Improving the outcome of the critically ill with cortico- and sex-steroids employing clinico-transcriptomics approaches

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Summary

Sepsis and septic shock exhibit a broad spectrum of immunological disorders with a very heterogeneous clinical manifestation. The heterogeneity makes understanding pathomechanisms extremely difficult, implicating therapeutic failures and complicating the discovery of novel treatments.

To better understand the pathomechanisms the underlying heterogeneity is required to be dealt with proper stratification. With an aim to improve the outcome with corticosteroids and sex-steroids independently in critically ill patients, this study employed clinico-transcriptomics approaches that implemented stratification at either clinical or molecular levels followed by an investigation of pathomechanisms using high throughput transcriptomic analyses.

In septic shock patients, corticosteroids treatment is one of the crucial supportive therapies known to resolve the septic shock effectively. However, large-scale clinical trials point towards ambiguous conclusions on the effect of corticosteroids (mainly hydrocortisone) on the patient outcome. The critical task remains on how to identify the subset of patients that can potentially benefit from the treatment. Addressing this issue, in this thesis, a blood serum ratio of IFNγ and IL-10 was discovered as a promising new theranostic marker for the treatment of hydrocortisone, and it was validated on three further independent datasets. A high IFNγ/IL-10 ratio in patients indicated better recovery-cum-survival without hydrocortisone treatment while a benefit was observed for patients with low ratios when hydrocortisone treatment was initiated. The recovery was seen regarding reduced serum lactate and norepinephrine requirement levels over time. Furthermore, *ex vivo* blood culture experiments indicated that the ratio negatively associates with the pathogen load in spiked human blood in an 'on-off' manner. The pathomechanisms behind the recovery were then characterized using transcriptomics data. High ratios indicated the upregulation of activation signatures of macrophages and T-cells in the patients. Moreover, the immune recovery was observed when the hydrocortisone treatment was initiated in patients with low ratios.

Much like the role of corticosteroids, a lack of consensus exists on the role of sex-steroids during sepsis. Although modulation of sex-hormones has been proven effective in experimental sepsis models, their usage is far away from clinics, mostly because the literature lacks consensus on explaining the underlying sex-based pathomechanisms. The lack of stratification approaches is apparent, and the underlying heterogeneity seems to have contributed vastly to these studies. To address the issue, in this thesis a statistical framework for stratification of patient-severity was developed. Clinical and transcriptional data from a larger longitudinal study of critically ill patients after trauma was investigated. The

stratification enabled the identification of pre-acute, acute and post-acute phases of the organ-dysfunction. During each phase, the gene sets explaining the sex-based pathomechanisms in peripheral leukocytes were identified. The transcriptomic profiles revealed that after critical injury, pre-menopausal female and male patients exhibited a distinctively different transcriptomic response. Before the most severe day (i.e. the day with the highest Multiple-organ-failure score), female patients showed differential regulation for a stronger innate immune response, before and at the most severe day better bioenergetic tolerance and better oxidative damage resistance. They showed early upregulation of wound healing mechanisms and, after the most severe day, a distinct upregulation of the adaptive immune system. These observations were enabled due to the synchronization of patients' severity profiles before transcriptomic investigations.

The benefits in pre-menopausal female patients were reported mostly to be due to sex hormones. Animal studies conducted previously in the group have shown that sex-hormone modulation by treating with Atraric acid or Flutamide improves survival in male septic mice. These treatments are known to induce anti-androgenic and pro-estrogenic regulation. To decipher the underlying pathomechanisms, the transcriptomic response of the liver of septic mice to the sex-hormone modulation was studied. The transcriptomics of the liver showed a regulatory shift in septic male mice towards female upon treatment. Compared to septic males, liver of females showed the properties of tolerance in which protection from antigenic overload is gained by receptor desensitization, reduced immunoreactivity, and inhibition of cell death. The female-like response of the treatments largely mimicked the tolerance properties seen in females. The transcriptomics of female and female-like response of the treatments support a strong possibility that the survival advantage could be from enhanced tolerance as a defense strategy.

In summary, the clinico-transcriptomics approaches can serve as vital means in overcoming therapeutic failures and aiding the discovery of novel treatments for critically ill patients.

Zusammenfassung

Sepsis und die häufig damit zusammenhängende Folge der schweren Sepsis, sowie der septische Schock beschreiben ein breites Spektrum an Störungen des Immunsystems des Wirts, an der endogene Hormone maßgeblich beteiligt sind. Das Ziel dieser Arbeit war es deshalb, den Einfluss von Kortikosteroiden oder Sexualhormonen, besser zu verstehen und durch die Behandlung mit diesen Hormonen das Überleben der Patienten zu verbessern.

Die Kortikosteroidtherapie von Patienten mit septischem Schock ist eine wichtige supportive Maßnahme. Trotzdem zeigten diverse klinische Studien keinen eindeutigen Effekt von Kortikosteroiden auf das Überleben der Patienten. Die Herausforderung hierbei ist es, die Patientensubpopulation zu definieren, die von der Therapie profitiert. Die vorliegende Arbeit identifizierte den Quotienten der gemessenen Zytokine IFNγ und IL-10 aus Blutserum als neuen und vielversprechenden theranostischen Marker. Ein hohes IFNγ/IL-10-Verhältnis im Patientenblut korrelierte mit verbessertem Überleben, sofern diese nicht mit Kortikosteroiden behandelt wurden, während Patienten mit einem niedrigen IFNγ/IL-10-Quotienten von der Behandlung profitierten, was sich in beiden Fällen sowohl in den sinkenden Serum-Lakat-Werten als auch in der verringerten Norepinephrin-Bedarf widerspiegelte. Die zugrundeliegenden Pathomechnismen der besseren Genesung wurden mittels Trasnkriptomdaten charakerisiert und zeigten bei einem hohen Verhältnis eine Hochregulierung der Makrophagen- und T-Zell-Aktivierung in den Patienten. In Patienten mit niedrigem Quotienten wurde diese vorteilhafte Aktivierung der Immunzellen nur in den Patienten beobachtet, die mit Hydrokortison behandelt wurden.

Ebenso kontrovers wie die Behandlung mit Kortikosteroiden wird die Rolle von Sexualhormonen während der Sepsis diskutiert. Obwohl die Modulation von Sexualhormonen in Sepsis-Tiermodellen einen positiven Effekt zeigte, liegt deren klinische Anwendung noch in weiter Ferne, vor allem da der zugrundeliegende geschlechtsspezifische Pathomechanismus und dessen Beeinflussung bisher nicht aufgeklärt ist. Hierfür wurden klinische Parameter und Transkriptomdaten einer großen longitudinalen Studie schwerkranker Traumapatienten untersucht. Die eigens dafür in dieser Arbeit entwickelte zeitliche Stratifizierung erlaubte die Identifikation markanter differenzieller Pathways in der prä-akuten, akuten und post-akuten Phase der Organdysfunktion, im Vergleich prä-menopausaler Frauen und Männer. Vor dem schwerwiegendsten Krankheitstag (Tag mit dem höchsten Wert für multiples Organversagen, MOF-Score) wiesen die Frauen eine höhere Expression des angeborenen Immunsystems auf und vor dem und am schwerwiegendsten Krankheitstag außerdem eine höhere Expression von Genen für bioenergetische

Toleranz und oxidative Stressresistenz. Des Weiteren zeigten Frauen eine frühe Hochregulation von Wundheilungsmechansimen und nach dem schwerwiegendsten Krankheitstag eine differenzielle Erhöhung des adaptiven Immunsystems.

Veröffentlichungen verknüpfen den vorteilhaften Verlauf bei prä-menopausalen Frauen meistens mit den Sexualhormonen. Tierstudien bestätigten die Hypothese der Modulation von Sexualhormonen zur Überlebensverbesserung männlicher septischer Mäuse durch eine Kombination aus antiandrogener und proöstrogener Behandlung aufgrund der Gabe von Atrarsäure oder Flutamid. Zur Untersuchung des Pathomechanismus wurden die Transkriptomdaten der Leber dieser septischen Mäuse verglichen. Die Transkriptomprofile der behandelten männlichen Mäuse zeigten eine Verschiebung zu den weiblichen Transkriptomprofilen im Vergleich zu den unbehandelten Männchen. Die Weibchen zeigten eine erhöhte Toleranz, die durch den Schutz vor Antigenüberlastung mittels Rezeptordesensitisierung erreicht wird, eine geringere Immunreaktivierung und geringeren Zelltod. Die Weibchen-ähnlichen Transkriptomprofile der behandelten Männchen wiesen in weiten Teilen eben diese Toleranz-Anzeichen auf. Diese Modulation könnte das Überleben der septischen männlichen Mäuse aufgrund einer daraus resultierenden erhöhten Toleranz als Verteidigungsmechanismus verbessert haben.

Zusammenfassend ergaben sich ein neuer Ansatz zur Entscheidungsunterstützung von Kortikosteroidgabe bei septischem Schock und die Identifizierung entscheidender Unterschiede der Regulationsmuster von Männern und Frauen bzw. männlichen und weiblichen Mäusen in Immunzellen von Sepsis Patienten und Leberzellen in septischen Mäusen. Diese Ergebnisse werden dazu beitragen, Fehltherapien zu verhindern und neue Behandlungen schwerkranker Patienten zu finden.

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List of Abbreviations

AA	Atraric acid
ACCP/SCCM	American college of chest physicians/Society of critical care medicine
ACTH	Adrenocorticotropic hormone
ADRENAL	Adjunctive corticosteroid treatment in critically ill patients with septic shock
AIS	Abbreviated injury scale
ANOVA	Analysis of variance
APROCCHSS	Activated protein-C and corticosteroids for human septic shock
AR	Androgen receptor
ARR	Absolute risk reduction
ATP	Adenosine triphosphate
CARS	Compensatory anti-inflammatory response syndrome
CLP	Cecal ligation and puncture
CORTICUS	Corticosteroid therapy of septic shock
DGE	Differential gene expression
DHT	5a-dihydrotestosterone
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FDA	Food and drug administration
FDR	False discovery rate
Flu	Flutamide
GEO	Gene expression omnibus
GR	Glucocorticoids receptor
HC	Hydrocortisone
HGNC	HUGO gene nomenclature committee
HMOX-1	Heme oxygenase 1
HP	Haptoglobin
HSSG	Hellenic sepsis study group

HUGO	Human genome organisation
ICU	Intensive care unit
IFNg	Interferon-gamma
IL	Interleukin
ISS	Injury severity score
LDA	Linear discriminant analysis
LOD	Log of odds
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MAQC	Microarray quality control study
MARS	Mixed anti-inflammatory response syndrome
MHC	Major histocompatibility complex
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
OR	Odds ratio
OxPhos	Oxidative phosphorylation
PC	Principal component
PCA	Principal component analysis
PCI	Polymicrobial contamination and infection
PDGF	Platelet-derived growth factor
PL	Placebo
RMA	Robust multi-array average
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SISPCT	Sodium selenite and procalcitonin guided antimicrobial therapy in severe sepsis
SOFA	Sequential organ failure assessment
TBSA	Total body surface area
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VASST	Vasopressin and septic shock trial

Chapter 1

Introduction

1.1 Sepsis

Sepsis is one of the oldest and most complex diseases. Hippocrates in his journals described sepsis as a process by which flesh rots, swamps generate foul airs and wounds fester [Majno, 1991]. Galen later considered sepsis a worthy and necessary step for wound healing [Funk et al., 2009]. With the confirmation of germ theory by Bassi, Pasteur, and others, sepsis was redefined as a systemic infection, often described as 'blood poisoning', and assumed to be the consequence of the host's invasion by pathogens that then spread in the bloodstream [Majno, 1991, Funk et al., 2009]. All these paradigms were not adequate since sepsis patients died despite successful clearance of the initiating pathogen. Work over the past three decades has revealed sepsis as a clinical syndrome or disease continuum of organ dysfunction [Gotts and Matthay, 2016]. In addition to its initiation by pathogens, the host response to sepsis is more focused, considering that this drives the pathogenesis [Marshall, 2014].

Our understanding of the pathophysiology of sepsis has evolved substantially over the last three decades [Gotts and Matthay, 2016]. For many years, the inflammatory dynamics of sepsis have been incompletely explored. Early septic deaths were presumed to be a consequence of exaggerated host's proinflammatory immune response [Bone, 1992]. Excess release of systemic cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-1 (IL-1) was well-documented in the septic human response and animal models. These findings drove the dominant concept of sepsis as a hyperinflammatory state and consequently resulted in many unsuccessful anti-inflammatory studies [Angus, 2011]. Later a multimodal hypothesis of sepsis was proposed in which an initial systemic inflammatory response syndrome (SIRS) in sepsis was believed to be a temporary

phenomenon followed by a compensatory anti-inflammatory response syndrome (CARS) often increasing the risk of secondary infections and other adverse outcomes [Hotchkiss et al., 2016]. Subsequently, simultaneous production of circulating proinflammatory and anti-inflammatory cytokines has been demonstrated in a model of polymicrobial sepsis, supporting that a continuously, highly mixed anti-inflammatory response syndrome (MARS) is present [Osuchowski et al., 2012]. Patient studies showed similar results and demonstrated that both classes of cytokines have an integral role in sepsis from the onset and onwards [Novotny et al., 2012]. Inadequate understanding of the pathophysiology has created fundamental problems in tackling it and its related complications that ultimately are leading to death.

Today, sepsis and septic shock are the most common causes of death among critically ill patients in non-coronary intensive care units [Hotchkiss et al., 2016]. In Germany, an approximate hospitality death rate for severe sepsis was reported to be 55% [Engel et al., 2007]. Case numbers of severe sepsis exceed 750,000 per year and were considered to rise steadily in the United States [Angus et al., 2001, Lagu et al., 2012]. A recent estimation made in 2015 for the worldwide population was more than 31 million sepsis and 19 million severe sepsis cases per year [Fleischmann et al., 2016]. Several factors contribute to sepsis susceptibility and outcome such as the initial site of infection, causative microbe, the pattern of acute organ dysfunction, co-morbidities of the patient and the amount of delay in initiation of antimicrobials [Angus and van der Poll, 2013]. For example, hourly delays in the first appropriate antimicrobial administration were associated with higher mortality [Weiss et al., 2014]. Unfortunately, identifying the infection imposes hurdles on detecting if the patient has sepsis. Blood cultures, which is the standard diagnostic method for microbe detection, are typically negative in more than half of the severe sepsis cases [Dellinger et al., 2008].

Besides the recognition of sepsis, its treatment is the primary challenge for clinicians. Despite many years of intensive research 'sepsis drug' is yet to be found. Therefore, sepsis was called as 'graveyard for pharmaceutical companies' [Riedemann et al., 2003]. The seemingly promising therapy with recombinant activated protein C (drotrecogin alpha) was unfortunately removed from the market in 2011 since it increased severe bleeding and ultimately did not decrease mortality [Martí-Carvajal et al., 2007]. Activated protein C has pleiotropic biological effects on the inflammatory response and coagulation, and it was shown to be effective in the preclinical animal models of sepsis in reducing organ damage and mortality

[Martí-Carvajal et al., 2007]. Apart from this trial, there have been more than 100 randomized clinical trials testing strategies to modify the septic response to improve survival but failed. These strategies included nonspecific suppression of inflammation using corticosteroids or ibuprofen; the specific blockade of microbial products such as endotoxin or host inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1 (IL-1), toll-like receptor 4 (TLR4), platelet-activating factor, and nitric oxide; nonspecific targeting of inflammatory mediators using polyclonal immunoglobulins; the administration of immunity-enhancing protein, including granulocyte colony-stimulating factor (G-CSF) and interferon gamma (IFN-γ); and the administration of anticoagulant molecules such as tissue factor pathway inhibitor (TFPI), anti-tissue factor antibodies, antithrombin, and thrombomodulin [Marshall, 2014].

Due to the significant impact of sepsis on global health, several initiatives were taken to improve the survival of patients with sepsis. One such initiative is the Surviving Sepsis Campaign (SSC) with an objective of reducing mortality from sepsis by 25% [Dellinger et al., 2008]. Several strategies have been implemented to achieve this goal, such as early goal-directed therapy towards infection, early use of broad-spectrum antibiotics, support for failing organs and low tidal volume ventilation. Although the sepsis mortality rate has subsequently decreased, the SSC goal is far from being achieved [Dellinger et al., 2013]. Even though there is no common effective therapy due to high variability among sepsis patients, general predispositions exist and, these include age (higher in newborns and elderly), ethnic background (higher in blacks compared to whites) and gender (higher in men compared to women) [Angus et al., 2001, Mayr et al., 2010].

1.1.1 Gender dimorphism in sepsis

In a large study observing 681,730 trauma patients, female patients showed significantly fewer complications and 21% lower death rate compared to male patients, despite the same average of injury severity scores (ISS) [Haider et al., 2009]. A recent meta-analysis involving 19 studies with 140,328 trauma patients found less mortality, shorter duration of hospitalization, and fewer complications in female patients [Liu et al., 2015]. Consistent with these findings, male gender was associated with a higher risk of major infections and Multiple-Organ-Failure (MOF) following trauma [Offner et al., 1999, Gannon et al., 2004, George et al., 2003]. Mostafa *et al.* [Mostafa et al., 2002], Deitch *et al.* [Deitch et al., 2007] and Trentzsch *et al.* [Trentzsch et al., 2014, Trentzsch et al., 2015] observed that premenopausal female patients are

better protected from MOF and sepsis after critical trauma. In particular, Trentzsch *et al.* analyzed 3,887 matched-pairs of male and female severe-trauma patients and observed that premenopausal females develop significantly less MOF despite matching injury, i.e. a matched Abbreviated Injury Scale score (for the thorax, head, abdomen, extremities), age and co-morbidities [Trentzsch et al., 2015].

It was also reported that female patients respond better to supportive treatments. In a study observing a cohort of 4,106 trauma patients, premenopausal women required less blood transfusion and showed lower serum lactate levels despite more severe injuries [Deitch et al., 2007]. In another, prospective clinical study, female patients required lower resuscitation volumes (12 L vs 8 L), less inotrope and vasopressor support (36% vs 10%) and less intervention (42% vs 15%) based on the Starling curve to maintain oxygen delivery in the heart compared to similarly injured male patients. The authors concluded that female patients responded better to standardized resuscitation compared to male patients [McKinley et al., 2002].

Besides these clinical differences, male and female individuals differ fundamentally in regulating immune response and metabolism. Female individuals elicit a more robust innate and cell-mediated immune response, making them less prone to certain infections, like hepatitis-B, tuberculosis, leptospirosis and more [Klein and Flanagan, 2016]. They develop a higher antibody response to vaccination providing a better anti-infection response; however, they are more prone to autoimmune diseases [Klein and Flanagan, 2016]. Regarding metabolic differences, studies have shown that energy metabolism differs between healthy male and female individuals. Female individuals oxidize more lipids preferably over carbohydrates, and they utilize less glycogen from skeletal muscles thereby producing less hepatic glucose [Tarnopolsky and Ruby, 2001]. Notably, muscle cells of female individuals have a significantly lower capacity for anaerobic glycolysis and aerobic oxidation [Green et al., 1984].

Critical trauma-hemorrhage can lead to immune dysfunction and metabolic derangements. It is often followed by the onset of sepsis. The hyper inflammation during sepsis leads to tissue damage, which, in turn, evokes the concomitant release of pro- and anti-inflammatory cytokines, but can also suppress a variety of cell-mediated immune responses leading to immunosuppression [Xiao et al., 2011]. Trauma-hemorrhage is also known to trigger hyperglycemia, increased fatty acid oxidation, and decreased ATP production resulting from mitochondrial dysfunction [Marik and Raghavan, 2004, Singer, 2014]. In summary, the immune and metabolic responses are severely altered after trauma-hemorrhage. Hence, studying the implications of gender is crucial to understand the underlying pathomechanisms of

trauma-hemorrhage.

1.1.2 Therapeutic potential of sex-steroids

Experimental studies in septic animal models have demonstrated the modulation of immune responses by sex-steroids. Overall, testosterone, the primary male sex hormone appears to have anti-inflammatory and immunosuppressive effects. It promotes the synthesis of anti-inflammatory cytokines such as IL-10 by macrophages [D'Agostino et al., 1999], reducing NK cell activity and the synthesis of pro-inflammatory cytokines, such as TNF-α, via inhibition of nuclear factor kappa B (NFκB) [Hou and Zheng, 1988, McKay and Cidlowski, 1999]. Testosterone has also been associated with the decreased expression of toll-like receptor 4 (TLR4) in macrophages which is involved in the activation of the innate immune system and production of inflammatory cytokines [Rettew et al., 2008].

In contrast, the dominant female hormone, estrogen appears to enhance cell-mediated and humoral immune responses. It accelerates NK cell cytotoxicity, as well as stimulates the production of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β [Miller and Hunt, 1996, Sorachi et al., 1993] and inhibits the synthesis of anti-inflammatory cytokines such as IL-10 [Kahlke et al., 2000]. Also, estrogens have been shown to increase survival and prevent apoptosis of immune cells [Straub, 2007, Vegeto et al., 1999]. Elevated systemic levels of estradiol in pre-estrus female mice played a pivotal role in post-trauma and hemorrhage immunocompetence [Knöferl et al., 2002]. Furthermore, administration of 17β-estradiol (E2) was associated with marginal improvement in the survival rates in male septic mice [Knöferl et al., 2000]. A single dose of estradiol following trauma-hemorrhage and resuscitation was shown to restore depressed immune responses [Knöferl et al., 2001].

Several murine studies have shown depressed immune responses in males as well as ovariectomized females following trauma-hemorrhage and sepsis [Ananthakrishnan et al., 2005, Angele et al., 2000]. Interestingly, pretreatment of female mice with 5a-dihydrotestosterone (DHT) prior to trauma-hemorrhage resulted in depressed macrophage function and cell-mediated immune responses [Angele et al., 1998]. Moreover, castration and depletion of male sex hormones prior to trauma-hemorrhage resulted in enhanced immune responses [Angele et al., 2001, Wichmann et al., 1996]. Furthermore, castrated male animals treated with DHT resurfaced the depressed cell-mediated immune responses, whereas additional administration of estradiol prevented the depression of immune responses [Angele

et al., 1999, Angele et al., 2001].

To clarify whether testosterone by itself is responsible for the depression of cell-mediated immune responses in male mice, studies were conducted in which female mice were pretreated with DHT for two weeks before the induction of trauma-hemorrhage [Angele et al., 1998]. The results showed that female mice that had artificially elevated plasma testosterone levels (comparable to males) displayed similar depression of splenic and peritoneal macrophage function as in males [Angele et al., 1998]. Furthermore, pretreatment of female mice with DHT suppressed the release of Th1 lymphokines, IL-2 and IFN-γ by splenocytes following trauma-hemorrhage to levels comparable to healthy male animals [Angele et al., 1998].

Integrating the lessons from male and female sex-hormone modulation experiments, in males, the simultaneous suppression of male hormonal response and activation of female hormonal response has been reported to significantly increase survival following the induction of severe polymicrobial sepsis (PCI) [Röll, 2015]. Pre-estrus females showed significantly increased survival while all septic male mice died within 28 hours post-PCI induction. A clinically used prostate cancer agent, Flutamide (Flu) and the novel natural compound Atraric acid (AA) extracted from the fruit of the plant *Sabal serrulate* were used independently to treat males at time points 0, 4, 8, and 16 hours following induction of polymicrobial sepsis. The results displayed a significant increase in the rate of survival for up to 20-25% (Figure 1.1 adapted from [Röll, 2015]). Flu and AA are androgen receptor (AR) antagonists; moreover, Flu is an indirect activator of estrogen receptor (ER) through the induction of aromatase enzyme, while AA is a more potent activator of ER [Röll, 2015]. These findings indicated that the simultaneous inhibition of AR and the activation of ER could induce stronger femalization in male mice upon treatment, following the polymicrobial sepsis. To determine the supportive cause of enhanced survival, clinical severity scores and cytokine levels (IL-6, IL-10, TNF-α, MCP-1, IFN-γ, IL-12p70) were measured in blood serum and in liver tissue at 10 hours post-PCI, together with liver function parameters (Lactate dehydrogenase, Creatinine, Aspartate transaminase, Bilirubin). However, no significant tendency was found associated with either the treated group or with the females. Perhaps, transcriptomic investigations at this time-point may identify the possible regulatory mechanisms.

