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Klinik für Anästhesiologie, operative Intensivmedizin und Schmerztherapie

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Systemische Inflammationsreaktionen bei operativen Intensivpatienten

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Implikationen aus der Biomarkerforschung: Von der Diagnose bis zur Langzeitsurveillance

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Verzeichnis der Anlagen

1. **The impact of early surgical intervention in free intestinal perforation: A Time-to-Intervention Pilot Study.**

   Hecker A*, **Schneck E***, Röhrig R, Roller F, Hecker B, Holler J, Koch C, Hecker M, Reichert M, Lichtenstern C, Krombach GA, Padberg W, Weigand MA. World J Emerg Surg. 2015 Nov 6;10:54.

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2. **Comparison of qSOFA score, SOFA score, and SIRS criteria for the prediction of infection and mortality among surgical intermediate and intensive care patients.**

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3. **Longitudinal Evaluation of Plasma Concentrations of Presepsin in Patients after Severe Trauma: A Prospective Observational Study.**
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4. ***Host-Derived Delta-Like Canonical Notch Ligand 1* as a Novel Diagnostic Biomarker for Bacterial Sepsis – Results from a Combinational Secondary Analysis.**
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5. **Plasma DNA and RNA Differentially Impact Coagulation during Abdominal Sepsis – An Explorative Study**
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9. **Trauma-Induced Long-Term Alterations of Human T Cells and Monocytes – Results of an Explorative, Cross-Sectional Study.**
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   ***Geteilte Erstautorenschaft, #Korrespondierender Autor**

Anlagen sind in der Reihenfolge aufgeführt, in der sie im Weiteren bearbeitet werden.

Inhaltsverzeichnis

Abkürzungsverzeichnis

AUCROC	Area under the Curve einer Receiver Operator Curve
BTLA	B- and T- Lymphocyte Attenuator
CARS	Compensatory Anti-Inflammatory Response Syndrome
CD	Cluster of Differentiation
CRP	C-reaktives Protein
CT	Clotting Time
CTLA-4	Cytotoxic T-lymphocyte-Associated Protein 4
DAMP	Damage-associated Molecular Pattern
DIC	Disseminated Intravascular Coagulation
DNA	Deoxyribonucleic Acid
DLL-1	Delta-Like Canonical Notch Ligand 1
ELISA	Enzyme-linked Immunosorbent Assay
GCS	Glascow Coma Scale
GDT	Goal-directed Therapy
GE	Genomic Equivalents
HLA-DR	Humanes Leukozyten Antigen - DR
HMGB-1	High-Mobility-Group-Box Protein 1
IL	Interleukin
IFN-γ	Interferon-γ
INR	International Normalized Ratio
IQR	Interquartile Range
ISS	Injury Severity Score
LPS	Lipopolysaccharid
MAP	Mean Arterial Pressure
MCF	Mean Clot Firmness

MESF	Molecules to Equivalent Soluble Fluorophore
MMP-9	Matrix Metalloproteinase 9
MODS	Multiple Organ Dysfunction Syndrome
MPO	Myeloperoxidase
NE	Neutrophile Elastase
NETs	Neutrophil Extracellular Traps
N.S.	Nicht signifikant
PAI-1	Plasminogen-Aktivator-Inhibitor 1
PAMP	Pathogen-associated Molecular Pattern
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death 1
PD-1L	Programmed Cell Death Ligand 1
PICS	Persistent Inflammation and Immunosuppression
PMA	Phorbolmyristatacetat
PRR	Pattern Recognizing Receptors
PTT	Partielle Thromboplastinzeit
RAGE	Receptors for Advanced Glycation End Products
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Realtime-Polymerase Chain Reaction
sCD14-ST	sCD^{14+} Subtype (Presepsin)
SIRS	Systemic Inflammation Response Syndrome
SOFA	Sequential Organ Failure Assessment
TNF-α	Tumor Necrosis Factor
TLR	Toll-Like-Rezeptoren
TU	Transcriptional Units

1 Einleitung

1.1 Präambel

Die Sepsis und der septische Schock sind trotz intensiver Forschungsbemühungen weiterhin mit einer hohen Mortalität von bis zu 60 % und einer langfristigen Morbidität assoziiert[1–4]. Zwar konnte die Weiterentwicklung von zielgerichteten diagnostischen und therapeutischen Verfahren die Mortalität senken, dennoch bleibt aber die frühzeitige Diagnose der Sepsis einer der hauptsächlichen Prädiktoren für das Überleben septischer Patienten[†]. Zu diesem Zweck wurde eine Vielzahl neuartiger Biomarker zur Identifikation septischer Erkrankungen evaluiert, ohne jedoch Eingang in die klinische Routinediagnostik zu erhalten[5–7]. Ein Grund hierfür ist häufig die mangelnde Trennschärfe der Parameter bei der Unterscheidung von septischen Erkrankungen zu anderen Ursachen von systemischen Inflammationszuständen. Insbesondere auf operativen Intensivstationen ist dieses Problem bekannt, da große operative Eingriffe häufig mit ausgeprägten postoperativen Inflammationsreaktionen einhergehen. So ähneln die klinischen Symptome eines postoperativen Inflammationssyndroms denen einer Sepsis und die etablierten Biomarker erscheinen dabei in der Regel ebenfalls erhöht[8]. In der Konsequenz kann dies zur Verschleierung einer Sepsis im postoperativen Verlauf und somit zu einer verzögerten Diagnose führen. Um dieser Problematik entgegen zu treten, werden alternative Biomarker benötigt, welche im Idealfall neben den immunologischen Veränderungen auch Störungen in weiteren Organsystemen anzeigen. Diese kumulative Habilitationsschrift fasst neun Studien zu diesem Themenkomplex zusammen, in welchen neben den akuten Veränderungen auch die langfristigen immunologischen Reaktionen septischer Erkrankungen im Vergleich zu sterilen Inflammationsreaktionen untersucht werden.

[†] Aus Gründen der besseren Lesbarkeit wird in dieser kumulative Habiliationsschrift die Sprachform des generischen Maskulinums verwendet. Es wird an dieser Stelle ausdrücklich darauf hingewiesen, dass die Verwendung der männlichen Form geschlechtsneutral verstanden werden soll.

1.2 Definition und Epidemiologie der systemischen Inflammationsreaktionen auf der operativen Intensivstation

1.2.1 Systemische Inflammationsreaktionen bei operativen Intensivpatienten

Systemische Inflammationsreaktionen treten regelhaft nach großen operativen Eingriffen, schweren Traumata, Verbrennungen und anderen kritischen Erkrankungen auf. Dabei werden sie durch das Vorliegen von mindestens zwei von insgesamt vier physiologischen Kriterien des *Systemic Inflammation Response Syndrome* (SIRS) definiert (Tabelle 1)[2,9,10]. Zwar werden die SIRS-Kriterien seit der Einführung der Sepsis-3-Definition nicht mehr zur Diagnose der Sepsis verwendet, dennoch sind sie weiterhin für die Identifikation von systemischen Inflammationszuständen valide[2,11,12]. Sie sollen klinische Befunde reflektieren, welche auf Alterationen des Immunsystems, der hämodynamischen Funktion, des respiratorischen und Gerinnungssystems sowie der metabolischen und hormonellen Homöostase basieren[13,14]. Validierungsstudien wiesen nicht nur eine erhöhte Mortalität bei operativen Patienten auf, welche die SIRS-Kriterien erfüllten, sondern auch eine Assoziation zwischen der Mortalitätserhöhung und der Menge an erfüllten SIRS-Kriterien[2,15,16]. Auf Basis der zu Grunde liegenden komplexen Reaktionen, welche im Rahmen eines postoperativen SIRS entstehen, wurde die Charakterisierung des SIRS basierend auf nur vier Kriterien kritisiert, ohne allerdings eine einheitliche Definition für die postoperative Situation zu etablieren. Hauptkritikpunkt ist die geringe Sensitivität der SIRS-Kriterien in Bezug auf die Identifikation eines SIRS sowie in der Diskriminierung des SIRS von manifesten Infektionen. Dies resultiert beispielsweise in einer hohen Varianz der beschriebenen Inzidenz des postoperativen SIRS (16 – 89 %) nach großen allgemeinchirurgischen Eingriffen[17,18]. Auf Grund dieser hohen Varianz ist die Diskrimination von etwaigen Infektionen im Rahmen des postoperativen SIRS schwierig und werden neue Methoden zur Beschreibung der postoperativen systemischen Inflammation mit möglichst hoher Diskrimination in Bezug auf Infektionen gesucht. Eine Lösung für diese Problematik könnte die Erhebung der klinischen Befunde mittels des ausführlicheren *Sequential Organ Failure Assessement* (SOFA) Score darstellen (Tabelle 2). Dieser ist allerdings lediglich für die Prognoseabschätzung septischer Patienten validiert und wird daher bisher auch nur für die Definition der Sepsis und nicht für die postoperative systemische Inflammationsreaktion empfohlen[1]. So konnte

beispielsweise an einem großen Kollektiv von 1.942 traumatologischen Patienten keine ausreichende Prädiktion für die Detektion von Infektionen nach operativen Eingriffen mittels des SOFA-Scores nachgewiesen werden[19]. Eine weitere Strategie stellt die Evaluation neuer Biomarker zur Erkennung von systemischen Inflammationsreaktionen nach operativen Eingriffen dar. Hierzu wurde eine Vielzahl unterschiedlicher Biomarker auf Basis verschiedener operativer Trigger (z.B. abdominal- und herzchirurgische Eingriffe) oder aber auch schwere Traumata untersucht[20–23]. Bisher konnte sich keiner der neuen Biomarker in die Definition der postoperativen systemischen Inflammationsreaktion durchsetzen.

SIRS Kriterien

Körpertemperatur	< 36 °C oder > 38 °C
Herzfrequenz	> 90/min
Atemfrequenz	> 20/min und ein p_aCO_2 ≤ 33 mmHg **oder** ein Oxygenierungsindex < 200 (bei invasiver Beatmung)
Anzahl der Leukozyten	< 4.000/mm^3 **oder** > 12.000/mm^3 **oder** > 10 % unreife Leukozyten

Tabelle 1 Darstellung der SIRS-Kriterien nach[2,9].

1.2.2 Sepsis und septischer Schock

Die Sepsis stellt eine lebensbedrohliche Krankheit sowie eine weltweite Gesundheitsbedrohung dar[24]. So lag 2015 beispielsweise die Inzidenz der Sepsis in der britischen Bevölkerung bei 19,3 je 100.000 Einwohnern und war bei den 55 – 64-jährigen Patienten mit einer Mortalität von 28,2 % assoziiert. Erlitten gleichaltrige Patienten einen septischen Schock, verstarben sogar 52,3 % der Menschen[16]. Die Sepsis ist dabei als eine „*lebensbedrohlichen Organdysfunktion, hervorgerufen durch eine fehlregulierte Wirtsantwort auf eine Infektion*“ definiert und stellt damit nicht mehr die Schwere der Infektion, sondern die Immunantwort des Menschen in das Zentrum der Definition[2]. Auf Grund des zunehmenden Verständnisses der immunologischen Pathomechanismen der Sepsis sowie mangelnder Spezifität der SIRS-Kriterien wurde im Rahmen der Einführung der Sepsis-3-Definition der qSOFA- und SOFA-Score für die Definition der Sepsis etabliert[2]. Beide Scores werden zur Identifikation der Sepsis

empfohlen, wobei der SOFA-Score bei intensivmedizinischen und der qSOFA-Score bei Patienten außerhalb der Intensivstation verwendet werden sollte. Aus diesem Grund besteht der qSOFA-Score nur aus drei Parametern (Vorliegen einer Atemfrequenz ≥ 22/min, einer Vigilanzminderung und eines systolischen Blutdrucks ≤ 100 mmHg), welche eine schnelle Evaluation ohne weitere diagnostische Hilfsmittel erlauben[2]. Werden bei der Berechnung mehr als zwei Kriterien als positiv gewertet, sollte der Patient als potentiell septisch angesehen werden und der SOFA-Score kalkuliert werden[1]. Dieser ist in der Berechnung aufwendiger, da die Schädigung sechs verschiedener Organsysteme charakterisiert wird. Er ist ab einer neuaufgetretenen Erhöhung von mehr als zwei Punkten als positiver Nachweis einer Sepsis zu werten (Tabelle 2). Der SOFA-Score ermöglicht eine Prädiktion der Letalität septischer Patienten[1,2]. Allerdings ist er als Werkzeug für die Diagnosestellung der Sepsis nicht gänzlich unumstritten, obwohl er auch für diesen Zweck durch die aktuellen Leitlinien der *Surviving Sepsis Campaign* und der Deutschen Sepsis Gesellschaft empfohlen wird[1,25–27]. Hauptkritikpunkte stellen dabei zum einen die ausschließlich retrospektive Validierung des SOFA-Scores sowie zum anderen der erhöhte Aufwand zur Berechnung (z.B. durch notwendige Blutentnahmen) dar, welcher zu einer Verzögerung der Diagnosestellung führen könnte[26,28]. Des Weiteren ist der SOFA-Score für die Erkennung einer Sepsis zwar sehr spezifisch, aber im Vergleich zu den SIRS Kriterien weniger sensitiv. Entsprechend ist die Frage zu stellen, ob eine falsch-positive Behandlung schwerer wiegt als eine falsch-negative Diagnose. Für operative Intensivpatienten ist darüber hinaus von hoher Relevanz, dass der SOFA-Score etwaige Vorerkrankungen mit entsprechenden vorbestehenden Alterationen der Organsysteme nicht berücksichtigt, so dass analog zu den SIRS-Kriterien falsch-positive Diagnosestellungen auftreten können. Obwohl mit der Leukozytenzählung, dem C-reaktiven Protein (CRP), dem Procalcitonin und dem Interleukin 6 (IL-6) validierte Biomarker für die Sepsis sowie eine Vielzahl an neuen bisher nicht validierten Biomarker existieren, werden lediglich der Laktat-Spiegel sowie die Konzentrationen von Kreatinin und Bilirubin im Rahmen der SOFA-Score-Berechnung erhoben[5,7].

Der septische Schock wird durch das Vorliegen einer Sepsis sowie den Einsatz eines Vasopressors für den Erhalt eines mittleren arteriellen Blutdrucks ≥ 65 mmHg sowie einem Laktat-Spiegel ≥ 2 mmol/l definiert[2]. Dies ist von hoher Bedeutung, da die

erhöhten Laktatspiegel Ausdruck einer Hypoperfusion, Gewebshypoxie und mitochondrialen Dysfunktion infolge des septischen Schocks sind und somit eine Verringerung des Laktatspiegels mit einer Verbesserung dieser Zustände assoziiert ist[29].

1.2.3 Epidemiologie der Sepsis bei operativen Intensivpatienten

Im klinischen Alltag entsteht sowohl bei der Anwendung der SIRS-Kriterien als auch des SOFA-Scores ein Dilemma, da beide Diagnosetools durch die physiologische postoperative Inflammationsreaktion alteriert werden. Gleichzeitig besteht aber ein hoher Behandlungsdruck, da insbesondere abdominalchirurgische Patienten ein erhöhtes Risiko für postoperative Infektionen aufweisen[13,30]. Seit der wegweisenden Studie von Kumar et al. wird angenommen, dass die Letalität septischer Schockpatienten je Stunde verzögerter antibiotischer Therapie um ca. 8 % steigt[31]. So wurde beispielsweise unabhängig der Todesursache eine Mortalität von bis zu 41,7 % bei notfallmäßigen abdominalchirurgischen Patienten beschrieben[32]. Darüber hinaus wiesen 66 % aller septischen Patienten nach einem abdominalchirurgischen Eingriff einen intraabdominalen Fokus auf[33,34]. In Abhängigkeit vom beschriebenen Fokus wurden dabei Mortalitätsraten zwischen 29 – 48 % berichtet[3,35,36]. Weiterhin untersuchte das *National Surgical Quality Improvement Project* mehr als 360.000 abdominalchirurgische Patienten und konnte eine, im Vergleich zum Myokardinfarkt, achtfach höhere Inzidenz des septischen Schocks nachweisen, welche wiederum mit einer signifikanten Erhöhung der 30-Tage-Mortalität assoziiert war[37]. Bei kardiochirurgischen Patienten ist die Inzidenz einer Sepsis auf Grund der fehlenden operationsbedingten Translokation von Darmbakterien geringer. Entwickeln kardiochirurgische Patienten allerdings im postoperativen Verlauf eine Sepsis, ist diese mit einer hohen Mortalität von 65 – 79 % assoziiert[38,39].

Die hohe Sepsis-assoziierte Mortalität bei operativen Patienten unterstreicht den Bedarf an diskriminativen Biomarkern. Die etablierten Biomarker CRP, Procalcitonin, IL-6 und Leukozytenanzahl sind für die Diagnosestellung einer akuten Infektion ausreichend validiert und können auch zur Verlaufskontrolle bei operativen Patienten eingesetzt werden. Allerdings kann mit keinem der genannten Biomarker eine Sepsis nach einem großen abdominal- oder herzchirurgischen Eingriff frühzeitig diagnostiziert

werden[5,7,20]. Der Grund hierfür ist einmal mehr der SIRS-induzierte postoperative Anstieg dieser Parameter.

SOFA Score

Organsystem	***Kriterium***	**Punkte**
Respiratorische Funktion	P_aO_2/F_iO_2 < 400 mmHg	1
	P_aO_2/F_iO_2 < 300 mmHg	2
	P_aO_2/F_iO_2 < 200 mmHg und Beatmung	3
	P_aO_2/F_iO_2 < 100 mmHg und Beatmung	4
Zentrales Nervensystem	GCS 13 – 14	1
	GCS 10 – 12	2
	GCS 6 – 9	3
	GCS 3 – 5	4
Herz-Kreislauf-System	MAP < 70 mmHg	1
	Dopamin ≤ 5 µg/kg/min **oder** Dobutamin	2
	Dopamin > 5 µg/kg/min **oder** Adrenalin ≤ 0.1 µg/kg/min **oder** Noradrenalin ≤ 0.1 µg/kg/min	3
	Dopamin > 15 µg/kg/min **oder** Adrenalin > 0.1 µg/kg/min **oder** Noradrenalin > 0.1 µg/kg/min	4
Leber	Bilirubin 1,2 – 1,9 mg/dl	1
	Bilirubin 2,0 – 5,9 mg/dl	2
	Bilirubin 6,0 – 11,9 mg/dl	3
	Bilirubin >12,0 mg/dl	4
Niere	Kreatinin 1,2 – 1,9 mg/dl	1
	Kreatinin 2,0 – 3,4 mg/dl	2
	Kreatinin 3,5 – 4,9 mg/dl	3
	Kreatinin ≥ 5,0 mg/dl	4
Gerinnung	Thrombozyten < 150.000/µl	1
	Thrombozyten < 100.000/µl	2
	Thrombozyten < 50.000/µl	3
	Thrombozyten < 20.000/µl	4

Tabelle 2. Darstellung des SOFA-Scores. *Abkürzung: GCS = Glascow Coma Score, MAP = Mean Arterial Pressure.*

1.3 Pathophysiologische Grundlagen der systemischen Inflammationsreaktionen bei operativen Intensivpatienten

1.3.1 Die initiale Immunantwort einer systemischen Inflammationsreaktion

Die komplexen Akutreaktionen der Sepsis basieren auf einer pathologischen Immunantwort des menschlichen Körpers. Dabei ist bisher nicht eindeutig geklärt, weshalb einige Menschen vulnerabler für die Entwicklung einer septischen Komplikation sind. Vor der Einführung der Sepsis-3-Defintion wurde im Rahmen der sog. *„Germ"*-Theorie angenommen, dass alleine die Moleküle, Proteine und Nukleinsäuren der mikrobiologischen Pathogene (*Pathogen-associated Molecular Pattern*, PAMPs) einen Zytokinsturm verursachen, der letztlich zu einer Erschöpfung des Immunsystems führt[40,41]. Zunehmend zeigte sich aber, dass insbesondere bei operativen Intensivpatienten auch *Damage-associated Molecular-Pattern* (DAMPs) in der Lage sind, *Pattern Recognizing Receptors* (PRRs) zu aktivieren, welche die weitere Immunantwort triggern[42,43]. Im Gegensatz zu der mikrobiologischen Herkunft der PAMPs, stammen DAMPs aus den körpereigenen Zellen und bestehen aus nukleären oder zytoplasmatischen Molekülen. Bekannte Trigger für ihre Freisetzung sind schwere Traumata, große abdominalchirurgische Eingriffe, herzchirurgische Operationen mit Einsatz eines kardiopulmonalen Bypasses sowie Krebserkrankungen[23,43–50]. Weitreichend untersuchte DAMPs stellen das *High-Mobility-Group-Box Protein 1* (HMGB-1), Histone und zellfreie Nukleinsäuren dar[41].

Die Freisetzung von HMGB-1 in das Blut erfolgt im Rahmen eines Zellschaden passiv durch Zellnekrose oder aktiv per Vesikelfreisetzung via einer Inflammasom-vermittelten Pyroptose[41,51]. Dabei kann ein steriles chirurgisches Trauma (z.B. durch eine Operation) ebenso wie eine Infektion die aktive Freisetzung verursachen, welche dann ihren Höhepunkt nach ca. 16 – 32 Stunden erfährt[52]. HMGB-1 triggert die Freisetzung weiterer proinflammatorischer Kaskaden und bindet Neutrophile am Ort ihrer Freisetzung. Zudem aktiviert es u.a. die *Receptors for Advanced Glycation End Products* (RAGE) sowie die Toll-Like-Rezeptoren (TLR)-2, -4 und -9, welche zu einer weiteren Makrophagenmigration führen[41,53,54]. Auf Grund seiner herausragenden Rolle als DAMP, wurde gezeigt, dass HMGB-1 sowohl bei septischen Patienten als auch im Rahmen des postoperativen SIRS erhöht ist[55–59].

Histone stammen aus dem Nukleosom menschlicher Zellen und kondensieren physiologischerweise das Chromatin des Zellkerns. Weiterhin sind sie wichtige Regulatoren der Genexpression und der genetischen Reparaturmechanismen[60]. Zudem können sie ebenso wie HMGB-1 aktiv sezerniert oder passiv durch einen Zelltod freigesetzt werden. Histone stellen dabei nicht nur klassische DAMPs dar, sondern sind auch wesentlicher Bestandteil von *Neutrophil Extracellular Traps* (NETs, siehe Kapitel 1.3.2)[61]. An Hand verschiedener Experimente konnte die toxische Wirkung von Histonen auf das Immunsystem dargestellt werden: So führt eine Applikation von Histonen zum Tod von Versuchstieren, während die Gabe von anti-Histon-Antikörpern zu einer Verbesserung einer Lipopolysaccharid (LPS)-induzierten Sepsis führte[41,62]. Auch beim Menschen konnte eine Assoziation zwischen einer erhöhten Konzentration zirkulierender Histone und einem Zellschaden bei septischen Patienten nachgewiesen werden[63]. Allerdings ist auch die antimikrobielle Wirkung von Histonen zu beachten, da sie verschiedene Bakterien wie z.B. Escherichia *coli*, Schigellen, Pseudomonaden und Klebsiellen neutralisieren können[64,65].

Analog zu den Histonen entstammen frei zirkulierende Nukleinsäuren dem menschlichen Nukleosom. Allerdings können sie durch eine Vielzahl an Pathomechanismen in das Blut freigesetzt werden. So führen neben der Zellnekrose und Apoptose auch die NETose zu ihrer Freisetzung[41]. Dabei sind nicht nur menschliche Nukleinsäuren als DAMPs relevant, sondern ebenso bakterielle, virale oder mitochondriale Nukleinsäuren. Insbesondere mitochondriale Nukleinsäuren sind dabei auf Grund ihrer bakteriellen Herkunft („Endosymbionten-Theorie“) in der Lage, die Immunantwort sowohl im Rahmen der Sepsis als auch des SIRS (z.B. nach Polytrauma) zu triggern[66,67]. Auf Grund ihrer stark negativ geladenen Oberfläche können sie auch zelluläre Bestandteile wie Neutrophile und Thrombozyten binden[67]. Des Weiteren führen sie zu einer Aktivierung der intrazellulären TLR-9 und anderer Alarmine, welche wiederum proinflammatorische Signalkaskaden induzieren[66]. Das Vorhandensein von frei zirkulierenden Nukleinsäuren ist mit einer höheren Krankheitsschwere bei septischen Patienten assoziiert[44,68,69]. Ein direkter Vergleich zwischen einem postoperativen SIRS und einer Sepsis, bezüglich der Menge an Nukleinsäuren, ist dagegen bisher nicht beschrieben.

1.3.2 NETs als Promotor der initialen Immunantwort

Neben der Freisetzung von DAMPs wird die initiale Immunantwort maßgeblich durch die Reaktion der Neutrophilen beeinflusst, da sie nicht nur Pathogene inaktivieren und phagozytieren können, sondern auch NETs freisetzen. Seit der Erstbeschreibung der NETose im Jahr 2004 durch Brinkmann et al. wurden NETs als wichtige Strukturen für die simultane Aktivierung des Immun- und Gerinnungssystems identifiziert[65,70].

NETs werden von den Neutrophilen entweder über einen suizidalen- oder vitalen Weg freigesetzt. Die suizidale NETose erfolgt nur kurzfristig für zwei bis vier Stunden und unterliegt einer Aktivierung durch Peptidylarginasedeaminase 4, welche zu einer Deaminierung des nukleären Chromatins der Neutrophilen führt. Im Anschluss kommt es zu einem *Reactive Oxygen Species* (ROS)-abhängigen Verlust der nukleären Zellmembran und Freisetzung des Chromatins in das Zytosol. Zuletzt erfolgt per Zelllyse die Freisetzung in das Blut[71,72]. Im Gegensatz zur suizidalen NETose gehen im Rahmen der vitalen NETose die neutrophilen Granulozyten bei der Produktion von NETs nicht unter. Dieser Prozess ist unabhängig von ROS und involviert eine Teildekondensation des Chromatins, welches per Enveloping an die äußere Zellmembran verbracht und kontrolliert in das Blut freigesetzt wird[65,73–75]. Der Stimulus für die vitale NETose erfolgt von immunologischer Seite via TLR-4 und Komplement C3 Protein, während es von Seiten der Thrombozyten via Glykoprotein Ib und β_2-Integrin (*Cluster of Differentiation*, $[CD]^{18+}$) aktiviert wird[76,77]. Eine Sonderform der vitalen NETose stellt die Freisetzung von mitochondrialer Desoxyribonukleinsäure (*Desoxyribonucleic Acid*, DNA) dar, welche durch Stimulation mit LPS erfolgt und abhängig von ROS ist[78].

Sobald das Chromatin der Neutrophilen in das Blut freigesetzt wird, formieren sie sich zusammen mit einer Vielzahl an antimikrobiellen Proteinen, wie beispielsweise Neutrophile Elastase (NE), Cathepsin G und Myeloperoxidase (MPO), Nukleinsäuren und Thrombozyten zu netzartigen Strukturen, welche den NETs entsprechen. In diesen Netzen verfangen sich Pathogene, welche schließlich phagozytiert werden, aber auch Thrombozyten und weitere Neutrophile[41]. Diese physiologische Reaktion weist eine hohe Heterogenität in Abhängigkeit von den Grunderkrankungen und der aktuellen Krankheitssituation auf. So können Diabetes mellitus, Krebserkrankungen und Arteriosklerose zu einer chronischen Aktivierung der NETose führen, während es im Rahmen von schweren Traumata, größeren Operationen und der Sepsis zu einer akut überschießenden NETose kommen kann[65,79–86].

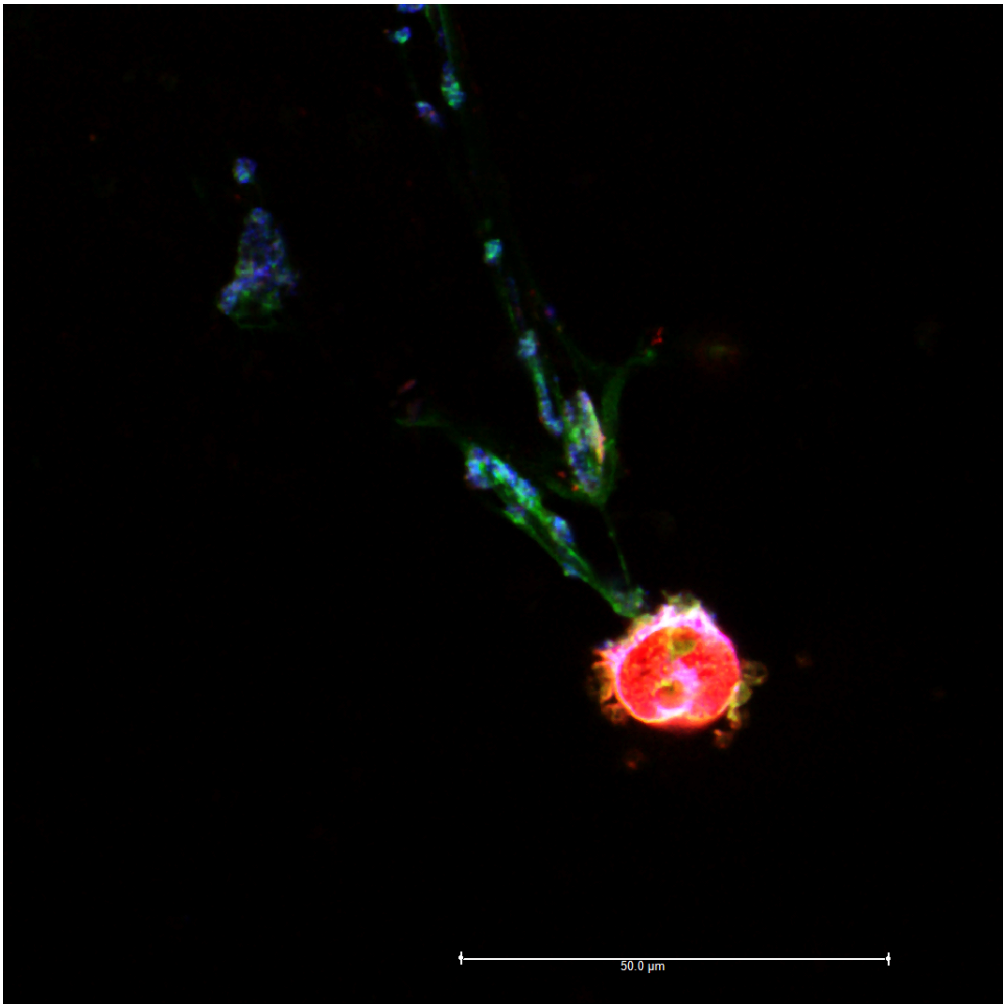

Abbildung 1 Fluoreszenzmikroskopische Overlayaufnahme eines Neutrophilen, welches NETs in typischer Weise (*„NETs-Tail“*) ausstößt. Die Färbung erfolgte mit MPO-, anti-Histon-, und Neutrophilen-spezifischen Anti-CD^{15+}-Antikörpern. Die Induktion der NETose erfolgte mit Phorbolmyristatacetat (PMA).

1.3.3 Immunhämostase

Das pathophysiologische Zusammenspiel von Immun- und Gerinnungssystem ist einer der wichtigen Motoren in der Entwicklung einer Sepsis. Einerseits zeigt das Gerinnungssystem eigene Immunkompetenzen auf, wie zum Beispiel die thrombozytäre Beteiligung an der NETose oder die antimikrobiellen Eigenschaften der Thrombinaktivierung[80,87]. Anderseits führt eine überschießende Aktivierung des Immunsystems zu einer Hyperkoagubilität in den Kapillaren, welche zu Mikrozirkulationsstörungen in den Organen und letztlich zu Organversagen führen kann[80,88,89]. Außerdem haben die induzierten Mikrozirkulationsstörungen starke Auswirkungen auf das hämodynamische System, in dem sie eine Vasoplegie verursachen. Hier schließt sich der *Circulus vitiosus*, denn diese führt wiederum zu einer Aggravierung der Organperfusion[4]. Aus diesem Grund sind NETs in der Entwicklung der septischen Koagulopathie und ihrem Endstadium, der disseminierten intravasalen Gerinnung (*Disseminated Intravascular Coagulation,* DIC), von hohem Interesse. Dabei führen sie nicht über die negative Ladung der Nukleinsäuren und Histone zu einer Kontaktaktivierung der Thrombozyten und des Gerinnungsfaktors XII, sondern auch zur einer NE-induzierten Inhibierung des *Tissue Factor Pathway Inhibitor* und des Thrombomodulins[80,87]. In wieweit die NETs selbst oder die negativ geladenen Anteile zur Kontaktaktivierung führen, ist aktuell in Diskussion[90]. Diese Mechanismen führen zur Endothelschädigung, welche wiederum durch das Freisetzen des Gewebsfaktors („*Tissue Factor*“) zur Aktivierung der Gerinnung führt. Diese Prozesse führen zu einem Verbrauch an Gerinnungsfaktoren („*Consumptive Coagulopathy*“), aber alleine noch nicht zur DIC. Entscheidend hierfür ist der Verlust der physiologischen Balance zwischen der Gerinnungsaktivität und Fibrinolyse[91]. Dies geschieht sowohl im Rahmen der postoperativen systemischen Inflammation als auch der Sepsis durch die beschriebene Schädigung des Endothels, welche eine physiologische antikoagulatorische Wirkung aufweist. Des Weiteren verringert sich durch den Untergang von Endothelzellen die Konzentration des Plasminogen-Aktivator-Inhibitors 1 (PAI-1), was zu einer gesteigerten Fibrinolyse führt[80,91]. Zuletzt wirken im Rahmen der Sepsis auch die Pathogene an sich fibrinolytisch, in dem sie Plasminogen direkt stimulieren oder PAI-1 inhibieren können[80]. Ist diese vulnerable Balance zwischen Gerinnung und Fibrinolyse gestört, kommt es neben der

Verbrauchskoagulopathie zur Hyperfibrinolyse, was zur Endstrecke der DIC führt und mit einer hohen Mortalität der Patienten assoziiert ist[89,92].

1.3.4 Immunologische Langzeitfolgen der Sepsis

Neben den akuten immunologischen Reaktionen steigt das Interesse an den Langzeitfolgen von sterilen und septischen systemischen Inflammationsreaktionen, da diese infolge des gesteigerten Überlebens der Patienten zunehmend demaskiert werden[93,94]. So wurde der Begriff des *Post Intensive Care* bzw. *Chronic Critical Illness Syndroms* etabliert, welches die Steigerung der allgemeinen Morbidität und Mortalität sowie die Verschlechterung chronischer Erkrankungen nach Durchleben eines kritischen Zustandes beschreibt[95–97]. So stellen langfristige Beeinträchtigungen verschiedener Organsysteme auch in der aktuellen *Coronavirus Disease-19*-Pandemie zunehmend ein relevantes Problem dar, da sie mit einer Einschränkung der Gesundheit und körperliche Leistungsfähigkeit über mehrere Monate einhergehen können[98,99].Während lange im Rahmen der Sepsis die Annahme eines Erkrankungskontinuum bestand, das eine initial proinflammatorische Reaktion gefolgt von einem Stadium der Immunsuppression beschrieb, zeigten aktuelle Publikationen wesentliche komplexere Abläufe[100,101]. Zwar besteht Anhalt für eine postseptische persistierende Immunsuppression, allerdings entsteht diese nicht durch eine Erschöpfung des Immunsystems, sondern durch ein dysfunktionales Immunsystem basierend auf epigenetische Veränderungen, welche das adaptive Immunsystem nachhaltig verändern[102,103]. Im Vergleich zu den akuten Sepsis-induzierten immunologischen Veränderungen sind die Langzeitalterationen in geringerem Maße erfasst. Es ist beschrieben, dass das angeborene Immunsystem durch eine reduzierte Produktion von Neutrophilen im Knochenmark, eine alterierte Chemotaxis der Neutrophilen und des ROS Systems langfristig beeinträchtigt wird[101,104,105]. Des Weiteren ist das antiinflammatorische Zytokin IL-10 vermehrt im Blut nachweisbar sowie das Zusammenspiel mit dem erworbenen Immunsystem gestört, da die Funktion Antigen-präsentierender Zellen gestört ist[104]. Insbesondere die monozytäre Präsentation des humanen Leukozyten Antigen (HLA-DR) ist reduziert, welches eine wichtige Rolle in der Aktivierung von T-Helferzellen nach Kontakt mit Pathogenen spielt[101,105]. Dies führt zu einer Reduktion an Lymphozyten, welches mit einer erhöhten

Langzeitmortalität septischer Patienten assoziiert ist. Darüber hinaus sind auch die lymphozytären Rezeptormerkmale langfristig verändert[106]. So konnte in der Milz von an einer Sepsis verstorbenen Patienten eine Reduktion der CD^{4+} und CD^{8+}-Lymphozyten nachgewiesen werden[103]. Es ist noch ungeklärt, inwieweit diese Veränderungen auch klinische Manifestationen wie beispielsweise virale Erkrankungen beeinflussen.

1.3.5 Immunologische Langzeitfolgen des Polytraumas und der postoperativen Inflammation

Das Polytrauma stellt eine weitere Ursache für die Entwicklung einer systemischen Inflammationsreaktion bei operativen Intensivpatienten dar. Dabei spielen die kurz- bis mittelfristigen immunologischen Reaktionen auf das Trauma eine ebenso wichtige Rolle wie die Verletzungsschwere an sich[47,107–110]. Es ist zudem bekannt, dass die langfristige Morbidität und Mortalität von polytraumatisierten Patienten steigt, wenn sie neben dem SIRS einen zusätzlichen komplikativen Verlauf (z.B. Infektionen) aufweisen[111–113]. Im Vergleich zur Sepsis sind die immunologischen Langzeitfolgen der nicht-septischen systemischen Inflammation nach einem Polytrauma allerdings nur im geringen Maße untersucht. Um diese Folgen besser zu charakterisieren wurde daher der Begriff des „*Persistent Inflammation and Immunosuppression*“-(PICS)-Syndroms geprägt. Immunologische Veränderungen könnten dabei im Rahmen einer Schwächung des Immunsystems zu Symptomen des PICS führen. So sind zum Beispiel für polytraumatisierte Patienten langfristige Veränderungen der TLR-2 und TLR-4 Rezeptoren auf Monozyten beschrieben, welches mit einer gestörten Identifikation von DAMPs und PAMPs assoziiert ist[114]. Dies führt zu einer gestörten Aktivierung der o.g. proinflammatorischen Signalkaskaden, was eine reduzierte Immunkompetenz gegenüber bakteriellen Erregern verursachen kann[115,116]. Des Weiteren lassen sich auch langfristig nach Polytraumatisierung epigenetische Veränderungen nachweisen, welche mit einer Reduktion des *Tumor Necrosis Factors* (TNF-α) und IL-10 assoziiert waren[108,117]. Polytraumatisierte Patienten stellen häufig auch jüngere Patienten ohne chronische Erkrankungen wie z.B. Karzinome oder Arteriosklerose dar. Dagegen weisen abdominal- und herzchirurgische Patienten regelhaft diese Begleiterkrankungen auf,

so dass ein Vergleich der langfristigen immunologischen Veränderungen zwischen diesen Patientenkohorten schwierig ist. So ist es bisher ungeklärt, ob die bekannten immunologischen Langzeitveränderungen nach operativen Eingriffen tatsächlich ausschließlich auf das SIRS oder andere begleitende Komorbiditäten zurückführen sind[118–121].

1.4 Differenzierung zwischen systemischen Inflammationsreaktionen und Sepsis bei operativen Intensivpatienten

1.4.1 Differentialdiagnostik der postoperativen Inflammationsreaktion

Wie oben beschrieben sind sich die klinischen Manifestationen des postoperativen SIRS und der Sepsis ähnlich. So kann zum Beispiel eine Tachykardie sowohl Symptom einer Sepsis als auch von Schmerzen oder eines Volumenmangels nach einem großen operativen Eingriff sein. Aus diesem Grund kommt der regelmäßigen und gewissenhaften klinischen Untersuchung des operativen Intensivpatienten sowie der kontinuierlichen Messung der Vitalparameter eine hohe Bedeutung zu. Nur auf diesem Wege können auch kleinere Änderungen der Symptome detektiert und entsprechend die Diagnosestellung erleichtert werden. Insbesondere Veränderungen der Vigilanz, eine verlängerte Rekapillierungszeit sowie eine Oligurie sind klinische Warnzeichen einer Sepsis oder anderer kritischer Erkrankungen (wie zum Beispiel eine Anämie oder Hypovolämie)[1,13]. Vorsicht ist bei der Bewertung von Peritonitiszeichen geboten, da diese bei sekundärer postoperativer Peritonitis seltener als bei der primären Form auftreten[122]. Neben den klinischen Befunden kommt der Beurteilung laborchemischer Parameter eine hohe Bedeutung zu, auch wenn diese in der Regel durch den Eingriff alteriert sind. Dabei stellt der am meisten untersuchte Parameter das CRP dar, welches am zweiten bis dritten postoperativen Tag den maximalen Anstieg zeigt und sich ab dem fünften postoperativen Tag wieder normalisiert. Fortbestehende Erhöhungen des CRPs sind verdächtig für eine Infektion und sollten zu einer erweiterten Diagnostik führen[123]. So wird zum Beispiel in der Kolorektalchirurgie ein Grenzwert von 100 mg/dl am fünften postoperativen Tag vorgeschlagen[13]. Allerdings sollte dies nicht als absoluter Grenzwert verstanden werden, sondern in Zusammenhang mit den klinischen und apparativen Befunden interpretiert werden. Die Messung des Procalcitonins stellt eine weitere laborchemische Methode für die Differenzierung des postoperativen SIRS von einer Sepsis dar. Allerdings sollte die Kinetik und nicht die Absolutwerte des Procalcitonins für die Detektion einer Sepsis entscheidend sein, da auch dieses perioperativ erhöht ist[13]. So sind dauerhafte Erhöhungen des Procalcitonins mit dem Vorliegen einer Sepsis assoziiert, während lokal begrenzte Infektionen auch ohne einen relevanten Anstieg nachweisbar sind[7,124]. Des Weiteren ist es für die

Verlaufsbeschreibung septischer Patienten, aber auch zum Überwachen des antibiotischen Therapieerfolgs geeignet[124]. Allerdings zeigte die *Combined Approach for The eArly diagnosis of INfection in sepsis* (CAPTAIN) Studie, dass die Messung des Procalcitonins für die Diskriminierung zwischen Sepsis und postoperativem SIRS nicht der Messung des CRPs überlegen ist[125]. Zusammenfassend ist die Kombination aus laborchemischen und klinischen Befunden sowie eine interdisziplinäre Bewertung trotz der Limitationen der individuellen Untersuchungsmethoden wegweisend für die frühzeitige Detektion einer Sepsis bei postoperativen Intensivpatienten.

1.4.2 Innovative Biomarker zur Diskriminierung der Sepsis von postoperativen systemischen Inflammationszuständen

Der ideale Biomarker weist eine hohe Sensitivität und Spezifität für die Identifikation einer Sepsis auf, wird nicht durch Begleiterkrankungen, Operationen oder andere Umstände beeinflusst und ist im intensivmedizinischen Kontext implementierbar. Um dieses Ziel zu erreichen, wurden bereits eine Vielzahl von Biomarkern untersucht, ohne aber einen idealen Kandidaten zu identifizieren[7,126,127]. Pierrakos et al. zeigten in ihrer aktuellen Metaanalyse, dass nur eine geringe Anzahl der 258 untersuchten Biomarker an Hand einer ausreichenden Anzahl von Patienten und im Vergleich mit den etablierten Biomarkern validiert wurden[7]. So wiesen nur neun der untersuchten Biomarker eine vergleichbare oder höhere Prädiktion als das CRP oder Procalcitonin auf. Insbesondere in der postoperativen Phase scheitern moderne, ebenso wie die etablierten Biomarker an einer ausreichenden Prädiktion einer Infektion oder Sepsis. So wurde zum Beispiel für IL-6, welches sich bereits in der klinischen Versorgung etablieren konnte, nach wie vor kein einheitlicher Grenzwert festgelegt. In der Folge variiert dieser zwischen 12 und 2.760 pg/ml[13,128]. Für abdominalchirurgische Patienten konnten die Messung der Matrix Metalloproteinase 9 (MMP-9), des *Tissue Inhibitor of Matrix Metalloproteinase-1*, des Glutamins und Syndecan-1 potentielle Kandidaten zur Identifikation von septischen Patienten nach großen abdominalchirurgischen Eingriffen darstellen[128–131]. Auch für kardiochirurgische Patienten wurden verschiedene Biomarker untersucht, ohne dass sich einer davon klinisch etablierte. Der lösliche CD^{14+}-Subtyp (*Soluble CD^{14+} Subtype*, (sCD14-ST, Presepsin)) stellte einen vielversprechender Ansatz dar, war aber in einer prospektiven

Validierungsstudie an herzchirurgischen Patienten den etablierten Biomarker nicht überlegen, während es in anderen Studien mit einer hohen Sensitivität und Spezifität für die Identifikation septischer Patienten assoziiert war[132–137]. Ein weiterer Vorteil des Presepsins besteht darin, dass es mittels einer *Point-of-Care*-Messung bereits auf der Intensivstation quantifiziert werden kann. Allerdings ist es bisher bei polytraumatisierten Patienten nur in wenigen Studien evaluiert worden, so dass die Frage nach der Diskrimination der Sepsis von systemischen Inflammationsreaktionen bei operativen Intensivpatienten bisher nicht ausreichend geklärt ist. Neben Presepsin stellt auch der lösliche *Delta-Like Canonical Notch Ligand 1* (DLL-1) einen weiteren vielversprechenden Kandidat für die Diskrimination der Sepsis von systemischen Inflammationszuständen dar, da er insbesondere bei dem Vorliegen von bakteriellen Infektionen vermehrt auf Monozyten exprimiert wird[138]. Da dieser bisher nicht klinisch validiert wurde, kann er im Gegensatz zu Presepsin nur mittels einem *Enzyme-linked Immunosorbent Assay* (ELISA) gemessen werden[‡].

Neben Proteinen können auch Nukleinsäuren und NETs als Surrogatparameter für die Identifikation der Sepsis genutzt werden. Dies ist insbesondere aus pathophysiologischer Sicht sinnvoll, da sie wie oben beschrieben wichtige Aktivatoren des Immun- und Gerinnungssystems darstellen[65,80,139]. Aus diesem Grund wurden bereits eine Vielzahl von Nukleinsäuren unterschiedlicher Herkunft zur Identifikation der Sepsis untersucht, allerdings meist im Hinblick auf ihren generellen Nutzen als Diagnosetool der Sepsis und weniger als diskriminativen Biomarker bei operativen Intensivpatienten[41,69,140–142]. Allerdings steigen sowohl die Konzentration der Nukleinsäuren als auch die der NETs im Rahmen eines (operativen) Traumas an, so dass auch hier die Diskrimination von systemischer Inflammation und Sepsis erschwert sein könnte[65,79,84,143–145]. Studien, welche die verschiedenen Inflammationsursachen direkt vergleichen, liegen allerdings bisher nicht vor. Während die Konzentration an Nukleinsäuren in der Regel mittels *Polymerase Chain Reaction* (PCR) quantifiziert werden können, existieren unterschiedliche Methoden zur Messung von NETs. Neben der etablierten aber auch zeitaufwendigen und untersucherabhängigen Fluoreszenzmikroskopie zur direkten Visualisierung der NETs, stehen durchflusszytometrische, ELISA-, und PCR-Methoden zur

[‡] Die detaillierten Methoden werden in den einzelnen Publikationen im Anhang dargestellt.

Quantifizierung von NETs-Surrogaten zur Verfügung[146–149]. Bisher konnte aber keine dieser Methoden ausreichend für die Identifikation der Sepsis klinisch etabliert werden[146,149].

Zuletzt könnte die durchflusszytometrische Charakterisierung der Rezeptoreigenschaften von Lymphozyten und Monozyten zur Differenzierung von septischen und nicht-septischen Inflammationszuständen hilfreich sein. Als ein Beispiel kann das monozytäre HLA-DR genannt werden, welches auf Grund seiner vermehrten Expression während der Sepsis und des Polytraumas als diagnostisches Tool diskutiert wurde[109,150,151]. Allerdings fehlen in Hinblick auf operative Intensivpatienten weiterhin valide Daten zum diskriminativen Nutzen des HLA-DR. Es ist aber weiterhin von besonderem Interesse, da HLA-DR ein wichtiger Indikator für eine vorliegende Immunsuppression darstellt[45,103,152,153]. Dies könnte für operative Intensivpatienten interessant sein, da diese häufig von einer kompromittierten Immunfunktion bedroht sind[14,103,154,155]. Zusammen mit der Charakterisierung von weiteren Rezeptoreigenschaften von Immunzellen könnte eine individuelle Abschätzung der Immunfunktion und somit des Risikos für die Entwicklung einer Sepsis erfolgen[156,157].

Eine probate Möglichkeit, die diskriminative Prädiktion für die Identifikation der Sepsis bei operativen Intensivpatienten zu erhöhen, erschien daher das Kombinieren der vielversprechenden Biomarker. So wies ein diagnostisches Panel, welches IL-6, IL-1α, TNF-α, HMGB-1, MMP-9, Vascular *Endothelial Growth Factor, Intercellular Adhesion Molecule 1*, MPO, Methylglyoxal sowie die Caspase-3 beinhaltete, eine hohe Sensitivität für die Identifikation einer Sepsis auf[158]. Eine weitere Studie wies mit der Kombination sieben unterschiedlicher Biomarker eine hohe Prädiktion für die Detektion einer beatmungsassoziierten Pneumonie bei intensivmedizinischen Patienten nach[159]. Zuletzt konnte die Early PREdiction of Sepsis-(EXPRES)-Studie mit Hilfe der durchflusszytometrischen Quantifizierung von 42 Leukozytenantikörpern darstellen, dass die kombinierte Messung von immunsupprimierenden Leukozytenrezeptoren mit einer guten Sepsisprädiktion assoziiert waren[160]. Hinsichtlich der Frage, ob durch den Einsatz dieser Biomarker-Panels eine Sepsis von anderen Inflammationsursachen effektiv unterschieden werden kann, existieren nur eine geringere Anzahl von Studien. McHugh et al. nutzten ein Microarray von verschiedenen RNA-Biomarkern, um

septische Patienten in einem Kollektiv von kritisch kranken Patienten zu identifizieren. Mit dieser Methode konnten septische Patienten mit einer hohen diskriminativen Prädiktion identifiziert werden (*Area under the Curve* der *Receiver Operator Curve* [AUCROC] 0,89 [95% Konfidenzintervall 0,85 – 0,93])[161]. Eine weitere Studie konnte zudem nachweisen, dass die Kombination dieses Microarrays mit anderen klinischen und laborchemischen Parametern (inklusive Procalcitonin) keinen weiteren Vorteil erbrachte[162]. Andererseits wiesen Sweeney et al. an Hand eines weiteren Panels, welches die Expression von elf verschiedenen Genen quantifizierte, zwar eine ausreichende Sensitivität von 94 %, aber nur eine geringe Spezifität von 60 % für die Diskrimination von bakteriellen Infektionen in einem Kollektiv von SIRS-Patienten nach[163]. Die geringe Spezifität der Panels entspricht derjenigen der etablierten Biomarker und ist am ehesten durch die Reaktionen im Rahmen der systemischen Inflammationsreaktionen bei Intensivpatienten zu erklären. So konnte beispielsweise im Rahmen einer prospektiven Studie an Intensivpatienten für die Diskrimination der Sepsis eine Sensitivität von 82,9 % und eine Spezifität von 53,9 % durch die Messung des Procalcitonins bzw. 90,7 % und 49,7 % des CRPs sowie 66,4 % und 61,7 % durch die Berechnung des SOFA-Scores nachgewiesen werden. Dagegen zeigte die Messung des IL-6 eine geringe Sensitivität von 28,2 % bei einer höheren Spezifität von 78,7 %[164]. Aus diesen Gründen konnte sich der Einsatz von Multipanels noch nicht in den klinischen Alltag etablieren. Dennoch sollten neue und innovative Biomarker auch im Kontext von kombinierten Diagnosepanels evaluiert werden. Des Weiteren ist festzustellen, dass kaum Studien zur immunologischen Langzeitsurveillance von operativen Intensivpatienten existieren. Ziel einer langfristigen Überwachung der Immunfunktion wäre eine möglichst frühzeitige Identifikation von Patienten, welche für Folgeinfektionen gefährdet sind.

1.5 Prinzipien der Therapie der postoperativen Sepsis

Das erste Ziel der Behandlung der postoperativen Sepsis stellt ihre möglichst frühzeitige Diagnose dar, um ihr leitliniengerecht mit zielgerichteten Therapie-*„Bundles“* effektiv begegnen zu können[1]. Dabei ist gerade die Identifikation septischer Patienten auf der operativen Intensivstation aus den o.g. Gründen eine besondere Herausforderung. Im Anschluss basiert die Therapie der postoperativen Sepsis, ebenso wie die allgemeine Behandlung der Sepsis, auf vier Säulen:

1. Supportive Therapie
2. Antibiotische Therapie
3. Fokussanierung
4. Adjuvante Therapie

Unter der supportiven Therapie wird zum Beispiel die hämodynamische Stabilisierung der Patienten mit einer Volumen- und differenzierten Katecholamintherapie verstanden. Auch wenn mehrere große Studien entgegen der Erstbeschreibung durch Rivers et al. keinen Effekt auf das Überleben der Patienten durch die Anwendung einer *Goal-directed Therapy* (GDT) nachweisen konnten, haben sich Algorithmen-basierte Therapiestrategien für die schnelle und effiziente Initialtherapie etabliert[165,166]. Aus diesem Grund erhält die individualisierte GDT zunehmende Beachtung[167].

Die antibiotische Therapie sollte entsprechend der Tarragona-Strategie (*„Hit hard and early!“*) möglichst frühzeitig und empirisch erfolgen[1,168]. Nach Nachweis eines Erregers sollte eine kalkulierte Antibiose appliziert werden[1].

Der chirurgischen Fokussanierung kommt bei der Therapie der Sepsis auf der operativen Intensivstation eine bedeutende Rolle zu, da eine Verzögerung zu einem signifikanten Anstieg der Mortalität führt[1,15,169]. Sie umfasst das Debridement und die Entfernung von infiziertem Gewebe, die Drainage von eitrigen Verhalten, die Dekompression der Abdominalhöhle, aber auch das Entfernen von infizierten Kathetern. Zum Teil können diese Maßnahmen durch einen interventionellen Ansatz erfolgen, häufig ist aber eine Operation notwendig[13]. Diese sollte initial auf ein Mindestmaß reduziert werden, um eine weitere Eskalation der Entzündungsreaktion zu vermeiden (*Damage Control Surgery*). Nach 36 – 48 Stunden kann dann eine sog.

Second-Look-Operation erfolgen und je nach Befund im Verlauf eine endgültige Versorgung gelingen[1,170].

Zur adjuvanten Therapie der Sepsis wurden in den letzten Jahren unterschiedliche Therapiestrategien untersucht. Allerdings zeigte weder die Gabe von Antithrombin III zur Behandlung der DIC noch von intravenösen Immunglobulinen oder Spurenelementen zur Immunonutrition (z.B. Selen) einen Effekt auf das Überleben septischer Patienten, so dass ihr Einsatz aktuell nicht empfohlen wird[1].

Zusammenfassend bereitet die frühzeitige Identifikation der Sepsis bei operativen Intensivpatienten die Basis für ihre effektive Therapie und zur Senkung der Sepsis-assoziierten Mortalität und Morbidität[1,13].

2 Fragestellungen und Zielsetzungen der Arbeit

Die Sepsis bei operativen Intensivpatienten stellt eine besondere Herausforderung dar. Wie in der Einleitung dargestellt, führen die physiologischen Anpassungsreaktionen auf ein (chirurgisches) Trauma zu Alterationen der Vital- und Laborparameter, welche die Diskrimination zwischen einer Sepsis und einer systemischen Inflammationsreaktion erschwert. Daher stellt sich die Frage, wie die Prädiktion einer Sepsis bei operativen Intensivpatienten verbessert werden kann. Aus diesem Grund wurden in dieser kumulativen Habilitationsschrift zunächst zwei Fragestellungen aus der klinischen Praxis bearbeitet:

- Ist eine kürzere Dauer bis zur chirurgischen Intervention bei Patienten mit intraabdomineller Sepsis mit einer Verringerung der Mortalität assoziiert?
- Weist der qSOFA-Score bei operativen IMC-Patienten eine höhere Prädiktion der Sepsis und Mortalität auf als der SOFA-Score?

Diese beiden klinischen Fragestellungen suggerierten die Notwendigkeit einer frühzeitigen und möglichst spezifischen Identifikation von septischen Patienten auf der operativen Intensivstation. Aus diesem Grund wurden im zweiten Abschnitt dieser Habilitationsschrift verschiedene immunologische Biomarker der Sepsis identifiziert und ihre Expression im Rahmen von septischen und postoperativen Inflammationszuständen untersucht. Hierzu wurden folgende Fragestellungen bearbeitet:

- Wie entwickelt sich die Kinetik des Presepsin über sieben Tage nach einem Polytrauma im Vergleich zu den etablierten Inflammationsparametern?
- Können mit Hilfe der Konzentration des Presepsin Patienten mit einem SIRS identifiziert werden?
- Können anhand der Plasmakonzentration von DLL-1 auch *in-vivo* bakterielle Infektionen detektiert werden?
- Kann mit Hilfe der Quantifizierung der Plasmakonzentration des DLL-1 ein posttraumatisches SIRS von einer Sepsis unterschieden werden?

In den nachfolgenden Untersuchungen wurde bei der Suche nach potentiellen Biomarkern für die Detektion der Sepsis bei operativen Intensivpatienten die Ebene der Proteine verlassen und im Folgenden Nukleinsäuren und deren Derivate

untersucht. Dabei wurden nicht nur die Biomarker bei verschiedenen Ursachen der systemischen Inflammationsreaktion, sondern auch ihre Assoziation zur Gerinnungsfunktion analysiert. Es wurden folgende Fragestellungen bearbeitet:

- Können anhand der Konzentration von frei zirkulierender DNA als auch von extrazellulärer Ribonukleinsäure (*Ribonucleic Acid*, RNA) abdominalchirurgische Patienten mit postoperativer systemischer Inflammation von septischen Patienten unterschieden werden?
- Sind diese Nukleinsäuren mit Veränderungen der Gerinnungsfunktion assoziiert?

Auf Grund dessen, dass die Ergebnisse zur Freisetzung der Nukleinsäuren Ausdruck einer NETose sein könnten, stellte sich im Anschluss die Frage, ob sich die Menge an NETs für die Diskrimination von septischen und postoperativen Inflammationszuständen eignet[65,171]:

- Weisen Patienten mit septischem Schock höhere Plasmaspiegel an frei zirkulierenden NETs als Patienten nach großen operativen Eingriffen auf?
- Wie stellt sich der Verlauf der freizirkulierenden NETs bei septischen und postoperativen Patienten dar?
- Sind frei zirkulierende NETs mit Alterationen der Gerinnungsfunktion assoziiert?

Im Anschluss wurde der potentielle Nutzen von mitochondrialer DNA (mtDNA) zur Diskrimination der Sepsis bei operativen Patienten untersucht. Es wurden folgende Fragestellungen bearbeitet:

- Können mit Hilfe der Konzentration an frei zirkulierender mtDNA septische Patienten von Patienten mit postoperativer systemischer Inflammation unterschieden werden?
- Weist die Konzentration an frei zirkulierender mtDNA eine Assoziation zu Störungen der Gerinnungsfunktion auf?

Im letzten Abschnitt wurde schließlich die immunologische Langzeitfunktion von ehemals septischen und polytraumatisierten Patienten untersucht. Es stellte sich die Frage, ob diese Patienten auch langfristig nach ihrem Intensivaufenthalt noch immunologische Folgeerscheinungen aufweisen und inwieweit diese mit klinischen Symptomen assoziiert sind. Daher war es zunächst Ziel der Studie, den klinischen

Immunstatus der Patienten zu erfragen und die Rezeptoreigenschaften der T-Lymphozyten und Monozyten dieser Patienten auf Basis folgender Fragestellungen zu charakterisieren:

- Wiesen Patienten noch mehrere Monate nach einer überlebten Sepsis eine höhere Inzidenz an Infektionen auf?
- Können mehrere Monate nach einer überlebten Sepsis morphologische Veränderungen auf den T-Lymphozyten und Monozyten nachgewiesen werden?
- Weisen stimulierte T-Lymphozyten und Monozyten von ehemals septischen Patienten eine reduzierte Sekretionskapazität von Interleukinen auf?

Die letzte Studie konzentrierte sich auf die immunologischen Langezeitfolgen von polytraumatisierten Patienten, da auch diese Patienten eine langfristig erhöhte Morbidität und Mortalität aufweisen[172–174]. Es sollten folgende Fragestellungen beantwortete werden:

- Sind ehemals polytraumatisierte Patienten auch langfristig häufiger an Infektionen erkrankt?
- Weisen Patienten auch noch mehrere Monate nach einem überlebten Polytrauma morphologische Veränderungen auf Ihren T-Lymphozyten und Monozyten auf?
- Ist die Sekretionskapazität von stimulierten T-Lymphozyten und Monozyten bei ehemals polytraumatisierten Patienten reduziert?

3 Zusammenfassung der Ergebnisse eigener Arbeiten

3.1 Retrospektive Untersuchung zum Einfluss der Dauer bis zur chirurgischen Intervention bei Patienten mit freier intestinaler Perforation (Anlage 1)

Auf Grund der persistierend hohen Mortalität der Sepsis und des septischen Schocks haben zielgerichtete diagnostische und therapeutische Strategien einen hohen Stellenwert für das Management kritisch kranker Patienten[1,175]. Insbesondere die frühzeitige Gabe einer empirischen antibiotischen Therapie ist in diesem Zusammenhang etabliert[1,13,31,176]. Zusätzlich sollten Patienten, welche eine Sepsis mit intraabdominellem Fokus aufweisen, eine frühzeitige Fokussanierung innerhalb von sechs bis zwölf Stunden erhalten[1]. Diese Empfehlung basiert allerdings auf einer geringen Evidenz (Grad 1C). Das Ziel dieser Pilotstudie war es daher, den Einfluss der Dauer zwischen Diagnosestellung und chirurgischer Intervention auf die Mortalität septischer Patienten mit freier intestinaler Perforation zu untersuchen.

Zu diesem Zweck wurde eine retrospektive, monozentrische Untersuchung bei Patienten durchgeführt, welche an einer Sepsis auf Grund nachgewiesener freier intestinaler Perforation litten. Ausgeschlossen wurden Patienten, welche eine postoperative Anastomoseninsuffizienz aufwiesen, einen interventionellen therapeutischen Ansatz (z.B. endoskopische Vakuumsaugverbände) erhielten, bei denen intraoperativ keine intestinale Perforation nachweisbar war, sowie minderjährige Patienten. Die Interventionszeit entsprach der Zeit zwischen der radiologischen Diagnosestellung bis zum Beginn der chirurgischen Intervention (operative Schnittzeit). Entsprechend der Dauer bis zur chirurgischen Versorgung wurden drei Gruppen gebildet (Interventionszeit < 3 Stunden, 3 - 9 Stunden und > 9 Stunden). Es wurde eine maximale Verzögerung von neun Stunden gewählt, da zuvor eine prospektive Studie an abdominalchirurgischen Patienten bereits nach einer Verzögerung von sechs Stunden eine Erhöhung der Mortalität nachwies[177].

Von 179 Patienten, welche in der Computertomographie eine intestinale Perforation aufwiesen, erfüllten 76 Patienten alle Einschlusskriterien. Von dieser Studienpopulation wurden 32,9 % (n = 25) Patienten innerhalb von drei Stunden, 47,4 % (n = 36) innerhalb von drei bis neun Stunden und 19,7 % (n = 15) nach neun

Stunden operativ versorgt. Das Patientenkollektiv war durch eine hohe Krankheitsschwere gekennzeichnet (Median [Interquartile Range (IQR)], SOFA < 3 Stunden: 6 [3 – 9,5]; SOFA 3 – 9 Stunden: 7 [5 – 10]; SOFA > 9 Stunden: 6 [4 – 9]; p = 0,479). Sowohl die Analyse der 30-Tage-Sterblichkeit (< 3 Stunden: 20% [n = 15]; 3 – 9 Stunden: 27 % [n = 21]; > 9 Stunden: 27 % [n = 21]; n.s.) als auch die Analyse des Gesamtüberlebens (< 3 Stunden: 80% [n = 61]; 3 – 9 Stunden: 75 % [n = 57]; > 9 Stunden: 73 % [n = 56], n.s.) zeigte einen Trend zu höherem Überleben in der frühen Interventionsgruppe von unter drei Stunden, welche jedoch nicht statistisch signifikant war. Es zeigte sich bzgl. der Sekundärparameter ebenfalls kein signifikanter Unterschied.

