR to identify 19 bacterial pathogens, 5 yeasts and 3 resistance markers directly from positive blood cultures. In addition to pathogen identification, this assay also tests for the presence of *mecA, vanA/B* and *Klebsiella pneumoniae* carbapenemase genes in the blood culture broth. It provides results in about 1 hour with 2 minutes for assay setup. A large clinical study revealed a sensitivity of 97.3% and a specificity of 99.8% for the identification of Gram positive bacteria, 98.1% and 99.9% for Gram negative bacteria and 99.2% and 99.9% for the identification of *Candida* sp. (67). The BioFire® filmarray system also provides testing for samples from the respiratory tract (sputum, including endotracheal aspirate and bronchoalveolar lavage) and from the gastrointestinal tract (stool samples).

But there are several possible sources of false positive results in nucleic acid-based assays, as molecular methods may detect the genetic material present in non-viable organisms. Unfortunately, most of these test methods are unable to be applied directly from blood. Therefore, incubating the blood cultures until they turn positive is still necessary. For the correct interpretation of the obtained results, a close collaboration between clinical microbiologists and clinicians is mandatory.

1.3. Circulating nucleic acids (CNA)

Circulating nucleic acids are cell-free DNA (cfDNA) or RNA (cfRNA) which is found in the liquid portion of biological fluids, like serum or plasma (68). CNA were first described by Mandel and M'Etais in 1948. Almost twenty years later, in the late sixties and early seventies of the last century, the appearance of cell-free DNA in adult humans was estimated to be pathological (69,70). In 1977 Leon et al. detected elevated CNA levels in cancer patients and described them as possible prognostic marker (71). One of the first successful applications of CNA-based assays occurred in 1997 for non-invasive prenatal diagnostic purposes, when Lo et al. first detected fetus-derived Y sequences in plasma samples of pregnant women (72). However, only the introduction of new technologies like Next-Generation DNA Sequencing has made it possible to characterize CNA in detail for the first time. CNA are present in a number of clinical disorders like cancer, stroke, trauma, myocardial infarction, autoimmune disorders, sepsis and pregnancy-associated complications (73–78). This opens a wide field for research. Using high-throughput sequencing with subsequent bioinformatics analysis of CNA molecules, the origin of particular CNA in the human genome can be determined, and differences in the pattern sets between healthy and diseased individuals can be characterized.

1.3.1. CNA as diagnostic tool

The amount of CNA is increased in patients compared to healthy controls, which indicates that elevated levels of CNA are a marker for pathophysiological conditions in general (79). The detection and isolation of circulating tumor DNA is a revolutionary technique in oncology and leads to detection and monitoring approaches, which have been termed "liquid biopsies" (80). Circulating tumor DNA (ctDNA) offers information about tumor burden, tumor evolution, mutations, copy number alterations, epigenetic alterations and fusion genes (80,81). CtDNA is a helpful tool in disease monitoring in terms of prediction of treatment response or recurrence (82). Analysing ctDNA enables the detection of mutations associated with resistance to different anti-cancer therapies (83). In prenatal diagnostics, CNA-based assays are used for fetal sex assessment, Rhesus genotyping, and fetal chromosomal aneuploidy detection via non-invasive procedures (84,85), even though false positive and false negative results are possible (86).

1.3.2. Size and Source

CNA are highly fragmented molecules. The size of the majority of them ranges between 80 and 200 base pairs (bp) (Fig. 9) (87). CNA mostly appear in a complex of histones, called nucleosome, which protects CNA from enzymatic degradation (88). In healthy individuals the most common size of histone complexed cell free DNA is around 166 bp but there have been found molecules in size of more than 10,000 bp, indicating that length may depends on the frequency of cell apoptosis and necrosis, as well as different metabolic processes out of the cells (89).

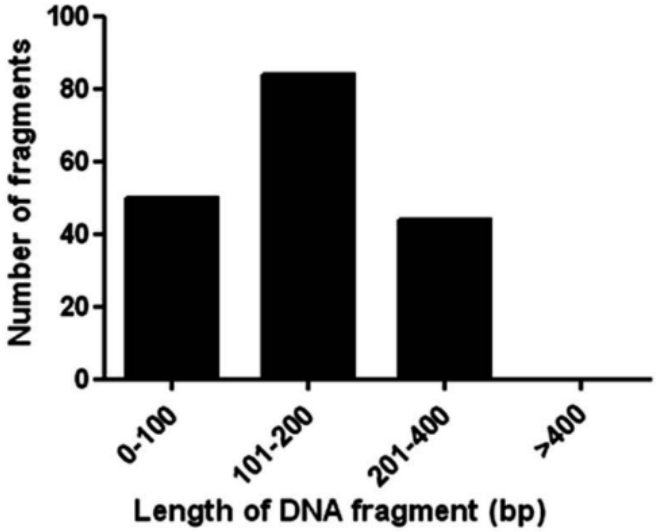


Fig. 9. Size distribution of cfDNA isolated from plasma in transplanted patients. Reproduced from (87) with permission of Elsevier.

Another major fraction of CNA is packaged in extracellular membrane vesicles where they are protected from degradation. Based on their size and origin they are divided into apoptotic bodies, microvesicles, and exosomes (Fig.10) (90). Production of microvesicles is associated with imbalances in the distribution of lipids on the plasma membrane. Apoptotic bodies are secreted by apoptotic cells, whereas exosomes are released via fusion of multivesicular bodies with the plasma membrane of cells (91). These circulating exosomes contain large fragments of DNA ranging from 100 bp to 17 kbp (92). Figure 11 shows that CNA can be actively released by secretion as well as through apoptosis and necrosis (93). Furthermore, CNA can arise from tumor cells, immune cells or other body organs. Genetic and epi-genetic alterations like point mutations, rearrangements and copy number alterations can be detected

in CNA (Fig. 11; see also section 1.3.1.). Biology and function of cell free DNA molecules are still not fully understood.

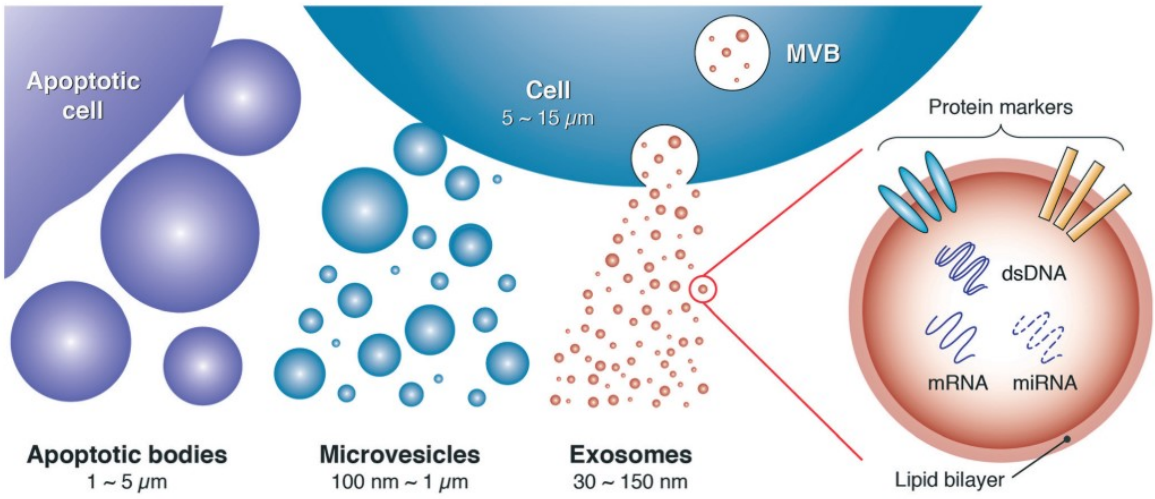


Fig. 10. Exosomes represent a subset of extracellular vesicles with characteristic size in the ∽30–150 nm range. MVB endosome-multivesicular body; dsDNA double stranded DNA; mRNA messenger RNA, miRNA micro RNA; Reproduced from (91) with permission of The Royal Society of Chemistry.

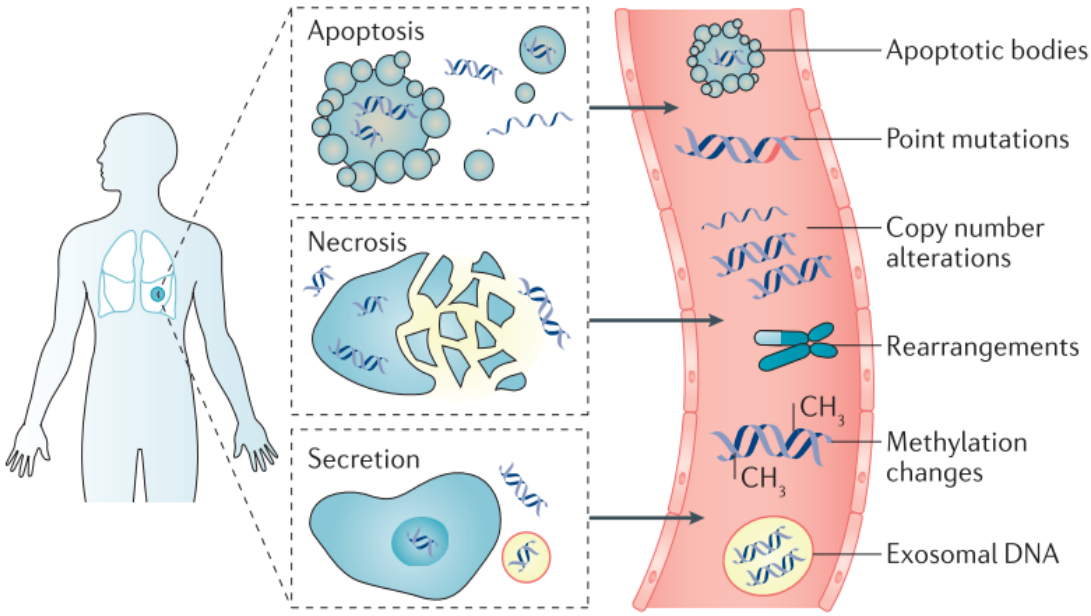


Fig. 11. Origins of cfDNA. Reproduced from (93) with permission of Springer Nature.

1.3.3. Function

One major task of CNA is cell-to-cell communication. Extracellular vesicles like exosomes represent an important vehicle for transfer of molecular structures (94,95). In 2015 it has been shown that these DNA molecules might be taken up by other cells in the body and that they are most likely being integrated into the host genome, thus having a profound effect on the receiving cell, probably stimulating a host response to the stress, which caused the apoptosis of the cell from which the CNA molecules originated (96). DNA molecules contained in exosomes can integrate into the nucleus when added to cell culture, thus supporting the hypotheses that CNA may act as mutagenic agents supporting ageing-related disabilities or aging itself (97).

2. Hypothesis and aim of the study

2.1. Previous work

Elevated levels of extracellular nucleic acids in blood can be used to detect specific disease conditions, such as sepsis (73). Via Next-Generation DNA Sequencing and bioinformatics these informative genomic regions (motifs) have been identified and clustered into three groups: (i) universal motifs (motifs which have similar counts in affected and non-affected persons), (ii) disease specific motifs (motifs which are much higher in septic patients) and (iii) control motifs (motifs which are found in higher counts in non-septic patients).

2.2. Hypothesis

1.) Specific DNA motifs exist in the serum and plasma of patients at the early onset of sepsis caused by bacteremia/fungemia. Motifs derive from host response and so they are unaffected by the pathogen which drives sepsis.

2.) A combination of DNA motifs exists, which covers most affected patients and can therefore be used to distinguish them from controls, who are characterized by an absence of sepsis.

3.) A combination of motifs exists, which allows the differentiation of patients who will develop sepsis at an early stage of disease from non-septic patients. Furthermore, it will be possible to determine specificity and sensitivity of this approach.

2.3. Aim

The aim of this thesis is to establish sensitivity, specificity, the positive predictive value (PPV) and the negative predictive value (NPV) of CNA markers derived from human blood plasma samples of septic patients. A further aim is to detect sepsis at an early stage, where clinical signs are not yet noticeable.

Ultimately, the results obtained through this thesis could lead to a low-throughput testing system for sepsis in high-risk patients. Short-term objective is to create a Multiplex PCR assay to detect sepsis-specific CNA motifs at an early point in time.

2.4. Ethics

The study was approved by the local ethics committee (No. 19-322 ex 07/08 and 21-469 ex 09/10) and was performed according to the standards of "Good Scientific Practice". Written informed consent was obtained from all patients eligible for the study. If patients were not able to give written informed consent at the time of study inclusion (e.g. intubated and mechanically ventilated patients), they were informed and asked for their consent after their arousal.

3. Material and Methods

Parts of the section “Material and Methods” were published recently in Ullrich et al. (1).

Figure 12 summarizes the workflow of this study. CNA have been isolated and amplified randomly from the plasma of patients with sepsis, drawn concomitantly to positive blood cultures and compared to samples from healthy controls. Via Illumina paired-end high-throughput sequencing with subsequent bioinformatics analysis of the CNA molecules, their origin in the human genome and their differences in the pattern sets between healthy controls and patients were characterized.

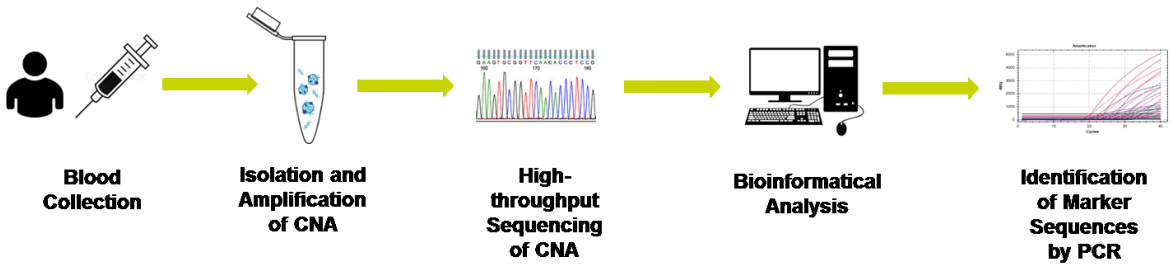


Fig. 12. Workflow of this study. With permission of Petra Heidinger, acib GmbH, Graz.

3.1. Study cohort

3.1.1. NOBI(C)S (novel biomarker invasive (candidiasis) and sepsis) cohort

The NOBI(C)S cohort consists of 218 human blood plasma samples from 127 individuals. 147 samples derived from 69 patients diagnosed with bacterial or fungal bloodstream infection (i.e. one of the most common causes of sepsis) and sepsis and 71 samples from 58 healthy individuals or patients suffering from diseases other than sepsis, like influenza or lymphoma (i.e. controls). For blood collection, Lithium Heparin tubes (green cap with black ring; Greiner VACUETTE, Kremsmünster, Austria) were used. The samples have been collected between 2015 and 2018 at the University hospital Graz, Austria. Therefore, the definition of Sepsis changed during the sampling period. The times of sample collection in the sepsis group ranged from four days before the day on which the blood cultures were taken (designated as day 1) to three days afterwards. All patients of the sepsis group in this cohort had bloodstream infection, which means that they all had positive blood cultures with a relevant pathogen (Tab. 8). Gram-negative or Gram-positive bacteria, as well as fungi,

were identified as the sepsis-causing pathogens by routine measures including BACTEC blood cultures machines (BACTEC FXTM, Becton Dickinson, Heidelberg, Germany). Clinical data (age and sex) and parameters of the SOFA Score like PaO_2/FiO_2 (mmHg), platelets count, bilirubin, creatinine and requirement of vasopressors were obtained retrospectively. In the context of descriptive statistics median and range (age) and mean and standard deviation (laboratory values) were calculated. No data regarding the Glasgow coma scale was available. The comparison and control samples represent patients with three disease conditions, including influenza, preeclampsia and lymphoma, as well as healthy individuals. Hence, this cohort was carefully designed to facilitate the development of early DNA markers that are specific to sepsis due to bacteremia or fungemia, not just general disease conditions, as opposed to healthy conditions.