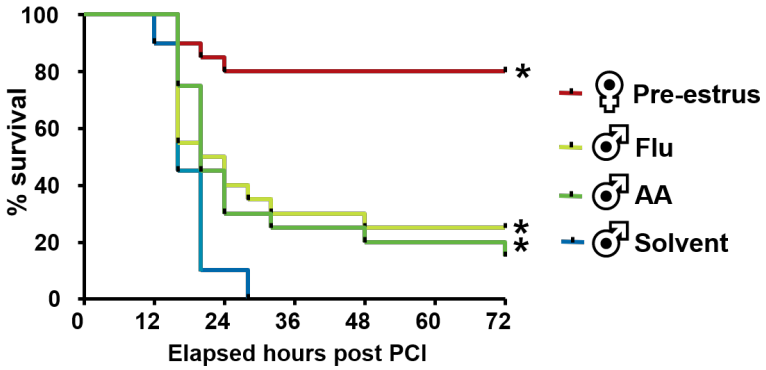

Figure 1.1: For induction of sepsis a 100% lethal doses (2 μl/g body weight) of a pre-adjusted (referred to male mouse survival) and microbiologically characterized human feces batch was injected intraperitoneally. Treatment with the indicated compounds or solvent (0.5% TWEEN80 in 0.9% NaCl) for female mice and male solvent control group at time points 0, 4, 8 and 16 hours post-PCI induction was performed. Kaplan-Meyer survival curve and statistical analyses (Mantel-Cox test) were performed with GraphPad PRISM5 and asterisks (*) indicate significant changes compared to male solvent control (dark blue) with a p-value <0.05. The curves demonstrate a total animal number of n=20 (except for solvent group n=10) whereat experiment was performed twice for each group with n=10. Pre-estrus females (red curve) survive sepsis far better compared to males (dark blue curve) (p=0.0002). Flu treatment (light green curve) or AA treatment (dark green curve) led to significant enhanced septic male survival compared to solvent treated male group (dark blue curve) (pFlu=0.032; pAA=0.021). Source [Röll, 2015]

1.2 Septic shock

A deadlier sequelae of sepsis is septic shock. The current sepsis definitions (i.e. Sepsis-3) describe septic shock as a subset of sepsis in which underlying circulatory and cellular metabolic abnormalities are profound enough to increase mortality substantially [Singer et al., 2016]. Patients with septic shock can be identified with persisting hypotension requiring vasopressors to maintain mean arterial pressure (MAP) $\geq$65 mmHg and having a serum lactate level >2 mmol/L (18 mg/dL) despite adequate volume resuscitation [Singer et al., 2016]. Norepinephrine is favored as the first-line vasopressor for septic shock in the Surviving Sepsis Guidelines [Dellinger et al., 2013]. It increases mean arterial pressure primarily through vasoconstriction, with little effect on heart rate, stroke volume, and cardiac output [Dellinger et al., 2013]. Serum lactate is produced by anaerobic respiration mainly because of the insufficient circulation during septic shock. Both, serum lactate level and the norepinephrine requirement reflect the shock severity in patients and are used for monitoring patients [Dellinger et al., 2013].

Early supportive therapy with fluid resuscitation and vasopressors to restore hemodynamics and reduce tissue hypoxia is decisive for the patient's outcome

[Dellinger et al., 2013]. However, mortality rates for septic shock may reach 60% even with early recognition and treatment [Fleischmann et al., 2016]. The limited improvement in septic shock survival can be explained by the inability to prospectively identify the patient subset that is most likely to benefit or be harmed from a specific therapy. We are becoming increasingly aware that the response to therapy is crucial and precision medicine is already an important research topic for acute illnesses and septic shock [Wong et al., 2015]. Using a digital messenger RNA quantification platform that can generate gene expression data in about 8–12 hours; Wong *et al.* could subclassify children with septic shock based on a 100-gene gene expression signature into subclasses that have clinically relevant phenotypes [Wong et al., 2015]. The class-defining genes corresponded to adaptive immunity and glucocorticoid receptor signaling, thus raising the possibility of a theranostic approach to pediatric septic shock.

Therapy options for septic shock patients are very limited. Among them, treatment with corticosteroids is one of the promising supportive therapy, known to effectively resolve the septic shock, while some patients were reported to develop adverse secondary infections [Sprung et al., 2008]. More research is required for identifying the patient subsets that are most likely to benefit or can get harmed from corticosteroids therapy during septic shock.

1.2.1 Therapeutic role of corticosteroids

In septic shock patients, treatment with corticosteroids, mainly hydrocortisone (HC) has been consistently reported to help in faster shock resolution [Annane et al., 2009, Venkatesh et al., 2018], whereas a survival benefit with HC treatment was demonstrated only in two French randomized controlled multicenter trials when administered together with fludrocortisone [Annane et al., 2002, Annane et al., 2018]. Conflicting results regarding the mortality endpoint were obtained in two other multinational trials, ADRENAL [Venkatesh et al., 2018] and CORTICUS [Sprung et al., 2008]. Possible explanations for this disparity included differences in the patient population with a two-fold higher risk of mortality in the French control group (61%) compared to CORTICUS (31%), and an increase in secondary infections, including new episodes of sepsis or septic shock recorded with corticosteroid application in the CORTICUS trial. The concern about the side effects of corticosteroids such as infections in patients with less severe septic shock stipulated more restrictive recommendations by the Surviving Sepsis Campaign. HC application is currently recommended for severe courses if patients do not respond

to adequate vasopressor therapy and fluid resuscitation [Dellinger et al., 2008, Dellinger et al., 2013]. To evaluate the effects of hydrocortisone on the balance between pro- and anti-inflammation, a randomized controlled study ("Crossover" study) was performed investigating immune effects of a three-day treatment in patients with septic shock followed by three days placebo, or vice versa (HC and placebo were "crossed over") [Keh et al., 2003].

The crossover study demonstrated that HC restored hemodynamic stability and modulated the immune response to stress by means of antiinflammation rather than immunosuppression [Keh et al., 2003]. Another, larger randomized trial (VASST) [Russell et al., 2008] hypothesized that low-dose vasopressin improves the therapy of septic shock patients compared to norepinephrine (conventional catecholamine). As the main result, low-dose vasopressin did not reduce 28-days mortality rate. Notably, in the studied cohort, about 75% of patients were treated with corticosteroids in addition to vasopressin or norepinephrine. Bentzer *et al.* followed up investigating corticosteroid-treated versus non-treated (only vasopressin or catecholamine vasopressors) patients. They identified a signature of three cytokines (Interleukin-3, Interleukin-6 and C-C-motif-chemokine-4) suggesting a response to corticosteroid treatment [Bentzer et al., 2016]. However, these results were based on a study which was not randomized, blinded or protocolized according to corticosteroids treatment, nor the signature was validated on another independent dataset. Further, Bentzer *et al.* neither distinguished between vasopressor and norepinephrine treatment, nor elaborated on how these three cytokines interact with corticosteroid treatment.

Corticosteroids are widely known for their immune suppressing effects [Coutinho and Chapman, 2011, Barnes, 2011], mediated by the glucocorticosteroid receptor (GR) which represses the pro-inflammatory acting transcription factors like AP-1 or NFκB [Baschant et al., 2013]. Nevertheless, increasing evidence indicates corticosteroids and GR to be involved in the activation of pro-inflammatory processes. Indeed, corticosteroid treatment was shown to induce the expression of innate immune-related genes, like TLRs in human mononuclear cells as well as anti-inflammatory genes, displaying the pivotal role of corticosteroids [Chinenov and Rogatsky, 2007, Galon et al., 2002]. In primary macrophages, corticosteroids induced a central component of the inflammasome resulting in secretion of pro-inflammatory cytokines such as interleukin-6 [Busillo et al., 2011]. The immune activating role of corticosteroids has been described as a response to acute stress enhancing the peripheral immune response, whereas chronic corticosteroid exposure

leads to immune-suppression [Dhabhar, 2002, Cruz-Topete and Cidlowski, 2015]. Hence the context and the biotype identification of patients is required for supporting the HC treatment decision of patients with septic shock.

1.3 Clinico-transcriptomics approaches

Transcriptomic analyses have been successfully used for diagnostics, identifying novel virulence factors, predicting antibiotic resistance and studying host-pathogen interactions [Lowe et al., 2017, Leonor Fernandes Saraiva et al., 2017]. It has also been used to investigate the gender dimorphism in the gene regulatory response to trauma [van Vught et al., 2017, Lopez et al., 2016]. Vaught *et al.* compared gene expression profiles of septic and healthy male and septic and healthy female individuals. Blood of septic patients was drawn at the day of admission. They identified that ERK and MAPK signaling, leukocyte extravasation signaling, PDGF signaling, and ephrin receptor signaling are specifically upregulated in males but not in females [van Vught et al., 2017]. However, this indirect way of finding male and female-specific gene sets by comparing septic versus healthy individuals of male and female patients separately may be sensitive to significance cutoffs and low expressed genes in healthy individuals. Another transcriptomic study by comparing transcription profiles of patients from 12 hours to 28 days after trauma, identified sex-specific differences being related to lymphocyte regulation, response to TGF-β stimulus, ubiquitin-dependent protein catabolic processes, and protein/macromolecule catabolic processing [Lopez et al., 2016]. Notably, the authors compared the data from all time points together to identify differentially expressed genes between male and female patients. The temporal progression of the disease was not accounted while identifying these differences. These two studies reported gender-dimorphism on the transcriptional level. However, they showed very different results which may be due to neglecting the individual temporal progression of the disease. This implies that transcriptomic investigations without profound stratification can lead to non-reproducible observations.

The stratification can be achieved by implementing established scoring systems such as Marshalls' Multiple Organ Dysfunction Score (MODS) and Sequential Organ Failure Assessment (SOFA) score that are repetitive and designed for daily bedside evaluation [Marshall et al., 1995, Vincent et al., 1996]. The scoring is based on six different scores, one each for the respiratory, cardiovascular, renal, hepatic, hematological and neurological systems. A high score correlates with increased

hospital mortality [Marshall et al., 1995, Vincent et al., 1996]. The timely score also reflects the disease status of the patients such as pre-acute, acute and post-acute phases of the disease. The patients can also be stratified based on their longitudinal severity profiles. Apart from organ dysfunction scores, variables of predisposition (e.g. age), insult (e.g. blunt/penetrating trauma, infection) and response (e.g. high/low biomarker levels, gene expression pattern, sepsis/septic shock) can be used in the stratification model [Marshall, 2014]. The approach of stratification coupled with transcriptomics is crucial in improving the reproducibility and interpretability of the findings.

An example of a clinico-transcriptomics approach was demonstrated by Rittirsch *et al.* [Rittirsch et al., 2016]. They aimed at investigating mechanisms linked to infection-related complications and sepsis-associated pathways after trauma. Infection-related complications were clinically defined by the presence or the absence of nosocomial infections. Using transcriptome analysis, the authors showed that the genes of the heme-degradation pathway such as HP (Haptoglobin) and HMOX1 (heme oxygenase-1) were significantly upregulated in patients with sepsis as compared to patients with systemic inflammation without infection. Further, the correlation with the clinical data revealed that patients who received allogeneic blood transfusions had a higher incidence of nosocomial infections and sepsis. The higher blood transfusion contributed to free heme which triggered higher expression levels of genes from the heme-degradation pathway.

With the focus on identifying molecular responses associated with longer-term post-injury complications in trauma patients, Desai *et al.* [Desai et al., 2011] also implemented a clinico-transcriptomics approach in their study. They utilized the MOF clinical score trajectories to cluster the patients into five clinical categories of increasingly poor outcome. Later, for each gene, fold change in the gene expression was calculated per hour, per patient. Fold changes for each gene were then correlated with the five clinical categories to identify significantly associated genes. In particular, early downregulation of MHC-class II genes and upregulation of the p38 MAPK signaling pathway were found to be strongly associated with longer-term post-injury complications.

In summary, the combination of clinical and transcriptomic markers improves the translational impact of molecular data and it represents a useful means for individual risk stratification in trauma and sepsis patients.

Chapter 2

Objectives

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. It manifests in complex forms, and the lack of proper understanding further adds to a larger-scale failure in maximizing benefits from existing therapies and the discovery of novel effective treatments.

This thesis deals with sepsis and its sequelae to address three objectives: one associated with the use of corticosteroids and the other two related to the sex-steroids.

The application of corticosteroids particularly hydrocortisone in septic shock patients is controversial. A subset of patients is reported to benefit in terms of shock resolution and increased survival from the treatment while another is reported to develop adverse effects including superinfections. The objective of this section was to identify a theranostic marker that can guide the application of hydrocortisone in septic shock patients in such a way that the benefits will significantly outweigh the risks. If such a marker exists, then investigate the associated molecular mechanisms to improve the understanding of the marker further.

The biological sex and sex-steroids in patients shape the basis for the underlying immune and metabolic responses. However, previous studies exploring transcriptomic sex differences show only a minimal agreement among each other, possibly due to neglecting the temporal progression and the synchronization of organ dysfunction severities over time. To address this issue, the objective was to investigate a time series of transcription profiles of critically ill patients after trauma and develop a statistical framework to synchronize these time series by considering the severity of each patient. The goal was also to describe the evolving temporal transcriptional regulation in peripheral immune cells of critically ill patients after trauma to explain the better physiological response in premenopausal female patients.

The third objective of the thesis was concerning the modulation of sex-hormones

during sepsis. The concomitant anti-androgenic and pro-estrogenic effects induced by Flutamide (Flu) and Atraric acid (AA) independently are shown to improve survival in male mice after polymicrobial sepsis. The goal of this study was to elucidate the extent of femalization introduced by the treatment at the transcriptomic level and identify the beneficial alterations explaining the survival advantages observed in male mice after polymicrobial sepsis.

Chapter 3

Materials and methods

3.1 The corticosteroids study

3.1.1 The CORTICUS cohort

The data for this study was collected by the CORTICUS Berlin study group who, in addition to the standard CORTICUS protocol, sampled blood for subsequent measurement of cytokines and other inflammatory mediators from 84 patients in 13 participating sites. The study was approved by the local ethics committee (ethics number 153/2001). Written informed consent was obtained from patients, proxies or their legal representatives. Eligible patients were enrolled if they met the following inclusion criteria: evidence of a systemic response to infection, clinical evidence of infection, and the onset of shock within the previous 72 hours and hypoperfusion or organ dysfunction attributable to sepsis. Major exclusion criteria included an underlying disease with a poor prognosis, life expectancy less than 24 hours, immunosuppression, and treatment with long-term corticosteroids within the past 6 months or short-term corticosteroids within the past 4 weeks. Detailed eligibility criteria is given in Appendix Tables A.1, A.2 and the original study [Sprung et al., 2008]. Patients were randomized (in a 1:1 ratio) to receive either a 50 mg intravenous bolus of hydrocortisone (HC) every 6 hours for 5 days, followed by a tapered dose of 50 mg HC every 12 hours until day 8, and then 50 mg HC once daily until day 11, or placebo. Data for baseline characteristics (before onset of the study) were extracted from the CORTICUS database. 79 out of 83 (95%) patients received norepinephrine at baseline.

Blood samples and serum assays of the CORTICUS Berlin sub-cohort

Blood samples were collected on day 0 (before the ACTH test and HC/placebo treatment), on day 2, on the morning of day 5 (end of full dose HC application), on day 12 (day after HC cessation), and on days 17 and 27. The short corticotropin test was performed immediately before HC/placebo application using blood samples taken before and 60 minutes after an intravenous bolus of 0.25 mg cosyntropin (Novartis). Blood samples were stored at 4°C for three hours to avoid time imbalances between blood collection at different sites and further processing. Serum and plasma were stored at -80°C until further analysis. Heparinized and EDTA whole blood samples were used for functional assays. At the time of the CORTICUS study, soluble mediators, interleukin-(IL)-6, 8, 10, 12 p70, interferon-γ (IFNγ), tumor necrosis factor alpha (TNF-α), soluble FAS, soluble TNF-receptor I (sTNF-RI) (all BD Biosciences OptEIA^{TM} Set Human), and E-selectin (R&D systems) were measured in serum, plasma, or culture supernatant with enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. All measurements were done in duplicate. For the cytokines, EDTA plasma was used. Hydrocortisol was measured from plasma. Surface antigens of leukocytes were measured using flow cytometry, leukocytes in EDTA, thrombocytes in citrate plasma (platelet enriched), caspase/BCL2 leukocytes with heparin. Serum lactate was measured by routine blood gas analytics. Serum lactate was measured for 51 patients at day 0. Among these, 41 patients were further observed daily for 3 days. All the blood and serum assays described above are performed by the CORTICUS Berlin study group.

Calibration of the IFNγ/IL-10 threshold

The validation cohorts used in this analysis had different death rates. The HSSG cohort had a much higher death rate compared to the discovery cohort (CORTICUS). In order to address this discrepancy, the optimal IFNγ/IL-10 threshold for deciding if the ratio is high or low was calibrated with the death rates. The CORTICUS data was used to establish the calibration curve. In CORTICUS, the optimal threshold was the 39.8 percentile of all 83 patients corresponding to the optimal ratio of 0.95 of IFNγ and IL-10 serum levels. This corresponds to the CORTICUS death rate of 27.7%. Several new datasets weighting the non-survived patients higher (or lower) mimicking a higher (or lower) overall death rate were generated. For every newly generated dataset, an optimal threshold is recalculated. A linear dependency of the

threshold according to the death rate was observed, leading to the following linear model,

$$threshold_{death\,rate} = 35\% + 0.215 \times death\,rate\,(in\,\%)$$

Using this model, an optimal threshold was selected for each validation set according to their death rates. The calibration curve applied to the validation datasets is depicted in the Figure 4.2.

Regression analysis for lactate and norepinephrine consumption

A regression analysis was performed to study the time course of lactate. To obtain the regression functions, patient-matched changes in lactate levels were calculated for day 1, 2 and 3, relative to the baseline (day 0) for the available 41 patients. These patients were grouped into four sets: High IFNγ/IL-10 with non-HC, high IFNγ/IL-10 with HC, low IFNγ/IL-10 with non-HC, and low IFNγ/IL-10 with HC. To assess if there is the rate of change is significant, a *linear regression t-test* was performed on the time series of each patient for each panel a) to d). In a *linear regression t-test*: A linear model,

$$y = b_0 + b_1 x \tag{3.1}$$

was set up for all 41 available patients in each group, in which y was the lactate level and x was the time point (day). The test tests if b_1 is not equal to zero, following a t-statistics.

A similar regression analysis was performed for norepinephrine consumption rates. The norepinephrine consumption at day 0, 1, 2 and 3 was investigated. These patients were grouped into four groups as described above. To find out if there is a significant rate of change, a *linear regression t-test* (as described above) was performed on the time series of each group.

Interaction analysis of treatment and IFNγ/IL-10 levels

Using CORTICUS data, a two-way ANOVA was carried out in R. A linear model for ANOVA was calculated using the 'lm' function. 28-day survival was the independent variable. Treatment and the IFNγ/IL-10 ratio were the dependent variables. An F-test was applied to assess the significance of the interaction coefficient (i.e. the coefficient for the product of the treatment variable and the IFNγ/IL-10 ratio variable).

Adrenocorticotropic hormone (ACTH) test analysis

Machine learning on ACTH test data was performed using a similar cross validation scheme as described in section 3.1.5. Two methods were applied, (1) using the same decision tree implementation as used for the discovery set, and (2) a linear discriminant analysis (LDA) employing the implementation from the 'caret' package of R [Kuhn, 2008]. The baseline cortisol level and the change in cortisol upon stimulation were the two features in these models.

Transcriptional profiling

Out of the 84 patients described above, 47 were randomly selected for transcription profiling with microarrays. Total RNA was isolated from whole blood using the PAXgene Blood RNA Isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. Alpha and beta globin mRNAs were depleted using the GLOBINclearkit (Ambion, Austin, TX). Transcriptomic profiling was conducted using Illumina Sentrix HumanWG-6 V2 BeadChip arrays containing $\sim$ 47,000 bead types (Illumina, Inc., San Diego). The platform, its reproducibility and sensitivity have been described in the FDA-guided Microarray Quality Control study (MAQC) [MAQC Consortium, 2006]. Hybridization was performed according to the "Gene Expression on Sentrix Arrays Direct Hybridization System Manual" (Illumina Inc.). For each sample, total RNA was converted to double-stranded cDNA, followed by amplification (*in vitro* transcription) to generate labeled cRNA. 1.5 μg cRNA of each sample was hybridized on a Sentrix BeadChip Array. After 16 h hybridization time at a temperature of 58°C for a volume of 30 μl label incorporation was performed by staining of hybridized arrays with 1μg/μl Streptavidin-Cy3 (FluoroLink Cy3, GE Biosciences, Pittsburgh, PA). Arrays were washed dried and scanned immediately on an Illumina BeadArray Reader. BeadChips were scanned by Gene Expression Module v3.2 according to the protocol of the manufacturer. Transcriptional profiling was performed by SIRS-Lab, Jena. The data was uploaded to Gene Expression Omnibus (GSE106878).

Data analysis of the transcription profiles

Normalization of gene expression profiles and statistical analysis were performed using R/Bioconductor (www.r-project.org). The data was log_2 transformed followed by quantile normalization using the 'lumi' package [Du et al., 2008]. Probes with a detection significance P $\leq$ 0.05 were selected for further analysis. Probe annotations

were processed using *lumiHumanIDMapping* and *lumiHumanAll.db* to get HGNC symbols via Illumina nuIDs. Probes with the same gene annotation were merged by averaging expression values using the function *avereps* of the 'limma' package [Ritchie et al., 2015]. The first analysis compared the samples of patients before placebo/HC treatment (0 hours) of 28 patients with IFNγ/IL-10 high versus 19 patients with IFNγ/IL-10 low values. Genes with standard deviation or average expression above the 25% percentile were selected and tested for significant up- or down-regulation using *two-sided t-tests*. Multiple testing correction was performed using the method by Benjamini-Hochberg [Benjamini and Hochberg, 1995]. For better resolution of functional gene sets, a lenient adjusted p-value cut-off of 0.2 was used for gene set enrichment analysis. In the second analysis, the effect of HC on 7 IFNγ/IL-10 low-ratio patients (benefitting from HC therapy, responders) was observed by comparing gene expression 48 h post start of the treatment versus pre-treatment (0 h). Genes with standard deviation or average expression above the 25% percentile were selected. Differentially expressed genes were obtained using *two-sided paired t-tests* followed by multiple testing correction (Benjamini-Hochberg [Benjamini and Hochberg, 1995], FDR 0.1). A similar comparison was made with 12 low-ratio patients treated with placebo. Genes distinctly up- or down-regulated in patients of the HC arm but not in the placebo arm were selected for gene set enrichment analysis.