Diese retrospektive Datenanalyse von septischen Patienten mit intestinaler Perforation konnte zwar ein höheres Überleben nach kürzerer Dauer bis zur chirurgischen Intervention (< 3 Stunden) nachweisen, verfehlte aber am ehesten auf Grund der zu geringen Fallgröße das statistische Signifikanzniveau. Vergleichbare Studien wiesen eine hohe Heterogenität hinsichtlich der untersuchten Lokalisation der Infektion, der Art der Perforation, der Dauer der chirurgischen Intervention, dem Ausmaß der Peritonitis sowie dem Studienkollektiv an sich, so dass ein direkter Vergleich mit unseren Studienergebnissen schwierig war[177–183]. In einer Studie an abdominalchirurgischen Patienten sank durch eine Verzögerung der chirurgischen Therapie von vier bis sechs Stunden die 60-Tage-Überlebensrate auf 55 % (adjustiertes relatives Risiko: 0,29 [0,16 – 0,47] pro Stunde Verzögerung), welches den Trend in unserer Studie unterstützt[177]. Um die Dauer zur chirurgischen Intervention zu reduzieren, sollten des Weiteren Strategien zur frühzeitigen Identifikation der Sepsis bei abdominalchirurgischen Patienten weiterentwickelt werden. Da diese häufig auf anästhesiologischen IMC-Stationen versorgt wurden, stellte sich die Frage, welcher Sepsis-Score bei diesem Patientenkollektiv die höchste Prädiktion für die Identifikation einer Sepsis aufweist[1].

Zusammenfassung

Diese retrospektive Datenanalyse von 76 septischen Patienten mit intestinaler Perforation weist auf ein höheres Überleben bei jenen Patienten, welche eine frühzeitige chirurgische Fokussanierung erhielten, erreichte aber auf Grund der geringen Kohortengröße keine statistische Signifikanz.

3.2 Retrospektive Analyse zum Vergleich des qSOFA- und SOFA-Scores im Hinblick auf die Prädiktion der Sepsis und Mortalität bei operativen Intensiv- und Intermediate Care Patienten (Anlage 2)

Für die Detektion der Sepsis bei intensivmedizinischen Patienten wird der SOFA-Score und bei allen Patienten außerhalb der Intensivstation der qSOFA-Score empfohlen[1]. Allerdings werden viele operativen Patienten auf einer IMC-Station behandelt, welches weder einer klassischen intensivmedizinischen noch einer normalstationären Versorgung entspricht[184–186]. Somit ist unklar, ob bei diesen Patienten der qSOFA- oder der SOFA-Score zur Prädiktion der Sepsis verwendet werden sollte. Aus diesem Grund wurden in der folgenden Studie beide Scores sowie die SIRS-Kriterien auf ihre Prädiktion hinsichtlich einer Sepsis sowie der Mortalität bei operativen Intensiv- und IMC-Patienten untersucht.

Bei der vorliegenden Studie handelte es sich um eine retrospektive 6-Jahres-Kohortenanalyse von 13.780 operativen Patienten, welche auf einer IMC- und/oder auf einer Intensivstation versorgt wurden. Die Patienten wurden auf das Vorliegen einer vermuteten Sepsis analysiert, welche durch den Beginn einer empirischen Breitspektrum-Antibiotikatherapie definiert wurde. Im Anschluss wurde zu diesem Zeitpunkt der qSOFA- und SOFA-Score sowie die SIRS-Kriterien berechnet. Die Sensitivität und Spezifität der Zielparameter wurde mittels der Berechnung der AUCROC dargestellt.

Bei 1.306 (18,3 %) aller IMC-Patienten und 1.365 (35,5 %) der intensivmedizinisch versorgten Patienten wurde eine Sepsis vermutet. Wurden die Patienten sowohl auf der Intensiv- als auch IMC-Station behandelt, wurde in 1.734 (62,0 %) Fällen ein Infektionsverdacht geäußert. Insgesamt starben 458 (3,3 %) der Patienten (IMC: 45 [0,6 %]; ICU: 250 [6,5 %]; IMC/ICU: 163 [5,8 %]). Unabhängig der Patientenkohorte erreichte keiner der analysierten Sepsis-Scores eine ausreichende Prädiktion für eine vermutete Sepsis (siehe Tabelle 2 und Abbildung 2, Anlage 2). Dagegen wies der qSOFA-Score eine ausreichende Prädiktion der Mortalität bei IMC-Patienten (AUCROC SIRS: 0,72 [0,71 – 0,72]; SOFA: 0,52 [0,51 – 0,53]; qSOFA: 0,82 [0,79 – 0,84]) und der SOFA-Score bei Patienten mit kombinierten IMC- und Intensivaufenthalt nach (AUCROC SIRS 0,54 [0,53 – 0,54]; SOFA 0,73 [0,70 – 0,77]; qSOFA 0,59 [0,58 – 0,59]).

Es konnte anhand einer großen Kohorte gezeigt werden, dass weder der qSOFA- noch der SOFA-Score oder die SIRS-Kriterien eine vermutete Infektion bei operativen Patienten mit ausrechnender Sensitivität und Spezifität anzeigen konnte. Dieses Ergebnis war unabhängig von einer Versorgung auf einer Intensiv- oder IMC-Station. Allein für die Mortalität konnte eine ausreichende Prädiktion des qSOFA-Scores bei IMC-Patienten sowie des SOFA-Scores bei Patienten, welche sowohl auf Intensiv- als auch auf IMC-Station behandelt wurden, nachgewiesen werden. Trotz des zunächst überraschenden Studienresultats zeigte sich, dass es mit der existierenden Literatur vereinbar war. So entsprachen sie mit Ausnahme des SOFA-Scores denen der Erstbeschreibung des qSOFA-Scores von Seymour et al.[187]. Darüber hinaus wies eine Vielzahl von Studien eine hohe Varianz in der Prädiktion für die Identifikation von septischen Patienten nach (siehe Tabelle 4, Anlage 2). Zum einen könnte dies durch die Untersuchung unterschiedlicher Patientengruppen erklärbar sein, zum anderen war der SOFA-Score ursprünglich nur für die Prädiktion der Mortalität entwickelt worden. Diese Studie konnte somit die Frage nach dem optimalen Scoring-Werkzeug für die Identifikation septischer Patienten auf der operativen IMC-Station nicht endgültig beantworten. Ein wichtiger Grund könnten dabei die operationsbedingten Alterationen der für die Scores relevanten Parameter darstellen. Diese führten häufig zum Erfüllen der erforderlichen Parameter und somit zu falsch positiven Ergebnissen (Tabelle 2, Anlage 2). Auf Basis dieser Überlegungen ist die Identifikation von diskriminativen Biomarkern von hohem Interesse.

Zusammenfassung

Die retrospektive Kohortenanalyse von 13.780 operativen Patienten wies weder für den qSOFA- oder SOFA-Score noch für die SIRS-Kriterien eine ausreichende Prädiktion für die Detektion einer Sepsis nach. Dies war unabhängig von einer Behandlung auf der IMC- oder Intensivstation. Dagegen konnte bzgl. der Mortalität eine ausreichende Prädiktion des qSOFA-Scores bei IMC-Patienten und des SOFA-Scores bei Patienten, welche sowohl auf der Intensiv- als auch der IMC-Station behandelt wurden, dargestellt werden.

3.3 Prospektive Observationsstudie zum Verlauf von Presepsin bei polytraumatisierten Patienten (Anlage 3)

Um sich dem klinischen Problem der Differenzierung einer Sepsis von einer posttraumatischen bzw. -operativen systemischen Inflammationsreaktion zu nähern, war die Analyse von innovativen Biomarkern nahe liegend, da die etablierten Entzündungsmarker für diese Fragestellung versagten[1,7,188]. Polytraumatisierte Patienten sind dabei von hohem Interesse, da sie zum einen häufig ein SIRS infolge der Verletzungen entwickeln und zum anderen besonders für infektiologische Komplikationen vulnerabel sind[189–191]. Der lösliche CD^{14+}-Subtyp (Presepsin) wurde für die Sepsis-Erkennung evaluiert und weist in verschiedenen klinischen Situationen eine suffiziente Sensitivität und Spezifität auf[133–137]. Interessanterweise konnten Hishino et al. keinen Anstieg des Presepsin innerhalb der ersten 24 Stunden nach Polytraumatisierung feststellen[192]. Auf Basis dieser Studie untersuchten wir die Kinetik der Presepsin Plasmakonzentration bei Patienten nach schwerem Trauma über die folgenden sieben Tage.

Zu diesem Zweck wurde eine prospektive, monozentrische Observationsstudie initiiert, in welcher wir 50 polytraumatisierte Patienten mit einem Injury Severity Score (ISS) > 16 einschlossen. Zusätzliche Einschlusskriterien waren ein Alter ≥ 18 Jahre und die Notwendigkeit für eine intensivmedizinische Behandlung. Außerdem musste ihre Anamnese bzgl. chronisch entzündlicher Erkrankungen unauffällig sein. Nach Einschluss erfolgten an sieben konsekutiven Tagen eine Blutentnahme und Erfassung der SIRS-Kriterien (nach Kriterien der *Surviving Sepsis Campaign* von 2013[193]). Neben den etablierten Infektionsparametern CRP und Procalcitonin wurden IL-6 und Presepsin quantifiziert.

Die inkludierten Patienten wiesen mit einem ISS im Median von 22 [IQR 17 – 34] eine hohe Verletzungsschwere auf. Drei Patienten verstarben, ohne dabei Anzeichen einer Infektion aufzuweisen. Einer der überlebenden Patienten erkrankte an einer Sepsis, während 30 Patienten ein SIRS entwickelten. Im Gegensatz zu IL-6, CRP und Procalcitonin, stieg der Presepsin-Plasmaspiegel nur in geringem Maße und erst verzögert ab dem dritten bis vierten Tag an (die folgenden Parameter sind als Median [IQR] angegeben, Presepsin nach Aufnahme: 487 [123 – 1.901] pg/ml, höchste Presepsin-Messung an Tag 6 nach Trauma: 802 [100 – 4.298] pg/ml). Das IL-

6 zeigte einen signifikanten Anstieg innerhalb der ersten 24 Stunden nach Trauma (siehe Anlage 3, Abbildung 1B, $p < 0{,}001$), während das Procalcitonin eine Erhöhung über die ersten drei Tage (siehe Anlage 3, Abbildung 1C, $p = 0{,}002$) und das CRP den höchsten Anstieg am zweiten Tag nach dem Trauma aufwies (siehe Anlage 3, Abbildung S1A, $p < 0{,}001$). Patienten, welche an einem SIRS oder einem Abdominaltrauma litten, wiesen zudem einen höheren Presepsin-Plasmaspiegel auf als Patienten ohne SIRS bzw. abdominelles Trauma (SIRS vs. kein SIRS: $p = 0{,}03$, siehe Anlage 3, Abbildung 3A; Abdominaltrauma vs. kein Abdominaltrauma: p = n.s., siehe Anlage 3, Abbildung 4A). Die Schwere des Traumas beeinflusste dagegen nicht den Presepsin-Plasmaspiegel (siehe Anlage 3, Abbildung 2A).

Ziel der vorliegenden Studie war die Beschreibung der Presepsin-Kinetik bei polytraumatisierten Patienten. Der Presepsin-Plasmaspiegel wurde dabei nicht durch die ausgeprägte Gewebstraumatisierung und der damit einhergehenden systemischen Inflammation beeinflusst, sondern stieg nur langsam im Verlauf über drei bis vier Tage an. Dies unterscheidet Presepsin von IL-6, CRP und Procalcitonin, welche signifikant nach dem Trauma anstiegen. In Zusammenschau mit den Ergebnissen von Hoshino et al. deutet dies darauf hin, dass Presepsin sich als diskriminativer Biomarker zur Detektion einer akuten Sepsis nach initialer Polytraumatisierung eignen könnte[192]. Allerdings stieg der Presepsin-Plasmaspiegel bei Patienten mit SIRS, aber ohne Infektionszeichen, signifikant an. Auf Grund der geringen Inzidenz von septischen Patienten in dem beobachteten Kollektiv, konnte kein Rückschluss auf die Sensitivität oder Spezifität von Presepsin in Bezug auf die Erkennung einer Sepsis gezogen werden. Dennoch hat auch die Detektion eines SIRS mittels Presepsin einen potentiellen klinischen Nutzen, da ein posttraumatisches SIRS mit einer erhöhten Morbidität und Mortalität assoziiert ist[194].

Zusammenfassung

In dieser prospektiven Observationsstudie blieb Presepsin im Gegensatz zu IL-6, CRP und Procalcitonin durch eine Polytraumatisierung unbeeinflusst, stieg aber bei Vorliegen eines SIRS signifikant an. Somit könnte Presepsin potentiell als Biomarker zur Erkennung von systemischen Inflammationszuständen sowie aber auch zur Detektion von Infektionen bei polytraumatisierten Patienten von Nutzen sein.

3.4 *Host-Derived Delta-Like Canonical Notch Ligand* 1 als potentieller neuer Biomarker zur Detektion einer bakteriellen Sepsis – Ergebnisse einer kombinierten Sekundäranalyse (Anlage 4)

Neben Presepsin wurde nach weiteren potentiellen Biomarkern zur Identifikation von septischen Patienten nach operativen Eingriffen gesucht. Der lösliche DLL-1 wurde in diesem Zusammenhang als potentieller Kandidat identifiziert, da er *in-vitro* bei Infektionen mit diversen Bakterien auf humanen Monozyten vermehrt exprimiert wird. Dies führt gleichermaßen zu einer Erhöhung des löslichen DLL-1 im Serum der Patienten sowie zu einer Aktivierung der proinflammatorischen Zytokinantwort[195,196]. Daher stellte sich die Frage, ob mit Hilfe der Messung der DLL-1-Plasmakonzentration septische Patienten mit ausreichender Prädiktion identifiziert werden können. Des Weiteren wurde untersucht, ob sich die DLL-1-Plasmaskonzentrationen bei postoperativen bzw. traumatologischen Patienten mit SIRS von septischen Patienten unterscheiden.

In dieser Sekundäranalyse wurden 80 septische Patienten [Sepsis], 50 operative Patienten [Chirurgie] und 36 polytraumatisierte [Trauma] Patienten aus drei unterschiedlichen prospektiven Observationsstudien analysiert. Um einen Einfluss von Alter und Geschlecht auszuschließen, dienten zudem 50 Patienten aus unterschiedlichen Altersklassen und verschiedenen Geschlechts als Kontrolle. Während klinische Daten sowie die CRP- und Procalcitonin-Plasmaspiegel aus dem Routinelabor entnommen wurden, erfolgte die Quantifizierung von DLL-1 mittels kommerziellen ELISA.

Zunächst konnte dargestellt werden, dass es bei septischen Patienten im Vergleich zu den Kontrollprobanden zu einem frühzeitigen signifikanten Anstieg des DLL-1 kam, welcher über die folgenden sieben Tage persistierte (die folgenden Parameter sind als Median und IQR angegeben, Kohorte 1: Kontrolle: 12,1 [10,6 – 13,2] ng/ml; Sepsis Zeitpunkt 0: 56,5 [47,8 – 72,8] ng/ml; Sepsis Zeitpunkt Tag 7: 34,3 [28,6 – 50,9] ng/ml). Dagegen wiesen die postoperativen (Chirurgie) und traumatologischen (Trauma) Patienten mit einem SIRS nur einen geringen DLL-1 Anstieg über vier Tage auf (Chirurgie Zeitpunkt 0: 13,6 [12,4 – 16,0] ng/ml; 48 Stunden: 17,6 [15,9 – 20,6] ng/ml; Trauma Zeitpunkt 0: 16,4 [16,2 – 20,1] ng/ml; 96 Stunden: 17,0 [14,5 – 19,7] ng/ml). Zuletzt konnte in der Kontrollkohorte kein

Einfluss des Alters oder Geschlechtes auf die Konzentration von DLL-1 im Blut festgestellt werden. In einer Posthoc-Analyse wies zudem DLL-1 im Vergleich zu CRP und Procalcitonin die höchste Sensitivität und Spezifität zur Identifikation septischer Patienten in den Kollektiven mit SIRS auf.

Vor dem Hintergrund, dass keiner der etablierten Biomarker eine ausreichende Diskriminierung septischer Patienten von Patienten mit postoperativem oder posttraumatischem SIRS erlaubt, konnte diese Sekundäranalyse erstmals DLL-1 als vielversprechenden Biomarker für diesen Zweck identifizieren. Die Konzentration von DLL-1 war bei septischen Patienten über sieben Tage nicht nur gegenüber den gesunden Kontrollprobanden signifikant erhöht, sondern auch gegenüber polytraumatisierten und postoperativen Patienten mit SIRS. Darüber hinaus hatten das Alter und Geschlecht der untersuchten Patienten keinen Einfluss auf die Konzentration von DLL-1. Bisher wurde für DLL-1 ein prognostischer Nutzen für die Evaluation von Patienten mit symptomatischer Aortenklappenstenose und Patienten mit chronischer Herzinsuffizienz auf Basis einer dilatativen Kardiomyopathie beschrieben[197,198]. Allerdings blieben die Grenzwerte in beiden Studien weit unter den DLL-1-Konzentrationen der septischen Patienten, so dass diese Erkrankungen auch keinen Einfluss auf die Diagnostik einer Sepsis haben dürften. Dagegen weisen Patienten nach Herztransplantation einen erhöhten DLL-1 Spiegel auf, welcher in den unteren Grenzbereich der Sepsis-Kohorte reichte[199].

Zusammenfassung

Zusammenfassend weist diese Studie erstmals die Eignung von DLL-1 als potentiellen Biomarker für die Identifikation septischer Patienten auch bei Patienten nach, welche an einer systemischen Inflammation auf Basis einer großen Operation oder eines Polytraumas litten. DLL-1 weist bei diesen Patienten zudem eine höhere Sensitivität und Spezifität als das CRP und Procalcitonin zur Erkennung einer Sepsis auf.

3.5 Explorative Studie zum Einfluss von frei zirkulierender DNA und RNA auf die Hämostase von Patienten mit intraabdomineller Sepsis (Anlage 5)

Während die vorherigen Studien Proteine als Zielparameter für die Detektion von systemischen Inflammationszuständen untersuchten, wurde in dieser Studie (Anlage 5) die Ebene der Nukleinsäuren gewählt. Dies geschah aus mehreren Gründen: Zunächst ist es erwiesen, dass eine Sepsis durch eine schwere Störung des Immunsystems verursacht wird und weniger durch die Schwere der Infektion[1,2]. In diesem Zusammenhang sind frei zirkulierende Nukleinsäuren von hohem Interesse, da sie während einer Sepsis durch den Zelluntergang und NETose freigesetzt werden und ihre Plasmakonzentration mit einer erhöhten Mortalität bei septischen Patienten einhergeht[68,69,200]. Des Weiteren spielen sie eine herausragende Rolle als Co-Aktivator des Gerinnungs- als auch des Immunsystems und könnten daher maßgeblich die Entwicklung der septischen Koagulopathie beeinflussen[80,201]. Dennoch existieren nur wenige Studien zum *in-vivo*-Effekt der frei zirkulierenden Nukleinsäuren auf die septische Koagulopathie. Während das Zusammenspiel zwischen der DNA und NETose in der letzten Dekade zunehmend in den Fokus der Sepsis-Forschung gelangt ist, ist der *in-vivo*-Einfluss der extrazellulären RNA weniger gut geklärt[74,79,80,202,203]. Zuletzt existieren bzgl. der Konzentration von frei zirkulierenden Nukleinsäuren nur eine geringe Anzahl an vergleichenden Untersuchungen zwischen septischen und abdominalchirurgischen Patienten. Aus diesen Gründen war es das primäre Ziel dieser Studie, die Menge an frei zirkulierender DNA und RNA aus dem Blutplasma von Patienten mit intraabdomineller Sepsis im Vergleich zu abdominalchirurgischen Patienten zu quantifizieren und eine etwaige *in-vivo* Assoziation zu Alterationen der Gerinnungsfunktion zu analysieren. Als sekundäre Fragestellungen sollten zudem eine Assoziation zwischen der Konzentration an Nukleinsäuren und Endorganschäden bzw. der Mortalität untersucht werden.

Zu diesem Zweck wurde eine prospektive, monozentrische Observationsstudie an insgesamt 35 Patienten durchgeführt. Es wurden 15 Patienten mit intraabdomineller Sepsis (definiert nach Kriterien der *Surviving Sepsis Campaign* von 2013[193]), zehn abdominalchirurgische und zehn gesunde Kontrollprobanden eingeschlossen. Die Blutentnahmen erfolgten unmittelbar nach Diagnosestellung der Sepsis bzw. nach Beendigung der Operation sowie nach 24, 72 und 168 Stunden, während bei den

Kontrollprobanden nur einmalig Blut entnommen wurde. Nach entsprechender Isolation der Nukleinsäuren aus dem Blutplasma wurde mittels TaqMan-Sonden-basierter *Realtime-Polymerase Chain Reaction* (RT-PCR) die Menge an frei zirkulierender DNA des ß-Globins (in *Genom Equivalents* [GE]) sowie von extrazellulärer RNA des ß-Aktins (in *Transcriptional Units* [TU]) gemessen. Um die *in-vivo* Gerinnungsfunktion zu charakterisieren, wurden neben der Globalgerinnung (Partielle Thromboplastinzeit (PTT), *International Normalized Ratio* (INR), Quick und Fibrinogen) eine Thrombelastographie durchgeführt.

Die septischen Patienten waren durch eine hohe Krankheitsschwere (SOFA Median [IQR]: 8 [1 – 14]) gekennzeichnet und wiesen zu 93 % einen abdominellen Fokus der Sepsis auf. Bei einem Patienten handelte es sich bei der Ursache um eine Infektion des Urogenitalsystems. Bei 70 % der operativen Patienten wurden große tumorchirurgische Eingriffe des Pankreas durchgeführt. Sowohl die Menge an frei zirkulierender DNA als auch die der extrazellulären RNA wiesen charakteristische Verläufe auf. Dabei zeigten, im Vergleich zu den operativen Patienten, septische Patienten innerhalb der ersten 24 Stunden signifikant höhere Werte beider Nukleinsäuren (die folgenden Parameter sind als Median angegeben, DNA: T_0: 52,1 vs. 15,9 GE, p = 0,012; T_{24h}: 29,1 vs. 10,1 GE, p = 0,035; RNA: T_0: 712,7 vs. 240,0 TU, p = 0,004; T_{24h}: 621,5 vs. 108,8 TU, p < 0.001). Gegenüber den Kontrollprobanden blieb die Konzentration der frei zirkulierenden DNA bei septischen Patienten auch im Verlauf über sieben Tage signifikant erhöht (T_0:52,1 vs. 2,4 GE, p < 0,001; T_{24h}: 29,1 vs. 2,4 GE, p < 0,001; T_{72}: 25,6 vs. 2,4 GE, p < 0,001; T_{168h}: 14,6 vs. 2,4 GE, p < 0,001) , während sie bei der extrazellulären RNA bereits nach 24 Stunden wieder abfiel (T_0: 712,7 vs. 220,0 TU, p < 0,05; T_{24h}: 621,5 vs. 222,0 TU; p = n.s.). Interessanterweise resultierten sie allerdings in gegenläufigen Veränderungen der Gerinnungsfunktion: Die Menge an frei zirkulierender DNA korrelierte positiv mit den Gerinnungszeiten, welche die Aktivierung der Gerinnselbildung abbilden (*Clotting Time* [CT]) (siehe Anlage 5, Tabelle 2) sowie positiv mit dem INR (r = 0,284, p = 0,043). Allerdings war die Menge an zellfreier DNA auch mit einem erhöhten Lyse Index in fast allen Reagenzien (mit Ausnahme des EXTEMs, siehe Anlage 5, Tabelle 2) assoziiert. Die Menge an extrazellulärer RNA korrelierte in der Nativgerinnung (NATEM) dagegen negativ mit der CT (r = -0,418, p = 0,002). Zuletzt zeigte sich, dass Patienten mit

erhöhten Kreatinin-Blutspiegeln (> 1,2 mg/dl) signifikant höhere Mengen an frei zirkulierender DNA, aber nicht an extrazellulärer RNA aufwiesen (72,7 vs. 24,0 GE, $p = 0{,}004$).

Ziel dieser Pilotstudie war es, den Verlauf zweier verschiedener Typen von Nukleinsäuren bei septischen und abdominalchirurgischen Patienten zu charakterisieren und einen *in-vivo*-Einfluss auf die Gerinnung darzustellen. Die Studie konnte zeigen, dass im Vergleich zu gesunden Probanden die Menge beider Nukleinsäuretypen schnell ansteigt, was die Ergebnisse anderer Studien bzgl. der Menge an zellfreier DNA bestätigt[67–69,141,204]. Interessanterweise wiesen septische Patienten dabei signifikant höhere DNA-Konzentrationen als operative Patienten auf, was auf eine potentielle Diskrimination zwischen SIRS und Sepsis hinweisen könnte. Bzgl. der extrazellulären RNA gab es bisher keine vergleichenden Studien zwischen diesen Kollektiven. Des Weiteren zeigte die Studie einen unterschiedlichen Einfluss der frei zirkulierenden DNA und RNA auf die Gerinnung der Patienten. Während die zellfreie DNA mit einer verlängerten CT entsprechend einer Verlängerung der Blutungszeit, aber auch mit einem erhöhten Lyse Index (hinweisend auf eine Hemmung der Fibrinolyse) in fast allen Reagenzien assoziiert war, zeigte sich in der Nativgerinnung eine Assoziation der extrazellulären RNA mit einer rein prokoagulatorischen Reaktion. Diese *in-vivo*-Ergebnisse fügen sich in die komplexe Diskussion zum Einfluss von Nukleinsäuren auf die septische Koagulopathie ein[67,80,201,205,206]. Es bleibt allerdings schwierig, den Einfluss einzelner Faktoren wie der Nukleinsäuren in dem komplexen Krankheitsbild der Sepsis zu definieren.

Sowohl die Ergebnisse zum Verlauf der Nukleinsäuren als auch die Korrelationsanalysen zum Einfluss auf die *in-vivo*-Koagulation weisen darauf hin, dass sie im Rahmen der Immunhämostase eine wichtige Rolle spielen könnten. Aus diesem Grund und weiterhin mit dem Ziel potentieller Biomarker für die Diskrimination von septischen zu sterilen systemischen Inflammationsreaktionen zu detektieren, konzentrierten sich die folgenden Arbeiten auf NETs, die „*Keyplayer*“ der Immunhämostase.

Zusammenfassung

Diese Observationsstudie zeigte zum einen, dass sowohl die Menge an frei zirkulierender DNA als auch an extrazellulärer RNA bei septischen Patienten höher war als bei abdominalchirurgischen Patienten mit SIRS. Zudem war die Konzentration der DNA bei septischen Patienten gegenüber der der Kontrollprobanden über sieben Tage signifikant erhöht. Des Weiteren korrelierte die Menge an frei zirkulierender DNA mit einer reduzierten Gerinnungsfunktion, während die Menge an extrazellulärer RNA mit einem prokoagulatorischen Zustand assoziiert war.

3.6 Neutrophil Extracellular Traps sind im septischen Schock mit einer Hyperkoagulation und in der postoperativen systemischen Inflammation mit einer Hypokoagulation assoziiert: Ergebnisse einer Proof-of-Concept-Studie zur durchflusszytometrischen Quantifizierung von Neutrophil Extracellular Traps (Anlage 6)

Seit ihrer Erstbeschreibung im Jahr 2004 wurde zunehmend die Bedeutung der NETs im Rahmen der initialen Aktivierung des Immun- und Gerinnungssystems erkannt[70,207]. So wurden sie bei einer Vielzahl von Erkrankungen, welche mit einer systemischen inflammatorischen Aktivierung des Immunsystems einhergehen, wie z.B. Tumor- und Autoimmunerkrankungen oder aber auch der Arteriosklerose, identifiziert[65,207,208]. Aus diesem Grund wurde vermehrt auch ihre Rolle in der Pathophysiologie der Sepsis untersucht. Eine Vielzahl von experimentellen Studien und Untersuchungen am Tiermodell konnten *in-vitro* die komplexe Kommunikation zwischen der initialen Immunantwort, der NETose, dem adaptiven Immunsystem und der Gerinnung im Rahmen der Sepsis teilweise entschlüsseln[47,74,76,80]. Allerdings existiert nur eine geringe Anzahl von Studien, welche die *in-vivo*-Effekte der NETs bei septischen und Patienten mit postoperativem SIRS untersuchen. Eine Ursache für dieses Problem stellen die komplexen Methoden zur Quantifizierung von NETs dar, weshalb für diese Studie eine neuartige Methode zur durchflusszytometrischen Quantifizierung von NETs aus Vollblut etabliert wurde[146,149]. Dabei sollte erstens die Methodik evaluiert werden, zweitens die Menge an NETs bei septischen und postoperativen Patienten mit SIRS und drittens mögliche Assoziationen zwischen der Menge an NETs und der Funktion des koagulatorischen Systems der Patienten dargestellt werden.

Für diese prospektive, monozentrische *Proof-of-Concept*-Studie wurden insgesamt 80 Patienten mit drei verschiedenen Genesen einer systemischen Inflammation (Septischer Schock, herz- und abdominalchirurgische Eingriffe [jeweils n = 20]) sowie einer zur Sepsis-Kohorte gematchten Kontrollgruppe [n = 20]) inkludiert. Die Durchflußzytometrie basierte auf anti-MPO- und anti-Histon-markierten Neutrophilen als Surrogate für NETs und wurde mit Hilfe der Fluoreszenzmikroskopie validiert. Im Anschluss wurden die NETs durchflusszytometrisch quantifiziert und im Hinblick auf weitere Inflammations- und Gerinnungsparameter analysiert. Letztere

wurden mittels Thrombelastographie, Thrombozytenimpedanzaggregometrie sowie aus dem Routinelabor gewonnen. Die Blutentnahme erfolgte bei septischen Patienten bei Aufnahme auf die Intensivstation sowie nach 24 und 72 Stunden, während sie bei operativen Patienten präoperativ, unmittelbar postoperativ sowie nach 24 und 72 Stunden durchgeführt wurde. Den Kontrollprobanden wurde einmalig Blut entnommen.

Im Anschluss an die Validierung konnte gegenüber den Kontrollprobanden eine signifikante Erhöhung der NETs-Konzentration sowohl bei den septischen als auch bei den operativen Patienten identifiziert werden (die folgenden Parameter sind als Median und IQR angegeben, Septischer Schock: (2,7 [1,9 – 3,9] %; Herzchirurgie: 2,7 [2,1 – 3,7] %; Abdominalchirurgie: 2,7 [2,1 – 3,9] %; Kontrolle: 1,6 [1 – 2] %; Kontrolle vs. Septischer Schock: $p = 0{,}001$; Kontrolle vs. Herzchirurgie: $p < 0{,}001$; Kontrolle vs. Abdominalchirurgie: $p < 0{,}001$). Des Weiteren zeigten sich zwischen den verschiedenen Kohorten Unterschiede im Verlauf der NETs. Während septische und herzchirurgische Patienten einen signifikanten Anstieg der NETs über 72 Stunden aufwiesen, stellte sich bei den abdominalchirurgischen Patienten nur eine signifikante Steigerung über 24 Stunden dar (Kontrolle vs. Septischer Schock Beginn: $p < 0{,}001$; Kontrolle vs. Septischer Schock 24 Stunden: $p < 0{,}05$; Kontrolle vs. Septischer Schock 72 Stunden: $p < 0{,}05$; Herzchirurgie präoperativ vs. postoperativ: $p < 0{,}001$, Herzchirurgie postoperativ vs. 24 Stunden: $p < 0{,}01$; Herzchirurgie postoperativ vs. 72 Stunden: $p < 0{,}05$; Abdominalchirurgie präoperativ vs. 24 Stunden: $p < 0{,}05$; siehe Anlage 5, Tabelle 2 und Abbildung 3). Es konnte allerdings kein statistisch relevanter Unterschied der NETs-Konzentration zwischen den postoperativen und septischen Messpunkten dargestellt werden. In der Korrelationsanalyse zwischen NETs und den thrombelastographischen Parametern stellten sich interessanterweise deutliche Unterschiede zwischen den postoperativen und septischen Patienten dar. Septische Patienten wiesen eine positive Korrelation zwischen der Menge an NETs und dem FIBTEM, welches die Fibrinogen-abhängige Gerinnselbildung repräsentiert, auf (FIBTEM *Mean Clot Firmness* [MCF]: $r = 0{,}37$, $p < 0{,}01$). Des Weiteren stellte sich eine negative Korrelation zur CT des FIBTEMs dar (FIBTEM CT: $r = -0{,}3$, $p = 0{,}02$), was in der Zusammenschau auf eine Assoziation zu einer Aktivierung der Gerinnung hinweist. Konträr hierzu korrelierte in beiden chirurgischen Kollektiven die MCF fast aller thrombelastographischen Reagenzien invers mit der Menge der NETs

(Herzchirurgie: $r = -0{,}28$, $p < 0{,}01$; Abdominalchirurgie: $r = -0{,}25$, $p = 0{,}03$) sowie in einigen Reagenzien mit Verlängerungen der Gerinnselbildungszeiten.

Das Ziel dieser Studie, eine valide durchflusszytometrische Methode zur Quantifizierung von NETs im intensivmedizinischen Setting zu etablieren, konnte erreicht werden. Des Weiteren zeigten sich charakteristische Verläufe im Rahmen der Sepsis und dem postoperativen SIRS nach abdominal- und herzchirurgischen Eingriffen. Allerdings stellten sich bzgl. der Menge an NETs keine Unterschiede zwischen den postoperativen Zeitpunkten der operativen und septischen Patienten dar, so dass ihr Nutzen als diskriminierender Biomarker für die Detektion der Sepsis im postoperativen Verlauf bezweifelt werden kann. Andererseits konnte die vorliegende Studie eine Korrelation zwischen der Menge an NETs und einem prokoagulatorischen Effekt auf die *in-vivo*-Gerinnung nachweisen, während die postoperativen Patienten eine negative Korrelation aufwiesen. Grundsätzlich müssen bei herzchirurgischen Patienten Gerinnungsanalysen auf Grund der notwendigen Antikoagulation während des kardiopulmonalen Bypasses mit Vorsicht bewertet werden. Dennoch wiesen diese Patienten zum einen eine adäquate Gerinnungsfunktion auf und zum anderen konnte dieselbe Korrelation bei abdominalchirurgischen Patienten nachgewiesen werden. Somit stellte die Studie die prokoagulatorischen *in-vivo*-Effekte der NETose dar, welche *in-vitro* bereits beschrieben wurden[76,139,202,209,210]. Die postoperative Assoziation der NETs zu einer Gerinnungsinhibierung war bisher nicht beschrieben, könnte aber eine Möglichkeit bieten, septische von SIRS Patienten zu differenzieren.

Im nächsten Schritt erfolgte eine Sekundäranalyse, welche den Einfluss von frei zirkulierender mtDNA als Teil der NETs auf die Gerinnung bei septischen und operativen Patienten untersucht.

Zusammenfassung

Diese Studie zeigte, dass die Durchflusszytometrie zur Messung von NETs genutzt werden kann. Anhand der Konzentration der NETs konnte nicht zwischen postchirurgischem SIRS und Sepsis unterschieden werden, aber die differente Wirkung der NETs auf die Gerinnungsfunktion septischer bzw. postoperativer Patienten könnte einen Ansatz für die Diskrimination zwischen diesen beiden Ursachen einer systemischen Inflammation darstellen.

3.7 Sekundäranalyse einer prospektiven Observationsstudie zum Einfluss frei-zirkulierender mitochondrialer DNA auf die Gerinnung bei septischen und postoperativen Patienten mit SIRS (Anlage 7)

Im nächsten Schritt sollte mit Hilfe einer Sekundäranalyse der vorherigen Studie (Anlage 6) die Konzentration an frei-zirkulierender mtDNA bei septischen und postoperativen Patienten mit SIRS untersucht werden. Die Analyse der mtDNA stellte dabei aus mehreren Gründen einen sinnvollen Ansatz dar, da ihre Freisetzung zum einen sowohl bei operativen als auch septischen Patienten beschrieben wurde und sie auf Grund ihrer negativen Ladung potentiell zu einer Aktivierung der Gerinnung führt[67,82,145,209,211,212]. Es ist allerdings noch nicht geklärt, ob mtDNA auch unabhängig von NETs eine messbare *in-vivo*-Aktivierung der Gerinnung verursachen kann. Zudem sollte diese Sekundäranalyse Unterschiede zwischen der Konzentration an frei zirkulierender mtDNA bei septischen Patienten und bei Patienten nach großen operativen Eingriffen identifizieren.

In diese Sekundäranalyse wurden die 80 Patienten der Primärstudie (Anlage 6) eingeschlossen (20 Patienten mit septischem Schock, je 20 Patienten mit SIRS nach herz- bzw. abdominalchirurgischen Eingriffen sowie Kontrollprobanden)[213]. Als Ziel-Gen diente die NADH-Dehydrogenase 1, welche mit Hilfe einer quantitativen Plasmid-basierten PCR quantifiziert wurde. Neben den Inflammationsparametern wurden zur Untersuchung etwaiger Assoziationen mit dem Gerinnungssystem thrombelastographische und globale Gerinnungsparameter aus der Primäranalyse verwendet.

Über alle Zeitpunkte kumuliert war die Konzentration der frei zirkulierenden mtDNA der septischen Patienten gegenüber beiden postoperativen Patientenkohorten signifikant erhöht (die folgenden Parameter sind als Median und IQR angegeben, Kontrolle: 1.208 [668 – 2.685] copies/µL; Septischer Schock: 3.823 [2.170 – 7.318] copies/µl; Herzchirurgie: 1.272 [417 – 2.720] copies/µl; Abdominalchirurgie: 1.356 [694 – 2.845] copies/µl; Kontrolle vs. Septischer Schock: $p < 0{,}001$; Septischer Schock vs. Herzchirurgie: $p < 0{,}001$; Septischer Schock vs. Abdominalchirurgie: $p = 0{,}006$; Herzchirurgie vs. Abdominalchirurgie: $p = 0{,}01$). Des Weiteren zeigten alle Kohorten einen charakteristischen Verlauf, welcher bei den septischen und abdominalchirurgischen Patienten mit einem Anstieg der mtDNA gekennzeichnet war,

während die kardiochirurgischen Patienten unmittelbar nach dem kardiopulmonalen Bypass einen Abfall aufwiesen (siehe Anlage 7, Abbildung 1). So unterschieden sich die postoperativen Messergebnisse beider chirurgischen Kohorten nicht von denen der Kontrollgruppe (die folgenden Parameter sind als Median und IQR angegeben, Kontrolle: 1.208 [668 – 2.685] copies/µl; Herzchirurgie: 1.272 [417 – 2.720] copies/µl; Abdominalchirurgie: 1.356 [694 – 2.845] copies/µl; Kontrolle vs. Herzchirurgie: $p = 0{,}660$; Kontrolle: vs. Abdominalchirurgie: $p = 0{,}190$; Herzchirurgie vs. Abdominalchirurgie: $p = 0{,}01$). Während sich im Plasma der septischen Patienten keine Assoziation zwischen der Konzentration der mtDNA und der Blutgerinnung identifizieren ließ, wiesen Patienten nach herzchirurgischen Eingriffen eine starke positive Korrelation mit dem Fibrinogen-Spiegel ($r = 0{,}57$, $p < 0.001$) und den Fibrinogen-abhängigen thrombelastographischen Parametern auf (MCF EXTEM: $r = 0{,}35$, $p = 0{,}01$; MCF INTEM: $r = 0{,}31$, $p = 0{,}02$; MCF FIBTEM: $r = 0{,}46$, $p < 0{,}001$).

Diese Sekundäranalyse wies bei septischen Patienten im Vergleich zu postoperativen Patienten mit SIRS signifikant höhere Konzentrationen an mtDNA der NADH-Dehydrogenase 1 nach. Während abdominalchirurgische Patienten postoperativ vergleichbar hohe Werte wie septische Patienten erreichten, fielen sie bei den kardiochirurgischen Patienten bereits unmittelbar nach dem kardiopulmonalen Bypass. Einerseits könnte dies bedeuten, dass die mtDNA potentiell zur Identifikation septischer Patienten nach herzchirurgischen Eingriffen genutzt werden könnte. Andererseits zeigten aber andere Studien nach der Beendigung des kardiopulmonalen Bypasses keinen signifikanten Abfall der Konzentration an mtDNA, so dass es sich um einen Dilutionseffekt handeln könnte[84,211,214]. Für eine abschließende Bewertung mangelt es allerdings an einer ausreichenden Anzahl an vergleichbaren Studien sowie an technischen Details zu den eingesetzten Herz-Lungen-Maschinen. Zudem muss der Einfluss zugrunde liegender Erkrankungen mit in die Bewertung inkludiert werden, da zum Beispiel das Vorliegen einer Arteriosklerose, Diabetes mellitus oder Hypertension die Konzentration an mtDNA erhöhen könnten[215–217]. Eine weitere Fragestellung dieser Sekundäranalyse war es, eine Assoziation zwischen der Gerinnungsaktivität und der Konzentration an mtDNA zu identifizieren. Während im Rahmen der primären Analyse eine positive Korrelation zwischen den NETs und der Gerinnungsaktivität bei septischen Patienten nachweisbar war, zeigte sich dies bzgl. der Konzentration der mtDNA nicht. Dagegen könnte ein *in-vivo*-Effekt der

kardiopulmonalen Bypass-induzierten Plättchenaktivierung beobachtet werden, da diese stark mit der Fibrinogen-abhängigen Aggregation assoziiert ist[67,211]. Allerdings sind weitere Studien mit höheren Fallzahlen notwendig, um diesen Zusammenhang zu bestätigen.

Zusammenfassung

Die Konzentration der frei zirkulierenden mtDNA war bei septischen Patienten höher als bei postoperativen Patienten mit SIRS. Während sich bei septischen Patienten keine Assoziation zwischen der Menge an mtDNA und einer erhöhten Gerinnungsaktivität feststellen ließ, stellte sich dies bei den kardiochirurgischen Patienten dar.

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3.8 Langzeit-Immunparalyse nach Sepsis – Ergebnisse einer prospektiven explorativen Studie (Anlage 8)

Neben dem Management der akuten Sepsis rücken die Langzeitfolgen der Sepsis in den Fokus der Wissenschaft, da trotz primärem Überleben die Patienten eine langfristig eingeschränkte Lebensqualität und erhöhte Sterblichkeit aufweisen[96,218–222]. Dieses sog. *Chronic Critical Care Syndrome* kann durch eine Beeinträchtigung fast aller Organsysteme, aber auch insbesondere des Immunsystems gekennzeichnet sein[97]. Hierfür könnte eine persistierende Immunparalyse verantwortlich sein, welche sich aus dem *Compensatory Anti-Inflammatory Response Syndrome* (CARS) entwickeln kann[223–225]. Das CARS zeichnet sich durch eine Erschöpfung der Immunfunktion nach einem Sepsis-bedingten Zytokinsturm aus. Hierdurch kommt es zu einem Anstieg der antiinflammatorischen bei simultanem Abfall der proinflammatorischen Zytokine. Als mittelfristige Folge der Immunsuppression können die Patienten das PICS sowie das *Multiple Organ Dysfunction Syndrome* (MODS) entwickeln[226]. Allerdings fehlen bislang langfristige Untersuchungen zum Immunstatus und den zellulären Immunkompetenzen von Patienten, welche eine Sepsis überlebt haben. Daher war es Ziel der vorliegenden Studie, sowohl die zellulären Bestandteile als auch die Zytokinexpression bei Patienten zu quantifizieren, welche langfristig einen septischen Schock überlebt haben.

Von 172 Patienten, welche langfristig (Median 26 [9 – 52] Monate) einen septischen Schock überlebt haben, wurden acht Patienten ausgewählt und mit acht Probanden nach Alter, Geschlecht und Vorerkranken gematcht. Zunächst wurde der Gesundheitszustand mit einem systematischen Fragebogen erfasst und im Anschluss die Lymphozyten mittels Durchflusszytometrie quantifiziert und charakterisiert. Von allen CD^{3+}-Lymphozyten wurden der CD^{4+}- und CD^{8+}-Lymphozytenanteil ermittelt, während die regulatorischen T-Zellen (CD^{4+}, CD^{25+}, CD^{127-}) aus der Fraktion der CD^{4+}-Lymphozyten kalkuliert wurden. Darüber hinaus wurden die Monozyten mittels Nachweises des CD^{14+}-Antigens identifiziert und zudem deren HLA-DR-Expression quantifiziert. Im nächsten Schritt wurden Oberflächenantigene auf den Lymphozyten (*Programmed Cell Death 1* [PD-1], *B- and T- Lymphocyte Attenuator* [BTLA], *Cytotoxic T-lymphocyte-Associated Protein 4* [CTLA-4] und Monozyten TLR 2, -4, und 5 sowie Dectin-1 und *Programmed Cell Death Ligand 1* [PD-1 L]) gemessen. Im letzten Schritt

wurde das Blut ex-vivo mit Zymosan, α-CD3/28 bzw. LPS stimuliert und die Zytokinsekretion (IL-2, IL-4, IL-6, IL-10, IL- 17A, TNF und IFN-γ) der Zellen quantifiziert.

Der Abstand zur Sepsis betrug im Median 26 [IQR 9 – 52] Monate. Keiner der Kontrollprobanden gab an, in den zwölf Monaten vor der Befragung eine Infektion erlitten bzw. eine Antibiose erhalten zu haben. Dagegen erlitten 62,5 % der Sepsis-Patienten im selben Zeitraum eine Infektion, welche bei vier der Patienten antibiotisch behandelt wurde. Darüber hinaus konnten drei Indikatorinfektionen für eine Immunsuppression (Herpes zoster, Infekt der unteren Atemwege, Candidose) nachgewiesen werden. Die Routineinfektionsparameter CRP und Procalcitonin waren bei den postseptischen Patienten leicht, aber signifikant erhöht (die folgenden Parameter sind als Median und IQR angegeben, CRP Kontrolle: 1,76 [0 – 32,53] mg/l; CRP Sepsis: 10,01 [0,88 – 23,65] mg/l; PCT Kontrolle: 0 [0] ng/ml; PCT Sepsis: 0 [0 – 0,2] ng/ml). Während keine Gruppenunterschiede bzgl. der Verteilung der CD^{3+} und CD^{4+}-Lymphozyten bzw. CD^{14+}-Monozyten nachgewiesen werden konnten, zeigte sich bei den ehemals septischen Patienten eine Reduktion der PD-1-Rezeptoren bei gleichzeitigem Anstieg der BTLA-Rezeptorendichte (siehe Anlage 8, Abbildung 1 und 2). Darüber hinaus konnte eine Reduktion der monozytären TLR-5-Rezeptorenexpression nachgewiesen werden, während sich die Expression der HLA-DR nicht unterschied (siehe Anlage 8, Abbildung 3 und 4). Zuletzt zeigten sich Unterschiede in der Kapazität der Zytokinsekretion. Gegenüber den gesunden Probanden war die Zymosan-induzierte Sekretion von IL-6, IL-10 und TNF-α bei den postseptischen Patienten inhibiert. Ebenso zeigten sich eine reduzierte Sekretion von IL-10 nach LPS-Stimulation sowie IFN-γ nach α-CD3/28-Applikation (siehe Anlage 8, Abbildung 5).

In dieser prospektiven Querschnittstudie konnte im Vergleich zu Kontrollprobanden eine erhöhte Anzahl an Infektionen bei Patienten festgestellt werden, welche einen septischen Schock überlebt haben. Darüber hinaus ließen sich diese Hinweise auf eine langfristige Immunsuppression auch auf der zellulären Ebene der Lympho- und Monozyten nachweisen. Während HLA-DR, als etablierter Biomarker für eine Immunsuppression, nicht zwischen den Gruppen unterschied, wiesen sowohl die Rezeptorexpression als auch die Zytokinsekretion bei den postseptischen

Patienten Alterationen auf. Bei weiterer Analyse der Rezeptoreigenschaften zeigte sich eine Reduktion der PD-1-Rezeptoren, während die Rezeptorendichte der BTLA-Rezeptoren stieg. Diese inhibierenden Modulatoren der T-Lymphozytenfunktion wurden bisher nur im Rahmen der akuten Sepsis untersucht und weisen zu Beginn einer Sepsis eine signifikante Erhöhung auf[227]. Allerdings war in der vorliegenden Studie der ebenso inhibierende T-Lymphozytenregulator BTLA hochreguliert, so dass sich aus dieser Studie keine eindeutige Kausalität zwischen der klinischen Immunsuppression und der T-lymphozytären Rezeptorexpression darstellen lässt. Eindeutiger lassen sich die Ergebnisse bzgl. der Reduktion der TLR-5-Rezeptordichte auf Monozyten einordnen, da TLR-5 bakterielle Flagelline erkennt und es somit zu einer eingeschränkten phagozytären Detektion von Bakterien kommen könnte. Da zum langfristigen postseptischen Verlauf keine Vergleichsdaten bzgl. der Zytokinsekretion existieren, bleiben nur Vergleiche zur akuten Sepsis. Während sich die kurzfristige Reduktion der α-CD3/28-induzierten IFN-γ-Sekretion auch langfristig nachwiesen ließ, zeigten sich bzgl. der α-CD3/28-induzierten IL-6-, IL-10- und TNF-α-Sekretion konträre Ergebnisse[228]. Im Gegensatz zu ihrer vermehrten Sekretion in der Akutphase, war ihre Expression im langfristigen Verlauf gehemmt[228].

Zusammenfassung

Patienten, welche einen septischen Schock überlebten, wiesen noch mehrere Monate später vermehrt Infektionen auf. Weiterhin konnten bei diesen Patienten Veränderungen der Rezeptorenexpression auf den Lympho- und Monozyten nachgewiesen werden. Insbesondere war die monozytäre TLR-5-Rezeptorexpression in der postseptischen Gruppe signifikant reduziert, was auf eine Inhibition der phagozytären Detektion von Bakterien hinweisen könnte. Zuletzt konnte diese Studie eine reduzierte Zytokinexpression nach Stimulation des angeborenen (LPS und Zymosan) und erworbenen Immunsystems (α-CD3/28) darstellen. Zusammenfassend weist die Studie somit auf eine persistierende Immunsuppression nach überlebter Sepsis hin, welche mit Alterationen der lympho- und monozytären Rezeptorexpression und Zytokinsekretion assoziiert sein könnte.

3.9 Langzeitveränderungen von humanen T-Lymphozyten und Monozyten nach Polytraumatisierung – Ergebnisse einer explorativen Querschnittsstudie (Anlage 9)

Auf Basis der Überlegungen und Ergebnisse der vorherigen Studie bei septischen Patienten entstand die Fragestellung, ob auch polytraumatisierte Patienten im Langzeitverlauf immunologische Veränderungen aufweisen. So könnte analog zur Sepsis die Hyperinflammation mit folgender Erschöpfung des Immunsystems zu langfristigen Veränderungen der Immunkompetenz führen. Es ist bekannt, dass epigenetische Modulationen zu einer reduzierten Funktion des Immunsystems führen können, welches sich dann klinisch in einem *Chronical Critical Illness Syndrom* äußert[140,229–231]. Während die Langzeitschäden der Sepsis zunehmend erkannt wurden, sind die immunologischen Folgen eines Polytraumas derzeit nur unzureichend untersucht[232]. Allerdings ist bekannt, dass ehemals polytraumatisierte Patienten über Jahre an erheblichen Beeinträchtigungen im Alltag leiden sowie eine erhöhte Mortalität aufweisen[172,173]. Immunologische Veränderungen mit eingeschränkter Immunkompetenz wären eine mögliche Erklärung für diese Langzeitfolgen.

In die vorliegende Studie wurden 24 Patienten eingeschlossen, von welchen zwölf Patienten innerhalb der sechs Monate (-12 bis +5 Tage) zuvor ein Polytrauma erlitten hatten (ISS ≥ 16) und wurden mit zwölf gesunden Kontrollprobanden verglichen. Während der aktuelle sowie der Gesundheitszustand der letzten sechs Monate mit Hilfe eines standardisierten Fragebogens erfasst wurde, erfolgte mit der Durchflusszytometrie die Quantifizierung des zellulären Immunstatus. Zunächst wurden die Zelltypen (CD^{4+}, CD^{8+}, CD^{14+}) charakterisiert und anschließend die Rezeptorexpression (in *Molecules to Equivalent Soluble Fluorophore* [MESF], HLA-DR, PD-1, BTLA, CTLA-4, TLR-2, -4, und -5, Dectin-1, PD-1L) quantifiziert. Im letzten Schritt wurden die Zellen mit unterschiedlichen Stimuli (LPS-, α-CD3/28, Zymosan) aktiviert und ihre Zytokinsekretion (IL-2, -4, -6, -10, TNF-α, und IFN-γ) erfasst.

Die Abfrage des Gesundheitsstatus ergab keine signifikanten Unterschiede zwischen den beiden Studiengruppen. Ebenso zeigten sich keine Unterschiede zwischen den Blutbildern bzw. den CRP- und Procalcitonin-Blutspiegeln, so dass die Immunzellen in klinisch gesundem Zustand charakterisiert wurden (die folgenden

Parameter sind als Median und IQR angegeben, Trauma: 7,2 [CI 6,6 – 7,6] giga/l, Kontrolle: 6,6 [CI 5,9 – 7,9] giga/l, *p* = 0,8; CRP Trauma: 0,3 [CI 0 – 1,6] mg/l, Kontrolle: 0,6 [CI 0 – 1,9] mg/l, *p* = 0,6; Procalcitonin: Trauma 0 [0 – 1,4] µg/l, Kontrolle 0 [0] µg/l). Während sich die T-Zell-Populationen von CD^{4+} und CD^{8+}-T-Zellen nicht unterschieden (die folgenden Parameter sind als Median und IQR angegeben, CD^{4+}: Trauma 60,5 % [55,3 – 65,5], Kontrolle 67,9 % [60,3 – 71,6], *p* = 0.1; CD^{8+} Trauma 32,6 [24,6 – 36,1], Kontrolle 25,8 [20,9 – 30,5], *p* = 0.1), ließen sich Veränderungen der Rezeptorexpression bei den ehemals polytraumatisierten Patienten nachweisen. So konnte auf CD^{4+}-T-Lymphozyten eine Erhöhung der PD-1-Expression festgestellt werden, während die BTLA-Expression sowohl auf CD^{4+} und CD^{8+}-T- Lymphozyten verringert waren (siehe Anlage 9, Tabelle 1). Auch zeigten sich Alterationen der monozytären Rezeptoren, da die Expression von TLR-2 und 4 signifikant reduziert war (die folgenden Parameter sind als Median und IQR angegeben, CD^{14+} TLR-2: Trauma 5.863 [3.687 – 20.779] MESF, Kontrolle 21.693 [18.054 – 26.069] MESF, *p* = 0,04; CD^{14+} TLR-4: Trauma 1.189 [928 – 3.664] MESF, Kontrolle 6.250 (5.461 – 8.205) MESF, *p* = 0,03). Die Expression des HLA-DR war dagegen nicht beeinträchtigt (siehe Anhang 9, Abbildung 3). Zwar konnte innerhalb der Traumakohorte eine Abnahme in der IL-6- und TNF-α-Sekretion dargestellt werden, allerdings erreichte diese Beobachtung keine statistische Signifikanz (die folgenden Parameter sind als Median und IQR angegeben, IL-6 Trauma: 21,1 [CI 16,7 – 43,1] pg/ml, Kontrolle 50,3 [CI 37,9 – 102,9] pg/ml, *p* = 0.16); TNF-α Trauma: 2,4 [CI 1,7 – 3,4] pg/ml, Kontrolle 3,8 [CI 2,9 – 5,2] pg/ml, *p* = 0,09).

Während die Mehrzahl der immunologischen Studien bei polytraumatisierten Patienten die Akutphase oder kurz- bis mittelfristige Folgen untersuchen, befasste sich die vorliegende Studie mit den immunologischen Langzeitfolgen. Obwohl die Patienten nicht mehr an Infektionen erkrankten als die Kontrollprobanden, wiesen sie Alterationen der T-lymphozytären und monozytären Zellrezeptoren auf. Die beobachtete Steigerung der PD-1-Rezeptoren auf CD^{4+}-Zellen wurde bei septischen Patienten im Kurzzeitverlauf beschrieben und war in dieser Situation mit einer erhöhten Mortalität assoziiert[233,234]. Andererseits zeigte sich in der vorherigen Studie an septischen Patienten eine Erhöhung der PD-1- und BTLA-Rezeptoren auf CD^{4+}-T-Lymphozyten, so dass eine definitive Wertung unserer Beobachtungen bei

polytraumatisierten Patienten schwierig ist[235]. Dagegen könnte die Alteration der Monozyten auf eine Immunsuppression hinweisen, da die Expression von TLR-2 und -4 signifikant reduziert waren. Beide TLR-Rezeptortypen sind für die Erkennung von DAMPs und PAMPs von hoher Bedeutung, da sie intrazelluläre proinflammatorische Signalkaskaden zur Bekämpfung von Bakterien aktivieren[115,236]. Zuletzt sollte eine gehemmte Funktionsfähigkeit der Immunzellen, definiert als ihre Fähigkeit zur Zytokinsekretion, identifiziert werden. Diesbezüglich zeigten sich zwar Trends zu einer reduzierten Ausschüttung von IL-6 und TNF-α, allerdings ohne eine statistische Signifikanz zu erreichen. Interessanterweise würde eine reduzierte TNF-α-Sekretion nach LPS-Stimulation ebenso in einer reduzierten Abwehrfähigkeit gegenüber Gram-positiven und -negativen Bakterien führen. Darum sollte das fehlende Erreichen des Signifikanzniveaus durch Studien mit höherer Fallzahl überprüft werden.

Zusammenfassung

Sechs Monate nach einer Polytraumatisierung waren die Zellrezeptor-expressionen sowohl auf den T-Zellen als auch auf den Monozyten alteriert. Insbesondere die Reduktion der monozytären TLR-2- und 4-Expression könnte auf eine Immunsuppression hinweisen. Hierauf weisen auch Trends zu einer reduzierten IL-6- und TNF-α- Sekretion hin. Allerdings litten die ehemals polytraumatisierten Patienten nicht häufiger an Infektionen, so dass die klinischen Implikationen dieser Beobachtungen noch zu klären bleiben.

4 Diskussion

4.1 Die Sepsis beim operativen Intensivpatienten als Motivation für die Suche nach neuen Biomarkern zur Differenzierung der Sepsis und des postoperativen SIRS

Die Sepsis stellte im Jahr 2017 mit einer Inzidenz von weltweit fast 49 Millionen Fällen und elf Millionen Toten eine globale Gesundheitsgefährdung dar[237]. Zwar sank dank intensiver Bemühungen die Mortalität der Sepsis vor allem in den westlichen Ländern, dennoch sind weiterhin Sterblichkeitsraten bis zu 35 % beschrieben[238]. Im Vergleich hierzu lag die Letalität in den 80er Jahren bei bis zu 80 %[4,239]. Ein besonderes Risikokollektiv stellen dabei operative Patienten dar. Zum einen sind sie auf Basis ihrer Grunderkrankung und der durchgeführten Operation immunkompromittiert und somit im Allgemeinen geschwächt, zum anderen maskieren die physiologischen Reaktionen auf den operativen Eingriff die Symptome einer Sepsis. Aus diesen Gründen ist die Mortalität bei operativen Intensivpatienten heutzutage weiterhin unverändert hoch. Während abdominalchirurgische Eingriffe in Europa mit einer Mortalität von 7,6 % assoziiert sind, weisen herzchirurgische Patienten mit 6,6 % eine leicht geringere 30-Tage-Letalität auf[240,241]. Allerdings steigt bei Vorliegen einer Sepsis die 30-Tage-Letalität bei herzchirurgischen Patienten um mehr als das 6-fache an[241]. Bei polytraumatisierten Patienten mit nachfolgender Sepsis ist darüber hinaus die Mortalität mit 19,5 % bis 23 % besonders hoch[191,242]. Vor diesem Hintergrund wurden seit der Jahrtausendwende eine Vielzahl an therapeutischen Optionen und Interventionen auf ihre Wirksamkeit im Rahmen der Sepsis evaluiert, ohne aber einen signifikanten Erfolg im Sinne einer Mortalitätsreduktion zu erreichen[243]. So formulierten Cohen et al. bereits 2015, dass der Rückgang der Sepsissterblichkeit nicht auf die mehr als einhundert therapeutischen Studien zurückzuführen ist, sondern auf eine höhere Vigilanz gegenüber der Sepsis und der Einführung standardisierter Behandlungspfade (sog. „*Sepsis-Bundles*“)[244]. Die Studien dieser kumulativen Habilitationsschrift konzentrieren sich daher nicht auf die Evaluation neuer Therapieoptionen, sondern auf die Früherkennung der Sepsis bei operativen Intensivpatienten.

Die erste Studie entstand aus einer klinischen Fragestellung, da im Gegensatz zur Evidenz für die möglichst frühzeitige empirische Antibiotikatherapie der optimale Zeitpunkt der chirurgischen Fokussanierung weniger genau definiert ist. Aktuell

empfiehlt die *Surviving Sepsis Campaign* eine Fokussanierung innerhalb von sechs bis zwölf Stunden nach Diagnosestellung einer Sepsis unter der Bedingung, dass die Operation medizinisch und logistisch umsetzbar ist (Empfehlungsgrad 1C)[1]. So entstand die Frage, ob eine frühzeitigere chirurgische Fokussanierung bei abdominalchirurgischen Patienten mit einem höheren Überleben assoziiert ist. Die retrospektive Analyse von 76 Patienten mit abdomineller Sepsis weist auf eine Reduktion der Mortalität hin, je kürzer die Dauer zur Intervention war (Anlage 1). Allerdings verfehlte die Studie auf Grund der limitierten Zahl inkludierter Patienten das statistische Signifikanzniveau. Dennoch wird dies durch die Ergebnisse anderer Studien gestützt. So konnten Gajic et al. einen signifikanten Mortalitätsanstieg von 41 auf 73 % durch eine zweitägige Verzögerung der operativen Versorgung eines akuten Abdomens bei internistischen Intensivpatienten nachweisen[179]. Neben der Notwendigkeit einer frühen chirurgischen Therapie unterstreicht dies die Bedeutung der interdisziplinären Bewertung von Intensivpatienten[179,245]. Zu ähnlichen Ergebnissen kamen auch weitere Analysen von Intensivpatienten[178,181,182]. Besondere Aufmerksamkeit galt der prospektiven Observationsstudie von Azuhata et al., da diese eine Verzögerung einer Operation im Rahmen eines septischen Schocks, auf Basis einer gastrointestinalen Hohlorganperforation, als unabhängigen Risikofaktor für eine Steigerung der 60-Tage-Letalität identifizierten[177]. Auf Grund ihrer Ergebnisse empfehlen die Autoren bei Vorliegen eines septischen Schocks eine operative Intervention innerhalb von sechs Stunden nach Diagnosestellung. Zwei Jahre später erschien eine dänische Kohortenstudie mit 2.803 inkludierten Patienten, welche überraschenderweise keinen Einfluss der Dauer bis zur chirurgischen Fokussanierung auf das Überleben der Patienten zeigen konnte[246]. Allerdings wies die Studie auch einige Limitationen auf. Zum einen enthielt sie auf Grund ihres Observationscharakters keine Informationen über das Vorliegen einer Sepsis sowie der präoperativ durchgeführten intensivmedizinischen Maßnahmen, welche maßgeblich das Überleben hätten beeinflussen können. Zum anderen blieb unklar, ob es sich bei allen Operationen um eine tatsächliche Notfallindikation handelte, da die Dauer bis zur chirurgischen Fokussanierung bis zu 48 Stunden betrug. Zudem wurden nicht ausschließlich septische Patienten mit Hohlorganperforation, sondern Patienten mit einem Ileus oder gastrointestinaler Blutung eingeschlossen. Zuletzt ist zudem festzustellen, dass die 90-Tage-Letalität bei einer Interventionsdauer von mehr als 17 Stunden auf 50 % stieg. Allerdings zeigte die Regressionsanalyse nach Adjustierung

der Einflussfaktoren keinen signifikanten Einfluss der chirurgischen Interventionsdauer auf die 90-Tage-Letalität. Entscheidend für die weitere Fragestellung dieser Habilitationsschrift waren die Ursachen der Zeitverzögerungen. So stellten North et al. in einer großen Querschnittsstudie ebenso eine Assoziation zwischen einer erhöhten Sterblichkeit und einer Verzögerung der operativen Intervention bei abdominalchirurgischen Patienten dar[183]. Darüber hinaus konzentrierte sich die Studie aber auf die Ursachen der Verzögerung und wiesen als Gründe die Dauer der diagnostischen Verfahren (41,7 %), Fehlinterpretation der erlangten Ergebnisse (21,6 %) sowie inkorrekte Anwendung der diagnostischen Verfahren (14,4 %) nach. Vor diesem Hintergrund stellte sich die Frage, wie die Diagnostik bei operativen Intensivpatienten optimiert werden kann, damit diese zügiger einer adäquaten Therapie zugeführt werden könnten.