Tab. 8. NOBI(C)S cohort

Sample class	Pathogen (sepsis) or condition (comparator/control)		Patients	Samples	Samples taken at day(s) relative to the day when blood culture was drawn (=day 1)							
					–4	–3	–2	–1	1	2	3	4
Sepsis	Gram-negative	*Escherichia coli*	21	41	1	1	3	1	16	17	0	2
	Gram-positive	*Staphylococcus aureus*	19	43	1	1	2	4	14	16	4	1
		Staphylococcus epidermidis	1	4	1	1	0	1	1	0	0	0
	Fungi	*Candida spp.*	28	59	0	1	6	9	22	21	0	0
		total	**69**	**147**	**3**	**4**	**11**	**15**	**53**	**54**	**4**	**3**
Control	Healthy		46	46	Not applicable							
	Influenza		10	19								
	Lymphoma		2	6								
	total		**58**	**71**								

Plasma collection

Blood collection was performed using the VACUETTE® blood collection system and Lithium heparin tubes (Greiner bio-one, Kremsmünster, Austria). Following blood

collection, all tubes were gently inverted 5–10 times. The samples were centrifuged using a horizontal rotor (swing-out head) or fixed angle rotor, respectively, for 10–15 minutes with a g-force of 1800 g to 2000 g. After the centrifugation step the separated plasma was aliquoted immediately and initially stored at +4°C, before being frozen and kept at -80°C for long-term storage.

3.2. Sequencing and Motif Identification

3.2.1. DNA Preparation for High-Throughput DNA Sequencing

Nucleic acids were extracted from the plasma using the High Pure Viral Nucleic Acid Kit (Roche Applied Sciences) according to the manufacturer's protocol. Subsequently, the extracted nucleic acids were amplified using the WGA4 GenomePlex Single Cell Whole Genome Amplification Kit (Sigma) according to the manufacturer's protocol. The amplified DNA was purified using the GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol and subsequently sent to Seq-IT GmbH, Kaiserslautern, Germany for high-throughput DNA sequencing.

3.2.2. Identification of Informative Genomic Regions (motifs)

Individual 150-bp long reads were extracted from the FASTQ sequence files produced by Seq-IT. The reads were then pair-merged with minimum overlaps of 10bp and a maximum of ambiguities within the overlapping region of three bp. Using software tools developed in-house the read pairs were clustered into clusters of highly similar reads. For each sample sequenced, abundance counts were generated for the clusters, which were used to identify those clusters that had similar counts across all samples (universal motifs), those that were more than twice higher in the abundance counts in sepsis patients (disease motifs) and also those that were more than twice higher counts in controls (control motifs). In a separate approach, the read pairs were mapped to the human genome assembly GRCh38.p12 (98) and universal, disease-specific and control-specific motifs were identified through map comparisons using in-house software tools.

3.3. Quantitative Real-time PCR (qPCR)

3.3.1. Primer design

Primer pairs were calculated using the Primer Quest Tool from Integrated DNA Technologies, USA, which resulted in PCR fragments of at least 170 bp in size. The primer size varied between 18 and 24 nucleotides, with a melting temperature (Tm) of approximately 62°C. The primers were synthesized at Integrated DNA Technologies, USA and shipped to Graz, Austria, in lyophilized form.

3.3.2. Generation of Real time-PCR *Cq* Values

Real-time PCR (qPCR) was performed using a BioRad CFX96 Touch™ Real-Time PCR Detection System operating with the CFX Maestro™ software (Bio-Rad Laboratories, USA) and using the LUNA qPCR reaction kit (New England Biolabs, Massachusetts, USA). The fluorescence signal of the PCR products was monitored continuously after each cycle, with the *Cq (*quantification cycle) value determination mode set to "Regression Mode". An aliquot of 2µl of a 1:40 diluted plasma sample was mixed with 10 µL of LUNA Master Mix, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM) and 7 µL of purified water (*Aqua bidest*, Fresenius). Table 9 provides the reaction mix. Each well contained 20 µL sample volume in total. After sealing, the plates were centrifuged at max. 3000 g for 3 seconds. The PCR protocol (Tab. 10) consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 45 sec of annealing/extension at 60°C. All data files derived from this study were combined and each *Cq* value generated was normalized by an inter-run calibration using a defined amount of gBlocks® which was included in every run, and used as calibrator to normalize differences between individual PCR runs. In particular, the mean of all *Cq* values gained from each calibrator amplification was used for data normalization.

Tab. 9. Reaction mix for qPCR

Components	Volume (µL)
Luna ®	10
Primer_Sepsis xy_fwd (10 µM)	0.5
Primer_Sepsis xy_rev (10 µM)	0.5
template	2
DNase free H_2O	7
total	20

Tab. 10. Protocol for qPCR

Protocol	time	cycles
95°C	2 min	
95°C	15 sec	plate read 40x
60°C	45 sec	
60°–95°C	melting curve	increment of 0.5°C for 5 sec, plate read

gBlocks® reference motifs

gBlocks® (IDT, Leuven, Belgium) Gene Fragments are chemically synthesized, double-stranded DNA. They consist of five different motifs derived from a former sequencing study (Sepsis 2, Sepsis 7, Sepsis 11, Sepsis 17 and Sepsis/Control 9, see also section 3.3.3) which are separated by five thymine bases with a total length of 564 bp. It was used as template in the first experiments to establish standard curves, annealing temperature and master mix evaluation. The sequence of gBlocks® is provided in Table 11. Motif 2 was also used later as a calibrator to normalize differences between individual PCR runs. The gBlocks® (IDT, Leuven, Belgium) and human genomic DNA (Sigma-Aldrich, St. Louis, Missouri, USA) were used as template for Multiplex RT- PCR and digital PCR experiments (primer list depicted in Table 12).

Tab. 11. gBlocks® sequence for normalizing PCR experiments

	sequence	Length (bp)	Conc.
gBlocks®	TTTTTCTCCCAAGTGCTGGGATTACAGATATGAGCCACCGCAACTGGCCCT GAACCTACTTTTAACATCCAGGAAAAATAGCCATATCATTGTTGTCCTAAA GTTTTTTTGGATACTGCTGCCAAGAAGTTGCTCTGAAGTCAGTTTCTATCAT TCTGCTCTTTGATTCAAAGCACTGTTTCTCTCACTGGGCCTCCAACCATGTT CCCTTCTTCTTAGCACCTTTTTACCAGGTCCCTGAAACAAAGGCTGGTCACA CTCGGCTGGCTTGTCCCCAACTTCTGCTCCTCCCATGAAGGCCCTCCACTCT CCCTCCGCTTCTCTGGACAGGTTGGGATTTTTCTAGTCCACAGTGTCTACG TAATGCCTAGCACATAATAGGCGCCTAGGAGACACCTGCGCATGAATGAA CAGTGTCTTCACTGCTTTGGTCCTGTTTTTCACTGATGGGTCTTGACTCTTT ATCCAACTTGCCAGTCTGTGTCTTTTAATTGGAGAATTTAGTCCATTTACAT TTAAAGTTAATATTGTTATGTGTGAATTTGATCCTGTCATTTTT	564	10 ng/µL; 1 722 145 962 copies/ng

A, adenine, T, thymine, G, guanine, C, cytosine; green, complementary bases for forward and reverse primer; purple, complementary bases for TaqMan probes

Tab. 12. Primer pairs for Sepsis 2, 7, 11, 17 and Sepsis/Control 9

motif ID	amplicon length	fwd Primer (5'-3')	Length	Tm	rev Primer (5'-3')	Length	Tm
Sepsis 2	99	CTCCCAAGTGCTGGGATT	18	61,4	ACTTTAGGACAACAATGATATGGC	24	61,5
Sepsis 7	114	TGGATACTGCTGCCAAGAAG	20	61,9	GGTGCTAAGAAGAAGGGAACA	21	61,9
Sepsis 11	109	CTGGCAACCTGGGATAACTT	20	62,1	AGCCAGATGCTAACGAAACA	19	62,3
Sepsis 17	94	CTAGTCCACAGTGTCTACGTAATG	24	61,9	CAGGACCAAAGCAGTGAAGA	20	62,0
Sepsis/Control 9	113	CACTGATGGGTCTTGACTCTTT	22	62,1	TGACAGGATCAAATTCACACATAAC	25	61,9

3.3.3. Motif evaluation

Evaluation of primer pair specificity was performed by agarose-gel analysis, as well as melting-curve analysis [60°C to 95°C; increment of 0.5°C for 5 sec; plate read]. Only primer pairs yielding a single fragment of the correct size during the agarose-gel analysis and resulting in a single peak of the expected melting temperature for the anticipated PCR fragment, respectively, were chosen for the subsequent analyses. Primer pairs resulting in a specific signal are referred to as biomarkers in this study. QPCR *Cq* values were only considered acceptable if they were in the 10–41 cycle range; values outside the acceptable range were set to "Out of Range (OR)".

Agarose Gel Electrophoresis

A 2% gel was poured by dissolving 4 g of agarose (Biozyme; Vienna, Austria) in 200 mL of 1X TAE buffer (4.84 g L^{-1} Tris, 0.292 g L^{-1} EDTA, 1.142 mL L^{-1} acetic acid). The agarose was solubilized by heating it in the microwave for approximately 3 min and after rinsing the flask with water to cool down the solution, Ethidium bromide was added to make DNA bands visible. Gels were run for 45 minutes at 120 Volt. 500 ng of the standard GeneRuler™ DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) were loaded onto the gel to determine the size and concentration of the DNA bands (Fig. 13).

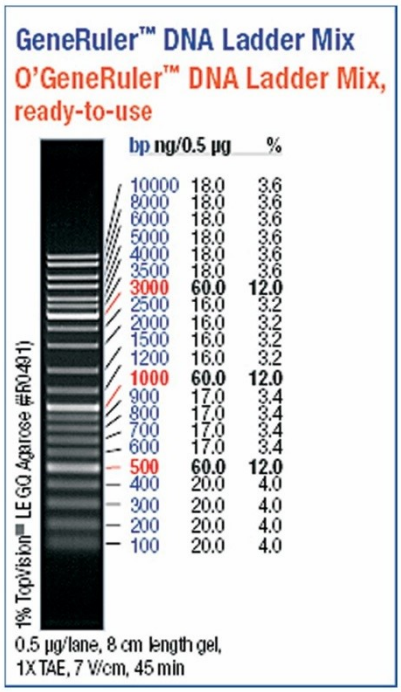


Fig. 13. GeneRuler™ DNA Ladder Mix. Reproduced with permission of Quentin Hoehe from Customer Service - Technical support, Fisher Scientific AG, part of Thermo Fisher Scientific; ch.tech@thermofisher.com

3.3.4. Data analysis and Statistics

3.3.4.1. Generation of Delta-*Cq* Values

Cq values were analyzed by using CFX Maestro™ software (Bio-Rad Laboratories Inc., Hercules, California, USA). The *Cq* value determination mode was set to "Regression Mode". This mode applies a multivariable, non-linear regression model to individual well traces and then uses this model to compute an optimal *Cq* value. For each sample, differences in *Cq* values are derived for all possible pairs of DNA biomarkers (=motifs). For example, given the *Cq* value for motif A (Cq_A) and the *Cq* value of motif B (Cq_B), the Delta

Cq (ΔCq) value for that pair of motifs is calculated as $\Delta Cq_{AB} = Cq_A - Cq_B$ (Fig. 14). In order to exclude ΔCq variables providing redundant information, only linearly independent and linearly uncorrelated biomarker pairs with a Pearson Correlation Coefficient of < 0.6 as cut-off were retained for subsequent classifier development.

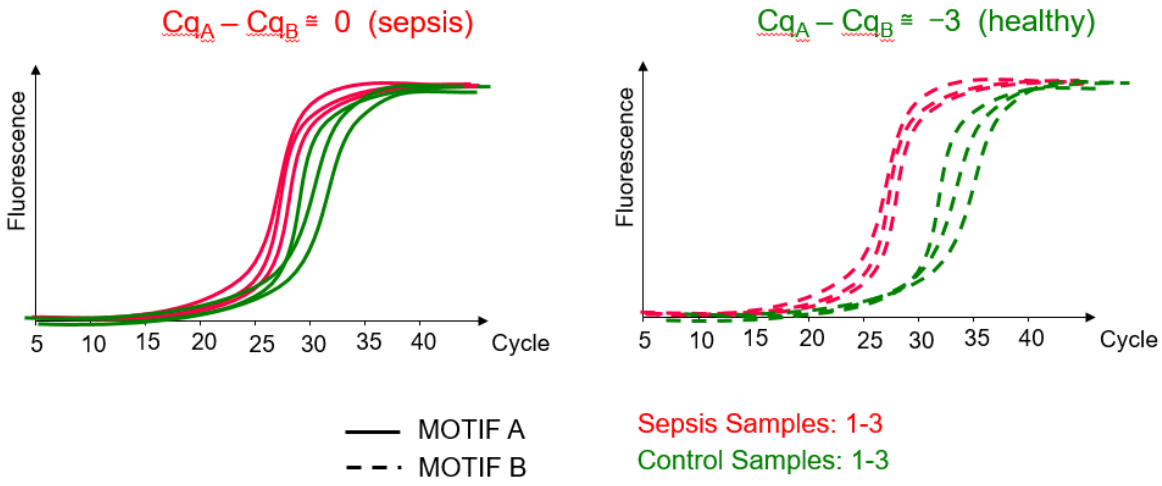


Fig. 14. Comparison of motif A (solid line) and B (dashed line) for three samples of sepsis patients and three samples of controls. For calculating the ΔCq value of motif A and B for sepsis samples, the *Cq* value of the red solid curve (motif A tested on sepsis samples) had to be compared to the red dashed line (motif B tested on sepsis samples). The same has to be done for the controls (green lines). With permission of Laura Villanova, Graz University of Technology.

3.3.4.2. Classifier

In machine learning, a classifier is a model of the distribution of class labels in terms of predictor features. The derived classifier is used to assign class labels to the testing instances where the values of the predictor features are known, but the value of the class label is unknown. The class label is the status of a sample (sepsis OR control), the predictor features are ΔCq values of the DNA biomarker pairs, and the instances are the samples. A total of 92 different classifier algorithms were tested and the five best-performing models were used, including Neural Network (99), Support Vector Machines with Class Weights (100), Localized Linear Discriminant Analysis (101), Self-Organizing Maps (102) and Mixture Discriminant Analysis (103), respectively.