Gene set enrichment analysis was carried out using the R-package 'gProfileR' with default parameters [Reimand et al., 2016]. A new method was developed to reduce the redundancy among the significant gene sets. Redundancy between two gene sets was quantified using Jaccard similarity coefficients, as defined below,

$$J(A, B) = \frac{A \cap B}{A \cup B} \tag{3.2}$$

where, A and B are gene sets containing significantly differentially expressed genes. Gene set pairs with $J(A, B)$ above a desired threshold were included in the model and represented as an undirected graph, $G = (X, E)$, with X as vertices and E as edges of the graph. A linear model was set up with a constraint to select at most one of the vertices of an edge:

$$X_i + X_j \leq 1, \ for\, every\, \{i, j\} \in E \tag{3.3}$$

$$X_i = 0, \ or\, 1, \ for\, 1 \leq i \leq n \tag{3.4}$$

With an objective function to:

$$maximize \sum w_i X_i \tag{3.5}$$

where, w_i is the weight of a gene set. The weight is derived from its significance (p-value) and calculated as $1 - log10(p\text{-}value)/100$. This maximization was done employing linear integer programming solved by the software Gurobi [Gurobi Optimization, 2016]. This lead to an optimal selection of at most one gene set from a pair in such a way that the overall number of non-redundant gene sets are maximized.

Assembling gene signatures of activated macrophages and T-cells

To get a gene signature of activated T-cells, normalized gene expression data from a study by Grigoryev et al. [Grigoryev et al., 2009, Grigoryev et al., 2011] (GEO accession number: GSE14352) was used. In their study, CD2+ T-lymphocytes were purified from peripheral blood mononuclear cells (PBMCs) of 10 healthy human donors. Non-activated CD2+ T-cells were resuspended in RPMI-1640 complete media and activated with CD3/CD28 Dynal beads. RNA was extracted using the mirVana miRNA Isolation Kit. The RNA was converted into labeled cDNA using the GeneChip WT Sense Target Labeling kit (Affymetrix) and the labeled cDNA was hybridized to Affymetrix Human Exon 1.0 ST arrays. Log_2 transformed and quantile normalized data was obtained from Gene Expression Omnibus. A comparison of 48 h post-activation samples with donor matching (non-activated) controls and tests was performed for differential expression using paired *two-sided Wilcoxon tests*. Multiple-correction was performed using the method of Benjamini-Hochberg [Benjamini and Hochberg, 1995]. The significant (adjusted $p \leq 0.05$) genes were assembled into a T-cell activation signature. The macrophage (M1) activation signature was obtained based on gene expression profiles published by Xue *et al.* (GEO accession number GSE46903) [Xue et al., 2014]. The authors purified monocytes from peripheral blood mononuclear cells (PBMCs) of 15 human donors. Resting monocytes were resuspended in RPMI-1640 complete media, activated with GM-CSF for 72 h and further activated with IFNγ. RNA was extracted with Trizol reagent, followed by clean-up and DNase I treatment with QIAGEN RNeasy mini kit in accordance with the prescribed protocol. Biotinylated cRNA were prepared with the Ambion MessageAmp kit for Illumina arrays and hybridized to Illumina HumanHT-12 V3.0 expression beadchip.

The raw data was downloaded from Gene Expression Omnibus, log_2 transformation and quantile normalization was performed in R using the 'lumi' package [Du et al., 2008]. A comparison between the expression profiles of 17 activated (24 h and 72 h post activation) samples with 19 non-activated samples was performed for differential expression using *two-sided Wilcoxon tests*. Multiple-correction was performed using the method of Benjamini-Hochberg [Benjamini and Hochberg, 1995]. The significant (adjusted $p \leq 0.05$) genes were assembled into a macrophage activation signature. *Two-sided Fisher's exact tests* were performed to test for enrichment of genes of interest in the T-cell and macrophage signatures.

Age stratification

In our CORTICUS sub cohort, the average age of the placebo patients was 69.4 years while for the HC patients it was 59.4 years. Therefore, the influence of age difference was tested on the overall results. The weighted stratification was performed as follows, the age of the youngest patient of the placebo group was 43 years. This patient was counted at 100%. For each year a placebo patient was older than 43 years we decreased the percentage of counting this patient by ΔP. In turn, the oldest HC patient was 87 years old. This was fully counted in the HC arm and we decreased the percentage of being counted for all HC patients by the same amount ΔP for each year they are younger than 87 years. The value ΔP was computed in such a way that the average age in both the HC and the placebo groups were equalled out.

3.1.2 The HSSG cohort

Validation was performed on data of septic shock patients from the Hellenic Sepsis Study Group (HSSG). This study included a prospective collection of clinical data and biomaterial since 2006 of patients admitted in 45 hospitals in Greece. The study protocol was approved from the Ethics Committees of all participating hospitals. Patients were enrolled after written informed consent provided either by themselves or by their immediate kin. Detailed eligibility criteria is given in Appendix Tables A.1 and A.2. All the enrolled patients were reclassified according to the Sepsis-3 classification criteria [Singer et al., 2016, Giamarellos-Bourboulis et al., 2017]. The HC treated patients were administered with 50mg hydrocortisone intravenously four times daily for 7 days followed by gradual tapering of the dose. From serum of 342 patients, secreted cytokines were measured using the LEGENDplex Human Inflammation Panel (13-plex) (Bio-Legend) according to

manufacturer's protocol with half of the reagents volume and sample incubation time at 4°C overnight. After quality control (discarding data from patients for which either (1) both IFNγ and IL-10 cytokine levels were below the detection limit, (2) the patient died or was discharged at the day of admission, (3) the serum was in an experimental batch with more than 40% of the serum samples with undetected IFNγ levels, or (4) bad consistency of the serum), a total of 162 eligible shock patients (HC: n=63, No HC: n=99) were selected. If only one of the cytokines (IFNγ or IL-10) was below the detection limit, the value of the detection limit was taken.

Propensity score matching of HSSG cohort

The propensity score matching is a statistical method that ensures that the distribution of baseline features (also called as 'covariates') will be similar between treated and untreated subjects in non-randomized clinical trials. For propensity score matching, first all available HSSG baseline clinical features (3 continuous, 11 binary and 1 categorical feature) were assessed for treatment bias using *Fisher's exact tests*. For this testing, the features were binarized as follows: For every continuous feature, four binary features were calculated based on their 25 percentiles. For example, for a continuous feature such as 'age', four bins were created (i.e. 1-25, 26-50, 51-75, 76-100 percentile). If a given patient has an age in the proposed percentile bin it is regarded as 1 or 0 otherwise. For categorical features, entry specific binary features were calculated. In total, this led to 25 binary features. Multiple-testing correction was performed employing the method by Benjamini Hochberg [Benjamini and Hochberg, 1995]. The multiple corrections did not lead to any significant ($p<0.1$) features associated with the treatment. However, two features, 'history of renal disease' and 'history of chronic heart failure' were the top contributors of bias before multiple corrections. An ad-hoc analysis was performed in order to study if the odds ratio (OR) is affected after propensity score matching based on these two features. The propensity score matching and selection of patients was performed using the 'matchIt' package in R [Ho et al., 2011] ('genetic algorithms', n=50,000 bootstrapping iterations).

3.1.3 The SISPCT cohort

The placebo-controlled, double-blind, randomized trial of Sodium Selenite and Procalcitonin-guided antimicrobial therapy in Severe Sepsis (SISPCT) was

performed in 33 hospitals in Germany. The purpose of this study was to determine whether the selenium (in the form of sodium-selenite) treatment can reduce mortality in patients with severe sepsis or septic shock. Additionally, it was investigated, whether the measurement of procalcitonin - a marker of infection - can be used to guide antimicrobial therapy during the disease course. Between the year 2009 and 2013, a total of 8,174 patients with septic shock or severe sepsis were screened and 1,089 eligible patients with informed consent were randomized. Among these, 109 patients (n=59 selenium-treated and n=50 placebo) were included in the Munich (Ludwig-Maximilians-University, LMU) sub-study for which the cytokine measurements of the blood samples (ethics vote amendment EudraCT: 2007–004333-42) were carried out. For this study, the selenium-treated patients, and one patient who died at the day of inclusion were excluded. Secreted cytokine levels in blood serum samples were measured by using the LEGENDplex Human Inflammation Panel (13-plex) (BioLegend) according to manufacturer's protocol with half of the reagents volume and sample incubation time at 4°C overnight. The cytokine measurements were carried out in collaboration with the University clinic Jena by Dr. Daniela Röll in the laboratory of Prof. Ralf Claus.

Propensity score matching of SISPCT cohort

For propensity score matching, first all available SISPCT baseline features (4 continuous, 18 binary and 1 categorical feature) were tested for treatment bias performing *Fisher's exact tests*. For this testing, the features were binarized as described in the section 3.1.2. The binarization led to 40 binary features. Multiple testing correction was performed employing the method by Benjamini Hochberg [Benjamini and Hochberg, 1995]. Significant features ($p<0.1$) were used for propensity score matching comprising age, serum lactate, application of inotrope/pressor drug, norepinephrine dosage, presence of septic shock (according to ACCP/SCCM criteria), presence of severe sepsis (according to ACCP/SCCM criteria) and kidney dysfunction. Finally, for propensity score matching and selection of patients, the 'matchIt' package in R [Ho et al., 2011] (genetic algorithms, n=50,000 bootstrapping iterations) was applied, leading to 24 patients with matched propensities.

3.1.4 The Crossover study

A second validation was performed applying data from a double-blinded, randomized, placebo-controlled, crossover study with 40 patients diagnosed with septic shock. The study protocol was approved by the institutional ethics committee [Keh et al., 2003]. Detailed eligibility criteria is given in Appendix Tables A.1 and A.2. Until day 3, one arm received first 100 mg loading dose of hydrocortisone and 10 mg per hour until Day 3 (n=20), followed by 3 days placebo. The other arm received the first three days placebo (n=20), followed by HC until day 6. Blood samples were collected on day 0 (before randomization), and every subsequent day until day 6. Enzyme-linked immunosorbent assays (ELISA) were used for measurement of interleukin 4, 8, 10, 12 p70, and IFNγ (BD PharMingen, Germany), soluble E-selectin (BenderMed Alexis, Austria), IL6 (R&D, Wiesbaden, Germany), and soluble tumor necrosis factor receptors I and II (Biosource, Germany). The cytokine measurements were requested from the authors of the study.

3.1.5 The rationale of the data analysis

The subcohort of CORICUS consisted of 83 patients and 137 patient features (potential predictors) that were used to perform an exploratory data analysis. The patient features also included the ratios of all cytokine combinations. The aim was to find one predictor which suits as a theranostic marker distinguishing HC responders from non-responders. Patients that can benefit from the HC treatment are referred to as 'HC responders' and others as 'non-responders'. The workflow is depicted in Figure 3.1. The analysis started with finding the best predictor of survival (28 days) for the placebo arm (n=41). For this, one-level decision trees (stumps) were calculated in a leave-one-out cross-validation scheme. The best decision trees were chosen by intelligent enumeration. Among these, the predictor and threshold that was present in the most of the trees (i.e. the predictor IFNγ/IL-10) was applied on the HC arm. The threshold value was represented in the percentile of IFNγ/IL-10. Patients whose predictor value ranked below the identified percentile were denoted as "low-ratio patients", the others as "high-ratio patients". When all non-surviving patients were weighted higher or lower, a linear relation between the optimal percentile and the corresponding death rates was established. Hence, these optimal cutoffs were calculated to gain a calibration curve with which the cutoff for the validation sets could be determined (details, 3.1.1). All analyses were carried out in R (www.r-project.org) using custom scripts. Odds ratio and statistical significance calculations

are described in the next section.

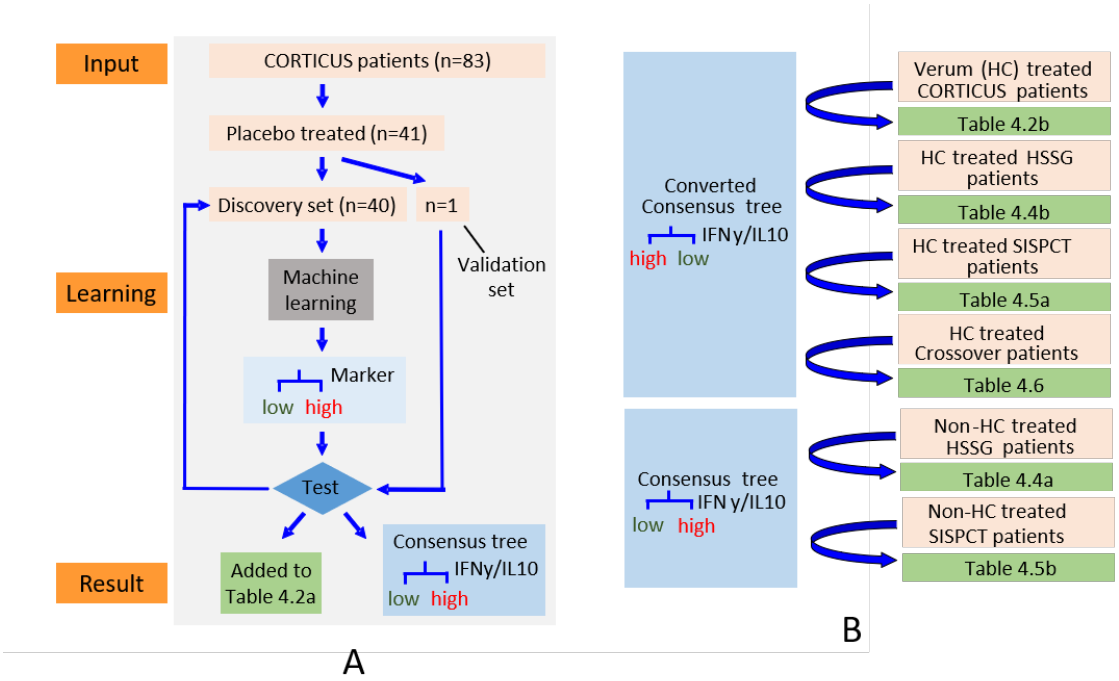

Figure 3.1: The workflow of theragnostic marker discovery and validation,

(A) The algorithm for the discovery of the theranostic marker, to note the term "validation set" is used here within the context of the cross validation scheme:

  (a) From all investigated CORTICUS patients, the placebo treated patients are selected

  (b) The selected patients are split into a training set (n=40) and a validation set (n=1)

  (c) Machine learning: Selection of the best predictor out of 137 available predictors to predict survival on the training set, using one-predictor based decision trees

  (d) Testing the performance of the selected predictor on the validation set

  (e) Adding the result from d) to the confusion matrix, and storing the tree

  (f) Going back to b). In b) the next patient is forming the validation set, and the rest of placebo patients are the training set

  (g) From all stored trees, a consensus tree is determined (the one which has been used most often, i.e. high IFNγ/IL-10 predicts survival, low IFNγ/IL-10 predicts non-survival)

(B) The converted consensus tree (low IFNγ/IL-10 predicts survival, high IFNγ/IL-10 predicts non-survival) is applied to the HC treated patients of CORTICUS, HSSG, SISPCT, and to the early arm of the Crossover study. The consensus tree is applied to the non-HC treated patients of HSSG and SISPCT.

Calculation of odds ratio

Odds ratios were calculated by the following method. The ratio of survivors to non-survivors that are treated according to the decision rule was computed. The analogous ratio was computed for the rest of the patients that were treated opposite to the rule. Finally, the odds ratio was computed by dividing these two ratios. Analogously to clinical studies that compare "treatment" with "no treatment", in this case "treated in compliance with the rule" were compared with "treated not in compliance with the rule". The significance of the odds ratio was calculated using a *one-sided Fisher's exact test*.

3.1.6 *Ex vivo* whole blood culture experiments

To assess the plausibility of the data-driven biomarker IFNγ/IL-10, *ex vivo* whole blood culture experiments were performed in which blood of healthy donors was spiked with a broad range of bacterial lysates to simulate distinct pathogen loads. 200 μL of diluted (HBSS (1:1, V/V) heparinized whole blood obtained from healthy volunteers (n=5, male, 20-25 years) was stimulated with serial dilutions of lysates from *E. coli* isolates obtained from septic patients upon ethics approval. *E.coli* lysates were created by sonification, following heat inactivation, and a serial dilution of the obtained fragments stock was performed. Following exposition (37°C, 18hrs, gently agitation 2rpm) plasma supernatant was prepared by centrifugation (2.500xg, RT, 10 min), secreted cytokine levels were measured using the LEGENDplex Human Inflammation Panel (13-plex) (BioLegend) according to the manufacturer's protocol with half of the reagents volume and sample incubation time at 4°C overnight. HBSS was used as vehicle control. The experiments were carried out in collaboration with the University clinic Jena by Dr. Daniela Röll.

3.2 The longitudinal sex-dependant transcriptome modulation study

3.2.1 The trauma cohort

The investigated cohort is a part of the retrospective observational study 'Inflammation and host response to injury' (IHRI) (ClinicalTrials.gov identifier: NCT00257231). The cohort data used in the thesis was obtained from the supplementary material of the original publication [Desai et al., 2011]. The most important inclusion criteria for the cohort were: blunt trauma without isolated head injury, blood transfusion within 12 hours of injury, base deficit $\geq$6 or systolic blood pressure <90 mmHg within 60 minutes after arrival at the emergency department and the emergency department arrival $\leq$6 hours from time of injury. The major exclusion criteria were: traumatic brain injury (defined as Glasgow Coma Scale (GCS) motor score <3 or Abbreviated Injury Scale (AIS) score for head >4 within 24 hours of injury), anticipated survival of <24 hours from injury, pre-existing immunosuppression or significant pre-existing organ dysfunction. All the patients were admitted within 12 h after injury and monitored for up to 28 hospital days. For each patient, the first blood sample was taken within 12 h after injury and approximately 1, 4, 7, 14, 21, and 28 days after the injury. Leukocytes from whole blood were isolated from peripheral blood samples (more details in ref. [Desai et al., 2011]). Total cellular RNA was extracted and hybridized onto an HU133 Plus 2.0 GeneChip (Affymetrix) according to the manufacturer's recommendations.

3.2.2 Patient selection

Among the available data of 168 patients, a subset of 132 (85 male; 47 female) was selected based on their age (between 16 to 50 years) and their maximum Multiple Organ Failure (MOF) score without neuronal component >1 during their first 28 days in the hospital. These 132 patients were included in the severity synchronization analysis. The upper age limit of 50 years was set to study the response of premenopausal female compared to male patients. Among these 132 patients, for 129 (83 male, 46 female) there was at least one transcriptomic profile available which could be mapped to the investigated time window of the synchronized profile. Hence, these patients were included in the transcriptomic analysis. A detailed patient selection scheme is given in Figure 3.2 below.

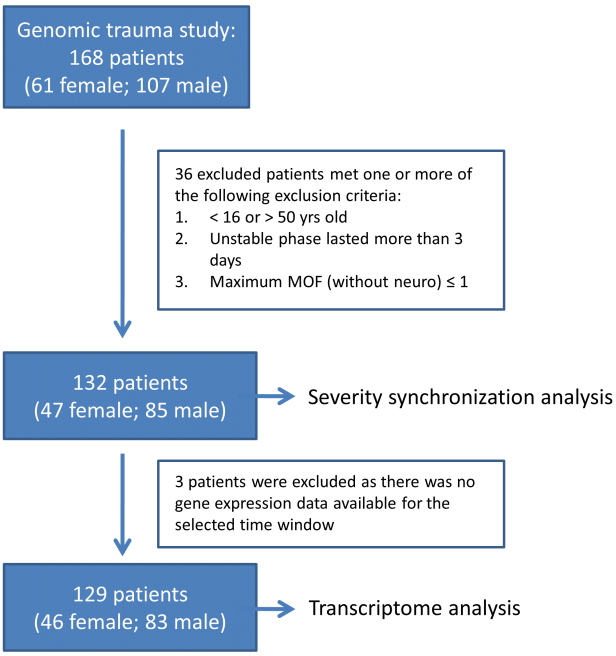

Figure 3.2: A subset of 132 (85 male; 47 female) out of 168 patients were included in the severity synchronization analysis. The selection was made based on their age, non-complicated recovery profiles (details in Figure 3.3) and the maximum MOF scores during 28-days from the time of injury. Among these, for 129 patients (83 male, 46 female) transcriptomic profiles were available which were matching to the time window of interest, i.e. from 3 days before the acute phase to 3 days after the acute phase.

Patients with complicated MOF profiles were excluded. A MOF score profile was defined to be a complicated one if it contained two or more days with the highest MOF score. An example of a complicated profile is shown in Figure 3.3A with an unstable phase of more than 3 days. An unstable phase was defined as the days between the first day of the highest MOF score and the last day of the highest MOF score. Patients were excluded if this unstable phase was >3 days. (B) The distribution of the lengths of unstable phases across all patients is shown in Figure 3.3B.

3.2.3 Data retrieval and pre-processing

Microarray normalization and statistical analysis were performed using R/Bioconductor (www.r-project.org). The raw data was background corrected and RMA normalized using the 'affy' package [Gautier et al., 2004]. Probe sets with a detection significance $P \leq 0.05$ were selected for further analysis. Probes were mapped to their corresponding gene names (HGNC symbols) via Illumina nuIDs using *lumiHumanIDMapping* and *lumiHumanAll.db* [Du et al., 2008]. Probes with the same annotated gene were merged by averaging expression values using the function *avereps* of the 'limma' package [Ritchie et al., 2015].

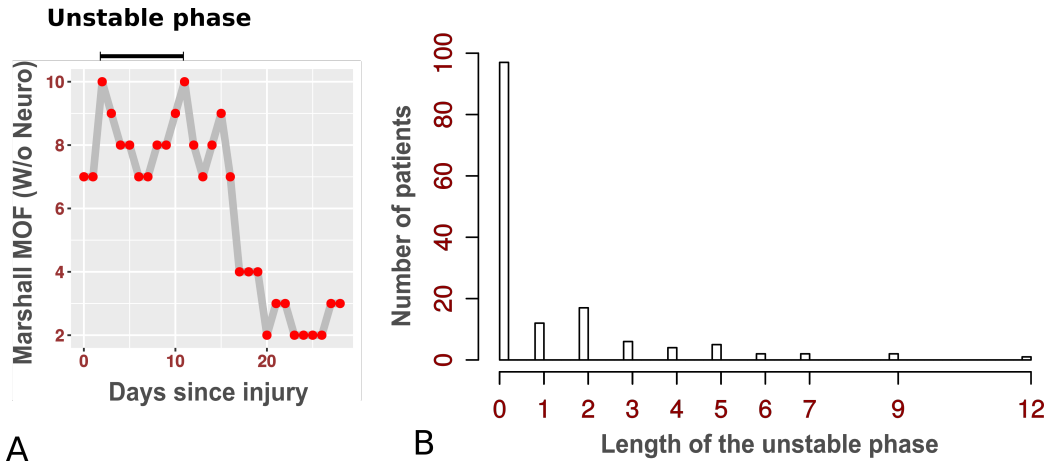

Figure 3.3: (A) An example of a complicated MOF score profile is shown here. An unstable phase was defined as the days between the first day of the highest MOF score and the last day of the highest MOF score. (B) The distribution of the duration of unstable phases in all patients.

3.2.4 The workflow

The workflow is depicted in Figure 3.4 summarizing the following steps:

1. Synchronization by severity to define the acute phase

To make the time series expression profiles comparable between patients, their severity profiles were synchronized. First, for each patient, the time point of the patient's most severe state of the disease was identified by selecting the maximal Marshall MOF score (without the neurological component, also in the following) within day 0 to day 28. This selected day, the day of the highest MOF score was set as the 'acute phase' for that patient. If a patient had multiple days with the highest MOF score, the time period between the first and the last day with the highest MOF score was defined as the 'unstable phase'. An example is given in Figure 3.3A. Only patients with an unstable phase shorter or equal to 3 days were included in the analysis (n=132) and the whole time from the first to the last day with highest MOF score was regarded as the acute phase. The acute phase was set as a reference for aligning the other days as explained in the next step.