Abgleitet von diesen Ergebnissen befasste sich die zweite Studie dieser Habilitationsschrift (Anlage 2) ebenso mit einer klinischen Fragestellung. Operative Patienten werden postoperativ häufig auf einer IMC-Station versorgt, da sie zwar einerseits keine klassische Intensivtherapie benötigen, andererseits aber überwachungspflichtig sind und einen höheren Pflegebedarf aufweisen[184,247–249]. Somit stellt die IMC-Station das Bindeglied zwischen der Normal- und Intensivstation dar. Es ist dabei erwiesen, dass alleine nur das Vorhandensein einer IMC-Station mit einer reduzierten Sterblichkeit von intensivmedizinischen Patienten assoziiert ist[250]. Darüber hinaus konnte eine weitere Studie nachweisen, dass selbst Patienten mit septischem Schock auf einer IMC-Station effizient behandelt werden können[251]. In diesem Zusammenhang ist aber wichtig festzuhalten, dass IMC-Stationen auf Grund fehlender Standardisierung eine heterogene Ausstattung und auch unterschiedliche Überwachungsmöglichkeiten aufweisen[184,185]. Da operative Patienten ein erhöhtes Risiko für die Entwicklung für infektiologische Komplikationen aufweisen, sollten sie konsequent mit Hilfe von standardisierten Sepsis-Scores evaluiert werden[1,19,252]. Allerdings kann bei anästhesiologischen IMC-Patienten nicht per se von einer intensivmedizinischen Versorgung gesprochen werden, so dass unklar ist, ob der (intensivmedizinische) SOFA-Score verwendet werden sollte. Alternativ bliebe der qSOFA-Score für Patienten außerhalb der Intensivstation[1,187]. Auf Basis dieser Fragestellung wurden 13.780 Patienten retrospektiv untersucht, welche entweder auf einer IMC- oder einer Intensivstation behandelt wurden. Die Studie konnte weder für den qSOFA noch für den SOFA-Score oder die SIRS-Kriterien eine ausreichende

Prädiktion für die Identifikation einer Sepsis (definiert als Beginn einer empirischen antibiotischen Therapie) nachweisen. Obwohl dies zunächst überraschend wirkte, waren diese Ergebnisse mit denen anderer Studien vergleichbar[11,187,253]. In der Validierungsstudie des qSOFA-Scores zeigte sich bereits mit Ausnahme des SOFA-Scores ein ähnliches Niveau für die Prädiktion von Infektionen[187]. Dies könnte damit begründet sein, dass der SOFA-Score ursprünglich zur Prädiktion der Mortalität und nicht von Infektionen entwickelt wurde. Die Empfehlung für die Nutzung des SOFA-Scores für diesen Zweck entstand im Rahmen eines Delphi-Verfahrens und war mit seiner weiten Verbreitung und suffizienten Beschreibung der Organdysfunktionen begründet[2]. Darüber hinaus wurde er auf Basis retrospektiver Daten erhoben und nicht mit prospektiven Daten validiert. Zuletzt können die physiologischen Alterationen der Vital- und Laborparameter nach einem großen chirurgischen Eingriff zu falsch positiven Ergebnissen führen, welche insbesondere die Spezifität der Scores senken. Zwar existieren keine vergleichbaren Studien, die speziell anästhesiologische IMC-Patienten untersuchen, dennoch weisen die Studienergebnisse von Kollektiven mit anderen zugrunde liegenden Ursachen für einen systemischen Inflammationszustand, wie z.B. (prä-)klinische Notfallpatienten oder auch traumatologische Patienten, eine ähnlich niedrige Prädiktion des qSOFA- und SOFA-Scores für die Identifikation septischer Patienten auf[11,19,253]. So konnte zum Beispiel die Studie von Krebs et al. an Hand von 1.942 prospektiven traumatologischen Intensivpatienten weder für den qSOFA- bzw. SOFA-Score noch für die SIRS-Kriterien eine ausreichende Vorhersagekraft für Infektionen nachweisen[19]. Dies könnte ebenso wie bei postoperativen Patienten mit den physiologischen Veränderungen der Vital- und Laborparameter erklärbar sein.

Während der qSOFA-Score sich für die Mortalitätsprädiktion bei IMC-Patienten eignete, konnte dies für den SOFA-Score bei Patienten nachgewiesen werden, welche sowohl auf der Intensiv- als auch IMC-Station behandelt wurden. Eine aktuelle Metanalyse von Lo et al. wies an einem allgemeinen Kollektiv von 380.920 kritisch kranken Patienten eine AUCROC von 0,68 in Bezug auf die Mortalitätsprädiktion des qSOFA-Scores nach, welches mit den Ergebnissen der Patienten vergleichbar war, die in unserer Studie sowohl auf der Intensiv- als auch IMC-Station behandelt wurden[254]. Auch eine weitere Metaanalyse von 229.480 Patienten bestätigte diese Ergebnisse, so dass die jetzigen Studienergebnisse plausibel erscheinen, auch wenn einige andere Studien eine höhere Prädiktion der Mortalität nachwiesen[255]. So wiesen

sowohl eine Studie an operativen und internistischen Patienten als auch eine weitere Untersuchung an herzchirurgischen Patienten eine AUCROC > 0,8 für die Prädiktion der Mortalität des qSOFA und des SOFA-Scores nach[12,256]. Allerdings war in der ersten genannten Studie von Kovach et al. die Analyse für das Versterben risikoadjustiert, so dass die prädiktive Qualität höher war[256]. In der zweiten Studie wurden dagegen ausschließlich herzchirurgische Patienten inkludiert, was eine Vergleichbarkeit mit einem gemischt chirurgischen Kollektiv erschwert, da es durch den Einsatz der Herz-Lungen-Maschine zu einer systemischen Kontaktaktivierung des Immun- und Gerinnungsystems kommt[12]. Im Gegensatz dazu ist das chirurgische Trauma bei nicht-herzchirurgischen Eingriffen regional begrenzt. Obwohl der SOFA-Score für die Prädiktion der Mortalität ausreichend validiert ist, zeigten neben unserer Studie auch andere Studien eine teils geringe Vorhersagekraft bezüglich der Mortalität[257–259]. Dies könnte mit unterschiedlichen Definitionen der Studienkollektive und der Sepsis bzw. schweren Infektionen erklärbar sein. In unserer Studie wurde der Beginn einer empirischen Antibiose als Nachweis eines Infektionsverdachts definiert. Diese Definition weist allerdings auch Schwächen auf, da sie auf Grund der unterschiedlichen Verschreibungspraktiken nicht universell einsetzbar ist. Des Weiteren reduzieren die operationsbedingten Alterationen der Vital- und Laborparameter die Spezifität der untersuchten Scores zur Mortalitätsprädiktion.

Zusammenfassend erscheinen auch die etablierten Scores nur eingeschränkt nützlich für die Identifikation von schweren Infektionen bei operativen Intensivpatienten. Dies könnte bei Patienten mit intraabdomineller Sepsis zur verzögerten Einleitung der operativen Therapie führen, welche sich negativ auf das Überleben der Patienten auswirkt. Diese Zusammenhänge weisen auf die Notwendigkeit für eine frühzeitige Identifikation der Sepsis bei operativen Patienten hin, weshalb in den folgenden Studien Biomarker für die Diskrimination von systemischen Inflammationszuständen und der Sepsis bei operativen Intensivpatienten untersucht werden.

4.2 Identifikation neuer Biomarker zur Differenzierung zwischen septischen Patienten und Patienten mit postoperativem SIRS

Durch diese eingeschränkte Verwertbarkeit klinischer Scores zur Prädiktion der Sepsis bei operativen Intensivpatienten rückte die Untersuchung unterschiedlicher Biomarker zur Identifikation der Sepsis in den Mittelpunkt unserer weiteren Untersuchung. Zu diesem Zweck wurde Presepsin mittels eines *Point-of-Care*-Messinstruments bei polytraumatisierten Patienten evaluiert (Anlage 3). Es zeigte sich, dass die Presepsin-Konzentration im Gegensatz zu CRP, Procalcitonin und IL-6 über sieben Tage nicht von einem Polytrauma-induzierten SIRS beeinflusst wurde und sich somit potentiell für die Differenzierung zwischen SIRS und Sepsis eignen könnte. Zum Zeitpunkt der Studie waren diese Ergebnisse nur für die ersten 24 Stunden nach einem Trauma bekannt[192]. Darüber hinaus war eine Diskrimination zwischen dem Vorliegen bzw. Fehlen eines SIRS möglich. Da für Presepsin eine hohe Sensitivität und Spezifität für die Detektion einer Sepsis beschrieben wurde, zeigten sich diese Ergebnisse hinsichtlich der Überwachung kritisch kranker Patienten vielversprechend. So konnten Vodnik et al. mit Hilfe des Presepins septische Patienten von abdominalchirurgischen Patienten mit postoperativem SIRS diskriminieren[260]. Auch eine Meta-Analyse von elf Studien wies eine hohe Sensitivität und Spezifität für die Identifikation septischer Patienten nach[137]. Allerdings waren in dieser Metaanalyse die Grenzwerte der Presepsin-Konzentrationen niedriger als in der vorliegenden Studie, so dass polytraumatisierte Patienten falsch positiv für das Vorliegen einer Sepsis gewertet werden könnten. Ein potentieller Lösungsansatz könnte somit die Bewertung des Verlaufs des Presepsin und nicht die Analyse einzelner Werte. Des Weiteren muss der prädiktive Nutzen von Presepsin zur Identifikation einer Sepsis mit abdominellem Fokus bei polytraumatisierten Patienten weiter evaluiert werden, da in der vorliegenden Studie kein Unterschied zwischen den Presepsin-Konzentrationen bei Patienten mit und ohne Abdominaltrauma nachgewiesen werden konnte. Dies wäre allerdings von hohem Nutzen, da die klinischen Symptome einer intraabdominellen Sepsis durch die Verletzungen maskiert werden können und polytraumatisierte Patienten mit Abdominaltrauma ein erhöhtes Risiko für die Entwicklung einer abdominellen Sepsis aufweisen[261]. Hier wäre die Inklusion von Presepsin in einem Multipanel-Ansatz zur Identifikation von septischen Patienten denkbar. Zusammenfassend könnte Presepsin ein potentieller Biomarker für die Detektion septischer Patienten nach Polytraumatisierung darstellen. Für die weitere Evaluation

müssen allerdings insbesondere polytraumatisierte Patienten mit Abdominaltrauma evaluiert werden.

Die nächste Studie evaluierte das Protein DLL-1 als diskriminierenden Sepsis-Biomarker (Anlage 4). Zum Zeitpunkt der Studiendurchführung war der Nutzen von DLL-1 nur zur Risikostratefizierung kardiovaskulärer und maligner Erkrankungen untersucht worden[197,198]. Die Entdeckung der monozytären Sekretion von DLL-1 führte zu der Idee, es auch hinsichtlich der Identifikation von septischen Patienten zu evaluieren. Seine physiologische Funktion im Rahmen des *Notch*-Signalwegs zur Aktivierung der Monozytenmaturation führte zur Annahme, dass es nur durch bakterielle DAMPs induziert wird und somit bakterielle Infektionen spezifisch anzeigt[196]. Im Rahmen der Studie konnte nachgewiesen werden, dass DLL-1 bei septischen Patienten in hohen Konzentrationen im Blut vorliegt, während es bei polytraumatisierten und abdominalchirurgischen Patienten mit SIRS nicht signifikant anstieg. Darüber hinaus wies DLL-1 im Vergleich zu Procalcitonin eine höhere Prädiktion für die Detektion einer Sepsis auf. Der herausragende Unterschied zwischen DLL-1 und den etablierten Biomarkern wie Procalcitonin oder CRP stellte die persistente Erhöhung über sieben Tage dar. Es ist dabei noch zu klären, ob dies durch eine lange Halbwertszeit oder eine stetige Produktion des DLL-1 im Rahmen einer Sepsis verursacht wird. Die persistierende Erhöhung könnte einerseits bei der Diagnose einer Sepsis bei postoperativen Patienten helfen, andererseits ist bei septischen Patienten mittels DLL-1 auf Grund der fehlenden Normalisierung (über den Beobachtungszeitraum von sieben Tage) keine Verlaufsbeurteilung des Therapieerfolgs möglich. In der Zusammenschau konnte DLL-1 als potentieller Biomarker für die Diskrimination einer Sepsis bei operativen Intensivpatienten mit SIRS identifiziert werden. Um mögliche Einflussfaktoren zu identifizieren, sollten zukünftige Validierungsstudien den Zusammenhang zwischen der Konzentration von DLL-1 und dem Vorliegen einer akuten Niereninsuffizienz bei septischen Patienten untersuchen, da eine Korrelation mit dem Kreatininplasmaspiegel beschrieben wurde[198].

Nachdem bereits zwei potentielle Biomarker identifiziert wurden, wurde im Rahmen der nächsten beiden Studien ein erweiterter Ansatz verwendet. Einerseits sollte von der Ebene der Proteine auf die der Nukleinsäuren gewechselt werden, andererseits sollte die Assoziation des Biomarkers mit einem MODS beurteilt werden. In diesem Zusammenhang erschien vor dem Hintergrund der Immunhämostase die

Analyse der septischen Koagulopathie sinnvoll. Die zu Grunde liegende Überlegung war dabei, dass eine Sepsis im Gegensatz zum postoperativen SIRS schwerere Alterationen des Gerinnungssystems verursacht. Somit war anzunehmen, dass Sepsis-bedingte Veränderungen des Immun- und Gerinnnungsystems messbar sind und zur Diskrimination zwischen Sepsis und SIRS nutzbar sein könnten. Daher war es Ziel, das Zusammenspiel zwischen der *in-vivo*-Gerinnung und den Nukleinsäuren bzw. in der Folgestudie den NETs darzustellen. In der ersten Studie wurden die Menge an zellfreier DNA für β-Globin sowie extrazellulärer RNA für β-Aktin bei septischen und abdominalchirurgischen Patienten quantifiziert (Anlage 5). Beide Nukleinsäuren codieren dabei für ubiquitär vorkommende Proteine, so dass sie einen allgemeinen Zellschaden anzeigen. Die Studie konnte sowohl für die Menge an DNA als auch an RNA signifikant höhere Konzentrationen bei septischen im Vergleich zu abdominalchirurgischen Patienten nachweisen. Allerdings war der signifikante Unterschied nur über die ersten 24 Stunden nachweisbar. So wäre grundsätzliche eine Diskrimination zwischen SIRS und Sepsis denkbar, allerdings tritt eine postoperative Sepsis bei elektiven operativen Eingriffen nur selten innerhalb der ersten 24 Stunden auf. Dennoch entspricht dies den Ergebnissen anderer Studien, welche eine Sepsis-induzierte Erhöhung von zellfreier DNA nachwiesen[68,69,141,204]. Im Gegensatz zu den eigenen Ergebnissen konnten Garnacho et al. allerdings keinen signifikanten Unterschied zwischen der Menge an zellfreier DNA bei septischen und SIRS-Patienten nachweisen[141]. Eine potentielle Ursache für diese Diskrepanz könnten Unterschiede im Patientenkollektiv darstellen. Während in der eigenen Studie alle Patienten einen abdominellen Fokus aufwiesen, war dies nur bei 45 % der Patienten in Garnacho et al.´s Studie der Fall. Da auch die Menge an RNA bei septischen Patienten gegenüber postoperativen Patienten mit SIRS signifikant erhöht war, stellt sich die Frage nach der Herkunft der Nukleinsäuren. Entspräche alleine der Zelluntergang dem Ursprung der frei zirkulierenden Nukleinsäuren, wäre auch bei großen chirurgischen Traumata ein Anstieg zu erwarten. Insofern erschien die Hypothese, dass es sich dabei um Surrogate für eine Sepsis-induzierte NETose handelt, plausibel. Dagegen sprach allerdings eine Studie von Hamaguchi et al., die nur einen geringen Anteil der NETose-assoziierten Nukleinsäuren bei septischen Mäusen nachweisen konnten[79]. Ob diese Beobachtung auf die humane Sepsis übertragen werden kann, ist weiterhin Bestandteil der wissenschaftlichen Diskussion. Für diese Hypothese spricht die messbare Assoziation zwischen der Konzentration der Nukleinsäuren und der *in-vivo*-

Gerinnungsfunktion der septischen Patienten. Dabei stellten sich interessanterweise, abhängig von der Art der Nukleinsäuren, unterschiedliche Effekte auf die Gerinnung dar: Auf Grund der Korrelation zwischen der Menge an zellfreier DNA und des gesteigerten Lyseindexes in fast allen thrombelastographischen Reagenzien (mit Ausnahme des EXTEM) könnte die Hemmung der Fibrinolyse eine potentielle Erklärung für die prokoagulatorischen Effekte sein. Dies wäre kongruent mit den Ergebnissen von Gould et al., die eine DNA-induzierte Inaktivierung von Plasmin mit konsekutiver Degradierungsstörung des Fibrins nachwiesen[205]. Im Gegensatz zu diesen prokoagulatorischen Effekten war die Menge an zellfreier DNA bei septischen Patienten auch mit einer Verlängerung der CT und somit einer verzögerten Initiierung der Gerinnung assoziiert. Diese Ergebnisse wurden in einer Studie von Collins et al. bestätigt, so dass anzunehmen ist, dass zellfreie DNA komplexe Reaktionen im Gerinnungssystem verursacht[262]. Die vorliegende Studie weist dies bezüglich darauf hin, dass die ihnen zugeschriebene prokoagulatorische Wirkung auf einer Hemmung der Fibrinolyse basieren könnte[67]. Auf Grund der fehlenden Assoziation zwischen der Menge an zellfreier DNA und der Gerinnselfestigkeit (definiert als MCF) scheinen die Fibrinogen- und Thrombozytenwirkung in diesem Zusammenhang eine untergeordnete Rolle zu spielen. Dagegen zeigte sich aber ein starker Zusammenhang zwischen der MCF und der Menge an extrazellulärer RNA im NATEM als Surrogat der nativen Gerinnung. Darüber hinaus war die CT kürzer je höher die Menge an extrazellulärer RNA war, was in der Gesamtzusammenschau auf eine prokoagulatorische Wirkung, ausgehend von der extrazellulären RNA, hinweisen könnte. Da diese sowohl im intrinsischen Gerinnungsweg als Cofaktor des Gerinnungsfaktor XII, als auch im extrinsischen Gerinnungsweg als Cofaktor des Gerinnungsfaktor VII agiert, könnte dies die prokoagulatorische Funktion erklären[67,263,264]. Bei der Interpretation von Gerinnungsanalysen bei operativen Intensivpatienten muss allerdings immer die Wirkung von Heparin berücksichtigt werden. Zwar wurde mittels HEPTEM-Analysen die Wirkung von Heparin grundsätzlich ausgeschlossen, allerdings bedeutet dies nicht, dass Nukleinsäuren die Heparinwirkung stören. So ist es bekannt, dass Histone auf Grund ihrer negativen Ladung die Wirkung von Heparin neutralisieren können[265]. Eine weitere Limitation der Studie war die geringe Fallzahl, welche auf Grund des Pilotcharakters der Studie gewählt wurde. So konnte zwar ein Zusammenhang zwischen der Höhe des Serum-Kreatinins, als Surrogat für ein akutes Nierenversagen, und der Menge an zellfreier

DNA dargestellt werden, doch konnte kein Zusammenhang zur Leberfunktion, dem SOFA-Score und der Mortalität identifiziert werden. Da anzunehmen ist, dass die Nukleinsäuren auch im Rahmen der NETose freigesetzt werden, konzentriert sich die folgende Studie auf den Einfluss der NETs im Rahmen der Sepsis und des postoperativen SIRS.

Um die Folgestudie (Anlage 6) zu initiieren, stellte sich zunächst die Frage, mit welcher Methode die NETs quantifiziert werden sollten. Unbestritten stellt die Fluoreszenzmikroskopie mit NETs-spezifischen Farbstoffen (z.B. MPO und zellfreie DNA oder Histone) hierbei den Goldstandard dar[149,266]. Allerdings weist sie auch einige Limitationen auf: Zunächst ist sie technisch aufwendig und erfordert ein hohes Maß an Expertise und Erfahrung in der Probenaufbereitung sowie auch in der späteren Analytik. Des Weiteren können nur Ausschnitte einer Probe mittels der Fluoreszenzmikroskopie untersucht werden. Größere Probenmengen gehen unweigerlich mit einem hohen Zeitaufwand einher und sind daher teilweise nicht umsetzbar. Darüber hinaus erfordert die Mikroskopie eine Probenfixierung. Dies ist insbesondere für Blutproben relevant, da NETs zu Aggregatbildung neigen und somit evtl. zu falsch-negativen Befunden in der Analytik von Blutproben führen könnten. Zuletzt ist die Fluoreszenzmikroskopie in einem hohen Maß untersucherabhängig, da z.B. entschieden werden muss, ob die angefärbten Nukleinsäuren bzw. Histone noch intra- oder bereits extrazellulär vorliegen[149,266]. Dies erlaubte häufig einen Interpretationsspielraum. Als Alternative zur Fluoreszenzmikroskopie stehen PCR- oder ELISA-basierte Messungen von NETs-Surrogaten wie beispielsweise zellfreie Nukleinsäuren, Histone oder neutrophile Proteinkomplexe (z.B. Komplexe aus MPO, Neutrophile Elastase und DNA). Diese Methoden haben sich insbesondere in der klinischen Forschung etabliert, da sie objektivierbar sind und eine absolute Quantifizierung erlauben[149,266]. Allerdings stellt die Standardisierung der ELISA und somit die Vergleichbarkeit mit anderen Studien ein häufiges praktisches Problem dar. Darüber hinaus handelt es sich um Surrogatparameter, so dass keine definitive Aussage über das Ausmaß der NETose getroffen werden kann. Im Jahr 2015 wurde mit der Durchflusszytometrie eine weitere Alternative für die Quantifizierung von NETs eingeführt. Gavillet et al. konnten nachweisen, dass die Färbung der Neutrophilen mit citrullinierten Histonen und MPO in der Fluoreszenzmikroskopie NETs-exprimierenden neutrophilen Granulozyten entsprechen[267]. So konnten sie sowohl nach Phorbolmyristatacetat (PMA)-Stimulation in Mäusen als auch bei septischen Patienten

einen Anstieg der NETs beobachten. Allerdings entstand auch Kritik an der Methodik, da mit ihr die unterschiedlichen Stadien der NETose nicht differenziert werden können[268,269]. So können beispielsweise fast vollständig degenerierte Neutrophile auf Grund ihrer Größe nicht mehr vom *Forwardscatter* erfasst werden. Nichtsdestotrotz erscheinen die Vorteile plausibel, da hohe Zellmengen in kurzer Zeit gemessen werden können und die Quantifizierung objektivierbar ist. Somit könnte diese sich zukünftig auch potentiell für die klinische Routinediagnostik eignen. Die Methodik der vorliegenden Studie stellt eine Adaption der Studienmethodik von Lee et al. dar, welche ebenso eine Durchflusszytometrie auf Basis einer Histon- und MPO-Färbung nutzen, allerdings ohne Fixierung der Zellen[148]. Das Studienziel, diese durchflusszytometrische Methode zur Quantifizierung der NETs für die translationale Forschung in der Intensivmedizin zu nutzen, konnte durch die erfolgreiche Etablierung sowie Validierung mit der Fluoreszenzmikroskopie erreicht werden.

Darüber hinaus konnte ein signifikanter Anstieg der NETs bei septischen Patienten gegenüber den gematchten Kontrollprobanden nachgewiesen werden. Da der kardiopulmonale Bypass auf Grund seiner großen Kontaktfläche ein besonders hohes Risiko für die Entwicklung eines SIRS darstellt, wurden im Gegensatz zur Vorstudie auch herzchirurgische Patienten inkludiert. Es zeigte sich aber, dass es bzgl. der Menge an NETs keinen relevanten Unterschied zwischen den beiden operativen Gruppen und den septischen Patienten gab, so dass anhand der NETs nur zwischen den Kontrollprobanden und dem Vorliegen einer systemischen Inflammation unterschieden werden konnte. Die mangelnde Differenzierung zwischen den verschiedenen Genesen einer systemischen Inflammationsreaktion könnte den Nutzen der NETs als Biomarker einschränken, da sie hinsichtlich operativer Intensivpatienten dieselben Limitationen aufweisen wie die etablierten Biomarker. Ursächlich hierfür könnten neben einer SIRS-induzierten NETose auch die Grunderkrankungen der Patienten sein, da sowohl die Arteriosklerose bei den herzchirurgischen- als auch die malignen Grunderkrankungen des Gastrointestinaltrakts bei den abdominalchirurgischen Patienten eine NETose induzieren[270,271]. Dies spiegelt sich auch in den präoperativen Konzentrationen der NETs wider, da diese im Vergleich zu den Kontrollprobanden bereits erhöht waren. Als Konsequenz könnten diese Grunderkrankungen einen größeren Unterschied der NETs-Messungen gegenüber den septischen Patienten maskieren. Ein grundsätzliches Problem der Quantifizierung der NETs stellt ihre Messung aus

peripheren Blutproben dar, weil NETs zunächst in ihrer Funktion als Fangnetz für Pathogene am Ort der Infektion verbleiben, um die Infektion örtlich zu begrenzen[74,203,272]. Zwar wurde mit verschiedenen Surrogaten nachgewiesen, dass im Rahmen einer Sepsis mehr NETs im peripheren Blut nachweisbar sind, aber es ist nicht auszuschließen, dass im Bereich des Infektionsfokus wesentlich höhere Konzentrationen an NETs messbar sind[148,267,273–276]. Ein anderer Ansatz fokussiert sich auf die Funktionalität der Neutrophilen und misst deren Fähigkeit zur PMA-induzierten NETose. Mit dieser Methodik konnten Abram et al. eine signifikant höhere NETose-Aktivität bei Neutrophilen nachweisen, welche aus dem Blut septischer Patienten gewonnen wurden[147]. Als Referenzkollektiv dienten kritische kranke Erwachsene, so dass kein definitiver Rückschluss zum postoperativen SIRS aus der Studie gewonnen werden kann, dennoch erscheint dieser Ansatz aber vielversprechend.

Basierend auf dem Konzept der Immunhämostase war die Überlegung entstanden, dass NETs auch eine Koagulopathie anzeigen könnten. Zwar gelang dieser Nachweis, allerdings zeigte sich analog zu den Studienergebnissen der Nukleinsäuren, ein komplexes Bild. Während septische Patienten in Einklang mit anderen Studien sowie den Erkenntnissen der Grundlagenforschung eine Assoziation zu einem prokoagulatorischen Status zeigten, ließ sich bei postoperativen Patienten das Gegenteil darstellen[80,139,277,278]. Da sich die bisherigen Studien auf septische Patienten konzentrierten, existierten keine vergleichbaren Daten. Auf Grund der starken Kontaktaktivierung durch den kardiopulmonalen Bypass muss analog zur vorherigen Studie (Anlage 5) die Interpretation der Blutgerinnung bei herzchirurgischen Eingriffen trotz des Nachweises einer vollständigen Heparin-Antagonisierung kritisch hinterfragt werden. Allerdings ließ sich die Assoziation zwischen NETs und einer Gerinnungsinhibierung auch bei abdominalchirurgischen Patienten nachweisen, welche weder Heparin noch Antifibrinolytika (z. B. Tranexamsäure) erhielten oder einen kardiopulmonalen Bypass benötigten. Auf Grund ihrer negativen Ladung weisen Histone und Nukleinsäuren einen größeren Effekt auf die Gerinnungsaktivierung auf als die NETs und könnten daher die Unterschiede zur Vorgängerstudie (Anlage 5) erklären[80,139,209]. Eine weitere mögliche Erklärung für dieses Phänomen könnte die Neutralisation der negativen Ladung durch die Komplexbildung von Histonen und Nukleinsäuren innerhalb der NETs darstellen[90]. So könnte die Abwesenheit von Bakterien, welche eine direkte

gerinnungsstimulierende Wirkung aufweisen, eine NETs-assoziierte Gerinnungsinhibierung im Rahmen des postoperativen SIRS erklären[90,202,279].

Zusammenfassend konnte diese Studie die durchflusszytometrische Quantifizierung weiterentwickeln und nachweisen, dass NETs sich nicht zur Diskriminierung einer Sepsis von einem postoperativen SIRS eignen könnten. Septische Patienten zeigten einen fibrinabhängigen, prokoagulatorischen Einfluss der NETs, während diese im Rahmen des postoperativen SIRS mit einer Inhibierung der Gerinnung assoziiert waren. Aus dieser Studie ließen sich mehrere weitere Fragestellungen ableiten. Zum einen müssen die Limitationen der Durchflusszytometrie, wie zum Beispiel die der mangelnden Identifikation später NETose-Stadien, adressiert werden. Des Weiteren stellt sich die Frage nach der Kausalität der Gerinnungsinhibierung im Kollektiv der postoperativen Patienten. Und zuletzt blieb der Einfluss der mtDNA als einer der Promotoren der NETose unklar, welches mit der folgenden Sekundäranalyse beantwortet werden sollte[78].

Als Zielparameter dieser Sekundäranalyse (Anlage 7) wurde die Konzentration an mtDNA gemessen, welche für die NADH-Dehydrogenase 1 codiert. Während es bekannt ist, dass mtDNA bei septischen, operativen, kritisch kranken und polytraumatisierten Patienten in erhöhten Konzentrationen nachgewiesen werden kann, blieb unklar, ob anhand der Konzentration der mtDNA zwischen einer Sepsis und einem postoperativen SIRS unterschieden werden kann[23,44,47,84,145,212,214,280,281]. Des Weiteren war es Ziel der Sekundäranalyse, einen Zusammenhang zwischen der Konzentration der mtDNA und den NETs sowie der Gerinnungsfunktion zu identifizieren. Entsprechend der Untersuchung der NETs, zeigte sich, dass die Konzentration bei septischen Patienten über drei Tage erhöht blieb. Während sich innerhalb der ersten 24 Stunden kein signifikanter Unterschied zwischen den abdominalchirurgischen und septischen Patienten nachweisen ließ, zeigte sich bei herzchirurgischen Patienten ein signifikanter Abfall nach Beendigung des kardiopulmonalen Bypasses. Dies könnte auf einen diskriminativen Effekt hindeuten, allerdings stehen diese Ergebnisse in Kontrast zu denen anderer Studien an herzchirurgischen Patienten[84,211,214]. Ein direkter Vergleich der Studien ist schwierig, da keine Details zur Technik des kardiopulmonalen Bypasses vorliegen und nur die Studien von Qin et al. auch NADH-Dehydrogenase 1 als Ziel-DNA genutzt haben[214]. Es ist allerdings auch möglich, dass ein Dilutionseffekt durch die Herz-Lungen-Maschine diese Beobachtung verursachen könnte. In der Analyse der septischen

Kohorte gegenüber den kumulierten postoperativen Zeitpunkten, zeigte sich, dass nur septische Patienten eine signifikante Erhöhung der mtDNA aufwiesen. Dieses Studienergebnis weist darauf hin, dass sich die mtDNA für die Diskrimination der Sepsis bei operativen Intensivpatienten eignen könnte. Da allerdings die Streubreite der Daten hoch war und sich teils Überlappungen zwischen der Konzentration an septischen und operativen Patienten darstellten, könnte die Definition eines Grenzwertes schwierig sein. Aus diesem Grund könnte der Einsatz der mtDNA im Rahmen eines Multipanel-Ansatzes sinnvoll sein.

Ein weiteres Ziel der Sekundäranalyse war es, die Rolle der mtDNA im Rahmen der *in-vivo*-NETose zu identifizieren. Hierfür wurde ein Verhältnis aus der Konzentration der mtDNA und den NETs gebildet, welches mit der Menge an NETs korrelierte. Dies unterstützt den *in-vitro*-Nachweis einer mtDNA-induzierten NETose durch Yousefi et al.[78]. Allerdings war mit Ausnahme der Fibrinogen-abhängigen Reagenzien bei herzchirurgischen Patienten kein Zusammenhang zwischen der Konzentration an mtDNA und der Gerinnung festzustellen. Dies lässt zwei Hypothesen zu: Erstens, die Konzentration an mtDNA ist nicht der Promotor der NETs-assoziierten Gerinnungsaktivierung bei septischen Patienten und zweitens, die Gerinnungsaktivierung im Rahmen von herzchirurgischen Eingriffen könnte auf die thrombozytäre Freisetzung von mtDNA zurückzuführen sein[211]. Dies würde die Hypothese von Qin et al. stützen, die bei herzchirurgischen Patienten eine Assoziation zwischen der Konzentration an mtDNA und der Menge an aktivierten Thrombozyten nachwiesen[211]. Bhagirath et al. untersuchten dies bezüglich die zugrunde liegenden Mechanismen der mtDNA-induzierten Thrombozytenaktivierung und zeigten, dass der thrombozytäre $\alpha_{IIb}ß_3$-Rezeptor von nukleärer, mitochondrialer und bakterieller DNA in ähnlichem Ausmaß aktiviert wird[67]. Da diese aktivierenden Effekte sowohl durch DNAse als auch durch TLR-Rezeptormodulatoren antagonisierbar waren, könnte die DNA-induzierte Stimulation über TLR der Thrombozyten vermittelt werden.

Zusammenfassend zeigte diese Sekundäranalyse, dass mit Hilfe der Konzentration an mtDNA im Gegensatz zu den NETs eine Sepsis von einem postoperativen SIRS differenziert werden kann. Allerdings sind weitere Studien mit höheren Fallzahlen für die Validierung von Grenzwerten notwendig.

4.3 Identifikation von immunologischen Langezeitveränderungen nach systemischen Inflammationsreaktionen zur Identifikation potentieller Risikopatienten

Im letzten Abschnitt wurde die Frage untersucht, in wie weit sich die Rezeptoreigenschaften von Monozyten und T-Lymphozyten sowie deren Sekretionskapazität von Zytokinen im Langzeitverlauf nach einer Sepsis oder einem Polytrauma ändern. Ziel ist es, Risikopatienten für eine Immundysfunktion zu identifizieren, da beide Patientenkollektive mit einer höheren Langzeitmorbidität und -mortalität assoziiert sind[113,155,172,226,282–284].

Im Rahmen der ersten Studie zu diesem Thema wurden Patienten, welche einen septischen Schock langfristig überlebten, nachfolgend untersucht (Anlage 8). Diese Patienten gaben im Vergleich zu gematchten Kontrollprobanden eine höhere Inzidenz an bakteriellen Infektionen, die eine antibiotische Therapie notwendig machten, sowie an viralen und fungalen Erkrankungen an. Insbesondere Letztere stellen klassische opportunistische Infektionserkrankungen von immunsupprimierten Patienten dar[285]. Bemerkenswert war zudem, dass die etablierten Infektionsparameter bei den postseptischen Patienten geringgradig erhöht waren, so dass hieraus bereits eine chronische Inflammation ableitbar war. Interessanterweise zeigte sich eine Normalisierung der CD^{4+} und CD^{8+}-positiven T-Helferzellen, obwohl diese bereits in der Akut- und Intermediärphase deutlich herabreguliert werden[286]. Allerdings zeigte die weitere Analyse, dass die vermeintliche Normalisierung sich nicht auf Rezeptorebene verfolgen ließ. Während sich die Rezeptorexpression auf den T-Lymphozyten nicht eindeutig einem immunsupprimierenden Status zuordnen ließ, gelang dies aber bei den Monozyten. So war die Menge an T-lymphozytären PD-1-Rezeptoren reduziert, während die Rezeptorendichte der BTLA-Rezeptoren hochreguliert war. Beide Rezeptoren entsprechen wichtigen supprimierenden Modulatoren der T-Lymphozyten-Funktion, so dass an Hand dieser Studie kein einheitlicher Rückschluss auf die Immunfunktion gezogen werden kann[227,228]. Dagegen weist die Suppression der monozytären TLR-5-Rezeptoren auf eine Schwächung des Immunsystems hin, da dieser Rezeptor für die Erkennung bakterieller Flagelline von hoher Bedeutung ist. Dass die Rezeptorexpression des HLA-DR sich nicht zwischen postseptischen und gesunden Probanden unterschied, erscheint plausibel, da septische Patienten, welche eine hohe HLA-DR-Expression aufweisen, eine deutlich erhöhte Mortalität zeigen. Daher ist anzunehmen, dass diese

vor der Rekrutierung verstorben sein könnten[287–291]. Da die Rezeptoreigenschaften nicht alleinig zu einer Immunsuppression führen, wurde im nächsten Schritt die Funktionalität der Mono- und Lymphozyten untersucht. Hier zeigte sich, dass die für die Akutphase bekannte Hemmung der α-CD3/28-induzierten Sekretion von IFN-γ auch im Langzeitverlauf einer Sepsis messbar war[228]. Dagegen konnte im Langzeitverlauf, im Vergleich zur septischen Akutreaktion, keine erhöhte Sekretionskapazität von IL-6, IL-10 und TNF-α nach α-CD3/28-Stimulation nachgewiesen werden, so dass von einer Normalisierung ausgegangen werden konnte[228]. Dieselbe Arbeitsgruppe konnte in einer weiteren Studie eine erhöhte Mortalität bei jenen Patienten nachweisen, welche eine reduzierte Zytokinsekretion nach LPS und α-CD3/28-Stimulation aufwiesen, welches die Ergebnisse der jetzigen Studie (Anlage 8) unterstützt[103].

Zusammenfassend weist diese Studie auf eine langanhaltende Immunsuppression nach überlebter Sepsis hin. Allerdings sollten auf Grund der geringen Fallzahl weitere Studien mit dem Fokus auf die verschiedene Ätiologien der Sepsis und anderer Ursachen für systemische Inflammationen folgen. Da sich die Vorarbeiten auf die Differenzierung der Sepsis von einem SIRS konzentrierten, erschien es plausibel auch potentielle Unterschiede im Langzeitverlauf zu untersuchen.

Analog zur Sepsis existieren zur Charakterisierung der Rezeptoreigenschaften von Lymphozyten und Monozyten sowie der Sekretionskapazität bei polytraumatisierten Patienten überwiegend Studien zur Akut- und Intermediärphase, allerdings nicht zum Langzeitverlauf[110,152,232,292,293]. Da polytraumatisierte Patienten regelhaft ein SIRS entwickeln, wurden sie als Zielkollektiv für diese Folgestudie ausgewählt (Anlage 9). Zunächst konnte festgestellt werden, dass anders als in der Vorstudie an septischen Patienten, die ehemals polytraumatisierten Patienten keine höhere Inzidenz an Infektionserkrankungen aufwiesen. Auf Rezeptorebene ließen sich dagegen deutliche Veränderungen gegenüber den Kontrollprobanden feststellen. Während die lymphozytären PD-1-Rezeptoren hochreguliert waren, waren die BTLA-Rezeptoren supprimiert. Beide Rezeptortypen entsprechen negativen T-Zellregulatoren, so dass analog zur Vorstudie eine Interpretation im Sinne einer Immunsuppression nicht möglich ist[235]. Im Gegensatz zur Sepsis ist die Assoziation zwischen einer vermehrten PD-1-Rezeptorexpression und einer erhöhten Mortalität bei polytraumatisierten Patienten widersprüchlich[233,234]. Dagegen war die

Interpretation der monozytären Oberflächenrezeptoren eindeutiger, da alle TLR eine reduzierte Expression zeigten. Dies ist zwar ein bekanntes Phänomen aus der Akutphase nach einem Polytrauma, aber bisher für den Langzeitverlauf nicht beschrieben. Da TLR eine wesentliche Rolle in der Identifikation von DAMPs und PAMPs spielen und in der Folge die intrazellulären inflammatorischen Signalkaskaden aktivieren, könnte dies auf eine Immunsuppression hinweisen[115,236]. Dass diese nicht klinisch nachweisbar war, könnte zum einen mit der geringen Fallzahl begründet sein, so dass Folgestudien mit höheren Patientenzahlen notwendig sind. Zweitens konnten in der Studie keine Gruppenunterschiede bzgl. der Expression des HLA-DR dargestellt werden. Zuletzt konnten zwar Trends bezüglich einer Reduktion der LPS-induzierten Sekretion von IL-6 und TNF-α bei den ehemals polytraumatisierten Patienten nachgewiesen werden, allerdings verfehlten diese auf Grund der geringen Fallzahl das statistische Signifikanzniveau. Die Zytokinsekretion nach Stimulation mit α-CD3/28 und Zymosan zeigte sich zudem ebenfalls unauffällig, so dass dies zur klinisch suffizienten Immunkompetenz der ehemals polytraumatisierten Patienten beigetragen haben könnte. In weiteren Studien sollte daher insbesondere der Effekt der reduzierten LPS-induzierten TNF-α-Sekretion untersucht werden, da diese in Kombination mit der Reduktion der monozytären TLR-2 und -4-Rezeptorexpression auf eine Schwächung der Immunantwort bei bakteriellen Infektionen hinweisen könnte.

Zusammenfassend zeigt diese Studie, dass sich auch im Langzeitverlauf nach einer Polytraumatisierung Veränderungen der lympho- und monozytären Rezeptorexpression nachweisen lassen. Da sich die Inzidenz von klinisch apparenten Infektionen nicht gegenüber gesunden Probanden unterschied, sollten zukünftige Studien neben den oben genannten Fragestellungen auch die Rolle von Infektionen im Rahmen der erhöhten Langzeitmorbidität und -mortalität infolge eines Polytraumas untersuchen.

4.4 Bedeutung der Forschungsergebnisse für die klinische Versorgung septischer Patienten in der operativen Intensivmedizin

Im ersten Abschnitt dieser kumulativen Habilitationsschrift wurden zwei klinische Fragestellungen bearbeitet. Zunächst stellte sich die Frage, ob die Dauer bis zur chirurgischen Intervention einen Einfluss auf die Mortalität von septischen Patienten mit intraabdominellem Fokus hat. Zwar konnte eine Reduktion der Mortalität dargestellt werden, ohne aber eine statistische Signifikanz zu erreichen. Dennoch weisen die Ergebnisse auch in Zusammenschau mit anderen Studien darauf hin, dass septische Patienten mit intraabdominellem Fokus von einer schnelleren Intervention als die bisher empfohlenen sechs bis zwölf Stunden bis zur Fokussanierung profitieren könnten[1,177–183]. Somit sollte eine frühzeitige chirurgische Fokussanierung von Hohlorganperforationen erwogen werden. In der nächsten Studie stellte sich die Frage, mit welchem Sepsis-Score operative Patienten, die auf einer IMC-Station versorgt werden, auf das Vorliegen einer Sepsis evaluiert werden sollten. Da IMC-Stationen nicht zwangsläufig als eine intensivmedizinische Versorgung gewertet werden können, kann auch nicht automatisch der SOFA-Score als diagnostisches Mittel der Wahl vorausgesetzt werden. Die retrospektive Analyse von einer hohen Anzahl an Patienten zeigte, dass weder der qSOFA noch der SOFA-Score eine schwere Infektion bzw. Sepsis (definiert als Beginn einer empirischen Antibiose) mit einer ausreichenden Prädiktion vorhersagen konnte. Hauptsächlich könnte die geringe Spezifität der Scores mit den physiologischen Alterationen der Vital- und Laborparameter nach operativen Eingriffen oder anderen Traumata erklärbar sein. Aus diesem Grund lässt sich für die klinische Versorgung schlussfolgern, dass sowohl der qSOFA als auch der SOFA-Score für operative IMC-Patienten mit Vorsicht interpretiert werden sollten. Ihre Verwendung ist dennoch zwingend zu empfehlen, da mit ihrer Berechnung die wichtigsten Organfunktionen quantifiziert werden können, sie eine ausreichend validierte Prädiktion für die Mortalität aufweisen und durch die Behandlungsleitlinien der Sepsis empfohlen werden[1]. So könnte eine mögliche Strategie zur frühzeitigen Identifikation von septischen Patienten auf der operativen IMC ein Sepsis-Screening mit Hilfe des qSOFA-Scores und die weitere Evaluation mittels des SOFA-Scores und weiterer Biomarker darstellen. Des Weiteren könnte die Anwendung der SIRS-Kriterien auch in Zukunft Bedeutung für die Detektion von systemischen Inflammationszuständen bei operativen Patienten haben, da mit deren Hilfe Risikopatienten für eine Sepsis identifiziert werden könnten.

Vor dem Hintergrund, dass weder die Vital- und Laborparameter, noch die etablierten Scores eine ausreichende Diskrimination zwischen einer physiologischen systemischen Inflammationsreaktion und einer Sepsis erlauben, stellte sich für den klinischen Alltag die Frage nach einer Optimierung. Obwohl die Entwicklung innovativer Biomarker eine Herausforderung darstellt und eine Vielzahl an Sepsis-Biomarkern evaluiert wurden, stellt dies weiterhin eine vielversprechende Strategie dar[5,7]. In dieser kumulativen Habilitationsschrift wurden sowohl Proteine (Presepsin, DLL-1) als auch Nukleinsäuren (frei zirkulierende DNA, extrazelluläre RNA, mtDNA) und deren Derivate (NETs) evaluiert. Dabei konnten gegenüber Kontrollprobanden alle untersuchten Parameter das Vorhandensein einer systemischen Inflammationsreaktion anzeigen. Darüber hinaus waren mit Ausnahme der Konzentration der NETs die Biomarker zumindest in der Frühphase (innerhalb der ersten 24 Stunden) in der Lage, zwischen einer systemischen Inflammation operativer Genese und einer Sepsis zu unterscheiden. Allerdings weisen alle Biomarker in der detaillierten Betrachtung auch Limitationen auf, so dass sie sich zum jetzigen Zeitpunkt (wie viele andere Biomarker auch) alleine nicht für die klinische Implementierung eignen[7]. Aus diesem Grund wären Multipanelansätze mit verschieden Biomarkern eine mögliche Lösung für diese Problematik. Diese könnte die Sensitivität und Spezifität in Bezug auf die Sepsis bei operativen Intensivpatienten steigern[5,7,127]. Erste Studien an septischen Pateinten mit unterschiedlicher Genese zeigen vielversprechende Ergebnisse, welche eine frühzeitige Identifikation der Sepsis im klinischen Alltag erlauben könnten[159,294]. Inwieweit sich die hier untersuchten Biomarker für operative Intensivpatienten mit Alterationen der Vital- und Inflammationsparameter eignen, wird Gegenstand zukünftiger Untersuchungen sein müssen.

Im letzten Abschnitt wurden immunologische Langzeitveränderungen von ehemals septischen und polytraumatisierten Patienten untersucht. Die kausalen Zusammenhänge der langfristig erhöhten Morbidität und Mortalität dieser Patientenkollektive waren bisher nur in geringem Maße untersucht, so dass die beiden vorgestellten Studien wichtige Hinweise für eine langfristige Immunsuppression dieser Patienten gaben[96,174,220,283,284]. Für den klinischen Alltag bedeutet dies, dass diese Patientenkollektive auch im Hinblick auf ihren Immunstatus eine langfristige Nachsorge erhalten sollten. Falls überhaupt eine Nachsorge erfolgt, werden diese Patienten aktuell in erster Linie weniger auf Grund ihrer Infektionen, sondern überwiegend wegen den fortbestehenden Behinderungen (z.B. nach einem Trauma)

behandelt. So wird in einem Konsensuspapier der *Society of Critical Care Medicine* für Intensivmedizin gefordert, dass ehemals kritisch kranke Patienten nach ihrer Entlassung wenigstens nach zwei bis vier Wochen erneut auf ihre körperliche und geistige Funktion evaluiert werden sollten[295]. So könnte in spezialisierten Sprechstunden, welche ehemalige operative Intensivpatienten langfristig begleiten, neben diesen Fragestellungen auch der Immunstatus erfasst werden, um entsprechenden Folgeproblemen zu begegnen.

5 Zusammenfassung

Der septische Schock ist trotz intensiver Forschung weiterhin mit einer hohen Letalität assoziiert[1,2]. Insbesondere die Detektion der Sepsis bei operativen Intensivpatienten stellt weiterhin eine Herausforderung dar, weil diese Alterationen der Vital- und Laborparameter aufweisen, welche die Diagnosestellung erschweren und verzögern können. Somit war es Ziel dieser kumulativen Habilitationsschrift, die frühzeitige Identifikation der Sepsis bei operativen Intensivpatienten zu optimieren. Zu Beginn wurde untersucht, ob eine frühzeitige chirurgische Fokussanierung bei Patienten mit abdomineller Sepsis das Überleben der Patienten steigert. Auf Basis der Studienergebnisse stellte sich die Frage, wie bei operativen Intensivpatienten mit klinischen Zeichen einer systemischen Inflammation eine Sepsis frühzeitig detektiert und somit eine zielgerichtete Therapie initiiert werden kann. Da sowohl die Interpretation der klinischen Zeichen als auch die etablierten Biomarker keine definitive Differenzierung zwischen einem postoperativen SIRS und einer Sepsis erlauben, besteht ein Bedarf an neuen diskriminativen Biomarkern. Hierzu wurden auf Ebene der Proteine die Biomarker Presepsin und DLL-1 untersucht, welche bei septischen Patienten im Vergleich zu Patienten mit SIRS eine erhöhte Nachweisbarkeit zeigten, so dass sie potentielle Kandidaten für diesen Zweck sein könnten. Auf Grund ihrer herausragenden Rolle in der Immunhämostase wurde im nächsten Schritt die Expression von Nukleinsäuren und NETs bei septischen und postoperativen Patienten analysiert. Während sich sowohl die Menge an zellfreier DNA als auch an extrazellulärer RNA innerhalb der ersten 24 Stunden zwischen Sepsis und SIRS bei abdominalchirurgischen Patienten unterschied, konnte dies bei NETs nicht nachgewiesen werden. Dagegen zeigten sich differente Assoziationen zwischen den Nukleinsäuren bzw. NETs und der Gerinnungsfunktion der Patienten. Während sich im Rahmen der Sepsis eine überwiegend prokoagulatorische Korrelation darstellte, waren NETs bei abdominal- und herzchirurgischen Patienten mit einer Inhibierung des Gerinnungssystems assoziiert. Inwieweit diese Erkenntnisse für die Differenzierung der Sepsis von einem postoperativen SIRS helfen können, müssen Folgestudien klären. Während Presepsin und DLL-1 für primär diagnostische Ziele evaluiert werden können, könnten Nukleinsäuren und NETs sowohl ein diagnostischer als auch therapeutischer Angriffspunkt sein, da sie in der Entwicklung der Koagulopathie bei operativen Intensivpatienten auch *in-vivo* nachweislich eine Rolle spielen. Des Weiteren konnte im Rahmen der Arbeiten eine durchflusszytometrische Methode zur

Quantifizierung von NETs etabliert werden. Sie ermöglicht im Gegensatz zum Goldstandard der Fluoreszenzmikroskopie eine untersucherunabhängige Analyse von großen Probenmengen und könnte daher insbesondere für die klinische Analytik von Blutproben von Interesse sein. Zusammenfassend könnten die hier evaluierten Biomarker grundsätzlich für die Diskrimination septischer Patienten genutzt werden. Für den klinischen Einsatz könnten in Zukunft insbesondere Multipanelansätze von Interesse sein[5,7].

Der letzte Abschnitt fokussierte sich auf die Identifikation von immunologischen Langzeitschäden einer Sepsis bzw. eines Polytraumas. In diesen Studien konnte ein Zusammenhang zwischen der langfristigen Infektanfälligkeit von ehemals septischen Patienten und der Reduktion der monozytären Rezeptorexpression und deren Zytokinsekretion dargestellt werden. Ehemals polytraumatisierte Patienten zeigten ebenso Veränderungen der Rezeptoreigenschaften, ohne aber eine klinische Immunsuppression nachzuweisen. Bei beiden Studienkollektiven konnte hinsichtlich der Lymphozytenfunktion und der Rezeptorexpression keine einheitliche Funktionsschädigung im Langzeitverlauf dargestellt werden, so dass auch hier weitere Studien notwendig sind. Für den klinischen Alltag sollten als Resultat dieser Studien ehemals kritische kranke Patienten langfristig betreut und ihr Immunstatus erfasst werden.

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8 Anlagen

8.1 Anlage 1

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RESEARCH ARTICLE Open Access

The impact of early surgical intervention in free intestinal perforation: a time-to-intervention pilot study

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Abstract

Purposes: An abdominal inflammatory focus is the second most often source of sepsis with a high risk of death in surgical intensive care units. By establishing evidence-based bundled strategies the surviving sepsis campaign provided an optimized rapid and continuous treatment of these emergency patients. Hereby the hospital mortality decreased from 35 to 30 %. Sepsis treatment is based on three major therapeutic elements: surgical treatment (source control), antiinfective treatment, and supportive care. The international guidelines of the surviving sepsis campaign were updated recently and recommend rapid diagnosis of the infection and source control within the first 12 h after the diagnosis (grade 1c). Interestingly this recommendation is mainly based on studies on soft tissue infections.

Methods: In this retrospective analysis 76 septic patients with an intraabdominal inflammatory focus were included. All patients underwent surgery at different time-points after diagnosis.

Results: With 80 % patients of the early intervention group had an improved overall survival (vs. 73 % in the late intervention group).

Conclusions: Literature on the time dependency of early source control is rare and in part contradicting. Results of this pilot study reveal that immediate surgical intervention might be of advantage for septic emergency patients. Further multi-center approaches will be necessary to evaluate, whether the TTI has any impact on the outcome of septic patients with intestinal perforation.

Keywords: Time-to-intervention, Free intestinal perforation, Sepsis

Introduction

Despite modern diagnostic and therapeutic developments the in-hospital mortality of septic patients still remains inacceptably high. With 60 % mortality rates in cases of severe sepsis and septic shock a continuous optimization of treatment and rapid diagnosis is necessary and life-saving.

In up to 25 % of all septic patients an intraabdominal inflammation can be detected [1]. Results of the PROWESS study reveal, that in 66,5 % of the surgical patients the peritoneal cavity was affected by an inflammatory focus [2]. On a surgical intensive care unit the secondary peritonitis due to an intestinal perforation or an anastomotic leakage with extraintestinal air in the radiographic imaging is the main cause for an intraabdominal sepsis and sepsis-associated death. In a post mortem analysis of 235 patients, who died on surgical intensive care units, a persistent, continuous septic intraabdominal focus was found in 32,5 % of cases [1]. Compared to patients from medical intensive care units septic patients from surgical intensive care units have a 7 day longer length of hospital stay and higher cost rates [3].

While an intestinal perforation on its own leads to a mortality of about 14 %, a septic clinical progress is associated with an increase in mortality to 30 %. Among

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the patients suffering from secondary peritonitis the postoperative, secondary peritonitis bears the highest risk of a lethal outcome (1-year mortality >70 %) [4].

In February 2013 the new Surviving Sepsis Guideline was published, which underlines the multimodal, rapid treatment for the septic patient with an intraabdominal focus [5]. According to the recent guideline the sepsis therapy can be subdivided into four different subtypes: surgical source control, the antiinfectious therapy, the supportive intensive care medicine and adjunctive therapeutic approaches.

Surgical approaches for the treatment of intraabdominal infections are mainly based on principle and tradition. Over the last decade evidence-based medicine has emerged to assure best clinical practice, based on a review of literature, quality of research and therapeutic impact. Only few surgical strategies have been evaluated by randomized, prospective and controlled trials. Intraoperative circumstances are often unique and require a flexible, somtimes even unstandardized reaction by the surgeon.

Surgical source control is the only causal treatment option for patients suffering from peritonitis and intraabdominal sepsis. If source control is not possible during the initial emergency operation, mortality increases from 13 to 27 % [6].

In 2004 Barie et al. showed that an inadequate surgical removal of the intraabdominal inflammatory focus leads to a mortality of more than 90 % [7]!

The recent Surviving Sepsis Guideline from 2013 recommends an early source control within 12 h after diagnosis (evidence grade 1C) [5]:

> *"A specific anatomical diagnosis of infection requiring consideration for emergent source control be sought and diagnosed or excluded as rapidly as possible, and intervention be undertaken for source control within the first 12 hr after the diagnosis is made, if feasible."*

While a time-dependency in the early phase of hospital admission has been shown for an antimicrobial treatment at least in some studies, it remains nebulous, if a time-dependency of surgical source control and the outcome of the septic patient could be detected. These effects have never been analyzed before for the surgical patient with intestinal perforation. Interestingly, the guideline recommendation is based on literature mainly dealing with necrotisizing soft tissue infections [8–10]. Furthermore, the necrosectomy of peripancreatic necrosis is citated as one rationale for a conservative, delayed surgical intervention [11, 12]. To our surprise there is hardly any literature, analyzing the influence of the time to intervention on the outcome of critically ill septic patients with an intestinal perforation. For that reason we performed this time-to-intervention pilot study to investigate, if surgical source control in the very early phase of early-goal directed sepsis therapy is of benefit for our surgical intensive care patients.

Materials and methods

This study was approved by the local ethical committee and was designed as a retrospective cohort analysis of clinical data from 76 septic patients (45 males and 31 females) with a mean age of 59.64 years (range 21–88 years) suffering from intestinal perforation. Observational period was between August 2008 and February 2012. Subjects were included, if intestinal perforation was detected by radiographic imaging (computertomography scan or x-ray of the abdomen) and confirmed during surgery. Intestinal perforation was diagnosed in cases of free intraabdominal air and/or extravasation of contrast medium into the peritoneal cavity. Patients were included, if they showed either free intestinal perforation or an anastomotic leakage. They were excluded, if the intraoperative and radiologic findings were discordant, of if there was a contained perforation. In addition, patients must have met the sepsis criteria of the ACCP/SCCM consensus conference on sepsis [13] [Fig. 1].

Patients were excluded from the study if the radiological diagnosis of an intestinal perforation was discordant to the intraoperative finding. Further exclusion criteria were postoperative perforations due to anastomotic leakage, age < 18 years and perforations, which were treated interventionally (e.g. CT-guided drainage, Endo-VAC-Systems). Patients with an open abdomen were furthermore excluded from the study.

"Time-to-intervention" is defined as the time span between the diagnosis by radiographic imaging (CT scan/x-ray) and the start of surgical intervention ("cut of the scin"). Due to the time to intervention the study group was subdivided into three groups: under 3 h, 3 to 9 h and longer than 9 h. These time intervals were chosen in order to represent usual hospital procedures. In daily routine a time to intervention of less than 3 h reflects the situation, when the surgeon is waiting for the next operation room available (high urgency). Patients who were operated between 3 and 9 h represent urgent cases, which were operated within one hospital working shift. More than 9 h of time to intervention represent a situation of a delayed diagnosis and/or treatment of the intestinal perforation.

Clinical data were collected by the regular hospital documentation software (IMESO KisData and ICUData version 7.7.0.1590, KAOS desktop version 3.0.0.1, MEDOS Nexus .med RIS Client version 9.3.2276). Parameters of interest were length of hospital stay, ventilatory time, pre- and postoperative blood parameters, amount of cristalloids and catecholamine consumption. Furthermore, the

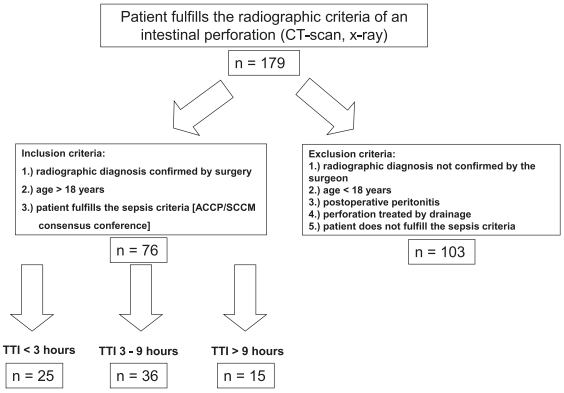

Fig. 1 Study protocoll of this retrospective analysis of patients with an intestinal perforation at our university hospital: Of 179 patients with the diagnosis of a perforation in the GI-tract 79 met the inclusion criteria of this pilot study. These patients were attributed to the three study arms according to their time-to-intervention

application of blood transfusions was analyzed. Different protein serum levels (lactate, pH, base excess, blood glucose, creatinine, urea, c-reactive protein, procalcitonin), hemoglobin and the amount of leukocytes were measured on admission in the emergency room and immediately after surgical source control. Both the in-hospital mortality and the overall motality were calculated. Additionally radiologic methods and surgical specifications (cause and location of perforation, surgical technique) were collected. The amount and type of infusion and transfusion were determined for the first 24 h after surgery. ICU prognostic scores (SAPS II, SOFA, APACHE II) were also calculated for the first postoperative 24 h according to the established protocols published before [14–16].

Baseline characteristics were expressed as mean ± standard deviation in normal and as median ± interquantile range in not normally distributed data. A p-value of $p < 0.05$ was considered as significant. Descriptive analysis was performed with contingency tables, while Chi-Squared-Test was used to describe the distribution of discrete parameters in proportion to the time to intervention. Mann–Whitney-U-Test was used to test metric values. All statistical analyses were performed with Microsoft® Excel® and IBM® SPSS® Statistics (version 21.0.0.0).

Results

From 179 patients with radiographic signs of an intestinal perforation 76 septic patients fulfilled the inclusion criteria. 31 female and 45 male septic patients with an intestinal perforation entered this retrospective monocenter study. Figure 2 shows the location of the intraabdominal inflammatory. With 36 cases most patients were operated between 180 and 540 min after diagnosis (study group II). In 25 patients surgery was performed in less than 180 min (study group I). In study group III (TTI > 540 min) 15 cases were included.

In the majority of cases (97 %) the diagnosis was based on an emergency computertomography scan. In two cases free intraabdominal air in the conventional x-ray of the abdomen led to the indication for surgical intervention.

Sixty patients developed respiratory failure requiring mechanical ventilation in the postoperative course. The duration of the operation did not differ between the three groups. In addition, the absolute time of mechanical ventilation and the amount of catecholamines applied did not depend on the time between diagnosis and surgical intervention (Fig. 3). Revisions had to be performed in 38 % of the cases (study group 1 44 % vs. study group 2 39 % vs. study group 3 27 %). In case of perforation of the small intestine segmental resection with a side-to-side anastomosis were performed. Perforations of the sigmoid colon were resected. Due to the impression of the emergency surgeon a Hartmann operation was done. Alternatively the colorectum was reconstructed by a descendorectostomy. In some cases (deep distal anastomosis) a diverting protective ileostomy was implemented.

Perforations of the ascending colon were reconstructed by a ileotransversostomy. In cases of anastomotic leakage either discontinuous or continuous reconstructions were performed due to the surgeon's impression.

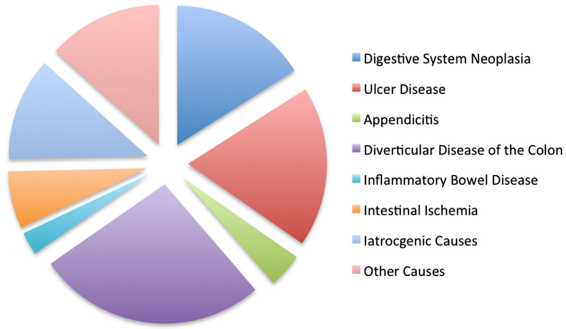

Fig. 2 The different reasons for the perforation reflects the typical pattern of a European university hospital

Concerning the 30-day in hospital mortality (non-significant) differences between the three study groups were detected: While patients of study group I had a 80 % survival, patients with a time-to-intervention of more than three hours (study group II and III) showed a survival of 73 % each. The overall survival was 80 % for the early intervention group, compared to 75 % (study group II) and 73 % (study group) respectively.

Tables 1, 2 and 3 provide the descriptive parameters, retrospectively analyzed in this study.

The time-courses of the serum CRP, urea, creatinine and lactate are presented in Fig. 4. Significant differences between the three study groups could not be detected.

Discussion

Despite the risks of a specific surgical intervention (fistulas, SIRS, bleeding) it is common surgical practice, that emergency patients with intestinal perforation are initially stabilized and transferred to the operation room.