Classifier Performance Assessment:

For each experiment, the classifier performance measures of sensitivity, specificity, accuracy, positive predictive value and negative predictive value were determined. For the characterization of each serum/plasma sample, there were four possible outcomes of the classification procedure:

• True positive: Sepsis sample correctly classified as sepsis,

• False positive: Control sample incorrectly classified as sepsis,

• True negative: Control sample correctly classified as control,

• False negative: Sepsis sample incorrectly classified as control.

To train a classifier, five-fold cross validation with five repetitions was performed (Fig. 15). In this training scheme, the training dataset was randomly partitioned into five folds (Fold 1, Fold 2, Fold 3, Fold 4 and Fold 5), where each fold contained approximately the same number of samples. Subsequently, the classifier was trained on four folds and tested on the remaining fold, *i.e.* the one not used for training. This procedure of training on four folds, followed by testing on the remaining fold was done in five iterations, each time using a different set of four folds for training, such that each of the samples was tested for classification only once. This approach gave rise to the complete set of results and performance measures. In addition, the whole process was repeated four times, each time with a different partitioning (five repetitions) to reduce the potential influence of any particular random partitioning of training samples into five folds on the performance measures. The performance measures from all repetitions were averaged to derive the combined performance measures.

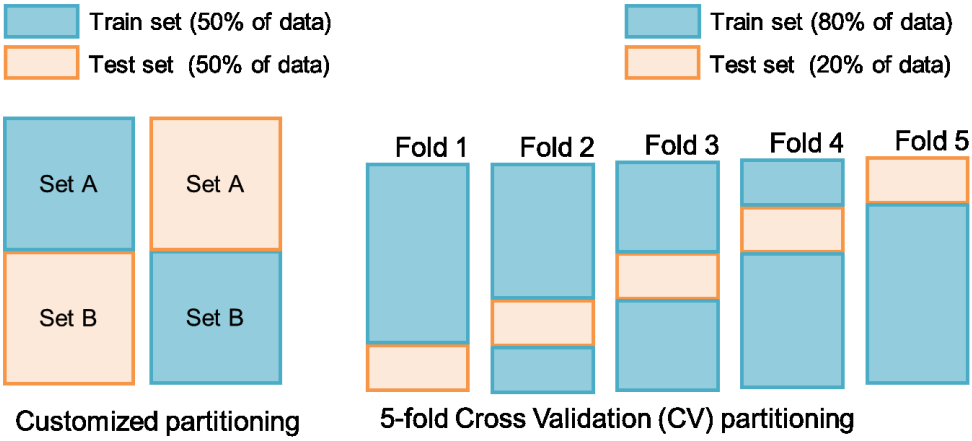


Fig. 15. Training a classifier performing five-fold cross validation with five repetitions. With permission of Laura Villanova, Graz University of Technology.

3.4. Multiplex qPCR

Multiplex PCR enables the simultaneous detection of multiple targets in one single reaction. This technique requires the use of different primers and two or more probes that can be distinguished from each other and detected simultaneously. The TaqMan™ probes are sequence-specific oligonucleotides and labelled with a fluorophore, which permits detection only after hybridization of the probe with its complementary sequence (Fig. 16).

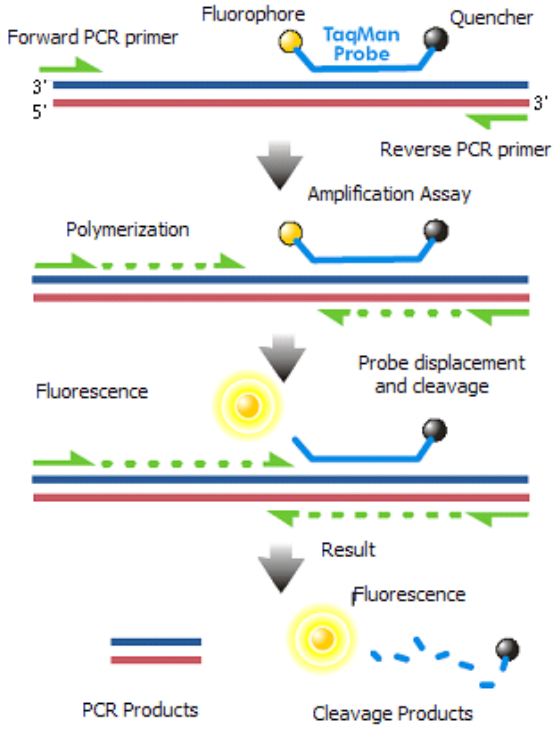


Fig. 16. Principle of TaqMan Probes, from https://commons.wikimedia.org/wiki/File:Taqman.png

First PCR experiments with SYBRGreen® and TaqMan™ probes were performed: (i) to find the perfect melting temperature by performing temperature gradient PCR experiments; (ii) to evaluate the performance of different Master mixes (SsoAdvanced™ Universal Probes Supermix versus IQ™Multiplex Powermix, both Bio-Rad Laboratories, USA); (iii) to determine the influence of plasma on PCR efficiency; and, (iv) to define the influence of five PCR reactions in parallel in order to establish a Multiplex assay.

3.4.1. Motifs

Identification of possible motifs due to sequencing results was done in a former study, outwards. FASTQ sequence files were provided. From the 50 possible motifs, four sepsis-specific motifs (Sepsis 2, Sepsis 7, Sepsis 11 and Sepsis 17) and one universal motif (Sepsis/Control 9) were selected.

3.4.2. Primer and probe design

Primer and probes were calculated using the Primer Quest Tool from Integrated DNA Technologies, USA. The primer size varied between 18 and 24 nucleotides, with a melting temperature (Tm) of approximately 62°C. The primers were synthesized at Integrated DNA Technologies, USA and shipped to Graz, Austria in lyophilized form. The probes were synthesized at LGC Biosearch Technologies (Risskov, Denmark). Tm of probes had to be 5–10°C higher then Tm of primers. Table 13 provides the primer and probe list.

Tab. 13. Primer and probes used for Multiplex PCR

Multiplex qPCR and digital PCR							
	motif ID	fwd Primer (5'-3')	Length	Tm	rev Primer (5'-3')	Length	Tm
	Sepsis 2	CTCCCAAGTGCTGGGATT	18	61.4	ACTTTAGGACAACAATGATATGGC	24	61.5
	Sepsis 7	TGGATACTGCTGCCAAGAAG	20	61.9	GGTGCTAAGAAGAAGGGAACA	21	61.9
	Sepsis 11	CTGGCAACCTGGGATAACTT	20	62.1	AGCCAGATGCTAACGAAACA	19	62.3
	Sepsis 17	CTAGTCCACAGTGTCTACGTAATG	24	61,9	CAGGACCAAAGCAGTGAAGA	20	62.0
	Sepsis/Control 9	CACTGATGGGTCTTGACTCTTT	22	62,1	TGACAGGATCAAATTCACACATAAC	25	61.9

	motif ID	Probe (5‘-3‘)	Length	Tm	Dye 5‘	Quencher 3‘
	Sepsis 2	ACAGATATGAGCCACCGCAACTGG	24	68.1	CAL Fluor® Gold 540	BHQ1
	Sepsis 7	TGTTTCTCTCACTGGGCCTCCAAC	24	67.9	CAL Fluor Red 610	BHQ2
	Sepsis 11	ACAGGGTTCCTGAGCTCACTGAAA	24	68.0	Quasar 670	BHQ2
	Sepsis 17	AGGAGACACCTGCGCATGAATGAA	24	68.1	Quasar 705	BHQ2
	Sepsis/Control 9	TCCAACTTGCCAGTCTGTGTCTTT	24	66.4	FAM	BHQ1

3.4.3. Temperature Gradient qPCR Experiments

QPCR was performed using a BioRad CFX96 Touch™ Real-Time PCR Detection System operating with the CFX Maestro™ software (Bio-Rad Laboratories, USA). Five motifs (four sepsis-specific motifs: Sepsis 2, Sepsis 7, Sepsis 11, Sepsis 17 and one universal motif: Sepsis/Control 9) were detected by qPCR in Singleplex assays. Increasing concentrations of gBlocks® (see Tab. 10) (100–1 000 000 copies) and 40 ng human genomic DNA (Sigma-Aldrich, St. Louis, Missouri, USA) were used as templates for enabling standard curves and finding the temperature optimum. An aliquot of 2 µL of template was mixed with 10 µL of either SsoAdvanced™ Universal Probes Supermix or IQ™Multiplex Powermix (both Bio-Rad Laboratories, USA), 0.4 µL of forward primer (15 µM), 0.4 µL of reverse primer (15 µM), 1 µL EvaGreen® Dye20X (Biotium, USA) and 6.2 µL purified water (*Aqua bidest*, Fresenius, Germany). Each well contained 20 µL sample volume in total. After sealing, the plates were centrifuged at max. 50 rpm for 3 seconds. The PCR protocol consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 30 sec of annealing/extension at a temperature gradient from 55°C–60°C and from 60–65°C followed by melting-curve analysis (65–95°C; increment of 0.5°C for 5 sec; plate read), Figure 17 depicts the PCR protocol. The fluorescence signals of the PCR products were monitored continuously after each cycle, with the *Cq* (quantification cycle) value determination mode set to “single threshold”. A quantification cycle is then determined for each well based on the position of a single threshold line.

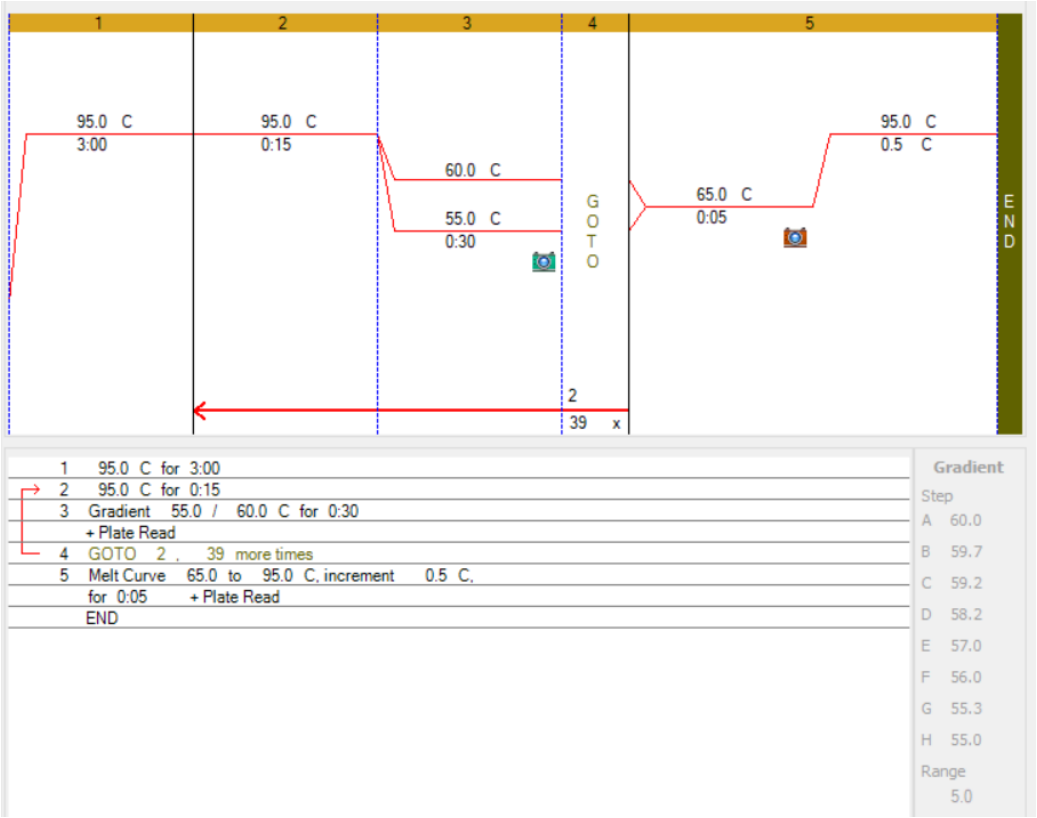


Fig. 17. PCR protocol for Gradient qPCR 55–60°C

3.4.4. Setup of Multiplex PCR

Multiplex qPCR was performed using a BioRad CFX96 Touch™ Real-Time PCR Detection System operating with the CFX Maestro™ software (Bio-Rad Laboratories, USA). Five motifs (Sepsis 2, Sepsis 7, Sepsis 11, Sepsis 17 and Sepsis/Control 9) were detected simultaneously. Increasing concentrations of gBlocks® (100–1 000 000 copies) and human genomic DNA (Sigma-Aldrich, St. Louis, Missouri, USA) were used as templates to define the influence of five PCR reactions in parallel. Templates were also spiked by human plasma, diluted in sterile water in a 1:40 and 1:10 ratio. An aliquot of 1 µL of template was mixed with 10 µL of either SsoAdvanced™ Universal Probes Supermix or IQ™Multiplex Powermix (both Bio-Rad Laboratories, USA), 0.4 µL of forward primer (15 µM), 0.4 µL of reverse primer (15 µM), 0.5 µL of probe (10 µM) and 2.5 µL purified water (*Aqua bidest*, Fresenius). Table 14 provides the reaction mix. Each well contained 20 µL of sample volume in total. The plates were sealed and centrifuged as described above. The PCR protocol (Figure 18) consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 1 min of annealing/extension at 60°C. The fluorescence signals of

the PCR products were monitored continuously after each cycle, with the *Cq* value determination mode set to “regression mode”.

Tab. 14. Reaction Mix for Multiplex PCR

Reaction Mix	Volume (µL)
SsoAdvanced™ Universal Probes Supermix or IQ™Multiplex Powermix	10
Primer_Sepsis 2_fwd (15 µM)	0.4
Primer_ Sepsis 2_rev (15 µM)	0.4
Probe_Sepsis 2 (10 µM)	0.5
Primer_Sepsis 7_fwd (15 µM)	0.4
Primer_ Sepsis 7_rev (15 µM)	0.4
Probe_Sepsis 7 (10 µM)	0.5
Primer_Sepsis 11_fwd (15 µM)	0.4
Primer_ Sepsis 11_rev (15 µM)	0.4
Probe_Sepsis 11 (10 µM)	0.5
Primer_Sepsis 17_fwd (15 µM)	0.4
Primer_ Sepsis 17_rev (15 µM)	0.4
Probe_Sepsis 17 (10 µM)	0.5
Primer_Sepsis/Control 9_fwd (15 µM)	0.4
Primer_ Sepsis/Control 9_rev (15 µM)	0.4
Probe_Sepsis/Control 9 (10 µM)	0.5
DNase free H_2O	2.5
template	1
total	20

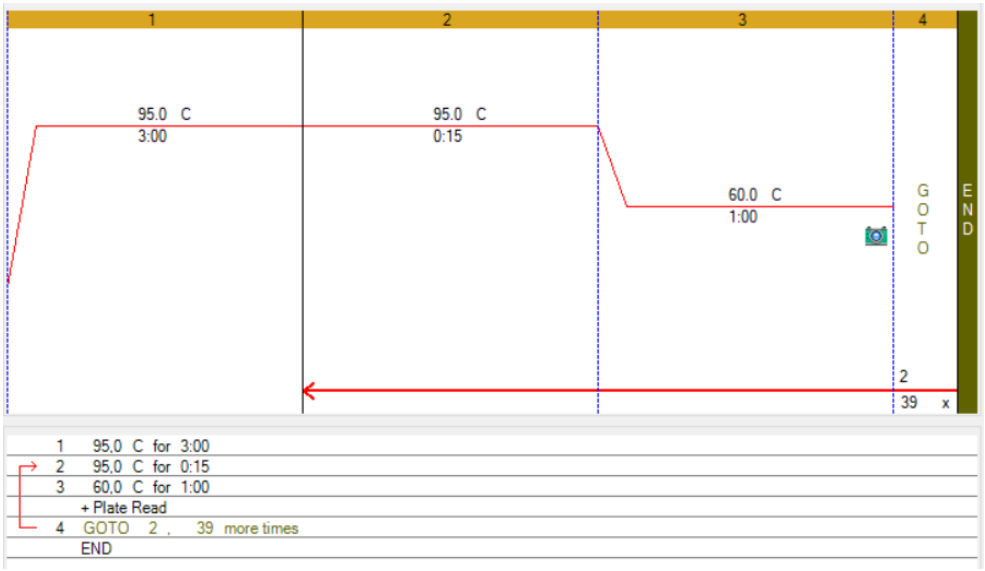


Fig. 18. PCR protocol for Multiplex PCR

3.5. Digital PCR (dPCR)

Digital PCR (dPCR) is a new approach to nucleic acid detection for absolute quantification of targets with high sensitivity. A sample of DNA is partitioned into millions of separate units (i.e., wells) using a nanofluidic chip with microchannels. Each well contains all reagents necessary for a PCR reaction (sample, master mix, TaqMan probes) and functions as a micro-PCR reactor. Each well is analyzed individually to detect the presence (positive=1) or absence (negative=0) of the target (Fig. 19). As positive wells may contain more than one copy of the target molecule, a simple summing of the number of positives will not yield the correct number of target molecules present across the partitions. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson formula (probability distribution).