2. Setting up the other phases

When regarding the transcription profiles according to this reference day, a strong increase and decrease in severity was observed within seven days, i.e. between day -3 to +3 according to the reference day. Hence, this time window was used for the analysis, i.e. seven days, 3 days before the acute phase, the day of the acute phase and 3 days after the acute day. For simplification and to get enough samples per analyzed time point, the window of seven days was categorized into five major

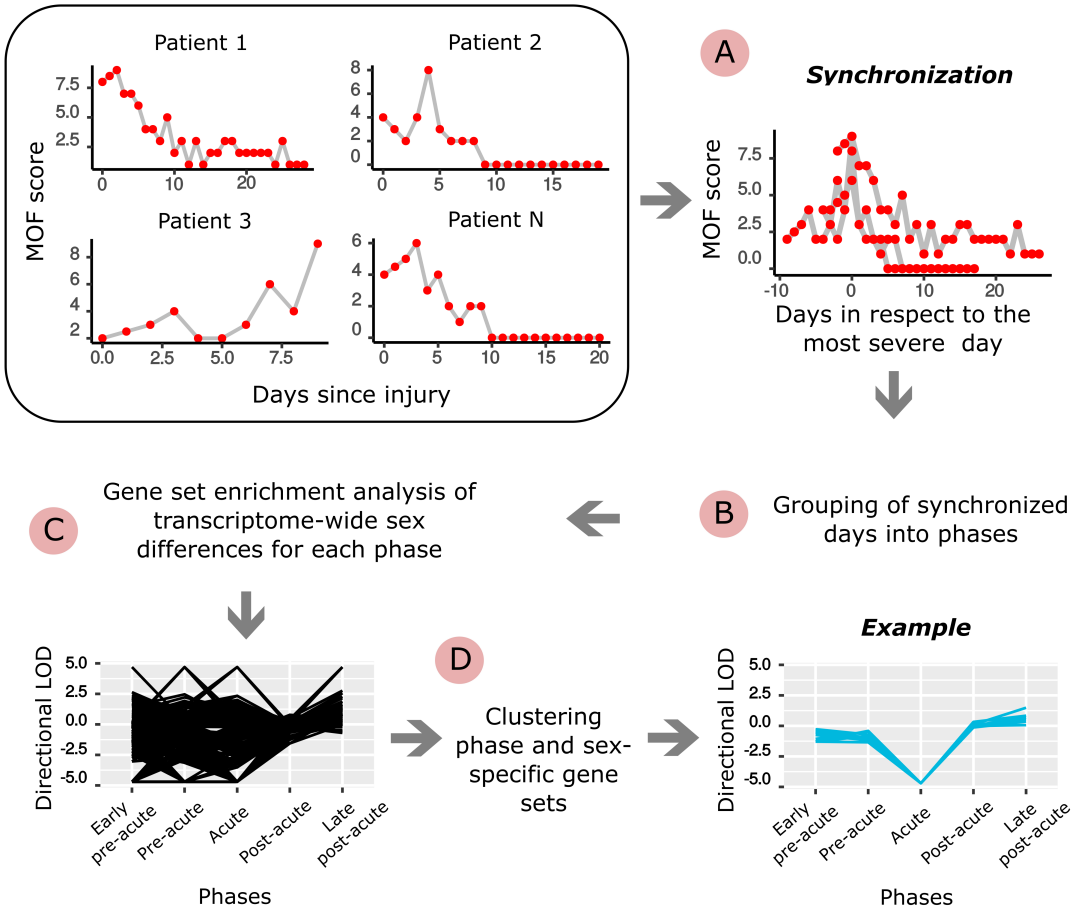

Figure 3.4: (A) The time series expression profiles of the patients were synchronized according to the day of maximal MOF (multiple organ failure) of the patients (denoted as acute phase). (B) The investigated days before, at and after the acute phase were binned into major temporal phases. (C) Sex-bias in the gene expression was quantified for each phase and tested for significant association with gene sets (D) The identified gene sets were grouped according to their temporal appearance and further into the similar cellular processes they describe.

phases. These phases were defined as 'early pre-acute' (2 to 3 days prior to the acute phase), 'pre-acute' (1 day prior to the acute phase), 'acute phase' (the most severe day), 'post-acute' (1 day after the acute phase) and 'late post-acute' (2 to 3 days after the acute phase). The distribution of the samples in each time point is given in Table 3.1, comprising data from altogether 330 samples of 129 patients.

3. Gene expression analysis

Each of the five temporal phases was analysed individually. The sex-bias in the expression of each gene summarized in t-values (i.e. t-statistics) by comparing transcription profiles of male and female patients. Only the genes from autosomal chromosomes were regarded. The t-values were used for gene set enrichment analysis to select Gene Ontology terms employing the 'piano' package [Väremo et al., 2013] (n=50,000 permutations, choosing 'mean' as the gene set statistics,

Table 3.1: The distribution of microarray samples across the phases.

#Samples	Early pre-acute	Pre-acute	Acute	Post-acute	Late post-acute
Male	63	29	52	21	46
Female	31	17	28	19	24

distinct directional, the t-statistic were used). Gene sets of 'Biological processes' were taken as defined by Gene Ontology [Ashburner et al., 2000]. Highly non-specific (containing more than 150 genes) and very small (less than 16 genes) Gene Ontology terms were omitted. P-values resulting from the gene set enrichment tests were corrected for multiple testing using the method by 'Benjamini-Hochberg' [Benjamini and Hochberg, 1995].

4. Clustering temporal profiles of enriched gene sets

Gene sets with similar profiles as per their temporal progression were clustered. For this only those gene sets were considered which had high significance ($p<0.025$) in at least one of the time phases. LOD scores for gene sets were calculated as LOD = $-log_{10}(p)$, where p were the adjusted p-values. These values were made directional by adding a minus sign for gene sets which were downregulated in female patients. The gene sets were separated into gene sets which were significantly enriched in only one phase (single phase cluster), and gene sets which were enriched in more than one phase (multiple phase cluster). From each of these groups of gene sets, clusters were formed according to the phase of their high significance. These phase-specific clusters were then separated into male and female clusters based on the directional LOD scores. The functional relevance of the clusters was derived based on the biological interpretation of their consisting gene sets.

3.2.5 Validating results by regarding transcription profiles from critically ill patients after burn injury

Validation was performed with a second publically available dataset. In this study, patients were recruited under the observational and prospective study conducted from 2000 to 2009 in four centres for burn injuries in the U.S. [Seok et al., 2013]. These patients had a burn injury of over 20% of the Total Body Surface Area (TBSA) and were admitted within 96 hours after the injury. Blood was drawn from the time of injury until one year. The patients underwent at least one excision and grafting surgery. Major exclusion criteria were: associated multiple injuries exclusive of burns (Injury Severity Score (ISS) $\geq$25) and several

pre-morbidity conditions (more details in ref. [Seok et al., 2013]). For validation, only those patients for which transcription profiles were available within the first week from the time point of injury were included. A subset of 103 (n=79 male; n=24 female) patients between the age of 16 to 50 years was used in the analysis. The raw data (downloaded from the NCBI GEO, accession number GSE37069) was pre-processed and normalized as described above.

3.2.6 Additional statistical analyses

Distributions were visualized using boxplots of z-transformed expression values of the gene sets of interest. *Two-sided t-tests* were performed to compare these distributions between male and female patients and between male and female healthy controls. The trend of MOF scores over time was studied using a *linear regression t-test*. *Wilcoxon rank-sum test* was used to compare injury severity scores, MOF scores and the distribution of day of maximum MOF between sexes after synchronization. As the transcriptomic profiling was originally carried out in four batches, the impact of batch effect was estimated by checking the distribution of male and female samples across batches. A *chi-squared test* was performed on the distribution of number of male and female samples across the batches. However, batch was not found to be a significant ($p>0.1$) confounder. For the analysis of severity-matched patients, a subset of acute phase samples of male and female patients with comparable MOF and AIS (at inclusion) scores was selected. The matching was performed using the 'MatchIt' package (algorithm: 'genetic') [Ho et al., 2011].

3.3 The sex-hormone modulation study in mice

3.3.1 Hormones and chemicals

Flutamide (Flu) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Atraric acid (AA) was kindly provided by Dr. Thomas Rösler from the Institute for Pharmaceutical Chemistry, Philipps-University Marburg, Germany. For *in vivo* experiments compounds were injected as homogenous suspensions in 0.9% NaCl (Fresenius Bad Homburg, Germany) supplemented with 0.5% polysorbate 80 (Tween 80) (Sigma-Aldrich Taufkirchen, Germany) in deionized sterile water. Solvent 0.9% NaCl supplemented with 0.5% Tween 80 was applied as a control in male and female mice.

3.3.2 Peritoneal contamination and infection (PCI) model of sepsis and transcriptome profiling

A severe form of PCI sepsis model as described by Gonnert *et al.* [Gonnert et al., 2011] was induced in C57BL/6 male and female mice aged between 12 to 16 weeks. For synchronization of the pre-estrus cycle, female mice were set on male mice dung three days before sepsis induction. For induction of sepsis, a 100% lethal dose (2 μl/g body weight) of a pre-adjusted (referred to male mouse survival) and microbiologically characterized human feces batch was injected intraperitoneally. Treatment with the compounds AA, Flu or solvent (0.5% Tween 80 in 0.9% NaCl) to male mice and only solvent to the female control group was given subcutaneously at the time points of 0 h, 4 h and 8 h after PCI induction. The compound administration was given coincidently to the volume-resuscitation.

The experiment was terminated 10 h post-septic insult to assure the collection of suitable samples of all animals (n=4 for each group). Mice were deeply anesthetized with isoflurane and sacrificed by heart puncture. The liver was dissected and snap frozen in liquid nitrogen for transcriptome profiling. Necropsy experiments were performed simultaneously for all group to assure comparability. The obtained liver tissues were thawed on ice and sections (50 mg $\leq$ 100 mg) were taken and placed in 1 ml of peqGOLD TriFast^{TM} (Peqlab, Erlangen, Germany). A stainless steel bead of 5 mm width was added to each tube. The reaction tubes were placed in a Tissue Lyser (Qiagen Hilden, Germany) which homogenized the tissue for 3 min at 30 Hz. Samples were verified to ensure that there was no visible debris and the bead was removed with sterile forceps. The total RNA was isolated from mashed liver using

the peqGOLD TriFast^{TM} (Peqlab, Erlangen, Germany) according to the manufacturer's instructions.

The transcriptome profiling was performed using (MouseRef-8 v2.0 Expression BeadChip containing $\sim$ 47000 bead types (Illumina Munich Germany). The integrity of the isolated RNA was assessed based on 28S:18S rRNA ratio using capillary electrophoresis system QIAxcel RNA QC Kit v2.0 (QIAGEN, Valencia, CA). A 200ng input RNA from each sample was prepared according to manufacturers protocol to obtain cRNA for chip hybridization using the TargetAmp^{TM}-Nano Labeling Kit for Illumina Expression BeadChip (Biozym, Hessisch Oldendorf, Germany). To remove disturbing enzymes and nucleotides, a clean-up step was executed using silica-based spin columns (Nucleospin RNA clean-up, Macherey-Nagel, Düren, Germany). A total sample volume of 15 μl was hybridized on the BeadChip array. The dry BeadChip was placed in an iScan reader to determine the transcriptional intensity of the respective gene, measuring fluorescence intensity as a surrogate.

The *in vivo* mouse study and transcriptome profiling experiments were performed by Dr. Daniela Röll. In this thesis, the analysis of these transcriptomic profiles was performed as described in the following sections.

3.3.3 Data analyses of the transcription profiles

Normalization of transcription profiles and statistical analysis were performed using R/Bioconductor (www.r-project.org). The data was log_2 transformed followed by quantile normalization using the 'lumi' package [Du et al., 2008]. Probes with a detection significance P $\leq$ 0.05 were selected for further analysis. Probes with the same gene annotation were merged by averaging expression values using the function *avereps* of the 'limma' package [Ritchie et al., 2015].

Principal component analysis

Principal component analysis (PCA) summarizes the complexity in high-dimensional data, such as transcriptome profiles, while retaining trends and patterns. It does it by an orthogonal transformation of several (possibly) correlated features into a (smaller) number of uncorrelated features called principal components. The first principal component summarizes for as much of the variance in the data as possible, and each following component accounts for as much of the remaining variance as possible. The PCA was performed to study the

transcriptomic shifts in the male mice as a consequence of the treatment. 10% of the genes with the lowest standard deviation were removed and the principal components (PCs) were derived using the *prcomp* function in R. The PCs being orthogonal to each other, their addition or subtraction produces a transformed vector that summarizes the two PCs. These transformed PCs were used to visualize the inherent sex-differences and female-like treatment effects in male septic mice.

Differential gene expression analysis and gene set enrichment analysis

Differential gene expression (DGE) analysis was carried out in R using the 'limma' package [Ritchie et al., 2015] using default parameters. This analysis was carried out for three comparisons: (*i*) septic female (n=4) *versus* septic male (n=3), (*ii*) AA treated male (n=4) *versus* solvent treated septic male (n=3) and, (*iii*) Flu treated septic male (n=4) *versus* solvent treated septic male (n=3). Genes with standard deviation or average expression above 25% percentile were selected and tested for significant up- or down-regulation. Multiple testing correction was performed using the method by Benjamini-Hochberg [Benjamini and Hochberg, 1995]. An adjusted p-value cut-off of 0.05 was used to obtain significantly differentially expressed genes. Co-directional genes (i.e. common up- or down-regulated genes) between the comparisons (*i*) and (*ii*), were extracted to study female-like effects of AA treatment and, similarly between (*i*) and (*iii*) to study female-like effects of Flu treatment. Gene set enrichment analysis was carried out using the R-package 'gProfileR' with default parameters [Reimand et al., 2016]. The redundancy among the significant gene sets was reduced (using the method described in the section 3.1.1 on page 18). After removing the redundancy, the gene sets were further categorized on the basis of cellular processes they describe, such as 'anabolic processes', 'cell death', 'catabolic processes', 'energy metabolism', 'growth response', 'hormonal response', 'homeostasis', 'immune response', 'metabolic processes', 'oxidative stress', 'response to stimuli', 'signaling processes', 'stress response', 'transport' and 'transcription and translation'.

Chapter 4

Results

4.1 The corticosteroids study

4.1.1 Patient characteristics

Patient characteristics such as height, weight, gender distribution, severities in terms of SOFA and SAPS II scores, duration of ICU- and hospital- stay were similar between the HC and placebo group. Age was significantly lower in the HC group (Table 4.1), but this was not found to be a confounder as described in section 3.1.1. Mean plasma concentrations of soluble mediators and leukocytes measured at the baseline (i.e. before treatment) were also similar between the HC and placebo group [Kolte et al., 2018].

Table 4.1: Patient characteristics of the studied CORTICUS sub-cohort

	HC (n=42)	**Placebo (n=41)**
Sex (male/ female, n)	29/ 13	30/ 11
Age [years]	59.4 (22-87)*	69.4 (43-88)
Height [cm]	171 (150-188)	172 (150-195)
Weight [kg]	79.5 (50-130)	77.1 (53-127)
Admission category (n)		
- Medical	1	0
- Emergency surgery	39	37
- Elective surgery	2	4
SOFA score at inclusion	10.4 (4-18)	9.8 (5-17)
SAPS II score		
- First 24 hours at ICU	47.9 (22-80)	48.0 (16-88)
- Last 24 hours before inclusion	43.7 (13-77)	47.2 (16-88)
Time: sepsis to inclusion [hours]	29 (3-71)	31 (1-67)
ACTH Responder/ Non-Responder (n)	26/ 16	29/ 12
Survival (n)		
- Day 28 (survivors/ non-survivors)	31/ 11	29/ 12

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Table 4.1 – *Continued from previous page*

	HC (n=42)	**Placebo (n=41)**
- ICU (survivors/ non-survivors)	27/ 15	28/ 13
- Hospital (survivors/ non-survivors)	23/ 17	26/ 15
ICU-stay [days] all patients	27.5 (3-160)	23.9 (3-89)
ICU-stay [days] survivors	23.3 (5-73)	26.3 (3-89)
Hospital stay [days] all patients	51.3 (11-207)	45.5 (3-135)
Hospital stay [days] survivors	50.5 (15-128)	53.9 (17-135)

** : $p < 0.05$; Range : minimum and maximum*

4.1.2 IFNγ/IL-10 stratifies CORTICUS patients

To identify the best treatment marker, the analysis of the baseline (blood samples were taken before treatment and pre-randomization) characteristics of the patients were considered. Baseline characteristics included 137 variables collected from 83 patients (60 survivors, 23 non-survivors) including basic patient characteristics and clinical variables, Sepsis-related Organ Failure Assessment (SOFA) scores, lymphocyte counts and plasma protein concentrations of cytokines. A leave-one-out cross-validation was performed with one-level decision trees (consisting of only one predictor at a time) on the placebo arm to discriminate between 28-day-survivors and non-survivors. This led to a high true positive rate (83%, Table 4.2a). In 95% of the cross-validation runs, the serum IFNγ/IL-10 ratio (also termed IFNγ/IL-10 in the following) with the same threshold (39.8 percentile of IFNγ/IL-10 ratio from all patients) was selected by the algorithm as the best predictor. Patients which IFNγ/IL-10 ratio ranked among the first 39.8% patients were denoted "low ratio patients", the others "high ratio patients". Upon applying the predictor to HC-treated patients, the reverse behaviour was observed with a high true negative rate, i.e. low IFNγ/IL-10 indicated a high likelihood of survival (85%) (Table 4.2b). A significant interaction effect (p=0.0083) was observed between the ratio and the treatment (Figure 4.1). These observations were then compiled into a decision rule: no HC treatment if the ratio was high, and HC treatment if low. Following the decision(/treatment) rule yielded the odds ratio (OR) of survival of 3.03 [95% Cl: 1.05-8.75], P=0.031. To note, IFNγ or IL-10 alone were not suitable for this prediction. The identified new treatment rule was applied to further, unseen data from additional clinical trials of septic shock (one larger validation set and two smaller datasets from two further clinical studies).

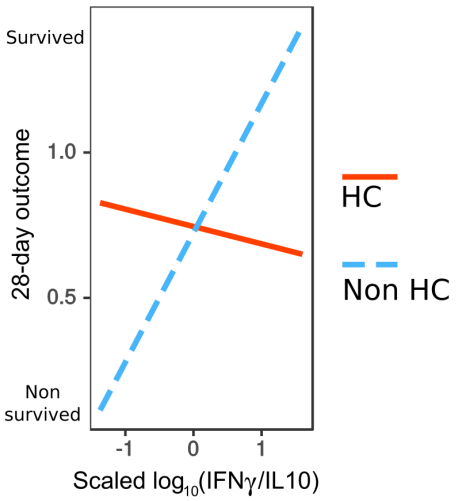

Figure 4.1: Interaction plot of treatment and IFNγ/IL-10 showed a significant interaction (p=0.0083) between treatment and the IFNγ/IL-10 ratio. Statistical details are given in the section 3.1.1 on page 17.

Table 4.2: Survival rates in CORTICUS according to high and low IFNγ/IL-10

a) CORTICUS patients treated with placebo

	Non-survivors	Survivors	% Survivors*
IFNγ/IL-10 high	**4**	**20**	**83%**
IFNγ/IL-10 low	8	9	53%

b) CORTICUS patients treated with HC (hydrocortisone)

	Non-survivors	Survivors	% Survivors
IFNγ/IL-10 high	9	20	69%
IFNγ/IL-10 low	**2**	**11**	**85%**

* **bold:** in compliance with the decision rule.

4.1.3 Validation based on patients from the Hellenic Sepsis Study Group (HSSG) and two further datasets

Table 4.3 shows the demographics of the patients used for validating the results.

Table 4.3: Patient characteristics of the studied HSSG cohort

	HC (n=63)	No HC (n=99)
Gender (male/ female, n)	32/31	38/61
Age [years]	71.10 (24.0-93.0)	73.10 (27.0-96.0)
SOFA	8.83 (2.0-20.0)	9.03 (2.0-21.0)
APACHE II	22.67 (7.0-41.0)	24.46 (3.0-52.0)
28 days survival (n, %)	20 (32%)	40 (40%)
Site of infection (n, %)		
- Community-acquired pneumonia	33 (52%)	62 (63%)
- Intrabdominal infection	30 (48%)	37 (37%)

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Table 4.3 – *Continued from previous page*

	HC (n=63)	**No HC (n=99)**
Co-morbidities (n, %)		
- Presence of acute kidney injury	22 (35%)	25 (25%)
- History of diabetes mellitus type 2	21 (33%)	26 (26%)
- History of renal disease	3 (5%)*	16 (16%)
- History of chronic heart failure	11 (17%)*	34 (34%)
- History of chronic obstructive pulmonary disease	11 (17%)	19 (19%)

** : $p < 0.05$; Range : minimum and maximum*

Applying IFNγ/IL-10 ratio to the group of HSSG patients, a similar stratification as for the CORTICUS patients was observed. High IFNγ/IL-10 indicated distinct higher survival of the HC untreated patients (50% versus 19%), as shown in Table 4.4a. While, in the HC treated group an opposite behaviour (28% versus 35%) was observed as shown in 4.4b, yielding an odds ratio of OR=2.01 [95% CI:1.04-3.88], P=0.026. As the death rate of the HSSG patients was much higher compared to CORTICUS, the threshold had to be adjusted according to a calibration scheme based on the CORTICUS data as explained in the methods section 3.1.1 (on page 16). The calibration curve is depicted in Figure 4.2.