In our pilot study 76 septic patients were analyzed concerning the time-dependency between surgical intervention and patient's mortality and morbidity. It thus provides insights into the surgical management in the very early phase after hospital admission:

More than 80 % of the patients with an intestinal perforation underwent surgical source control within the first 9 h after hospital admission. About one third of the patients was operated within 3 h. In the great majority of cases we met the 12-h intervall of surgical source

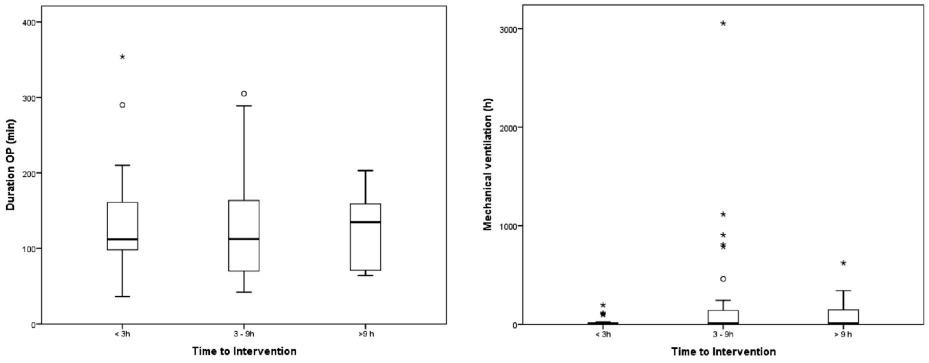

Fig. 3 All study groups were equal concerning the duration of surgical source control. The time-to-intervention had no significant impact on the time of mechanical ventilation or the amount of catecholamins adiministered after surgery. o and * represent single spike values

Table 1 Characteristics of the three study groups

		<3 h			3–9 h			>9 h			
	N	*n*	Mean	SD	Median [IQR]	*n*	Mean	SD	Median [IQR]	*n*	Mean	SD	Median [IQR]	*p*-Value
Age	76	25	59,64	18,775	63 [50, 74]	36	64,36	14,934	65 [56.25;76.5]	15	68,8	14,586	69 [61;80]	0,205
Length of hospital stay	76	25	20,64	14,731	22 [10, 28]	36	28,75	32,056	17,5 [11;29]	15	20,27	14,235	16 [11;27]	0,921
Duration of surgery (minutes)	76	25	135,2	73,005	112 [97, 165.5]	36	126,58	68,393	112,5 [70;170.25]	15	127,07	47,596	135 [70; 160]	0,744
Ventilatory time (hours)	76	25	29,524	51,3765	6,2 [0.5, 19.2]	36	228,253		9,9 [5.175; 148.75]	15	105,94	186,3957	7,7 [3.9;276]	0,141
Katecholamins (within 24 h)	76	25	7102,32	15541,449	450 [0, 9330]	36	31294,5	80932,459	1911 [0; 9700]	15	4570,8	8733,606	0 [0; 2340]	0,604
Bikarbonate	76	25	84	123,929	0 [0, 155]	36	65,56	91,634	50 [0; 100]	15	40	54,116	0 [0;100]	0,656
Kristalloids	76	25	2876	1416,945	2600 [1600, 4125]	36	2564,17	1364,581	2525 [1562.5; 31^37.5]	15	1990	978,373	1800 [1500; 2100]	0,184
Kolloids	76	25	854	1951,384	500 [0, 1000]	36	326,39	421,813	0 [0; 500]	15	400	430,946	500 [0; 500]	0,13
Substitution of erythrocyte concentrates	76	25	276	465,725	0 [0, 1020]	36	283,33	411,617	0 [0; 990]	15	260	0	437,199 [0; 1200]	0,839
Thrombocyte concentrates	76	25	20	70,711	0	36	20,83	71,088	0	15	33,33	129,099	0	1
Fresh frozen plasma	76	25	100	322,749	0	36	187,5	432,497	0	15	133,33	296,808	0	0,322
Glucose substitution within24h	76	25	15,2	76	0	36	15,2	76	0	15	40	129,835	0	0,987
Bicarbonate substitution within 24 h	76	25	70	119,024	0 [0, 100]	36	48,53	101,865	0	15	53,33	104,312	0 [0; 50]	0,335
Kistralloid substitution within 24 h	76	25	2990,8	2348,709	2800 [1720, 3700]	36	3198,61	1712,078	2800 [2000; 4500]	15	2536,67	1155,649	2250 [1700; 3000]	0,79
Kolloid substitution within 24 h	76	25	380	505,8	0 [0, 500]	36	328,57	404,076	0 [0; 500]	15	833,33	1531,417	0 [0; 1000]	0,869
Erythrocyte concentrates within 24 h	76	25	108	285,657	0	36	233,33	424,937	0 [0; 300]	15	260	337,639	0 [0; 600]	0,056
Thrombocyte concentrates within 24 h	76	25	12	60	0	36	12	60	0	15	8,33	50	0	0,604
FFP within 24 h	76	25	48	132,665	0	36	177,78	390,685	0 [0; 350]	15	26,67	103,28	0	0,283
SAPS_II score after source control	76	25	42,36	15,508	43 [29.5, 50.5]	36	44,28	15,161	46 [31.25; 55.75]	15	41,53	13,674	38 [34; 46]	0,894
APACHE II score after soruce control	76	25	21,08	8,026	21 [14, 26]	36	21,86	6,69	21,5 [17.25; 26.5]	15	19,87	5,718	20 [16; 25]	0,79
SOFA score after source control	76	25	6,32	4,059	6 [3, 9.5]	36	7,28	3,377	7 [5; 10]	15	6,27	3,127	6 [4; 9]	0,479
Gastric reflux within 24 h (ml)	76	25	166	225,074	30 [0, 300]	36	266,94	436,347	100 [0; 400]	15	163,33	227,272	80 [0, 250]	0,57

FFP fresh frozen plasma, *SAPS* Simplified Acute Physiology Score, *APACHE* Acute Physiology and Chronic Health Evaluation II-score, *SOFA* Sequential Organ Failure Assessment score

Table 2 Descriptive analysis of different parameters of the three study groups

		<3 h				3–9 h				>9 h				
	N	*n*	Mean	SD	Median [IQR]	*n*	Mean	SD	Median [IQR]	*n*	Mean	SD	Median [IQR]	*p*-Value
Before surgery														
Serum lactate	46	9	2,45	1,66193	1,9 [1.2, 3.2]	28	2,6404	2,67295	1,8 [1.175; 2.875]	9	1,9733	0,99991	1,7 [1.235; 2.645]	0,661
pH	49	17	7,34	0,05374	7,33 [7.3, 7.375]	23	7,3422	0,10904	7,38 [7.24; 7.41]	9	7,4144	0,04613	7,43 [7.4; 7.445]	0,074
BE	49	17	−3,3824	2,56131	−3,8 [−5.35, −0.9]	23	−4,8	6,70271	−3,8 [−8.5; 0.4]	9	−1,0778	6,41693	−2,2 [−5.4; 1.4]	0,95
Glucose	55	11	152,684	70,4683	138 [101, 166]	33	150,494	73,9948	136 [100; 165]	11	163,909	68,9775	148 [107; 176]	0,869
Hemoglobin	75	24	12,417	3,0039	12,6 [9.7, 14.65]	36	13,681	13,8924	11 [9.475; 13.575]	15	10,593	1,9036	9,9 [9.1; 11.8]	0,11
Creatinine	76	25	1,352	1,138	1 [0.8, 1.25]	36	7,136	34,2748	1 [0.8; 2.05]	15	1,347	0,9782	1 [0.8; 1.5]	0,781
Urea	76	25	59,68	54,4225	41 [25, 83]	36	64,764	62,7524	42,5 [32.25; 73.75]	15	76,133	41,8857	63 [43; 108]	0,175
Leukocytes	76	25	12,276	7,2799	12,3 [7.25, 17.2]	36	12,328	8,8553	9,25 [7.975; 15.775]	15	11,373	7,0199	9,3 [6.1; 12.9]	0,808
CRP	75	25	141,6148	157,91994	74,3 [9.05, 253]	36	168,0072	131,14877	149,175 [42.32; 250.28]	14	181,7514	137,22675	155,7 [56.28; 320.4]	0,176
PCT	12	3	13,267	14,584	10,6 [0.2, 0	7	8,714	12,3999	0,8 [0.4; 15]	2	12,95	15,3442	12,95 [2.1; 0]	1
After Surgery														
Serum lactate	66	15	2,9718	2,19183	2,25 [1.35, 4]	36	1,9333	1,41219	1,4 [1.1; 2.775]	15	2,3867	1,92423	1,8 [1.2; 2.2]	0,06
pH	74	23	7,302	0,082848	7,308 [7.231, 7.37]	36	7,346	0,079749	7,35 [7.31; 7.4]	15	7,33233	0,080053	7,31 [7.265; 7.4]	0,072
BE	74	23	−5,4348	3,49488	−5,1 [−8.4, −3]	36	−3,7389	3,98929	−3,8 [−5.4; −2.2]	15	−3,8467	5,18871	−4,5 [−6.2; −2.3]	0,059
Glucose	75	24	126,288	42,1621	122 [1104, 145.75]	36	130,717	51,9907	134,5 [107.25; 144.75]	15	172,467	54,1702	175 [138; 221]	0,118
Hemoglobin	75	24	16,438	22,6004	11,9 [10.25, 13.68]	36	17,181	28,0558	10,65 [9.425; 13.08]	15	10,367	2,4555	10,4 [9.6; 11.6]	0,065
Creatinine	75	24	1,304	1,1035	1 [0.9, 1.12]	36	1,45	0,7865	1,2 [0.825; 1.7]	15	1,267	0,8156	0,9 [0.8; 1.6]	0,319
Urea	75	24	54,67	47,416	39,5 [26.25, 49.5]	36	71,08	56,263	59 [36; 78]	15	74,13	43,083	67 [47; 83]	0,025
Leukocytes	75	24	10,129	5,9572	9,4 [4.95, 13.4	36	10,597	6,5088	8,45 [6.43; 13.2]	15	12,02	7,4694	12,9 [4.6; 16.9]	0,759
CRP	75	24	137,0608	110,5366	140,93 [31.2, 229.4]	36	168,595	107,2545	156,555 [76.8; 228.5]	15	182,9213	104,25169	184,2 [115; 256]	0,175
PCT	13	3	20,933	20,9381	17,7 [1.8, 0]	9	20,933	20,9381	17,7 [5.35; 29.6]	1	26,356	34,8009	14,4 [9.75; 30.5]	1

BE base excess, *CRP* C-reactive protein, *PCT* procalcitonin

Table 3 General description of the three study groups conerning OP technique, antibiotics and others

	Total		Up to 3 h.		3 to 9 h.		More than 9 h.	
Gender	n	%	n	%	n	%	n	%
Female	31	41 %	8	32 %	13	36 %	10	67 %
Male	45	59 %	17	68 %	23	64 %	5	33 %
Total	76	100 %	25	100 %	36	100 %	15	100 %
Ventilation	N	%	n	%	n	%	n	%
Not ventilated	16	21 %	5	20 %	8	22 %	3	20 %
Ventilated	60	79 %	20	80 %	28	78 %	12	80 %
Total	76	100 %	25	100 %	36	100 %	15	100 %
OP-method	N	%	n	%	n	%	n	%
Laparascopic	2	3 %	2	8 %	0	0 %	0	0 %
Open	74	97 %	24	92 %	35	97 %	15	100 %
Converted	2	3 %	0	0 %	1	3 %	0	0 %
Total	76	100 %	26	100 %	36	100 %	15	100 %
Antibiotics	N	%	n	%	n	%	n	%
No antibiotics	2	3 %	0	0 %	0	0 %	2	13 %
Antibiotics	74	97 %	25	100 %	36	100 %	13	87 %
Total	76	100 %	25	100 %	36	100 %	15	100 %
Revision	N	%	n	%	n	%	n	%
No revision	47	62 %	14	56 %	22	61 %	11	73 %
Revision	29	38 %	11	44 %	14	39 %	4	27 %
Total	76	100 %	25	100 %	36	100 %	15	100 %
Ischemia	N	%	n	%	n	%	n	%
No ischemia	67	88 %	22	88 %	31	86 %	14	93 %
Ischemia	9	12 %	3	12 %	5	14 %	1	7 %
Total	76	100 %	25	100 %	36	100 %	15	100 %

control strongly recommended by the Surviving Sepsis Campaign [5]. The overall survival was 80 % for study group I and decreased to 75 % (study group II) and 73 % (study group III) respectively.

In a multivariate analysis of septic patients with necrotizing soft tissue infections Boyer et al. was able to show that a delayed surgical intervention > 14 h after diagnosis was a negative prognostic predictor, associated with a 34-fold increase in hospital mortality [9]. Hospital mortality was 40.6 %. Forty-one percent of the patients were septic on admission.

Delayed operation is recognized as a contributor to adverse outcome in many fields of emergency surgery. A prospective trial from Kollef could determine the delay in diagnostics followed by surgical intervention as one independent negative predictor for the survival of intensive care patients [17]. Reasons for delayed surgical intervention were analyzed in a retrospective study by North et al. and by the Danish National Indicator Project in 2009: out-hospital perforation, masked clinical signs of peritonitis, late surgical attendance and a missing pulseoxymetry on admission were independent prognostic factors for a bad outcome.

In a study from the field of pediatric surgery all children, who survived a necrotizing fasciitis had underwent surgical source control within three hours after admission [8]. These data are supported by a retrospective cohort study by Gajic et al., who could identify delays in surgical evaluation and therapy as critical contributors to mortality on medical intensive care units: A delay of more than 48 h led to a dramatic decline in patients' survival (41 % vs. 73 % in the early intervention group) [18].

Despite some evidence for a conservative treatment of perforated peptic ulcera [19] surgery is still the gold standard for gastric perforation [20]. For patients with perforations of the upper GI tract Buck et al. could detect a strong dependency of the delay of surgical treatment after hospital admission on the 30 day survival rates [21]. Over the first 24 h after admission each hour

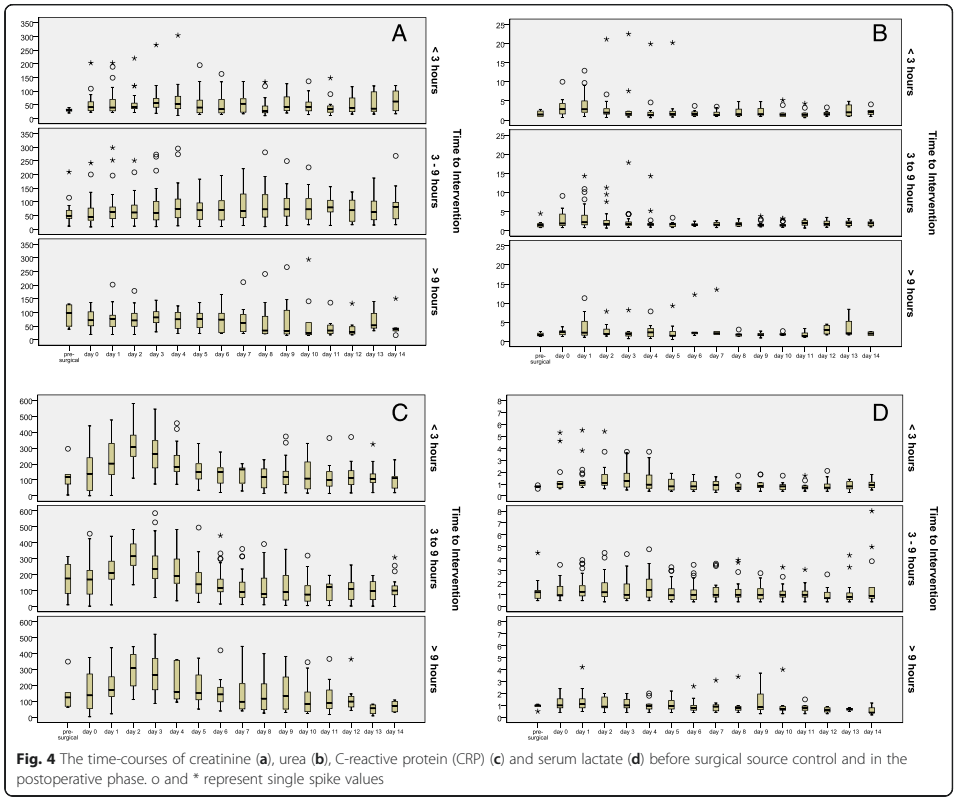

Fig. 4 The time-courses of creatinine (**a**), urea (**b**), C-reactive protein (CRP) (**c**) and serum lactate (**d**) before surgical source control and in the postoperative phase. o and * represent single spike values

of surgical delay was associated with a 2 % decrease in patient survival [21]. The amount of septic patients in the study group remains unclear. About 16 % were hypotensive on admission.

This strong time-dependency conflicts the recommendation of the new guidelines of the Surviving Sepsis Campaign, which advises a time-to-intervention of 12 h from diagnosis to source control. These very early hours after hospital admission are still nebulous concerning the influence of the velocity of diagnostics and surgical source control on patients'survival.

Due to the very limited number of septic patients included in this pilot study we potentially failed to find a significant influence of the TTI on the mortality. Nevertheless with prolonged TTI mortality rates showed an increasing tendency to higher levels. While 30-day mortality was 20 % in Study group I, it was 27 % in group III. Hospital mortality confirmed this tendency (study group I 20 % vs. Study group II 33 % vs. Study group III 33 %).

In contrast to Wachta et al., who investigated 14 % mortality for patients with peritonitis after intestinal perforation, our study revealed a higher overall mortality (31 %). This can be explained by the inclusion of only septic patients with increased APACHE II scores > 20 (vs. APACHE II 14.3). Besides the lack of prospective randomized studies a comparison of studies on surgical source control ist nearly impossible. This is due to incoherence in the group of patients with intestinal perforation: While patients with perforations requiring intensive care have a very poor prognosis, patients with iatrogenic perforation show an improved survival [18, 22]. Perforations due to an ischemia of the bowel have a very bad prognosis. With a 1 year-mortality of more than 70 % patients with secondary peritonitis due to postoperative complications have the worst outcome [4]. Generally peritonitis is a negative predictor for patients' outcome after intestinal perforation. In our study an early surgical intervention tends to result in lower rates of

peritonitis (group I 88 % vs. Group II 92 % vs. Group III 100 %). This goes in line with the differences in mortality we could determine.

To minimize the incoherence of the study group the patients with complications after surgery like anastomotic leakage or ischemia were excluded from the study cohort. Nevertheless this important group of patients for the surgeons everyday-life should be analyzed in future studies.

This incoherence in the group of septic patients in general also reflects the contradictory results on the impact of a rapid or delayed initiation of antimicrobial therapy on the mortality of septic patients: The prospective, objective multicenter study from Ferrer et al. revealed, that only an early broad-spectrum antibiotic treatment within one hour is life-saving for the septic patient [23]. However, source control was not tested in this study. These data underline the results of Kumar et al., who determined the impact of delays in initiation of an effective antibiotic treatment on the mortality of septic shock patients. Each hour of delay in the administration of antimicrobials was associated with a decreased survival of 5–10 % [24]. Despite its wide adoption, whether this practice is of benefit for all septic patients is still controversial. Hranjec et al. analyzed 1483 patients concerning an aggressive (early start of antimcirobial treatment) vs. conservative (start of antibiotics as soon as the infection objectively was confirmed) treatment modality. Interestingly, the benefitial effects of an early administration of antibiotics were not detectable in the hemodynamic stable surgical patient [25]. Detailed analysis of the study protocols reveals that the beneficial effects of a rapid initiation of antimicrobial pharmacotherapy especially can be found for the subgroup of patients with a septic shock, a group, which also received antibiotics in the Hranjec study immediately.

From these data one could deduce that not the standardized, strictly time-dependent, but the personalized antimicrobial therapy could be trend-setting.

The originality of our study is supported by several differences to trials published previously: First only those patients were included, who met the sepsis criteria of the ACCP/SCCM consensus conference on sepsis. Second, all patients had an intestinal perforation. The locations of the perforations and its reasons reflect the spectrum of a typical European university hospital. Third, all study groups showed no differences concerning their catecholamine consumption or APACHE II scores. Thus the groups are comparable concerning the severity of sepsis.

This small unicenter study is a pilot-study to turn the focus on citically ill, septic surgical patients, which – so far – are not reflected by the recent sepsis guidelines.

Due to the retrospective study design the analysis of the patients suffering from severe abdominal sepsis bears the risk of an important bias: It is probable, that those patients, who were staged as very critical in the emergency room were transferred into the operation room more rapidly than those, who presented as relatively stable concering their clinical situation. We tried to minimalize this bias by analysing the consumption of catecholamines, the analysis of preoperative labarotory parameters and clinical scores (e.g. APACHE II). Our study was designed and performed in only one surgical center and thus only a very limited number of septic patients were included. Results can only show tendencies, which underlines, that multicenter approaches are necessary to analyze this group of critically ill surgical patients in the very early phase of emergency and intensive care treatment.

Conclusions

Despite its relevance literature on the time dependency of early surgical intervention in intestinal perforation is rare and contradictory. In this pilot study there is a tendency that immediate surgical intervention might be of advantage for septic emergency patients. Further multicenter approaches will be necessary to evaluate, whether the TTI has any impact on the outcome of septic patients with intestinal perforation.

Abbreviations

APACHE: acute physiology and chronic health evaluation; SAPS: simplified acute physiology score II; SOFA: sepsis-related organ failure assessment; TTI: time to intervention.

Competing interests

Andreas Hecker and other co-authors have no conflict of interest.

Authors' contributions

AH, ES, RR and RF designed the study and wrote the manuscript. BH, JH, CK and MR were involved in statistical analysis of the data. MR and CL participated in the coordination and helped to draft the manuscript. GAK, WP and MAW were involved in the final corrections. All authors read and approved the final manuscript.

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8.2 Anlage 2

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RESEARCH ARTICLE Open Access

Comparison of qSOFA score, SOFA score, and SIRS criteria for the prediction of infection and mortality among surgical intermediate and intensive care patients

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Abstract

Background: It is crucial to rapidly identify sepsis so that adequate treatment may be initiated. Accordingly, the Sequential Organ Failure Assessment (SOFA) and the quick SOFA (qSOFA) scores are used to evaluate intensive care unit (ICU) and non-ICU patients, respectively. As demand for ICU beds rises, the intermediate care unit (IMCU) carries greater importance as a bridge between the ICU and the regular ward. This study aimed to examine the ability of SOFA and qSOFA scores to predict suspected infection and mortality in IMCU patients.

Methods: Retrospective data analysis included 13,780 surgical patients treated at the IMCU, ICU, or both between January 01, 2012, and September 30, 2018. Patients were screened for suspected infection (i.e., the commencement of broad-spectrum antibiotics) and then evaluated for the SOFA score, qSOFA score, and the 1992 defined systemic inflammatory response syndrome (SIRS) criteria.

Results: Suspected infection was detected in 1306 (18.3%) of IMCU, 1365 (35.5%) of ICU, and 1734 (62.0%) of IMCU/ICU encounters. Overall, 458 (3.3%) patients died (IMCU 45 [0.6%]; ICU 250 [6.5%]; IMCU/ICU 163 [5.8%]). All investigated scores failed to predict suspected infection independently of the analyzed subgroup. Regarding mortality prediction, the qSOFA score performed sufficiently within the IMCU cohort (AUCROC SIRS 0.72 [0.71–0.72]; SOFA 0.52 [0.51–0.53]; qSOFA 0.82 [0.79–0.84]), while the SOFA score was predictive in patients of the IMCU/ICU cohort (AUCROC SIRS 0.54 [0.53–0.54]; SOFA 0.73 [0.70–0.77]; qSOFA 0.59 [0.58–0.59]).

Conclusions: None of the assessed scores was sufficiently able to predict suspected infection in surgical ICU or IMCU patients. While the qSOFA score is appropriate for mortality prediction in IMCU patients, SOFA score prediction quality is increased in critically ill patients.

Keywords: Sepsis, Critical care, qSOFA, SOFA, Mortality, Infections

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Background

Sepsis is defined as a life-threatening disease complex characterized by severe organ dysfunction resulting from a dysbalanced host response to an infection [1]. Despite modern treatment protocols, sepsis-related mortality remains highly associated with delays in adequate treatment [2]. For this reason, modern clinical concepts have focused on the development of criteria aiming for the rapid identification of sepsis [3, 4].

For 24 years, sepsis has been defined as suspected or proven infection, together with two or more systemic inflammatory response syndrome (SIRS) criteria [5]. However, during the last decade, clinical characteristics that serve to define sepsis changed due to an improved understanding of the underlying pathobiology. Therefore, in 2016, the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) introduced a significant change in the approach to the definition and diagnostic criteria of sepsis [1].

Nevertheless, a highly sensitive and specific diagnostic test for the detection of sepsis is currently still lacking. Among ICU encounters with suspected infection, the Sepsis-3 Task Force recommended the use of the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score for the identification of septic patients [1, 6]. For the rapid identification of patients with suspected infection outside of the ICU, on the other hand, Seymour et al. introduced the quick Sequential Organ Failure Assessment (qSOFA) score [7]. The qSOFA score is a simple score consisting of three items: respiratory rate (RR) ≥ 22 breaths per minute, altered mentation (Glasgow Coma Scale [GCS] < 15), and systolic blood pressure (SBP) < 100 mmHg. A qSOFA score ≥ 2 was found to be significantly predictive of increased all-cause mortality in patients outside of the ICU [7]. Therefore, the authors of the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) recommended the use of the qSOFA score for the identification of adult septic patients in out-of-hospital, emergency department, or general hospital ward settings [1].

Intermediate care units (IMCUs) are logistically situated between ICUs and general wards and serve as an alternative care setting for patients deemed too unstable to be cared for on the general ward, but without requiring the resources of an ICU [8–10]. Lacking a unitary definition of IMCUs, their nomenclature varies from high dependency, progressive care, medium care, or step-down units, resulting in a high variability of organizational practice [8]. While most IMCUs offer continuous monitoring of vital signs, the ability to provide mechanical ventilation, renal replacement therapy, and differentiated catecholamine therapy is normally limited [11]. Although IMCU patients commonly demand a higher level of nursing compared to the normal ward, the severity of illness is lower than on the ICU [12, 13]. It is worth noting that the mere presence of an IMCU is associated with a significantly reduced hospital mortality in ICU patients, underlining the impact of an IMCU as a bridge between the ICU and the regular ward [14]. Furthermore, by demonstrating that septic shock patients can be successfully treated on an IMCU, Meaudre et al. proposed the potential of this critical care resource [15]. Surgical patients, in particular, are often treated in IMCUs because they are commonly extubated shortly after surgery and are therefore not mandatorily eligible for ICU treatment. However, surgical patients are also at risk for postoperative infections. Clinical signs of infection in these patients are challenging, since they might also be caused by the surgery itself, implicating the need for thorough risk stratification [16, 17]. Lacking evidence, it is not yet defined whether these patients should be evaluated as ICU or non-ICU patients when it comes to the identification of sepsis, respectively severe infection, raising the question as to whether the SOFA or qSOFA score should be used. For this reason, there are currently no specific recommendations for the screening of septic patients treated on IMCU. Therefore, the aim of our study was to compare the predictive power of qSOFA and SOFA scores, as well as the 1992 defined SIRS criteria, for mortality or infection in a large sample of surgical ICU and IMCU patients. We hypothesized that the qSOFA score would perform superiorly to the SOFA score and SIRS criteria in predicting mortality or infection among IMCU patients.

Methods

Study design and patient recruitment

This retrospective, 6-year cohort study was approved by the local ethics committee (Justus-Liebig-University, Giessen, Germany, trial code 240/16). The methods and results are presented in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Data of all patients aged ≥ 18 years with suspected infection who were treated at the surgical ICU and/or IMCU of the University Hospital of Giessen between January 01, 2012, and September 30, 2018, were included.

Data acquisition

After identification of patients, study data were automatically extracted from the local patient data management system (ICU-Data®, IMESO® GmbH, Giessen, Germany) with Structured Query Language and Procedural Language (SQL/PL-SQL)-based scripts. Patients' characteristics included age, body mass index (BMI), treatment unit (ICU, IMCU, or both), Acute Physiology and Chronic Health Evaluation (APACHE) II score, and, if applicable, the type of performed surgery. Episodes of suspected

infections were defined as the first 72 h after starting treatment with broad-spectrum antibiotic agents, which included carbapenems, glycopeptides, quinolones, piperacillin/sulbactam, ceftazidime, cefepime, linezolid, tigecycline, daptomycin, and fosfomycin. Contrarily, the following antibiotics were excluded because they did not meet the definition of broad-spectrum antibiotic treatment, according to the European and local sepsis guidelines [18]: ampicillin, cefazolin, cefuroxime, colistin, metronidazole, erythromycin, trimethoprim/sulfamethoxazole, and azithromycin.

While the SOFA score was recorded daily throughout the patient's ICU treatment by the attending physician, SIRS criteria and qSOFA score were not registered systematically and therefore needed to be calculated retrospectively. First, relevant vital signs (respiratory rate, systolic blood pressure, heart rate, temperature), which were automatically recorded every 15 min, were systematically analyzed for outliers. For this purpose, a second data table was built, and the median for each parameter was calculated. For the calculation of the median of the respiratory and heart rates as well as the systolic blood pressure, three values of each time point were included (i.e., corresponding time point and two values aside). Since extreme values of both parameters were possible in critically ill patients, no absolute thresholds were defined as outliers. Secondly, the median for each temperature time point was calculated out of seventeen values (i.e., corresponding time point and sixteen values aside) to equalize incorrect measurements, which can be caused by a dislocated temperature probe. Body temperature measurements ≤ 31 °C were defined as artefacts and therefore excluded. If GCS was not available, Richmond Agitation Sedation Scale (RASS) was used for the assessment of consciousness (where RASS ≠ 0 was defined as the fulfillment of "altered mental status," respectively as GCS ≤ 15). Leucocyte count was derived from the daily routine blood cell count, while arterial carbon dioxide partial pressure ($paCO_2$) was extracted from the blood gas analyses, which was most recent to the analyzed time frame.

In accordance with their definitions, the SIRS criteria and the qSOFA score were rated positive if at least two criteria were fulfilled during a minimum of 30 min [7, 19]. The SOFA score of each day was compared with the value of the previous day. An increase of at least two points was rated positive.

Furthermore, the outcome analysis included the need for and duration of invasive ventilation, requirement for catecholamines, length of hospital stay, and hospital mortality.

Statistical analysis

All encounters were divided into three subgroups, according to their location of treatment (IMCU only, ICU only, or both [IMCU/ICU]). In cases of normal distribution of the data, the results are expressed as mean ± standard deviation (SD) and, in cases where data were not normally distributed, as median (interquartile range [IQR]). Receiver operating characteristic curves (ROC) were used for calculation of the predictive validity of the SIRS criteria, qSOFA score, and SOFA score. The primary aims of these analyses were defined as the identification of suspected infection and the prediction of mortality, assessed by means of the area under the ROC curve (AUCROC). Furthermore, sensitivity and specificity of both primary aims were calculated. AUCROCs were considered to be poor at 0.51–0.69, adequate at 0.7–0.79, sufficient at 0.8–0.89, and excellent at 0.9 or higher. AUCROCs are displayed with the 95% confidence interval. Data were tested for statistically significant differences using chi-squared test or Fisher's exact test, when appropriate. A two-tailed value of $p < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the R statistical software version 3.5.1 (www.r-project.org).

Results

Characteristics of the study cohorts

For the observational period, 13,780 patients were identified. Of these, 7133 (51.8%) were treated only at the IMCU, 3850 (27.9%) at the ICU, and 2797 (20.3%) at both the ICU and IMCU (Fig. 1). Patients' characteristics, underlying departments, and outcome parameters are shown in Table 1. Overall, 458 (3.3%) subjects died within the observation period (IMCU 45 [0.6%]; ICU 250 [6.5%]; IMCU/ICU 163 [5.8%]). Suspected infections were identified in 4405 (32.0%) patient encounters (IMCU 1306 [18.3%]; ICU 1365 [35.5%]; ICU/IMCU 1734 [62.0%]; Fig. 1).

Performance of clinical scores in the IMCU

Among 1306 IMCU patients with suspected infection, 1023 (78.3%) fulfilled at least two positive SIRS criteria. Furthermore, a SOFA score increase was detected in 65 (5.0%) cases, while qSOFA scoring was positive in 735 (56.3%) patients.

Overall, the predictive performance of the scores of interest was low. However, compared to the SOFA score, the SIRS criteria and qSOFA score performed superiorly regarding their discrimination between suspected infection and the use of broad-spectrum antibiotics (SIRS: AUCROC = 0.63 [0.62–0.65]; SOFA: AUCROC = 0.52 [0.51–0.53]; qSOFA: AUCROC = 0.63 [0.62–0.65]; SIRS vs. SOFA: $p < 0.001$; qSOFA vs. SOFA: $p < 0.001$; SIRS vs. qSOFA: $p = 0.833$; Fig. 2). While the highest sensitivity for the detection of presumed sepsis was achieved by means of the SIRS criteria, the maximum specificity was found with the SOFA score (Table 2).

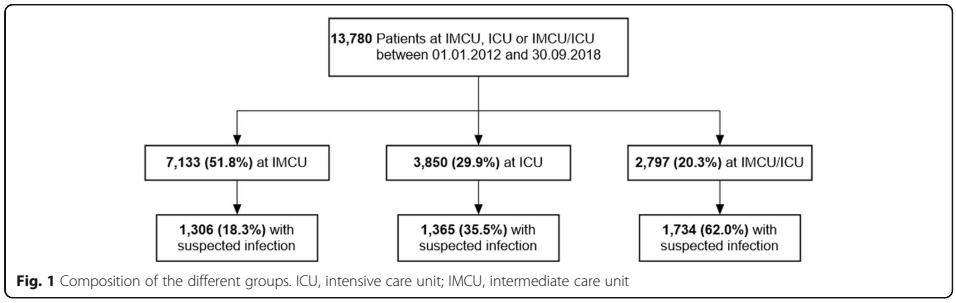

Fig. 1 Composition of the different groups. ICU, intensive care unit; IMCU, intermediate care unit

All IMCU patients with suspected infection who died (45 [3.4%]) fulfilled at least two SIRS criteria, while the SOFA score was positive in 12 (26.7%) lethal cases and the qSOFA score in 44 (97.8%) of those who died. The highest predictive validity for hospital mortality was achieved by calculating the qSOFA score, while SIRS criteria and SOFA score performed significantly inferiorly regarding their predictive validity (SIRS: AUCROC = 0.72 [0.71–0.72]; SOFA: AUCROC = 0.63 [0.56–0.69]; qSOFA: AUCROC = 0.82 [0.79–0.84]; SIRS vs. SOFA: $p = 0.006$; qSOFA vs. SOFA: $p < 0.001$; SIRS vs. qSOFA: $p < 0.001$; Fig. 3). SIRS criteria and qSOFA score reached high sensitivity and low specificity regarding mortality, while the SOFA score revealed contrary results (Table 3).

Performance of clinical scores in the ICU

Of 1635 ICU encounters with suspected infection, a SOFA score increase was identified in 446 (32.7%) encounters, while qSOFA scoring was positive in 1111 (81.4%) cases. In 1276 (93.5%) encounters, at least two SIRS criteria were fulfilled.

Overall, the discriminative power for the identification of patients receiving broad-spectrum antibiotic treatment in the cohort of patients with suspected infection was poor (SIRS: AUCROC = 0.63 [0.62–0.64]; SOFA: AUCROC = 0.65 [0.64–0.66]; qSOFA: AUCROC = 0.66 [0.65–0.68]; SIRS vs. SOFA: $p = 0.008$; qSOFA vs. SOFA: $p = 0.098$; SIRS vs. qSOFA: $p < 0.001$; Fig. 2). SIRS criteria and qSOFA score were highly sensitive but not specific for presumed sepsis, while contrary results were demonstrated for the SOFA score (Table 2).

Overall, 250 (15.3%) ICU patients with suspected infection deceased. A majority of these subjects showed positive SIRS criteria (241 [96.4%]) and qSOFA score (240 [96.0%]), while positive SOFA was detected only in 122 (48.8%) encounters. SIRS criteria and qSOFA score reached high sensitivity but low specificity regarding the prediction of mortality, while SOFA score revealed contrary results (Table 3). Overall, the predictive validity of all included scores was poor. However, compared to SIRS criteria, SOFA and qSOFA scores performed superiorly regarding the prediction of mortality (SIRS: AUCROC = 0.60 [0.59–0.62]; SOFA: AUCROC = 0.69 [0.66–0.72]; qSOFA: AUCROC = 0.69 [0.68–0.71]; SIRS vs. SOFA: $p < 0.001$; qSOFA vs. SOFA: $p = 0.92$; SIRS vs. qSOFA: $p < 0.001$; Fig. 3).

Clinical scores in patients treated at the ICU and IMCU

Among the 1734 (62.0%) encounters with suspected infection in patients who were admitted to the IMCU and ICU, 1676 (96.7%) showed at least two positive SIRS

Table 1 Basic patient characteristics

Parameter	IMCU	ICU	IMCU/ICU	All
Age (years)	61 [41–76]	64 [52–75]	69 [57–78]	64 [49–76]
BMI (kg/m^2)	26.8 [23.9–31.0]	26.2 [23.4–29.8]	26.7 [23.9–30.9]	26.6 [23.7–30.7]
APACHE II	4 [0–12]	12 [0–19]	18 [14–23]	10 [0–17]
Invasive ventilation	1.9%	52.3%	67.0%	29.2%
Need for catecholamines	4.3%	38.0%	57.1%	24.4%
Hospital stay	3.12 ± 11.91	4.80 ± 12.34	23.11 ± 33.79	7.65 ± 20.22
Hospital mortality	0.6%	6.5%	5.8%	3.3%
Infection	18.3%	35.5%	62.0%	32.0%

Data are expressed as median with interquartile range (IQR), percentage, or, if normally distributed, as mean with standard deviation (±)
APACHE II Acute Physiology and Chronic Health Evaluation, *BMI* Body mass index, *ICU* intensive care unit, *IMCU* Intermediate care unit

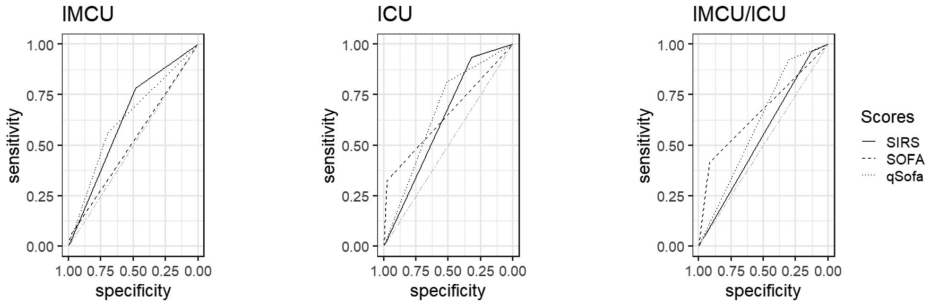

Fig. 2 Predictive validity for suspected infection using clinical scores. Receiver operating characteristic curves for positive SIRS criteria, SOFA score, and qSOFA score are pictured. ICU, intensive care unit; IMCU, intermediate care unit; qSOFA, quick Sequential Organ Failure Assessment; SIRS, systemic inflammatory response syndrome; SOFA, Sequential Organ Failure Assessment

criteria, while SOFA and qSOFA scores were positive in 721 (41.6%) and 1607 (92.7%) encounters, respectively.

The predictive validity for presumed sepsis of all scores was poor (SIRS: AUCROC = 0.55 [0.54–0.56]; SOFA: AUCROC = 0.67 [0.65–0.68]; qSOFA: AUCROC = 0.61 [0.60–0.63]; SIRS vs. SOFA: $p < 0.001$; qSOFA vs. SOFA: $p < 0.001$; SIRS vs. qSOFA: $p < 0.001$; Fig. 2). While the SIRS criteria and qSOFA score revealed high grades of sensitivity and low specificity, contrary results were demonstrated for the SOFA score (Table 2).

Moreover, mortality among the encounters with suspected infection on the IMCU/ICU amounted to 163 (9.4%). All of them offered a positive qSOFA score and at least two positive SIRS criteria (163 [100%]), while the SOFA score was increased in 119 (73.0%) encounters.

Regarding hospital mortality, the SIRS criteria and qSOFA score revealed only poor predictive validity, whereas the SOFA score was predictive for the patients' death (SIRS: AUCROC = 0.54 [0.53–0.54]; SOFA: AUCROC = 0.73 [CI, 0.70–0.77]; qSOFA: AUCROC = 0.59 [0.58–0.59]; SIRS vs. SOFA: $p < 0.001$; qSOFA vs. SOFA: $p < 0.001$; SIRS vs. qSOFA: $p < 0.001$; Fig. 3). SIRS criteria and qSOFA score reached high sensitivity but low specificity regarding mortality, whereas the SOFA score performed adequately (Table 3).

Table 2 Sensitivity and specificity of clinical scores for infection

Parameter	IMCU	ICU	IMCU/ICU
Sensitivity of SIRS	0.78	0.93	0.97
Specificity of SIRS	0.48	0.32	0.13
Sensitivity of SOFA	0.05	0.33	0.42
Specificity of SOFA	0.99	0.97	0.92
Sensitivity of qSOFA	0.56	0.81	0.93
Specificity of qSOFA	0.70	0.51	0.30

ICU Intensive care unit, *IMCU* Intermediate care unit, *SIRS* Systemic inflammatory response syndrome, *SOFA* Sequential Organ Failure Assessment, *qSOFA* Quick Sequential Organ Failure Assessment

Discussion

The rapid identification of sepsis serves as the basis for its successful management. According to the current recommendations of the Surviving Sepsis Campaign, the SOFA score should be used as a predictive tool for the detection of sepsis as well as for the risk stratification of critically ill patients. In addition, the qSOFA score has been introduced for the identification of septic patients outside of the ICU [7, 20, 21]. However, to our knowledge, both scores have not been evaluated in the context of surgical IMCU patients. Therefore, this is the first study comparing the predictive value for presumed sepsis of the SOFA and qSOFA scores, as well as the 1992 defined SIRS criteria, in a large cohort of 13,780 surgical IMCU and ICU patients of a tertiary university hospital.

Overall, among encounters with suspected infection in IMCU patients, none of the analyzed scoring tools showed sufficient predictive validity for severe infection (defined as the use of broad-spectrum antibiotics), whereas the qSOFA score was able to predict mortality in a sufficient manner. Interestingly, even though the assessment with the historical SIRS criteria does not meet the current practice guidelines, they were more predictive than the SOFA score within the IMCU patient cohort. Furthermore, among ICU patients as well as patients who underwent a combined IMCU and ICU treatment, all analyzed scoring systems failed to provide sufficient validity for the prediction of infection and mortality. Only in patients who underwent a combined IMCU and ICU treatment the SOFA score was able to adequately predict mortality.

At first glance, these study results might be surprising. However, in comparison to previous findings, the

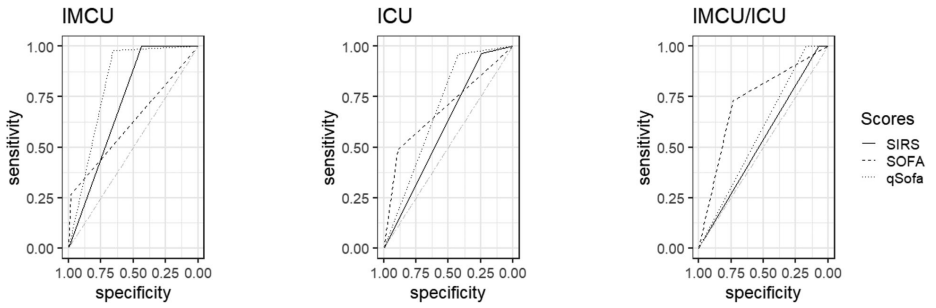

Fig. 3 Predictive validity for hospital mortality using clinical scores. Receiver operating characteristic curves for positive SIRS criteria, SOFA score, and qSOFA score are pictured. ICU, intensive care unit; IMCU, intermediate care unit; qSOFA, quick Sequential Organ Failure Assessment; SIRS, systemic inflammatory response syndrome; SOFA, Sequential Organ Failure Assessment

performance of the qSOFA score and the SIRS criteria remain agreeable. The qSOFA score was first developed and validated by Seymour et al., who analyzed 148,907 unselected patient encounters with suspected infection, consisting of a validation cohort of 7932 ICU and 66,522 non-ICU patients. With the exception of the SOFA score, the predictive value of the qSOFA and SIRS criteria could be matched to our study results within the ICU cohort (AUCROC SOFA 0.74 vs. 0.52; AUCROC qSOFA 0.66 vs. 0.63; AUCROC SIRS 0.64 vs. 0.63) [7]. Their findings have been validated in several studies featuring large numbers of patients (Table 4), resulting in a varying performance of the mentioned scores. However, it has to be stressed that originally Seymour et al. aimed to validate the qSOFA and SOFA scores as predictors for mortality and not for the identification of sepsis. The Sepsis-3 definition indicates that, due to their predictive value for mortality, both scores can be used for sepsis risk stratification (either at the ICU or non-ICU), but also emphasizes that the underlying data was derived from retrospective studies and requires further validation. However, until now, no prospective data, with sufficient numbers of patients, is available.

Table 3 Sensitivity and specificity of clinical scores for mortality

Parameter	IMCU	ICU	IMCU/ICU
Sensitivity of SIRS	1.00	0.96	1.00
Specificity of SIRS	0.44	0.24	0.07
Sensitivity of SOFA	0.27	0.49	0.73
Specificity of SOFA	0.98	0.89	0.74
Sensitivity of qSOFA	0.98	0.96	1.00
Specificity of qSOFA	0.65	0.42	0.17

ICU Intensive care unit, *IMCU* Intermediate care unit, *SIRS* Systemic inflammatory response syndrome, *SOFA* Sequential Organ Failure Assessment, *qSOFA* Quick Sequential Organ Failure Assessment

Lo et al. performed a literature review and retrospective data analysis of 380,920 patients, demonstrating an AUCROC of 0.68 for the predictive value of in-hospital mortality for the qSOFA score, which is in line with our study findings in surgical ICU and IMCU patients [20]. Furthermore, a meta-analysis of 229,480 patients compared the qSOFA score and SIRS criteria for their ability to predict patient mortality and revealed only a slightly better performance of the qSOFA score, which supports the findings of our study [22]. However, some studies revealed a high power for the prediction of mortality: Kovach et al. analyzed hospital mortality in a retrospective data set of 3749 surgical and medical ICU patients with suspected infection, while Zhang et al. investigated retrospectively 5109 cardiac surgical patients, with both studies resulting in AUCROC > 0.8 for the prediction of mortality by using the SOFA and qSOFA scores [21, 23]. However, it must be highlighted that, contrary to our approach, the patients of Kovach's study were adjusted for a baseline risk factor for death, which increased the predictive quality of the SOFA score, while Zhang et al. only included cardiac surgical patients, which are hardly comparable with the sources of systemic inflammation in our study. During cardiac surgery, systemic inflammation is mainly induced by cardiopulmonary bypass, which leads to strong activation of the inflammatory response through the blood's foreign surface contact with the components of the heart-lung machine, reperfusion injury/reperfusion injury [24]. Contrarily, local surgical trauma is causative for the onset of inflammation during non-cardiac surgery.

Even though qSOFA and SOFA scores are widely accepted as tools for the identification of septic patients, they failed to predict suspected infection in each individual group of patients in our study. These findings are supported by Krebs et al., who also evaluated the qSOFA and

Table 4 Overview studies regarding clinical criteria

Author	Patients	Collective	Primary outcome	Suspected infection	SIRS	SOFA	qSOFA
Lo et al. [20]	*n* = 380,920	Mixed	Mortality	No	n.a.	n.a.	0.68
Kovach et al. [21]	*n* = 10,981	ED; ICU; mixed	Mortality	Yes	0.79	0.90	0.84
Seymour et al. [7]	*n* = 7932	ICU; mixed	Mortality	Yes	0.64	0.74	0.66
Zhang et al. [23]	*n* = 5109	Surgical ICU	Mortality	No	0.95	0.96	0.95
Falcao et al. [31]	*n* = 3008	Surgical ICU	Mortality	No	n.a.	0.742	n.a.
Gando et al. [25]	*n* = 1045	ED; mixed	Infection	Yes	0.647	n.a.	0.582
Basile-Filho et al. [32]	*n* = 847	Surgical ICU	Mortality	No	n.a.	0.791	n.a.
Mungan et al. [33]	*n* = 233	Surgical ICU	Mortality	No	n.a.	0.631	n.a.
Innocenti et al. [34]	*n* = 135	ED-HDU; mixed	Mortality	Yes	n.a.	0.80	n.a.

ICU Intensive care unit, *ED* Emergency department, *HDU* High-dependency unit, *qSOFA* Quick Sequential Organ Failure Assessment, *SIRS* Systemic inflammatory response syndrome, *SOFA* Sequential organ failure assessment

SOFA scores as well as the SIRS criteria in 1942 prospective patient days within a cohort of surgical trauma ICU patients, concluding that all scores failed to predict the development of new infections [17]. But, also in an out-of-ICU setting, a failure of the qSOFA score (and SIRS criteria) has already been described in a collective of patients attending the emergency room (n = 1045) [25]. Moreover, another large retrospective analysis failed to prove a high predictive power of the qSOFA score and the SIRS criteria in patients admitted to the emergency department [26].

These opposing results might be partially explainable, as already discussed above, by the choice of the study population, which might strongly influence the study results because only postsurgical patients were investigated in our study, in contrast to medical and surgical patients in the underlying study. Further, the variable predictive validity between the studies might be caused by the differences in the study designs. Considering the original publication of Seymour et al., the lower predictive capacity of the SOFA score in our study might be caused by the varying definition of suspected infection. While it was defined as the combination of antibiotics and body fluid cultures by Seymour et al., the administration of broad-spectrum antibiotics was used in our approach. It has to be noted that the prescribing behavior of antibiotics varies between physicians depending on their clinical experience, qualification, and specialty. Charani et al. compared the antibiotic prescribing between medical and surgical specialties. Besides more frequent and longer prescription, antibiotics were more likely to be escalated in surgical patients [27]. A recent systemic review offers a potential explanation for these findings by identifying nine determinants that influenced antibiotic prescription behavior including the fear of risking an adverse outcome [28]. Surgical patients are challenging when it comes to identifying infectious complications, and the consequences of sepsis are more devastating in these patients which potentially leads to a more liberal application of broad-spectrum antibiotics [29, 30]. This might offer an explanation for the low specificity of the analyzed scores for detecting a presumed sepsis. Furthermore, even in an isolated analysis of studies that only investigated surgical patients, the predictive performance varies strongly: Falcao et al. analyzed 3008 surgical ICU patients and showed a sufficient predictive validity of the SOFA score regarding mortality (AUCROC of 0.74) [31]. Similar results are published by Basile-Filho et al., who revealed an AUCROC of 0.79 by using the SOFA score for the prediction of mortality within 847 surgical ICU patients [32]. Contrarily, Mungan et al. showed a lower predictive validity of 0.63 of the SOFA score in surgical ICU patients [33]. Although authors of these studies described their population as "surgical patients," it must be highlighted that their calculations comprised all kinds of surgical patients, independently of their risk for infection, including those without suspicion of infection. By contrast, our study only focused on the investigation of postsurgical patients with suspected infection. In our opinion, this issue is of high relevance because the majority of postsurgical patients following major surgery regularly show clinical signs of systemic inflammation such as tachycardia, fever, and tachypnea, which commonly represent signs of a surgery-induced systemic inflammation rather than an infection. For this reason, it is not only challenging to discriminate between postsurgical sterile systemic inflammation and infection, but the predictive ICU sores might also become distorted into false positive results. This may explain the high sensitivity but low specificity of the qSOFA score and SIRS criteria because both systems include only clinical criteria for easy assessment. Since these criteria are often fulfilled during the postsurgical phase, the chance that they are truly positive is high (sensitivity). On the other hand, this leads to a low rate of true false cases (specificity). Since the SOFA score consists of much more detailed intensive care variables than the qSOFA score and the

SIRS criteria, the specificity is higher, but sensitivity remains low. These arguments are in accordance with the findings of Gando et al. as well as Krebs et al., who demonstrated that the SIRS criteria, SOFA score, and qSOFA score were not able to predict sepsis in the emergency department or surgical ICU [17, 25].

These limitations of the ICU scores are of high interest for their use on surgical IMCUs because of the increasing demand of IMCU capacity. Therefore, the importance of the surgical IMCU, as a bridge to the normal ward, is rising. Patients attending the IMCU commonly represent those surgical patients at moderate to high risk of developing postsurgical complications. Analogous to ICU patients, the rapid identification of infectious complications is altered by surgery-induced signs of systemic inflammation, underlining the need for specific IMCU scores. Lacking studies that focus on surgical IMCUs, other high-dependency units (HDUs) (but not ICUs) have to be analyzed for the interpretation of our study results. Innocenti et al. analyzed 3311 patients admitted to HDUs and demonstrated that the SOFA score, in opposition to our results, showed a good discriminatory ability for HDU mortality [34]. However, contrary to our approach, no cutoff values for SOFA scores were used, and no postsurgical patients were included. In our study, the prediction of mortality was sufficient using the qSOFA score in IMCU patients. Another study showed that these scores are also not able to predict infection in the emergency room [25]. Based on the findings of our study, the use of the qSOFA score as a predictor of mortality can be supported, while its predictive power for the detection of suspected infection can be doubted in postsurgical IMCU patients, which might be caused by surgery-induced systemic inflammation.

However, due to the retrospective study design, further prospective studies that include high numbers of postsurgical IMCU patients are necessary to validate these findings. Due to the fact that most critically ill patients are regularly transferred to the IMCU during their medical treatment, a subgroup of these patients was created. The increased APACHE II score reflects the serious illness of the included patients. Since clinical scores were not able to distinguish for suspected infection in this subgroup, severity of disease seems not to improve the quality of these scores. While adequate prediction for mortality was calculated with the SOFA score, this could indicate its better quality in critically ill patients. Since these patients are missing in the ICU subgroup, this could also explain our lower results for the SOFA score in the ICU.

Nevertheless, our study features some limitations. First, this retrospective analysis is not able to draw conclusions regarding the underlying causalities. Second, due to the retrospective design, no sample size calculation was performed. Third, lacking of more specific alternatives, the administration of broad-spectrum antibiotic agents was used as a surrogate for the diagnosis of suspected infection. While the clinical symptoms and inflammatory parameters are physiologically altered by the surgery, body fluid cultures result in negative samples in a majority of cases (e.g., due to the perioperative antibiotic treatment) [28, 35, 36]. Furthermore, even if sepsis was assessed by intensivists, its diagnosis remains subjective [37]. Nonetheless, it has to be highlighted that the administration of a broad-spectrum antibiotic agent serves only as a surrogate for the true presence of sepsis. Fourth, the RAAS was used as a surrogate parameter for GCS < 15 in the absence of the GCS, which is problematical since the qSOFA score was developed and validated with the use of GCS.

Conclusions

In summary, neither SOFA nor qSOFA score was able to distinguish for suspected sepsis (defined by the application of broad-spectrum antibiotics) in surgical patients, independently of IMCU, ICU, or IMCU/ICU stay. Nevertheless, the qSOFA score revealed sufficient prediction for mortality in the IMCU. Further, as the SOFA score showed the best results regarding mortality in IMCU/ICU patients, its predictive quality depended on the severity of the disease. Summarizing, it remains unclear whether qSOFA or SOFA score should be used in surgical IMCU patients for risk stratification.

Abbreviations

APACHE II: Acute physiology and chronic health evaluation II; AUROC: Area under the receiver operating curve; GCS: Glasgow coma scale; HDU: High-dependency unit; ICU: Intensive care unit; IQR: Interquartile range; IMCU: Intermediate care unit; qSOFA: Quick sequential organ failure assessment; RAAS: Richmond agitation sedation scale; RR: Respiratory rate; SBP: Systolic blood pressure; SD: Standard deviation; SIRS: Systemic inflammatory response syndrome; SOFA: Sequential organ failure assessment; STROBE: Strengthening the reporting of observational studies in epidemiology

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Authors' contributions

CK, FE, ES, and MS were responsible for the study design and conduct, CK, FB, and TF data acquisition, statistical analysis and interpretation, and writing of the manuscript. MM performed the statistical analysis and CK, FE, ES, AH, and CK interpretation, and approved and helped to draft the manuscript. All authors approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics approval and consent to participate

This study was approved by the local ethics committee (Justus-Liebig-University, Giessen, Germany, trial code 240/16). The study methods and results are presented in accordance with STROBE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

8.3 Anlage 3

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Original Articles

Longitudinal Evaluation of Plasma Concentrations of Presepsin in Patients after Severe Trauma: A Prospective Observational Study

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Abstract

Background: The high mortality rate of patients suffering from severe trauma is based not only on the mechanism of injury, but also on the higher risk for development of subsequent infections. Therefore, the early recognition of infection after severe trauma is of particular importance for patient outcome. However, early diagnosis is often masked by the consequences of the sterile, damage-triggered immune response. Our study sought to analyze the course of soluble CD14-subtype (sCD14-ST, presepsin) compared with clinically established inflammatory and infectious biomarkers in a cohort of patients with severe trauma.
Patients and Methods: Between January 2015 and February 2016, 50 patients suffering from severe trauma (Injury Severity Score [ISS] > 16) were enrolled and followed up for seven consecutive days after intensive care unit (ICU) admission. Clinical routine data, signs of infection, and the inflammatory biomarkers presepsin, C-reactive protein (CRP), procalcitonin (PCT), and interleukin-6 (IL-6) were assessed.
Results: Regarding the well-established biomarkers CRP, PCT, and IL-6, we observed trauma-associated alterations (day 1: CRP 13 mg/L, interquartile range [IQR] 0–129; PCT 1.1 μg/L, IQR 0–13; IL-6 108 pg/mL, IQR 29–795), which did not correlate with the clinical development of systemic inflammatory response syndrome (SIRS), whereas elevated plasma concentrations of presepsin in the clinical course were associated with the presence of SIRS (presepsin: no-SIRS vs. SIRS $p = 0.03$).
Conclusion: Our study investigates systematically the kinetic of presepsin compared with established inflammatory and infectious markers after severe trauma. Presepsin is neither affected by the early post-traumatic nor the delayed immune response over seven days after trauma, making it a possible option as a diagnostic biomarker of infection worth further evaluation.

Keywords: biomarker; sepsis; SIRS

Severe trauma is the fifth leading cause of death in Germany [1]. In addition to the acute traumatic damage of tissues and resulting direct complications such as hemorrhage, pneumothorax, or traumatic brain injury, trauma-induced alterations on cellular and molecular levels can affect patient's morbidity, mortality, and long-term outcome.

Tissue hypoperfusion during shock and subsequent cell death as well as the direct physical damage result in the release of immunogenic damage-associated molecular patterns (DAMPs), capable of triggering a systemic inflammatory response syndrome (SIRS) [2,3]. Damage-associated molecular patterns (DAMPs) are recognized by a variety of pattern recognition receptors (PRRs) [4]. Among these receptors, especially the activation of toll-like receptors (TLRs) leads to the release of pro-inflammatory and anti-inflammatory mediators, which can trigger excessive inflammation, sepsis, and multiple organ failure (MOF) [5,6].

Currently the development of infection and sepsis after severe trauma represents a major challenge to modern intensive care medicine. Despite continuous progress in

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antibiotic therapy, surgical source control, and adjuvant therapies, sepsis remains one of the most common disease entities in intensive care units (ICUs) and is reported as a main cause of death among critically ill patients [7–9].

The high mortality rate of patients with severe trauma is based not only on the trauma mechanism, but also on the higher risk for the development of infections [10]. An epidemiologic study reports an incidence of 4% of trauma-associated infectious complications, especially blood stream infections [11]. Therefore, the early diagnosis of infection and immediate administration of anti-infective therapy is of particular importance for the severely injured patient's outcome. However, early and rapid diagnosis of infection is often impeded by the physiologic reactions of sterile damage-triggered immune response [12]. In order to differentiate post-traumatic SIRS from nosocomial infections, the predictive value of intensive care scoring systems such as Acute Physiology And Chronic Health Evaluation (APACHE II) score or Injury Severity Score (ISS) have been validated [13], but are still discussed controversially [14]. Biomarkers are seen as a supplemental approach within this context, whereas the acute phase proteins C-reactive protein (CRP), procalcitonin (PCT), and interleukin 6 (IL-6) are the most commonly used biomarkers for early assessment of infection in daily clinical routine [14]. The new biomarker soluble CD14 subtype (sCD14-ST, presepsin) exhibits a high diagnostic value of detecting patients with sepsis within different clinical contexts [15,16]. Furthermore, plasma presepsin levels indicate a more specific infectious origin of inflammation among different cohorts of patients in comparison to the established infectious biomarkers CRP, PCT, and IL-6, which regularly show alterations resulting from non-infectious origins [15,17–22]. In a pilot cohort study, Hoshino et al. [23] found no trauma-associated elevation of presepsin in patients 24 hours after severe trauma. This study sought to analyze the course of presepsin compared with established inflammatory biomarkers (CRP, PCT, IL-6) in patients suffering from severe trauma.

Patients and Methods

Study Design

After approval by the local ethics committee (No. 164/14), we performed a prospective observational study (German Clinical Trials Register, Trial registration: DRKS00010991). Between January 2015 and February 2016, 50 consecutive trauma patients were enrolled at the surgical intensive care unit (ICU) of the University Hospital of Giessen (Germany). All enrolled patients gave written informed consent or written informed consent was obtained from the nominated legally authorized representative on behalf of participants in accordance with ethical standards. Inclusion criteria were defined as at least 18 years of age, severe trauma (ISS >16), and admission to the surgical ICU. Patients were excluded in cases of positive history of chronic viral diseases (human immunodeficiency virus [HIV], hepatitis B/C). Patients were observed for seven consecutive days after ICU admission and screened for signs of infection and SIRS according to the criteria of the Society of Critical Care Medicine (SCCM) and the American College of Chest Physicians (ACCP) [24].

Management of blood samples

Blood samples were collected at study inclusion and in the morning on the seven consecutive days in the ICU. Quantification of presepsin was performed with 7.5 mL heparinized blood at each time point of extraction using a point-of-care analyzer (PATHFAST™ Presepsin, Mitsubishi Chemical, Tokyo, Japan) according to the manufacturer's instructions. Additional laboratory data were obtained from clinical routine blood analysis and contained inflammatory parameters PCT, CRP, IL-6, and leucocyte count. For CRP, PCT, and IL-6 quantification lithium heparin plasma samples were used. C-reactive protein was measured with the Siemens ADVIA® system (Siemens, Erlangen, Germany) using the manufacturer's reagent, whereas PCT was quantified by the Siemens Centaur® system and Thermo Fischer Scientific Brahms reagent (Brahms, Henningsdorf, Germany). Interleukin-6 was measured by the Siemens Immulite® system using the Siemens reagent.

Data management and statistical analysis

Clinical routine data were extracted from an electronic patient data management system (ICUData®, Imeso GmbH, Giessen, Germany) and included patient's baseline data, vital signs, and relevant intensive care therapy information. For multiple group comparisons, an initial global Kruskal-Wallis test was performed, followed by Mann-Whitney U test for pairwise comparisons in case of rejected global null hypothesis ($p < 0.05$). Correlation analysis included non-parametric Spearman ρ testing; SPPS (version 23, IBM, Armonk, NY) was used for statistical analysis.

Results

Study population

During the recruitment period, we included 50 adult patients suffering from severe trauma. A first blood sample was taken after initial resuscitation and, if necessary, after damage control surgery. The maximum elapsed time between hospital admission and first blood sample was 15 hours. The cohort consisted mainly of male patients (72%) with a median age of 47 years (interquartile range [IQR] 19–83 y; Table 1). The patients presented with a median body mass index (BMI) of 24.7 kg/m^2 (IQR 20.2–32.9 kg/m^2) and the high severity of injuries was reflected by a median ISS of 22 (IQR 17–34).

In the majority of cases, patients presented with thoracic injuries (90%), followed by injuries to the external tissue (86%) and limbs (70%). Three patients (6%) died during the 30-day observation period from a trauma-related non-infectious cause, of whom one patient (2%) died within the first 24 hours of treatment. The median length of ICU stay was six days (IQR 2–34 d) and the overall median hospital stay was 18 days (IQR 3–46 d). Thirty patients (60%) developed SIRS at some point during their hospital stay. One case of sepsis was observed within our study cohort during the subsequent seven-day clinical observation period. We registered two cases of acute kidney failure and one hemostasis dysfunction based on liver injury. There was no chronic liver or kidney disease in the patient's medical history.

Table 1. Baseline Characteristics of the Study Cohort

	Whole study cohort n = *50*
Age	
years 47 (19–83)	
Gender	
male 36 (72)	
BMI	
kg/m^2 24.7 (20.2–32.9)	
Comorbidities	
History of COPD	0
History of myocardial infarction	1 (2)
History of congestive heart failure	1 (2)
History of renal failure	0
History of immunosuppression	0
Current smokers	10 (5%)
Scores at admission	
ISS	22 (17–34)
NISS	27 (17–34)
RISC	3.18 (0–4.22)
APACHE II	14 (7–31)
SOFA	5.5 (2–15)
Injuries	
Head injury	28 (56)
Thorax trauma	45 (90)
Abdominal trauma	28 (56)
Extremity trauma	36 (72)
External injuries	43 (86)
Cause of injury	
Car accident	24 (48)
Motorcycle accident	10 (20)
Bicycle accident	3 (6)
Fall	10 (20)
Unknown or other	3 (6)
Outcome	
Hospital mortality	3 (6)
30-d mortality	3 (6)
Duration of treatment	
ICU stay	
days 6 (2–34)	
Hospital stay	
days 18.5 (3–46)	
Mechanical ventilation	
hours 14 (0–291)	

Values are given as number (% total) or median (interquartile range) for age, BMI, clinical scores, and durations.