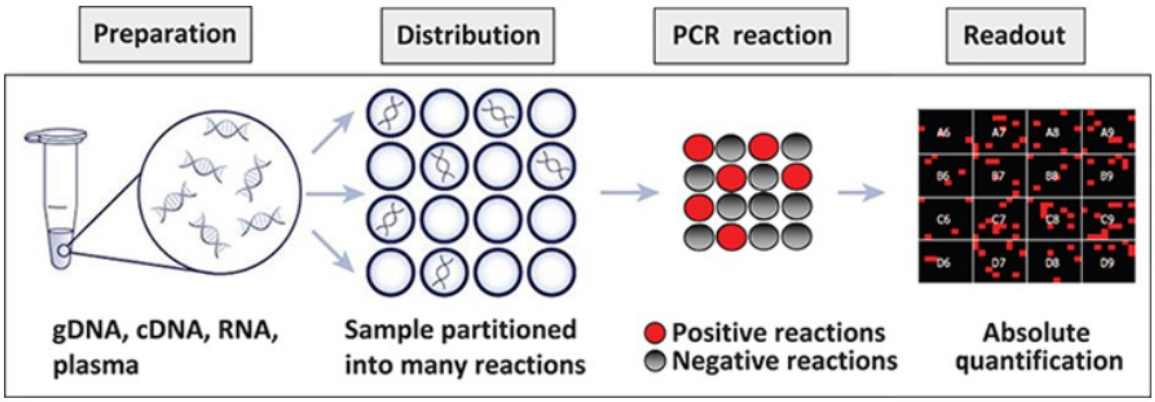


Fig. 19. Basic principle of digital PCR

gDNA, genomic DNA; cDNA, complementary DNA; RNA, ribonucleic acid. Reproduced with permission of Quentin Hoehe from Customer Service - Technical support, Fisher Scientific AG, part of Thermo Fisher Scientific; ch.tech@thermofisher.com

3.5.1. Setup of dPCR

Digital PCR was performed using a QuantStudio® 3D Digital PCR System operating with the QuantStudio® 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Two motifs (Sepsis 2 and Sepsis/Control 9) were detected and quantified simultaneously. The motif Sepsis 2 was labeled with VIC and the motif Sepsis/Control 9 with FAM. Different concentrations of gBlocks® and human genomic DNA (Sigma-Aldrich, St. Louis, Missouri, USA) were used as templates (Tab. 15). Experiments were also performed by adding human plasma instead of purified water. Experiments were performed with and without purification of gBlocks® with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, Missouri, USA) according to manufacturer protocol. An aliquot of 1 µL of template was mixed with 7.5 µL of QuantStudio® 3D Digital PCR V2 master mix, 1 µL of TaqMan Assay and 5.5 µL purified water (*Aqua bidest*, Fresenius) or human plasma dilution. Table 16 provides the components of the TaqMan assay. Chip loading was performed with the QuantStudio™ 3D Digital PCR Chip Loader (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's instructions. Each chip was loaded with 14.5 µL of the reaction mix (Tab. 17). The PCR protocol is shown in Table 18.

Tab. 15. Overview of used templates

gBlocks® (copies)	human genomic DNA (haploid genomic equivalents)
30000	10000
20000	2000
10000	1000
2000	100
1000	
200	
100	

Tab. 16. Components of the TaqMan assay for digital PCR

Components	Concentration of stock solution (µM)	Volume of stock in master mix (µl)	Concentration in master mix (µM)	Concentration in PCR reaction (nM)
Primer_Sepsis 2_fwd	100	5,0	13,5	900
Primer_Sepsis 2_rev	100	5,0	13,5	900
Primer_Sepsis/Control 9_fwd	100	5,0	13,5	900
Primer_Sepsis/Control 9_rev	100	5,0	13,5	900
Probe_Sepsis 2 (VIC)	100	1,4	3,8	250
Probe_Sepsis/Control 9 (FAM)	100	1,4	3,8	250
DNase free water		14,2		
total		37		

Tab. 17. Reaction mix for digital PCR

Components	1x reaction Volume [µl]
TaqMan assay mix	1
QuantStudio™ 3D Digital PCR Master Mix v2	7.5
template (gBlocks® or genomic DNA)	1
DNase free H_2O (or plasma 1:40 dilution)	5.5
total	15

Tab. 18. Protocol for digital PCR

Protocol	time	cycles
96°C	10 min	
60°C	2 min	45x
98°C	0.5 min	
60°C	2 min	

4. Results

4.1. Baseline characteristics NOBI(C)S sepsis cohort

The NOBI(C)S sepsis cohort consists of 147 samples from 69 patients. All patients had confirmed bacteraemia or fungemia, which means all patients had positive blood cultures with a relevant pathogen. 28 patients had invasive candidemia (IC; 20 *C. albicans*, 5 *C. glabrata*, 3 *C. parapsilosis*), 19 patients *Staphylococcus aureus* bacteremia (SA), 21 patients *Escherichia coli* bacteraemia (EC) and one patient had positive blood cultures with *Staphylocoocus epidermidis* (SE). Blood cultures were ordered by the treating physicians due to clinical hints of bacteremia, fungemia and sepsis. The SOFA score was calculated retrospectively. Blood values (platelets count, bilirubin and creatinine) were determined for the day when blood cultures have been taken and compared to the values determined up to 72 before and 48 hours after. Glasgow Coma Score data was not systematically documented for all patients and therefore not included in the analysis. The median age was 68 years (range 18–96), 35 patients (50.7%) were male. Fourteen patients (20.3%) were ventilated and 16 (23.2%) needed vasopressors. For four patients (5.8%) data regarding ventilation was missing. The mean platelets count was 190 G/l (SD ± 126.4), for four patients (5.8%) data was missing. The mean bilirubin was 2.3 mg/dl (SD ± 3.4), 31 patients (44.9%) had no bilirubin determination on dedicated examination days. The mean creatinine was 2.1 mg/dl (SD ± 2), nine patients (13%) had no creatinine determination on dedicated examination days. Four patients were already diagnosed with chronic kidney disease (CKD). A SOFA Score ≥ 2 points was present in 65 of 69 patients which therefore fulfilled the current Sepsis-3 Definition criteria. Within the bacteremia/fungemia groups 100% of patients with *Candida spp.* fungemia, 95% (18/19) of patients with *S. aureus* bacteremia and 86% (18/21) of patients with *E. coli* bacteremia had data available that fulfilled Sepsis-3 criteria. The remaining four patients fulfilled the previous sepsis definition with positive SIRS criteria. Table 19 depicts the baseline characteristics of the NOBI(C)S sepsis cohort patients in detail. All laboratory values shown had been determined on the day when blood cultures had been taken.

Tab. 19. Baseline characteristics of the NOBI(C)S sepsis cohort patients.

total n=69	IC (n=28)	SA (n=19)	EC (n=21)	SE (n=1)
age years median (range)	66.5 (19–90)	67 (52–81)	74 (18–96)	50
Sex male n (%)	15 (53.6%)	12 (63.2%)	7 (33.3%)	1 (100%)
Ventilated n (%)	13 (46.4%) 2 n.d.	1 (5.3%) 2 n.d.	0	0
Vasopressors n (%)	15 (53.6%)	1 (5.3%)	0	0
platelets count (G/l) mean (±SD)	220 (±146.4) 1 n.d.	199 (±126.8) 1 n.d.	148 (±74.8) 2 n.d.	12 (haematological malignancy)
bilirubin (mg/dl) mean (±SD)	3.2 (± 4.6) 9 n.d.	1.3 (±1.3) 13 n.d.	1.65 (±1.5) 9 n.d.	0.3
creatinine (mg/dl) mean (±SD)	1.3 (±0.8) 5 n.d.	3.5 (±3.1) 2 n.d. 3 with CKD	1.7 (±1.2) 2 n.d. 1 with CKD	0.65
GCS	n.d.	n.d.	n.d.	n.d.
SOFA Score ≥2 n (%)*	28 (100%)	18 (95%)*	18 (86%)*	*

IC, invasive candidemia; SA, *S. aureus* bacteremia; EC, *E. coli* bacteremia; SE, *S. epidermidis* bacteremia; n.d., not documented; CKD, chronic kidney disease; GCS, Glasgow Coma Scale

*One *S. aureus* und three *E. coli* bacteremia patients had positive SIRS criteria. One *S. epidermidis* bacteremia patient had underlying haematological malignancy, and showed an acute febrile disease with elevation of CRP. Due to the underlying malignancy, the decrease of thrombocytes from 99 to 12 G/L could not be clearly attributed to the bloodstream infection, and leukopenia could not be used in combination with fever for calculation of the SIRS criteria.

4.2. Baseline characteristics control cohort

Table 20 depicts the baseline characteristics of the NOBI(C)S control cohort patients. All laboratory values shown had been determined on the day of admission. The control cohort consisted of 71 samples from 58 individuals. This cohort included 19 samples from 10 patients with infection but without sepsis (influenza cohort) as well as 6 samples from 2 patients with other underlying severe diseases but without infection (lymphoma) as well as 46 samples from 46 healthy individuals without underlying disease (healthy controls). Of the 46 healthy controls, 31 patients had been admitted to hospital for elective plastic surgery (e.g. mamma augmentation or reduction surgery). The patients had no infectious disease, determined by a thorough investigation of clinical signs and symptoms, as well as lab results. Blood sampling was performed prior to surgery. Most of the surgeries were done in outpatient's clinics and patients brought laboratory results from their general practitioner. There were no clinical or microbiological signs of infection at the time of blood sampling. Another 15 persons were healthy volunteers and no further blood values were determined. The median age of this cohort was 44.5 years (range 19–86) and 31 patients (53.4%) were male.

Tab. 20. Baseline characteristics of NOBI(C)S control cohort patients.

total n=58	Influenza (n=10) 19 samples from different timepoints	Lymphoma (n=2) 6 samples from different timepoints	Healthy elective surgery (n=26)	Healthy volunteers (n=20)
age years median (range)	64.5 (40–80)	75 (73–77) mean	47.5 (19–86)	32.5 (23–58)
Sex male n (%)	8 (80%)	2 (100%)	9 (34.5%)	12 (60%)
Ventilated n (%)	0	0	0	0
Vasopressors n (%)	0	0	0	0
platelets count (G/l) mean (±SD)	173 (± 82.3) 2 n.d.	202 (± 52.9) 1 n.d.	n.d.	n.d.

bilirubin (mg/dl) mean (±SD)	1.05 (±0.6) 8 n.d.	0.5 (± 0.3) 1 n.d.	n.d.	n.d.
creatinine (mg/dl) mean (±SD)	2.8 (±2.8) 2 n.d. 3 with CKD	1.0 (± 0.1) 1 n.d	n.d.	n.d.
GCS	n.d.	n.d.	n.d.	15
SOFA Score ≥2 n (%)	2 (20%)	0	0	0

4.3. Identification of motifs

Sequencing and bioinformatics methods revealed 60 sepsis-specific motifs, meaning that they occurred more often in bacteremia/fungemic septic patients than in patients without. After primer designing, motifs were evaluated by qPCR including melting curve analysis followed by agarose gel electrophoresis. Only primer pairs yielding a specific amplification product, represented as a single fragment of the correct size during the agarose-gel analysis and resulting in a single peak of the expected melting temperature for the anticipated PCR fragment, were chosen for the subsequent analyses (Fig. 20 shows as example motif Sepsis SC 2, which fulfilled these criteria). Figure 21 depicts a negative result with two bands in agarose-gel and two peaks in melting curve, why this motif was discarded. Acceptable *Cq* values were in the 10–41 cycle range. Values outside the acceptable range were set to "Out of Range (OR)" and discarded. 23 primer pairs revealed a specific amplification product and acceptable *Cq* values between 10–41 cycles. With the 24 motifs for sepsis identified, a total of 276 biomarker pairs (variables) were obtained (binomial coefficient $276 = n!/(n-k)!*k!$ with $n=24$ and $k=2$). Of the 276 motif pairs that were formed from 24 biomarkers, after removing redundant pairs, 23 biomarker pairs remained (Tab. 22), which were used for the subsequent classifier development. In Table 21 the identified 24 motifs with corresponding primer pairs are listed.