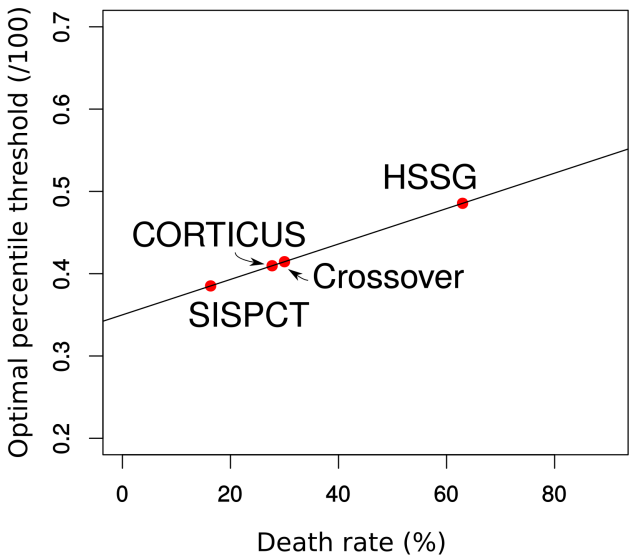
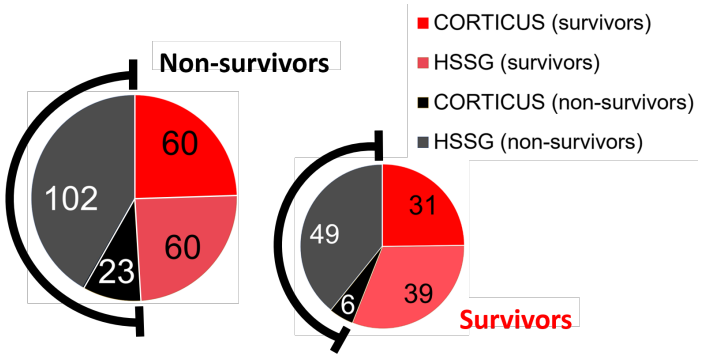


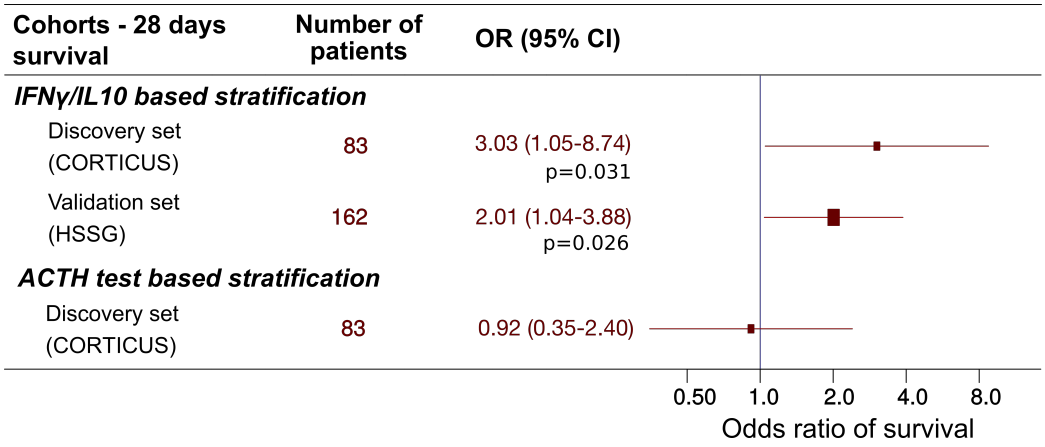
Figure 4.2: The calibration curve is depicted to set the percentile threshold of the predictor, depending on the death rate of the study. The positive slope hints that as the death rate increases more patients are needed to be treated with HC in order to obtain better survival. HSSG had a death rate of 62.96%, hence the corresponding optimal threshold of 48.55 percentile was set. For SISPCT and the crossover study, the death rates were 16.33% and 30.00% corresponding to the 38.51 and 41.50 percentile, respectively

Figure 4.3a illustrates the enhanced survival of both studies and Figure 4.3b shows

the forest plot. The HSSG cohort had an imbalance of two co-morbidities in the HC treated and non-treated group. These co-morbidities were 'history of chronic heart failure' and the 'history of renal disease'. To account for this imbalance in co-morbidities, propensity score matching was performed (as described in section 3.1.2 on page 22) leading to the selection of 126 patients with survival OR=2.17 (95% CI: 1.02-4.63), p=0.032 in the patients following the rule. This confirmed that the existing bias bias in the co-morbidities were not the contributing factors in the performance of the predictor, IFNγ/IL-10.



(a)



(b)

Figure 4.3: Pie chart and Forest plot of CORTICUS and HSSG a) Survivors (red to light red) and non-survivors (black to grey) of all patients (left) and of patients complying with the proposed decision rule (right), patient numbers are given in the pies. Treatment according to the rule led to an absolute risk reduction (ARR) of 0.14 [95% CI: 0.03-0.26]. b) Forest plot for the sub-cohorts of CORTICUS (discovery set), HSSG (validation set). Below, the performance of the ACTH test is shown (details are given in section 4.1.9).

Furthermore, the performance of the marker was tested on two unseen smaller datasets. The first dataset included the patients from the placebo arm of the randomized multi-center, placebo-controlled, trial of Sodium Selenite and ProCalciTonin-guided antimicrobial therapy in Severe Sepsis (SISPCT)[Bloos et al., 2016]. For this dataset, selenium-treated patients, and one patient who died at the

Table 4.4: Survival rates in HSSG according to high and low IFNγ/IL-10
a) HSSG patients, untreated with HC

	Non-survivors	**Survivors**	**% Survivors***
IFNγ/IL-10 high	**27**	**27**	**50%**
IFNγ/IL-10 low	32	13	29%

b) HSSG patients treated with HC

	Non-survivors	**Survivors**	**% Survivors**
IFNγ/IL-10 high	21	8	28%
IFNγ/IL-10 low	**22**	**12**	**35%**

* **bold:** in compliance with the decision rule.

day of inclusion were excluded. After propensity score matching, n=24 patients were included in the analysis (details about the study and statistics, see section 3.1.3 on page 22). The treatment rule was found to be valid: 100% of the patients survived (n=3) if treated according to the rule, compared to 77% if not treated according to the rule, as shown in Table 4.5. In the second dataset the patients of the early arm of the Crossover study [Keh et al., 2003] were included (for details about this study and the crossover scheme, see section 3.1.4 on page 24). In this study, the early arm got a comparable HC application as the HC arm of CORTICUS, and hence was used for validating the marker. In line with the results from CORTICUS, HSSG and SISPCT, low IFNγ/IL-10 showed better survival (88% survivors), whereas high IFNγ/IL-10 was an indicator for considerably worse outcome (57% survivors). The result is shown in Table 4.6. Due to the small sample numbers, the odds ratio of survival from SISPCT and the crossover study were not significant. In summary, the investigated patients from all studies evidenced IFNγ/IL-10 as a theranostic marker for HC application in septic shock.

4.1.4 Time courses of serum lactate and norepinephrine requirement reflect better recovery in patients treated in compliance with the treatment rule

High serum lactate levels have been demonstrated to reflect the degree of metabolic derangements and increased mortality in sepsis patients [Singer et al., 2016], hence lactate levels were included as an additional criteria in the new definition of septic shock [Singer et al., 2016]. As measured in the CORTICUS study, the median initial lactate in serum was 1.89 mmol/L in patients with high

Table 4.5: Survival rates in SISPCT according to high and low IFNγ/IL-10
a) SISPCT patients, untreated with HC

	Non-survivors	**Survivors**	**% Survivors***
IFNγ/IL-10 high	**0**	**8**	**100%**
IFNγ/IL-10 low	0	6	100%

b) SISPCT patients treated with HC

	Non-survivors	**Survivors**	**% Survivors**
IFNγ/IL-10 high	3	4	57%
IFNγ/IL-10 low	**0**	**3**	**100%**

* **bold:** in compliance with the decision rule.

Table 4.6: Patients from the early arm of the Crossover study (treated with HC, similar to the CORTICUS HC arm)

	Non-survivors	**Survivors**	**% Survivors**
IFNγ/IL-10 high	5	7	54%
IFNγ/IL-10 low	**1**	**7**	**88%**

* **bold:** in compliance with the decision rule.

IFNγ/IL-10, compared to 2.89 mmol/L in patients with low IFNγ/IL-10 suggesting IFNγ/IL-10 to indicate the severity of cellular derangement in sepsis, analogous to the lactate levels. To note, although lactate levels correlated inversely to the IFNγ/IL-10, serum lactate itself performed worse as a theranostic marker, compared to IFNγ/IL-10. Studying the time course of the first 3 days after inclusion, lactate levels overall decreased in all patient groups reflecting their recovery. Notably, a significant rate of lactate decrease was explicitly identified in the group of patients which were treated in compliance with the rule (Figure 4.4). Shock recovery reduces the need for norepinephrine (NE) requirement. In line, the rate of norepinephrine requirement decreased significantly (P=2.0e-05) specifically in the group of patients in compliance with the rule (Figure 4.5). In summary, time series of serum lactate and NE requirement reflected better recovery of septic shock in patients treated in compliance with the treatment rule.

4.1.5 IFNγ/IL-10 reflects the pathogen load

In light of the observations described in the literature associating IFNγ and IL-10 with the severity of parasitic and tuberculosis infection [Jamil et al., 2007], it was hypothesized if IFNγ/IL-10 reflects the immunological load of immune cells when challenged with typical pathogens found in sepsis. The blood from healthy

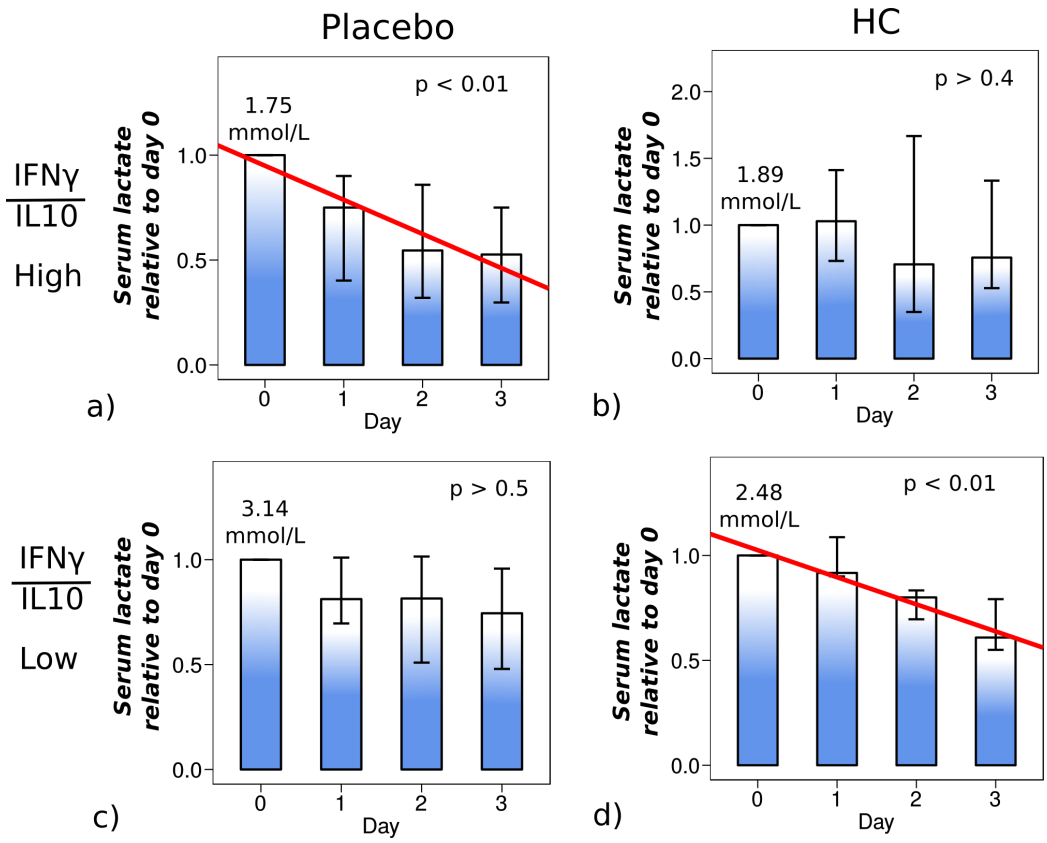


Figure 4.4: Baseline adjusted lactate levels, i.e. values for days 1-3 relative to day 0 for each patient are presented. Absolute serum lactate levels and p-values for the change over time in all available patients of the corresponding sub-group are reported in each panel. Patients in panels (a) and (d) reflect those in compliance with the treatment rule. For patients with high IFNγ/IL-10, serum lactate decreased significantly in the placebo arm (P<0.01 n=11) (a), while its time course was rather heterogenous in the HC arm (n=13) (b). Among the low-ratio patients, there was a tendency of decrease in the placebo arm, however, not significant (n=12) (c), in contrast to a significant decrease in the HC arm (P<0.01, n=5) (d).

donors was spiked with *E. coli* fragments from clinical isolates across a broad range of concentrations, mimicking the immunological load. As expected, with increasing load, IFNγ and IL-10 levels increased, but at varying degree. In terms of the ratio kinetics, the inverse behaviour was observed, i.e. a high IFNγ/IL-10 was observed for low pathogen concentrations and *vice versa* with an "on-off" kinetics (Figure 4.6). This ratio kinetics confirms the hypothesis that a higher ratio associates with a lower immunological load.

4.1.6 Genes for immune response and proliferation are upregulated in patients with initial high IFNγ/IL-10

In the following, patients with an initial high ratio of IFNγ/IL-10 will be denoted as "high ratio" patients. Using microarrays, the differential gene expression

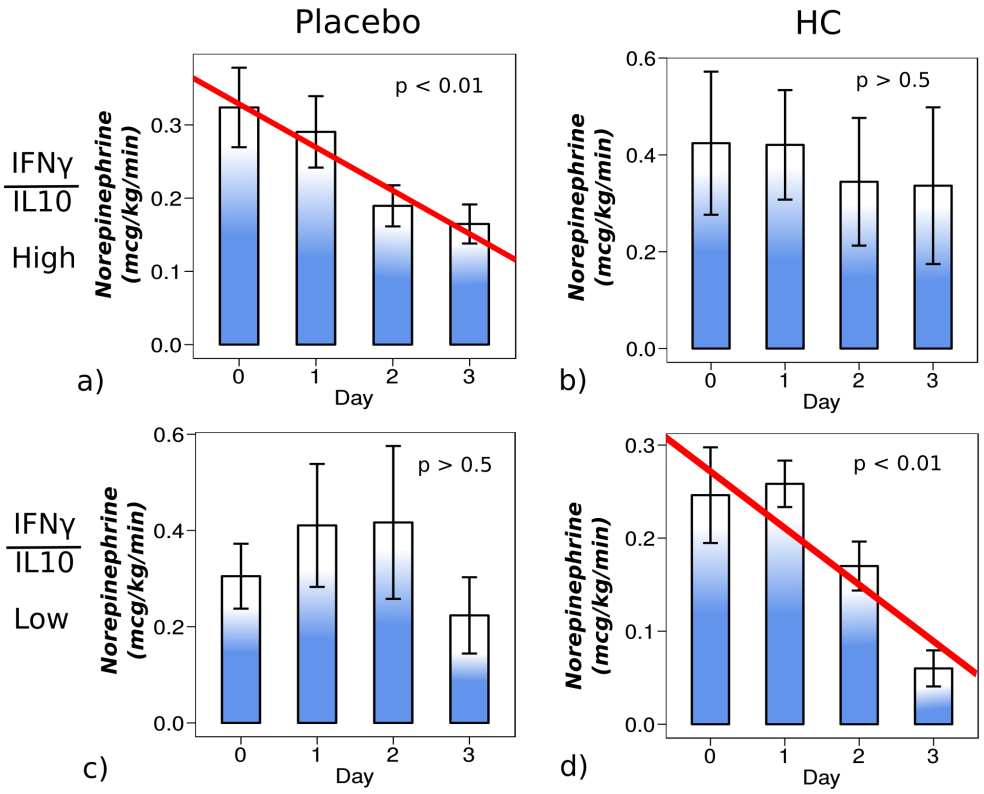


Figure 4.5: Norepinephrine requirement for upto 3 days from enrollment is presented. Patients in panels (a) and (d) reflect those in compliance with the treatment rule. For patients with high IFNγ/IL-10, NE requirement decreased significantly in the placebo arm (P<0.01 n=21) (a), while its time course was rather heterogenous in the HC arm (n=29) (b). Among the low-ratio patients, there was a heterogeneous trend in the placebo arm (n=20) (c), in contrast to a significant decrease in the HC arm (P<0.01, n=21) (d). Putting the compliant arms together (a and d), the rate of decrease over time was highly significant (p<2e-05), but not significant in the non-complaint arm (panel b and c), p>0.5).

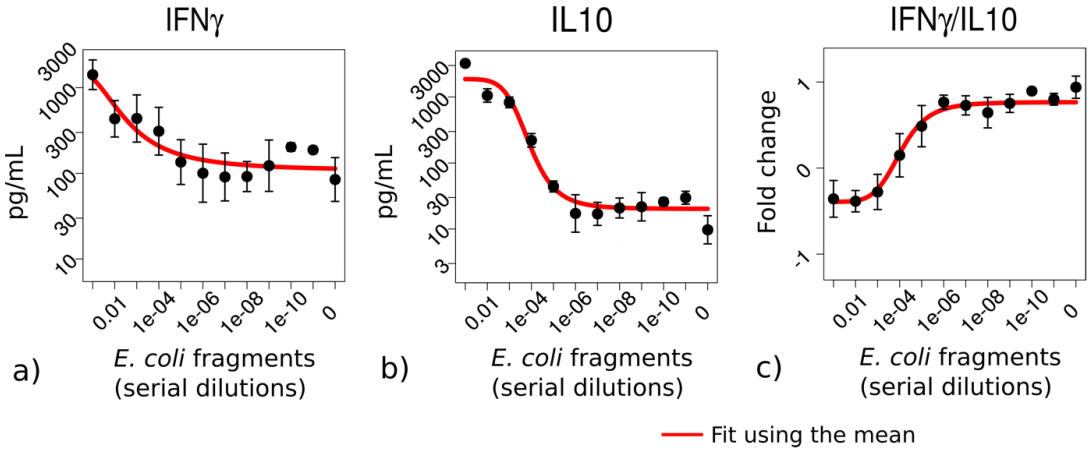


Figure 4.6: Whole blood from five healthy donors was challenged with serial dilutions of *E. coli* fragments mimicking the immunological load. (a) IFNγ and (b) IL-10 elevated with increasing bacterial load while (c) their ratio showed the opposite behaviour, i.e. a high load was associated with a lower ratio.

of whole blood samples (drawn at baseline) was studied between high-ratio and low-ratio patients. 689 genes were up- and 359 down-regulated in high-ratio patients. Most prominently, upregulated genes in high-ratio patients were significantly enriched in immune response (14 gene sets, 151 genes), cell division (15 gene sets, 128 genes) and DNA repair (11 gene sets, 45 genes) categories. The five topmost significant gene sets from each category are shown in Table 4.7. Interestingly, about 20% of the upregulated genes were associated with proliferative processes. There were not any gene sets being enriched with down regulated genes.

Table 4.7: Enriched sets of upregulated genes in initial *high-ratio* versus *low-ratio* patients

*	Gene set	ID	Adjusted p-value	Sig. genes
IM	viral transcription	GO:0019083	6.80E-08	25
IM	type I interferon signaling pathway	GO:0060337	8.04E-05	13
IM	defense response	GO:0006952	8.65E-04	79
IM	innate immune response	GO:0045087	1.69E-03	49
IM	immune response	GO:0006955	3.25E-03	80
CD	DNA replication	GO:0006260	1.40E-09	36
CD	telomere maintenance via recombination	GO:0000722	2.43E-07	11
CD	mitotic cell cycle	GO:0000278	1.70E-06	67
CD	G1/S transition of mitotic cell cycle	GO:0000082	3.24E-06	26
CD	cell cycle	GO:0007049	5.52E-06	98
DR	DNA repair	GO:0006281	9.40E-07	44
DR	DNA synthesis involved in DNA repair	GO:0000731	6.48E-06	14
DR	translesion synthesis	GO:0019985	2.87E-04	9
DR	mismatch repair	GO:0006298	3.29E-04	8
DR	nucleotide-excision repair, DNA gap filling	GO:0006297	3.98E-04	7
DR	error-prone translesion synthesis	GO:0042276	1.04E-03	6
TR	rRNA processing	GO:0006364	1.19E-04	25
TR	translational initiation	GO:0006413	1.53E-04	21
TR	ribonucleoprotein complex biogenesis	GO:0022613	1.93E-04	36
TR	translation	GO:0006412	1.42E-03	42
TR	translational termination	GO:0006415	1.88E-02	11
SR	cellular response to stress	GO:0033554	2.28E-04	94
SR	endoplasmic reticulum unfolded protein response	GO:0030968	1.50E-02	13
SR	response to endoplasmic reticulum stress	GO:0034976	2.50E-02	20
SR	cellular response to stress	GO:0033554	2.28E-04	94
SR	endoplasmic reticulum unfolded protein response	GO:0030968	1.50E-02	13
SR	response to endoplasmic reticulum stress	GO:0034976	2.50E-02	20
MC	DNA metabolic process	GO:0006259	1.94E-08	72
MC	protein targeting to membrane	GO:0006612	8.60E-05	21
MC	nuclear-transcribed mRNA catabolic process	GO:0000956	2.66E-04	21
MC	single-organism intracellular transport	GO:1902582	6.83E-04	52
MC	aromatic compound catabolic process	GO:0019439	1.53E-03	32

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Table 4.7 – *Continued from previous page*

*	Gene set	ID	Adjusted p-value	Sig. genes

** Categories: IM - Immune response, CD - Cell division, DR - DNA repair, TR - Translation, SR - Stress response, MC - Miscellaneous*

4.1.7 Upregulated genes in patients with high IFNγ/IL-10 overlap significantly with gene signatures of activated T-cells and macrophages

To elucidate if innate or adaptive immune cells express IFNγ and IL-10, their expression in different immune cell types from healthy donors (data was taken from [Petryszak et al., 2016]) was studied. IL-10 was found to be mainly expressed in innate immune cells like macrophages and neutrophils, while, IFNγ was more expressed in T-cells, together with natural killer and dendritic cells linking innate and adaptive immunity (Figure 4.7), suggesting both immune systems to be involved in the regulation of IFNγ/IL-10.

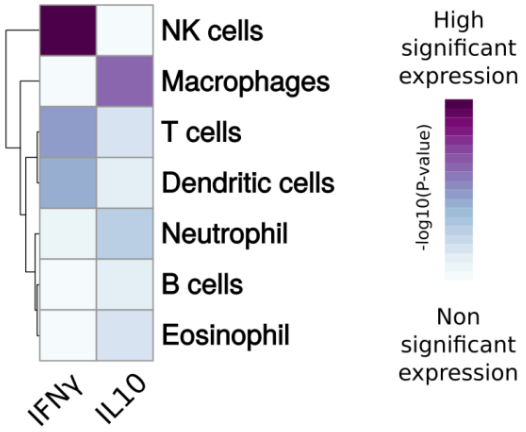

Figure 4.7: Data was taken from the Expression Atlas [Petryszak et al., 2016]. It was used to compare gene expression in different immune cell types testing upregulation using *one sided t-tests*. The intensity levels for the heatmap are represented as $-log_{10}(p - values)$.

Given these observations, it was hypothesized that the active state of these major immune cell types might have contributed to the list of upregulated genes in high-ratio patients. To test this hypothesis, the gene list was compared to gene expression signatures of activated macrophages (representing innate immunity) and activated T-cells (representing the adaptive immune system). Strikingly, genes of interest were highly represented in the signatures of both cell types (T-cells: 301 genes, P<3e-22; Mγ macrophages: 71 genes, P<2e-10, Table 4.8). The observed overlap with activated T-cells was mainly due to cell division and DNA repair (The

five topmost significant gene sets from each category are shown in Table 4.9). In contrast, the gene overlap with activated macrophages showed gene sets of immune response, such as interferon-gamma-mediated signalling, cytokine-mediated signalling and antigen processing and presentation (The ten topmost significant gene sets from each category are shown in Table 4.10).