APACHE II = Acute Physiology And Chronic Health Evaluation Score; BMI = body mass index; COPD = chronic obstructive pulmonary disease; ICU = intensive care unit; ISS = Injury Severity Score; NISS = New Injury Severity Score; RISC = Revised Injury Severity Classification Score, SOFA = Sequential Organ Failure Assessment Score.

Time course of presepsin, PCT, CRP, and IL-6 after trauma

Presepsin, IL-6, and PCT were measured daily over a period of seven days after hospital admission (Fig. 1). We observed initial presepsin concentrations of 487 pg/mL (IQR 123–1901) with no obvious kinetic over time (Fig. 1A; $p = 0.746$). On day 6, the concentrations of presepsin were highest within the observation period (802 pg/mL, IQR 100–4298). In contrast, IL-6 values were increased early after trauma (108 pg/mL, IQR 29–795) and gradually decreased over time until day six (36 pg/mL, IQR 0–364; Fig. 1B; $p < 0.001$). We also found PCT concentrations above the local hospital cutoff (> 0.5 µg/L) during the first three days after admission, with highest concentrations on day one (median: 1.1 µg/L, IQR 0–13) and an average decrease below pathologic concentrations at day four (Fig. 1C; $p = 0.002$). Lowest CRP values were found at hospital admission (13 mg/L, IQR 0–129), peaking on day 2 (166 mg/L, IQR 65–351) (Supplementary Fig. S1A; $p < 0.001$; see online supplementary material at http://www.liebertpub.com/sur), whereas leucocytes increased late on day six and later (Supplementary Fig. S1D; $p < 0.001$).

Presepsin, PCT, and IL-6 at hospital admission according to the severity of trauma

Baseline values of presepsin, IL-6, and CRP at hospital admission were clustered into three groups according to trauma severity (reflected by the ISS; Fig. 2A–2C). An ISS <20 is a predictor for lower mortality [25] and an ISS >30 indicated approximately 10% worst-injured patients in our study cohort. Regarding presepsin or PCT, we did not observe differences between the groups (Fig. 2A and 2C). In contrast, we found differences in IL-6 between patients of the lowest (ISS 16–20) and highest ISS (ISS >30; $p = 0.05$) as well as between the population of moderate (ISS 21–30) and highest ISS ($p = 0.022$; Fig. 2B). Median IL-6 values at hospital admission were considerably elevated in all three groups (local hospital cutoff value ≥50 pg/mL) (ISS 16–20: 66 pg/mL, IQR 0–550; ISS 21–30: 105 pg/mL, IQR 29–795; ISS >30: 435 pg/mL, IQR 165–1203).

Association of biomarkers with the clinical presence of SIRS

To evaluate the association of biomarker plasma concentrations with the presence of SIRS, all available samples were stratified by the occurrence of SIRS or no-SIRS. Changes between presepsin and IL-6 were identified (presepsin: no-SIRS vs. SIRS $p = 0.03$; IL-6: no-SIRS vs. SIRS $p = 0.03$; Fig. 3A and 3B). In contrast, PCT values did not differ between SIRS and no-SIRS patients ($p = 0.504$; Fig. 3C).

Presepsin and PCT values in patients with or without abdominal trauma

Differentiating patients with or without abdominal injuries, higher median values of presepsin and PCT were observed during the clinical course after abdominal trauma. For presepsin, plasma levels between groups of patients with and without abdominal trauma began to increase from day five (not significant; Fig. 4A). In contrast, PCT was increased in patients with abdominal trauma from day two and persisted throughout the observation period (Fig. 4B).

Correlation of investigated biomarkers

Upon stratification of the samples into four groups according to their PCT concentrations, a clear trend of increasing presepsin concentrations can be observed (Fig. 4C). Moreover, the plasma concentrations of the biomarker presepsin exhibited a positive and significant correlation to all examined classic biomarkers (IL-6: $p < 0.001$; PCT: $p < 0.001$; CRP: $p = 0.003$), but only with IL-6 and PCT to a

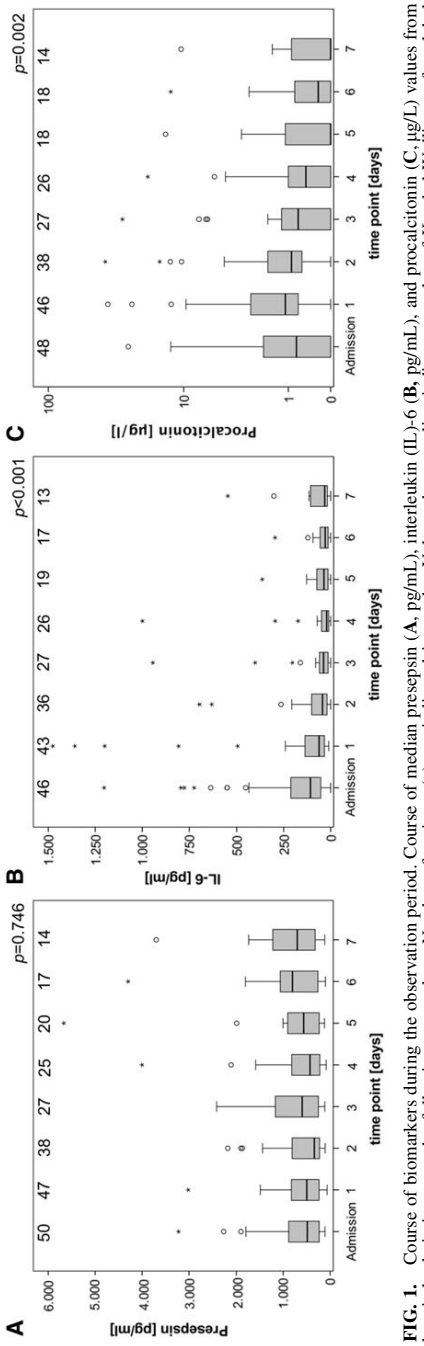

FIG. 1. Course of biomarkers during the observation period. Course of median presepsin (**A**, pg/mL), interleukin (IL)-6 (**B,** pg/mL), and procalcitonin (**C**, µg/L) values from hospital admission over the following seven days. Number of patients (n) are indicated in graphs. Values above outline indicate p -value of Kruskal-Wallis test for global differences.

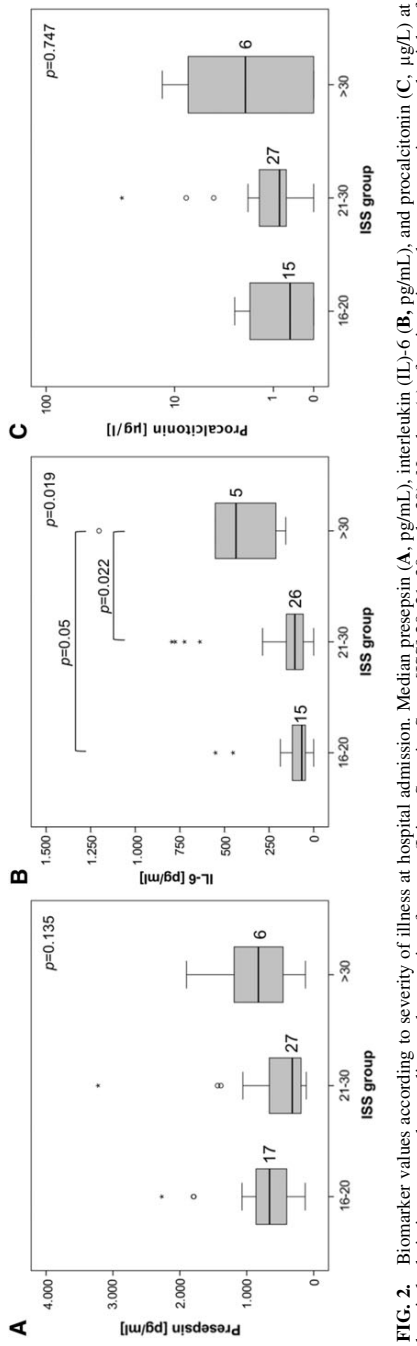

FIG. 2. Biomarker values according to severity of illness at hospital admission. Median presepsin (**A**, pg/mL), interleukin (IL)-6 (**B,** pg/mL), and procalcitonin (**C**, µg/L) at hospital admission, grouped according to the severity of trauma (Injury Severity Score [ISS] 20, 21–30 and >30). Number (n) of patients in each group are given on the right of the boxes. Values in the upper right corner within outlines indicate p value of Kruskal-Wallis test for global differences.

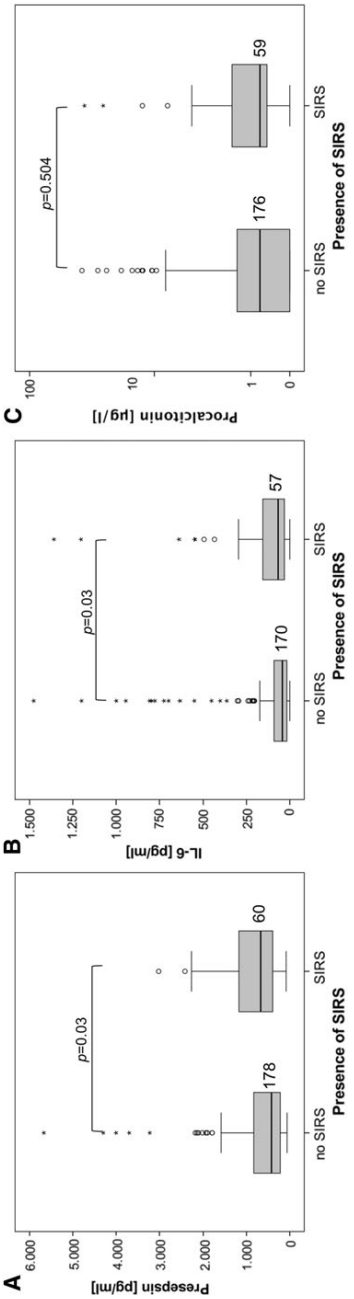

FIG. 3. Association of biomarkers with the clinical presence of systemic inflammatory response syndrome (SIRS). Comparison of median presepsin (**A**, pg/mL), interleukin (IL)-6 (**B**, pg/mL), and procalcitonin (**C**, μg/L) over the entire observation period, grouped according to presence of SIRS. Number (n) of samples in each group are given on the right of the boxplots.

moderate extent (p = 0.479 and p = 0.449, respectively; Fig. 4D).

Discussion

Our study sought to examine the kinetics of established and novel biomarkers of inflammation and infection in patients suffering from severe trauma to evaluate their usefulness in this context. Trauma patients are at high risk for developing infectious complications during their hospital stay. Therefore, early and accurate differentiation between sterile inflammation and infection might reduce delayed trauma-associated mortality [10,11].

Biomarkers are used for the prediction of inflammatory and infectious processes among different cohorts of patients. Previous studies have shown a trauma-associated elevation of routinely used biomarkers [26–28], rendering them not sensitive enough to detect infection among trauma cohorts with high values of trauma-associated sterile inflammation and increasing the need for novel markers. Former study data support presepsin as a powerful marker for the diagnosis of infection, especially sepsis among different cohorts of patients [15,29–31]. Hoshino et al. [23] found no trauma-associated elevation of presepsin in trauma patients 24 hours after severe trauma [23]. Our study also proves presepsin to be uninfluenced by the early sterile inflammatory response after trauma, whereas it still adheres to the clinical presence of SIRS.

The systemic immune response after major trauma has been shown to cause severe damage to multiple organs caused by the initial cascade of inflammation aggravated by subsequent infection—and in the worst case, sepsis—to which the body has become susceptible [32]. Among our study cohort, no initial trauma-induced increase of presepsin was detected, but a delayed increase of its plasma concentrations, especially on the third and fourth day after trauma. Regarding the results from earlier study cohorts, normal values for presepsin were reported as 517 pg/mL [33] and 58–339 pg/mL [34]. Investigating surgical patients with suspected abdominal sepsis, Vodnik et al. [35] observed higher presepsin values in patients with confirmed sepsis (1508.3 ± 866.6 pg/mL) compared with patients representing isolated SIRS (430.0 ± 141.33 pg/mL; $p < 0.0001$). Additionally, Zhang et al. [36] identified presepsin as an effective biomarker for the diagnosis of sepsis in a systematic meta-analysis. Among the 11 included studies, the threshold ranged from 317 to 729 pg/mL, the area under the curve (AUC) ranged from 0.70 to 1.00, sensitivity from 0.70 to 1.00, and specificity from 0.62 to 0.93. Regarding these findings, compared with the values observed in our study cohort (time of admission 487 pg/mL [IQR 123–1901] with no obvious change over time), we conclude that longitudinal marker assessment might be superior to single-point or cross-sectional assessment with a binary cutoff value in the discrimination between sterile SIRS and infection.

Grotz et al. [37] hypothesized that SIRS in patients presenting with severe abdominal trauma is based on the translocation of bacteria through the intestinal wall as a result of tissue hypoperfusion [38]. Activation of the gastrointestinal immune system results in an irritation of visceral nerves leading to a neuro-humoral mediated SIRS. Our data do not support presepsin as being sensitive for SIRS after abdominal trauma. This may be explained by the neurohumoral genesis

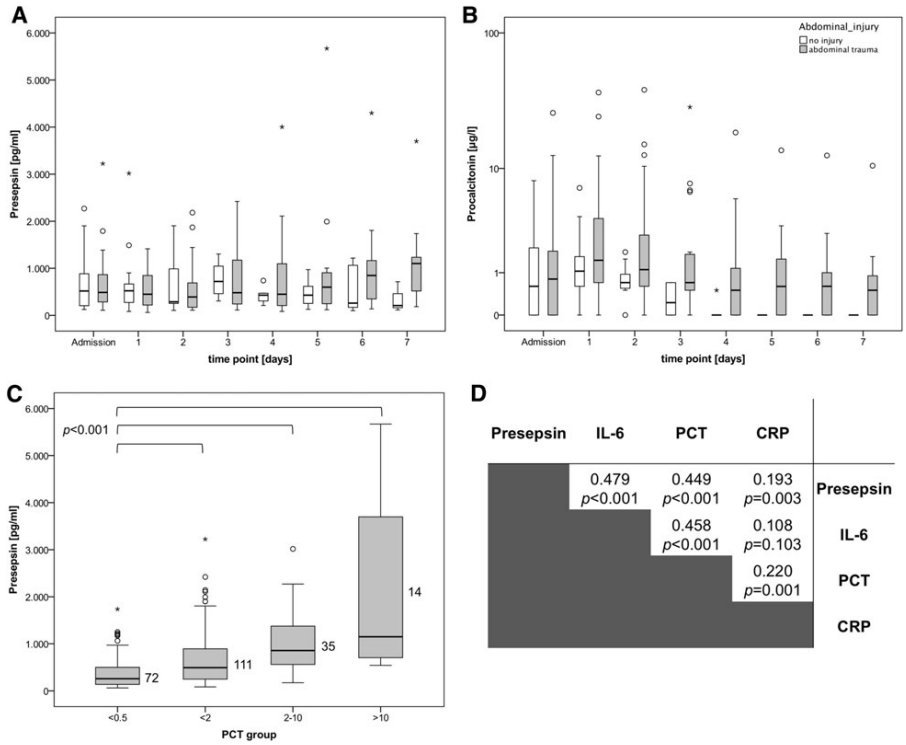

FIG. 4. Correlation analyses of the investigated biomarkers Comparison of median presepsin (**A**, pg/mL) and procalcitonin (**B**, µg/L) values according to the presence of an abdominal injury. Correlation of presepsin values and grouped procalcitonin (PCT) values (< 0.5/< 2/2–10/> 10 µg/L) (**C**). Number (n) of patients in each group are given on the right of the boxplots. Correlation of presepsin, IL-6, PCT, C-reactive protein (CRP) (**D**).

of non-sterile SIRS after abdominal injury, which may alter the concentrations of presepsin. In contrast, PCT issued sensitivity for SIRS within the clinical course after traumatic abdominal injury. Nevertheless, patients with a high level of PCT presented a trend toward higher levels of presepsin (considering all time points). For PCT, we can observe a trauma-induced increase, which was according to our data, irrespective of the trauma severity or the presence of SIRS.

Among the established biomarkers of inflammation and infection, IL-6 is known to predict an early phase of inflammation before the increase of circulating CRP and fever occurs. It has been proven that during bacterial infections, IL-6 concentrations increase as early as within two hours after endotoxin administration and then gradually decrease [39]. Interleukin-6 is also useful as a negative prognostic marker in patients newly admitted to ICUs across the entire spectrum of SIRS severity [40]. For example, current guidelines for the diagnosis and treatment of sepsis among pediatric patients recommend the clinical use of IL-6 [41]. In contrast, in our study we were not able to identify an association of IL-6 with trauma patients experiencing a SIRS. In line with the study of Gebhard et al. [42], we showed an early increase of IL-6 after severe trauma. Moreover, consistent with previous studies, patients with the most severe injuries presented with the highest IL-6 plasma levels [42,43].

The available literature for the course of CRP after severe trauma reported near-normal serum CRP levels on admission (median 8.5 mg/L vs. 7.5 mg/L), reaching peak values (median 110 mg/L) three days after trauma [44], corresponding with our findings. Giannoudis et al. [44] reported no correlation between CRP and ISS in a trauma population, which is also in line with our current data.

Serum PCT levels increase in severe systemic infections but may also be elevated in non-infectious conditions [18,45]. Whereas only a minimal increase of PCT plasma levels is observed in cases of SIRS, significant elevation occurs in (gram-negative) sepsis [46]. PCT plasma re-induction indicates possible septic complication during SIRS after major trauma. In addition, high PCT concentration in trauma patients at ICU admission indicates an increased risk of septic complications [47]. Nevertheless, we are not able to

demonstrate an association between PCT and the occurrence of SIRS after severe trauma.

This study has some limitations. With only 50 patients, the statistical power of the study is limited, but nevertheless, to our knowledge, this is the first prospective study that evaluates systematically the course of presepsin levels compared with clinically used biomarkers of inflammation and infection. Furthermore, the measured mortality among our study cohort (6%) seemed low. That might be because we did not include pre-hospital and trauma room deaths. Further studies enrolling a larger number of patients need to be conducted to clarify the usefulness of presepsin in this context.

Conclusion

Our study investigates systematically the kinetic of presepsin compared with established inflammatory and infectious markers following severe trauma. Presepsin is neither affected by the early post-traumatic nor the delayed immune response over seven days after trauma, making it a possible option as diagnostic biomarker of infection worth further evaluation.

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Author Disclosure Statement

No competing financial interests exist.

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8.4 Anlage 4

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Host-Derived *Delta-Like Canonical Notch Ligand 1* as a Novel Diagnostic Biomarker for Bacterial Sepsis—Results From a Combinational Secondary Analysis

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Hildebrand D, Decker SO, Koch C, Schmitt FCF, Ruhrmann S, Schneck E, Sander M, Weigand MA, Brenner T, Heeg K and Uhle F (2019) Host-Derived Delta-Like Canonical Notch Ligand 1 as a Novel Diagnostic Biomarker for Bacterial Sepsis—Results From a Combinational Secondary Analysis. Front. Cell. Infect. Microbiol. 9:267. doi: 10.3389/fcimb.2019.00267

Background: Sepsis is a life-threatening syndrome, resulting from a dysbalanced host response to infection. However, especially the early, pro-inflammatory immune response in sepsis is similar to other inflammatory conditions without infectious cause, e.g., trauma or surgery. This aspect challenges the value of current biomarkers for diagnosis, as these are often broadly induced. We earlier identified *Delta-like Protein 1* (DLL1), a canonical Notch ligand, to be released from monocytes upon bacterial stimulation. Considering the importance of monocytes in the pathophysiology of sepsis, we hypothesized that this mechanism might occur also in the clinical setting and DLL1 might serve as a biomarker of life-threatening bacterial infection.

Methods: We combined samples from three different studies, including subgroups of patients with sepsis ($n = 80$), surgical patients ($n = 50$), trauma patients ($n = 36$), as well as healthy controls ($n = 50$). We assessed plasma concentrations of DLL1 using ELISA. We performed Area-under-receiver-operator-curve (AUROC) analysis to evaluate the diagnostic performance of DLL1 compared to leucocytes, C-reactive protein (CRP), and procalcitonin (PCT).

Results: Plasma concentrations of DLL1 were strongly elevated already at sepsis onset and maintained elevated until day 7. In contrast, neither surgical patients nor patients after severe trauma presented with elevated levels, while conventional biomarkers of inflammation (e.g., leucocytes and CRP), responded. AUROC analysis revealed a cut-off of 30 ng/ml associated with the best diagnostic performance, yielding a superior accuracy of 91% for DLL1, compared to 75, 79, and 81% for CRP, leucocytes, and PCT.

Conclusion: DLL1 is a novel host-derived biomarker for the diagnosis of sepsis with a better performance compared to established ones, most likely due to its high robustness in non-infectious inflammatory responses.

Clinical Trial Registration: POCSEP-Trial DRKS00008090; MIRSI DRKS00005463; SPRINT DRKS00010991.

Keywords: trauma, SIRS, sepsis, monocytes, shock, inflammation, infection, DLL1

INTRODUCTION

Since decades, extensive research is conducted to identify biomarkers of sepsis. In 2010, a total of 178 published biomarkers were identified, and new ones are emerging nearly on a daily base (Pierrakos and Vincent, 2010). Most of the markers solely possess prognostic values and thereby lacks the actionable result a clinician is urgently expecting from a biomarker by aiding diagnosis, response prediction or therapeutic monitoring. Considering the few markers with diagnostic value, close to none have come the long way to clinical routine. In 2016, sepsis has been re-defined as life-threatening organ dysfunction, arising from the dysregulated host response to infection (Singer et al., 2016). Therefore, the purpose of a biomarker is to clarify, if the critical condition of the patient is a result of infection demanding antibiotic treatment, or—most probably of greater importance—caused by other reasons. Latter will make unnecessary exposure to antibiotics obsolete and will free resources to proceed with extended diagnostics. However, the urgent clinical need for rapid diagnosis derives from the evidence that each hour of delayed antibiotic treatment increases mortality between 2 and 4% (Bloos et al., 2017; Seymour et al., 2017).

Today, the most abundant biomarkers measured in clinical routine are C-reactive protein (CRP) and procalcitonin (PCT) (Biron et al., 2015). PCT is often claimed as "gold standard" for the diagnosis of infection (or even sepsis). However, while evidence for PCTs usefulness in the decision for the discontinuation of antibiotic treatment is steadily growing (Rhee, 2017), its diagnostic value is under steady debate and largely depends on the clinical context of assessment (Tang et al., 2007; Wacker et al., 2013). Compared to PCT, the acute phase protein CRP is often supposed to be of lower specificity, as also conditions as surgery, cancer or severe trauma can increase its plasma levels. However, this kinetic also holds true for PCT (Parli et al., 2018). This unravels a statistical misconception and dilemma: the essential indices of a diagnostic test, above all sensitivity and specificity, are a result of a dichotomous grouping of patients according to an arbitrary threshold. By adapting this, sensitivity and specificity inevitably change as well. In real life, however, adaptions of thresholds are hardly manageable, rising the need for the identification of robust biomarkers for diagnosing life-threatening infection (alias sepsis) in as diverse as possible clinical settings and patient populations.

Delta-like canonical Notch ligand 1 (DLL1), among others, belongs to the Delta/Jagged family of transmembrane proteins (Kovall et al., 2017). As a ligand of the Notch receptors, cell-surface DLL1 activates downstream signaling pathways upon receptor binding, which are critically involved in a plethora of processes during embryonic development, angiogenesis and hematopoiesis (Bray, 2016). Contrastingly, uncontrolled activation of Notch signaling has been connected to disturbances in development and cancer (Penton et al., 2012; Capaccione and Pine, 2013), hinting toward Notch and its ligand as therapeutic targets as well (Briot and Iruela-Arispe, 2015). As a result of the interaction, receptor and its ligand DLL1 are cleaved enzymatically from the surface, resulting in the generation of soluble DLL1 (sDLL1).

We recently found an upregulation of DLL1 in primary human monocytes in response to *in vitro* infection with various bacteria (Hildebrand et al., 2018). As a consequence, DLL1 triggered Notch signaling in neighboring cells and amplified the pro-inflammatory cytokine response. Not surprisingly, also large amounts of sDLL1 were detectable in the cell supernatant. Considering the central role of monocytes and macrophages in the pathophysiology of sepsis, we asked if this might occur in the clinical setting during human sepsis as well.

To address this, we conducted a secondary analysis of plasma samples from three independent studies, combining patients with sepsis as well as patients after surgery, trauma, and healthy volunteers. We aimed to unravel the kinetic of DLL1 and its diagnostic value as a host-derived response biomarker to discriminate sepsis from sterile systemic inflammatory processes compared to established markers.

MATERIALS AND METHODS

Study Cohorts

The secondary analysis contains samples from three prior observational cohort studies, conducted to evaluate different biomarkers in sepsis or severe trauma. All studies have received clearance from the responsible ethics committees before recruitment. If necessary, the secondary analysis of samples has been amended. In all studies, written informed consent was obtained from the patients before inclusion. If the patient was not able to give consent, its legal representative was asked instead.

Cohort 1 (POCSEP-Trial; German Clinical Trials Register ID: DRKS00008090; Ethical committee of the Medical Faculty of the University Heidelberg: S-247/2014) contains samples from 30 adult patients with sepsis or septic shock according to the 2001 consensus criteria (Levy et al., 2003), drawn on onset (0 h), 24 h, 48 h, and 7 days later. As control groups, 30 healthy volunteers, as well as 30 patients after extensive visceral surgery (e.g., Whipple procedure or hemihepatectomy) were included with samples available from 0, 24, and 48 h after end of surgery. Exclusion criteria were recent cardiac surgery, severe trauma or therapy with tranexamic acid (Schmitt et al., 2019).

Cohort 2 (MIRSI-Trial; German Clinical Trials Register ID: DRKS00005463; Ethical committee of the Medical Faculty of the University Heidelberg: S-097/2013) contains samples from 50 patients with septic shock according to the 2001 consensus criteria (Levy et al., 2003), drawn on onset (0 h), 24 h, 48 h, and 7 days later. Same control groups as described in Cohort 1 have been recruited with 20 patients and 20 healthy volunteers, respectively. No exclusion criteria were applied (Decker et al., 2017).

Despite patients with sepsis were recruited under the consensus criteria valid at time of study conduct, all patients also fulfilled Sepsis-3 consensus criteria as evaluated retrospectively. Importantly, relevant organ dysfunction as a key element of definition was present in all patients, indicated by the Sequential Organ-Failure Assessment score (SOFA) (**Table 1**).

Cohort 3 (SPRINT; German Clinical Trials Register ID: DRKS00010991; Ethical committee of the Medical Faculty of the Justus-Liebig-University Giessen: 164/14) contains samples of 36 adult patients (as available from originally 50 patients) with severe traumatization (Injury Severity Score (ISS) $\geq$ 16), drawn on admission (initial), 24, 48, 72, and 96 h later. Patients with known chronic viral infections have been excluded (Koch et al., 2018).

To rule out an influence of age and sex on sDLL1 plasma concentrations, 90 anonymized healthy control samples of both sex and different age groups (18–27, 28–37, 38–47, 48–57, and 58–67 years, $n = 9$ each group and sex) were obtained from the Blood Donor Biobank (Bavarian Red Cross Blood Donor Service) and analyzed.

Measurement of DLL1

Quantification of soluble DLL1 in plasma has been performed using a commercially available ELISA kit (RayBiotech Life, Inc., Norcross, USA) according to the manufacturer's instructions. Importantly, all samples were diluted 1:30 (or higher if demanded by the concentration) with the supplied Assay Diluent A before quantification to lie within the calibration curve and to minimize interfering matrix effects. Absorbance measurements have been performed on an ELx808 microplate reader (BioTek Instruments, Inc., Winooski, USA) with a subsequent automatized calculation of concentrations within the corresponding Gen5 software (BioTek Instruments, Inc., Winooski, USA).

Measurements of leucocytes, CRP and PCT have been performed in the routine laboratories of each study site.

Statistical Analysis

All visualizations and statistical analysis have been conducted with GraphPad Prism (version 8.1.2, GraphPad Software, Inc., La Jolla, USA). All CRP and PCT values below internal reference range (2 mg/l and 0.05 μg/l) were set to 2 and 0.05 μg/l, respectively. Scatter plots containing single data points are used for visualization, with medians indicated within. If not stated otherwise, numbers reported are median values (95% confidence interval).

Group comparisons between corresponding timepoints of postoperative and sepsis patients within cohort 1 and 2 were conducted using Kruskal-Wallis test, followed by pairwise comparison with Dunn's post-test (corrected for multiple testing). Healthy controls were compared to timepoints 0, 24, and 48 h of patients with sepsis. Influence of age and sex was assessed by ordinary two-way-ANOVA in the dataset of healthy donors.

To assess the diagnostic performance of DLL1 in comparison to leucocytes, CRP and PCT, a pooled *Area Under Receiver Operator Characteristic* (AUROC) analysis was performed: (1) all samples from patients with sepsis and after surgery (0, 24, and 48 h) as well as from patients with trauma (all time points) and healthy volunteers were selected, from which measurements of all four biomarkers were available. (2) Samples were grouped into "Sepsis" ($n = 148$) or "Control" ($n = 201$; healthy volunteers, surgical and trauma patients). Area under curve (AUC) and the 95% confidence interval were reported as global indicators of discriminatory performance. Same approach was repeated to evaluate the prognostic value of DLL1 in regard to 28-day mortality within the patients with sepsis. *Post-hoc* power calculation was performed using G*Power (version 3.1.9.3., free from University of Düsseldorf) (Faul et al., 2007).

To identify the cut-off value corresponding to the best combination of sensitivity and specificity, Youden index was calculated and the maximum value selected. Subsequently, the accuracy of each marker was calculated by applying the identified cut-off to the full ROC analysis cohort ($n = 349$). Furthermore, the correlation between markers was assessed by Spearman-Rho test.

RESULTS

The Study Cohorts

In total, 80 patients with sepsis were available for this secondary analysis. In both cohorts 1 and 2, the majority of these patients were male and suffered from abdominal, respectively, surgical site-associated infection as the source of sepsis (**Table 1**), with a total of 15 patients (18.6%) also having evidence for a (co-)infection of the lung. Both cohorts were similar regarding disease severity as depicted by SOFA score. However, mortality was higher in Cohort 2 (C2, 40%) compared to Cohort 1 (C1, 22%).

Groups of patients with sepsis and patients after surgery ($n = 50$) were comparable in age, while healthy volunteers ($n = 50$), and trauma patients ($n = 36$) were substantially younger. CRP, leucocytes, and PCT were partly available from post-surgery patients and healthy volunteers, while biomarkers were consistently available for patients with

TABLE 1 | Baseline characteristics of patients and healthy volunteers.

	Cohort 1			Cohort 2			Cohort 3
	Sepsis	**Post-OP**	**Healthy**	**Sepsis**	**Post-OP**	**Healthy**	**Trauma**
n=	50	20	20	30	30	30	36
Age (years)	66 (41–88)	65 (49–80)	30 (23–44)	60 (20–84)	64 (36–85)	23 (19–45)	49 (18–85)
Male sex	38 (76)	10 (50)	5 (25)	21 (70)	23 (77)	12 (40)	27 (75)
BMI (kg/m2)	27.2 (18.8–45.9)	24.9 (29.2–37.2)	–	24.8 (18.2–47.8)	27.4 (16.6–41.2)	23.0 (18.0–28.4)	25.4 (19.5–38.6)
CLINICAL SCORES							
SOFA	11 (6–18)	–	–	14 (6–20)	–	–	6 (1–15)
ISS	–	–	–	–	–	–	24 (17–34)
SITE OF INFECTION (MULTIPLE NAMING POSSIBLE)							
Surgical site	30 (60)	–	–	12 (40)	–	–	–
Abdominal	45 (90)	–	–	11 (36.7)	–	–	–
Urinary tract	1 (2)	–	–	1 (3.3)	–	–	–
Lung	10 (20)	–	–	5 (16.7)	–	–	–
Other	1 (2)	–	–	6 (20)	–	–	–
LABORATORY VALUES							
Leucocytes ($\times 10^3/\mu l$)	12.1 (1.7–76.0)	6.9 (4.8–11.2)	–	19.2 (1.2–52.6)	12.2 (4.4–32.8)	6.6 (4.1–11.4)	8.9 (4.5–19.2)
CRP (mg/l)	190.7 (19.2–430.3)	5.5 (2.0–92)	–	218.9 (49.6–522.6)	2.7 (2.0–74.2)	2 (2.0–5.0)	16.7 (0.0–161.0)
PCT (µg/l)	8.3 (0.1–288.5)	–	–	17.5 (0.3–185.9)	0.1 (0.05–1.5)	0.05 (0.05–0.05)	0.7 (0.05–13)
OUTCOME							
28-day-mortality	11 (22)	0 (0)	0 (0)	12 (40)	2 (7)	0 (0)	1 (3)

Data are presented either as median (min–max) or as number (percentage) in case of "Male sex" and "28-day-mortality." BMI, Body mass index; APACHE II, Acute physiology and chronic health evaluation score II; SAPS II, Simplified acute physiology score II; SOFA, Sequential organ failure assessment score; ISS, Injury severity score; Post-OP, postoperative.

sepsis (**Supplementary Figure 1**). Most importantly, surgical patients exhibited a delayed increase of CRP, PCT, and leucocytes (**Supplementary Figures 1A–F**), while trauma patients primarily increased CRP, as well as PCT plasma levels (**Supplementary Figures 1G–I**). That kinetics are indicative of a sterile systemic inflammatory response syndrome (SIRS), blurring the biomarker-based diagnosis of emerging infections.

The Kinetics of DLL1 in Sepsis and Trauma

We evaluated the kinetics of DLL1 in different groups of patients as well as healthy volunteers. Latter exhibited median plasma concentrations 12.1 ng/ml (CI: 10.6–13.2 ng/ml; C1) and 8.2 ng/ml (95%CI: 7.7–10.1 ng/ml; C2) (**Figures 1A,B**). We found a strong increase in plasma concentrations already at the onset of sepsis [0 h, 56.5 ng/ml (C1), 60.5 ng/ml (C2)] (**Figures 1A,B**, **Supplementary Table 1**). DLL1 concentrations remained elevated after 24 h (48.6 and 64.7 ng/ml), 48 h (48.2 and 58.1 ng/ml), and even 7 days later (34.3 and 37.4 ng/ml), compared to healthy reference values (**Figures 1A,B**, **Supplementary Figure 2**, **Supplementary Table 1**). To examine the impact of sterile inflammation on DLL1 concentrations, we measured two stress test cohorts: patients after surgery and patients with severe multiple injuries, both groups involving extensive tissue damage and subsequent sterile inflammation as described above. In patients after surgery, DLL1 increased slightly from 13.6 to 17.4 ng/ml after 48 h (in C1) and from 12.8 to 15.2 ng/ml after 24 h (in C2) (**Figures 1A,B**, **Supplementary Table 1**). In line, patients with multiple injuries presented—compared to healthy volunteers—slightly increased plasma concentrations from admission (0 h, 16.4 ng/ml; 16.2–20.1 ng/ml) until day 4 after trauma (17.0 ng/ml; 14.5–19.7 ng/ml) (**Figure 1C**, **Supplementary Table 1**). Importantly, after analysis of the healthy donor cohort, age and sex did not exert an effect on sDLL1 concentrations, neither individually, nor additive (**Supplementary Figure 3**).

In summary, DLL1 is increased exclusively in sepsis, while sterile insults as major surgery and severe trauma do not influence its generation.

DLL1 Has Superior Diagnostic Performance

Next, we investigated the value of DLL1 as a diagnostic marker for the differentiation between sterile inflammation and sepsis. Therefore, we pooled all samples from which leucocytes, CRP and PCT data were available and grouped them into "sepsis"

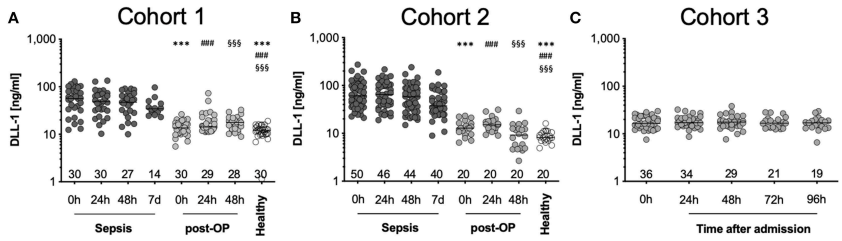

FIGURE 1 | Distribution of DLL1 plasma concentrations in the study cohorts. **(A,B)** depicts measurements of sepsis studies and **(C)** of trauma patients. Bottom numbers indicate available samples on each timepoint. Horizontal line depicts median, y-axis has been set to log-scale. ***/###/§§§ $p < 0.001$ vs. "Sepsis 0 h," "Sepsis 24 h," or "Sepsis 48 h," respectively. Post-OP, postoperative.

($n = 148$) and "controls" ($n = 201$). An correlation analysis of all patients with sepsis (0, 24, and 48 h) yields no correlation of DLL1 with leucocytes (−0.03411 (CI: −0.1674–0.1004; $p < 0.00001$; $n = 227$), and only weakly with CRP (0.2253 (CI: 0.0938–0.3490; $p < 0.00001$; $n = 226$), as well as with PCT (0.3655 (CI: 0.2140–0.4998; $p < 0.00001$; $n = 151$). Next, AUROC analysis was conducted and revealed AUCs of 0.8236 (0.7812–0.8660) for CRP (**Figure 2A**), 0.7573 (0.6949–0.8125) for leucocytes (**Figure 2B**), 0.8705 (0.8318–0.9092) for PCT (**Figure 2C**), and 0.9303 (95%CI: 0.8997–0.9610) for DLL1 (**Figure 2D**). *Post-hoc* analysis revealed a power of 1.0 for the discriminatory value of DLL1. The best cut-off values (and the corresponding combination of sensitivity/specificity) of the individual markers were extracted by maximum Youden index procedure: 159.4 mg/l CRP (72.3% sensitivity/76.6% specificity), 15.2 μl leucocytes (60.1% sensitivity/92.0% specificity), 2.5 μg/l PCT (74.3% sensitivity/85.6% specificity), and 29.7 ng/ml DLL1 (81.8 sensitivity/97.0 specificity). Finally, the accuracy of each marker was calculated by applying the given thresholds to the full cohort, yielding a superior accuracy of 91% for DLL1, compared to 75, 79, and 81% for CRP, leucocytes, and PCT, respectively. Regarding outcome prediction (28-day survival), plasma DLL1 failed to be of prognostic value (**Supplementary Figure 4**). However, statistical power of these analysis is low, with 0.0641 (Onset) and 0.195 (24 h), needing replication in larger cohorts.

DISCUSSION

In line with our prior experimental finding of DLL1 induction upon monocyte activation (Hildebrand et al., 2018), we found plasma levels of DLL1 to be strongly elevated in septic patients. In contrast, neither surgical trauma nor severe injuries lead to an increase of it. As these conditions are well-known to induce sterile inflammatory responses of the body, DLL1 represents a robust and specific biomarker for sepsis. Compared to routine laboratory markers, especially to PCT, DLL1 exhibits a superior accuracy for diagnosis, but not for prognosis, of bacterial infection.

The syndrome sepsis can result from various infectious encounters of patients already embedded in complex clinical contexts, fueling an interwoven machinery of disturbed response systems of the host (Angus and van der Poll, 2013). Importantly, phenotypically similar host responses can be mounted upon, e.g., surgery (Dabrowska and Słotwinski, 2014), severe injury (Lord et al., 2014), and ischemic stroke (Courties et al., 2014), hampering the performance of existing biomarkers to discriminate an infection-related host response to sterile responses. The most prominent biomarker PCT, has been approached in a plethora of studies and despite the availability of large, comparative datasets, its real-life utility is controversially debated since many years (Simon et al., 2004; Tang et al., 2007; Wacker et al., 2013; Wu et al., 2017). Only recently, a study including patients presenting with SIRS on ICU revealed that no humoral biomarker currently available is capable to stratify patients with critical illness of non-infectious and infectious origin (Parlato et al., 2018). Among all markers examined, CRP levels and HLA-DR expression showed the best diagnostic performance, but both still exhibit values too low for routine use.

The intriguing difference of sDLL1 compared to CRP and PCT is its persistence over time. While established marker substantially respond to intensive care therapy, DLL1 remains elevated for 7 days. It is unclear, if this phenomenon is due to steady formation of new sDLL1 or if the half-life is just substantially longer. In any case, DLL1 remains informative for diagnosis over a sustained period of time, thereby limiting its use for therapy guidance. Many markers used in clinical routine exert redundant informational content. As we are able to show its weak correlation to other markers, the use of sDLL1 might add further value to diagnosis.

While the cellular origin of the high levels of sDLL1 observed in our patients with sepsis remains speculative, the mechanism of formation is known: transmembrane DLL1 is cleaved by a combined ADAM protease and γ-secretase activity upon binding to Notch receptor and therefore, sDLL1 is a surrogate of recent Notch signaling (Six et al., 2003; Dyczynska et al., 2007). Biological processes of utmost importance in host defense are monocyte activation and maturation in the circulation. Activation of the DLL1-Notch axis by endothelial cells has been shown to be a crucial trigger for the conversion of classical into alternative (non-classical) monocytes under steady state

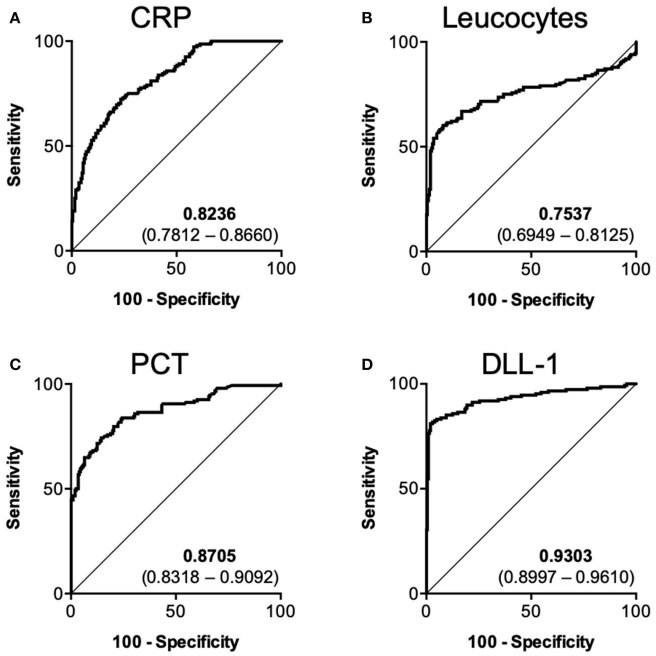

FIGURE 2 | Diagnostic performance of DLL1 in comparison to established biomarkers. Pooled ROC analysis containing 148 sepsis samples vs. 201 control samples. **(A)** CRP (C-reactive protein), **(B)** Leucocytes, **(C)** PCT (Procalcitonin), **(D)** DLL1 (Delta-like 1). Numbers within subpanels indicate area under curve (lower–upper 95% confidence interval).

(Gamrekelashvili et al., 2016), as well as for the maturation of tissue macrophage upon ischemia toward a pro-resolving and repair phenotype (Krishnasamy et al., 2017). The resulting non-classical monocytes exhibit an impaired antigen-presentation capacity, but increased expression of the immune checkpoint *Programmed Death*-1 (PD-1) and its ligand PD-L1 (Ferreira da Mota et al., 2018). This is in line with our finding of an inter-monocyte activation of the Notch axis, resulting in a comparable phenotype (Hildebrand et al., 2018). Taken together, beside monocytes one might speculate the endothelium as a relevant source of cleaved DLL1 observed in plasma of patients with sepsis as a consequence of signaling. Considering the cytokine-mediated capillary leakage syndrome as a well-known clinical hallmark of sepsis (Siddall et al., 2017), the Notch axis might be a critical molecular hub in the pathophysiology, warranting further research.

A series of publications evaluated the use of sDLL1 as a biomarker in different diseases. In a cohort of 136 patients with symptomatic aortic stenosis, Abraityte and colleagues found a prognostic value of sDLL1, with low and high plasma levels indicating poor outcome (Abraityte et al., 2015). However, "high" plasma levels were already defined as ≥6.93 ng/ml, which is substantially below the threshold we proposed for the diagnosis of infection and lies well within the range of the healthy volunteers we discovered.

The same group also showed elevated levels in a cohort of patients with chronic heart failure compared to healthy volunteers (Norum et al., 2016). Again, sDLL1 negatively correlated with diastolic function, exercise capacity, as well as outcome. The reported concentrations were well below the values we observed in our cohorts, with 7.4 ng/ml indicated as upper tertile of the cohort. Based on these findings, Norum et al. (2017) further examined plasma of patients suffering from dilated cardiomyopathy, as these commonly present with diastolic dysfunction. Importantly, in contrast to the initial publications, levels of sDLL1 in healthy controls were identical to the values we observed, with patients of high disease severity presenting slightly elevated levels (approximately median 12–13 ng/ml, as results are not given in publication). Furthermore, in both patient groups with dilated cardiomyopathy and chronic heart failure, sDLL1 correlated well with markers of impaired kidney function (e.g., creatinine). Patients with sepsis often impose with renal dysfunction as a consequence of inflammation-induced acute kidney injury, potentially leading to an accumulation of sDLL1

(Zarbock et al., 2014). Further studies need to unravel the association between DLL1 and kidney function during sepsis. Also, looking in urine might be of interest to evaluate its use as biomarker of kidney function.

Besides heart diseases, also patients with non-small cell lung cancer (NSCLC) and chronic obstructive pulmonary disease (COPD) were recently assessed for sDLL1 (Berg et al., 2018). Plasma levels of DLL1 differed between the two cohorts, ranging from 3.8 to 22.5 ng/ml in NSCLC and from 8.1 to 27.8 ng/ml in COPD patients. Apart from internal medicine, patients with schizophrenia, as well as bipolar disorders were shown to possess elevated levels of sDLL1 compared to healthy controls (Hoseth et al., 2018). Conflicting, this study reports substantially lower levels of sDLL1 (median 4.5 ng/ml) in healthy volunteers, as compared to their own earlier studies and to our results. Two challenges might be underlying this phenomenon: first, there is no standardized assay for the quantification of DLL1 and secondly, no indication is given how the assay was performed in terms of sample dilution. As plasma and serum are complex matrixes, interferences cannot be excluded, potentially introducing a technical bias.

In a combined study on two cohorts of patients after heart transplantation, sDLL1 concentrations have been found to be elevated compared to healthy controls (Norum et al., 2019). Moreover, time since transplantation, as well as the immunosuppressive medication used (everolimus or calcineurin inhibitor) were shown to influence sDLL1 concentrations. The authors reported median values between 12.8 and 26.6 ng/ml in different subgroups. If looking in detail, the highest values were reported in patients with acute rejections on baseline (median: 26.6 ng/ml; IQR: 23.1–31.0 ng/ml). These values are ranging into the threshold proposed from us for diagnosis of infection and therefore might represent a limitation of our marker. Importantly, in their publication Norum and colleagues also identified expression of DLL1 on T cells, endothelial cells, and vascular smooth muscle cells and its release upon activation with cytokines. This further substantiates the hypothesis of endothelial cells as relevant source of circulating sDLL1.

Results of one earlier study hinted, comparable to ours, toward a diagnostic value of DLL1 for infection: By assessing sDLL1 levels in cerebrospinal fluid of patients with HIV with suspected *Mycobacterium tuberculosis* infection, the researchers found a cut-off value of 1.15 ng/ml associated with an excellent specificity of 98%, but a low sensitivity of only 32% (Bahr et al., 2018). However, this clinical context is very special and CSF is not as easily obtainable as blood. Nevertheless, one important conclusion can be drawn from this study, despite in need of clarification in the blood: chronic HIV infection does not seem to elevate DLL1 levels *per se*, but only when bacterial infection occurs in addition.

Our study implies several limitations, not lastly due to its characteristic as a secondary analysis of three independent cohorts. No matching for sex and age was performed in the primary studies, leading to skewed demographic compositions. Using a separate dataset of healthy donors, we are able to prove no clear influence of either age or sex on sDLL1 concentrations. However, larger cohorts are necessary to define definite reference values. From a technical perspective, to prove DLL1's potential as a biomarker of bacterial infection (and other pathologic conditions), several aspects need to be ensured: First, a standardized assay needs to be developed and used for quantification, enabling definite cut-off determination. Secondly, sample pre-analytic must be harmonized with respect to anticoagulation of drawn blood, processing, and storage. Especially analytical stability is a crucial aspect of biomarker assessment und clinical usability. It must be carefully revisited for DLL1, as our samples have been stored for an extended time frame before analysis and we cannot rule out degradation of sDLL1. However, as all cohorts recruited patients and controls during the same period, bias within the cohorts is limited. Also, considering the high amounts of sDLL1 consistently present in patients with sepsis, the value of DLL1 as a biomarker for the identification of bacterial infection in critically ill patients remains unchallenged, with the potential exception of patients after heart transplantation. This limit needs to be verified in further studies, including also patients after transplantation of other solid organs. Importantly, the real-life approach of Parlato and colleagues should serve as a role model for the design of future biomarker studies, evaluating the marker of interest directly within the setting of interest.

In conclusion, by combining several cohorts of patients, we identified plasmatic sDLL1 to be a potential new host-derived biomarker with high diagnostic accuracy for sepsis. Its superior sensitivity and specificity compared to CRP and PCT must be confirmed in independent cohorts. As it results from a specific process inherent to the syndromes' pathophysiology, the DLL1-Notch axis might serve as a potential theranostic target in sepsis as well.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study protocol of each study contained was assessed and positively evaluated by the local ethics committee (S-247/2014 and S-097/2013, Ethical Committee I of the Medical Faculty Heidelberg; 164/14, Ethical Committee of the Medical Faculty of the Justus-Liebig-University Giessen). Furthermore, the studies were registered in the German Clinical Trials Register (IDs: DRKS00008090, DRKS00005463, DRKS00010991). Informed consent was obtained from the patients or, if not possible due to sedation or mental deterioration, from the legal representative.

AUTHOR CONTRIBUTIONS

DH contributed to the study concept, conducted sample measurements, interpreted the results, and drafted and wrote the manuscript. SD, CK, and FS came up with the

primary study concept, recruited patients, and interpreted results. SR and ES recruited patients, interpreted results, and drafted the manuscript. MS contributed to the primary study concept, recruited patients, interpreted results, and drafted the manuscript. KH and MW contributed to the study concept, interpreted results, and drafted the manuscript. TB contributed to the primary and secondary study concepts, interpreted results, and drafted the manuscript. FU contributed to the study concept, conducted sample measurements, data analysis, and drafted and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00267/full#supplementary-material

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Conflict of Interest Statement: DH, KH, MW, and FU hold the worldwide intellectual property rights for the use of DLL1 as diagnostic marker for severe infections (PCT/EP2018/079273 and EP17198330).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8.5 Anlage 5

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Plasma DNA and RNA differentially impact coagulation during abdominal sepsis—an explorative study

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ABSTRACT

Background: Cell-free DNA (cfDNA) and extracellular RNA (exRNA) are both suspected to activate coagulation cascades in sepsis. Therefore, our study investigated the influence of plasmatic nucleic acids on coagulation in septic patients in comparison to patients after major abdominal surgery.

Materials and methods: A total of 15 patients with sepsis, 10 postoperative patients, and 10 healthy volunteers were included in this longitudinal study. Blood was collected at sepsis onset and after surgery respectively, as well as after 24, 72 and 168 h. Levels of cfDNA and exRNA were measured by quantitative probe-based polymerase chain reaction. In addition, thromboelastography for coagulation as well as thromboaggregometry for platelet function was conducted.

Results: Both cfDNA and exRNA were elevated in patients with sepsis compared with postoperative patients and healthy volunteers. While higher exRNA levels correlated with a faster clotting time and more stable clots, cfDNA correlated with a shorter clotting time but also less fibrinolysis. In addition, higher cfDNA seems to be associated with kidney dysfunction as well as with general markers of cell damage (lactate dehydrogenase and lactate).

Conclusions: Both nucleic acid species might be associated with different effects on coagulation during sepsis, with an overall procoagulatory influence. For this reason, individualized therapeutic approaches in patients suffering from coagulation-associated organ dysfunction might be feasible.

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Background

Despite modern therapeutical concepts and ongoing innovations in intensive care medicine as well as organ support techniques, sepsis and septic shock remain severe conditions with increasing incidence rates and associated with mortality rates between 20% and 60%.[1,2] As a central feature, a dysregulated response of the immune system takes places, leading in its extent to tissue damage and organ failure.[3] Particularly after abdominal surgery, patients are at high risk to develop severe infections and subsequent sepsis. The National Surgical Quality Improvement Project analyzed more than 360,000 patients after abdominal surgery and ascertains an eightfold increased higher incidence of septic shock compared with myocardial infarction leading to a commensurably 30-d mortality.[4,5] It is well-examined and in recent ongoing discussion that early recognition and aggressive algorithm–based treatment of septic shock improves survival.[6] Especially postoperative infections are at risk of delayed identification because of the concealment of infection signs by regular body reactions triggered by the surgical procedure.

Sepsis and septic shock are associated with extensive cell death leading to a loss of cell integrity and subsequently to a release of cell-free DNA (cfDNA), consisting of small DNA fragments, into the circulation.[7,8] Nevertheless, the source of cfDNA during sepsis is not yet clear. Both necrosis and apoptosis occur with no knowledge which type of cell death dominates. In addition, also the generation of neutrophil extracellular traps (NET) during a special form of cell death, called NETosis, has been hypothesized as a major source of cfDNA. Overall, it seems to be proven that elevated levels of cfDNA are associated with higher mortality in septic patients, indicating a crucial role of free nucleic acids in the pathophysiology of sepsis.[8-10] In the last decade, evidence arose suggesting that cfDNA represents a major crosslink between inflammation and coagulation. Recently, the term "immunothrombosis" was implemented, underlining the critical role of cfDNA in the initial activation of the immune as well as the coagulatory system.[11,12] First, together with histones, cfDNA constitutes a main part of NETs, which catch and eliminate microbes in early immune response. On the other hand, *in vitro* cfDNA has been shown to activate coagulation via the intrinsic pathway and impairs fibrinolysis.[13,14] However, the *in vivo* influence of cfDNA on coagulation in sepsis is not thoroughly evaluated yet.

As distinguished from cfDNA, the physiological and pathophysiological role of exRNA is not well understood in sepsis, but some evidence exists that presume exRNA to be another important link between coagulation and inflammation.[15,16] Apart from sepsis, exRNA has been proven to be an activator of factor seven–activating protease, a pro-coagulant key player.[17] Both, cfDNA and exRNA, feature a negative electric charge derived from their glucose–phosphate backbone, which might be the structural feature rendering them capable of activating the coagulation system *in vitro* as well as *in vivo*.

Exaggerating inflammation as fundamental part of septic shock leads to a strong activation of the procoagulatory pathways with simultaneous inhibition of anticoagulatory compensatory mechanisms, resulting in a condition of high thrombotic risk.[18] Free nucleic acids influence the activation of the coagulatory system, but their *in vivo* influence on coagulation in sepsis has not been proven so far.

The evaluation of septic coagulopathies remains a challenging task because of the fast and dynamic reactions, ranging from extensive hypercoagulation to a two-faced disseminated intravascular coagulopathy (DIC). Thromboelastography and thromboaggregometry are proven useful tools, which allow rapid point-of-care assessment of coagulatory and platelet (mal-) functions.[19,20] Therefore, we chose both methods to evaluate the *in vivo* effect of nucleic acids on coagulation during sepsis and framed five core hypotheses, which were the objectives of this study:

1. The amount of circulating nucleic acids in peripheral blood is elevated in patients suffering from sepsis.
2. The amount of circulating nucleic acids in peripheral blood influences coagulation in patients with sepsis.
3. Circulating nucleic acids in peripheral blood in patients suffering from sepsis influences the stability of clot firmness.
4. The amount of circulating nucleic acids correlates with the degree of organ damage.
5. Circulating nucleic acids have a prognostic value for patients suffering from sepsis.

Materials and methods

Study design

This study was designed as a single center, prospective, observational study. After approval of the local ethics committee was given (University of Giessen, Germany, Ref.-No. 100/12), patients were enrolled in the surgical intensive care unit (ICU) of the University Hospital of Giessen and Marburg (site Giessen), Germany between October 2013 and April 2014. All patients and healthy volunteers gave informed consent. If the patient was not able to consent, the consent of the patient's legal representative was obtained. Sepsis was defined according to the criteria of the Surviving Sepsis Campaign,[6] while surgical patients had to undergo elective and major abdominal surgery. Only nonpregnant patients of at least 18 y were included. Further exclusion criteria were autoimmune diseases (detailed inclusion and exclusion criteria are shown in Supplementary Table 1). Septic patients were only included within 6 h after clinical diagnosis of sepsis, whereas the first blood sample of surgical patients was taken after surgery. In case of a present systemic inflammatory response syndrome (SIRS; >2 criteria) at this time point, the patients were excluded. After inclusion, blood samples of surgical and septic study patients were collected at baseline, after 24, 72, and 168 h. Healthy volunteers donated blood only once. At each time point, levels of cfDNA and exRNA as well as Rotational thromboelastometry (ROTEM; TEM International GmbH, Munich, Germany), Multiplate (Roche Diagnostics International Ltd, Grenzach-Wyhlen, Germany), and routine blood analysis were performed. In addition, clinical data were collected.

Thromboelastography and platelet impedance aggregometry

Thromboelastography and platelet impedance aggregometry were performed immediately after blood draw at the ICU. Blood was asserved in citrate tubes (2.9 mL), and thromboelastography was conducted subsequently by the use of ROTEM according to the manufacturer's instructions. ROTEM analysis involved the use of six reagents (star-tem, in-tem, ex-tem, fib-tem, ap-tem, and hep-tem) to assess the following pathways: NATEM as method relying on endogenous activators of coagulation triggered by a sole recalcification of the citrated plasma sample, INTEM to measure contact activation ("intrinsic"), EXTEM to measure *Tissue Factor* activation of coagulation ("extrinsic"), FIBTEM to measure platelet-independent activation of coagulation, APTEM to measure hyperfibrinolysis, and HEPTEM to measure heparin-independent contact activation. The analysis included the measurement of clotting time (CT), clot formation time, maximum clot firmness (MCF) and 60-min lysis index (LI). Concomitant, thrombocyte function was analyzed in hirudinea-stabilized blood samples (2.7 mL) with the aid of impedance aggregometry (Multiplate) and the use of ASPItest, ADPtest, and TRAPtest reagents according to the manufacturer's instructions.

Blood sampling

Blood was collected in ethylenediaminetetraacetic acid, and subsequently plasma was separated by centrifugation (1.500 RCF, 10 min), aliquoted in three parts and stored frozen at −80°C until further use. All blood samples were processed within 1 h after blood collection.

Isolation and quantification of cfDNA

QIAamp DNA Blood Mini Kit (Product no. 51,304; Qiagen, Venlo, Netherlands) was used to isolate cfDNA from 500 μL plasma according to the manufacturer's protocol. Five microliter of the isolate was subsequently used for semi-quantitative quantification by quantitative polymerase chain reaction as described previously.[8,21] Briefly, a quantitative polymerase chain reaction targeting a region of the β-globin locus was run using TaqMan methodology. To quantify the circulating amount of β-globin DNA, we compared the results of the PCR against a calibration curve, constituted from serial dilutions of DNA extracted from the cell line A549 using the same kit as for plasma samples. Two replicate isolations were performed once from 5×10^6 cells each and pooled after spectrophotometric quantification and quality control (NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific, Wilmington, Delaware). All samples exhibited a 260/280 ratio >1.9. The undiluted isolate was stored aliquoted and undiluted at −80°C until further use. Before each sample run, one aliquot was gently thawed and serially diluted nine times (1:2) in nuclease-free water. Taking 6.6 pg as average "weight" of a genome, we got an equivalence number of 46,765 genomes (genomic equivalents [GE]) per 5 μL isolate. Dilutions 3-9 (equivalent to a dynamic range of 1732-2.4 GE) were run as calibration curve on every plate containing samples. All runs were performed on a StepOnePlus cycler (Applied Biosystems, Waltham, MA) using TaqMan Gene Expression Master Mix Kit (Applied Biosystems). Primer and probe sequences and final concentrations are given in Supplementary Table 2. All samples and standards were run in triplicate, and automatic calculation was performed by the instrument's software (StepOne Software V2.2.3; Applied Biosystems, Waltham, MA).

Isolation and quantification of exRNA

Isolation of exRNA was done using Norgen Biotek RNA Purification Mini Kit (Product no. 51,000; Norgen Biotek, Thorold, Canada), consistent to manufacturer's instructions. Afterward, quantification was done by quantitative TaqMan PCR targeting β-actin transcripts using a commercially available TaqMan assay from Applied Biosystems (assay ID: Hs01060665_g1) as well as one-step reagents to minimize variance (Applied Biosystems AgPath-ID One-Step-RT-PCR Kit; Life Technologies, Carlsbad, CA). Analogous to DNA quantification, we used an arbitrary standard curve also for exRNA quantification. For generation, we isolated RNA from 10^7 A549 lung epithelial cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified it spectrophotometrically. The isolate contained 119,580 transcriptional units (TU) per microliter when 0.01 ng RNA was assumed as RNA content of one cell. This isolate was diluted 1:1 to have a "top standard" concentration of 59,790 TU/μL and stored in aliquots at −80°C until further use. Before each run, an eight times serial dilution (1:2) was done and dilutions 3-8 (equivalent to a range from 2214 to 9 TUs) were used as standards on plate. All samples and standards were run in triplicate, automatic calculation was performed by the instrument's software (StepOne Software V2.2.3).

Clinical data and statistical analysis

Relevant clinical and demographic data were collected from the electronic patient data management system while statistical analysis was performed with SPSS (version 22; IBM, Armonk, NY). Global analysis of nonparametric correlations was processed using Spearman-Rho test (two sided, level of significance $P < 0.05$). For group comparisons, global Kruskal–Wallis test was performed first, followed by Mann–Whitney U test for two-group comparison. In this case, a P value < 0.05 was taken as statistically significant. Significance levels are depicted in the figures as asterisks/hashes with $^{*/\#}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Study population

In total, 35 subjects were included in this study, of which 10 were healthy volunteers, 10 postoperative patients, and 15 patients suffering from sepsis. Except for one patient, who had a urogenital infection, the source of sepsis was identified as an abdominal focus ($n = 14$) (Table 1). Septic patients were more severely ill reflected by higher median ICU scores at onset compared with postoperative patients (Acute

Table 1 – Baseline characteristics of the study groups

Item	Healthy volunteers	Surgical patients	Septic patients
N	10 (100)	10 (100)	15 (100)
Age (y)	62 (32-77)	61 (44-76)	66 (33-79)
Male sex	5 (50)	6 (60.0)	8 (53.3)
BMI (kg/m^2)	–	23.3 (19.6-27.9)	26.3 (18.4-37.0)
28-d survival	–	10 (100)	9 (60)
Site of infection			
Abdominal	–	–	14 (93.3)
Urogenital	–	–	1 (6.7)
Type of operation			
Esophageal resection	–	2 (20)	–
Pancreatic resection	–	7 (70)	–
Extended gastric resection	–	1 (10)	–
Clinical scores at onset			
APACHE II	–	16 (7-19)	22 (8-30)
SOFA	–	7 (0-10)	8 (1-14)
SAPS II	–	29 (17-41)	49 (26-55)
cfDNA (GE)			
Onset	2.4 (2.4-6.3)	15.9 (9.4-54.1)	52.1 (2.4-1099.2)
24 h	–	10.1 (3.3-61.6)	29.1 (4.23-428.4)
72 h	–	13.8 (4.0-72.3)	25.6 (4.2-84.2)
168 h	–	8.4 (4.5-50.6)	14.6 (2.4-1283.3)
exRNA (TU)			
Onset	222.0 (65.1-917.2)	240.0 (38.2-565.2)	712.7 (94.2-2704.7)
24 h	–	108.8 (26.7-550.8)	621.5 (57.2-2862.6)
72 h	–	353.6 (73.7-685.6)	543.9 (68.6-2557.6)
168 h	–	719.9 (264.8-1560.8)	367.3 (70.5-5345.2)

Values represent median (range) except for N, male sex, site of infection, type of operation, and 28-d survival, for which count (percentage of all) are given.