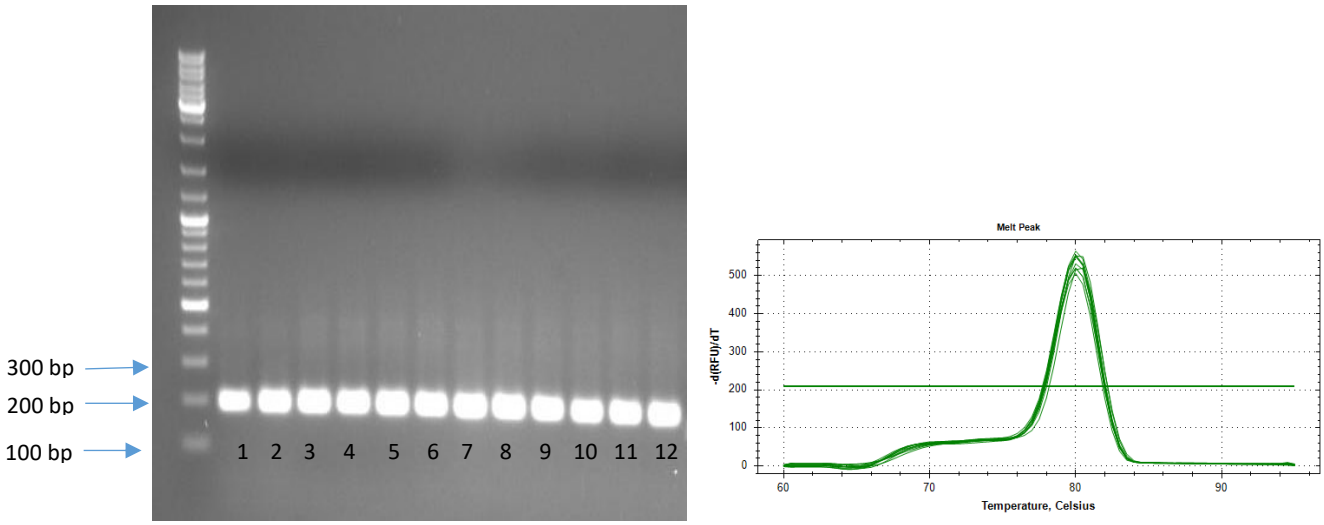


Fig. 20. Sepsis SC 2 (186 bp) yielded a specific amplification product in 2% agarose gel electrophoresis (left) and a single peak in melting curve analysis (right). Mastermix: LUNA qPCR (New England Biolabs, Massachusetts, USA), templates: lanes 1-3: invasive candidiasis, lanes 4-6: *S. aureus*, lanes 7-9: *E. coli*, lanes 10-12: healthy, elective surgery group. Standard: GeneRuler^{TM} DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

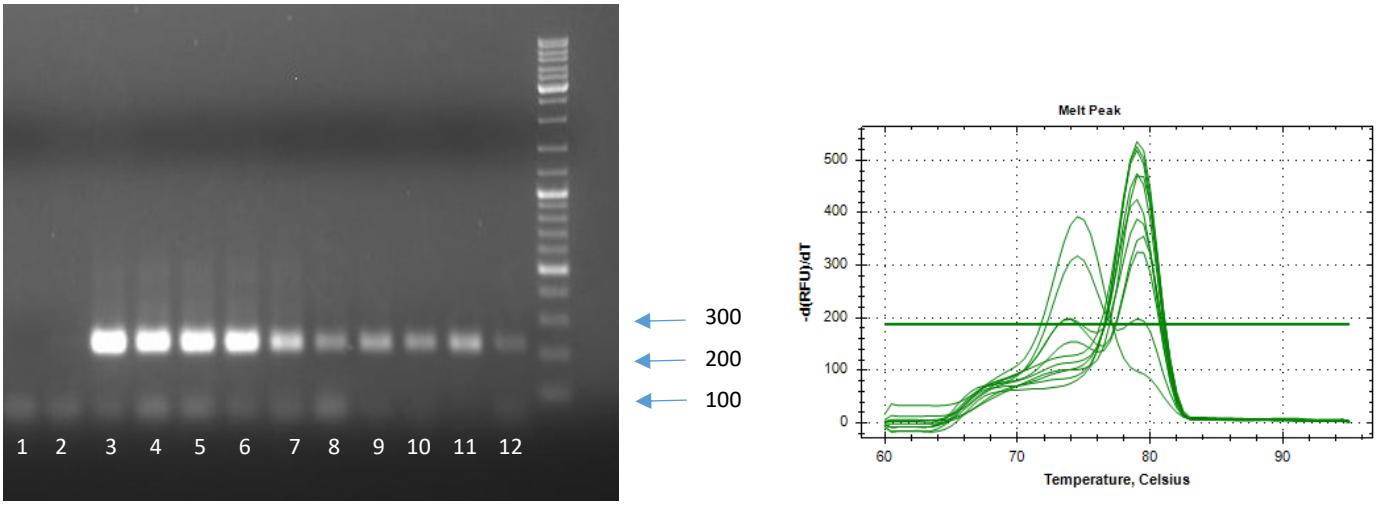


Fig. 21. Sepsis JC 15 (113 bp) yielded two bands in 2% agarose gel electrophoresis (left). Corresponding melting curve showed two peaks (right). Mastermix: LUNA qPCR reaction kit (New England Biolabs, Massachusetts, USA), templates: lanes 1-3: invasive candidiasis, lanes 4-6: *S. aureus*, lanes 7-9: *E. coli*, lanes 10-12: healthy, elective surgery group. Standard: GeneRuler^{TM} DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Tab. 21. Sequences of the 24 identified motifs with corresponding primer pairs, From Ullrich et al., 2020 (1)

	Final motif ID	Length (bp)	Sequence	fwd Primer	rev Primer
1	Sepsis J7	232	TTTTTGTTGCGATAGTTTACTGAGAATGATGGTTTCCAATTTCATCCATGTCCCTACAAAGGACATGAACTCATCATTTTTTATGGCTGCATAGTATTCCATGGTGTATATGTGCCACATTTTCTTAATCCAGTCTATCATTGTTGGACATTTGGGTTGGTTCCAAGTCTTTGCTATTGTGAATAATGCCGCAATAAACATACGTGTGCATGTGTCTTTAGAGCAGCATGAT	TTGTTGCGATAGTTTACTGAGAATG	TGCTGCTCTAAAGACACATGC
2	Sepsis J15	244	AAACATACGTGTGCATGTGTCTTTATAGTAGAATGATTTATAATCCTTTGGGTATATACCCAGTAATGGGATTGCTGGGTCAAATGGTATTTCTGGTTCCAGATCCTTGAGGAATCACCCACTGTCTTCCACAATGGTTGAACTAATTTACACTCCCACCAACAGTGTAAAAGCATTCCTGTTTCTCCACATCCTCTCCAGCATCTGTTGTTTTCTGACTTTTTAATGATCATCATTCTAACTG	ACATACGTGTGCATGTGTCTT	AAACAACAGATGCTGGAGAGG
3	Sepsis J16	238	ATTCAACATAGTATTGGAAGTTCTGGCCAGGGTAATTAGGCAGGAGAAGGAAATAAAGGGTATTCAATTGGGAAAAGAGGAAGTCAAATTGTTCCTGTTTGCAGATGACATGATTGTATATCTAGAAAACCCCATTGTCTCAGCCCAAAATCTCCTTAAGCTGATAAGCAACTTCAGCAAAGTCTCAGGATACAAAATCAATGTACAAAAATCATAAGCATTCTTATACACCAACAAC	TCAACATAGTATTGGAAGTTCTGG	GTATCCTGAGACTTTGCTGAAG
4	Sepsis J17	198	GGTCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACCCCATCTCTACTAAATATACAAAAATTACCCGGGCATGGGGACGGGTGCCTGTAATCCCAGCTGTTCAGGAGGCTGAGGCAGGGGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGAACAGAGATCGTGTCACTGCACTCCAGCCTGGGTGATAG	GGTCAGGAGTTCGAGACCA	CAGGCTGGAGTGCAGTG
5	Sepsis J19	160	TCTTTGAAACCAACAAGAACAAAGACACAACATACCAGAATCTCTGGGACACATTCAAAGCAGTGTGTAGAGGGAAATTTATAGCACTAAATGCCCACAAGAGAAAGCAGGAAAGATCCAAAATTGACACCCTAACATCACAATTAAAAGAACTAGAAAA	GAAACCAACAAGAACAAAGACAC	AATTGTGATGTTAGGGTGTCAA
6	Sepsis J21	202	TTTTTTGTTCTTGTGATAGTTTGCTGAGAATGATGGTTTCCAGCTTCATCCATGTCCCTACAAAGGACATGAACTCATCATTTTTATGGCTGCATAGTATTCCATGGTGTATATGTGCCACATTTTCTTAATCCAGTCTATCATTGTTGGATATTTGGGTTGGTTCCAAGTCTTTGCTATTGTCAGTAATGTCTCAATAAAC	TTGTTCTTGTGATAGTTTGCTGAG	GAGACATTACTGACAATAGCAAAGAC

7	Sepsis J23	233	GAGAGATCCACTGTTAGTCTGATGGGCTTCCATTTGTG GGTAACCCGACCTTTCTCTCTGGCTGCCCTTAACATTAT TTCCTCATTTCAACTTTGCTGAATCTGACAATTATGTGT CTTGGAGTTGCTCTTCTCGAGGAGTATCTTTGTGGCATT CTCTGTATTTCCTGAATTTGATTGTTGGCCTGCCTTGCT AGATTGGGGAAGTTCTCCTGGATAATATCCTGCAGAGT G	GAGAGA TCCACT GTTAGT CTGATG G	CAATCT AGCAAG GCAGGC CAA
8	Sepsis J36	227	AGTTGGCTTCATCCCTGGGATGCATGGCTGGTTCAACA TACACAAATCAATAAACGTAATCCATCATATAAACAG AACCAAAGACAAAAACCACATGATTATCTCAATAGAT GCAGAAAAGGCCTTTGACAAAATTCAACAACCCTTCAT GCTAAAAACTCTCAATAAATTAGGTACTGATGGGACGT GTCTCAAAATAATAAGAGCTATCTATGACAAACCCAC AC	GGATGC ATGGCT GGTTCA A	GTGTGG GTTTGT CATAGA TAGCTC
9	Sepsis JU2	256	ATATGAACTTTAAAGTAGTTTTTTCCAATTCTGTGAAG AAAGTCATTGGTAGCTTGATGGGGATGGCATTGAATCT ATAAATTACCTTGGGCAGTATGGCCATTTTCATGATAT TGATTCTTCCTATCCATGAGCATGGAATGTTCCATTTGT TTGTGTCCTCTTTTATTTCATTGAGCAGTGGTTCGTAGT TCTCCTTGAAGAGTCCTTCACATCCCTTGTAAGGTGGA TTCCTAGGTATTTTATTCTCTGAAGC	TCCAAT TCTGTG AAGAAA GTCATT G	AAATAC CTAGGA ATCCAC CTTACA A
10	Sepsis JU4	277	ACCTTGGGCAGTATGGCCATTTTCAGGATATTGATTCT TCCTACCCATGAGCATGGAATTTTCTTCCATTTGTTTGT ATCCTCTTTTATTTCACTGAGCAGTGGTTTGTAGTTCTC CTTGAAGAGGTCCTTCACATCCCTTGTAAGTTGGATTC CTAGGTATTTTATTCTCTTTGAAGCAATTGTGAATGGG AGTTCACTCATGATTTGGCTCTCTGTCTGTTGTTGGTGT ATAAGAATGCTTGTGATTTTTGTACATTGATTTTGTATC CTGAGAC	ACCTTG GGCAGT ATGGC	GCATTC TTATAC ACCAAC AACAGA
11	Sepsis JU11	188	TTTTGGTATCAGTACCATGCTGTTTTGGTTACTGTAGCC TTGTAGTATAGTTTGAAGTCAGGTAGCGTGATGCCTCC AGCTTTGTTCTTTTGGCTTAGGATTGACTTGGCGATGC GGGCTCTTTTTTGGTTCCATATGAACTTTAAAGTAGTTT TTTCCAGTTCTGTGAAGAAAGTCATTGGTAGCTT	TGGTAT CAGTAC CATGCT GTTT	GCTACC AATGAC TTTCTTC ACAG
12	Sepsis JC1	166	AAACGTCCGCTTGCAGATACTACAAAAAGAGCGTTTC AAACCTGCTCTATGAAAGGCAATGTTCAACTCTGTGAC TTGAATGCAGACATCACAGAGCAGTTTCTGAGAATGCT TCTGTCTAGATTTTATAGGAAGATATTCCCGTTTCCAA CGAAATCTTCACAGC	AAACGT CCGCTT GCAGAT AC	GCTGTG AAGATT TCGTTG GAAAC
13	Sepsis JC2	146	GGAAATATGGCAAAGTATTTTCTGAGTATGCTGCTGTG TACGTTTTATATTGCATCCCGTTTCCAACGAAATCCTCA AAGCGATCCAAATATCCACTTGCAGATTCCAAAAAAA GAGTGTTTCAAACTGCTCTGTCAGTACAAAGG	AGTATG CTGCTG TGTACG TTT	CCTTTG TACTGA CAGAGC AGTT

14	Sepsis JC4	199	TGTGAACTCAGCTAACAGAGGTGGATCTTTCTTTTGATAGAGCAGTTCTGAAAAACACTTTTTGTTGAATCTGCAAGTGGACATTTGGATAGATTTGAAGATTTCGTTGGAAACGGGAATATCTTCATATCAAATCTAGACAGCAGCATTCCCAGAAATTTCTTTCGGATATTTCCATTCAACTCATAGAGATGAACAT	TCAGCTAACAGAGGTGGATCT	CTATGAGTTGAATGGAAATATCCGAAAG
15	Sepsis JC5	214	CTTGTGGCCTTCGTTGGAAACGGGATTTCTTCATATTATGCTAGACAGAAGAATTCTCAGTAACTTCCTTGTGTTGTGTGTATTCAACTCACAGAGTTGAACGATCCTTTACACAGAGCAGACTTGAAACACTCTTTTTGTGGAATTTGCAAGTGGAGATTTCAGCCGCTTTGAGTTCAATGGTAGTATAGGAAATATCTTCCTATAGAAACTA	CTTGTGGCCTTCGTTGGAAA	ATTGAACTCAAAGCGGCTGAA
16	Sepsis JC6	171	ATATTTGGATAGCTGTGAAGATTTCGTTGGAAACGGGAATATCTTCCTATAAAATCTAGACAGAAGCATTCTCAGAAACTGCTCTGTGATGTTTGCATTCAAGTCACAGAGTTGAACATTGCCTTTCCTAGAGAGGTTTGAAACGCTCTTTTGGTAGTATATGGAAGTGGA	GATAGCTGTGAAGATTTCGTTGG	AGCGTTTCAAACCTCTCTAGG
17	Sepsis JC34	221	GTTTGGAAACACTCTGTCTGTAAAGTCTGCAAGCAGATATTTGGATCTCTTTGAGCCCTTCGTTGGAAACGGGGTTTCTTCATATTATGCTAGACAGAAGAATTCTCAGTAACTTCCTTGTGTTGTGTGTATTCAACTCACAGAGTTGAACGATCCTTTACACAGAGCAGACTTGAAACACTCTTTTTGTGGAATTTGCAAGTGGAGATTTCACAAAAAAC	TGGAAACACTCTGTCTGTAAAGT	CTCCACTTGCAAATTCCACAAA
18	Sepsis JC35	171	GCTCTGCGATGTGTGCGTTCAACTCTCAGAGTTTAACTTTTCTTTTCATTCAGCAGTTTGGAAACACTCTGTTTGTAAAGTCTGCACGTGGATAATTTGACCACTTAGAGGCCTTCGTTGGAAACGGGATTTCTTCATATTCTGCTAGACAGAAGAATTCTCAGAATCTTC	TCTGCGATGTGTGCGTTC	TCTGTCTAGCAGAATATGAAGAAATCC
19	Sepsis JC42	238	AGTTCAACCATTGTGGAAGACAGTGTGGTGATTCCTCAAGGATCTAGAACTAGAAATACCATTTGACCCAGCCATCCCATTACTGGGTATATACCCAAAGGATTATAAATGATTCTACTATAAAGATACATGCACATGTATGTTTATTGTAGCACTCTTCACAATAGCAAAGACTGGGAACCAACCCAAATGCCCATCAATAATAGACTGGATAAAGAAAATGTGGCACATAGATACC	GTTCAACCATTGTGGAAGACAG	CCAGTCTATTATTGATGGGCATTT
20	Sepsis JC48	151	GAATCGAATGGAATCATCGAATGGACTCGAATGGAATAATCATTGAACGGAATCGAATGGAATCATCATCGGATGGAAATGAATGGAATCATCATCGAATGGAATCGAATAGAATTATGGAATGAAATCCAGTGTGATCATCATCGAATGG	TCGAATGGACTCGAATGGAATAA	TCGATGATGATCACACTGGATTT