Table 4.8: Overlapping upregulated genes of initially IFNγ/IL-10 high patients with T-cell and IFNγ-macrophage activation signatures

P < 3e-22	**Upregulated genes in IFNγ/IL-10 high**	**Not upregulated genes in IFNγ/IL-10 high**
Upregulated genes in activated T-cells	301	409
Not upregulated genes in activated T-cells	312	9,573

P < 2e-10	**Upregulated genes in IFNγ/IL-10 high**	**Not upregulated genes in IFNγ/IL-10 high**
Upregulated genes in activated IFNγ macrophages	71	679
Not upregulated genes in activated IFNγ macrophages	48	11,593

Table 4.9: Enriched sets of upregulated genes in patients with high IFNγ/IL-10 overlapping with upregulated genes in activated T-cells

*	**Gene set**	**ID**	**Adjusted p-value**	**Sig. genes**
IM	type I interferon signaling pathway	GO:0060337	8.09E-04	8
IM	regulation by virus of viral protein levels in host cell	GO:0046719	1.12E-03	4
IM	somatic hypermutation of immunoglobulin genes	GO:0016446	1.73E-03	4
IM	viral transcription	GO:0019083	1.01E-02	10
CD	DNA-dependent DNA replication	GO:0006261	7.53E-11	20
CD	cell cycle G1/S phase transition	GO:0044843	7.06E-10	25
CD	DNA strand elongation	GO:0022616	1.51E-06	7
CD	regulation of cell cycle	GO:0051726	1.69E-05	42
CD	DNA biosynthetic process	GO:0071897	3.46E-05	16
DR	interstrand cross-link repair	GO:0036297	7.38E-03	6
DR	error-prone translesion synthesis	GO:0042276	1.12E-02	4
DR	DNA ligation	GO:0006266	1.77E-02	4
DR	strand displacement	GO:0000732	4.45E-02	4

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Table 4.9 – *Continued from previous page*

*	Gene set	ID	Adjusted p-value	Sig. genes
SR	cellular response to stress	GO:0033554	1.70E-05	64
TR	mitochondrial translation	GO:0032543	2.53E-05	13
MC	protein modification by small protein conjugation	GO:0032446	1.01E-03	35
MC	positive regulation of protein ubiquitination involved in ubiquitin-dependent protein catabolic process	GO:2000060	1.28E-03	9
MC	positive regulation of gene expression, epigenetic	GO:0045815	9.26E-03	7
MC	protein-DNA complex assembly	GO:0065004	1.10E-02	12
MC	macromolecular complex subunit organization	GO:0043933	1.15E-02	55

** Categories: IM - Immune response, CD - Cell division, DR - DNA repair, TR - Translation, SR - Stress response, MC - Miscellaneous*

Table 4.10: Enriched sets of upregulated genes in patients with high IFNγ/IL-10 overlapping with upregulated genes in activated macrophages

*	Gene set	ID	Adjusted p-value	Sig. genes
IM	cytokine-mediated signaling pathway	GO:0019221	3.02E-10	18
IM	type I interferon signaling pathway	GO:0060337	5.66E-10	9
IM	immune system process	GO:0002376	2.47E-08	34
IM	regulation of innate immune response	GO:0045088	3.57E-08	14
IM	defense response to virus	GO:0051607	3.43E-07	10
IM	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	GO:0002479	1.20E-06	7
IM	cytokine production	GO:0001816	1.80E-05	14
IM	interferon-gamma-mediated signaling pathway	GO:0060333	3.94E-05	6
IM	tumor necrosis factor-mediated signaling pathway	GO:0033209	2.12E-04	7
IM	activation of immune response	GO:0002253	4.50E-04	11
MC	protein deubiquitination	GO:0016579	4.46E-04	8
MC	regulation of cellular ketone metabolic process	GO:0010565	1.56E-03	6
MC	protein polyubiquitination	GO:0000209	9.50E-03	7
MC	negative regulation of protein modification process	GO:0031400	2.46E-02	9

** Categories: IM - Immune response, MC - Miscellaneous*

4.1.8 HC upregulates immune response in patients with low IFNγ/IL-10

According to the treatment rule, HC treatment improves survival in patients with low IFNγ/IL-10 ("responders" in the following). To study the change in regulation in this group upon HC treatment, gene expression at 24 h after the first HC treatment was compared to 0 h, specifically in the responders (and not differentially expressed in the non-responders). The comparison identified 301 up- and 210 genes being down-regulated. Gene set enrichment analysis revealed that most of the upregulated genes belonged to the immune system. The up- and down-regulated enriched gene sets are shown in Table 4.11 and Table 4.12, respectively.

Table 4.11: Enriched sets of upregulated genes (24h versus 0h) of the responders

*	Gene set	ID	Adjusted p-value	Sig. genes
IM	defense response	GO:0006952	2.76E-03	44
IM	regulation of defense response	GO:0031347	3.21E-03	26
IM	cytokine production	GO:0001816	4.00E-03	25
IM	immune response	GO:0006955	1.06E-02	44
IM	innate immune response	GO:0045087	2.76E-02	27
IM	cellular response to biotic stimulus	GO:0071216	3.46E-02	11
IM	immune system process	GO:0002376	3.47E-02	59
IM	response to molecule of bacterial origin	GO:0002237	3.79E-02	15
IM	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent	GO:0002480	3.95E-02	3
IM	response to abiotic stimulus	GO:0009628	2.27E-02	34
MC	organic cyclic compound catabolic process	GO:1901361	3.04E-02	19
MC	regulation of multicellular organismal process	GO:0051239	4.78E-02	63

** Categories: IM - Immune response, MC - Miscellaneous*

Table 4.12: Enriched sets of downregulated genes (24h versus 0h) of the responders

*	Gene set	ID	Adjusted p-value	Sig. genes
MC	cellular modified amino acid biosynthetic process	GO:0042398	4.86E-02	5

** Categories: MC - Miscellaneous*

Strikingly, this was very similar to the list from the previous section when comparing initial IFNγ/IL-10 high- versus low- patients. Indeed, a significant overlaps (Table 4.13) of the two gene lists for upregulation (n = 24, P<3e-03) and downregulation (n = 23, P<1e-09) was observed. Again, mainly genes for the

immune response were commonly upregulated. Notably, this included all three members of the butyrophilin subfamily 3, named BTN3A1, BTN3A2 and BTN3A3. Commonly downregulated genes did not show any significantly enriched gene set. In summary, gene expression of the responders suggests recovery of immune cell function on the gene regulatory level, similar to the initial high-ratio patients for which no HC treatment is recommended by the treatment rule.

Table 4.13: Overlapping upregulated genes of initially IFNγ/IL-10 high patients with the upregulated genes of the responders after 24 hours

P < 3e-03	**Upregulated genes in patients with high IFNγ/IL-10 before treatment**	**Not upregulated genes in patients with high IFNγ/IL-10 before treatment**
Upregulated genes in the responders	24	277
Not upregulated genes in the responders	656	15072

P < 1e-09	**Downregulated genes in patients with high IFNγ/IL-10 before treatment**	**Not downregulated genes in patients with high IFNγ/IL-10 before treatment**
Downregulated genes in the responders	23	187
Not downregulated genes in the responders	336	15492

4.1.9 The corticotropin stimulation test

The corticotropin stimulation test (a.k.a ACTH test) has been used to assess the adrenal functions and hence in the decision-making of hydrocortisone treatment. However, poor response to corticotropin stimulation is no longer recommended for hydrocortisone treatment in septic shock patients [Annane et al., 2002]. To improve the performance of this test, additional machine learning concepts were implemented to stratify for treatment using serum hydrocortisone at baseline and after corticotropin stimulation. In line to the results of the original study [Sprung et al., 2008], the corticotropin test failed to predict a better response to treatment.

The best performance resulted in OR = 0.92 [95% CI: 0.35-2.40] as shown in Figure 4.3b.

4.2 The longitudinal sex-dependent transcriptome modulation study

4.2.1 Stratifying severity in patients followed by transcriptomic analysis

For the studied 132 patients, the difference in their Abbreviated Injury Scale (AIS) score at admission was non-significant between sexes (male patients: AIS=3.99 (95% CI: 3.81-4.17); female patients: AIS=4.15 (95% CI: 3.91-4.39), 4.8A). Independent from gender, heterogeneous severity profiles were observed when comparing them in respect to the temporal progression. To address this, synchronization was performed in such a way that the day of highest disease severity (peak of the MOF scores, the most severe day) was matched for all profiles. After synchronization, the distribution of the day of highest severity was comparable between male and female patients (Figure 4.9). Accordingly, also the transcription profiles were synchronized by the day with the maximal MOF score. The synchronization provided a distinct phase of increasing severity (increasing MOF), highest severity (MOF peak) and declining severity (declining MOF), as shown in Figure 4.8B.

As the patients showed this strong increase and decrease of severity within a seven days window, this window of 7-days, i.e. 3 days before the most severe day, the most severe day and 3 days after the most severe day was the focus in the following analysis. During this window, male patients showed a significantly ($p<0.01$) higher degree of organ dysfunction than female patients. For simplicity, the phases were binned into five sets comprising 'early pre-acute' (2 to 3 days prior to the most severe day), 'acute phase' (the most severe day), 'pre-acute' (1 day prior to the acute phase), 'post-acute' (1 day after the acute phase) and 'late post-acute' (2 to 3 days after the acute phase).

For each phase differential expression analysis was carried out to obtain differentially expressed gene sets between male and female patients. These gene sets were grouped according to their significance across the temporal phases. The functional relevence of the clusters was derived based on the biological interpretation of their consisting gene sets [Kolte and König, 2019].

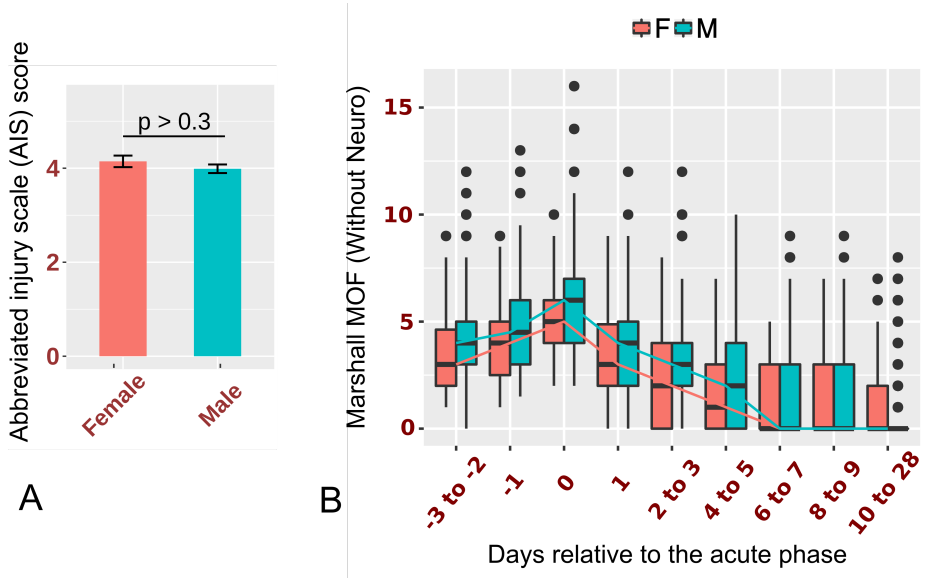


Figure 4.8: (A) At admission, the abbreviated injury scale (AIS) score was comparable between the sexes (male patients: AIS=3.99 (95% CI: 3.81-4.17), female patients: AIS=4.15 (95% CI: 3.91-4.39; p > 0.3)). (B) The Marshall MOF score (without neurological components) of the synchronized profiles was higher in male patients at the later time points.

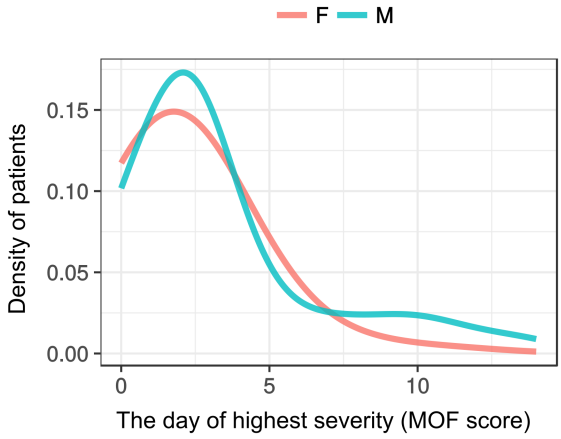


Figure 4.9: Male and female patients showed a similar distribution of the highest MOF score after baseline. To note there was no significant (p>0.1, *Wilcoxon rank-sum test*) difference in the distributions.

4.2.2 The innate immune response is upregulated in the early pre-acute phase of female patients

The early pre-acute phase was defined as the period of 2 to 3 days prior to the acute phase. As expected from the synchronization, there was a significant (p<0.0001) rate of increase in the MOF score from this phase to the acute phase across male and female patients. The transcriptomics comparison at this phase revealed a distinct higher regulation of a broader set of genes associated with an *acute-phase response* in

female patients compared to male patients. A total of seven gene sets related to the innate immune response were identified in this cluster specific to females in the early pre-acute phase. These included *positive regulation of NF-κB transcription activity*, *myeloid dendritic cell differentiation* and *chemotaxis*, *cytokine response*, and, more specifically *IL-7 mediated signaling*. Figure 4.10A shows the temporal profiles of all identified gene sets corresponding to the innate immune response. Appendix Figure A.2 shows all gene sets of this cluster which were upregulated in females during this phase.

In line, another cluster was identified consisting of upregulated gene sets in female patients. In this cluster, gene sets were upregulated in at least two of the first three temporal phases of the disease, i.e. in the early pre-acute, pre-acute and acute phases. Also, this cluster contained innate immunity-related gene sets, such as *cellular response to tumour necrosis factor*, *positive regulation of ERK1-ERK2 cascade*, and *neutrophil chemotaxis*. Appendix Figure A.1 shows the temporal profiles of all gene sets in this cluster. The expression of genes from these seven gene sets of innate immunity from the first cluster was compared to their expression in healthy controls and observed that in both sexes these gene sets were upregulated, and in the female patients, these sets were significantly more upregulated (Figure 4.10B). The other gene sets of both clusters contained sets for further signaling processes like phosphatidylinositol mediated signaling, transcriptional regulatory processes, protein modification and neural processes (Appendix Figure A.2 and A.1).

4.2.3 Wound healing and recovery processes are upregulated early in female patients

During the pre-acute and the acute phase, a distinct upregulation of recovery processes was observed in female patients. The upregulated gene sets at the pre-acute phase were predominantly associated with the stages of early wound healing such as tissue restoration and blood coagulation. These included *positive regulation of vasoconstriction*, *platelet activation*, *blood coagulation, intrinsic pathway* and *platelet degranulation*. The process of *fibrinolysis* was also upregulated in female patients. Simultaneously, an upregulation of cellular processes governing *leukocyte migration* and *positive regulation of phagocytosis*, together with signaling processes such as *positive regulation of MAPK cascade* in female patients was observed. There were eleven gene sets identified in this cluster related to the early

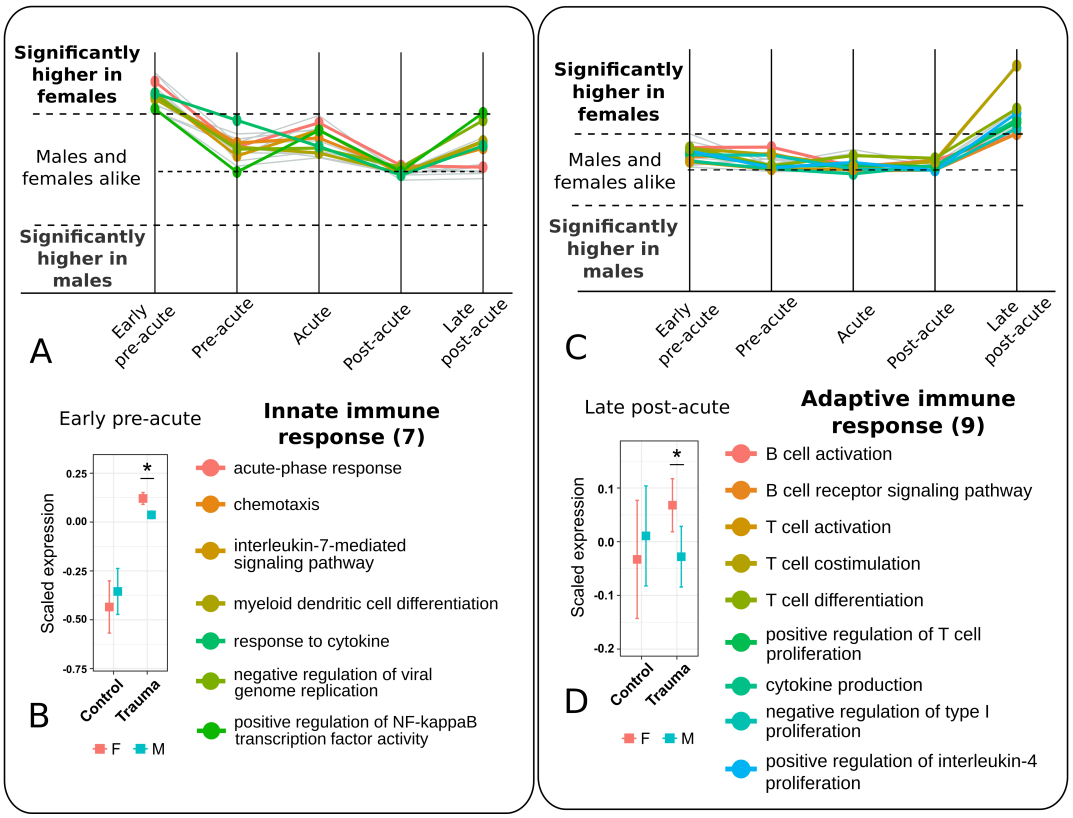


Figure 4.10: (A) The cluster of the early pre-acute phase consisted of 16 gene sets being upregulated in female patients. They comprised seven gene sets of the innate immune response. These gene sets are shown here. The complete list is given in Appendix Figure A.2. (B) For the same phase, in comparison to healthy controls, leukocytes of both sexes showed a higher expression of genes for the innate immune response. A distinctively higher expression was observed in female patients. (C) Gene set clusters of the late post-acute phase consisted of 17 gene sets being upregulated in female patients, nine were related to the adaptive immune response. These gene sets are shown here. The complete list is given in Appendix Figure A.3. (D) For the same phase, in comparison to healthy controls, leukocytes of female patients showed upregulation of the adaptive immune response. These gene sets were similarly expressed in male patients and healthy controls. The significance of difference between male and female was tested using *two-sided Student's t-tests* (* $P<0.05$, mean bar and Whiskers of the boxplots: mean±se).

wound healing response. Figure 4.11A shows the temporal profiles and lists of representative gene sets. The complete cluster is given in Appendix Figure A.4. During the acute phase, the most prominent upregulated gene sets in leukocytes of female patients were growth and development related gene sets. These included *anterior/posterior pattern specification*, *artery morphogenesis*, *skeletal system development* and gene sets for several neural processes. Upstream to these, *response to IL-1 signaling* and *cytokine-mediated signaling* were also upregulated. This cluster consisted of eleven gene sets for growth and developmental processes. Figure 4.11C shows the temporal profiles and lists of representative gene sets, while the complete cluster is given in Appendix Figure A.5. To note, the eleven gene sets for

early wound healing in the pre-acute phase and another eleven gene sets for growth and developmental processes in the acute phase were upregulated in male and female patients, when compared to healthy individuals. Still, these gene sets showed significantly higher expression in female compared to trauma male patients (Figure 4.11B, D).

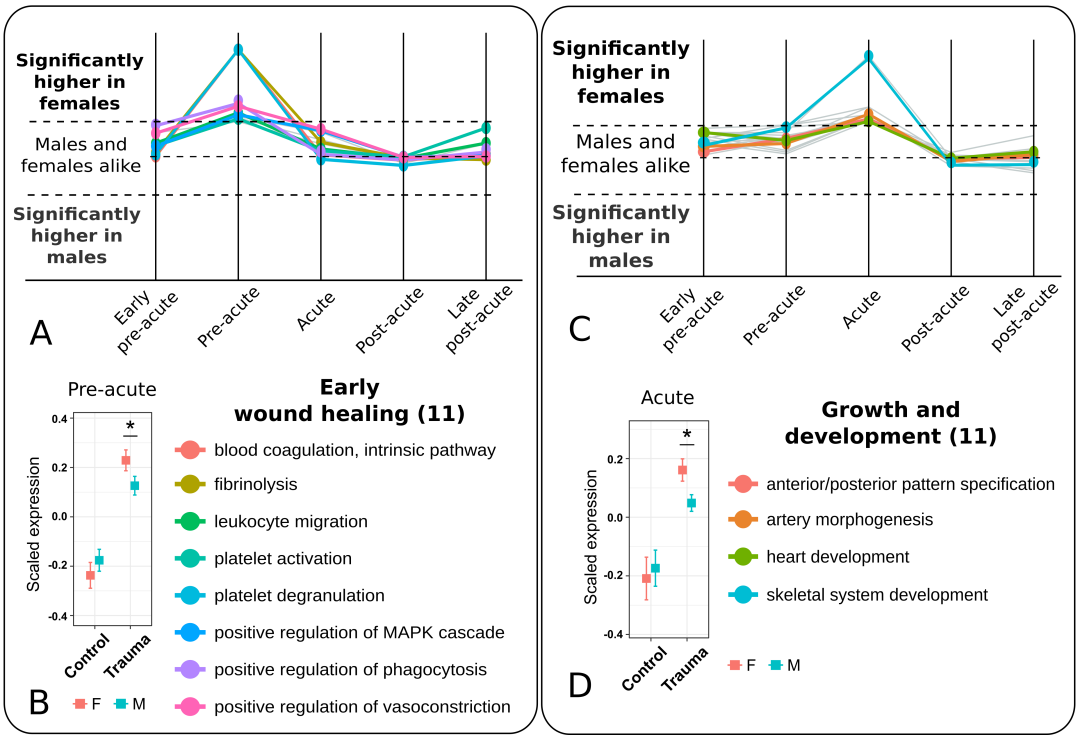


Figure 4.11: (A) The cluster of the pre-acute phase contained 11 gene sets being upregulated in leukocytes of female patients. These gene sets are all related to wound healing processes. Eight representative gene sets are shown here. The complete set is given in Appendix Figure A.4. (B) In comparison to healthy controls, the gene sets were higher expressed in the leukocytes of both sexes. A distinctively higher expression was observed in the leukocytes of female patients. (C) The cluster of the acute phase contained 19 gene sets being upregulated in female patients. Eleven of these were associated with growth and development. Four representative gene sets are listed here. The complete list is given in Appendix Figure A.5. (D) In comparison to healthy controls, leukocytes of both sexes showed a higher expression of these gene sets, distinctively higher expression was observed in leukocytes of female patients. The significance of difference between male and female was tested using *two-sided Student's t-tests* (* $P<0.05$, mean bar and whiskers of the boxplots: mean±se).

4.2.4 During the pre-acute and acute phases, genes for transcription and translation, cell cycle, DNA damage and repair and oxidative phosphorylation are downregulated in female patients

Two third of the identified sex-specific transcriptomic gene sets were downregulated in female compared to male patients. Most of these gene sets were observed within the first three phases, i.e. during the early pre-acute, pre-acute and acute phases. These gene sets were grouped into two larger clusters. One cluster comprised gene sets that were downregulated in at least two of first three phases i.e. early pre-acute, pre-acute and acute phase, (Figure 4.13), and the other cluster comprised gene sets which were downregulated only in the acute phase (Appendix Figure A.6).

The first cluster contained gene sets related to cell cycle, transcription and translation, DNA damage and repair and energy metabolism including oxidative phosphorylation (OxPhos). Compared to healthy individuals, the expression of these gene sets was downregulated in both sexes of the patients. Figure 4.13 shows the temporal profiles and their representative gene sets. The complete cluster is given in Appendix Figure A.6. Still, these gene sets were distinctively more downregulated in female than in male patients.

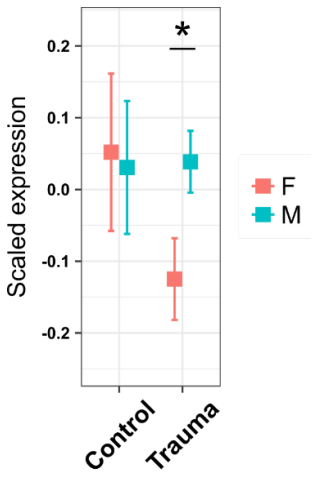


Figure 4.12: In comparison to healthy individuals, energy metabolism gene sets were downregulated in female patients during the acute phase. The expression of the genes from these gene sets in male patients was comparable to healthy controls (* P<0.05, *two-sided Students' t-test*; mean±se).