Physiology and Chronic Health Evaluation [APACHE] II: 22 *versus* 16, Sequential Organ Failure Assessment Score [SOFA]: 8 *versus* 7, Simplified Acute Physiology Score II [SAPS II]: 49 *versus* 29). Most of the surgical patients (70%) underwent a pancreatic resection. While all surgical patients were alive at day 28 after inclusion, 40% of the patients with sepsis died.

Hypothesis I—plasma cfDNA and exRNA levels

At baseline and 24 h after inclusion, cfDNA blood levels were significantly higher in patients with sepsis compared with postoperative patients (onset: 52.1 *versus* 15.9 GE, $P = 0.012$; 24 h: 29.1 *versus* 10.1 GE, $P = 0.035$) as well as healthy volunteers (onset: 52.1 *versus* 2.4 GE, $P < 0.001$; 24 h: 29.1 *versus* 2.4 GE, $P < 0.001$; Fig. 1A, Table 1). After 72 and 168 h, despite an overall decrease of the GE in the patients with sepsis, the difference to the healthy group remained significant (72 h: 25.6 *versus* 2.4 GE, $P < 0.001$; 168 h: 14.6 *versus* 2.4 GE, $P < 0.001$), whereas the difference to the surgical patients did not keep relevant (72 h: 25.6 *versus* 13.8 GE, $P = 0.446$; 168 h: 14.6 *versus* 8.4 GE, $P = 0.529$). Comparable, postoperative patients presented significant higher plasma cfDNA levels as healthy volunteers at all time points (onset: 15.9 *versus* 2.4 GE, 24 h: 10.1 *versus* 2.4 GE, 72 h: 13.8 *versus* 2.4 GE, 168 h: 8.4 *versus* 2.4 GE, all $P < 0.001$).

Similarly to cfDNA, also plasma exRNA was significantly elevated at study onset and 24 h later in the sepsis group in comparison to the surgical population (onset: 712.7 *versus* 240.0 TU, $P = 0.004$, 24 h: 621.5 *versus* 108.8 TU, $P < 0.001$) and to the healthy population (222.0 TU; Fig. 1B).

Hypotheses II and III—influence of free nucleic acids on clot formation and stability

For further analysis, measurements from all time points of patients with sepsis were combined to facilitate correlation to other parameters. We found a moderate negative correlation of cfDNA with thrombin time as well as a positive correlation with international normalized ratio (Table 2). These findings were substantiated by ROTEM analysis: all tests except of NATEM found moderate, positive correlations of cfDNA with CT (Fig. 2), questioning the role of cfDNA as strong activator of coagulation. Moreover, all assays except EXTEM revealed an association of cfDNA with an increased LI after 60 min. In sharp contrast, higher

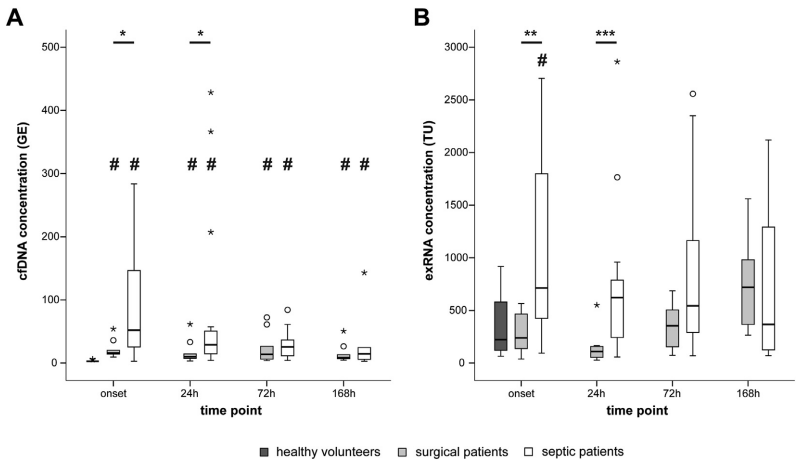

Fig. 1 – Concentrations of cfDNA (A) and exRNA (B) of the three study groups at different time points. Boxplots show the median with whiskers spanning the 1.5× interquartile range. *//***above bar: $P < 0.05/0.01/0.001$, comparing septic *versus* surgical patients on same time point; $^{\#}P < 0.05$, septic/surgical patients *versus* healthy volunteers. Asterisks and circles represent outliers.**

exRNA concentrations correlated negatively with NATEM CT as well as with NATEM, INTEM, and APTEM MCF (Fig. 3), without influencing other assay's CT or LI. Despite a positive correlation of exRNA (but not cfDNA) with thrombocyte numbers, no functional impact was found in Multiplate analysis (Table 2), and no correlation was found neither for cfDNA nor exRNA with any assay's clot formation time (data not shown). Overall, there is a strong hint toward an opposing role of exRNA and cfDNA in coagulation, independent of thrombocytes.

Hypothesis IV—influence of free nucleic acids on organ dysfunction

To evaluate the impact of plasmatic nucleic acids on organ dysfunction, we performed a subgroup analysis according to the reference values of established markers (Table 3). Regarding established markers of renal function, we found significantly higher levels of cfDNA in sepsis patients with an elevated creatinine (>1.2 mg/dL) compared with patients within or below the reference range (Fig. 4A; 72.7 *versus* 24.0 GE, $P = 0.004$). This effect was not visible for exRNA as well as when urea was taken for stratification (Fig. 4B-D). In addition, we evaluated markers of liver function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin. No differences were found for any parameter with regard to cfDNA or exRNA levels (Fig. 5A-F). Finally, we looked for associations to general tissue damage and metabolic markers. Patients with an elevated lactate or lactate dehydrogenase (LDH) also had higher cfDNA levels (lactate: 57.8 *versus* 24.6 GE, $P = 0.009$; LDH: 58.6 *versus* 15.1 GE, $P = 0.028$) (Fig. 6A and B). Less pronounced but yet significant, also exRNA was elevated in patients with sepsis presenting with abnormal lactate (912.9 *versus* 445.1 TU, $P = 0.027$; Fig. 6C and D).

Hypothesis V—prognostic value of free nucleic acids

Neither cfDNA nor exRNA levels showed a significant difference between nonsurviving and surviving patients at any time point within the observation period (Supplementary Fig. 1). Nevertheless, nonsurviving patients showed a tendency toward higher levels of cfDNA, especially at onset and 24 h later. In line with this, cfDNA showed a moderate to strong positive correlation with ICU scores (APACHE II: $\rho = 0.489$, $P < 0.001$; SOFA: $\rho = 0.572$, $P < 0.001$; SAPSII: $\rho = 0.673$, $P < 0.001$; $n = 48$ for all analysis; data not shown).

Discussion

Our pilot study enrolled 35 subjects overall (15 septic patients, 10 surgical patients, and 10 healthy volunteers) and is the first one to our knowledge that reports both cfDNA as well as exRNA levels in patients with sepsis side-by-side with the comprehensive evaluation of their association to coagulation. In addition, we provide insight into the plasma kinetic of nucleic acids not only in patients with sepsis but also in surgical patients.

The levels of cfDNA increase early in sepsis and after surgery compared with control patients. The difference between both the groups diminishes over time, although it stays elevated compared with controls. Our results are in line with earlier publications that demonstrated significant higher levels of cfDNA in peripheral blood of septic patients,[7,8,10,22]

Table 2 – Correlation of routine parameters, ROTEM, and Multiplate with plasma concentrations of cfDNA and exRNA

Parameter	Median (range)	cfDNA		exRNA		N
		ρ	P	ρ	P	
Thrombin time	69 (49-100)	−0.351	0.011	−0.149	0.296	51
INR	1.2 (1-1.5)	0.284	0.043	0.053	0.713	51
PTT	42 (27-70)	0.274	0.051	−0.244	0.085	51
Platelets	210 (20-555)	−0.154	0.282	0.357	0.010	51
Multiplate						
ADP	42 (8-186)	−0.219	0.114	0.100	0.475	53
ASPI	56 (16-209)	−0.137	0.329	0.185	0.184	53
TRAP	60 (16-264)	−0.210	0.132	0.177	0.204	53
EXTEM						
CT	84 (53-137)	0.473	0.000	0.134	0.341	53
MCF	73 (59-88)	0.028	0.840	0.259	0.061	53
LI	96 (91-100)	0.115	0.418	−0.177	0.209	52
INTEM						
CT	187 (131-390)	0.440	0.000	−0.014	0.923	53
MCF	69 (51-82)	0.071	0.615	0.290	0.035	53
LI	98 (90-100)	0.309	0.025	−0.112	0.423	53
FIBTEM						
CT	78 (52-130)	0.434	0.001	0.004	0.980	53
MCF	37 (18-48)	−0.108	0.442	−0.046	0.741	53
LI	100 (94-100)	0.424	0.001	−0.012	0.934	53
APTEM						
CT	89 (35-138)	0.463	0.000	−0.009	0.951	53
MCF	73 (54-81)	0.001	0.995	0.284	0.039	53
LI	97 (93-100)	0.312	0.024	−0.037	0.796	52
HEPTEM						
CT	2013 (144-281)	0.520	0.000	0.121	0.387	53
MCF	70 (53-80)	0.058	0.679	0.198	0.155	53
LI	97 (89-100)	0.395	0.003	−0.054	0.700	53
NATEM						
CT	666 (370-5144)	0.134	0.339	−0.418	0.002	53
MCF	69 (53-78)	−0.092	0.537	0.563	0.000	47
LI	97 (90-100)	0.465	0.002	0.204	0.195	42

INR = international normalized ratio; PTT7 = partial thromboplastin time.
Results of PTT and CTs are given in seconds; international normalized ratio without unit; ADP, ASPI, TRAP as area under curve; MCFs in millimeter; thrombin time and LI as percentage; Platelets as $\times 10^3$ cells/μL. Light grey fill: $P < 0.05$, medium grey fill: $P < 0.01$.

but our study is the first reporting also data of healthy volunteers. Rhodes *et al.* examined patients after ICU admission for any reason and found higher cfDNA in the subgroup of critically ill patients who developed sepsis.[8] In another study from Garnacho *et al.*, this early observation was confirmed, but their results do not show a difference between patients with SIRS and sepsis, while our own findings show a significant difference between patients after surgery and sepsis. The reason might be that the studies differed in the group composition: all our patients suffered from abdominal sepsis, while Garnacho's study included heterogeneous patients with varying sites of infection (with only 45% of patients suffering from abdominal sepsis). In addition, only 23% of their SIRS patients was subjected to prior surgery,[7] while we excluded these from our post-operative group. Taken together, the underlying pathophysiological processes taking part during sepsis seem to lead to a strong release of cfDNA, while even extensive surgical procedures with concomitant destruction of cells only lead to a minor, yet significant release. The source of cfDNA is currently under debate. For a long time, the extensive formation of NETs in the capillary system, triggered by the exaggerated and systemic immune reaction during sepsis, was believed to be the major source.[11] However, a recent preclinical study by Hamaguchi *et al.* revealed only a minor role of NET formation for the systemic levels of cfDNA.[23] Taken into account the differences between animal models and human sepsis, the truth might lie in between: NET formation *per se* contributes DNA into the bloodstream. The

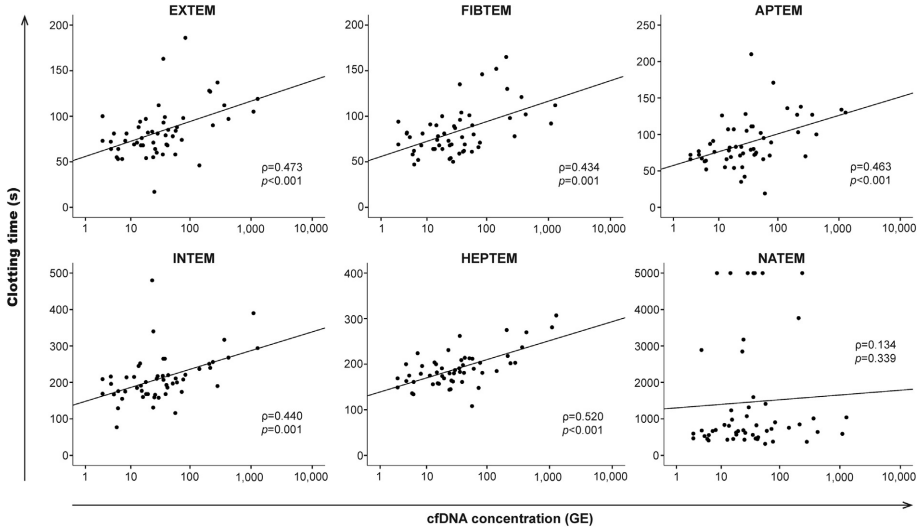

Fig. 2 – Correlation of cfDNA concentration to ROTEM CT using different assays. Combined data from all time points of septic patients was used for analysis. ρ: Spearman's Rho correlation coefficient. X axis is adjusted to logarithmic scaling.

occlusion of small vessels by NETs in synergy with coagulatory processes initiated via activated endothelial cells then results in a hypoxic microenvironment within the tissue, fostering cell death to a tremendous higher degree than defined surgical procedures and thereby a further passive DNA release.

Analogous to cfDNA, also exRNA levels were elevated in septic patients compared with surgical patients and healthy volunteers, but only in the first 24 h. This finding might be explained by the rapid degradation of exRNA by endogenous plasmatic ribonucleases, which have been shown to be increased during sepsis.[24] In line with our findings and supporting the idea of dying cells as origin, Kannemaier *et al.* stated earlier to "expect an increase of exRNA under conditions of tissue injury in association with sepsis, bacterial, or viral infection."[17] Nevertheless, until now only distinct intracellular and extracellular miRNAs have been assessed for their potential use as diagnostic and prognostic biomarkers of sepsis.[25,26] The total amount of RNA has been shown to be elevated during trauma-induced sterile inflammation, also correlating with organ dysfunction,[27] but our study is the first to prove the same kinetic during sepsis.

To examine a potential influence of the nucleic acids on coagulation (our hypotheses II and III), we correlated our measurements with a broad panel of laboratory marker as well as results of ROTEM and Multiplate point-of-care tests. Several publications certify thromboelastography a high specificity and sensitivity for the identification of DIC and

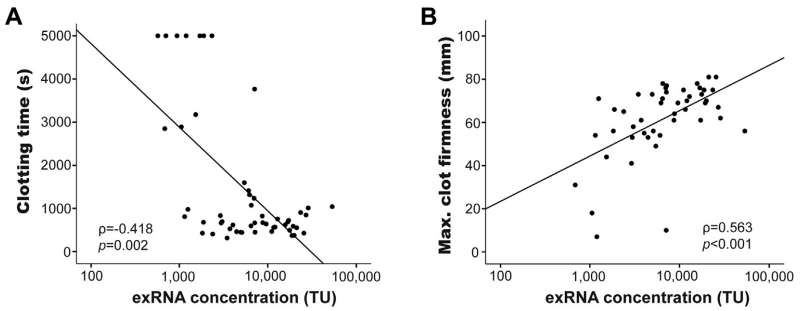

Fig. 3 – Correlation of exRNA to NATEM CT (A) and MCF (B). Combined data of septic patients over all time points was used for analysis. ρ: Spearman's Rho correlation coefficient. X axis is adjusted to logarithmic scaling.

Table 3 – Characteristics of liver, kidney, and general tissue damage parameters in correlation to nucleic acids in septic patients over all time points

Parameter	cfDNA (GE)	P	exRNA (TU)	P	N
LDH					
Normal range (90-250 U/L)	15.1		712.7		9
Elevated (>250 U/L)	58.6	0.028	1250.3		14
Lactate					
Normal range (0.5-2.2 mmol/L)	24.6		445.1		29
Elevated (>2.2 mmol/L)	57,8	0.009	912.9	0.027	22
AST					
Sub-normal (<35 U/L)	2.8		646.1		1
Normal range (10-35 U/L)	15.6		710.2		9
Elevated (>35 U/L)	37.4		558.5		30
ALT					
Normal range (10-35 U/L)	26.4		445.1		23
Elevated (>35 U/L)	38.4		1163.6		17
Bilirubin					
Normal range (0-1 mg/dL)	25.1		545.6		22
Elevated (>1 mg/dL)	39.6		1227.5		16
Creatinine					
Sub-normal (<0.6 mg/dL)	24.6		238.6		7
Normal range (0.6-1.2 mg/dL)	24.0		527.0		26
Elevated (>1.2 mg/dL)	72.7	0.004	918.6		18
Urea					
Normal range (10-50 mg/dL)	22.2		490.1		18
Elevated (>50 mg/dL)	36.2		701.6		33

hyperfibrinolysis, whereas in other prospective studies, thromboelastography failed to detect septic coagulopathy.[20] If coagulopathy was identified, both hypocoagulatory and hypercoagulatory states were found.[28-30] One plausible reason might be the different pathophysiological stages of sepsis, leading first to a pronounced procoagulatory state and later to excessive bleeding due to consumption of coagulation factors—a fulminant condition named DIC. Taken this dynamic staging into account, we combined the measurements over all time points to enable a meaningful analysis. A previous study using thromboelastometry demonstrated that septic patients had lower fibrinolysis[31] and tended toward a more pronounced clot formation once initiated.[32] Recently, light was shed on the molecular mechanisms by the study of Gould *et al.* who found an impaired fibrinolysis in sepsis patients, mediated by the binding of cfDNA to plasmin, rendering it incapable to degrade fibrin.[13] Our results in part confirm these earlier findings: our patients show an increased LI (equals a decreased clot breakdown) in correlation to cfDNA throughout all assays except EXTEM. Although no correlation of cfDNA with any assay's MCF could be observed, CT is as well prolonged like shown before by Collins *et al.*[32] These findings might at first be counter-intuitive, as DNA does not seem to imply a clear cut procoagulatory function as shown before *in vitro*,[33] but rather to participate in clot structure and as a negative regulator of fibrinolysis. Nevertheless, as extensive and uncontrolled coagulation might be harmful, this aspect might possess a pathophysiological relevance in the context of sepsis. The effect of cfDNA seems to depend heavily on its association with other factors such as, for example, histones, which have been shown to diminish the effects of both unfractionated and low–molecular weight heparin, even when in complex with DNA.[34] This might also be one important aspect contributing to the fact that our patients, despite receiving routine heparin treatment, presented with a heterogeneous response, for example, in terms of partial thromboplastin time. Moreover, depending of the subcellular origin (mitochondrial or nuclear), especially mitochondrial DNA is capable to activate immune cells, mediated by structural similarities to bacterial DNA and leading to secondary effects on coagulation.[35] In line with this, a recent cross-sectional study was able to show a correlation of endotoxin activity to native coagulation in early sepsis.[36] This makes it obvious that immune cells are confronted with a variety of stimuli, capable to activate the cells and, considering the tight interaction, indirectly also coagulation. However, the individual contribution of each factor can hardly be dissected in a complex system like our patients. Interestingly and strengthening our findings of a missing association with MCF, changes in platelet function as measured by Multiplate did not adhere to cfDNA level, pointing toward a platelet-independent effect as suggested also by Collins *et al.*[32] However, our exRNA results again tell another, contrary story. We found a procoagulatory tendency as shown by shorter CTs in native

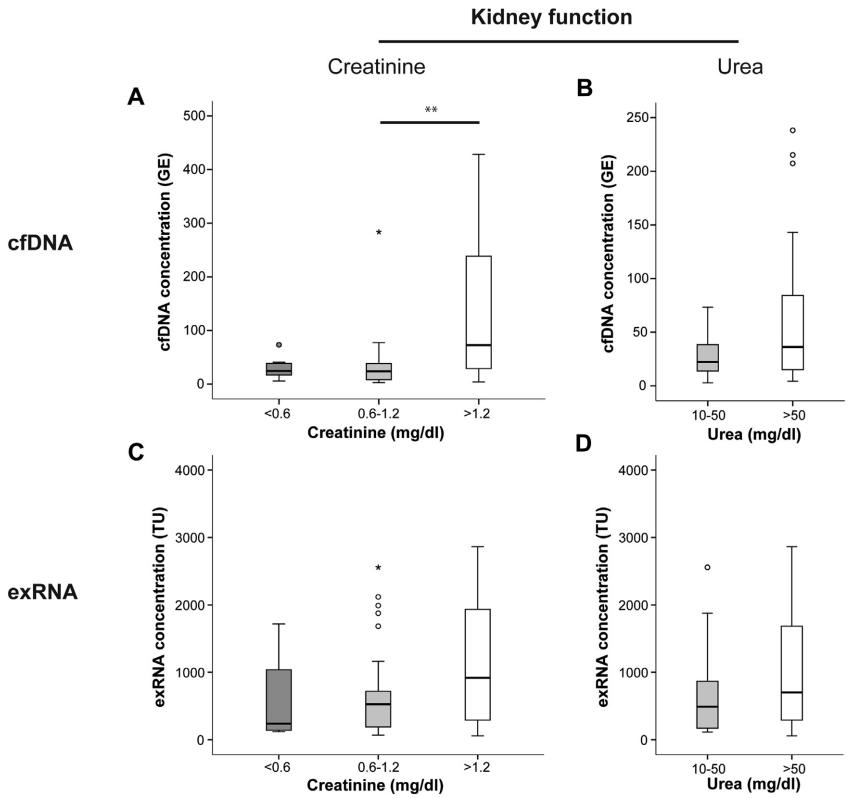

Fig. 4 – Concentrations of cfDNA (upper panel) and exRNA (lower panel) in patients with sepsis grouped according to markers of kidney function. (A and C) Creatinine (n = 7/26/18) and (B and D) urea (n = 18/33) were used for grouping, with boxplots showing the median and whiskers spanning the 1.5× interquartile range. $^{**}P < 0.01$, comparing "0.6-1.2" *versus* "> 1.2". Asterisks and circles represent outliers.

coagulation (NATEM) with increasing exRNA levels. In addition, also MCF correlates to exRNA. Overall, our findings hint toward a dual role exRNA, distinct from cfDNA. As reported earlier, RNA represents a cofactor for central components of both extrinsic (Factor XII) and intrinsic (factor VII–activating protease) coagulation pathways.[17,37]

Early containment of microorganisms is one crucial component for host survival during infection but constitutes a double-edged sword, as mechanisms like NETs and thrombus formation within capillaries also lead to a reduced blood supply of tissues. In a dysbalanced, systemic condition like sepsis, this might initiate the fatal stretch of organ dysfunction.[14] We aimed to address the question of an association of nucleic acids with organ (dys-)function in sepsis. We found higher levels of cfDNA in patients with impaired renal function, high lactate or LDH concentrations, indicative for persisting organ damage, and metabolic alterations. Only in the case of increased lactate, also a correlation to plasma exRNA concentrations was found. Illness severity scores adhered to levels of cfDNA as well, while liver function seems to be independent. Because of the high blood perfusion, the liver is in general vulnerable for bloodstream infections. Recently, NETs have been proven as critical mediators of liver injury.[38] Interestingly, DNA was dispensable, as under DNAse treatment, the DNA content of NETs diminished, leaving a robust histone structure behind. Nevertheless, the liver capillaries possess the largest diameter of all capillaries, and therefore, a full occlusion is not easy to achieve. In addition, because of the anatomical characteristic of the liver with high vessel density, an occluded vessel might be dispensable, as collaterals can ensure, for example, oxygen supply. In contrast, the smaller fenestrated capillaries of the kidney are at high risk during sepsis, at worst manifesting in acute kidney injury.[39] DNAse treatment has been proposed as therapeutic approaches to prevent organ dysfunction. In animal studies, early treatment of animals with DNAse led to a dissemination of bacteria and

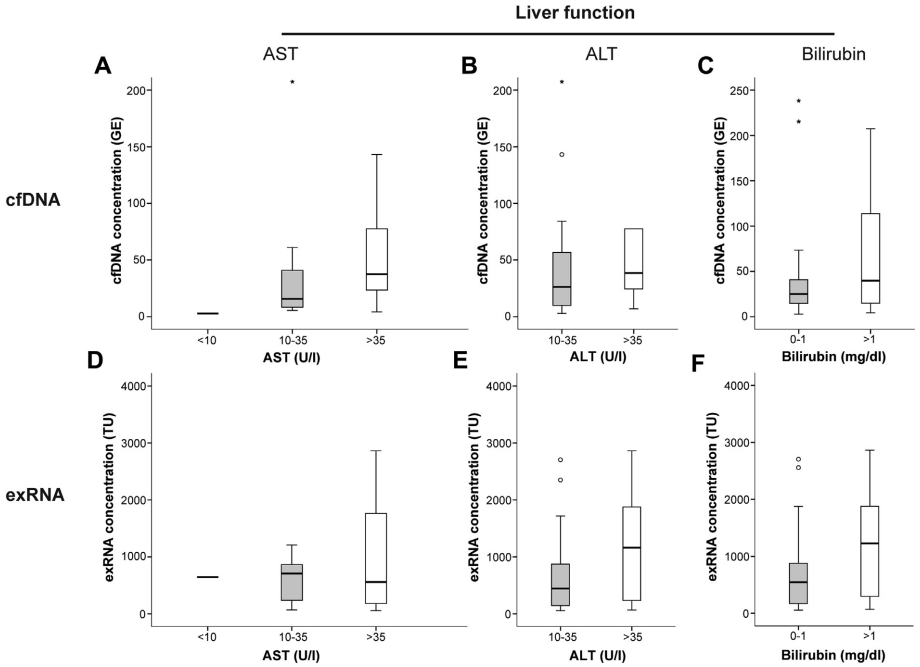

Fig. 5 – Concentrations of cfDNA (upper panel) and exRNA (lower panel) in patients with sepsis grouped according to markers of liver function. (A and D) AST (n = 1/9/30), (B and E) ALT (n = 23/17), and (C and F) bilirubin (n = 22/16) were used for grouping, with boxplots showing the median and whiskers spanning the 1.5× interquartile range. Asterisks and circles represent outliers.

impaired survival, whereas delayed treatment improved organ function, especially of lung and kidney.[40,41] Taken together, earlier preclinical findings of other groups together with our clinical results bridge both worlds and underline the potential usefulness of DNAse treatment in conditions of septic organ dysfunction for biomarker-stratified patient populations. Also, RNAse treatment might be feasible as a therapeutic approach, depending on the net coagulatory tendency of the individual patient.

The prognostic value of free circulating nucleic acids has been discussed recently and some evidence for cfDNA exist.[7,8,10,42] Despite no significant differences likely because of sample size, our results in general confirm these data. Again, levels of exRNA did not follow the kinetic of cfDNA.

Our study implies several limitations. Above all, our study is solely observational and on this basis correlative. Potential causal interrelations arising from our data need to be proven in defined and controlled cell culture or animal experiments. Next, because of the explorative character of our study, we only included a small, but yet very homogenous number of patients with sepsis. To weaken this point, we performed a longitudinal sampling for each patient, aiming to assess the patient's condition in different stages of the disease. Not surprisingly, patients with sepsis showed higher clinical scores at baseline compared with our surgical patients. Finally, the methods in our study have been used in earlier studies, nevertheless the detailed methodology differs remarkably between available studies, making a direct comparison of quantitative measurements impossible and raising the need for a consensus method in the future. Importantly, the method should be independent of PCR amplification, which requires long stretches of RNA/DNA and therefore might underestimate the true amount of circulating nucleic acids by rather detecting only the top of the iceberg. Emerging from this idea, further studies are needed to evaluate the dependency of nucleic acid function on size.

Conclusions

Our study proves the presence of both species of nucleic acids in the plasma of patients with sepsis (and surgical patients) and their potential influence on coagulation. While exRNA on the one hand might act as a classical (co-)activator of

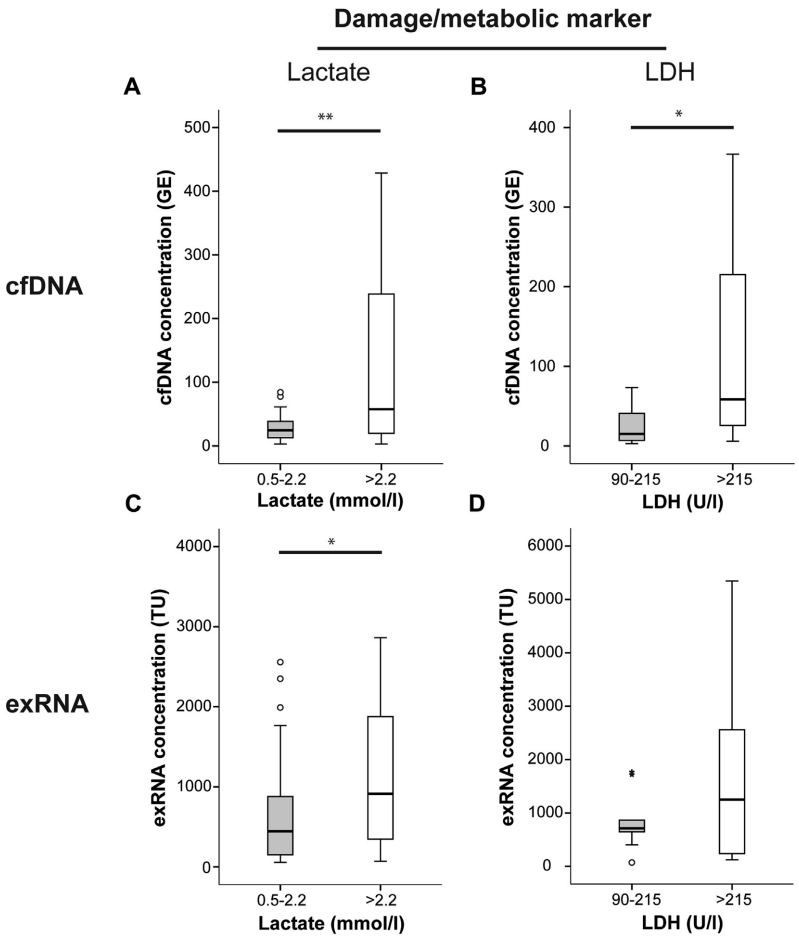

Fig. 6 – Concentrations of cfDNA (upper panel) and exRNA (lower panel) in patients with sepsis grouped according to markers of cell damage and metabolic alterations. (A and C) Lactate ($n = 29/22$) and (B and D) LDH ($n = 9/14$) were used for grouping, with boxplots showing the median and whiskers spanning the 1.5× interquartile range. */ $P < 0.05/0.01$. Asterisks and circles represent outliers.**

coagulation, cfDNA on the other hand might modulate decay and stability of established clots by acting as a constituent as well as an interacting factor with, for example, plasmin. Our results foster the current concept of immunothrombosis in sepsis and put it into a clinical setting. Early detection of immunothrombosis incorporating multidimensional and rapid assessment of relevant factors resulting in clinical pathways might be a possible basis for future treatment strategies so urgently needed to master the dynamic coagulatory changes occurring during sepsis.

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Authors' contributions: E.S., O.S., C.L., M.A.W, and F.U. were responsible for the study initiation, study design, data and sample acquisition, statistical analysis and interpretation, and for creating this article. C.K., D.S., F.S., A.H., W.P., B.S., S.W., and C.A. contributed to the data and sample acquisition and gave general advice. All authors have revised the article and approved the final version of article.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jss.2016.11.044.

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8.6 Anlage 6

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Flow Cytometry-Based Quantification of Neutrophil Extracellular Traps Shows an Association with Hypercoagulation in Septic Shock and Hypocoagulation in Postsurgical Systemic Inflammation—A Proof-of-Concept Study

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Abstract: This proof-of-concept study aimed to evaluate a novel method of flow cytometry-based quantification of neutrophil extracellular traps (NETs) in septic shock patients and to identify possible interactions between the number of free-circulating NETs and alterations of the coagulatory system. Patients suffering from septic shock, a matched control group (CTRL), and patients suffering from systemic inflammation after cardiac (CABG) or major abdominal surgery (MAS) were enrolled in this prospective proof-of-concept study. Compared to the matched controls, free-circulating NETs were significantly elevated in septic shock and postsurgical patients (data are presented in median (IQR)); septic shock: (2.7 (1.9–3.9); CABG: 2.7 (2.1–3.7); MAS: 2.7 (2.1–3.9); CTRL: 1.6 (1–2); CTRL vs. septic shock: $p = 0.001$; CTRL vs. CABG: $p < 0.001$; CTRL vs. MAS: $p < 0.001$). NETs correlated positively with FIBTEM mean clot firmness (MCF) in septic shock patients ($r = 0.37$, $p < 0.01$) while they correlated negatively in surgical patients (CABG: $r = -0.28$, $p < 0.01$; MAS: $r = -0.25$, $p = 0.03$). Flow-cytometric quantification of NETs showed a significant increase in free-circulating NETs under inflammatory conditions. Furthermore, this study hints to an association of the number of NETs with hypercoagulation in septic shock patients and hypocoagulation in surgery-induced inflammation.

Keywords: inflammation; sepsis; coagulopathy; cardiopulmonary bypass; major abdominal surgery

1. Introduction

Despite tremendous efforts to develop new diagnostic and therapeutic approaches, septic shock still remains associated with high mortality. Particularly, patients suffering from septic coagulopathy are considered to be at high risk for limited outcome [1,2]. This is recognized by the Sepsis-3 definition, which highlights that the host´s pathophysiological reactions to a pathogen are determined by the severity of organ failure [3]. Furthermore, tissue hypoperfusion is defined as the main symptom in septic shock; it is caused by vasoplegia, endothelial damage, and leakage, as well as capillary thrombotic occlusion [2]. Next to sepsis-induced alterations of the renal, hepatic, cardiovascular, respiratory, and central venous system, coagulopathies display a common and hazardous complication of sepsis [4,5]. Lyons et al. investigated 6148 septic patients and identified the presence of sepsis-associated coagulopathy (SAC) as an independent predictor for increased mortality [4]. Until today, despite ambitious approaches, no specific treatment of SAC could be successfully established [6–8]. For this reason, early detection of sepsis and SAC are crucial for the survival of patients. Diagnostic management should be based on the combined scoring of clinical signs of coagulopathy and blood coagulation parameters [1]. However, despite innovative approaches, there is still no specific biomarker for the early detection of SAC available for clinical routine use [9–13]. By investigating the underlying interactions between the innate immune and coagulatory systems, immunothrombosis has been identified as an important trigger of systemic inflammation and offers new diagnostic and therapeutic approaches for the management of septic coagulopathy [14–17].

Neutrophil extracellular traps (NETs) were identified as major players in immunothrombosis [16,17]. Consisting of nucleic acids, histones, and granule contents, such as myeloperoxidase (MPO) and neutrophil elastase, NETs are released by neutrophils. Once set free in the capillary vasculature, they form web-like formations that trap pathogens and closely interact with platelets. The capture of pathogens decelerates their spread and concentrates neutrophil antimicrobial activity. However, NETs might contribute to negative effects, such as excessive activation of coagulation, which eventually results in disseminated intravascular coagulation [18–20]. Several determinants lead to an NET-induced activation of the coagulatory system: Due to their polyanionic surface, NETs activate the intrinsic plasmatic system while the extrinsic pathway is stimulated by tissue factor presentation of NETs and platelets are strongly activated by DNA/histones complexes [21–24]. However, despite an increasing number of studies hinting towards a procoagulatory effect of NETs, the clinical relevance of these findings remains controversial [18,19,23,25].

Elevated plasma levels of NETs were first identified in autoimmune and cancerous diseases, but current studies proved their crucial role in the development of sepsis [26–32]. Furthermore, some studies indicate that NETs are also elevated in the peripheral plasma of patients suffering from systemic inflammation after major surgery [27,33–35]. Nevertheless, even though the amount of free-circulating NETs is increased in sepsis and surgery-induced systemic inflammation, it remains unknown if NETs offer discriminative value in distinguishing between septic shock and postsurgical systemic inflammation [31,36]. Particularly, in the early postsurgical phase, discrimination of severe infectious complications from sterile postsurgical inflammation remains challenging in the clinical routine [37,38].

Since NETs may offer an opportunity for novel diagnostic and therapeutic approaches, the need for robust and clinically feasible quantification of NETs arises. Until today, fluorescence microscopy remains the most established method for NET quantification; however, this method has some limitations. First, it only analyzes an abstract of a sample; second, it is not feasibly for daily intensive care unit (ICU) routine; and third, it is highly dependent on expert scientists' skills and interpretations. In contrast, flow cytometry offers fast and reliable counting of a high number of neutrophils. In 2015, two methods of flow cytometry-based NET quantification were published by two separate research facilities. While Zhao et al. established a combination of high-speed multi-spectral imaging and morphometric image analysis, Gavillet et al. used a direct flow cytometry-based assay for NET identification and quantification [39,40]. Although both approaches are able to count a high number of cells and perform

morphological analysis, they also feature some limitations. First, they are highly specialized and require expert knowledge, which makes them less suitable for research in the clinical routine. Second, the appropriate gating strategy to identify NET-releasing neutrophils is still under discussion [41,42]. In 2018, Lee et al. published an optimized method of Gavillet et al.'s protocol, which aimed to quantify NET-releasing neutrophils by using whole blood probes without cell fixation [43]. Therefore, this method seems more feasible for implementation in an ICU.

This proof-of-concept study aimed to investigate the flow cytometry-based quantification of NETs in a clinical intensive care setting. First, we hypothesized that flow cytometry-based quantification of NETs is able to distinguish between matched control and septic shock patients as well as patients suffering from surgery-induced inflammation. Second, we aimed to identify possible interactions between the number of free-circulating NETs and coagulatory dysfunctions using thrombelastography as a practicable solution for use in the clinical routine.

2. Experimental Section

2.1. Study Design

This single-center, prospective, observational proof-of-concept study included 80 patients who were enrolled from October 2018 to March 2019 at the University Hospital of Giessen. The study was approved by the local ethics committee (Justus-Liebig-University of Giessen, trial code: 86/18) and registered in the German Clinical Trials Register (trial code: DRKS00013584). The methods and results are presented in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines and the Declaration of Helsinki.

Patients of legal age were enrolled at the University Hospital of Giessen after signing an informed consent form. If patients were not able to consent to the study, consent was obtained through their legal representatives. General exclusion criteria comprised an age <18 years, current pregnancy or nursing, history of recent severe trauma, autoimmune disease, severe valvular heart disease, active hematological disease, the need for immunomodulatory medication, or having undergone therapy with extracorporeal life support or renal replacement therapy prior to study inclusion. Septic shock patients must have met the Sepsis-3 definition of septic shock [3]. Cardiac surgical patients underwent coronary artery bypass graft surgery (CABG) while visceral surgical patients received major abdominal surgery (MAS), such as the Whipple procedure, oncological esophageal and gastric resection, or colectomy. Surgical patients had to meet at least two criteria of the systemic inflammatory response syndrome (SIRS) within 24 h after surgery [44]. Since no baseline values were achievable in septic patients, control (CTRL) patients were matched to them. CTRL patients were matched to septic patients based on age and gender as well as pre-existing conditions, such as a history of malignant solid or hematologic diseases, arteriosclerosis (coronary, cerebral, or peripheral artery disease), chronic renal disease, or diabetes mellitus.

Blood was collected shortly prior to surgery as well as immediately, 24, and 72 h postoperatively in surgical patients. From septic shock patients, blood was first drawn after admission to the ICU and again after diagnosis of septic shock, followed by blood collections after 24 and 72 h. CTRL patients were asked for blood donation at a single time point. Blood was collected in ethylenediaminetetraacetic acid (EDTA) for flow cytometry, fluorescence microscopy, and ELISA, while citrate tubes were used for thrombelastometry and hirudin tubes for whole blood ristocetin-induced platelet impedance aggregometry. Plasma samples were stored at −80 °C and only thawed once for the ELISA analyses. Clinical data were extracted from the local patient data management system (IMESO GmbH, Giessen, Germany).

2.2. Flow Cytometry

The flow cytometry protocol was adapted from the protocol published by Lee et al. [43]. In brief, 100 µL of whole blood were incubated with 10 µg immunoglobin G (IgG, 10% solution, Grifols,

Barcelona, Spain) for 10 min in order to eliminate unspecific binding sites. Afterwards, 5 µL of directly-conjugated anti-H3-Histone antibody (Alexa Fluor 647 Anti-Histone, BioLegend, San Diego, CA, USA) were incubated for 30 min in darkness at room temperature followed by the application of 5 µL of anti-human cluster of differentiation (CD) 15^+- (Pacific Blue™ anti-human CD15 antibody, BioLegend, San Diego, CA, USA) and 10 µL of myeloperoxidase-(MPO)-antibody (ab11729, Abcam, Cambridge, UK). The mixture was then incubated again for 30 min at room temperature in darkness. In the next step, 1 mL of lysis buffer (1:10 dilution, BD Pharm Lyse™, Frankin Lakes, NJ, USA) was incubated for 10 min under the same conditions for red blood cell lysis. Next, 1 mL of 2% bovine serum albumin in phosphate-buffered saline (PBS) was applicated followed by centrifugation (200 g for 10 min). Following this, the supernatant was separated and 300 µL of PBS were applied. Impairment of membrane integrity by red blood cell lysis was checked in healthy controls using the application of 7-aminoactinomycin (7-ADD, application five minutes prior to measurement, #559925, BD Biosciences, Franklin Lakes, NJ, USA, Supplement 1 in Supplementary Materials). Furthermore, in preliminary tests, a positive control was performed by stimulating the whole blood of healthy subjects with phorbol 12-myristate 13-acetate (100 nmol/L, PMA, Sigma, St. Louis, MO, USA) for 4 h (Supplement 2 in Supplementary Materials) [43,45].

BD FACS Canto II and BD FACSDIVA software (version 6.1.3, Franklin Lakes, NJ, USA) were used for flow cytometric analysis. In order to avoid detection bias, samples were blinded for flow cytometry. The gating strategy involved three steps: First, leucocytes were targeted, and neutrophils were identified as $CD15^+$-cells. Second, the fluorescence-minus-one (FMO) technique was used to set the gating borders of MPO- and anti-H3-Histone-antibody. Finally, MPO- and anti-H3-Histone-antibody-positive cells were defined as surrogates for NETs (Figure 1). In order to exclude neutrophil aggregates, we checked for neutrophil duplicates. Furthermore, we quantified NETs with isotype controls in preliminary experiments to exclude relevant background fluorescence signals and found comparable results to the original description of Lee et al. (Supplement 2 in Supplementary Materials) [43]. Results were shown as the percentage of NETs for all gated neutrophils. Gating results ≤0.5% were excluded due to the impossibility of exact discrimination.

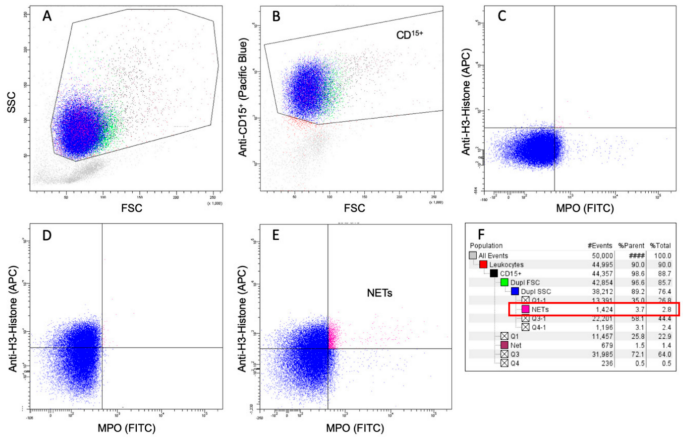

Figure 1. Description of the flow-cytometric gating strategy. First, leucocytes were targeted (**A**) and neutrophils identified as $CD15^+$-cells (**B**). Second, the fluorescence-minus-one (FMO) technique was used to set the gating borders of MPO- and anti-H3-Histone-antibody (**C**,**D**) and last, MPO- and anti-H3-Histone-positive cells were defined as surrogates for NETs (**E**). Results are shown as the percentage of gated neutrophils (**F**, red box). Abbreviations: APC: Allophycocyanin; CD: Cluster of Differentiation; FITC: Fluorescein isothiocyanate; FSC: Forward Scatter; MPO: Myeloperoxidase; NETs: Neutrophil Extracellular Traps; SSC: Side Scatter.

2.3. Fluorescence Microscopy

In order to validate MPO- and anti-H3-Histone-antibody-positive cells as NETs surrogates, confocal fluorescence microscopy was performed. In total, 100 µL of whole blood were incubated with 10 µL of PMA (100 nmol/L) for four hours, followed by red blood cell lysis (incubation of 1 mL of Pharm Lyse™ (1:10 dilution) for 10 min). Afterwards, lysis was stopped using 1 mL of PBS, and centrifugation was performed with 200 g at 20 °C for 10 min. Then, the supernatant was decanted and 3 min of Cytospin® (Cellspin 1, Tharmac, Wiesbaden, Germany) centrifugation was used for fixation on cover slips followed by staining with 5 µL of anti-H3-histone (Alexa Fluor 647 Anti-Histone, BioLegend, San Diego, CA, USA), 5 µL of anti-human-CD15 (Pacific Blue™ anti-human CD15 antibody, BioLegend, San Diego, CA, USA), and 10 µL of MPO-antibody (ab11729, Abcam, Cambridge, UK) was performed. After incubation for 30 min in darkness, samples were washed with PBS and analyzed with fluorescence microscopy (Leica TCS SP5, Wetzlar, Germany) (Supplement 3 in Supplementary Materials).

2.4. ELISA

ELISA analyses included measurements of High-Mobility-Group-Protein B1 (HMGB1, Human HMGB1 ELISA Kit, Aviva Systems Biology, San Diego, CA, USA), MPO (Human MPO Instant ELISA, eBioscience, Frankfurt a.M., Germany), and interleukin-8 (IL-8, Human IL-8/CXCL8 Quantikine HS ELISA, R&D Systems, Minneapolis, MN, USA); these analyses were performed according to the manufacturer's instructions. The optical density of samples was measured with the recommended absorbance (HMGB1: 450 nm; MPO: 450 nm, IL-8: 490 nm) and analyzed using an automated plate reader (Epoch; BioTek Instruments GmbH, Heilbronn, Germany).

2.5. Coagulatory Analyses

Point-of-care devices were used for thrombelastography (ROTEM®, Matel Medizintechnik, Hausmannstaetten, Austria) and whole blood ristocetin-induced platelet impedance aggregometry (Multiplate®, Roche Diagnostics, Rotkreuz, Switzerland), while the results of all other coagulatory tests were derived from the local clinical laboratory routine. Thrombelastographic assays included NATEM®, INTEM®, FIBTEM®, and EXTEM®, while the clotting and clot formation time (CT and CFT, respectively, both in seconds), mean clot firmness (MCF, mm), and lysis index after 60 min (LI60, %) were analyzed. Whole blood ristocetin-induced platelet impedance aggregometry was performed after stimulation by adenosindiphosphate (ADPtest®), thrombin-receptor activator protein 6 (TRAPtest®), and arachidonic acid (ASPItest®). Furthermore, the severity of coagulopathy was scored using the SAC score [46].

2.6. Statistical Analysis

First, values were tested for normal distribution using the Shapiro–Wilk test. Parametric data were described with mean and standard deviation while the median and interquartile range were used for non-parametric data. For statistical analysis of differences in the amount of free-circulating NETs between the study groups, the Kruskal–Wallis test was performed followed by the Wilcoxon test for analysis of intergroup differences. The analysis of the variation in the number of free-circulating NETs according to the timepoint was accomplished by applying the Friedman test followed by the pairwise Wilcox test for an analysis of the differences between timepoints within each study group. For this purpose, septic shock patients were compared to their matched controls. The correlation of NETs with respective parameters was expressed as a Pearson´s correlation coefficient. Clinical data, laboratory routine data, and experimental data were recorded in an external database (Microsoft Excel, Redmond, WA, USA). Data were processed and analyzed using R statistical software version 3.4.2 (www.r-project.org).

3. Results

Patient characteristics are shown in Table 1. All data are presented as median (interquartile range (IQR)).

Table 1. Description of the study cohorts.

	Septic Shock (n = 20)	Cardiac Surgery (CABG, n = 20)	Major Abdominal Surgery (MAS, n = 20)	Control Patients (CTRL, n = 20)
General Characteristics				
Age (years)	69 (64.3–74)	70 (62–79)	68 (54–70)	69 (66.3–74.3)
Sex (% male)	70	75	60	70
BMI ($kg \cdot m^{-2}$)	27.9 (21.7–32.6)	30 (27.6–36.5)	24 (22.4–26.9)	27 (23.2–29.2)
ASA				
I	0	0	1 (5%)	1 (5%)
II	0	0	8 (40%)	6 (30%)
III	10 (50%)	18 (90%)	11 (55%)	13 (65%)
IV	9 (45%)	2 (10%)	0	0
V	1 (5%)	0	0	0
SOFA onset	10.5 (10–12.5)	NA	NA	NA
SOFA 24 h	11.5 (8–13)	3 (1–3.8)	2 (0–3)	NA
SOFA 72 h	9 (5.5–14.5)	3.5 (1–4.8)	3.5 (1.8–4.8)	NA
Focus of infection				
Abdominal	12 (60%)			
Pulmonary	3 (15%)	NA	NA	NA
Urological	3 (15%)			
Soft tissue	2 (10%)			
Type of abdominal surgery				
Whipple Procedure			8 (40%)	
Open Partial colectomy	NA	NA	4 (20%)	NA
Esophagus resection			4 (20%)	
Other major abdominal surgery			4 (20%)	
Duration of Cardiopulmonary bypass	NA	93 (74.8–111)	NA	NA
In-hospital death (%)	35	0	5	0
Preexisting Diseases				
Diabetes mellitus	9 (45%)	12 (60%)	1 (5%)	8 (40%)
Chronic kidney failure	4 (20%)	5 (25%)	1 (5%)	3 (15%)
Arteriosclerosis	14 (70%)	20 (100%)	5 (25%)	14 (70%)
Malignant cancerous disease	7 (35%)	0	13 (65%)	7 (35%)
Anticoagulatory Therapy				
Prophylactic heparinization onset/preoperative	10 (50%)	20 (100%)	20 (100%)	15 (75%)
Prophylactic heparinization postoperative		0	0	
Prophylactic heparinization 24 h	12 (60%)	18 (90%)	18 (90%)	
Prophylactic heparinization 72 h	11 (55%)	16 (80%)	18 (90%)	
Therapeutic heparinization onset/preoperative	8 (40%)	0	0	5
Therapeutic heparinization postoperative		0	1 (5%)	
Therapeutic heparinization 24 h	6 (30%)	2 (10%)	1 (5%)	
Therapeutic heparinization 72 h	7 (35%)	3 (15%)	1 (5%)	

Data are shown as median (interquartile range) or as an absolute number and percentage (n (%)) of the study group. Abbreviations: ASA: American Society of Anesthesiology Score; BMI: Body Mass Index; SOFA: Sepsis-related Organ Failure Assessment; NA: not applicable.

3.1. Quantification of Free Circulating NETs

Compared to matched control patients, levels of free-circulating NETs were statistically significantly elevated in all patient samples independently of the study group and time point (Figure 2, Table 2, septic shock: 2.7 (1.9–3.9); CABG: 2.7 (2.1–3.7); MAS: 2.7 (2.1–3.9); CTRL: 1.6 (1–2); CTRL vs. septic shock: $p = 0.001$; CTRL vs. CABG: $p < 0.001$; CTRL vs. MAS: $p < 0.001$). Preoperative values of both surgical groups were significantly higher compared to those of the matched control group (Figure 3, Table 2, CTRL: 1.6 (1–2); CABG: 2 (1.7–2.6); MAS: 2.6 (1.7–3.3); CTRL vs. CABG preoperative: $p = 0.034$; CTRL vs. MAS preoperative: $p = 0.004$; CABG preoperative vs. MAS preoperative: $p = 0.354$). Septic shock patients showed a significant increase at onset and over three days compared to their matched control patients (Figure 3, Table 2, septic shock onset: 3.2 (2.3–4.2); septic shock 24 h: 2.5 (1.8–3.7); septic shock 72 h: 2.3 (1–3.8); CTRL vs. septic shock onset: $p < 0.001$; CTRL vs. septic shock 24 h: $p = 0.02$; CTRL vs. septic shock 72 h: $p = 0.05$). In cardiac surgical patients, the amount of free-circulating NETs peaked immediately after the surgery and decreased significantly after 24 and 72 h, respectively (Figure 3, Table 2, CABG preoperative: 2 (1.7–2.6); CABG postoperative: 3.5 (2.7–4.6); CABG 24 h: 2.7 (2.1–3.5); CABG 72 h: 2.8 (2.1–3.8); CABG preoperative vs. CABG postoperative: $p < 0.001$; CABG postoperative vs. CABG 24 h: $p = 0.0014$; CABG postoperative vs. CABG 72 h: $p = 0.01$). MAS led to the lowest increase of NETs but gained statistical significance immediately after surgery (Figure 3, Table 2, MAS preoperative: 2.6 (1.7–3.3); MAS postoperative: 2.9 (2.3–5.2); MAS 24 h: 2.6 (2–3.8); MAS 72 h: 2.7 (2.3–3.9); MAS preoperative vs. MAS postoperative: $p = 0.03$). The postsurgical levels of free-circulating NETs did not differ compared to septic shock patients (Figure 3, Table 2).

Table 2. Results of inflammatory parameters.

		Septic Shock (n = 20)		Cardiac Surgery (CABG, n = 20)		Major Abdominal Surgery (MAS, n = 20)		Control Patients (CTRL, n = 20)	
Leucocytes (L^{-1})	onset 24 h 72 h	11.9 (7.1–19.7) 13.5 (9.3–20.9) 14.2 (10.7–17.3)	Preop Postop 24 h 72 h	8.1 (6.6–9.4) 11 (7.9–15) 10.7 (8.2–12.2) 10.6 (8.2–11.8)	Preop Postop 24 h 72 h	7.6 (6–9] 10.3 (9.4–12.5) 11.5 (9.3–12.9) 7.4 (6.5–11.6)	Ctrl	5.9 (5.3–7.9)
CRP (mg/L)	onset 24 h 72 h	229.5 (117.2–277.3) 244.6 (166.5–287.7) 236.5 (139.5–268.8)	Preop Postop 24 h 72 h	3.8 (1.9–10.6) 4.3 (2.6–9.2) 75.1 (67.2–109.8) 202.4 (156.3–241.2)	Preop Postop 24 h 72 h	5.1 (1.7–10.3) 6.5 (2.5–11.4) 68 (46.6–88.5) 149 (115.7–200)	Ctrl	1.1 (0–6.4)
PCT (µg/L)	onset 24 h 72 h	9.2 (5.2–38.1) 10.4 (4.9–29.2) 7 (2.2–25.6)	Preop Postop 24 h 72 h	0.2 (0.1–0.2) N.A. N.A 1.6 (1.6)	Preop Postop 24 h 72 h	N.A. 0.6 (0.4–0.7) 0.7 (0.3–0.9) 0.8 (0.4–0.9)	Ctrl	N.A.
NETs (%)	onset 24 h 72 h	3.2 (2.3–4.2) 2.5 (1.8–3.7) 2.3 (1–3.8)	Preop Postop 24 h 72 h	2 (1.7–2.6) 3.5 (2.7–4.6) 2.7 (2.1–3.5) 2.8 (2.1–3.8)	Preop Postop 24 h 72 h	2.6 (1.7–3.3) 2.9 (2.3–5.2) 2.6 (2–3.8) 2.7 (2.3–3.9)	Ctrl	1.6 (1–2)
HMGB1 (pg/mL)	onset 24 h 72 h	40,332.1 (25,079.6–51,674.9) 32,692.3 (21,563.6–50,421.8) 25,496.2 (23,125.4–33,421.3)	Preop Postop 24 h 72 h	25,241.3 (20,953.1–46,031.4) 23,982.5 (17,353.2–49,133.1) 30,440.2 (22,238.5–41,098.5) 26,584.3 (20,870.2–38,988.1)	Preop Postop 24 h 72 h	31,126.8 (20,032.8–38,097.8) 25,343.5 (21,913.1–41,784.2) 28,800.1 (21,687.7–39,665.6) 21,780.6 (16,867–34,755.6)	Ctrl	26,297.5 (22,149.3–34,710.9)
MPO (ng/mL)	onset 24 h 72 h	700,905.7 (285,135.5–886,644) 542,611.2 (303,891–832,728.9) 498,553 (381,058.9–610,573.3)	Preop Postop 24 h 72 h	392,102.8 (199,581–571,528,04) 438,502.8 (341,657.5–638,995.4) 595,820.4 (275,593.4–892,010.7) 529,317.3 (306,869.6–885,046)	Preop Postop 24 h 72 h	367,381.5 (187,582–499,310.8) 480,111 (344,182.5–885,513.8) 713,023.1 (433,356.9–913,219.4) 351,888,.5 (235,179.9–711,455.7)	Ctrl	214,472.6 (136,124.2–296,626.7)
Interleukin 8 (pg/mL)	onset 24 h 72 h	470.4 (105.9–1462,30) 206.6 (100.1–489.9) 165.1 (90.2–195.5)	Preop Postop 24 h 72 h	39.2 (26.1–49) 85.3 (57.7–127.9) 67.1 (40.7–99) 55.2 (42.9–72.2)	Preop Postop 24 h 72 h	35 (20.4–49.8) 71.1 (58.2–129.1) 60.9 (41.2–110.4) 41.9 (27.1–63.6)	Ctrl	35.8 (25–40.5)

Data are shown as median (IQR). Abbreviations: CRP: C-Reactive Protein; DNA: Deoxynucleic Acid; HMGB1: High-Mobility-Group-Protein B1; MPO: Myeloperoxidase; NETs: Neutrophil Extracellular Traps; PCT: Procalcitonin.

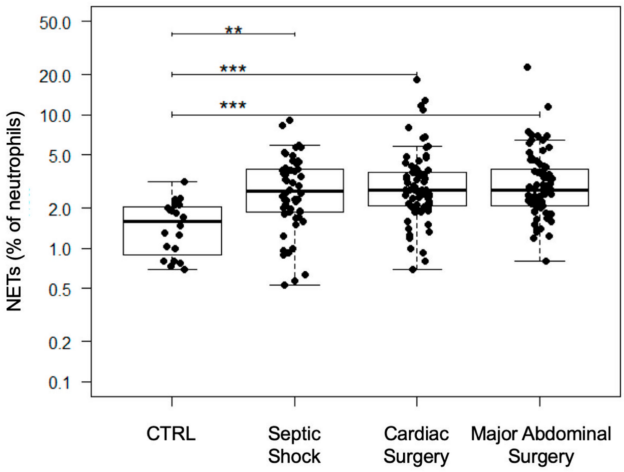

Figure 2. Results of the NET quantification of the study groups. With the exception of preoperative values, all time points per group were summarized. Results are shown in boxplot diagrams. Asterisks display the degree of statistical significance: **: $p < 0.01$; ***: $p < 0.001$. Abbreviations: NETs: Neutrophil Extracellular Traps.

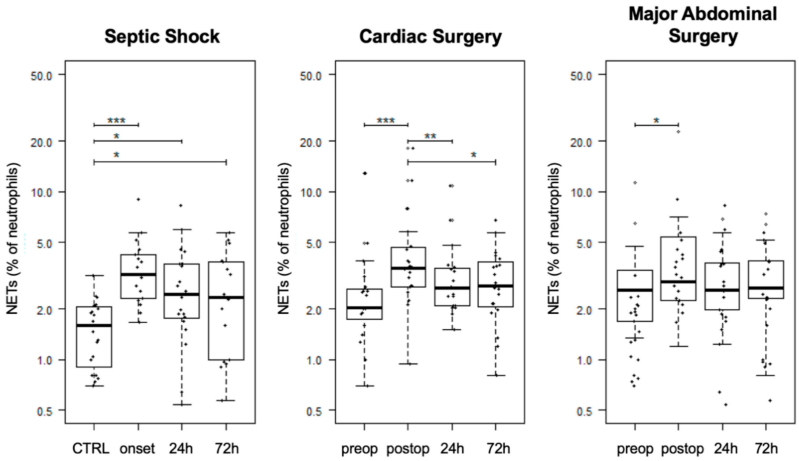

Figure 3. Time courses of free-circulating NETs. Results are shown in boxplot diagrams. Asterisks display the degree of statistical significance: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Abbreviations: CTRL: Control group; NETs: Neutrophil Extracellular Traps.

3.2. Association of NETs to Inflammatory Parameters

Compared to the control group, plasma levels of MPO and IL-8 increased significantly, beginning from the onset of septic shock, and remained significantly elevated over 24 h (Table 2, MPO: CTRL vs. septic shock onset: $p < 0.01$; CTRL vs. septic shock 24 h: $p < 0.01$; CTRL vs. septic shock 72 h: $p = 0.12$; IL-8: CTRL vs. septic shock onset: $p < 0.001$; CTRL vs. septic shock 24 h: $p < 0.01$; CTRL vs. septic shock 72 h: $p = 0.58$). While MPO showed a significant postoperative increase only in

MAS patients (Table 2, preoperative vs. postoperative: $p = 0.02$; preoperative vs. 24 h: $p = 0.004$), no detectable changes were found in CABG patients. With the exception of a significant elevation of IL-8 immediately after CABG, similar results were found for IL-8 expression in CABG patients (Table 2, CABG preoperative vs. postoperative: $p = 0.008$), while MAS patients presented a significant postoperative increase in IL-8 (Table 2, MAS preoperative vs. postoperative: $p = 0.008$, preoperative vs. 24 h: $p = 0.05$). Compared to the control group, changes of HMGB1 levels in septic shock patients almost reached statistical significance (at the onset of septic shock), but showed a significant increase after 24 and 72 h after septic shock onset (Table 2, control vs. septic shock onset: $p = 0.08$, control vs. septic shock 24 h $p = 0.07$; septic shock onset vs. septic shock 24 h: $p = 0.04$; septic shock onset vs. 72 h: $p = 0.04$). The other study groups did not present significant changes in HMGB1 plasma levels (Table 2).

While plasma levels of MPO and IL-8 did not correlate with the amount of free-circulating NETs in any study group, plasma levels of HMGB1 in septic shock patients showed a positive correlation to NETs (Table 3, $r = 0.3$; $p = 0.03$). Free-circulating NETs did not correlate to plasma levels of CRP and PCT nor to the leucocyte count (Table 3).

Table 3. Correlation of NETs to coagulatory and inflammatory parameters.

Parameter	Septic Shock ($n = 20$)		Cardiac Surgery (CABG, $n = 20$)		Major Abdominal Surgery (MAS, $n = 20$)		Control Patients (CTRL, $n = 20$)	
	Correlation Coefficient	*p*-Value	Correlation Coefficient	*p*-Value	Correlation Coefficient	*p*-Value	Correlation Coefficient	*p*-Value
Parameter	**Septic Shock ($n = 20$)**		**Cardiac Surgery (CABG, $n = 20$)**		**Major Abdominal Surgery (MAS, $n = 20$)**		**Control Patients (CTRL, $n = 20$)**	
	Correlation Coefficient	***p*-Value**	**Correlation Coefficient**	***p*-Value**	**Correlation Coefficient**	***p*-Value**	**Correlation Coefficient**	***p*-Value**
Thrombelastography								
EXTEM CFT (s)	−0.10	0.47	0.31	<0.01	0.27	0.02	0.04	0.87
FIBTEM CFT (s)	−0.15	0.31	0.00	1.00	0.25	0.05	−0.22	0.50
INTEM CFT (s)	0.07	0.61	0.26	0.02	0.20	0.09	−0.23	0.34
NATEM CFT (s)	−0.12	0.41	−0.09	0.44	−0.01	0.91	0.14	0.55
EXTEM CT (s)	−0.20	0.14	0.01	0.91	0.12	0.30	−0.43	0.06
FIBTEM CT (s)	−0.31	0.02	−0.02	0.85	0.00	0.99	−0.42	0.07
INTEM CT (s)	0.00	0.98	0.24	0.04	0.12	0.33	−0.33	0.16
NATEM CT (s)	−0.04	0.80	−0.10	0.38	−0.04	0.74	−0.06	0.80
EXTEM LI60 (%)	−0.08	0.55	−0.25	0.03	0.01	0.97	−0.02	0.94
FIBTEM LI60 (%)	−0.36	<0.01	−0.04	0.70	0.06	0.59	0.32	0.17
INTEM LI60 (%)	−0.12	0.38	−0.21	0.06	0.02	0.85	0.12	0.62
NATEM LI60 (%)	−0.16	0.30	−0.32	<0.001	0.03	0.84	0.11	0.65
EXTEM MCF (mm)	0.15	0.27	−0.38	<0.001	−0.28	<0.01	−0.25	0.28
FIBTEM MCF (mm)	0.37	≤0.01	−0.28	<0.01	−0.25	0.03	−0.38	0.10
INTEM MCF (mm)	0.18	0.19	−0.41	<0.001	−0.32	<0.01	−0.21	0.38
NATEM MCF (mm)	0.20	0.16	−0.23	0.04	-0.09	0.46	−0.33	0.15
Impedance Aggregometry								
ASPItest (Units)	0.24	0.08	0.019	0.87	−0.063	0.6	−0.1	0.67
TRAPtest (Units)	0.17	0.22	−0.058	0.61	−0.085	0.48	−0.11	0.64
ADPtest (Units)	0.07	0.64	−0.12	0.3	−0.07	0.56	−0.05	0.82

Table 3. *Cont.*

Global Coagulatory Parameters								
PTT (s)	−0.15	0.28	0.03	0.79	−0.09	0.5	0.09	0.7
INR	−0.21	0.12	0.18	0.1	0.08	0.52	0.16	0.53
Platelet count (L^{-1})	0.39	0.004	−0.032	0.78	−0.16	0.17	0.048	0.84
Fibrinogen (g/L)	0.31	0.101	−0.26	0.07	−0.1	0.7	NA	NA
Inflammatory Parameters								
Leucocytes (L^{-1})	0.007	0.96	−0.016	0.89	−0.12	0.33	−0.21	0.37
CRP (mg/L)	−0.1	0.47	−0.14	0.24	−0.12	0.34	−0.51	0.32
PCT (µg/L)	0.059	0.69	0.6	0.59	0.12	0.68	N.A.	N.A.
HMGB-1 (pg/mL)	0.30	0.03	0.04	0.76	−0.08	0.51	−0.43	0.06
MPO (ng/mL)	−0.16	0.24	0.04	0.75	−0.06	0.6	−0.19	0.41
Interleukin 8 (pg/mL)	0.01	0.93	0.16	0.16	0.04	0.70	−0.21	0.37

Data were derived from Pearson's correlation analysis. Significant p-values are highlighted in bold. Abbreviations: CABG: Coronary Artery Bypass Graft; CRP: C-Reactive Protein; CFT: Clot Firmness Time; CT: Clotting Time; CTRL: Control group; DNA: Deoxynucleic Acid; HMGB1: High-Mobility-Group-Protein B1; INR: International normalized ratio; LI60: Lysis Index after 60 min; MAS: Major Abdominal Surgery; MCF: Mean Clot Firmness; MPO: Myeloperoxidase; NA: not applicable; NETs: Neutrophil Extracellular Traps; PCT: Procalcitonin; PTT: Partial Thromboplastin Time.