21	Sepsis JC50	245	AGATATAGAAAAGGCCTTTGACAAAATTCAACAACTC TTCATGCTATAAACTCTCAGTAAATTAGGTATGGATGG GAAATATCTCAAAATAATAGGAGCTATCTATGACAAA CCCACAGCCAATATCATACTGAATGGGCAAAAACTGG GAGCATTCCCTTTGAAAACTGGCACAAGACAGGGATG CCCTCTCTCACCACTCCTATTCAACATAGTGTTGGAAG TTCTGGCCAGGGCAATTAGGC	CAACAA CTCTTC ATGCTA TAAACT CTC	GGCCAG AACTTC CAACAC TAT
22	Sepsis SC2	186	GTGGATATTCGGACCTCTTTGAGGCCTTCGTTGGAAAC GGGATTTCTTCATATTATGCTAGACAGAAGATTTCTCA GTAACTTCTTTGCGTTGTGTGTATGCAACTCACAGAGT TCAACCTTCCTTTAGACAGAGCAGATTTGAAACACTCT TTTTGTGGAATTTGCAAGTGGAGATTTCAAGCGCTTCG ATGCCAATGG	GTGGAT ATTCGG ACCTCT TTGA	GCGCTT GAAATC TCCACT TG
23	Sepsis SC5	180	CAATAACTTGACCAACGGAACAAGTTACCCTAGGGAT AACAGCGCAATCCTATTCTAGAGTCCATATCAACAATA GGGTTTACGACCTCGATGTTGGATCAGGACATCCCGAT GGTGCAGCCGCTATTAAAGGTTCGTTTGTTCAACGATT AAAGTCCTACGTGATCTGAGTTCAGACCGGAGTAATCC AGGTCGGTTTC	CCAACG GAACAA GTTACC CTA	CTGGAT TACTCC GGTCTG AAC
24	Sepsis SC7	162	CTGGCATTTTGTAGATGTGGTTTGACTATTTCTGTATGT CTCCATCTATTGATGAGGGTCTTACTCTTTTAGTATAAA TAGTACCGTTAACTTCCAATTAACTAGTTTTGACAACA TTCAAAAAAGAGTAATAAACTTCGCCTTAATTTTAATA ATCAACACCCTCCTAGCCTTACTACTAATAATTATTAC ATTTTGAC	GTAGAT GTGGTT TGACTA TTTCTGT ATG	GGCTAG GAGGGT GTTGAT TATT

Tab. 22. Combination of motif pairs

motif pair	combination	
1	Sepsis J15	Sepsis JC42
2	Sepsis J15	Sepsis SC2
3	Sepsis J16	Sepsis J17
4	Sepsis J16	Sepsis J23
5	Sepsis J16	Sepsis SC7
6	Sepsis J17	Sepsis JC5
7	Sepsis J19	Sepsis JC6
8	Sepsis J21	Sepsis J23
9	Sepsis J36	Sepsis JC34

10	Sepsis J36	Sepsis SC2
11	Sepsis J7	Sepsis JC50
12	Sepsis JC1	Sepsis JC5
13	Sepsis JC1	Sepsis JC6
14	Sepsis JC1	Sepsis JU11
15	Sepsis JC2	Sepsis JC4
16	Sepsis JC2	Sepsis JC48
17	Sepsis JC34	Sepsis JC50
18	Sepsis JC35	Sepsis JC6
19	Sepsis JC35	Sepsis SC2
20	Sepsis JC48	Sepsis JC6
21	Sepsis JU11	Sepsis JU2
22	Sepsis JU2	Sepsis JU4
23	Sepsis SC2	Sepsis SC5

4.4. Diagnostic classification performance of the quantitative qPCR

The five best-performing algorithms were used for classifier training. The five algorithms used in this study achieved 89.6% overall balanced accuracy and resulted in balanced accuracy values that were stable across multiple points in time. Overall sensitivity was 93.8 % and specificity 85.4%. An overall positive predictive value (PPV) of 93% and a negative predictive value (NPV) of 87.1% was achieved. For these five algorithms, the performance data is shown in Table 23. Table 24 shows the balanced accuracy results ranging from three days before to 1 day after the blood culture was drawn. A sub-analysis regarding differences in sensitivity between bacterial and fungal sepsis did not reveal significant results (data not shown).

Tab. 23. NOBI(C)S Cohort Classification Results (ranked according to "Balanced accuracy")

Classifier trained	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Balanced accuracy
Support Vector Machines with Class Weights	94.6%	87.6%	94.1%	88.6%	**91.1%**
Self-Organizing Map	95.5%	85.8%	93%	90.6%	**90.6%**
Localized Linear Discriminant Analysis	90.9%	90.1%	95%	82.5%	**90.4%**
Mixture Discriminant Analysis	95.2%	83.4%	92.2%	89.4%	**89.3%**
Neural network	92.7%	80.3%	90.7%	84.2%	**86.5%**

Tab. 24. NOBI(C)S Cohort Balanced Accuracy Results across time points (from 3 days prior to 2 days after the blood culture was drawn)

Classifier trained	Day -3 (n=4)	Day -2 (n=11)	Day -1 (n=15)	Day 1 (n=53)	Day 2 (n=54)
Mixture Discriminant Analysis	96.5%	91.9%	96.5%	96.5%	95.6%
Self-Organizing Map	98.6%	98.6%	98.6%	98.6%	98.6%
Support Vector Machine with Class Weights	100%	100%	100%	100%	100%
Localized Linear Discriminant Analysis	100%	100%	100%	100%	100%
Neural network	80%	90.9%	93.3%	94.7%	94.8%

The boxplots in Figure 22 depict the *Cq* value differences (ΔCq) from four motif combinations between bacteremic/fungemic sepsis and control cohort. If there is a difference between *Cq* values the motif pair can be used to distinguish between sepsis and control. All depicted marker pairs (boxplot A-D) show higher ΔCq values for sepsis samples, which mean they can be used to predict sepsis. For example, boxplot B shows the ΔCq value for the primer pair J16 and SC7, following the formula $\Delta Cq = Cq_{J16} - Cq_{SC7}$. The ΔCq value for this primer pair in the sepsis group is around -5, indicating that motif J16 comes five cycles earlier than motif SC7, if the patient is suffering from sepsis. If he or she is not, the difference is only around three cycles. Boxplot C shows the ΔCq values for the primer pair JC2 and JC4, which is for sepsis samples around 3, indicating that motif JC2 comes three cycles later than motif JC4, if the patient is suffering from sepsis. Only the detection of a difference represented as ΔCq value leads to a prediction if the patient will develop sepsis, whether or not the value is positive or negative.

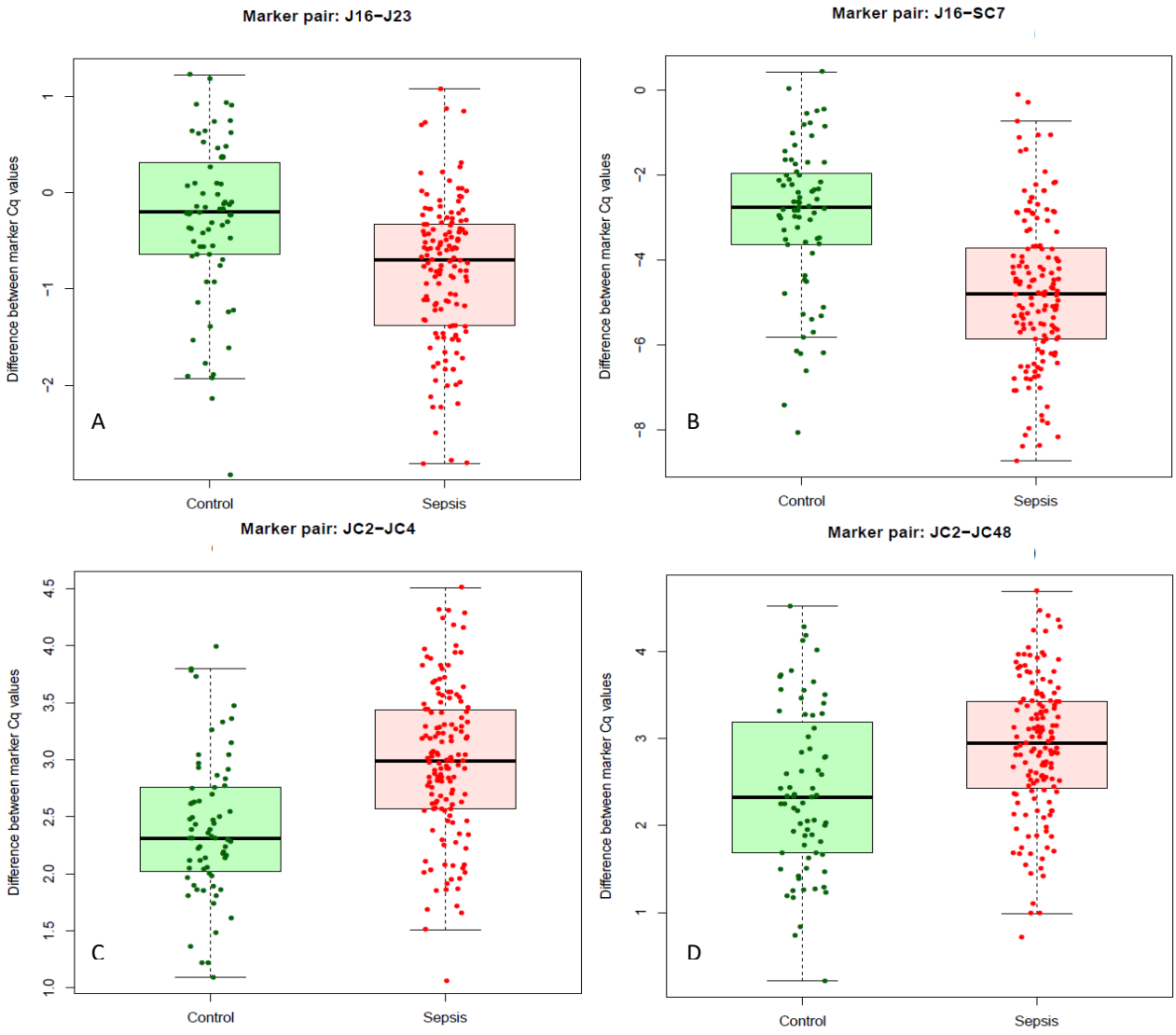


Fig. 22. The boxplots depict the distribution of differences between *Cq* values (Δ*Cq* on y-axis) for the two classes (control and sepsis) for four different marker pairs. The dots represent the samples with their horizontal positions randomly assigned to avoid overlapping. Green box: control group, red box: sepsis group. A: Δ*Cq* value in the sepsis group is -1 versus 0 in the control group. B: Δ*Cq* value in the sepsis group is around -5 versus -3 in the control group. C: Δ*Cq* value in the sepsis group is 3 versus around 2.3. D: Δ*Cq* value in the sepsis group is 3 versus around 2.2 in the control group. With permission of Jung Soh, CNA Diagnostics Inc., Calgary, Canada, 2020.

4.5. Multiplex qPCR

QPCR experiments with SYBR Green® and TaqMan™ probes (Singleplex and Multiplex assays) were performed: (i) to find the perfect melting temperature by performing gradient PCR experiments; (ii) to evaluate the performance of different Master mixes (SsoAdvanced™ Universal Probes Supermix versus IQ™Multiplex Powermix, both Bio-Rad Laboratories, USA); (iii) to determine the influence of plasma on PCR efficiency; and,

(iv) to define the influence of five PCR reactions in parallel in order to establish a Multiplex assay.

4.5.1. Melting Temperature

Gradient qPCR experiments with temperatures from 55°C to 65°C were performed. Five motifs (Sepsis 2, Sepsis 7, Sepsis 11, Sepsis 17 and Sepsis/Control 9) were detected by qPCR in Singleplex and Multiplex assays. Increasing concentrations of gBlocks® (IDT, Leuven, Belgium) and human genomic DNA (Sigma-Aldrich, St. Louis, Missouri, USA) were used as templates. Figure 23 depicts agarose gel electrophoresis results for a gradient temperature PCR experiment with temperatures from 55°C to 60°C. Single bands for all tested motifs were shown at a temperature around 60°C. These results were confirmed by melting curve analysis, which revealed single peaks for all tested motifs at 60°C (Fig. 24). Therefore, all further experiments were done at 60°C.

Fig. 23. 2% Agarose gel electrophoresis of temperature gradient PCR experiments. Temperature ranged from 55°C (left bars) to 60°C (right bars). Single bands are shown at 60°C (last lane) for all tested motifs. Motifs: Sepsis 2 (99 bp), Sepsis 7 (114 bp), Sepsis 11 (109 bp), Sepsis 17 (94 bp), Sepsis/Control 9 (113 bp);

Mastermix: SsoAdvancedTM Universal Probes Supermix (Bio-Rad Laboratories, USA) Template: 40 ng human genomic DNA. Standard: GeneRuler™ DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

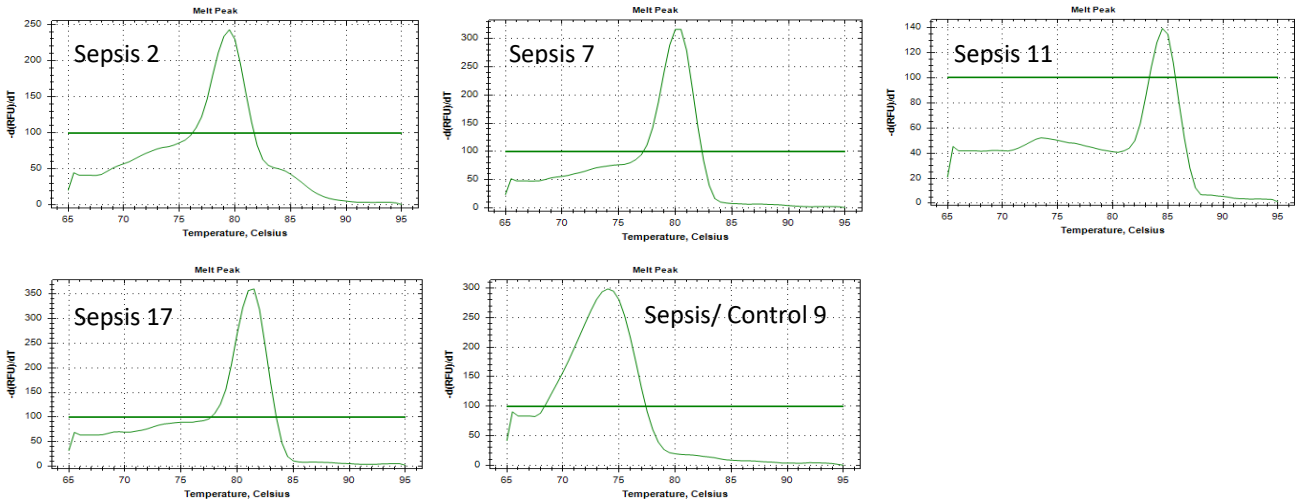


Fig. 24. Corresponding melting curves with single peaks for all tested motifs at 60°C. Template: 40 ng human genomic DNA. Mastermix: SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, USA) Template: 40 ng human genomic DNA.