ATP producing processes such as *regulation of glycolytic processes*, *TCA cycle*, *fatty acid beta-oxidation* and *electron transport chain* were significantly down regulated in female, compared to male patients. In line, ATP consuming processes were also downregulated in female patients, such as *DNA repair by excision mechanisms*, *RNA processing*, and specifically *transcription via RNA polymerase I, II and III*, *RNA transport*, *RNA splicing*, *tRNA processing*, *tRNA modification*, *RNA metabolism* etc. and gene sets for cell cycle comprising *DNA replication to telomerase maintenance*, *transition of mitotic cell cycle*, *mitotic spindle assembly checkpoint*, *chromosome segregation* and

anaphase promoting complex-dependent catabolic process.

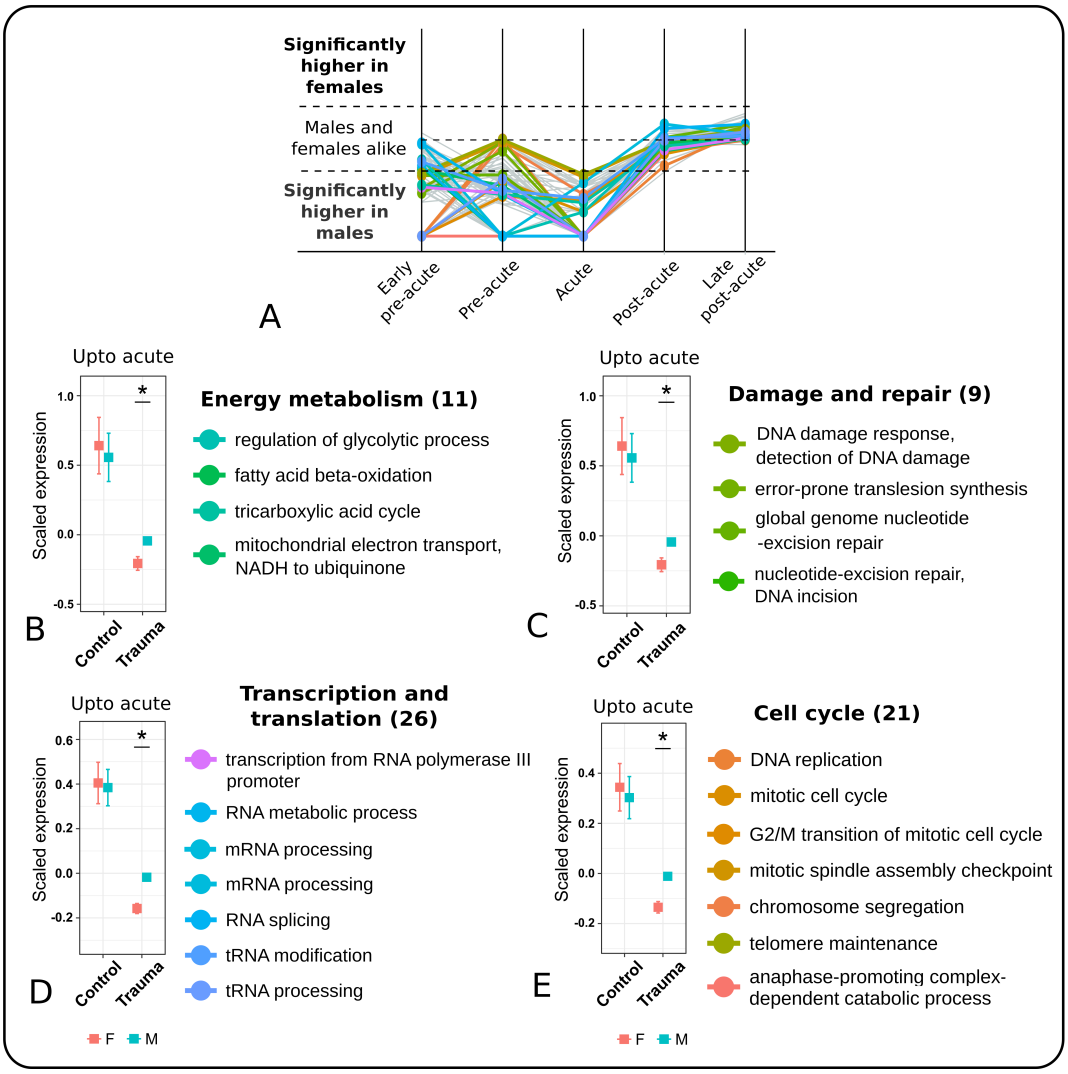


Figure 4.13: (A) The cluster consisted of 85 gene sets being downregulated in female patients. Most of these were associated to (B) energy metabolism and the mitochondrion (11 gene sets), (C) cell cycle (21 gene sets), (D) transcription and translation (26 gene sets), and (D) damage and repair (9 gene sets). For each category, three representative gene sets are listed here. The complete list is given in A.7. In comparison to healthy controls, for all categories, leukocytes of both sexes showed a lower expression of these gene sets, but relatively lower expression was observed in female patients. The significance of this difference between male and female was tested using *two-sided Student's t-tests* (* P<0.05, mean bar and whiskers of the boxplots: mean±se).

The second cluster comprised gene sets which were again related to transcription and translation, DNA damage and repair, and energy-metabolism (Appendix Figure A.6). In comparison to healthy individuals, these gene sets were downregulated in male and female patients during the acute phase, except for energy metabolism related gene sets. The gene expression of energy metabolism related gene sets in male patients was comparable to the healthy individuals (Figure 4.12). In summary, cell cycle, repair, and energy producing and ATP consuming processes were

distinctively downregulated in female, compared to male patients in the pre-acute and acute phases.

4.2.5 Genes coding for the adaptive immune response are distinctively higher expressed in the late post-acute phase of female patients

After the acute phase, as expected from the synchronization, the MOF scores declined at a significant rate ($p<0.0001$) reflecting a phase of recovery in male and female patients. In female patients, the recovery was observed also at the molecular level. In the late post-acute phase i.e. 2 to 3 days after the acute phase, a distinct upregulation of genes of the adaptive immune system was observed in female patients. This comprised *T cell activation*, *T cell co-stimulation*, *T cell differentiation* and *positive regulation of T cell proliferation*. Additionally, in female patients, a distinct upregulation of *cytokine production*, and particularly of *IL-4 production* was observed, together with the upregulation of *negative regulation of type I interferon production*. Also, gene sets related to *B cell activation*, *B cell receptor signaling* was upregulated in female, compared to male patients. Figure 4.10C shows the temporal profiles of gene sets from the adaptive immunity cluster. This cluster consisted of nine gene sets out of which five representatives are listed. The complete cluster is given in Appendix Figure A.3. Comparing the expression profiles with the profiles of healthy individuals, showed that these nine gene sets for adaptive immunity were upregulated only in female patients. Male patients expressed these comparable to the controls (Figure 4.10D). In summary, leukocytes of female patients showed a distinct higher expression of genes being responsible for restoring the adaptive immune system during this late post-acute phase.

4.2.6 The divergent regulation can be attributed to gender dimorphism rather than to the different disease severities

Male patients showed more MOF compared to female patients in the acute phase. Thus, the identified transcriptomic differences between male and female patients could have been due to the differences in the severity but not directly associated with gender dimorphism. To justify if the identified transcriptomic differences can be attributed to the sexes but not to the severity alone, an analysis

was performed in which a subset of transcription profiles (n=28 female, n=23 male patients) from the acute phase were selected in such a way that the MOF scores and AIS scores of the corresponding male and female patients were comparable at the acute phase. Comparing the transcriptional profiles of male versus female samples showed 123 gene sets to be significantly differentially expressed, out of which 114 (93%) were identical to the previously identified gene sets during the acute phase (Figure 4.14). As expected, the downregulated gene sets in female patients were associated with transcription and translation, cell cycle, DNA damage and repair, and oxidative phosphorylation, while upregulated gene sets were related to innate immune response, and growth and development. In summary, the identified gene sets by comparing male versus female patients were not due to the differences in the severities but the gender dimorphism itself.

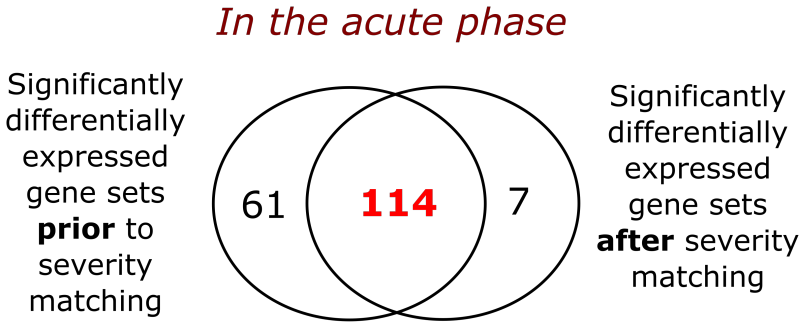


Figure 4.14: Comparison of differentially expressed gene sets prior and after severity matching in the acute phase.

4.2.7 Similar gender dimorphism of gene regulation in the blood of trauma patients and patients with severe burn injury

The transcription profiles of patients with severe burn injuries were studied to validate the main findings from the trauma study. The patients were 16-50 years old with injury severity scores above 25. As the longitudinal severity scores were not available for synchronization, the transcriptomic response of the first week after burn injury was investigated. The transcriptomic gender dimorphism in these patients was found comparable to the pre-acute and acute phase of trauma patients. This included upregulation of the innate immune response, wound healing and growth processes, and downregulation of transcription and translation, cell cycle, DNA damage and repair and energy metabolism in female patients with burn injury (Figure 4.15).

The adaptive immune response in these patients was overall suppressed and identical between sexes during the first week of burn injury. Notably, female patients showed significant upregulation of the adaptive immune response after the first week. These results support the main findings observed for patients after blunt trauma.

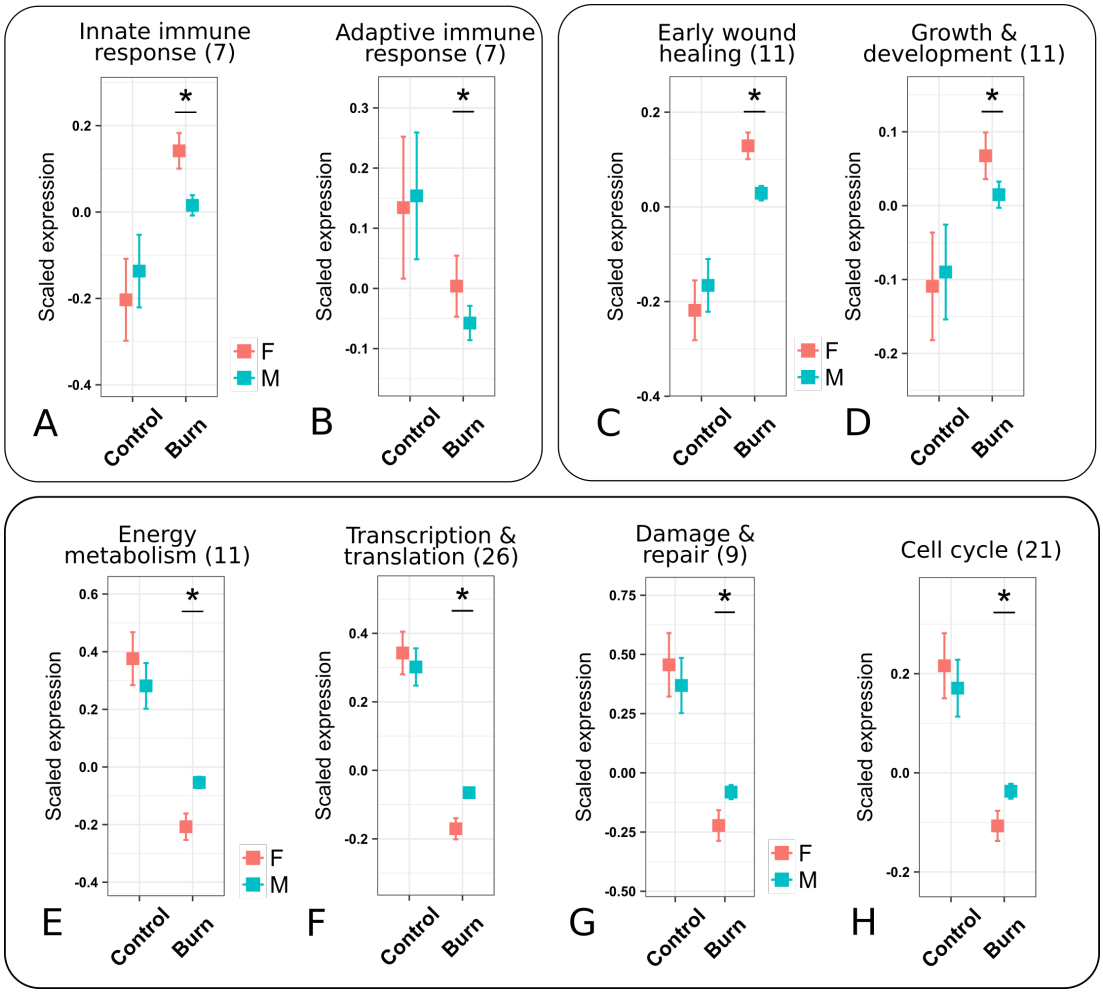


Figure 4.15: The figure depicts the expression levels in patients with severe burn injury and healthy individuals for the identified sex-specific gene sets in the early pre-acute, pre-acute, acute and late post-acute phases of the trauma patients. A) and B) represent the immune response related modules and their expression levels. C) and D) represent the healing response and, growth and development modules. E) to H) represent the downregulated gene sets related to the bioenergetics and housekeeping molecular processes. The significance of difference between male and female was checked using *two-sided Student's t-tests* (* P<0.05, mean bar and whiskers of the boxplots: mean±se).

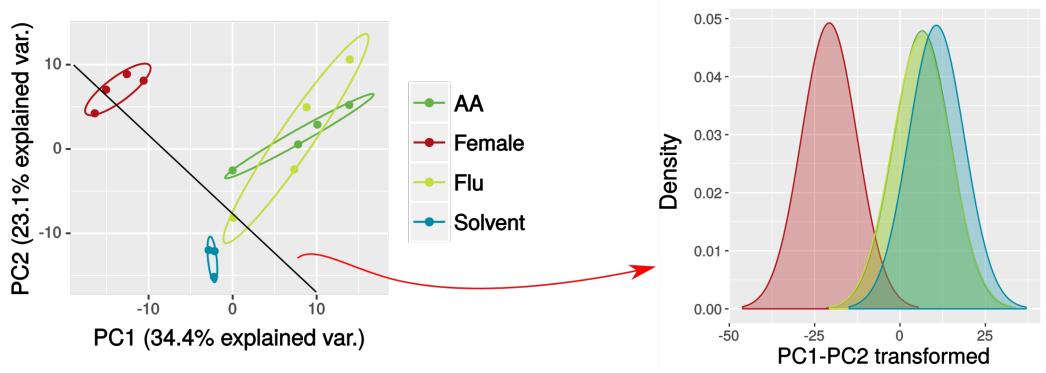
4.3 The sex-hormone modulation study in mice

4.3.1 The treatments induce a transcriptomic shift in septic male mice towards septic female mice

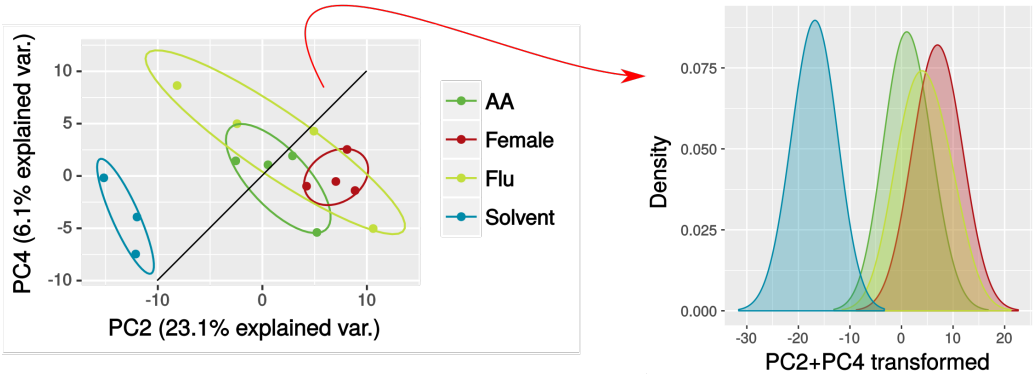
The gender dimorphic septic response and the effects of Atraric acid (AA) or Flutamide (Flu) treatment on septic male mice was interrogated at the transcriptomic level. The total variance in the entire dataset was summarized using principal component analysis (PCA). The first four principal components (PCs) explained nearly 75% variance of the whole dataset. Both, the first and second PCs distinctly separated solvent treated septic male and septic female mice (Figure 4.16). The difference between the treatments was captured in the third component. Furthermore, the first component marginally partitioned between female mice against solvent or compound treated males. The second component, in addition to the male-female separation, clustered the treated male mice to the female side; thereby, indicating transcriptomic shift introduced by compounds in septic males towards females. To further refine the transcriptomic shift caused by the treatments and gender differences, the combination of PCs were used as shown in Figure 4.16. As PCs are linear orthogonal axes, diagonal axis to any two PCs can summarize the information ascribed in them. The gender differences were distinct along the $PC1 - PC2$ axis (Figure 4.16a), while female-like effects of the treatment were apparent from the $PC2 + PC4$ axis (Figure 4.16b). It is important to note that the 'female-like' effects of the treatments are essentially the subset of the gender differences that are influenced by the treatments.

4.3.2 Septic female mice downregulate immune response, redox processes, energy processes and cell death mechanisms in liver

Under septic conditions, the liver transcriptomic response of pre-estrus females differed from males in immune, metabolic and cellular processes. The comparison led to 467 up- and 367 down-regulated genes in females. The immune response was predominately downregulated with distinct downregulation of gene sets such as *acute-phase response, response to lipopolysaccharides* and interleukin response particularly *response to interleukin-1*. This was accompanied by downregulation of energy metabolism. Gene sets such as *generation of precursor metabolites and*



(a)



(b)

Figure 4.16: a) PC1 (explaining 34.4% variance) - PC2 (explaining 23.1% variance) highlights the presence of inherent gender differences unaffected by the treatments. b) while, PC2 (explaining 23.1% variance) + PC4 (explaining 6.1% variance) highlights the proportion of transcriptomic shift induced by the treatments in septic male mice towards septic females. The term 'solvent' here represents the septic male mice treated with solvent.

energy, glucose metabolic processes were downregulated. Apart from glucose metabolism, metabolism of other energy precursors such as *acetyl-CoA, alpha-amino acids* and *fatty acids* were evidently downregulated. The synthesis of energy precursors and break down of complex organic molecules are mainly carried out by mechanisms of *oxidation-reduction process* that result in the synthesis of reactive oxygen species. This cascade was downregulated in the liver of septic females. Despite the downregulation of the energy metabolism, the cell death mechanisms were not upregulated, on contrary, gene sets such as *apoptic process* and the *programmed cell death* were downregulated. There were a plethora of gene sets ascribed to response to various stimuli, such as *organic substances, lipids, oxygen-containing compounds* etc. being downregulated indicating an overall state of desensitization of hepatocytes. Among the upregulated gene sets in septic females were the transcription and translation associated processes such as *RNA metabolic processes, RNA splicing, RNA processing* and *translation initiation*. At most five

top significant gene sets from each category are given below in Table 4.14 and Table 4.15. In summary, septic female mice downregulated immune response, redox processes, energy processes and cell death mechanisms in liver.

Table 4.14: Enriched sets of upregulated genes in the liver of septic females

*	Gene set	ID	Adjusted p-value	Sig. genes
AP	amide biosynthetic process	GO:0043604	2.63E-07	40
AP	organonitrogen compound biosynthetic process	GO:1901566	8.46E-06	52
AP	cellular component biogenesis	GO:0044085	6.22E-03	73
AP	ribonucleoprotein complex biogenesis	GO:0022613	8.55E-03	20
CP	ER-associated ubiquitin-dependent protein catabolic process	GO:0030433	1.94E-04	9
CP	cellular macromolecule catabolic process	GO:0044265	1.63E-03	33
CP	macromolecule catabolic process	GO:0009057	1.84E-03	38
CP	catabolic process	GO:0009056	3.35E-03	54
CP	cellular catabolic process	GO:0044248	3.57E-03	46
HR	cellular response to gonadotropin stimulus	GO:0071371	1.57E-03	5
HR	response to thyroid hormone	GO:0097066	3.38E-03	5
HR	cellular response to follicle-stimulating hormone stimulus	GO:0071372	5.20E-03	4
HR	response to hormone	GO:0009725	1.00E-02	31
MP	cellular amide metabolic process	GO:0043603	3.67E-07	46
MP	cellular lipid metabolic process	GO:0044255	7.82E-05	39
MP	fatty acid metabolic process	GO:0006631	4.41E-04	21
MP	organonitrogen compound metabolic process	GO:1901564	5.32E-04	69
MP	monocarboxylic acid metabolic process	GO:0032787	1.08E-03	26
SR	cellular response to stress	GO:0033554	4.60E-04	57
SR	response to endoplasmic reticulum stress	GO:0034976	1.01E-03	15
TT	RNA processing	GO:0006396	8.96E-08	42
TT	translation	GO:0006412	7.80E-06	34
TT	RNA splicing	GO:0008380	2.11E-05	23
TT	RNA export from nucleus	GO:0006405	4.85E-04	9
TT	regulation of translational initiation	GO:0006446	2.31E-03	8
MC	protein N-linked glycosylation via asparagine	GO:0018279	1.11E-03	6
MC	single-organism catabolic process	GO:0044712	9.73E-03	28

** Categories: AP - Anabolic processes, CP - Catabolic processes, HR - Hormonal response, MP - Metabolic processes, SR - Stress response, TT - Transcription and translation, MC - Miscellaneous*

Table 4.15: Enriched sets of downregulated genes in the liver of septic females

*	Gene set	ID	Adjusted p-value	Sig. genes
AP	lipid biosynthetic process	GO:0008610	2.50E-09	31
AP	carboxylic acid biosynthetic process	GO:0046394	1.53E-05	16
AP	regulation of gluconeogenesis	GO:0006111	1.27E-04	7

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Table 4.15 – *Continued from previous page*

*	Gene set	ID	Adjusted p-value	Sig. genes
AP	protein oligomerization	GO:0051259	1.44E-03	19
AP	organonitrogen compound biosynthetic process	GO:1901566	6.59E-03	36
CD	cell death	GO:0008219	1.07E-04	52
CD	programmed cell death	GO:0012501	4.07E-04	48
CD	apoptotic process	GO:0006915	1.06E-03	46
CD	regulation of cell death	GO:0010941	1.06E-03	41
CD	positive regulation of apoptotic process	GO:0043065	3.44E-03	19
CP	organic acid catabolic process	GO:0016054	4.47E-14	24
CP	carboxylic acid catabolic process	GO:0046395	2.59E-13	22
CP	cellular amino acid catabolic process	GO:0009063	2.33E-10	13
CP	fatty acid beta-oxidation using acyl-CoA dehydrogenase	GO:0033539	3.00E-03	4
CP	nucleobase catabolic process	GO:0046113	3.02E-03	3
EM	generation of precursor metabolites and energy	GO:0006091	7.64E-08	22
EM	hexose metabolic process	GO:0019318	1.31E-07	18
EM	glucose metabolic process	GO:0006006	2.65E-06	15
EM	cellular respiration	GO:0045333	2.85E-04	11
EM	aerobic respiration	GO:0009060	2.87E-04	8
GR	liver development	GO:0001889	2.46E-05	12
GR	regulation of centromere complex assembly	GO:0090230	2.27E-04	3
GR	lipid modification	GO:0030258	4.00E-04	12
GR	aging	GO:0007568	3.83E-03	13
HR	steroid biosynthetic process	GO:0006694	4.06E-07	14
HR	response to peptide hormone	GO:0043434	2.02E-05	23
HR	response to glucocorticoid	GO:0051384	3.56E-04	11
HR	response to insulin	GO:0032868	5.69E-04	16
HR	estrogen biosynthetic process	GO:0006703	1.91E-03	3
HS	chemical homeostasis	GO:0048878	4.45E-06	38
HS	cellular iron ion homeostasis	GO:0006879	9.32E-05	7
HS	cellular transition metal ion homeostasis	GO:0046916	1.10E-04	9
HS	cation homeostasis	GO:0055080	4.18E-03	21
HS	cellular cation homeostasis	GO:0030003	4.33E-03	19
IR	response to interleukin-1	GO:0070555	1.89E-04	10
IR	response to molecule of bacterial origin	GO:0002237	7.06E-04	16
IR	cellular response to lipopolysaccharide	GO:0071222	1.19E-03	11
IR	immune system process	GO:0002376	2.76E-03	54
IR	acute-phase response	GO:0006953	5.61E-03	5
MP	organic acid metabolic process	GO:0006082	1.76E-24	64
MP	organic hydroxy compound metabolic process	GO:1901615	1.42E-09	26
MP	acetyl-CoA metabolic process	GO:0006084	1.07E-04	6
MP	cellular modified amino acid metabolic process	GO:0006575	4.35E-04	10
MP	alpha-amino acid metabolic process	GO:1901605	4.75E-04	12
OS	oxidation-reduction process	GO:0055114	9.40E-04	79
OS	reactive oxygen species metabolic process	GO:0072593	9.75E-04	15