3.3. Association of NETs to Coagulatory Parameters

In the initial analysis of the association of NETs to global coagulatory parameters, no significant correlation between NETs and PTT, INR, and fibrinogen was revealed for any study group. However, thrombocytes were positively correlated with NETs in septic shock patients (Table 3, $r = 0.39$, $p = 0.004$). While CTRL patients did not show any alterations of thrombelastographic parameters, septic shock patients showed a statistically significant negative correlation of the FIBTEM CT to free-circulating NETs and a significant positive association with the MCF (Table 3, Figure 4, FIBTEM CT: $r = -0.3$, $p = 0.02$; FIBTEM MCF: $r = 0.37$; $p < 0.01$). Other thrombelastographic assays did not reach statistical significance in septic shock patients (Supplement 4 in Supplementary Materials). Contrarily, after abdominal and cardiac surgery, a significant negative correlation of MCF with NETs could be detected in almost all assays (Table 3, Figures 5 and 6). Furthermore, cardiac surgical patients showed a significant correlation of NETs with prolonged INTEM CT and CFT as well as EXTEM CFT (INTEM CT: $r = 0.24$; $p = 0.04$; INTEM CFT: $r = 0.26$; $p = 0.02$; EXTEM CFT: $r = 0.31$; $p < 0.01$). Patients who underwent MAS showed the same tendencies but only reached statistical significance in the association of EXTEM and FIBTEM CFT to NETs (EXTEM CFT: $r = 0.27$; $p = 0.02$; FIBTEM CFT: $r = 0.25$; $p = 0.05$). A significant association of NETs to a reduced LI60 could be shown in CABG and septic shock patients; however, the amount of change was minimal (Table 3, Supplement 1 in Supplementary Materials; FIBTEM LI60 septic shock: r = −0.36; $p < 0.01$; NATEM LI60 CABG: $r = -0.32$, $p < 0.001$; EXTEM LI60 CABG: $r = -0.25$, $p = 0.03$). The results of whole blood ristocetin-induced platelet impedance aggregometry did not reveal any correlations with NETs (Table 3). Furthermore, NETs did not correlate with the results of the SAC score ($r = 0.07$, $p = 0.64$).

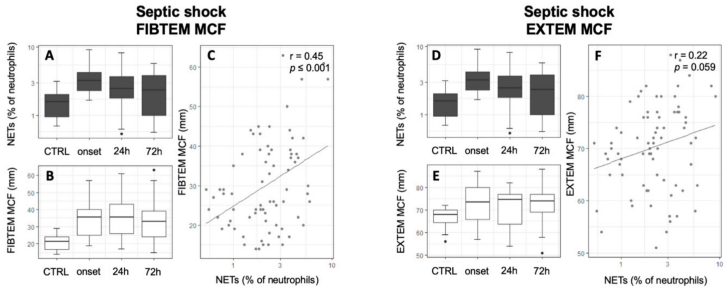

Figure 4. Positive correlation of free-circulating NETs to FIBTEM MCF in septic shock patients. Time courses of NETs (**A**,**D**), FIBTEM MCF (**B**), and EXTEM MCF (**E**) are shown as boxplot diagrams while scatter plots are uses to present correlations between NETs and FIBTEM MCF (**C**) and EXTEM MCF (**F**). Abbreviations: CTRL: Control group; MCF: Mean Clot Firmness; NETs: Neutrophil Extracellular Traps; *r*: Pearson's Correlation Coefficient.

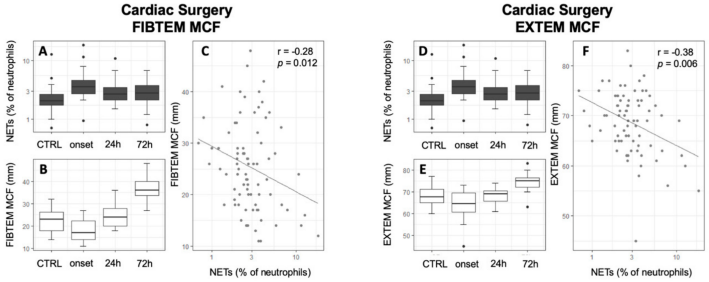

Figure 5. Inverse correlation of free-circulating NETs to FIBTEM MCF in cardiac surgical patients. Time courses of NETs (**A**,**D**), FIBTEM MCF (**B**), and EXTEM MCF (**E**) are shown as boxplot diagrams while scatter plots are used to present correlations between NETs and FIBTEM MCF (**C**) and EXTEM MCF (**F**). Abbreviations: CTRL: Control group; MCF: Mean Clot Firmness; NETs: Neutrophil Extracellular Traps; r: Pearson's Correlation Coefficient.

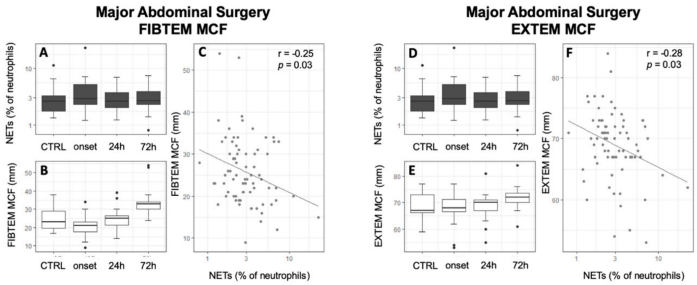

Figure 6. Inverse correlation of free-circulating NETs to FIBTEM MCF in major abdominal surgical patients. Time courses of NETs (**A**,**D**), FIBTEM MCF (**B**), and EXTEM MCF (**E**) are shown as boxplot diagrams while scatter plots are uses to present correlation between NETs and FIBTEM MCF (**C**) and EXTEM MCF (**F**). Abbreviations: CTRL: Control group; MCF: Mean Clot Firmness; NETs: Neutrophil Extracellular Traps; r: Pearson's Correlation Coefficient.

3.4. Association of NETs to Outcome Parameters

Neither the SOFA scores of septic and surgical patients nor the in-hospital death rates of septic shock patients correlated with the number of free-circulating NETs (SOFA: septic shock: $r = -0.1$, $p = 0.49$; CABG: $r = 0.17$, $p = 0.36$; MAS: $r = -0.2$, $p = 0.38$; in-hospital death: septic shock: $r = 0.05$, $p = 0.46$).

4. Discussion

This explorative proof-of-concept study evaluated a novel flow cytometry-based approach of quantifying free-circulating NETs in an intensive care setting. Furthermore, to our knowledge, this study is the first study to directly compare NET generation in patients suffering from septic shock to that in those suffering from surgery-induced inflammation. In accordance with other studies that have used various methods to investigate NET release, flow cytometry was able to prove a significant elevation of free-circulating NETs in septic shock and postsurgical patients [18,19,26,27,31,33]. However, flow cytometry-based NET quantification did not show significant differences in the NET release between septic and postsurgical sterile systemic inflammation within the first three days following surgery and at septic shock onset. Although sepsis in the early postsurgical phase remains a challenging and severe complication, little data is available on NETosis in postsurgical infections [18,26,27,35,47]. Some studies investigated SIRS following cardiopulmonary bypass and showed an elevation in NETs after cardiac surgery, thereby supporting our study results [34,35,48]. Furthermore, trauma-induced SIRS and SIRS caused by medical conditions were associated with increased blood levels of free-circulating NETs [18,26,27]. However, to our knowledge, no other study has addressed the question of whether the degree of NETosis differs between cases of sterile postsurgical systemic inflammation and septic shock. Therefore, we directly compared the number of free-circulating NETs in septic and surgical patients and found no differences between these patient cohorts. It must be highlighted that this study was not designed to evaluate NETs as a potential biomarker; however, the results of our study question the value of flow cytometry-based NET quantification for the detection of septic complications after cardiac surgery and MAS. This may be caused by the selection of patients in our study. While all cardiac surgical patients suffered from arteriosclerosis, a high proportion of patients undergoing MAS displayed a history of malignant diseases. In contrast, a smaller proportion of septic and matched control patients suffered from arteriosclerosis and cancer. Since both diseases are associated with NETosis, baseline levels of free-circulating NETs were higher in the surgical groups compared to the (septic shock) matched controls [49,50]. Therefore, these underlying diseases may mask a relatively higher increase of NET release in septic patients. Furthermore, NETs are known to consist of nucleic acids (e.g., mitochondrial DNA), histones, platelets, and other damage-associated patterns, making them potent proinflammatory components, which are also released during cardiac surgery, trauma, and medical SIRS [26,27,34,35,48,51]. Lastly, not only might the amount of free-circulating NETs cause the association of NETs to adverse outcomes in septic surgical patients, but it may be also affected by the patient's neutrophils' capability to release NETs. Abram et al. induced NETosis with PMA in the blood samples of septic shock patients and showed a significantly higher capacity of releasing NETs in neutrophils deriving from septic patients compared to those from non-septic, critically ill patients [18]. Future studies must investigate whether patients suffering from postsurgical inflammation also depict a reduced capacity for PMA-induced NETosis.

Since NETs are associated with sepsis-associated coagulopathy, our study also aimed to investigate whether the amount of free-circulating NETs is associated with clinically relevant coagulopathies in septic shock and postsurgical systemic inflammation [15,18,20,21,52,53]. In accordance with Yang et al., we were able to show a procoagulatory effect of NETs in septic shock patients [19]. Yang et al. used fluorescence microscopy for NET quantification while coagulation was assessed with the measurement of thrombin–antithrombin complexes and fibrin formation. Their results showed an NET-induced hypercoagulation in septic patients. The detailed mechanisms leading to activation of coagulation during sepsis are yet not well understood; however, a tight connection between NETs, thrombin

and platelet activation, and endothelium adhesion has already been described [14,15,23,54,55]. Our study focused on thrombelastographic analyses in order to reflect a high practicability for intensive care physicians and revealed a positive correlation of the number of NETs to clot firmness in fibrinogen-dependent assays as well as to the number of platelets in septic shock patients. Interestingly, in contrast to other publications, we were not able to find a correlation between NETs and the results of SAC scoring, which might be explained by our limited number of patients [18,20]. Furthermore, this study also revealed a significant negative correlation of NETs with FIBTEM and EXTEM MCF in postsurgical patients. Although interpretation of coagulatory status following cardiopulmonary bypass should be performed with caution, these results remain resilient. First, thrombelastography and global coagulatory parameters did not show severe alterations after cardiopulmonary bypass, and second, similar associations of NETs to reduced coagulatory function were also measured in MAS patients who were at low risk for postsurgical coagulopathy. Since this was the first description of this phenomena and this study did not investigate the underlying causalities, the reasons behind these associations remain unclear. Noubouossie et al. recently showed that individual histone proteins and nucleic acids, rather than NETs, directly activate the coagulatory system. The authors assumed that the procoagulatory effect of negatively charged nucleic acids might be neutralized by the histone–DNA complexes [56]. Due to the fact that bacteria can not only stimulate NETosis but also trigger the release of free-circulating nucleic acids and histones as well as directly activate the coagulatory system, the findings of Noubouossie et al. may play a role in the lack of pathogens in postsurgical SIRS [14,56,57].

Analogous to the original descriptions of this method, our validation experiments showed a positive proof of PMA-induced NET formation in flow cytometry and fluorescence microscopy [40,43]. Based on the validation experiments, we adjusted the original flow cytometry protocol of Lee et al. [43]. First, we used directly conjugated antibodies in order to further simplify the method and to reduce the risk of background staining, and second, gating was performed using the FMO technique after blocking unspecific binding sites with immunoglobin G instead of isotype controls. We chose to use the FMO technique in order to reduce the fluorescence spillover from other channels caused by the use of multiple colors, and thereby minimize errors in compensation. Furthermore, FMO offers detailed discrimination of stained cell populations while isotypes might not stain specifically. In our opinion, this adjusted protocol represents an investigator-independent and practicable approach for fast and reliable quantification of NETs which is practicable in an intensive care setting. However, flow cytometry has its limitations: First, due to the morphologic changes of neutrophils during NETosis, it should be recognized that flow cytometry may not be able to detect all NET-releasing neutrophils; in particular, swollen and degrading cells may fall out of the scatter range [41]. Therefore, later stages of NETosis might not be detected by flow cytometry. Second, with the exception of serum-based samples, blood samples must be processed immediately in order to minimize neutrophil autoactivation and cannot be frozen. Third, correlation analysis to other serum plasma surrogate parameters revealed a significant positive correlation of NETs to HMGB1 only in septic shock patients while neither HMGB1 in surgical patients nor MPO in any of the other study groups showed an association with the number of NETs as measured using flow cytometry-based quantification. This may be caused by the lack of an increase of HMGB1 in surgical patients while MPO plasma release underlies a high number of influencing factors, such as arteriosclerosis as well as systemic heparin-application, which is highly prevalent in severely ill and cardiac surgical patients [58,59]. Although NETs are induced by IL-8 via the mitogen-activated protein kinase pathway, we were not able to detect a correlation of IL-8 with circulating NETs [18]. This may be caused by a rapid decrease of IL-8 plasma levels or a varying expression of IL-8 caused by unknown influencing factors.

Furthermore, our study has other limitations: First, due to the proof-of-concept study design, we did not perform a sample size calculation. This may offer an explanation as to why this study failed to correlate the number of quantified NETs with outcome parameters. Second, the study does not allow for a conclusion for patients suffering from sepsis without signs of shock. In order to observe a high degree of NETosis, this proof-of-concept study concentrated only on septic shock patients. However, since

septic patients lacking shock symptoms are also associated with adverse outcomes, the role of NETs should be further investigated in these patients [60]. Third, until today, flow cytometry-based NETs quantification remains a method requiring high expertise and technical equipment. Therefore, from a practical and financial point of view it is not yet suitable for daily routine blood analysis. However, a recent review underlines the potential for computational and automatized flow cytometry-based quantification of NETs, which is supported by the findings of this study [61]. Fourth, this study did not investigate the causal context of NETosis within the different study groups. Finally, although therapeutic heparinization occurred only in a small number of cases, a bias effect cannot be ruled out.

5. Conclusions

This proof-of-concept study investigated the value of flow-cytometric NET quantification in septic shock patients as well as in patients suffering from postsurgical systemic inflammation. The methodology was able to detect NETs in a reliable manner and showed a significant increase of NETs under inflammatory conditions. However, flow cytometry-based NET quantification did not distinguish between septic shock and postsurgical inflammation, casting doubt on the discriminative power of this method. Furthermore, this study showed a clinically apparent procoagulatory shift in septic patients that was associated with the free-circulating NETs. In contrast, NETs deriving from surgical patients were negatively correlated to fibrinogen-associated thrombelastographic assays. Particularly, the association of free-circulating NETs to a procoagulatory shift in septic shock may offer a therapeutic target worthy of further research. In summary, flow cytometry offers a practicable solution for the quantification of NETs in an intensive care setting. Further investigations are necessary to explain the underlying mechanisms leading to the opposing coagulatory reactions in septic and postsurgical inflammation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/1/174/s1, Supplement 1 Demonstration of preliminary tests with 7-AAD in order to prove vitality of neutrophils (red box). First, leucocytes were targeted (**A**) and identified as $CD15^+$-cells (**B**). Then, MPO- and anti-H3-Histone-positive cells were defined as surrogates for NETs (**C**). 7-AAD negative cells represent vital cells (**D**) and are quantified to a high proportion (**E**). Abbreviations: APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; FSC: Forward Scatter; MPO: Myeloperoxidase; NETs: Neutrophil Extracellular Traps; SSC: Side Scatter, 7-AAD: 7-aminoactinomycin. Supplement 2 Comparison of FMO and isotype controls. FMO (**A**,**C**) and isotypes (**B**,**D**) show comparable gating results. PMA leads to a strong activation of NET release (**E**). Results are shown in percentage of gated neutrophils (**F**). Abbreviations: APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; FMO: Fluorescence-minus-one; FSC: Forward Scatter; MPO: Myeloperoxidase; NETs: Neutrophil Extracellular Traps; PMA: phorbol 12-myristate 13-acetate; SSC: Side Scatter. Supplement 3 Fluorescence microscopy of native and PMA stimulated neutrophils. Native neutrophils are shown in the upper row with marked stainings (**A**–**C**), while in the lower row, NET-releasing neutrophils are shown after PMA stimulation with the accordant stainings (**D**–**F**). NETs show a typical comet tail configuration. G displays the overlay of all staining antibodies. Abbreviations: MPO: Myeloperoxidase; NETs: Neutrophil Extracellular Traps; PMA: phorbol 12-myristate 13-acetate. Supplement 4 Results of thrombelastography. Data are shown as median (IQR). Abbreviations: CFT: Clot Firmness Time; CT: Clotting Time; LI60: Lysis Index after 60 min; MCF: Mean Clot Firmness.

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Conflicts of Interest: The authors declare no conflict of interest.

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8.7 Anlage 7

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Blood Levels of Free-Circulating Mitochondrial DNA in Septic Shock and Postsurgical Systemic Inflammation and Its Influence on Coagulation: A Secondary Analysis of a Prospective Observational Study

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Abstract: Major surgery is regularly associated with clinical signs of systemic inflammation, which potentially affects the rapid identification of sepsis. Therefore, this secondary analysis of an observational study aims to determine whether NADH dehydrogenase 1 (ND1) mitochondrial DNA (mtDNA) could be used as a potential biomarker for the discrimination between septic shock and postsurgical systemic inflammation. Overall, 80 patients were included (septic shock (n = 20), cardiac artery bypass grafting (CABG, n = 20), major abdominal surgery (MAS, n = 20), and matched controls (CTRL, n = 20)). Quantitative PCR was performed to measure ND1 mtDNA. Thromboelastography was used to analyze the coagulatory system. Free-circulating ND1 mtDNA levels were significantly higher in septic shock patients compared to patients suffering from post-surgical inflammation ({copies/µL}: CTRL: 1208 (668–2685); septic shock: 3823 (2170–7318); CABG: 1272 (417–2720); and MAS: 1356 (694–2845); CTRL vs. septic shock: $p < 0.001$; septic shock vs. CABG: $p < 0.001$; septic shock vs. MAS: $p = 0.006$; CABG vs. MAS: $p = 0.01$). ND1 mtDNA levels in CABG patients showed a strong positive correlation with fibrinogen (correlation coefficient [r]= 0.57, $p < 0.001$) and fibrinogen-dependent thromboelastographic assays (maximum clot firmness, EXTEM: $r = 0.35$, $p = 0.01$; INTEM: $r = 0.31$, $p = 0.02$; FIBTEM: $r = 0.46$, $p < 0.001$). In conclusion, plasma levels of free-circulating ND1 mtDNA were increased in septic shock patients and were discriminative between sepsis and surgery-induced inflammation. Furthermore, this study showed an association between ND1 mtDNA and a fibrinogen-dependent pro-coagulatory shift in cardiac surgical patients.

Keywords: sepsis; DAMPs; SIRS; inflammation; coagulation; coagulopathy

1. Introduction

Sepsis is a hazardous complication that can follow major abdominal and cardiovascular surgery, and is associated with increased morbidity and mortality [1]. Because major surgery is regularly associated with clinical signs of systemic inflammation (e.g., fever, tachycardia, and altered mental status), the rapid identification of sepsis can be difficult. Furthermore, established biomarkers of inflammation cannot sufficiently discriminate septic conditions from regular post-surgical inflammation, leaving the clinician in a challenging situation [2].

Over the last decade, neutrophil extracellular traps (NETs) have been identified as major players in the development of septic shock and postsurgical systemic inflammation [3,4]. Released by neutrophil granulocytes, NETs consist of histones, myeloperoxidase (MPO), neutrophil elastase, platelets, and nucleic acids, such as mitochondrial DNA (mtDNA) [3]. Once released into the vascular system, NETs form web-like structures to trap pathogens, but can also snare platelets, resulting in the activation of the coagulatory system [5,6]. Furthermore, the plasmatic coagulatory system may be stimulated by the NET's polyanionic surfaces and direct tissue factor presentation [6,7].

The primary study on which this secondary analysis is based aimed to identify NETs, measured by flow cytometry, as a potential discriminative biomarker between sterile and septic inflammation [8]. However, although NETs significantly increased after major surgery and during sepsis, the blood levels of free-circulating NETs did not differ between these conditions. Additionally, an association between NETs and an anticoagulatory pattern was observed in patients suffering from postsurgical systemic inflammation, although the primary study demonstrated the well-known pro-coagulatory properties of NETs in septic shock patients.

Because NETs failed to discriminate between septic shock and postsurgical inflammatory responses, this secondary analysis focused on mitochondrial DNA (mtDNA) as a potential discriminatory biomarker. Compared with NETs and other nucleic acids, mtDNA might represent a reasonable target for differentiation between inflammatory conditions caused by varying origins (e.g., sterile vs. infectious causes). First, NETs are mainly released locally in capillaries, organs, and other tissues, whereas free-circulating mtDNA can be found systemically [5]. Second, mtDNA differs from nuclear DNA, due to an increased ability to induce inflammatory responses via Toll-like receptor 9 (TLR-9), which in turn is pivotal for neutrophil activation. Moreover, mtDNA release has also been associated with the TLR-9-dependent activation of platelets, suggesting the possibility that mtDNA may be able to activate the coagulatory system [9]. Lastly, elevated mtDNA levels have been associated with increased intensive care unit (ICU) mortality [10]. MtDNA may therefore be able to differentiate between varying inflammatory conditions, such as sepsis and postsurgical inflammatory responses. Although mtDNA is known to be released during trauma, major abdominal and cardiovascular surgery, and sepsis, direct comparisons among these patient cohorts remain lacking [3,11,12]. Due to the mitochondrial origins of NADH dehydrogenase 1 (ND1), the plasma levels of free-circulating ND1 mtDNA have been used as a surrogate marker for mitochondrial damage under inflammatory conditions, and were therefore chosen as the target for this secondary analysis [13–15].

The goal of this follow-up analysis was to quantify the plasma levels of free-circulating ND1 mtDNA associated with septic shock and surgery-induced systemic inflammation. Furthermore, we aimed to identify any potential in vivo interaction between plasma levels of ND1 mtDNA and the coagulatory system [8,16,17].

2. Experimental Section

2.1. Study Design

This study is a secondary analysis, based on a single-center, prospective, observational proof-of-concept study that included 80 patients at the University Hospital of Giessen [8]. The local ethics committee approved the primary study, as well as the secondary data analysis (Justus-Liebig-University of Giessen, trial code: 86/18), and both were registered in the German Clinical Trials Register (trial code:

DRKS00013584). Both the original study and this secondary analysis were performed in accordance with the Helsinki Declaration, and all methods and results are presented in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Between October 2018 and March 2019, consecutive patients of legal age were enrolled in the study, after signing informed consent forms. If patients were not sui juris, consent was obtained from their legal representatives. Exclusion criteria included current pregnancy or nursing, age < 18 years, history of severe valvular heart and/or autoimmune disease, recent suffering of severe trauma, the need for immunomodulatory medication, hematological disease, any prior need for extracorporeal membrane oxygenation, or renal replacement therapy. Septic shock patients were enrolled according to the Sepsis-3 definition of septic shock [18]. Because no baseline parameters were available in septic patients, control patients (CTRL) were matched based on age, gender, and underlying health conditions (arteriosclerosis, cancerous diseases, renal insufficiency, and diabetes mellitus). These pre-existing diseases were chosen because they are associated with increased NET generation [19,20]. Since NETs represented the target parameter of the primary study, these conditions might have biased the study results, and were therefore matched in the control and septic shock group. Since these conditions are also connected to an impaired mitochondrial function, the matching strategy is also appropriate for this secondary analysis [21–24]. Visceral surgical patients underwent major abdominal surgery (MAS), such as Whipple's procedure, oncological gastric or esophageal resection, or colectomy, whereas all cardiac surgical patients received coronary artery bypass graft (CABG) surgery. Surgical patients had to fulfill at least two criteria associated with the definition of systemic inflammatory response syndrome (SIRS), within 24 h after surgery [25].

2.2. Sample Processing

In surgical patients, blood was collected prior to and immediately after surgery, as well as 24 and 72 h post-operatively. In patients who suffered from septic shock, blood was first taken after admission to the ICU and then again after 24 and 72 h. Furthermore, blood was drawn only at a single time point in CRTL patients. Blood was collected in citrate tubes for thromboelastometry and hirundine tubes for whole-blood, ristocetin-induced, platelet impedance aggregometry, whereas ethylenediaminetetraacetic acid (EDTA) tubes were used for the flow cytometry, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA). Plasma samples were stored at −80 °C and thawed only once for ELISA analyses. Clinical data were obtained from the patient data management system (IMESO GmbH, Giessen, Germany).

2.3. Quantitative Polymerase Chain Reaction

The quantification of ND1 mtDNA by quantitative PCR (qPCR) was performed as previously described [10,13]. First, blood was collected (in a 7.5 mL EDTA tube) and centrifuged to separate plasma (200 *g* for 10 min at room temperature). Afterwards, 100 µL plasma were diluted with 100 µL phosphate-buffered saline (PBS), and the mixture was centrifuged again at 5000 *g* (10 min at 4 °C). The supernatant was frozen at −20 °C. After thawing, the mtDNA was purified with a commercial purification kit, according to the manufacturer´s instructions (QIAquick PCR Purification Kit, Qiagen, Venlo, The Netherlands). Next, the samples were diluted 1:20 with nuclease-free, deionized–distilled H_2O before qPCR analysis. A StepOnePlus cycler (Thermo Fisher, Waltham, MA, United States) was used to quantify ND1 mtDNA in all samples. The ND1 mtDNA primers used were as follows: "ND1 mtDNA FW: 5´-CCA CCT CTA GCC TAG CCG TTT A-3´" and "ND1 mtDNA RW: 5´-GGG TCA TGA TGG CAG GAG TAA T-3´" (synthesized by Eurofins, Luxembourg, Luxembourg).

Samples were quantified using the mean values of triplicate measurements. The results were converted to copies/µL, according to Chiu et al. [26], using a standard curve. A plasmid containing human ND1 mtDNA (OriGene Technologies, Rockville, MD, United States) was used to establish the standard curve. The number of plasmid copies was calculated by a NanoDrop 2000 spectrophotometer

(Thermo Fisher Scientific). Serial dilutions of the corresponding copy number of plasmid (30–300,000 copies per PCR reaction) were used.

2.4. Flow Cytometry

The primary analysis successfully evaluated a novel flow cytometry-based method to quantify NETs, which has been described in detail within the publication of the primary analysis [8]. In brief, after the identification of CD15+ neutrophils (Pacific BlueTM anti-human CD15 antibody, BioLegend, San Diego, CA, United States), NETs were identified by the positive staining of myeloperoxidase (MPO, ab11729, Abcam, Cambridge, United Kingdom) and anti-H3-Histone antibody (Alexa Fluor 647 Anti-Histone, BioLegend, San Diego, CA, United States) within the CD15+ cell population, in red-cell lysis samples processed for flow cytometry (BD FACS Canto II with BD FACSDIVA software, version 6.1.3, Becton Dickinson, Franklin Lakes, USA). Details regarding the gating strategy are explained in the primary analysis. Data are presented as the percentage of NETs for all gated neutrophils.

2.5. ELISA

ELISA analyses were used to measure interleukin-8 (IL-8; Human IL-8/CXCL8 Quantikine HS ELISA, R&D Systems, Minneapolis, MN, United States), high mobility group protein B1 (HMGB1; Human HMGB1 ELISA Kit, Aviva Systems Biology, San Diego, CA, United States) and MPO (Human MPO Instant ELISA, eBioscience, Frankfurt, Germany). All analyses were performed according to the manufacturer's instructions. An automated plate reader (Epoch, BioTek Instruments GmbH, Heilbronn, Germany) was used, and the probes were measured in accordance with their recommended absorbances (IL-8: 490 nm, HMGB1: 450 nm, MPO: 450 nm).

2.6. Inflammatory Parameters

Plasma levels of C-reactive protein (CRP) and procalcitonin (PCT), as well as the blood cell count were performed during clinical routines in the local laboratory of the university hospital of Giessen.

2.7. Coagulatory Analysis

For thromboelastography (ROTEM, Matel Medizintechnik, Hausmannstaetten, Austria) and whole-blood, ristocetin-induced platelet impedance aggregometry (Multiplate, Roche Diagnostics, Rotkreuz, Switzerland), point-of-care devices were used, and all other coagulatory tests were performed by the local clinical laboratory. Thrombelastographic assays included NATEM, INTEM, FIBTEM, and EXTEM. For each assay the clot formation time (CFT; seconds), clotting time (CT; seconds), mean clot firmness (MCF; mm), and lysis index after 60 min (LI60; %) was measured. For whole-blood, ristocetin-induced platelet impedance aggregometry, platelets were stimulated with ADP (ADPtests), thrombin-receptor activator protein 6 (TRAPtest), and arachidonic acid (ASPItest).

2.8. Statistical Analysis

Values were tested for normal distribution using the Shapiro–Wilk test. Parametric data were expressed as the mean and standard deviation, whereas the median and interquartile range (IQR) were used for non-parametric data. To identify a potential interaction between plasma levels of free-circulating NETs and ND1 mtDNA, the ratio of ND1 mtDNA and NETs was calculated (ND1 mtDNA/NETs). Differences in mtDNA quantities between the study groups were analyzed by ANOVA, followed by a pairwise *t*-test for the analysis of intergroup differences. The analysis of variations in mtDNA levels across different time points within the same group was performed using the Friedmann test, followed by the pairwise *t*-test for paired groups. For this purpose, septic shock patients were compared with their matched controls. A *p*-value of $p < 0.05$ was considered to be statistically significant. Correlations between mtDNA levels and various parameters were analyzed with Pearson's correlation coefficient. Experimental data, laboratory routine data, and clinical data were stored in

an external database (Microsoft Excel, Redmond, WA, United States). Data were analyzed using R statistical software version 3.6.2 (12 December 2019; www.r-project.org).

3. Results

Overall, 80 patients were included (20 patients per study group). Patient characteristics are presented in the primary study, in detail (8). No differences between the four groups were observed for age, sex, or body mass index (BMI) values. The ages of each group, expressed as the median (IQR), were 69 (64–74) for septic shock, 70 (62–79) for CABG, 68 (54–70) for MAS, and 69 (66–74) for CTRL. In the septic shock group, 70% (n = 14) were male, while in the CABG group it was 75% (n = 15), MAS was 60% (n = 12), and CTRL was 70% (n = 14). The BMI values were 27.9 (21.7–32.6) for septic shock, 30 (27.6–36.5) for CABG, 24 (22.4–26.9) for MAS, and 27 (23.2–29.2) for CTRL. Regarding the sepsis-related organ failure assessment (SOFA) score, septic shock patients suffered from a severe condition (SOFA onset: 10.5 (10–12.5); 24 h: 11.5 (8–13); 72 h: 9 (5.5–14.5)), leading to an in-hospital mortality of 35% (n = 7). Sepsis derived from abdominal origins in 60% (n = 12) of cases, whereas a pulmonary or urological source of infection were identified in 15% of cases (n = 3), each, and a soft-tissue infection was identified in 10% (n = 2) of cases. The MAS group included 40% (n = 8) of patients who underwent Whipple's procedure, open partial colectomy and esophagus resection were each performed in 20% (n = 4) of patients, and the remaining patients underwent other types of MAS. The matching of CTRL individuals with septic patients was sufficient to control for pre-existing disease (see details in prior publication [8]).

The anticoagulatory therapy did not differ significantly between the surgical study groups, while septic shock patients received prophylactic heparinization in fewer patients (prophylactic heparinization at onset/24 h/72 h in septic shock, respectively, and preoperative/postoperative/24 h/72 h in surgical patients (% of all patients in each group): septic shock = 50%/60%/55%; CABG = 100%/0%/90%/80%; MAS = 100%/0%/90%/90%; CTRL = 75%). After onset, 24 and 72 h septic shock patients received therapeutic heparinization in 40%, 30%, and 35% of cases, respectively (see details in the primary study [8]).

3.1. Quantification of Free-Circulating ND1 mtDNA Plasma Levels

3.1.1. Time Course

Compared with the CTRL group, septic shock patients showed a significant increase in ND1 mtDNA plasma levels, expressed as copies/µL, at onset and over three following days (Figure 1; CTRL: 1208 (668–2685); septic shock onset: 3865 (2092–6332); septic shock 24 h: 3650 (1992–9129); septic shock 72 h: 3177 (2680–7318); CTRL vs. septic shock onset: $p = 0.017$; CTRL vs. septic shock 24 h: $p = 0.013$; CTRL vs. septic shock 72 h: $p < 0.001$; all other analyses regarding the analyzed timepoints resulted in p-values > 0.05).

Plasma levels of ND1 mtDNA decreased immediately after CABG and increased over the following 72 h (Figure 1; CABG preoperative: 1042 (296–2859); CABG postoperative: 431 (204–1159); CABG 24 h: 2042 (834–3096); CABG 72 h: 1517 (861–3922); CABG postoperative vs. CABG 24 h: $p = 0.005$, CABG postoperative vs. CABG 72 h: $p = 0.005$; all other analyses regarding the analyzed timepoints resulted in p-values > 0.05). The preoperative values did not differ from the CTRL group (CABG preoperative vs. CTRL: $p = 1.0$).

In contrast, patients undergoing MAS presented a significant elevation in free-circulating ND1 mtDNA immediately after surgery, followed by a continuous increase over 72 h (Figure 1; MAS preoperative: 650 (333–1139); MAS postoperative: 1716 (727–4346); MAS 24 h: 1780 (1189–2715); MAS 72 h: 2083 (1225–3107); MAS preoperative vs. MAS postoperative $p = 0.021$; MAS preoperative vs. MAS 24 h: $p < 0.001$; MAS preoperative vs. MAS 72 h: $p < 0.001$; all other analyses regarding the timepoints resulted in p-values > 0.05). The preoperative values did not differ from the CTRL group (MAS preoperative vs. CTRL: $p = 1.0$).

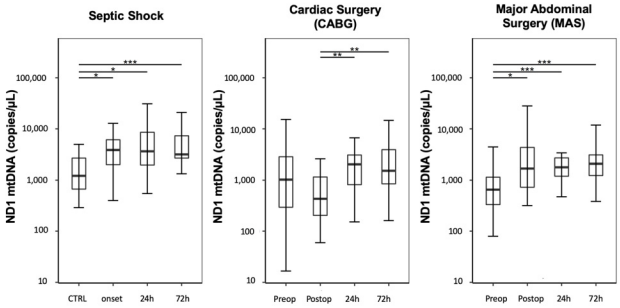

Figure 1. Time course of plasma free-circulating NADH dehydrogenase 1 (ND1) mitochondrial DNA (mtDNA) levels. The results are displayed as boxplot diagrams. Asterisks display the degree of statistical significance: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. CTRL: control group.

Compared with post-CABG patients, the free-circulating ND1 mtDNA levels were significantly higher at onset in septic shock patients; however, septic shock levels did not differ significantly compared with those in patients undergoing MAS (septic shock onset vs. CABG postoperative: $p < 0.001$; septic shock onset vs. MAS postoperative: $p = 1.0$; CABG postoperative vs. MAS postoperative: $p = 0.021$).

The pooled data analysis of the combined postsurgical timepoints of each group, respectively of all septic timepoints, revealed significantly higher levels of ND1 mtDNA for septic shock patients compared to CTRL, as well as to both other groups. Nonetheless, the ND1 mtDNA levels in both surgical cohorts were not different from CTRL, but differed significantly from each other, with higher levels in MAS. (Figure 2; CTRL: 1208 (668–2685); septic shock: 3823 (2170–7318); CABG: 1272 (417–2720); MAS: 1356 (694–2845); CTRL vs. septic shock: $p < 0.001$; CTRL vs. CABG: $p = 0.660$; CTRL: vs. MAS: $p = 0.190$; septic shock vs. CABG: $p < 0.001$; septic shock vs. MAS: $p = 0.006$; CABG vs. MAS: $p = 0.01$).

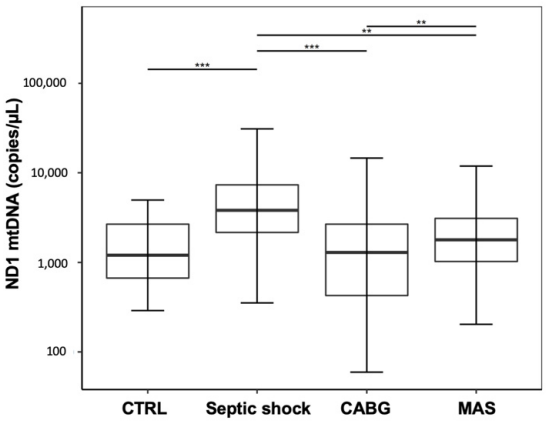

Figure 2. Pooled data analysis for the plasma free-circulating ND1 mtDNA levels for all post-surgical patients, compared with the pooled data for septic patients. Asterisks display the degree of statistical significance: **: $p < 0.01$, ***: $p < 0.001$. Abbreviations: CABG: coronary artery bypass graft; CTRL: control group; MAS: major abdominal surgery.

3.1.2. Ratio between Plasma Levels of Free-Circulating ND1 mtDNA and NETs

Compared with the CTRL group, septic shock patients presented increased ND1 mtDNA/NET ratios, expressed as (copies/µL)/%, starting at onset and lasting over 72 h (Figure 3; CTRL: 708 (368–1684); septic shock onset: 1450 (530–2807); septic shock 24 h: 1762 (1139–4250); septic shock 72 h: 1682 (849–3229)). However, only the measurement after 72 h reached significance, compared with the control group ($p = 0.021$).

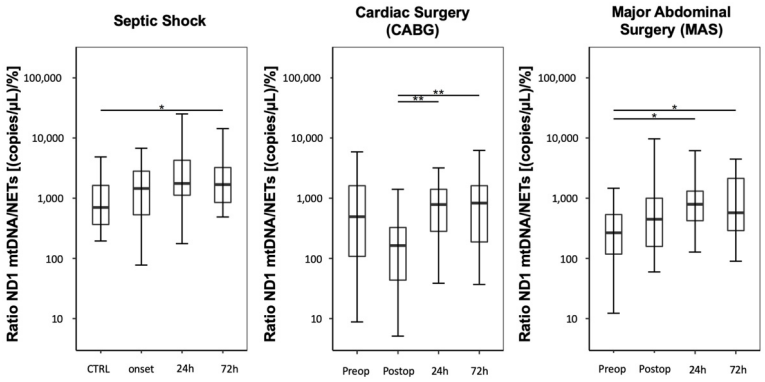

Figure 3. Time course showing the ratio between free-circulating ND1 mtDNA and NETs levels. The results are displayed as boxplot diagrams. Asterisks display the degree of statistical significance: *: $p < 0.05$; **: $p < 0.01$. CTRL: Control group.

Immediately after CABG, patients showed a significant decrease in the mtDNA/NET ratio, followed by increases at 24 and 72 h (Figure 3; CABG preoperative: 494 (109–1669); CABG postoperative: 164 (44–328); CABG, 24 h: 787 (296–1404); CABG, 72 h: 829 (188–1615); CABG postoperative vs. CABG 24 h: $p = 0.002$; CABG postoperative vs. CABG 72 h: $p = 0.002$).

Compared to preoperative values, MAS led to a postsurgical increase in the mtDNA/NET ratio over three days (Figure 3; MAS preoperative: 271 (118–526); MAS postoperative: 452 (160–997); MAS, 24 h: 791 (425–1309); MAS, 72 h: 573 (290–2160); MAS preoperative vs. MAS 24 h: $p = 0.013$; MAS preoperative vs. MAS 72 h: $p = 0.013$).

A pooled data analysis of the postsurgical and septic data regarding the mtDNA/NET ratio revealed a statistically significant decrease in the CABG patients compared to CTRL, and significantly lower values in CABG and MAS patients compared to septic shock. In contrast, the increase in septic shock patients compared to CTRL was not significant. (Figure 4; CTRL: 708 (368–1684); septic shock: 1566 (822–3377); CABG: 487 (94–1180); MAS: 499 (228–1259); CTRL vs. septic shock: $p = 0.106$, CTRL vs. CABG: $p = 0.042$, CTRL vs. MAS: $p = 0.535$, septic shock vs. CABG: $p < 0.001$, septic shock vs. MAS: $p = 0.003$, CABG vs. MAS: $p = 0.042$).

3.2. Correlations between Plasma Free-Circulating ND1 mtDNA Levels and Inflammatory and Coagulatory Parameters

The plasma levels of HMGB-1, IL-8, MPO, procalcitonin, and leukocyte counts showed no significant associations with ND1 mtDNA levels within any of the study cohorts (Table 1; details in Supplementary Materials). Only ND1 mtDNA and C-reactive protein (CRP) in patients undergoing CABG displayed a significant correlation (Table 1).

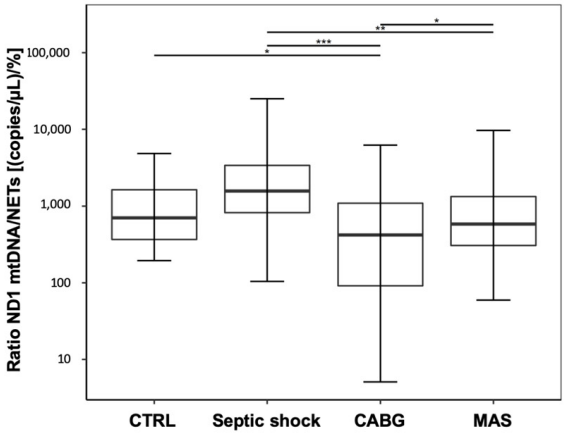

Figure 4. Pooled data analysis for the ratio between free-circulating ND1 mtDNA and NET levels for all post-surgical patients, compared with the pooled data for septic patients. Asterisks display the degree of statistical significance: *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$. Abbreviations: CABG, coronary artery bypass graft; CTRL, control group; MAS, major abdominal surgery.

Table 1. Correlations between free-circulating plasma ND1 mtDNA levels and inflammatory parameters. Data were derived from Pearson's correlation analysis.

Parameters	Septic Shock		Cardiac Surgery (CABG)		Major Abdominal Surgery (MAS)		Control (CTRL)	
	r	*p*-Value	*r*	*p*-Value	*r*	*p*-Value	*r*	*p*-Value
MPO (ng/L)	−0.01	0.92	0.09	0.52	0.22	0.09	0.35	0.13
HMGB-1 (pg/L)	−0.14	0.31	−0.05	0.69	0.10	0.45	0.30	0.20
IL-8 (pg/L)	−0.07	0.63	−0.13	0.33	0.22	0.09	−0.02	0.94
NETs (%)	−0.15	0.26	−0.24	0.26	−0.03	0.85	−0.25	0.28
Leucocyte count (L^{-1})	−0.03	0.83	−0.21	0.11	−0.05	0.73	−0.30	0.20
CRP (mg/L)	0.17	0.22	0.44	*<0.001*	−0.01	0.92	−0.05	0.84
PCT (µg/L)	0.03	0.82	N.A.	N.A.	0.23	0.43	N.A.	N.A.

Significant *p*-values are highlighted in italics. Abbreviations: CABG: coronary artery bypass graft; CRP: C-reactive protein; CTRL: control group; HMGB1: high mobility group protein B1; IL-8: interleukin 8; MAS: major abdominal surgery; MPO: Myeloperoxidase; NETs: neutrophil extracellular traps; N.A.: not available; PCT: procalcitonin.

In septic shock patients, the plasma levels of ND1 mtDNA were not consistently associated with any alterations in the coagulatory system, as measured by thromboelastography (Table 2). However, cardiac surgical patients also showed a strong positive correlation between ND1 mtDNA and fibrinogen levels, as with the results of fibrinogen-dependent thromboelastographic assays (INTEM, EXTEM, and FIBTEM MCF; Table 2), and the international normalized ratio (INR) value correlated negatively with the plasma levels of ND1 mtDNA in cardiac surgical patients. Patients undergoing MAS only showed a positive correlation between ND1 mtDNA levels and the INR (Table 2). The platelet aggregometry measurements did not reveal any correlations with the ND1 mtDNA levels in any of the study groups.

Table 2. Correlation of free-circulating plasma levels of ND1 mtDNA to coagulatory parameters. Data were derived from Pearson's correlation analysis.

Parameters	Septic Shock		Cardiac Surgery (CABG)		Major Abdominal Surgery (MAS)		Control (CTRL)	
	r	*p*-Value	*r*	*p*-Value	*r*	*p*-Value	*r*	*p*-Value
Thromboelastography								
EXTEM CT (s)	0.01	0.96	−0.19	0.15	−0.33	*0.01*	0.15	0.54
INTEM CT (s)	0.10	0.48	−0.16	0.23	−0.10	0.47	−0.16	0.51
FIBTEM CT (s)	0.15	0.27	−0.05	0.71	−0.39	*<0.001*	0.16	0.50
NATEM CT (s)	0.20	0.14	−0.01	0.91	−0.13	0.33	0.01	0.96
EXTEM CFT (s)	−0.11	0.41	−0.18	0.19	0.29	*0.03*	0.52	*0.02*
INTEM CFT (s)	−0.09	0.53	−0.18	0.19	0.03	0.84	0.32	0.16
FIBTEM CFT (s)	−0.11	0.42	−0.08	0.63	−0.10	0.51	0.29	0.36
NATEM CFT (s)	0.17	0.23	0.23	0.08	−0.12	0.39	−0.18	0.44
EXTEM MCF (mm)	0.07	0.60	0.35	*0.01*	−0.20	0.14	−0.32	0.18
INTEM MCF (mm)	0.08	0.55	0.31	*0.02*	−0.17	0.21	−0.26	0.26
FIBTEM MCF (mm)	0.05	0.74	0.46	*<0.001*	−0.13	0.35	−0.12	0.62
NATEM MCF (mm)	−0.42	*<0.001*	0.24	0.08	0.03	0.82	−0.09	0.72
EXTEM LI60 (%)	0.08	0.55	*0.01*	0.93	0.28	*0.04*	0.07	0.76
INTEM LI60 (%)	0.06	0.64	−0.03	0.80	0.31	*0.02*	0.06	0.79
FIBTEM LI60 (%)	0.08	0.55	0.10	0.45	0.11	0.42	−0.12	0.63
NATEM LI60 (%)	0.25	0.09	0.07	0.61	0.14	0.31	0.06	0.81
Impedance Aggregometry								
ASPI (Units)	0.00	0.97	−0.06	0.64	−0.21	0.12	0.29	0.21
ADP (Units)	0.06	0.66	0.01	0.92	−0.23	0.09	0.11	0.65
TRAP (Units)	−0.01	0.93	−0.03	0.84	−0.19	0.16	0.29	0.22
Global coagulatory Parameters								
Platelet count (L^{-1})	0.01	0.96	−0.18	0.18	−0.11	0.42	−0.28	0.23
PTT (s)	0.09	0.53	−0.20	0.13	0.23	0.11	0.08	0.75
INR	0.07	0.63	−0.37	*<0.001*	0.30	*0.03*	−0.17	0.49
Fibrinogen (g/L)	0.12	0.54	0.57	*<0.001*	0.03	0.89	N.A.	N.A.

Significant *p*-values are highlighted in italics. Abbreviations: CABG: coronary artery bypass graft; CFT: clot firmness time; CT: clotting time; CTRL: control group; INR: international normalized ratio; LI60: lysis index after 60 min; MAS: major abdominal surgery; MCF: mean clot firmness; N.A.: not available; PTT: partial thromboplastin time.

4. Discussion

The quantification of plasma free-circulating mtDNA levels, particularly ND1 mtDNA, has previously been used to identify sepsis, surgical trauma, and critical illness [10,13,15,27–32]. To our knowledge, this is the first study to examine whether they can be used to discriminate sepsis and surgery-induced systemic inflammatory reactions. With this data analysis, subsequent to our previous study on NETs, we were able to show that distinct developments in the detectable levels of ND1 mtDNA occurred that were dependent on the underlying inflammatory trigger. Whereas septic shock patients presented the elevation of ND1 mtDNA levels consistently over 72 h, surgical patients showed different results. The plasma levels of ND1 mtDNA in patients undergoing MAS increased and remained comparable to the elevation observed in septic shock patients, whereas in cardiac surgical patients ND1 mtDNA plasma levels decreased significantly immediately after CABG. The observed drop in ND1 mtDNA plasma levels immediately after cardiopulmonary bypass stands in contrast with the findings of other studies that have investigated the course of free-circulating mtDNA during cardiac surgery. Yet, only the studies by Qin et al. [27,29,30] have examined ND1 mtDNA, whereas

human cytochrome B has been targeted more frequently. Because no detailed data regarding the cardiopulmonary bypass technique is available in most studies, the reason for the observed decrease in our study remains unclear, although a dilutional effect might offer an explanation. Moreover, although the absolute ND1 mtDNA level was higher in our study compared with that reported by Qin et al. [29], the increase in the ND1 mtDNA level was not significantly elevated compared with the baseline level. The increase in free-circulating mtDNA is a well-known characteristic of sepsis, and has previously been connected with organ failure and poor prognosis [10,13,15,31,32], but detailed data regarding changes in mtDNA plasma levels are lacking for abdominal surgical patients. Hu et al. [30] revealed an association between the quantities of various mtDNA fragments and the severity of intra-abdominal infections following major trauma. However, these data are not transferable to elective MAS patients, because the studied patients suffered from a trauma-induced severe inflammatory response prior to the surgical procedures. Although mitochondrial haplogroups have been identified as potential biomarkers for the prognosis of sepsis in abdominal surgical patients, the mtDNA level was not quantified in that study [33]. To detect differences between the study groups, we pooled the data from all postsurgical timepoints and compared them with the pooled data from septic patients. This analysis revealed that in comparison with the CTRL group, only septic shock patients showed significantly elevated plasma levels of ND1 mtDNA, whereas the surgical groups did not differ significantly from the CTRL group. Compared to septic shock patients, ND1 mtDNA levels were significantly lower within the surgical patients, most probably caused by the decrease of ND1 mtDNA immediately after cardiopulmonary bypass. This difference was stronger in CABG patients, and was also visible in comparison to the patients who underwent MAS. For this reason, this study supports the hypothesis that the amount of free-circulating plasma ND1 mtDNA levels is potentially helpful for the discrimination of septic shock from surgery-induced systemic responses, and is therefore a promising target for future studies, including larger numbers of patients. Furthermore, ND1 mtDNA could potentially be evaluated in context of a multimarker strategy for identifying postsurgical sepsis. Nonetheless, it has to be considered that the data was scattered with overlapping data ranges, not allowing us to easily define a cut-off value to be used for clinical discrimination between post-surgical inflammation and sepsis. The evaluation of such a threshold might be challenging, and will surely require further studies with larger numbers of patients included.

The primary study upon which the present data analysis was based aimed to quantify NETs using a novel flow cytometry-based method [8]. The interest in examining the additional mtDNA measurements is derived from the hypothesis that free-circulating mtDNA levels might represent a large proportion of NETs. Therefore, we examined the ratio between ND1 mtDNA and NETs, and found comparable time courses between the mtDNA/NET ratio and the ND1 mtDNA plasma levels, indicating that ND1 mtDNA levels likely represent a large proportion of NETs. This finding is supported by the study performed by Yousefi et al. [11], who identified mtDNA release as a pivotal component of vital NETosis. During vital NETosis, neutrophils release mtDNA through the stimulation of TLR-4 and complement factor 5a in a reactive oxygen species-dependent way, resulting in NETs containing solely mtDNA. However, very little in vivo data are available regarding mtDNA-containing NETs, and the available data was primarily derived from trauma patients [3,12,34]. The mtDNA/NET ratio results in our study indicate that mtDNA-containing NETs derived from vital NETosis might play an in vivo role during septic shock and surgery-induced systemic inflammation. However, because mtDNA is also well-known as a damage-associated molecular pattern DAMP that is released during sepsis, the simultaneous release of NETs and mtDNA (independent of vital NETosis) might also explain our findings [3,10,31,32]. Therefore, further research efforts remain necessary to differentiate between mtDNA-containing NETosis and NET-independent mtDNA release.

NETs play a pivotal role in the interaction between the inflammatory and coagulatory system (called immunothrombosis) [16,35]. Especially under septic conditions, NETs can activate platelets and the plasmatic coagulatory system, which is also supported by our previous studies [6,8,16,17,36]. However, except for fibrinogen-dependent parameters in cardiac surgical patients, no consistent

associations between the levels of free-circulating ND1 mtDNA and any coagulatory changes were detectable in any of the study groups. On the other hand, this study was not designed for this purpose of investigation. Interestingly, all fibrinogen-dependent thromboelastography assays and the plasma levels of fibrinogen were positively correlated with ND1 mtDNA levels, whereas the INR showed a negative association with ND1 mtDNA levels in cardiac surgical patients, indicating the in vivo pro-coagulatory and fibrinogen-dependent influence of ND1 mtDNA. These results might agree with the findings from Qin et al. Based on the detection of a close relationship between plasma mtDNA levels and activated platelets in cardiac surgical patients, Qin et al. [27] suggested that mtDNA might also be partially derived from activated platelets. Therefore, our findings might describe the in vivo effects of CABG-induced platelet activation, which is strongly associated with fibrinogen-dependent platelet aggregation. On the other hand, it should be mentioned that in patients suffering from coronary artery disease, fibrinogen is also released as an acute phase protein, which might explain the positive correlation of fibrinogen and ND1 mtDNA plasma levels in this particular study group. The same mechanism might also explain the association of CRP and ND1 mtDNA plasma levels in CABG patients. Nonetheless, our findings could support the conclusion by Bhagirath et al. [9], who postulated that free-circulating DNA of any type (including mtDNA) leads to the activation of platelets and thrombin generation. Interestingly, our primary study identified a negative correlation between the detected NET levels and fibrinogen-dependent assays in cardiac surgical patients, indicating that ND1 mtDNA might activate the coagulatory system independent of NETs [8].

Our study features some limitations. First, this secondary analysis represents an observational study, and is not able to draw conclusions regarding the underlying causalities. Second, no sample size calculation was performed regarding either the quantification of ND1 mtDNA or the coagulatory analyses. Since the primary study was designed as a proof-of-concept study aiming to evaluate flow cytometry-based NET quantification, the necessary number of included patients was planned with only 20 patients per group. This might explain our failure to detect further interactions between ND1 mtDNA and inflammatory or coagulatory parameters in the other study groups. In particular, the lack of correlations between inflammatory parameters and ND1 mtDNA levels should be reevaluated using a larger number of patients. However, these findings might have resulted from an increase of the HMGB-1 and MPO levels observed in surgical patients and potentially unidentified influencing factors, such as arteriosclerosis and systemic heparin application, which were highly prevalent in the investigated patients [37,38]. Third, even though all patients received comparable anticoagulatory regimens without intergroup differences (details are presented in the primary study [8]), those regimens' influence on the performed coagulatory analyses cannot be ruled out. Furthermore, high-dose heparinization throughout cardiopulmonary bypass and its antagonization might have influenced the coagulatory analysis, even though antagonization was effective in terms of activated clotting time. Subsequently, the correlation of fibrinogen and CRP to ND1 mtDNA plasma levels in CABG patients might be triggered by arteriosclerosis, questioning the specificity of ND1 mtDNA plasma levels. Fourth, due to a methodological limitation, PCT values are lacking in the surgical groups. However, among the surgical groups, we did not reveal any postoperative (suspected) infections. That was one explanation for why we did not find PCT values among the post-surgical patients. Lastly, even though pneumonia represents the most common source of sepsis, it was only present in a minor part of the included patients. Due to the high clinical impact of septic shock caused by pneumonia, the role of free-circulating ND1 mtDNA in pneumogenic sepsis should be evaluated in future studies.

5. Conclusions

In summary, the herewith presented analysis identifies ND1 mtDNA as a potential biomarker for the discrimination of septic shock and postsurgical systemic inflammation. Plasma levels of free-circulating ND1 mtDNA were significantly higher in septic shock patients compared to all other study groups. Surgical patients showed no increase of ND1 mtDNA plasma levels compared to the CTRL group. These study results indicate that ND1 mtDNA might be useful as a discriminative

biomarker for perioperative sepsis. Furthermore, this study shows an association between ND1 mtDNA levels and a fibrinogen-dependent pro-coagulatory shift in cardiac surgical patients.

Supplementary Materials: The following can be found online at http://www.mdpi.com/2077-0383/9/7/2056/s1, Table S1: The results of the inflammatory parameters as presented in [8]. Data are shown as medians (IQR). Abbreviations: CRP: C-Reactive Protein; DNA: Deoxynucleic Acid; HMGB1: high mobility group protein B1; MPO: myeloperoxidase; NETs: neutrophil extracellular traps; PCT: procalcitonin.

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Sepsis-induced long-term immune paralysis – results of a descriptive, explorative study

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Abstract

Background: Long-lasting impairment of the immune system is believed to be the underlying reason for delayed deaths after surviving sepsis. We tested the hypothesis of persisting changes to the immune system in survivors of sepsis for the first time.

Methods: In our prospective, cross-sectional pilot study, eight former patients who survived catecholamine-dependent sepsis and eight control individuals matched for age, sex, diabetes and renal insufficiency were enrolled. Each participant completed a questionnaire concerning morbidities, medications and infection history. Peripheral blood was collected for determination of i) immune cell subsets ($CD4^+$, $CD8^+$ T cells; $CD25^+$ $CD127^-$ regulatory T cells; $CD14^+$ monocytes), ii) cell surface receptor expression (PD-1, BTLA, TLR2, TLR4, TLR5, Dectin-1, PD-1 L), iii) HLA-DR expression, and iv) cytokine secretion (IL-6, IL10, TNF-α, IFN-γ) of whole blood stimulated with either α-CD3/28, LPS or zymosan.

Results: After surviving sepsis, former patients presented with increased numbers of clinical apparent infections, including those typically associated with an impaired immune system. Standard inflammatory markers indicated a low-level inflammatory situation in former sepsis patients. $CD8^+$ cell surface receptor as well as monocytic HLA-DR density measurements showed no major differences between the groups, while $CD4^+$ T cells tended towards two opposed mechanisms of negative immune cell regulation via PD-1 and BTLA. Moreover, the post-sepsis group showed alterations in monocyte surface expression of distinct pattern recognition receptors; most pronouncedly seen in a decrease of TLR5 expression. Cytokine secretion in response to important activators of both the innate (LPS, zymosan) and the adaptive immune system (α-CD3/28) seemed to be weakened in former septic patients.

Conclusions: Cytokine secretion as a reaction to different activators of the immune system seemed to be comprehensively impaired in survivors of sepsis. Among others, this could be based on trends in the downregulation of distinct cell surface receptors. Based on our results, the conduct of larger validation studies seems feasible, aiming to characterize alterations and to find potential therapeutic targets to engage.

Keywords: Sepsis, Immunology, Immune system, Immunocompromised

Background

Sepsis remains a big challenge in modern intensive care medicine and is still a leading cause of death [1–3]. It is an interwoven immune reaction to distinct microorganisms, which comprises systemic inflammatory response syndrome (SIRS) with a "cytokine storm" accompanied by the counteracting, so-called compensatory anti-inflammatory response syndrome (CARS) [4, 5].

In the case of overwhelming CARS, the phenomenon of immune paralysis occurs due to immune cell apoptosis and functional impairment of lymphocytes and phagocytes, also associated with increased anti-inflammatory and decreased pro-inflammatory cytokine production [6–9]. Overshooting of the anti-inflammatory response predisposes the host to secondary bacterial infection, infection with opportunistic microorganisms and re-activation of latent viruses [7, 10–13]. In contrast to

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the initial causative insult of sepsis, which can be treated by source control and anti-infective therapy, no therapeutic or preventive strategies are established to combat the deleterious effects of immune paralysis during the period of CARS [14]. Furthermore, the immune cell phenotype of patients who die from sepsis has features consistent with immunosuppression [15]. Patients with impaired immune function are prone to incomplete recovery from sepsis. They often present with typical syndromes that follow the SIRS-CARS reaction: persistent inflammation-immunosuppression catabolism syndrome (PICS) and multiple organ dysfunction syndrome (MODS) [16]. Besides sophisticated intensive care treatment, a relevant number of survivors of the initial septic event ultimately fall victim to PICS or MODS in the long-term.

Even after complete recovery many patients who recover from sepsis have an impaired quality of life for years and are found to have increased mortality [17–19]. Studies have illustrated ongoing mortality of up to 43 % after one year [17], 44.9 % after two years [20] and 74.2 % after five years beyond hospital discharge [21]. Sepsis-related long-lasting impairment of the immune system is believed to be the underlying reason for these delayed deaths in those who initial survive sepsis. However, studies investigating the immune status of survivors of sepsis in humans are missing. In this prospective, cross-sectional pilot study we aimed to explore for the first time the phenomenon of sepsis-induced long-term immune paralysis (SLIP) in a limited number of patients who survived catecholamine-dependent sepsis.

Methods

Enrollment of patients who had previously survived sepsis

The local ethics committee at the Medical Faculty of the Justus-Liebig-University Gießen, Germany approved the study protocol (trial code: 291/13). We identified all patients who had been diagnosed with sepsis (ICD10: A41.X) and who had required vasopressors or inotropes (catecholamine-dependent sepsis), and had been treated on the interdisciplinary surgical intensive care unit at Gießen University Hospital between January 2011 and December 2013. Data were extracted from our patient data management system ICUData (IMESO® GmbH, Giessen, Germany). All patients identified were contacted by mail. After evaluation of possible exclusion criteria, written informed consent was collected and individuals were enrolled in the study. Exclusion criteria were: onset of sepsis less than nine months or more than 60 months prior to the date of investigation, pregnancy, participating in another interventional study, chronic viral infections (HIV, hepatitis), end-stage renal failure, autoimmune diseases and patients taking high-dose corticosteroids (hydrocortisone >200 mg/day or equivalent) or other immunosuppressive medications.

Eight individuals who had never suffered from sepsis were matched for age, sex, diabetes and renal insufficiency and checked for the presence of exclusion criteria, and were subsequently enrolled as controls after giving written informed consent. Blood (50 mL) was collected in heparinized, ethylenediaminetetraacetic acid (EDTA) or serum tubes by peripheral venipuncture from each study participant and immediately processed.

Questionnaire

All study participants were asked to complete a 63-item questionnaire on current and past morbidities, current medications and clinically apparent infections during the last twelve months. In addition, former data on the sepsis-associated hospital stay were extracted from our patient data management system (focus of infection and time since sepsis).

Standard laboratory parameters

For standard laboratory tests, blood samples were processed in the routine hospital laboratory. Blood count (white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelets) and additional parameters of relevance in the context of infections (fibrinogen, aspartate transaminase, alanine transaminase, γ-glutamyl transferase, C-reactive protein (CRP) and procalcitonin) were measured according to in-house standards.

Characterization of immune cell subsets

To assess the number of T cell subsets and monocytes, whole blood was stained and measured by flow cytometry as described in detail below. The percentage of $CD4^+$ and $CD8^+$ T cells was calculated as the fraction of all $CD3^+$ events, while the amount of regulatory T cells ($CD4^+$ $CD25^+$ $CD127^-$) was calculated as the fraction of all $CD4^+$ events. Last, the percentage of monocytes was calculated as the amount of $CD14^+$ cells from all measured cellular events.