4.5.2. Evaluation of Master Mixes

Two different master mixes (SsoAdvanced™ Universal Probes Supermix or IQ™ Multiplex Powermix both from Bio-Rad Laboratories, USA) were evaluated. Performing Multiplex assays with five reactions in parallel, both master mixes showed similar results as long as gBlocks® (IDT, Leuven, Belgium) was used as template. Performance of SsoAdvanced™ Universal Probes Supermix decreased when template changed to human genomic DNA, independent of the used amount (5-40 ng, Fig. 25, pictures A-D). In that case, IQ™Multiplex Powermix revealed better amplification results depending on the used template amount (Fig. 25, pictures E-H). Good amplification was shown for PCR using 40 ng of human genomic DNA as template (Fig. 5, picture H).

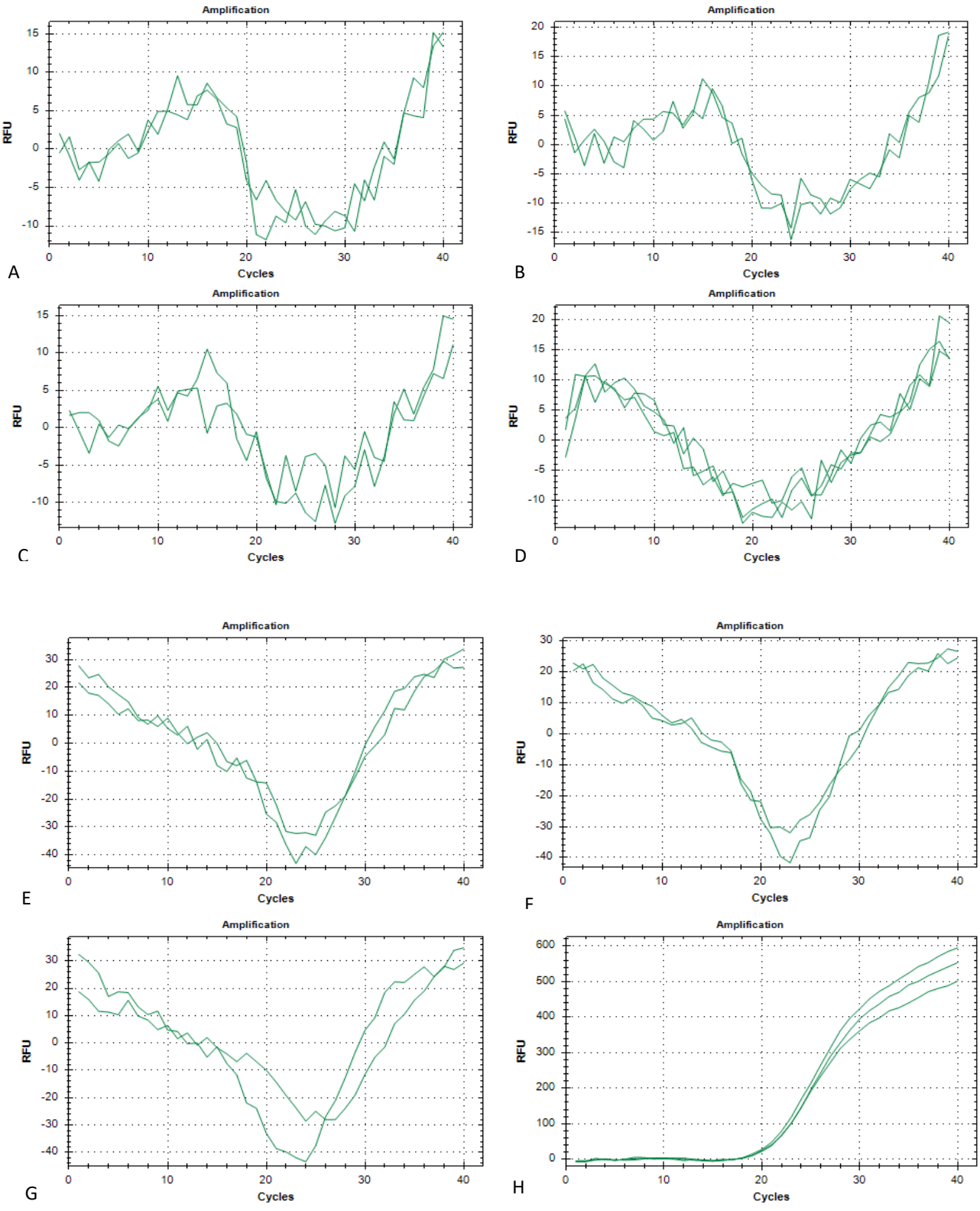


Fig. 25. A-D: no amplification of motif Sepsis 2 (99 bp) in a Multiplex assay with SsoAdvanced™ Universal Probes Supermix; Template: human genomic DNA, A: 5 ng, B: 10 ng, C: 15 ng, D: 40 ng F-G: no amplification of motif Sepsis 2 with IQ™ Multiplex Powermix; Template: humane genomic DNA, E: 5 ng, F: 10 ng, G: 15 ng; H: good amplification of motif Sepsis 2 with IQ™ Multiplex Powermix; Template: 40 ng humane genomic DNA

4.5.3. Multiplex PCR with five PCR reactions in parallel

Under the conditions described above (annealing temperature 60°C; IQ™ Multiplex Powermix) multiplex assay showed good performance for this marker set for both tested templates (100-1 000 000 copies of gBlocks® and 40ng human genomic DNA). All motifs revealed good amplification curves. *Cq* value for motifs Sepsis 2, Sepsis 7 and Sepsis 17 was 20 (template: 40 ng human genomic DNA); Sepsis 11 had a *Cq* Value of 18.5, motif Sepsis/Control 9 a *Cq* value of 9.5, indicating that this sequence appears much more often than the others (confirmed by using Bowtie2, an alignment search tool, to align the sequence against the human genome GRCh38; the sequence had 7879 perfect alignments). Figure 26 depicts amplification curves for the established multiplex assay (performed in triplicate).

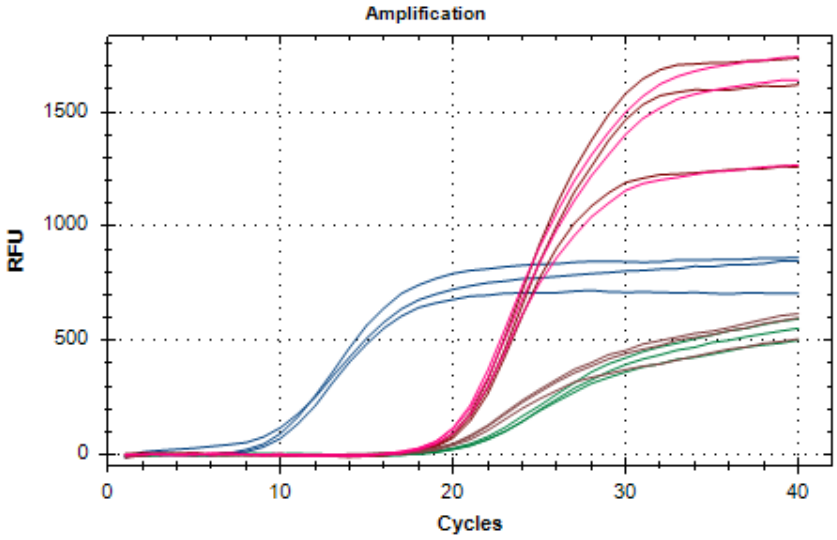


Fig. 26. Amplification curves for the Multiplex assay at 60°C for marker set 1 (green: Sepsis 2; pink: Sepsis 7; lower brown curve: Sepsis 11, brown (next to pink curve): Sepsis 17; blue: Sepsis/Control 9), Template: 40 ng human genomic DNA; Mastermix: IQ™ Multiplex Powermix

4.5.4. Influence of Plasma on PCR efficiency

To determine the influence of plasma on the PCR efficiency 2 µL of human plasma (undiluted, 1:10 and 1:40 dilution) were added to the PCR reaction. The used templates were human genomic DNA and gBlocks®. PCR efficiency remained stable after adding human plasma as long as the template was gBlocks® and plasma was diluted. When human genomic DNA was used as template, the amplification of motif Sepsis 2 deteriorated after adding human plasma (Fig. 27, Picture D). *Cq* values of all other motifs (with exception of motif Sepsis 2) remained nearly equal to the *Cq* values determined in experiments without adding

plasma. It seems that some components of human plasma interfere with the fluorophore (CAL Fluor® Gold 540) of motif Sepsis 2. Additionally, to determine the robustness of the established qPCR, singleplex experiments with bovine serum (undiluted, 1:10 and 1:40 dilution) were performed. *Cq* values were comparable to experiments without serum and experiments with diluted human plasma (template: different amounts of gBlocks®; target motif: Sepsis 2, see Fig. 28; performed in triplicate). The determined *Cq* values are shown in Table 25.

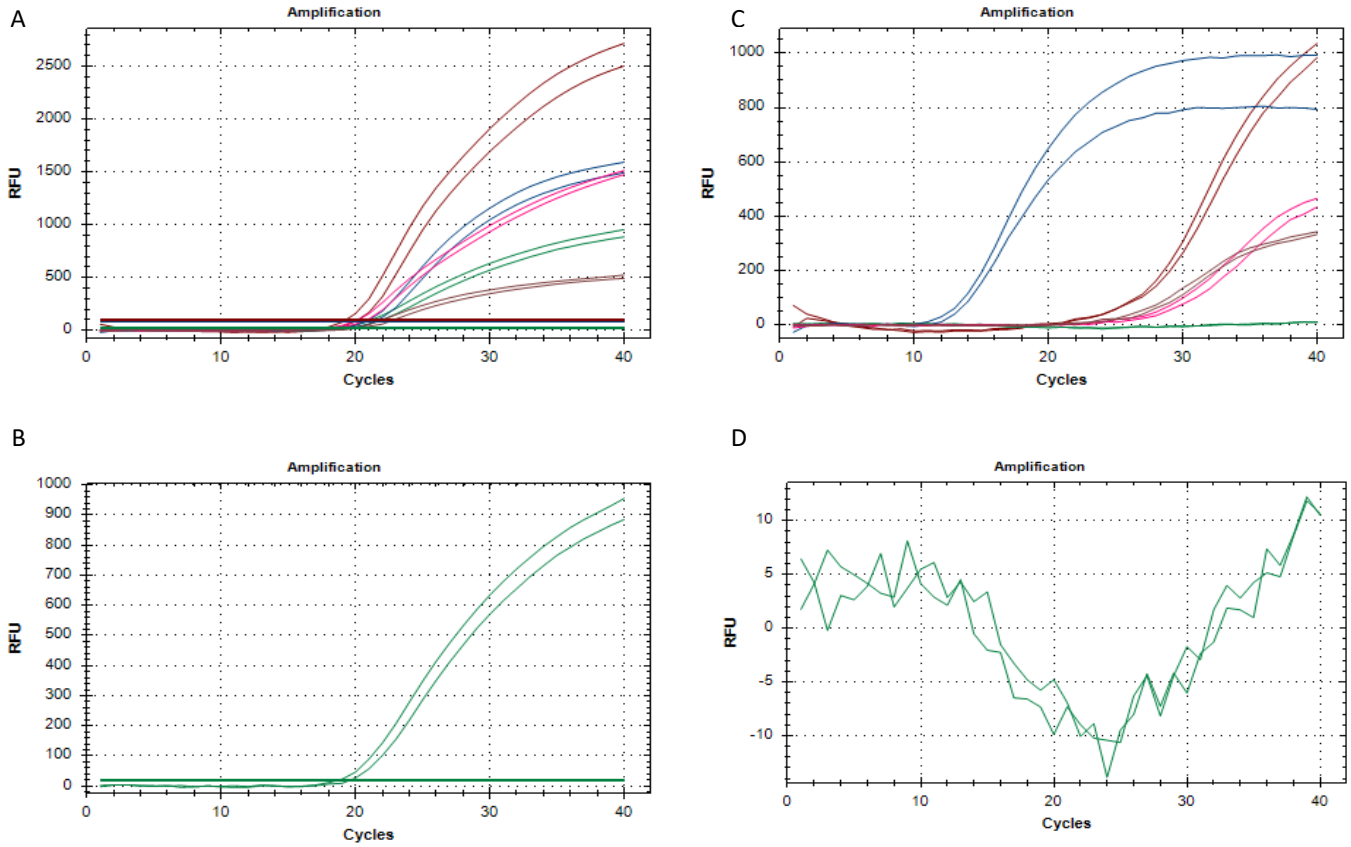


Fig. 27. Multiplex assay at 60°C, IQ™ Multiplex Powermix, A+B: Template: 2000 copies gBlocks®, 2µL of human plasma (1:10 dilution) added to the PCR reaction; A: all motifs, B: motif Sepsis 2; C+D: Template: 60 ng human genomic DNA, 2µL of human plasma (1:10 dilution) added to the PCR reaction, C: all motifs, D: motif Sepsis 2 (green: Sepsis 2; pink: Sepsis 7; lower brown curve: Sepsis 11, brown (next to pink curve): Sepsis 17; blue: Sepsis/control 9)

Tab. 25. Determined *Cq* values without plasma/serum, after addition of 2 µL of human plasma (undiluted, 1:10 and 1:40 dilution) and 2 µL of bovine serum (undiluted, 1:10 and 1:40 dilution); Target: motif Sepsis 2, Template: 10-10 000 000 copies gBlocks®

gBlocks® (copies)	without plasma/ serum	human plasma undiluted	human plasma 1:10 dilution	human plasma 1:40 dilution	bovine serum undiluted	bovine serum 1:10 dilution	bovine serum 1:40 dilution
10	30	37.5	33.5	32	38	33	30
100	28.5	38	33	31.5	39.5	32	30
1 000	26	37	30	29	37.5	29	27
10 000	22	39	27	26	34.5	26	24
100 000	19	38.5	23	22	32	22	20.5
1 000 000	16	38	20	19	29	19	17
10 000 000	12.5	38	17	16	27	15.5	14

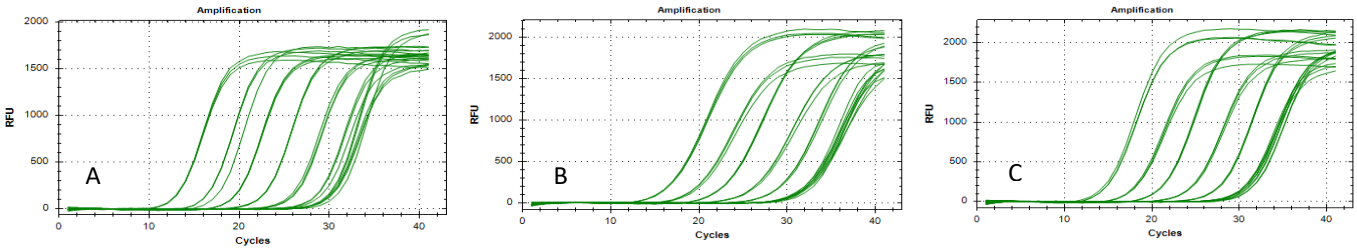


Fig. 28. Amplification curves without plasma/serum (A), with 2 µL of human plasma (1:40 dilution) added to the PCR reaction (B), with 2 µL of bovine serum (1:40 dilution) added to the PCR reaction (C); Experiment performed in triplicates; Target: motif Sepsis 2, Template: 10-10 000 000 copies gBlocks®; Mastermix: LUNA qPCR (New England Biolabs, Massachusetts, USA)

4.5.5. Diagnostic classification performance of the Multiplex qPCR

Diagnostic classification performance of the first marker set was quite low. The marker set using Multiplex qPCR contained four sepsis specific motifs (Sepsis 2, Sepsis 7, Sepsis 11 and Sepsis 17) and one so-called universal motif (Sepsis/Control 9). The five motifs could not distinguish between sepsis and control group. Hence, more attention was set on finding new motifs and establishing qPCR via Singleplex assay (see section 4.3. and 4.4.).

4.6. Digital PCR

First trials were performed by using digital PCR (QuantStudio® 3D Digital PCR System, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Motif Sepsis 2, labeled with VIC and motif Sepsis/Control 9 labeled with FAM were the targets. Experiments with gBlocks® as template revealed quantification rates between 55-66%. Figure 29 shows a scatter plot generated by QuantStudio® 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The software assesses whether the data on

a chip is reliable based upon loading, signal, and noise characteristics and displays quality indicators for each chip in a project (green, yellow or red flag). In the depicted plot 10 000 copies gBlocks® were used as template. Chip quality was good (green flag). Quantification results revealed 5520 copies of motif Sepsis/Control 9 (FAM) and 5750 copies of motif Sepsis 2 (VIC), corresponding to an error factor of 1.7.

Fig. 29. dPCR scatter plot shows signal from FAM reporter dye (motif Sepsis/Control 9) on the Y-axis against the signal from VIC reporter dye on the X-axis (motif Sepsis 2). The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow). Chip quality: good (green flag). Template: 10 000 copies gBlocks®. FAM: 5520 copies; VIC: 5750 copies

Quantification results decreased after adding human plasma. It seemed that components of plasma (i.e., proteins, salts, lipids) were inhibiting the PCR reactions. In figure 30, the dPCR scatter plot shows no amplification after adding human plasma. Due to these problems, establishing a digital PCR (dPCR) failed. Therefore, the primary focus of this study was put on qPCR experiments.

Fig. 30. dPCR scatter plot shows no amplification (yellow). Chip quality: medium (yellow flag). Template: 1000 copies gBlocks®, addition of 5.5µL human plasma.

5. Discussion

We identified 24 molecular motifs, based on circulating nucleic acids originating from the human genome, which in combination can be used in a quantitative qPCR assay to predict development of human sepsis due to bacteremia/fungemia at an early onset. We were able to identify genomic regions, which showed significant differences in fragment coverage when comparing sepsis patients and control individuals, thus enabling the use of these particular genomic regions as targets for qPCR assay development.

In comparison to other studies, we considered some new aspects in this study:

- Hard inclusion criteria regarding sepsis cohort
- Control cohort consisted also of diseased individuals
- Determination of host-related and not pathogen-related markers
- Possibility to predict onset of sepsis, two to three days before any clinical signs pointing towards bacteremic and/or fungemic sepsis
- First steps were made for establishing a Multiplex assay

Several studies have used very broad inclusion criteria, including patients with a wide range of illness severity, reducing the sensitivity of these studies (104). We included only patients who had a genuine infection, diagnosed by clinical and laboratory signs of infection like fever, chills and/or leucocytosis, CRP and/or PCT elevation. Infection was confirmed microbiologically for every patient. All patients of the study group had positive blood cultures with a relevant pathogen, which means they were suffering from bloodstream infection. According to the "The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)" sepsis is a severe and life-threatening response to infection with bacteremia/fungemia as one of the major causes (6). Clinicians are encouraged to use the SOFA score, with an increase of two points or more being associated with a higher in-hospital mortality (23,24). For all patients the SOFA score, which is more specific than the SIRS criteria, was calculated retrospectively. All but four patients had an increase in SOFA Score of ≥ 2 points compared to the values determined up to 72 prior and 48 hours after the indication of drawing blood cultures was evident. As stated in literature, specific inclusion criteria based on the current sepsis definition should be used for enrolment in sepsis trials (105). The use of inclusion criteria employing the severity of organ dysfunction may help with limiting selection bias (104). In this study all but four patients fulfilled the current

definition for sepsis due to confirmed infection by hard criteria (positive blood culture) and a change in SOFA Score ≥ 2 points. Values for calculating the SOFA Score were determined on day 1 (the day when blood cultures were drawn) and compared to the values up to days -3 and +3. We have to discuss that four patients did not fulfil sepsis-3 criteria at this time point, but had positive SIRS criteria as used prior to Sepsis-3 definitions. However, as Sepsis-3 guidelines were published in 2016 and we included patients starting in 2015 the mixture of SIRS and Sepsis-3 criteria is based on changing sepsis definitions during the study period. Another limitation was the lack of data, so we had no dedicated documentation of the GCS. As all patients fulfilled sepsis definitions, we did not retrospectively calculate GCS based on documented neurological examinations. Considering these circumstances, we postulate that some patients would have fulfilled higher SOFA values according to the Sepsis-3 definition if data had been complete. Our control group included 46 healthy volunteers as well as patients suffering from severe diseases like lymphoma (2 patients) or viral infectious disease like influenza (10 patients). Despite the fact that elevated CNA levels are also a marker for various diseases in general (79) our assay was able to distinguish between bacteremic/fungemic "sepsis" and "no sepsis" and not only between diseased and healthy. A limiting factor may be that the number of healthy volunteers was nearly four times higher than the number of diseased controls. Sub-analysis with higher sample sizes of diseased controls are advised. The median age of the control cohort was 44.5 years (range 19–86) in contrast to the sepsis cohort, where the median age was 68 years. Elevated CNA levels are associated with multiple aging-related diseases, such as cancer, diabetes, and Alzheimer's disease. It is unclear whether age alone has a big disease-independent influence on CNA levels.

Compared to tests based on the detection of the infectious organisms, we included patients suffering from infection due to different bacteria as well as fungi. Another advantage of this approach is that its validity is independent from the causative agent, as we determine host-related markers and not pathogen-derived structures. For generating the motifs, we include three different species of bacteria (*S. aureus, E. coli* and *S. epidermidis*) and three different species of *Candida* (*C. albicans, C. glabrata* and *C. parapsilosis*), all causative pathogens of bloodstream infection. At the moment, the test's performance in detecting sepsis caused by other pathogens is still unclear. Further studies in this field are needed. Sub-analyses regarding differences in sensitivity between bacterial and fungal sepsis did not reveal significant results (data not shown), but due to a limited sample size (59 samples of fungal

sepsis and 88 of bacterial sepsis) further examinations and studies with more samples are necessary.

Just a few studies have focused on markers for early detection of sepsis thus far (106–108). In our study, plasma samples drawn at an early time before patients developed symptoms were included. The main advantage of the approaches developed in this study is the possibility to predict the onset of severe infection, and subsequently sepsis, two to three days before any clinical signs pointing towards bacteremia/fungemia and/or sepsis emerge. The detection of the onset of sepsis with two to three days prior to first clinical signs is unique to our approach, as none of the other tests have been shown to predict the onset of human sepsis prior to first clinical signs. The small sample size of early plasma samples (4 samples from day -3 and 11 from day -2) may be a limiting factor. This early detection capability needs to be confirmed in future studies by including more samples from days before the onset of symptoms. However, this study included 15 samples from day -1 and 53 samples from the day when blood cultures were drawn. Considering that most patients at this point in time are still asymptomatic or oligosymptomatic, and results of blood cultures on day one are usually not immediately available, this test can lead to a rapid and early source identification and treatment. A future field of application for this assay may be patients who have an increased risk for developing sepsis like immuno-compromised, critically ill patients, or patients with severe burns. Regular testing of these patients may prompt early interventions and preserve this vulnerable group from developing sepsis. Future studies to evaluate the clinical benefit on patients' outcome of this test system are mandatory.

Multiplex experiments were essential in finding the right melting temperature, for master mix evaluation and to determine how five PCR reactions in parallel influence each other. The marker set we used for Multiplex qPCR contained four sepsis-specific motifs and one so-called universal motif. Unfortunately, this marker set was not useful in differentiating between sepsis and controls. We, therefore, circled back and focused on the sequencing of plasma samples from the described cohorts, followed by establishing qPCR as Singleplex assays (1). Nonetheless, the medium-term objective is to create a Multiplex qPCR assay to detect sepsis-specific CNA motifs. The advantages of Multiplex assays are a shorter hands-on-time in laboratory and the possibility to handle more samples concurrently. Using a set of 24 markers requires five wells in a qPCR multiplex assay (when using the BioRad CFX96 Touch™ Real-Time PCR Detection System or similar qPCR machine types), which would allow more than ten diagnostic tests to take place simultaneously on a single plate.

Compared to other routinely used diagnostic tools and parameters our assay expands the spectrum with some benefits.

Blood cultures are still the “gold standard” in diagnosing bacteremia, because pathogen identification and antimicrobial susceptibility testing is possible, but there are still some disadvantages of this method. Negative results are possible and common, especially in patients with invasive candidiasis where an estimate of 50% are not detected by blood cultures (64). Due to intermittent bacteremia (109), which is defined as recurring bacteremia due to discontinuous seeding of the same organisms, which can be caused by infections such as abscesses, cholangitis, pneumonia, osteomyelitis, spondylodiscitis and focal infections, blood cultures can reveal negative results. Because of low numbers of circulating bacteria drawing an appropriate volume of blood is crucial (62,110). Lee et al. had shown that drawing more blood cultures increases the percentage of detected pathogens. Detection of invasive candidiasis had been increased of 10% if three blood culture pairs in place of two were obtained (63). Sometimes repeated drawings must be performed and each venepuncture contains the risk of side effects like hematoma, thrombosis or infection. Concomitant antimicrobial treatment can decrease the yield of microbiological cultures significantly. Microbiological pathogen detection is significantly higher if blood cultures are taken prior to antimicrobial therapy (58). Despite the fact that most bacteria of severe bacteremia grow within two days, the blood culture incubation period can go up to five to seven days until they turn positive (59,111). Another problem is the existence of not cultivable bacteria such as *Bartonella sp.*, *Coxiella sp*., *Bordetella sp*., *Legionella sp*., *Mycoplasma sp*. etc., which are usually diagnosed serologically, by antigen detection assays or via PCR. Furthermore, detection of anaerobic bacteria can be more difficult regarding time and sensitivity (60). In a prospective observational cohort study of 1001 patients 41.5% had culture-negative sepsis (112).

In conclusion, blood culture results are often negative and even when they are positive there are some difficulties in interpretation. Contaminants are common, especially if microorganisms of questionable evidence, like bacteria of the skin or environmental flora, are cultivated (113). The identity of the organism, the number of positive culture sets, the number of positive bottles within a set, the time to positivity, clinical and laboratory data and patient’s condition are important hints in interpreting blood culture results and differentiating between true pathogen and contaminant (114).

Molecular tests, which are based on the detection of the infectious organisms, for example the T2Candida® and T2Bacteria® panels (T2 Biosystems, Lexington, Massachusetts, USA) or the BioFire® filmarray system (bioMerieux, Marcy-l'Étoile, France), have some limitations which narrow their clinical benefits.

The T2Candida® panel enables the detection of five *Candida* species consolidated into three groups (*C. albicans/C. tropicalis, C. parapsilosis, and C. krusei/C. glabrata*) from EDTA whole blood within 3-5 hours. A recent study evaluated 133 samples of 32 patients with candidemia and 22 patients with deep-seated invasive candidiasis. Eight of 25 candidemic patients (32%) had a negative T2 result on day 0 (day when blood cultures were drawn), despite positive blood cultures. Furthermore, the system revealed discordant *Candida* species identification in two candidemic patients. The authors of the study conclude that despite the advanced time-to-results the clinical value of T2Candida® in diagnosing candidemia seems to be limited (115). The bacterial panel (T2Bacteria®) from T2 Biosystems is able to identify five different bacterial strains (*Enterococcus faecium, S. aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *E. coli*) from EDTA whole blood within 3-5 hours. Beside the fact that these five organisms account only for around 50% of blood stream infections (116) sensitivity and specificity of T2Bacteria® reached just 90% within this limited spectrum (117). Only 48% of positive blood cultures were covered by T2Bacteria®, which means 52% of blood stream infection were missed due to the limited spectrum of the panel (117). This result was strengthen by another study which confirmed that 47.8% of bacteria isolated by culture belonged to species not covered by the T2 panel (118).

The Biofire® filmarray system (bioMerieux, Marcy-l'Étoile, France) identifies 19 bacterial pathogens, five yeasts and three resistance markers directly from positive blood cultures. It provides results in about 1 hour with 2 minutes for assay setup, as soon as blood cultures turn positive. As stated before, around 40% of patients have blood-culture negative sepsis and if blood cultures turn positive it may require a few days. Another major aspect is interpretation or rather misinterpretation of Biofire® results by clinicians. A study showed that nearly 50% of Biofire® results were misinterpreted due to incomplete understanding of the information contained in the microbiology report. This obviates the benefits of rapid pathogen identification and may result in ineffective treatment or overuse of broad-spectrum antibiotics (119).

In comparison to these molecular tests, our assay works independently from the causing pathogen and can be used to capture bacterial and fungal sepsis cases at a very early time point.

5.1. Limitations

As already discussed above, our study has some limitations. For motif generation we include only six pathogens (three bacteria and three *Candida ssp.*). Considering that this approach detects host-related CNA, we postulate that the influence of different pathogens will not be high. A second study including more samples with more pathogens trying to answer this question is currently being conducted (1). Another possible source of bias is the limited diversity of the cohorts. All patients from our cohorts were of Caucasian ethnicity, and all samples were collected at the Medical University of Graz (mono-center study). At the moment we do not know how much the results are influenced by race, ethnicity, and geographical location. To answer these very important questions, more studies including patients from different ethnicities and multi-center studies are needed. In a current study we included 98 African-American samples with similar performance (data are not published yet). Another limiting factor is the retrospective study design. Samples were collected prospectively but data collection, selection, preparation and calculation of data was performed retrospectively. Furthermore, the results of the five best-performing algorithms were based on the same sample set used for marker development and classification training. There is evidence that the balanced accuracy dropped by at least 10% for these algorithms when testing on a set of blinded samples, that had not been used for classifier training (1). Prospective studies and blinded testing of more samples are urgently needed.

6. Conclusion

The presented results are promising for the development of a future commercial assay. Given the high sensitivity and specificity values reached in this study, we are already conducting additional studies and plan to do additional experiments. A medium-term objective is to create a Multiplex qPCR assay. As our approach is based on the genetic system of mammalian species, it could also be used to develop diagnostic assays based on host-response markers for many other diseases. Long-term objectives are further clinical studies to evaluate the test's performance in clinical routine.

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