Quantitative HLA-DR analysis on monocytes

Expression of human leucocyte antigen-antigen D-related (HLA-DR) on $CD14^+$ monocytes was measured by flow cytometry according to the manufacturer's recommendations (Quantibrite HLA-DR/Monocyte antibody cocktail, BD Bioscience, Heidelberg, Germany). Briefly, 50 μL of EDTA-anticoagulated whole blood was incubated with 20 μl of the antibody cocktail and incubated for 30 min. Afterwards, erythrocyte lysis was performed by adding 450 μl FACS Lysing solution (BD Bioscience, Heidelberg, Germany) and further incubation for 15 minutes. Measurement was immediately done on a FACSCalibur flow cytometer (BD Bioscience). In addition, a 4-point

calibration curve (Quantibrite PE Beads, BD Bioscience) was also measured daily to enable the transformation of the measured sample values to molecules of HLA-DR.

Expression of cell surface proteins

Cell surface expression analysis was performed by flow cytometry. For each target of interest, 100 μL whole blood was incubated with the corresponding antibodies and incubated for 30 minutes at 4 °C, followed by a lysing step of 15 minutes at room temperature after addition of 2 mL fluorescence-activated cell sorting (FACS) lysing solution. Finally, the cells were washed twice (1.000 rpm, 5 minutes, 4 °C) with cold phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA) containing 1 % bovine serum albumin (fraction V, protease-free; Carl Roth, Karlsruhe, Germany).

The following antibodies were used for the identification of immune cell subsets: fluorescein isothiocyanate (FITC)-CD3 (BD Bioscience, #555916), FITC-/phycoerythrin (PE)-CD4 (BD Bioscience, #555346/555347), PE-CD8 (BD Bioscience, #555367), FITC-CD14 (BD Bioscience #555397), PE-CD25 (BD Bioscience, #555432), Alexa Fluor 647-CD127 (BD Bioscience, #558598). For the quantitative measurement of expression levels on T cells, the following antibodies were used: programmed cell death 1 (PD-1) (Biolegend, San Diego, USA, #329908), B- and T-lymphocyte attenuator (BTLA) (Biolegend, #344510), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Biolegend, #349908) (all conjugated to allophycocyanin (APC)). For the quantitative measurement of expression levels on monocytes, the following antibodies were used in addition: toll-like receptor (TLR)2 (Biolegend, #309708), TLR4 (Biolegend, #312806), TLR5 (Abcam, Cambridge, UK, #4.5119), Dectin-1 (Biolegend, #355404) or programmed cell death ligand 1 (PD-1 L) (Biolegend, #329706) (all conjugated to PE). Furthermore, for every antibody used, an according sample was stained with the same amount of isotype-matched non-binding antibody (isotype control). Percentages of positive cells represent the fraction of cells with fluorescence above isotype control.

To obtain robust and reliable quantitative results without bias due to the day-to-day variance of the instrument, we measured a 4-point calibration curve for both PE and APC (QUANTUM™ MESF beads, Bangs Laboratories, Fishers, IN, USA). Fluorescence intensity of each sample was normalized by subtracting the staining intensity of the according isotype control. Subsequently, conversion to molecules of equivalent soluble fluorophore (MESF) was done be recalculating the normalized intensity to the results of the calibration curve.

Cytokine secretion of ex vivo-stimulated whole blood

For the functional assessment of the immune cells, whole blood was diluted 1:1 with RPMI1640 containing proprietary GlutMAX™ (Thermo Fisher Scientific, Waltham, MA, USA) and 5 % fetal bovine serum (Ultra-low endotoxin; Cell Concepts GmbH, Umkirch, Germany) and incubated with α-CD3/28 (eBioscience, Frankfurt, Germany; T cell stimulation), ultrapure lipopolysaccharide (LPS) (0111:B4; TLR4 agonist, activates monocytes and dendritic cells) or depleted zymosan (β-1,3-D-glucan; TLR2/Dectin-1 agonist, activates monocytes and dendritic cells) (both Invivogen, San Diego, CA, USA).

For T cell activation, microplates were coated in advance by incubation of 50 μL α-CD3 antibody solution (10 μg/mL) for 24 h. After incubation, the wells were washed twice with sterile PBS and 300 μL of diluted blood and α-CD28 antibody (2 μg/Ml) were added. LPS and zymosan stimulation was performed directly in the blood by adding the agonists in a final concentration of 100 ng/mL and 25 μg/mL, respectively.

All stimulation was done in triplicate for 24 h. After incubation, the samples were centrifuged (3.000 rpm, 5 minutes) and supernatant recovered. As the readout, the secretion of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF and IFN-γ) were measured using a multiplex Th1/Th2/Th17 cytometric bead array (BD Bioscience).

Statistical analysis

The present pilot study was set up with an estimating intention to assess the variances of the endpoints to enable sample size estimation for further studies. Because of lacking data, no *a priori* sample size calculation was performed for this study. Based on the study intention and the small sample size, no group comparisons were performed. Visualization in scatter plots was done using GraphPad Prism (Version 5.0f, GraphPad Software, La Jolla, CA, USA).

Results

Enrollment and specimen collection

Long-term survivors of catecholamine-dependent sepsis were identified (n = 172) and contacted by mail 9–52 months after the septic event. Of all interested responders (n = 14), 8 former patients were eligible to be enrolled in the study. Afterwards, 8 individuals matched for age, sex, diabetes, and renal insufficiency, who had never suffered from sepsis, were enrolled as controls.

The clinical characteristics of the patients who had survived sepsis and the control group are shown in Table 1. In the post-sepsis group, the median period between sepsis and the study time point was 26 (9–52) months. The most frequent focus of infection was urogenital (50 %), followed by necrotizing fasciitis (25 %) and pulmonary or endoprosthesis infection (12.5 % each). Further clinical characteristics (especially age, sex, diabetes and renal insufficiency) were similar in the two

Table 1 Clinical characteristics of controls and patients who survived sepsis

	Control (n = 8)	Sepsis (n = 8)
Age, median (range)	59 (34–85)	60 (36–82
Male sex	5 (62.5)	5 (62.5)
Body mass index, kg/m^2, median (range)	26.4 (18.1–34.3)	24.4 (22.2–40.4)
Time since sepsis, months, median (range)		26 (9–52)
Infection focus responsible for sepsis		
Urogenital		4 (50)
necrotizing fasciitis		2 (25)
Pulmonary		1 (12.5)
Endoprothesis infection		1 (12.5)
Morbidities (current and past)		
Heart and circulation		
Myocardial infarction	2 (25)	2 (25)
Myocarditis	0 (0)	0 (0)
Stroke	1 (12.5)	0 (0)
Pulmonary artery embolism	0 (0)	0 (0)
Thrombosis	0 (0)	0 (0)
Airway and lung		
Asthma	0 (0)	0 (0)
chronic bronchitis	0 (0)	2 (25)
Pneumonia	0 (0)	2 (25)
Exacerbation of chronic obstructive pulmonary disease	0 (0)	1 (12.5)
Kidney		
Insufficiency	1 (12.5)	1 (12.5)
Pyelonephritis	0 (0)	1 (12.5)
Glomerulonephritis	0 (0)	0 (0)
Gastrointestinal tract and metabolism		
Chronic inflammatory bowel disease	0 (0)	0 (0)
Ulcus	0 (0)	1 (12.5)
Gastritis	1 (12.5)	1 (12.5)
Diabetes	3 (37.5)	3 (37.5)
Current medications	6 (75)	7 (87.5)
Anticoagulants	0 (0)	3 (37.5)
Anti-platelet agents	5 (62.5)	3 (37.5)
Antihypertensive medication	2 (25)	5 (62.5)
Diuretics	1 (12.5)	4 (50)
Diabetes medication	3 (37.5)	3 (37.5)
Insulin	2 (25)	3 (37.5)

Absolute number (percentage), if not otherwise specified

groups. However, distinct differences were found in prescribed medications e.g., anticoagulant, blood pressure and diuretic medication.

Characteristics of clinically apparent infections during the previous year

Of the sepsis survivors 62.5 % had experienced at least one infection during the previous 12 months (Table 2). One of those patients suffered five episodes of upper airway infection. At least one episode of antibiotic treatment was prescribed to four individuals in the sepsis survivors. Three different types of infection occurred (oral candidiasis, herpes zoster and lower airway infection), which are typically associated with an impaired immune system. None of the patients in the control group was diagnosed with an infection or was treated with anti-infective medication.

Exclusion of current acute infections

Standard inflammatory markers were determined to screen for current infectious diseases. White blood cell count was within the physiological range in all but one individual (13.2 cells/μL × 10^3) in the control group (Table 3). CRP was slightly elevated in eight sepsis survivors and seven control individuals. Despite all values being <50 mg/L (threshold for severe infection), there was a minor trend towards increased CRP levels in patients with a history of sepsis. Procalcitonin (PCT) was marginally increased (0.1; 0.1; 0.2 ng/ml) in three sepsis survivors. Thus, the presence of relevant but not clinically apparent infective disorders at the time of enrollment, which could affect our analysis, was unlikely. However,

Table 2 Characteristics of clinically apparent infections during the previous year

	Control (n = 8)	Sepsis (n = 8)
Individuals with ≥1 infection	0 (0)	5 (62.5)
Incidence per annum, number (range)		1 (0–5)
Outpatient		2 (25)
Stationary		2 (25)
Antibiotic therapy		4 (50)
Application of blood components		2 (25)
Site of infection		
Upper airway		3 (37.5)
Lower airway		2 (25)
Urogenital		0 (0)
Gastrointestinal		1 (12.5)
Central nervous		0 (0)
Special infectious entities		
Herpes zoster		1 (12.5)
Herpes simplex		0 (0)
Oral candidiasis		1 (12.5)
Cytomegalovirus (re)infection		0 (0)

Absolute number (percentage), unless specified otherwise

Table 3 Blood count and additional laboratory parameters

	Control (n = 8)			Sepsis (n = 8)		
	Median	Perc 05	Perc 95	Median	Perc 05	Perc 95
WBC, cells/μL × 10^3	5,6	3,8	13,2	7,7	5,3	9,9
Erythrocytes, cells/μL × 10^6	4,8	3	5,3	4,55	4,1	6,1
Hemoglobin, g/L	140	88	158	137	106	170
Hematocrit, %	0,41	0,26	0,48	0,42	0,35	0,51
MCV, fL	86,5	85	93	89,5	83	96
MCH, pg	29,65	28,7	31,9	28,9	25,8	31,7
MCHC, g/L	340,5	331	353	327,5	306	342
Platelets, cells/μL × 10^3	268	179	346	261	231	320
Fibrinogen, g/L	3,38	2,54	5,43	3,55	2,29	5,21
AST, U/L	28	16	33	20	11	55
ALT, U/L	25	12	52	22	16	73
GGT, U/L	13	7	81	34	9	155
CRP, mg/L	1,76	0	32,53	10,01	0,88	23,65
PCT, ng/mL	0	0	0	0	0	0,2

perc percentile, *WBC* white blood cell count, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *AST* aspartate transaminase, *ALT* alanine transaminase, *GGT* γ-glutamyl transferase, *CRP* C-reactive protein, *PCT* procalcitonin

the slightly elevated CRP and PCT values in sepsis survivors indicate a chronic low-level inflammatory status.

Characterization of circulating immune cells

$CD4^+$ and $CD8^+$ subsets of $CD3^+$ T cells were determined by flow cytometry analysis (Fig. 1). Additionally, the percentage of regulatory T cells ($CD25^+$ $CD127^-$ Tregs) and $CD14^+$ (antigen-presenting) monocytes as part of all leukocytes were determined. No substantial differences between the two groups were observed.

Furthermore, we determined expression levels for important negative regulators of lymphocyte function. Therefore we measured PD-1, CTLA-4 and BTLA receptor expression within the $CD4^+$ and $CD8^+$ T cell subsets by 1) quantifying the percentage of receptor-positive cells and 2) assessing a surrogate parameter of the cell surface receptor density (MESF) (Fig. 2). No expression of CTLA-4 was measurable (data not shown). In $CD4^+$ cells of sepsis survivors, the receptor density of PD-1 appeared to be downregulated, but was upregulated for BTLA. In $CD8^+$ cells no substantial changes were seen. In summary, this is indicative of two opposing mechanisms of negative immune cell activation feedback in $CD4^+$ cells in patients who have survived sepsis.

Next we sought to clarify whether the initiation of innate immune responses of monocytes via pattern recognition receptors and the negative regulatory molecule PD-1 ligand (PD-1 L) might be disturbed in sepsis survivors due to modified expression levels. Therefore, we determined the surface protein expression of different toll-like-receptors (TLR2, TLR4, TLR5), Dectin-1, and PD-1 L (Fig. 3). For TLR4 and PD-1 L there were no substantial differences between the two groups. Dectin-1-positive cells were more frequent but Dectin-1 density was not affected in the sepsis survivors. There was a slight increase in $TLR2^+$ cell count and receptor density. The most notable and coherent effect was seen for TLR5, which is a fundamental player in pathogen-associated molecular pattern recognition (bacterial flagellin) and therefore an activator of innate immunity. Its receptor-positive cell count and especially the receptor density were substantially decreased, which points towards a selectively impaired innate immune system in this special aspect.

No evident differences were seen in the monocytic HLA-DR receptor density as a surrogate marker of global immune function (Fig. 4).

Cytokine secretion of ex-vivo-stimulated whole blood

Last, we determined the immune competence of individual patients to react to distinct inflammatory stimuli. Whole blood from sepsis survivors and controls was either incubated with the T cell activator α-CD3/28, or the monocyte/macrophage-activators LPS (via TLR4) or zymosan (via TLR2/Dectin-1). Subsequently, effects on the expression of the cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α and IFN-γ were measured (Fig. 5). No IL-2, IL-4, and IL-17A secretion was detectable (data not shown). For the other parameters determined, the post-sepsis group had decreased cytokine responses. There were pronounced trends towards lower IFN-γ production after stimulation with α-CD3/28 and IL-10 production after LPS activation. Most impressively, stimulation with zymosan, a yeast surface protein, was followed

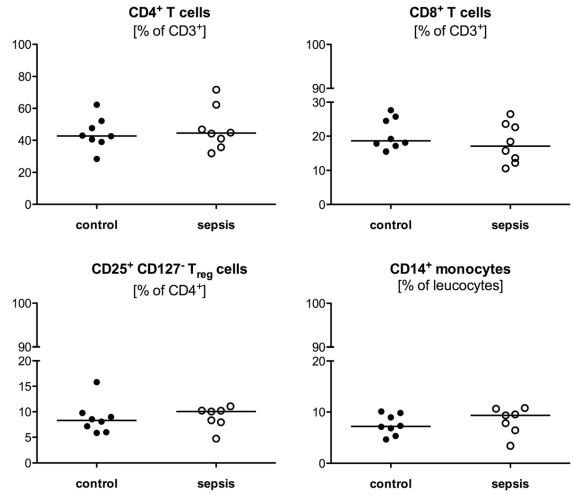

Fig. 1 Characterization of immune cells subsets. Peripheral blood was collected from eight survivors of sepsis and matched controls and analyzed by flow cytometry to determine the percentage of circulating CD4+ and CD8+ subsets of $CD3^+$ T cells, the amount of regulatory T cells ($CD25^+$ $CD127^-$ Treg) as a fraction of all $CD4^+$ cells, and $CD14^+$ monocytes of all leucocytes. The markers used are described in "Methods". Each data point represents an individual patient. *Horizontal lines* indicate median values

by substantially reduced production of IL-6, IL-10 and TNF-α. The observed responses did not depend on the sole number of leucocytes present in the sample. In fact, only the LPS-induced TNF secretion correlated significantly with the number of leucocytes, but paradoxically the relationship was negative (Spearman r −0.5303, $p = 0.0346$; data not shown). All of the abovementioned cytokines were also assessed in native plasma from the participants, to rule out the possibility of their presence as chronic immunogenic stressors, inducing maladaptation of the immune cells. We found no detectable concentration for any cytokine (data not shown).

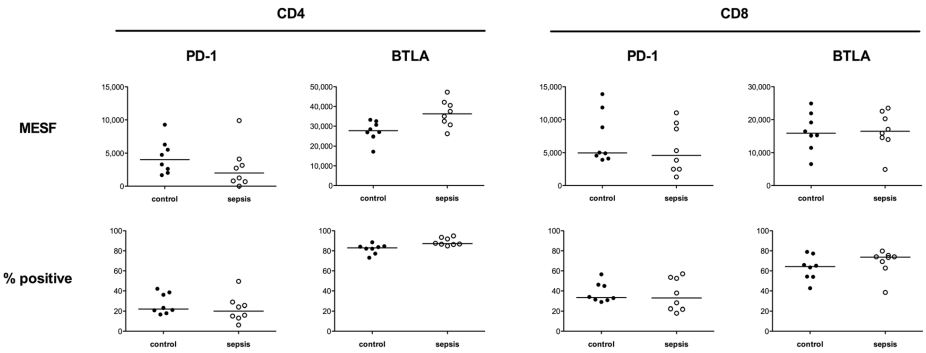

Fig. 2 Determination of the pattern of expression of specific receptors on $CD4^+$ and $CD8^+$ T cells. Peripheral blood was collected from eight survivors of sepsis and matched controls and stained for the indicated markers, followed by determination of percent positive relative to isotype control staining (*bottom line*) and quantification of fluorescence intensity by molecules of equivalent soluble fluorochrome (*MESF*) calculation (*upper line*) as described in "Methods". Each data point represents an individual patient. *Horizontal lines* indicate median values. *PD* programmed cell death, *BTLA* B- and T-lymphocyte attenuator

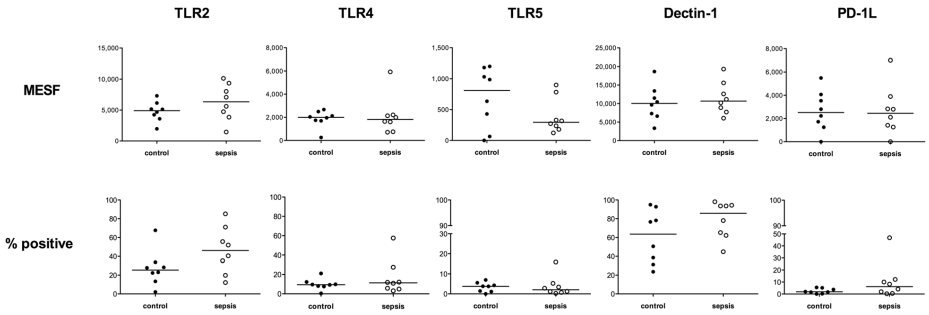

Fig. 3 Determination of expression pattern of specific receptors on monocytes. Peripheral blood was collected from eight survivors of sepsis and matched controls and stained for the indicated markers, followed by determination of percent positive relative to isotype control staining (*bottom line*) and quantification of fluorescence intensity by molecules of equivalent soluble fluorochrome (*MESF*) calculation (*upper line*) as described in "Methods". Each data point represents an individual patient. *Horizontal lines* indicate median values. *TLR* toll-like receptor, *PD-1 L* programmed cell death ligand

Taken together, the net effect of detection and reaction to important activators of both the innate and the adaptive immune system seems to be impaired in survivors of sepsis.

Discussion

In our prospective, cross-sectional study we aimed to explore the phenomenon of sepsis-induced long-term immune paralysis. We identified patients who survived an episode of catecholamine-dependent sepsis 9–60 months prior to enrollment. General health status, immune cell subsets, cell surface receptor expression, HLA-DR expression, and cytokine secretion of stimulated whole blood were determined. We conducted our study to shed light on the phenomenon of persisting high morbidity in survivors of sepsis. In their review Winters et al. reported 1-year mortality after hospital discharge, which differed between 3 % and 43 % in 17 distinct studies [17]. In one other study, the 2-year mortality rate after hospital discharge was 44.9 % among individuals surviving severe sepsis. This was 1.5 times higher than the in-hospital mortality rate in the same study [20]. A very late increment for sepsis-related mortality was observed in a study that reported 1-year mortality of 51.4 %, increasing to 74.2 % 5 years after hospital discharge [21]. An impaired immune response as one sequelae of previous sepsis is believed to be a major contributing factor in these delayed deaths [16, 22]. To our knowledge studies investigating this phenomenon are rare, especially in humans. In distinct murine models, Marwart et al. demonstrated sepsis-induced loss of naive T cells but no prolonged defects in T cell function [23]. On analysis of myeloid cells after polymicrobial sepsis in mice, precursors of dendritic cells in the bone marrow were found to develop into regulatory dendritic cells that mediated immunosuppression [24]. Our own investigations showed that human sepsis induces distinct epigenetic changes in immunologically relevant genes that could ultimately contribute to functional changes in monocytes [25]. As these modifications are persistent and can be propagated, changes in basal regulatory mechanisms might also affect the immune function after surviving sepsis [22]. Additionally, effects on behavioral, affective and molecular responses in the brain of mice are shown after surviving LPS-induced systemic inflammation [26, 27].

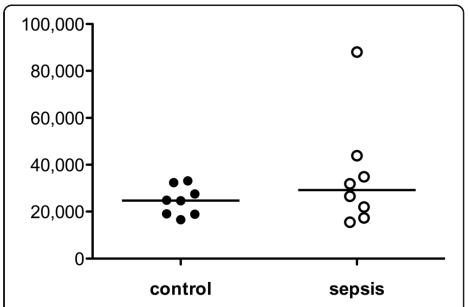

Fig. 4 Determination of human leukocyte antigen (HLA)-DR expression on monocytes. Peripheral blood was collected from eight survivors of sepsis and matched controls and HLA-DR expression was measured. Each data point represents an individual patient. Horizontal lines indicate median values

There was almost complete matching of clinical characteristics that likely modify immune function (age, sex, presence of diabetes and renal function). As a result, the

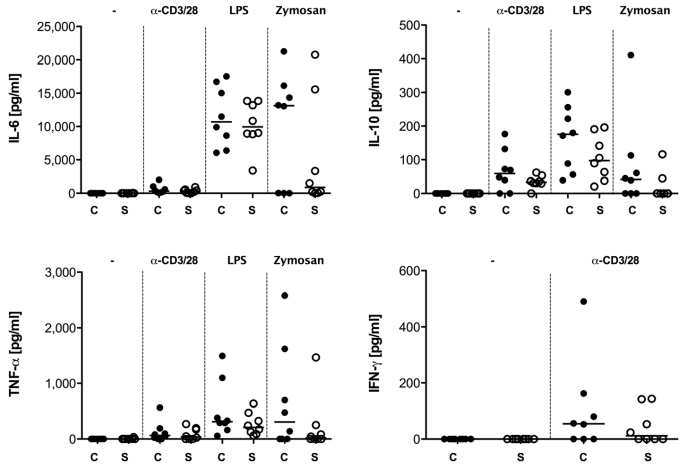

Fig. 5 Cytokine secretion of ex-vivo-stimulated whole blood. Peripheral blood was collected from eight survivors of sepsis (*S*) and matched controls (*C*). Whole blood was stimulated with either CD3/28 antibody (α-CD3/28), lipopolysaccharide (LPS) or Zymosan. Supernatants were harvested after 24 h and TNF-α, interferon-γ (*IFN-γ*), and IL-6 and IL-10 were measured by enzyme-linked immunosorbent assay. Each *data point* represents an individual patient. *Horizontal lines* indicate median values. *CD* cluster of differentiation

two groups were comparable in the context of the present study. The hypothesis of impaired immunity in survivors of sepsis is supported by the results of the clinical status questionnaire (Table 2). First, the observations revealed continuing infections even months and years after surviving sepsis. Second, the need for antibiotic drug prescriptions in four patients and two hospital admissions emphasize the severity and relevance of the infections. Third, we observed infections that are typically associated with impaired immune function: oral candidiasis, herpes zoster and lower airway infection. This is in line with mouse models, which indicate that survivors of septic shock are susceptible to infection with pathogens that are usually innocuous [24, 28–30]. Notably, these types of infection are likely to reduce the patient's quality of life, an assumption that is consistent with previous reports describing impaired quality of life secondary to serious illness [17, 20].

The standard inflammatory markers were in the normal range or slightly increased, sufficient to exclude clinically relevant infectious episodes at the time of enrollment (Table 3). However, comparing the two study groups, the sepsis survivors had low CRP values, but higher CRP values than controls, and more frequently had slightly elevated PCT. This observation also supports the perception that sepsis is followed by a long-lasting, low-grade chronic inflammatory state.

Sepsis induces a comprehensive loss of myeloid cells and lymphocytes including $CD4^+$ and $CD8^+$ T cells [8]. Boomer et. al. observed a reduction in $CD4^+$ and $CD8^+$ T cells in the acute phase and an increase of regulatory T cells in the process of sepsis [31]. As shown by our own results the distribution of subsets seems to normalize in the long term after surviving sepsis (Fig. 1). Nevertheless, the presence of certain cells does not imply the exertion of function and several studies, mainly conducted in animals, show the presence of sustained functional alterations after severe infection [22, 28].

For the next investigation we therefore determined the expression levels of cell surface receptors on different T cell subsets (Fig. 2) and on monocytes (Fig. 3). In a longitudinal study, Boomer et. al. observed a trend in the MESF increase in PD-1 at the onset of sepsis [31]. In another study there was upregulation of PD-1 on T cells and PD-L1 on monocytes in patients with septic shock [32]. Relevant to the potential for clinical implementation, the blockade of PD-1 and CTLA-4 improves survival in primary and secondary fungal sepsis in mice [33]. In our study, we found contrary results for PD-1 in sepsis survivors in the long term (Fig. 2). However, BTLA receptor expression trended towards upregulation. As both are members of the important negative regulators of T cell function, clear-cut conclusions are hard to draw. It appears that the resulting phenotype might be highly dependent on the individual immune system, raising a question about the influential variables.

In our study, patients who survived an episode of sepsis had alterations in monocyte surface expression of

pattern recognition receptors (Fig. 3). The most notable effect was in substantially decreased TLR5 expression. As TLR5 recognizes flagellin, which is the main component of bacterial flagella, the downregulation of TLR5 may in consequence contribute to impaired detection of certain bacteria by phagocytes.

Decreased surface HLA-DR expression on monocytes is a reliable surrogate marker of global immunosuppression and its value predicts mortality and development of nosocomial infection in patients suffering from sepsis [34–39]. As a reasonable consequence, survivors of sepsis in our study had no substantial differences in HLA-DR expression compared to controls (Fig. 4).

Boomer et al. have already showed that peripheral blood mononuclear cells isolated from whole blood 7 days after onset of sepsis have impaired secretion of IFN-γ when stimulated with α-CD3/28 [31]. According to our results, this trend appears to continue even months and years post-sepsis (Fig. 5). In contrast to our findings, the values for IL-6, IL-10 and TNF-α after α-CD3/28 stimulation were increased in that study [31]. As these patients were still hospitalized and in an acute immune condition, this could be one explanation for the difference. In another study from Boomer et. al., the cytokine secretions of TNF-α, IFN-γ, IL-6 and IL-10 were globally decreased after LPS and α-CD3/28 stimulation in patients who died from sepsis [15]. The same trends were observable in our group of sepsis survivors (Fig. 5).

Despite our valuable findings, there are several limitations in our study. First, our investigation is a pilot study and therefore we only examined a small number of patients. As a result we were only able to demonstrate pronounced trends and this is why our investigations need extensive revalidation with a larger sample size. Second, our control population consisted of mostly healthy individuals besides the described matching parameters. As so, we did not compare patients who had survived sepsis with patients who had survived non-septic critical illness. Therefore, our findings may be due to surviving an acute critical illness or non-matched co-morbidities and not necessarily from surviving sepsis. Third, we did not know the patients' immune status before sepsis. The septic event could have evolved from previous differences in immune system function. These differences could be still detectable after sepsis, but not necessarily be a consequence of surviving sepsis. As most individuals had previously had urosepsis, different types of sepsis (e.g., abdominal sepsis) might have another fundamental effect on immune function and need to be evaluated. Furthermore, each individual was only investigated once and therefore, we were not able to identify possible changes in immune cell function over time.

We conducted this study as a basis for further investigation of a poorly studied group of patients: the survivors of sepsis. Even though long-lasting impairment of the immune system is believed to be the underlying reason for delayed sepsis-related death and to impact quality of life, studies investigating the immune status of survivors of sepsis in humans are missing. In the future, comprehensive studies need to test the correlation between the reasons (alterations of the immune cells) and the effects (quality of life and survival). Post-septic patients may require specific diagnostic tools and the results could enable us to identify patients who might benefit from structured post-sepsis care. Individualized medicine does not stop at the hospital door and therefore, we need to reconsider what comes after acute critical care.

Conclusions

The reactivity of immune cells to different activators appears to be comprehensively impaired in survivors of sepsis. Among other reasons, this could be based on the downregulation of distinct cell surface receptors. Nevertheless, we believe that our findings might merely represent the tip of the iceberg, and further validation studies in larger cohorts are needed to clearly track the changes and the underlying molecular mechanism and their biological relevance.

Key messages

- In the long term, survivors of sepsis had increased numbers of clinically apparent infections and a low-level inflammatory status based on the standard inflammatory markers
- In the post-sepsis group, there were alterations in monocyte surface expression of distinct pattern recognition receptors, most pronouncedly observed in decreased TLR5 expression
- Cytokine secretion in response to important activators of both the innate (LPS and zymosan) and the adaptive immune system (α-CD3/28) appeared to be weakened in sepsis survivors

Abbreviations

ALT: alanine transaminase; AST: aspartate transaminase; BMI: body mass index; BTLA: B- and T-lymphocyte attenuator; CARS: compensatory anti-inflammatory response syndrome; CD: cluster of differentiation; CMV: cytomegalovirus; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; CTLA: cytotoxic T-lymphocyte-associated protein; EDTA: ethylenediaminetetraacetic acid; FITC: fluorescein isothiocyanate; GGT: γ-glutamyl transferase; HIV: human immunodeficiency virus; HLA: human leukocyte antigen; ICD: International Statistical Classification of Diseases and Related Health Problems; ICU: intensive care unit; IFN: interferon; IL: interleukin; LPS: lipopolysaccharide; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MESF: molecules of equivalent soluble fluorochrome; MODS: multiple organ dysfunction syndrome; PBMC: peripheral blood mononuclear cell; PBS: phosphate-buffered saline; PCT: procalcitonin; PD: programmed cell death; PICS: persistent inflammation-immunosuppression catabolism syndrome; SIRS: systemic inflammatory response syndrome; TLR: toll-like receptor; TNF: tumor necrosis factor; WBC: white blood cell count.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CA, CL, MAW and FU were responsible for study design and conduct, data and sample acquisition, statistical analysis and interpretation and writing the manuscript. SAB acquired samples and data, assisted in the statistical analysis and interpretation, and approved and helped to draft the manuscript. CK, BHS, ES, AH and SW supported the study conduct and data acquisition, discussed the data and participated in data interpretation and manuscript preparation. All authors approved the final version of the manuscript.

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8.9 Anlage 9

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TRAUMA-INDUCED LONG-TERM ALTERATIONS OF HUMAN T CELLS AND MONOCYTES—RESULTS OF AN EXPLORATIVE, CROSS-SECTIONAL STUDY

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ABSTRACT—**Background:** Major trauma leads to complex immune reactions, known to result in a transient immunodeficiency. The long-term consequences of severe trauma on immune function and regulation as well as its clinical impact remain unclear. **Methods:** Six months (ranging from −12 to +5 days) after a major trauma event, 12 former trauma patients (Injury Severity Score ≥ 16) and 12 healthy volunteers were enrolled. The current clinical status and infection history since discharge were assessed by a standardized questionnaire. Immune cell subsets (cluster of differentiation (CD)4^+, $CD8^+$, $CD14^+$), cell surface receptor expression (programmed cell death protein 1 (PD-1), B- and T-lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4, toll-like receptor (TLR)-2, -4, and -5, Dectin-1, programmed death ligand 1(PD-1L)), and human leucocyte antigen D-related receptor (HLA-DR)-expression were quantified by flow cytometry. Cytokine secretion (IL-2, -4, -6, -10, and 17A, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ) was assessed after stimulation of whole blood with LPS-, α-CD3/28, or zymosan. **Results:** Analysis of surface receptors on T cells revealed a significant elevation of PD-1 expression on $CD4^+$ T cells, whereas BTLA expression on $CD4^+$ and $CD8^+$ T cells was significantly suppressed in the trauma cohort. Monocytes showed a significantly reduced expression of TLR-2 and -4 as well as a reduced proportion of TLR-4 monocytes. HLA-DR receptor density revealed no significant changes between both cohorts. LPS-induced IL-6 and TNF-α secretion showed non-significant trends toward reduced values. No differences regarding clinical apparent infections could be detected. **Conclusions:** Six months following major trauma, changes of cell surface receptors on $CD4^+$ and $CD8^+$ T cells as well as on $CD14^+$ monocytes were present, hinting toward an immunosuppressive phenotype. Following major trauma, although IL-6 and TNF-α release after stimulation were reduced, they did not reach statistical significance. Overall, further studies are necessary to evaluate the clinical implications of these findings. **Trial registration:** DRKS00009876, Internet Portal of the German Clinical Trials Register (DRKS), registration date 11.08.2016, https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00009876.

KEYWORDS—CARS, immune tolerance, inflammation, multiple trauma, SIRS

ABBREVIATIONS—APC—allophycocyan; BTLA—B- and T-lymphocyte attenuator; CARS—compensatory anti-inflammatory response syndrome; CD—cluster of differentiation; CRP—C-reactive protein; CTLA-4—cytotoxic T-lymphocyte-associated protein 4; CTRL—control; DAMP—damage-associated patterns; EDTA—ethylenediaminetetraacetic acid; FITC—fluorescin isothiocyanate; HLA-DR—human leucocyte antigen D-related receptor; IL—interleukin; INF—interferon; ISS—injury severity score; LPS—lipopolysaccharide; MODS—multiple organ dysfunction syndrome; PCT—procalcitonin; PD 1—programmed cell death protein 1; PD-1 L—programmed death ligand 1; PDMS—patient data management system; PE—phycoerythrin; PICS—persistent inflammation, immunosuppression and catabolism syndrome; RAGE—receptors for advanced glycation end products; SIRS—systemic inflammatory response syndrome; TLR—toll-like receptor; TNF—tumor necrosis factor

INTRODUCTION

Trauma remains one of the leading causes of deaths worldwide and remains a challenging task for emergency physicians, surgeons, and intensivists (1). Over the last decades, trauma-associated survival increased as a consequence of improved performance in prehospital life support as well as in subsequent surgical and intensive care (2). However, data from trauma registers exhibit an increased mortality up to 3 years following major trauma (3,4). Therefore, long-term consequences of major trauma, such as posttraumatic immunodeficiency, gain rising interest.

Major trauma induces a complex immune response triggered by tissue damage, leading to a massive release of endogenous damage-associated patterns (DAMPs) (5,6). These activate the immune and the complement systems, via, e.g., toll-like receptors (TLR) and receptors for advanced glycation end products (RAGE) (7). As a consequence, the activation leads to the clinical signs of a noninfectious systemic inflammatory

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response syndrome (SIRS) due to a rapid increase of inflammatory cytokines (2). To prevent the human body from overwhelming immune reactions, negative feedback mechanisms, as the release of anti-inflammatory cytokines, are mounted simultaneously. Disbalance of pro- and anti-inflammatory regulatory mechanisms may result in a long-term state of immunosuppression (compensatory anti-inflammatory response syndrome (CARS)), leaving the patient at high risk for post-traumatic infection and sepsis (2,8,9). Furthermore, the excessive release of cytokines (''cytokine-storm'') may lead to a qualitative and quantitative change of gene expression (''gene-storm''). Gene expression of pro-inflammatory mediators increases rapidly after trauma while genes responsible of anti-inflammatory pathways are simultaneously suppressed, which was identified for up to 28 days in trauma patients suffering from nosocomial infections (10). Especially in septic patients, persistent inflammation, immunosuppression, and catabolism syndrome (PICS) and multiple organ dysfunction syndrome (MODS) are well-described complications of the SIRS-CARS continuum. The growing understanding of the pathophysiologic changes in sepsis led to the renewed Sepsis-3 definition, highlighting the organ dysfunction as a result of a dysregulated host response (11).

Septic patients exhibit a higher long-term morbidity and mortality, which might be associated with post-septic immunosuppression, potentially caused by relevant and long-lasting cytokine suppression (12). Interestingly, even though major trauma leads to a similar immune reaction and organ dysfunction and is associated with increased long-term mortality, no data is available describing the long-term immune status of patients after major trauma. Therefore, this explorative study aims to describe the cell surface receptor expression and function of lympho- and monocytes 6 months after major trauma for the first time.

METHODS

Study design and patient recruitment

This prospective, noninterventional, explorative cross-sectional study was approved by the local ethics committee (Justus-Liebig-University, Giessen, Germany, trial code 26/16). The study methods and results are presented in accordance with the STROBE guidelines.

The local patient data management system (PDMS) ICU-Data (IMESO GmbH, Giessen, Germany) of the surgical intensive care unit at the university hospital of Giessen and Marburg (site Giessen) was automatically screened for adult patients admitted with severe multiple injuries, objectified by an Injury Severity Score (ISS) $\geq$16 and the need for intensive care treatment. The search period encompassed September 2016 to March 2017. Patients with signs of acute infection, age $>$ 60 years, history of trauma associated infectious complications, chronic viral diseases (e.g., hepatitis, human immunodeficiency virus), diabetes mellitus, terminal kidney disease, need for immunomodulatory medication or pregnancy were excluded. Suitable patients were contacted via mail and asked for participation. After written consent, blood was collected and a standardized interview was performed covering information about comorbidities, infectious diseases, and medications throughout the last 6 months. Patients were paid an expense allowance. Healthy subjects, who never suffered from major trauma, were matched for age and gender and checked for exclusion criteria. After providing written consent, subjects were enrolled as already described.

Blood collection and standard laboratory parameters

Blood was drawn via peripheral venipuncture, split into serum, citrate, and ethylenediaminetetraacetic acid (EDTA) tubes (approximately 25 mL in total), and immediately processed. Leucocytes, C-reactive protein (CRP), procalcitonin (PCT), and fibrinogen as standard laboratory parameters were analyzed by the hospital core laboratory.

Flow cytometry

As published before in detail, flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany) was performed to characterize immune cell subsets and expression of cell surface proteins as well as to describe quantitative HLA-DR-characteristics of monocytes (12). First, T cell $CD4^+$ and $CD8^+$ subsets of the fraction of all $CD3^+$ events and monocytes subsets ($CD14^+$) were characterized.

Second, flow cytometric quantification of expression of human leucocyte antigen D-related receptor (HLA-DR) on $CD14^+$ monocytes was performed complying with manufacturer's instructions (Quantibrite HLA-DR/Monocyte antibody cocktail, BD Bioscience, Heidelberg, Germany). After incubation of the antibody cocktail in whole blood, erythrocytes were lysed by adding FACS Lysing Solution. Daily Four-point calibration (Quantibrite PE Beads, BD Bioscience, Heidelberg, Germany) was performed to transform the sample values to HLA-DR-molecules. Third, analysis of cell surface expression was performed. Preparation steps included incubation of whole blood with the antibody of interest for cell lysis and two washing steps. $CD4^+$ and $CD8^+$ T cell receptor characterization included programmed cell death protein 1 (PD-1), B- and T-lymphocyte attenuator (BTLA), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). For description of $CD14^+$ monocytes receptors, TLR 2-, 4-, and, 5, BTLA-, and Programmed death ligand 1 (PD-1 L) antibodies were used. Details of used antibodies are summarized in supplement 1, http://links.lww.com/SHK/A868. Quantified isotypes (IgG1 and IgG2a) were subtracted for further quantification and negative values were defined as zero. Four-point calibration curve for phycoerythrin (PE) and allophycocyanin (APC) (Quantum MESF beads, Bangs Laboratories, Fishers, Ind) was performed as part of the daily quality controls. Each sample's fluorescence intensity was normalized to the isotype control. The normalized intensity was calculated on the base of the calibration curve after conversion of molecules to equivalent soluble fluorophore (MESF).

Cell stimulation and cytokine secretion

Ex-vivo stimulation of whole blood was performed as already published in detail (12). After 1:1 dilution of whole blood (RPM 1640, mixture of GlutMAX, Thermo Fisher Scientific, Waltham, Mass) and 5% fetal bovine serum (Ultralow endotoxin, Cell Concepts GmbH, Umkirch, Germany), incubation with α-CD3/28 for T cell stimulation (concentration (CD3/CD28) 5/2 μg/mL, eBioscience, Frankfurt, Germany), ultrapure lipopolysaccharide (concentration 100 ng/mL, LPS, Invivogen, San Diego, Calif, or depleted zymosan for monocyte stimulation (concentration 10 μg/mL, β-1,3-D-glucan, Invivogen, San Diego, Calif) were performed. Subsequently, cytokine secretions of IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ were quantified using a multiplex Th1/Th2/Th17 cytometric bead array (BD Bioscience, Heidelberg, Germany). Furthermore, high-sensitive ELISA was performed to quantify IL-8 (R&D Systems, Minneapolis, Minn), IL-10 (R&D Systems, Minneapolis, Minn), and IFN-γ (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions.

Clinical data and statistical analysis

Clinical data of the initial intensive care therapy was obtained from the hospital's patient data management system ICU-Data (IMESO GmbH, Giessen, Germany). Caused by its explorative character, a calculation of an *a priori* sample size was not rational. Descriptive analysis was performed for demographic data, clinical characteristics, and infectious laboratory parameters. All variables are presented as medians with interquartile ranges, and categorical variables as numbers and percentages. For detection of intergroup differences, a Kruskal–Wallis test was performed followed by the *post-hoc* pairwise Mann–Whitney *U* test. No correction for multiple testing was applied. Comparisons of categorical variables were generated by the Pearson v2 test. Statistical significance was defined as $P \leq 0.05$. Statistical analysis was performed using *R* statistics, version 3.4.2 (www.r-project.org).

RESULTS

Characteristics of the study cohorts

A total of 14 former trauma patients were included and matched with 14 healthy volunteers. The trauma event occurred 6 months (ranging from −12 to +5 days) prior to study inclusion. The maximum deviation of −12 days occurred in

TABLE 1. **Baseline characteristics of trauma patients and controls.**

	Control (n = 12)		Former trauma patients (n = 12)	
Age in years	38 (24–46)		33 (22–44)	
Male sex (%)	8 (66.7)		8 (66.7)	
BMI in kg/m^2	23.2 (22–26.7)		24.8 (21.4–27.2)	
Trauma characteristics				
ISS			27 (22–35.8)	
Length of stay ICU in days			7.5 (2.8–20)	
Length of stay hospital in days			24.8 (13–27.2)	
Invasive ventilation in hours			15.7 (7–119.5)	
Need for blood transfusion (%)			9 (75)	
Pre-existing disease				
Smoking (%)	2 (16.7)		3 (25)	
Coagulatory disorder (%)	0		1 (8.3)	
Arterial hypertension (%)	2 (16.7)		2 (16.7)	
Asthma bronchiale (%)	1 (8.3)		0	
Medication				
Anticoagulative substances (%)	0		1 (8.3)	
Diuretics (%)	1 (8.3)		0 (0)	
Antihypertensive drugs (%)	2 (16.7)		2 (16.7)	
Current work status				
Trauma induced work disability (%)	0		8 (66.7)	
Infectious status in last 6 months				
Number of infections (%)	No infections	4 (33.3)	No infections	4 (33.3)
	n = 1	5 (41.7)	n = 1	6 (50)
	n = 2	2 (16.7)	n = 2	2 (16.7)
	n > 2	1 (8.3)	n > 2	0
Professional medical treatment (%)	3 (25)		3 (25)	
Hospital admission due to infectious cause (%)	1 (8.3)		2 (16.7)	
Need for antibiotic treatment (%)	3 (25)		2 (16.7)	
Upper airway infection (%)	7 (58.3)		7 (58.3)	
Pneumonia (%)	0		1 (8.3)	
Gastrointestinal infection (%)	0		0	
Lower urinary tract infection (%)	0		1 (8.3)	
Herpes simplex (%)	1 (8.3)		2 (16.7)	

Values are presented as median and interquartile range or if labeled with (%) in absolute number and percentage. Numbers of infections were defined as infectious diseases which occurred during the last months and were either suspected by patients and were treated by themselves or diagnosed and treated by a physician.
BMI indicates body mass index; ICU, intensive care unit; ISS, injury severity score.

two cases and −9 days in one case, while all other participants ranged from 1 to 5 days of 6 months after the date of trauma. Two patients were excluded because of unsuspected pathological laboratory parameters in one case and a methodological error in the second case. Severe trauma was reflected by an initial median ISS of 27 (range: 22–36). Patient's characteristics and their questionnaire's answers are shown in Table 1.

Past and current infectious status

Patients and healthy subjects were asked for signs of diseases caused by different types of bacterial (e.g., meningitis), viral (e.g., *herpes simplex*, *cytomegalovirus*), and fungal infections (e.g., thrush,). Positive answers are shown in Table 1. No obvious differences between the two study cohorts could be found. To exclude current infections, WBC, CRP, and PCT were quantified. Except for one patient, all patients and subjects exhibited regular levels of WBC (trauma: 7.2 [CI 6.6–7.6] giga/L, control: 6.6 [CI 5.9–7.9] giga/L, $P = 0.8$) and CRP (trauma: 0.3 [CI 0–1.6] mg/L, control: 0.6 [CI 0–1.9] mg/L, $P = 0.6$). All PCT levels with the exception of one subject (1.4 μg/L) remained in the normal range. The single patient with elevated WBC also featured the highest CRP plasma level (30.13 mg/L). As this indicated an acute infection, he or she was excluded from further analysis.

Characterization of circulating immune cells

Proportion of T cells subsets ($CD4^+$ and $CD8^+$) did not differ between both study cohorts ($CD4^+$: trauma 60.5% [55.3–65.5], control 67.9% [60.3–71.6], $P = 0.1$; $CD8^+$: trauma 32.6 [24.6–36.1], control 25.8 [20.9–30.5], $P = 0.1$). Further analysis of receptor expression of T cell subsets showed significant reduction of BTLA receptor cell density on $CD4^+$ and $CD8^+$ T cells following major trauma (Fig. 1, Table 2). Interestingly, compared with healthy subjects, PD-1 density on $CD4^+$ T cells was significantly elevated. Furthermore, $CD4^+$ T cells trended to a higher percentage of PD-1-positive cells in the trauma cohort, whereas PD-1-analysis of $CD8^+$ T cells showed trends to the contrary direction (Fig. 1, Table 2). CTLA-4 was not measurable in both cell types (Table 2).

Monocytes, as a surrogate for the innate immune system, showed significant changes of TLR expression in the trauma cohort. TLR-2 and -4 cell surface receptor density as well as the

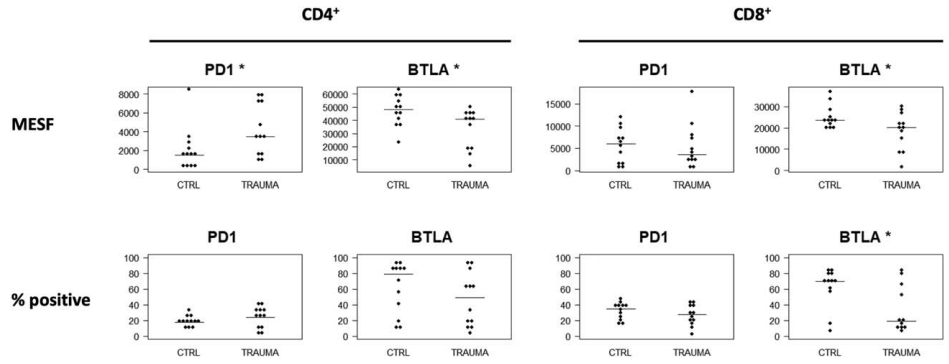

Fig. 1. **Expression analysis of specific receptors on CD4+ and CD8+ T cells.** Each data point represents an individual patient. Horizontal lines indicate median values. $P \leq 0.05$ is characterized by * next to the graph's upper labeling. CTRL indicates control; PD, programmed cell death; BTLA, B- and T-lymphocyte attenuator.

count of TLR-4 positive monocytes were significantly reduced in the trauma cohort. Also, TLR-5 receptor density and the amount of TLR-2 and -5 positive monocytes were reduced but did not reach statistical significance (Fig. 2, Table 2). Furthermore, no significant changes could be detected for cell surface expression of Dectin-1 and PD-L1 (Fig. 2) as well as for HLA-DR receptor density (Fig. 3) between trauma patients and healthy subjects.

In summary, we observed a significant up-regulation of PD-1-receptor cell density on $CD4^+$ T cells with simultaneous down-regulation of BTLA-receptors on $CD4^+$ and $CD8^+$ T cells. Monocytes presented significant down-regulation of TLR-2 and -4-receptors and a trend toward suppression in TLR-5 density in the trauma cohort.

Ex-vivo stimulation of cytokine expression

Compared with unstimulated probes, LPS stimulation caused a significant ($P < 0.01$) increase of IL-6 and TNF-α secretion in trauma (20-fold/2.7-fold) as well as control (49-fold/4-fold) patients. Though IL-6 release showed a strong tendency toward

Table 2. **Results of the receptor cell characterization of $CD4^+$ and $CD8^+$ T cells and $CD14^+$monocytes.**

Cell characterization	Control (n = 12)	Trauma (n = 12)	*P* value
$CD4^+$			
BTLA (MESF)	48,272 (39,197–55427.5)	40778.5 (19845.8–46,282)	0.04
BTLA/$CD4^+$ T cells (%)	79 (33–89)	48.8 (17–72.2)	0.53
PD-1 (MESF)	1536.5 (406.5–2477.3)	3501.5 (1799.3–7456.5)	0.03
PD-1/$CD4^+$ T cells (%)	17.9 (15.4–20.8)	24.4 (10.8–34.1)	0.37
CTLA-4 (MESF)	0 (0–446.3)	99 (0–932.5)	0.54
CTLA-4/ $CD4^+$ T cells (%)	0.1 (0–0.4)	0.1 (0–0.5)	0.81
$CD8^+$			
BTLA (MESF)	23685.5 (22262.3–25757.3)	20119.0 (13898.8–22,974)	0.04
BTLA/$CD8^+$ T cells (%)	70.1 (61.6–78.5)	18.8 (12.5–56.9)	0.04
PD-1 (MESF)	6060.5 (2,028–8,081)	3641.5 (2492.3–7536.5)	0.67
PD-1/$CD8^+$ T cells (%)	35 (23.8–39.8)	27.9 (19–38)	0.44
CTLA-4 (MESF)	149 (0–331.5)	913.5 (231.3–1905.3)	0.06
CTLA-4/ $CD8^+$ T cells (%)	0 (0)	0.2 (0–0.8)	0.02
$CD14^+$			
TLR-2 (MESF)	21,693 (18,054–26,069)	5,863 (3686.5–20,779)	0.04
TLR-2/$CD14^+$ monocytes (%)	81.8 (36.3–87.2)	18.2 (0.2–75.6)	0.08
TLR-4 (MESF)	6,250 (5,461–8205.3)	1189.5 (928–3663.8)	0.03
TLR-4/$CD14^+$ monocytes (%)	20 (5.3–43.5)	0.9 (0.1–16.1)	0.04
TLR-5 (MESF)	3045.5 (162.8–5299.8)	0 (0–908.8)	0.06
TLR-5/$CD14^+$ monocytes (%)	10.3 (1.1–19)	0 (0–11.5)	0.17
Dectin-1 (MESF)	10218.5 (8774.5 – 11606.5)	9,717 (5,942–11098.3)	0.4
Dectin-1/$CD14^+$ monocytes (%)	10.5 (5.3–62.2)	1.4 (0–47.6)	0.37
PD-L1 (MESF)	3,314 (2443.3–3,802)	2,566 (1,237–3238.8)	0.09
PD-L1/$CD14^+$ monocytes (%)	7.8 (5.6–11.7)	6.5 (4.6–7.7)	0.19

For detection of intergroup differences Kruskal–Wallis test was performed followed by *post-hoc* pairwise Mann–Whitney *U* test. No correction for multiple testing was applied.
BTLA indicates B- and T-lymphocyte attenuator; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; PD-1 L, programmed death ligand 1; TLR, toll-like receptor.

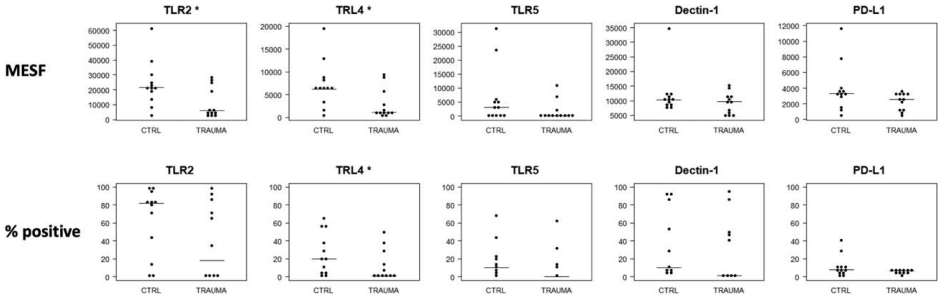

FIG. 2. **Expression analysis of specific receptors on monocytes.** Each data point represents an individual patient. Horizontal lines indicate median values. $P \leq 0.05$ is characterized by * next to the graph's upper labeling. CTRL indicates control; TLR, toll-like receptor; PD-1 L, programmed death ligand.

a reduced secretory capacity in the trauma cohort, statistical significance was not achieved (IL-6, trauma: 21.1 [CI 16.7–43.1], control 50.3 [CI 37.9–102.9], $P = 0.16$). Analogous effects were detected in TNF-α-secretion (TNF-α, trauma 2.4 [CI 1.7–3.4], control 3.8 [CI 2.9–5.2], $P = 0.09$). All other cytokines (IL-2, IL-4, IL8, IL-10, IL- 17A, and IFN-γ) did not show a significant difference between both study cohorts following LPS-stimulation (Fig. 3, supplement 2, http://links.lww.com/SHK/A869).

Neither α-CD3/28 nor Zymosan-stimulation resulted in an increased cytokine secretion at all (Fig. 4, supplement 2, http://links.lww.com/SHK/A869).

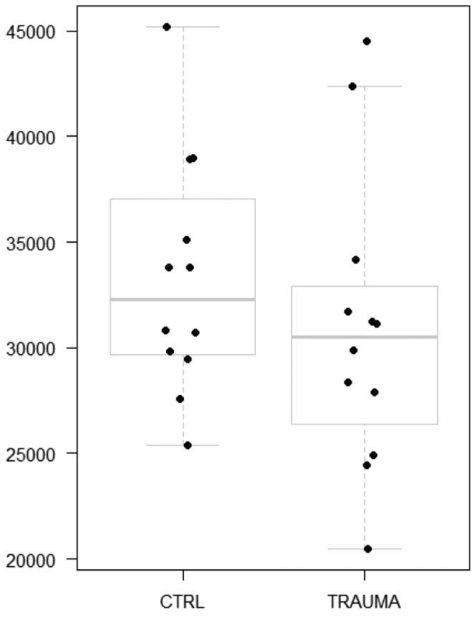

FIG. 3. **Quantification of human leukocyte antigen (HLA)-DR expression on monocytes.** Each data point represents an individual patient. Horizontal lines indicate median values. CTRL indicates control.

DISCUSSION

Major trauma leads to long-term impairment of general health status and life quality (13,14). Even though the initial in-hospital mortality has decreased over the last decades, the 3-year mortality is still elevated up to 16% following major trauma (3). Infectious-related causes of death range from 2.4% to 6% and might be explainable by long-term immunosuppression, based on the short to mid-term immune paralysis following severe trauma (2,15–17). Therefore, the aim of this explorative, cross-sectional study was to identify long-term alterations of lympho- and monocyte cell surface receptor expression and function as well as their association to clinically relevant infections. We identified 12 patients who suffered from major trauma 6 months prior to study inclusion and matched them for age and gender to 12 healthy controls.

Several studies addressed the short to mid-term effects of trauma-induced immune paralysis but to our knowledge, our study is the first to give a detailed description of long-term alterations of the lympho- and monocyte cell surface receptor expression (2,5,18–20). First, we were able to identify changes in lymphocyte surface receptor expression of the negative T cell regulators PD-1 and BTLA. While PD-1 receptor expression was up-regulated in $CD4^+$ T cells, the relative number of BTLA-receptors and its cell surface receptor expression was consistently down-regulated in $CD4^+$ and $CD8^+$ T cells. Lymphocyte PD-1 up-regulation is a known short-term consequence of sepsis and associated with increased mortality, but in trauma patients data remains inconsistent in trauma patients (21,22). Interestingly, in a study including former septic patients, we revealed inverse reactions of PD-1 and BTLA cell surface receptor expression in $CD4^+$ T cells, while $CD8^+$ T cells remained unaffected (12).

TLR-cell surface receptor expression of monocytes was consistently down-regulated, while PD-L1 and Dectin-1 were not affected. Especially, TLR-2 and -4 play a crucial role in recognition of DAMPs and PAMPs and activation of intracellular pro-inflammatory signaling pathways (e.g., activation of nuclear factor "kappa-light-chain-enhancer" of activated B cells), indicating a long-term impairment of immune reactions to gram-positive and gram-negative bacteria after trauma-induced inflammation (23,24). Comparable suppression of

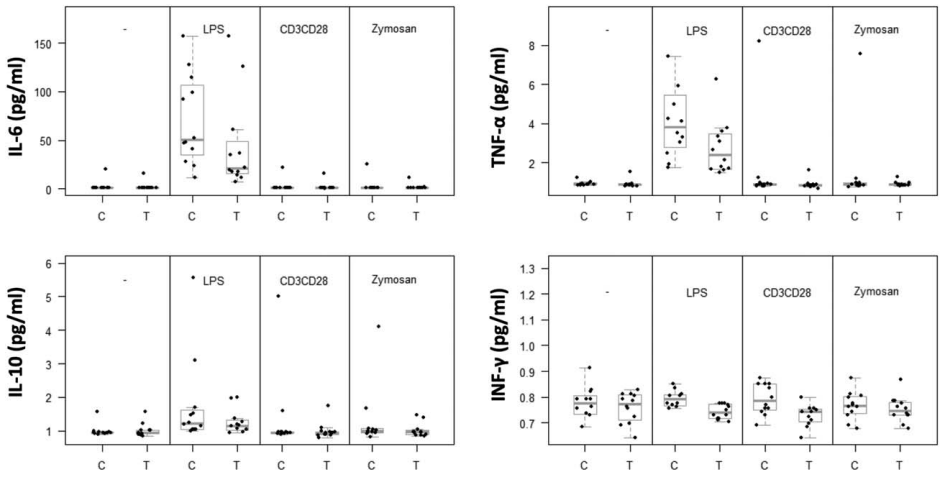

Fig. 4. **Quantification of cytokine secretion following whole blood *ex-vivo* stimulation.** Each data point represents an individual patient. Due to readability outliers have been excluded from graphical display (TNF-α: one patient (CTRL/non-stimulated > 15); one patient (CTRL/stimulated > 10), IL-10: one patient (CTRL/non-stimulated > 10)). Each first column shows results of unstimulated probe. Horizontal lines indicate median values. CD indicates cluster of differentiation; C, control; T, trauma.

TLR-2 and 4 cell surface receptor density on monocytes is known as short-term reactions of severe trauma, but to our knowledge this study is the first describing the same effects 6 months after major trauma (25). The alterations of monocyte TLR expression differ from findings in a cohort of septic patients, hinting toward different immune-modulatory mechanisms (12). Interestingly, suppression of HLA-DR is described in the early period following multiple trauma, but was not more suppressed after 6 months (25–27). This might be caused by its short life span of approximately 1 day for classical monocytes and 4 to 6 days for intermediate and non-classical monocytes (28). HLA-DR long-term convalescence also displays a possible reason for the lack of clinically apparent infections. PD-L1-detection did not reveal any changes in this study, which might be caused by its determination in peripheral blood since it is mainly expressed in organ tissue, e.g., alveolar cells (29).

Next to the description of changes in the immune cell receptor set, this study also aimed to identify possible functional impairments. Even though we investigated only 12 patients, LPS-induced cytokine secretion of IL-6 and TNF-α showed already strong trends toward a reduced secretory capacity in the trauma cohort. Induction with α-CD3/28 and zymosan did not result in changes of cytokine secretion. Especially, the reduced capacity of TNF-α secretion in combination with significant reduced TLR-2 and -4 receptor density on monocytes might lead to impaired immune competence regarding both gram-negative and gram-positive bacterial infections. However, these results were not associated with a clinical increase of clinical apparent infections in our cohort, which might be caused by the limited number of included patients.

There are some possible explanations for these findings. While naive T cells are long-living, several subsets of T cells (including $CD4^+$ and $CD8^+$ T cells) distinctly differ in their survival times (30). Therefore, other mechanisms might play a role in long-term changes of T cell and monocytes following major trauma. First, the early onset immune reactions include a peak of anti-inflammatory cytokines but not of pro-inflammatory cytokines, indicating a very early immunosuppressive effect (27). Analogous to the epigenetics of sepsis, these alterations might also cause long-ranged epigenetic changes of the innate and adaptive immune system after severe trauma (12,31). Long-term epigenetic modifications of the hematopoietic stem cells could also cause reduced cytokine secretion capacity. While acute epigenetic alterations of major trauma patients are described, data addressing long-lasting epigenetic alterations following severe trauma are rare and should be further studied (32,33). Second, immune cells might be influenced and paralyzed by a microenvironment as described by Roquilly et al. (34). Reduction of antigen presentation and cytokine secretion of dendritic cells and macrophages were identified in the lungs after resolved pneumonia, indicating a mechanism for long-term immunosuppressive effects after sepsis and trauma (34).

It is well known that severe trauma leads to a long-term increase of morbidity and mortality. However, the majority of long-term quality-of-life assessments of trauma patients aim to identify functional, socioeconomical, and psychological impairments and do not include infectious complications (3,4,13,35,36). Infections are only at high focus for the initial as well as for the short to mid-term phase after severe trauma during hospitalization and are related to a worse patient outcome (37–39). Especially, elderly patients seem to be at

increased risk for the development of a posttraumatic chronic critical illness syndrome, which is accompanied with an increased risk for infections. However, overall data of occurring infections of former trauma patients are lacking. Therefore, further studies investigating both the long-term infectious and immune status of trauma patients in large cohorts are necessary. The Reanimation Low Immune Status Markers (REALISM) project aims to identify alterations of the immune system over 2 months following severe conditions with a need for intensive care treatment, including severe trauma patients. This project started in December 2015 and might shed light into the short- to mid-term adaptive immune mechanism following multiple trauma (40).

Our study has some limitations. First, our findings imply only correlations and do not explain underlying mechanisms. Causative mechanisms for alterations of the T cell and monocyte receptor expressions, such as epigenetic or transcriptional changes between alterations of the T cell and monocyte receptor expressions, should be enlightened in further studies. Second, also caused by its explorative character and due to exclusion of two patients, the study size was too small to gain significant results in the experiments investigating cytokine secretion. Nevertheless, we were able to identify alterations of the receptor expressions of $CD4^+$ and $CD8^+$ T cells as well as monocytes and trends in decreased LPS-induced secretion of IL-6 and TNF-α in the trauma cohort. Third, even though we asked for a detailed medical history, we have no information of the initial immune set of T cells and monocytes. Last, we compared the former trauma patients with healthy volunteers and not with patients who survived a critical illness deriving from another reason (e.g., major abdominal surgery). Therefore, we are not able to differentiate between trauma- and critical-illness-induced alterations of the innate immune system. Lastly, we only included patients who were able to answer mail and phone calls. Therefore, we might have missed patients who were still hospitalized with infectious complications.

Overall, in context to the well-described chronic critical illness syndrome, our results contribute to a better understanding of the long-term reactions on T cells and monocytes following major trauma. Especially, the suppression of TLR-cell surface receptors might lead to a reduced innate immune competency to bacterial infections. In a former study, we were able to prove reduction of cytokine expression in former septic patients, which is explainable by epigenetic changes following severe sepsis (12,31,41). While these reactions were associated with an increase of clinical apparent infections in septic patients, former trauma patients did not suffer from more infections compared with their matched controls even though they showed still major impairments in daily routine and work.

CONCLUSIONS

Overall, we were able to detect alterations of receptor expression on $CD4^+$ and $CD8^+$ T cells as well as on $CD14^+$ monocytes. Furthermore, LPS-induced IL-6 and TNF-α release were reduced in former trauma, indicating a long-term immunosuppressive effect of major trauma. Due to the limited sample size and the corresponding low statistical power, we found no difference in clinically apparent infections. Future studies with higher patient numbers are needed to clarify the association as well as to identify the underlying causes and their clinical implications.

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