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Table 4.15 – *Continued from previous page*

*	Gene set	ID	Adjusted p-value	Sig. genes
OS	cellular response to oxygen-containing compound	GO:1901701	1.15E-03	34
OS	regulation of reactive oxygen species metabolic process	GO:2000377	2.36E-03	11
RS	response to organonitrogen compound	GO:0010243	1.90E-07	35
RS	response to external stimulus	GO:0009605	2.49E-06	63
RS	response to lipid	GO:0033993	1.34E-05	35
RS	response to oxygen-containing compound	GO:1901700	5.39E-05	49
RS	response to endogenous stimulus	GO:0009719	1.77E-04	47
SP	positive regulation of intracellular signal transduction	GO:1902533	2.54E-04	30
SP	regulation of biological quality	GO:0065008	9.84E-04	76
SP	intracellular signal transduction	GO:0035556	1.94E-03	56
SP	positive regulation of cell communication	GO:0010647	3.23E-03	39
SP	protein kinase B signaling	GO:0043491	4.22E-03	10
SR	response to wounding	GO:0009611	2.88E-05	23
TR	regulation of cellular localization	GO:0060341	2.11E-03	27
MC	single-organism carbohydrate metabolic process	GO:0044723	5.93E-08	26
MC	single-organism catabolic process	GO:0044712	4.35E-04	45
MC	macromolecular complex assembly	GO:0065003	2.58E-03	39
MC	protein complex assembly	GO:0006461	2.81E-03	32
MC	gland development	GO:0048732	3.45E-03	20

** Categories: AP - Anabolic processes, CD - cell death, CP - Catabolic processes, EM - Energy metabolism, GR - Growth response, HR - Hormonal response, HS - Homeostasis, IR - Immune response, MP - Metabolic processes, OS - Oxidative stress, RS - Response to stimuli, SP - Signaling processes, SR - Stress response, TR - Transport, TT - Transcription and translation, MC - Miscellaneous*

4.3.3 Atraric acid treatment induces female-like response by downregulation of immune response, oxidative stress and cell death in the liver of septic male mice

The significant differentially expressed genes that overlapped between the comparison of AA treated septic male *versus* solvent treated septic male and solvent treated septic female *versus* solvent treated septic male in the same up- or down-regulated fashion were referred to as codirectional genes. These were 157 up- and 107 down-regulated genes essentially describing the female-like response of the AA treatment. Female-like upregulated genes were not enriched among any gene sets, however, downregulated genes were found associated with immune response, oxidative stress and cell death mechanisms. The immune response was restricted to

the gene sets of innate immunity such as *response to lipopolysaccharide* and response to pro-inflammatory cytokines such as *interleukin-1 (IL-1)* and *interleukin-6 (IL-6)*. The redox processes were not downregulated however the associated reactive oxygen species metabolic process was present. Interestingly, by AA treatment, liver of male mice also downregulated *programmed cell death* in female-like fashion. The treatment also induced the desensitization of the hepatocytes by downregulation of response to various external stimuli such as *organic substances, endogenous stimuli, lipids, oxygen-containing compounds* etc. Table 4.16 lists the five topmost significant gene sets from each category. In summary, the AA treatment to septic male mice induced a female-like transcriptomic response in the form of downregulation of immune response, oxidative stress and cell death mechanisms in liver.

Table 4.16: Enriched sets of female-like downregulated genes in the liver of AA treated male septic mice

*	Gene set	ID	Adjusted p-value	Sig. genes
CD	cell death	GO:0008219	9.62E-06	25
CD	regulation of cell death	GO:0010941	1.30E-04	20
CD	programmed cell death	GO:0012501	1.35E-04	22
CD	apoptotic process	GO:0006915	3.42E-04	21
CD	positive regulation of apoptotic process	GO:0043065	3.30E-03	10
CP	positive regulation of catabolic process	GO:0009896	5.54E-05	9
CP	positive regulation of protein catabolic process	GO:0045732	4.48E-04	7
CP	positive regulation of cellular catabolic process	GO:0031331	6.39E-04	7
CP	organic substance catabolic process	GO:1901575	9.60E-04	20
CP	catabolic process	GO:0009056	9.90E-04	20
GR	regulation of cellular component movement	GO:0051270	1.09E-03	13
GR	cell migration	GO:0016477	1.33E-03	17
GR	aging	GO:0007568	1.89E-03	8
GR	regulation of cell shape	GO:0008360	8.49E-03	5
HR	response to hormone	GO:0009725	2.91E-03	12
HS	myeloid cell homeostasis	GO:0002262	9.09E-03	5
IR	immune system process	GO:0002376	4.68E-05	26
IR	cellular response to lipopolysaccharide	GO:0071222	9.11E-04	9
IR	response to interleukin-1	GO:0070555	1.03E-03	6
IR	cellular response to interleukin-6	GO:0071354	4.69E-03	3
IR	chemokine-mediated signaling pathway	GO:0070098	6.74E-03	4
MP	positive regulation of macromolecule metabolic process	GO:0010604	1.41E-05	31
MP	positive regulation of cellular metabolic process	GO:0031325	8.75E-04	27
OS	cellular response to oxygen-containing compound	GO:1901701	8.46E-05	17

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Table 4.16 – *Continued from previous page*

*	Gene set	ID	Adjusted p-value	Sig. genes
OS	regulation of reactive oxygen species metabolic process	GO:2000377	1.08E-03	6
OS	reactive oxygen species metabolic process	GO:0072593	1.65E-03	7
RS	response to external stimulus	GO:0009605	2.45E-05	26
RS	response to oxygen-containing compound	GO:1901700	1.05E-04	20
RS	response to lipid	GO:0033993	1.39E-04	15
RS	cellular response to organic cyclic compound	GO:0071407	5.16E-04	11
RS	response to organic cyclic compound	GO:0014070	6.63E-04	15
SR	regulation of response to stress	GO:0080134	6.05E-04	16
TR	movement of cell or subcellular component	GO:0006928	2.22E-04	21
TR	positive regulation of protein transport	GO:0051222	5.27E-03	9
TT	positive regulation of gene expression	GO:0010628	2.03E-03	19
MC	locomotion	GO:0040011	6.47E-05	21
MC	muscle cell cellular homeostasis	GO:0046716	1.27E-03	3
MC	hematopoietic or lymphoid organ development	GO:0048534	3.25E-03	13
MC	ossification	GO:0001503	6.40E-03	8
MC	blood vessel development	GO:0001568	9.49E-03	10

** Categories: CD - cell death, CP - Catabolic processes, GR - Growth response, HR - Hormonal response, HS - Homeostasis, IR - Immune response, MP - Metabolic processes, OS - Oxidative stress, RS - Response to stimuli, SP - Signaling processes, SR - Stress response, TR - Transport, TT - Transcription and translation, MC - Miscellaneous*

4.3.4 Flutamide treatment induces female-like response by downregulation of cell death mechanisms in the liver of septic male mice

The codirectional genes were identified for Flu treatment similarly as described in the above section. These were 113 up- and 112 down-regulated genes those essentially described the female-like response of Flu treatment. The female-like upregulated genes were solely enriched in the category of *translation* and *amide biosynthesis*. Interestingly, the Flu treatment also downregulated the *cell death* and *programmed cell death* gene sets in female-like manner. Significant gene sets from each category are given below in Table 4.17 and Table 4.18. In summary, the Flutamide treatment to septic male mice induced a female-like transcriptomic response in the form of downregulation of cell death mechanisms in the liver.

Table 4.17: Enriched sets of female-like upregulated genes in the liver of Flu treated male septic mice

*	Gene set	ID	Adjusted p-value	Sig. genes
AP	amide biosynthetic process	GO:0043604	1.97E-03	14
MP	cellular amide metabolic process	GO:0043603	1.81E-03	16
TT	translation	GO:0006412	2.22E-03	13

** Categories: AP - Anabolic processes, MP - Metabolic processes, TT - Transcription and translation*

Table 4.18: Enriched sets of female-like downregulated genes in the liver of Flu treated male septic mice

*	Gene set	ID	Adjusted p-value	Sig. genes
CD	cell death	GO:0008219	1.02E-03	23
CD	programmed cell death	GO:0012501	3.85E-03	21
CP	catabolic process	GO:0009056	8.35E-06	25
CP	organic substance catabolic process	GO:1901575	2.44E-04	22
CP	cellular catabolic process	GO:0044248	1.23E-05	22
MP	cellular lipid metabolic process	GO:0044255	3.89E-03	14
MC	ossification	GO:0001503	7.12E-03	9

** Categories: CD - Cell death, CP - Catabolic processes, MP - Metabolic processes, MC - Miscellaneous*

Chapter 5

Discussion

5.1 IFNγ/IL-10 acts as a theranostic marker for stratification of hydrocortisone therapy in patients with septic shock

The clinical community is divided regarding the use of 'moderate-dose' hydrocortisone (HC) to patients with septic shock. Though faster shock resolution is consistently reported in a meta-analysis [Annane et al., 2009] and in ADRENAL [Venkatesh et al., 2018] trial, the survival benefit with HC treatment was demonstrated only in two French randomized controlled trials, when administered together with fludrocortisone ([Annane et al., 2002] and APROCCHSS [Annane et al., 2018]). However, survival advantage with HC could not be replicated in CORTICUS [Sprung et al., 2008] and ADRENAL [Venkatesh et al., 2018] trails. Possible explanations for this disparity include differences in the patient population with a two-fold higher risk of death in the French control group (of [Annane et al., 2009]) compared to CORTICUS (61% versus 31%, respectively), and an increase in superinfections, including new episodes of sepsis or septic shock recorded with corticosteroid administration in the CORTICUS trial. Considering these observed disparities and differences in outcome, it was hypothesized that a subset of patients might benefit from the HC treatment while others may get harmed. With this aim to identify these subsets, a machine learning based study was performed.

Starting with a larger panel of predictors from septic shock patients treated with HC or placebo in CORTICUS, the ratio of serum IFNγ and IL-10 was identified as the promising new marker for HC treatment decision (odds ratio of 3.03, P=0.031). The treatment decision was formulated as: HC if the ratio is low and no HC treatment if

the ratio is high. The panel predictor included the patient demographic data, clinical features and serum blood cytokine measurements from 83 patients. The rule was further validated by applying the marker and threshold to three unseen datasets. For CORTICUS, the cutoff value for deciding if the ratio of IFNγ/IL-10 is low or high was found to be the 39.8 percentile (patients which IFNγ/IL-10 ratio ranked among the first 39.8% patients were denoted "low ratio patients", the others "high ratio patients"). As in particular the survival rate of the HSSG cohort was very different to CORTICUS, this value needed to be adjusted by a calibration curve calculated from the CORTICUS dataset mimicking higher death rates in CORTICUS by weighting non-survived patients higher. The serum cytokine measurements were performed by different assays, in different labs and at different times (HSSG: own data from 2018, CORTICUS: data from the publication of 2009, SISPCT: own data from 2017, Crossover study: data from the publication in 2003). Applying successfully the rule across differently generated data suggests this marker to be generic and relatively robust.

The functional implications of low and high IFNγ/IL-10 ratios were elucidated by performing blood culture assays. The ratio was observed to be negatively associated with the pathogen load in *ex vivo* blood culture experiments. This was supported by the clinical data from Matera *et al.* in their study regarding critically ill patients with bacteremia (higher bacterial load) and without bacteremia (none or non-detectable bacterial load). Critically ill patients with bacteremia showed a lower ratio compared to those without bacteremia [Matera et al., 2013]. Metabolically, elevated serum lactate indicates the severity of cellular disturbances [Singer et al., 2016] and is associated with poor outcome [Casserly et al., 2015]. Patients with low IFNγ/IL-10 showed high serum lactate. Furthermore, by stratifying patients in compliance with the proposed decision rule, a considerable decrease was observed over time in serum lactate specifically in the group of patients which were treated in compliance with the theranostic marker. The recovery from septic shock is indicated by reduced norepinephrine requirement [Dellinger et al., 2013]. Indeed, the patients treated in compliance with the proposed decision rule showed a significant rate of decrease in norepinephrine consumption indicating a better recovery of these patients.

IFNγ has been consistently documented to activate cells of adaptive immunity [Schoenborn and Wilson, 2007] and IL-10 as a suppressor of innate immune responses and inflammation [Sabat et al., 2010, Kühn et al., 1993]. Accordingly, high IFNγ/IL-10 reflects increased activated adaptive and innate immunity.

Transcriptomic profiles of patients with high ratios compared to patients with low ratios comprised of upregulated genes coding for the immune system and, surprisingly, for proliferation and cell cycle. Comparing this list to gene signatures of activated T-cells and macrophages associates upregulation of the immune response with activated macrophages, while upregulation of proliferation/cell cycle rather with T-cells. Together, this may reflect a recovery of immune cells in which corticosteroid treatment may slow down this process. In turn, low IFNγ/IL-10 may indicate the opposite scenario of a strong suppression of innate immunity and low activation of cells of adaptive immunity. Indeed, blood challenged with bacterial fragments *ex vivo* displayed low IFNγ/IL-10 at high loads and *vice versa*. Transferring these observations to septic shock patients with low IFNγ/IL-10, it reflects that HC treatment yielded a better outcome in HC-responders because HC may have allowed the highly loaded immune system more pace for recovery. This is evidenced when regarding time lapse expression profiles, as particularly in the leukocytes of the responders, genes for immunity and proliferation were upregulated, quite like the profiles of patients with initial high IFNγ/IL-10. Notably, this included all three members of the butyrophilin subfamily 3. The expression of these genes was reported to correlate with the severity of sepsis negatively [Andreu-Ballester et al., 2013]. The genes encode receptors of $\gamma\delta$T-cells and have been associated with tuberculosis protection and early IFNγ production [Chen, 2016]. This is evidenced, as written above, by the reduction of serum lactate and norepinephrine requirement as an indicator of recovery, particularly in these patients.

The concern about the side effects of corticosteroids such as infections in patients with less severe septic shock stipulated more restrictive recommendations by the Surviving Sepsis Campaign. HC application is currently recommended for patients not responding to adequate fluid resuscitation and vasopressor therapy [Dellinger et al., 2008, Dellinger et al., 2013]. It seems intuitively promising to include an immune biomarker, such as IFNγ/IL-10 reflecting the status of the patients' immune system. Consistent with this concept, Bentzer et al. performed a secondary analysis of the VASST trial investigating corticosteroid treated versus non-treated (only vasopressin or norepinephrine vasopressors) patients. A signature of three cytokines (IL3, IL6, CCL4) was identified suggesting a response to corticosteroid treatment [Bentzer et al., 2016]. However, these findings were based on a study which was not controlled, randomized or blinded according to corticosteroid treatment. Further, the study neither distinguished between the

vasopressin and norepinephrine treatment nor investigated on how these three cytokines interact with corticosteroid treatment. In turn, based on transcriptomic investigations, this thesis provides molecular reasoning that the identified ratio of serum cytokines IFNγ and IL-10 seems to stratify corticosteroid treatment for recovery of peripheral immune cells.

Limitations and strengths

The relatively small sample sizes along with the high dimensional data sets obtained imply that these results must be interpreted with caution. A further limitation of this study was that the use of corticosteroids was not randomized in the HSSG and the SISPCT study. The strengths or limitation of this study was that the validation of the marker based on serum cytokine data was measured very differently to CORTICUS suggesting the marker does not depend on a certain platform of the assay. Strengths of this study were that (i) the well-phenotyped cohorts of patients with septic shock were used, (ii) the marker showed very similar results across all studies, and (iii) not only the marker showed the potential clinical relevance but also provided a functional reasoning why this may support HC application by investigating *ex vivo* blood culture experiments.

5.2 Temporal progression of gene regulation of leukocytes explains gender dimorphism of critically ill patients after trauma

Previous clinical trials showed that female (in their premenopausal phase) compared to male trauma patients are better protected from organ failure, show fewer complications, better tolerate critical trauma and develop less severe organ failure. As differences in gene regulation of healthy individuals have been described [Klein and Flanagan, 2016, Tarnopolsky and Ruby, 2001, Wu and O'Sullivan, 2011, Green et al., 1984], in this thesis it was hypothesized that gender dimorphism is also prominent in the gene regulation of peripheral leukocytes of the critically ill and may explain the better clinical course of female patients. As a case study, critically ill patients after blunt trauma were investigated. A statistical framework synchronizing the transcription profiles based on the severity of the patients was developed which enabled the tracking of the differences in male and female patients before, at, and after the most acute phase of organ dysfunction. The analytical approach detected a very divergent regulation of male and female patients. The main findings were validated by a second, independent dataset of transcription profiles from peripheral leukocytes of patients with severe burn injury.

In the early pre-acute phase, leukocytes of female patients showed a stronger innate immune response at the transcriptional level. Particularly the upregulation of NF-κB transcription activity and the ERK1-ERK2 signaling cascade. The role of NF-κB and ERK1-ERK2 signaling are well described in the survival, activation and differentiation of innate immune cells independent of gender [Buscà et al., 2016, Liu et al., 2017]. Distinct gender dimorphism had been described for components of innate immunity such as higher efficiency of antigen presenting cells (APCs), higher activation and phagocytotic activity of macrophages and neutrophils of female patients [Klein and Flanagan, 2016]. This supports the observed findings on a systems-view. In addition, the upregulation of genes for higher myeloid dendritic cell differentiation and neutrophil chemotaxis was observed in female patients. Myeloid dendritic cells are the bridge linking innate and adaptive immunity. They comprise a heterogeneous population of cells presenting antigens to T-cells [Chistiakov et al., 2015]. During the same phase, IL-7 mediated signaling was observed to be upregulated in female patients. IL-7 signaling has been extensively studied in the context of survival and differentiation of B-cells, and proliferation of

B- and T-cells [Sammicheli et al., 2012, Corfe and Paige, 2012, Guimond et al., 2009]. Immunotherapy by application of IL-7 has been shown to enhance the immune response in patients with limited naïve T-cells [Unsinger et al., 2010, El-Kassar et al., 2012, Tuckett et al., 2014]. Furthermore, high levels of IL-7 have been reported to affect the selection of the T- versus the B-cell lineage [El-Kassar et al., 2012]. These early triggers may support the recovery of adaptive immunity which was found to be upregulated in female patients later in the late post-acute phase.

During the pre-acute phase, the upregulation of early wound healing and recovery processes was observed in female patients. Better wound healing in premenopausal females has been reported in a few studies before [Jorgensen et al., 2002, Ashcroft and Ashworth, 2003, Gilliver et al., 2008]. Wound healing is a highly organized process involving several characteristic overlapping steps including skin and vessel integrity restoration, inflammation for attracting leukocytes, proliferation to diminish the lesioned tissue area and remodelling of the extra cellular matrix [Gonzalez et al., 2016]. Indeed, in the pre-acute phase, leukocytes of female patients showed upregulated processes associated with restoring tissue and vessel integrity. These included upregulation of platelet activation/degranulation and blood coagulation to restrict losing blood. The second stage of wound healing is characterized by localized swelling and clearance of damaged cells and pathogens from the wound area. In line, female patients showed an upregulation of genes for leukocyte migration and regulation of phagocytosis. Higher activation and phagocytotic activity of macrophages and neutrophils have been previously reported in females [Klein and Flanagan, 2016]. Upregulation of the MAPK cascade and required protein phosphorylation gene sets was observed in female patients, which play a predominant role for cell proliferation, cell-cell adhesion and growth during early wound healing [Thuraisingam et al., 2010]. It is reasonable that, at the transcriptomic level, healing processes are initiated before the acute phase. These processes may form the functional basis to initiate wound healing processes. Upregulation of these processes was followed by upregulated gene sets related to cell growth and development at the acute phase. Leukocytes of female patients showed an upregulation of IL-1 signaling. The family of IL-1 cytokines activates the innate immune system and also supports the activation and proliferation of T-cells [Sims and Smith, 2010]. In line, an upregulation of the adaptive immune system and T-cell growth was observed in the late post-acute phase.

The differential regulation of energy metabolism and housekeeping processes

covered almost two-third of the identified sex-specific gene sets. From the pre-acute to the acute phase, leukocytes of female patients downregulated energy metabolism and, in particular, oxidative phosphorylation. The downregulation was observed for ATP producing and consuming processes. Surprisingly, peripheral leukocytes of female patients may sustain a limited metabolism by such downregulation without being driven into apoptosis, as upregulation of cell death associated processes was not observed compared to male patients.

During the pre-acute phases of increasing multiple organ failure, a reduced energy production may be advantageous by limiting the production of reactive oxygen species (ROS). It was reported that cells of females are less exposed to oxidative stress in healthy conditions. Ide *et al.* observed a lower abundance of *in vivo* biomarkers for oxidative stress in premenopausal women [Ide et al., 2002]. In an animal model, lower oxidative stress was observed [Barp et al., 2002], and ROS production was lower in human umbilical endothelial cells of female compared to male [Zhang and Lingappan, 2017]. Hence, higher exposure to oxidative stress may be an intrinsic risk factor for male individuals when getting critically ill. In his review about the discrepancy between the need for energy and the potential risk of cell damage when producing energy, Mervyn Singer describes that a hallmark of survivors of sepsis is to better preserve ATP and mitochondrial functions [Singer, 2014]. He suggests that in these patients, cells may enter a "hibernating state in the face of overwhelming inflammation". Our observation supports this as particularly in the critical pre-acute phases, as a distinct downregulation of these energy producing and consuming processes in the leukocytes of female patients was observed. As described above, these female patients showed a better recovery, as, despite no differences between the sexes in the injury score (AIS) at baseline, the MOF score was higher in male patients at and after the acute phase. Taken together, the leukocyte transcriptomic response to trauma point to a better bioenergetic tolerance and oxidative damage resistance in female patients.

The gene sets of the adaptive immune response were distinctively higher expressed in leukocytes of female, compared to male patients during the late post-acute phase, and also compared to healthy controls. Additionally, female patients showed a distinct upregulation of IL-4 production. The presence of IL-4 during the response of naïve T helper cells has been shown in the development of Th2 cells [Zhu et al., 2010]. Interestingly, negative regulation of type I interferon production was also upregulated in female patients. Type I IFNs are known to be important for host defence against viruses. However, *in vivo* studies have also

identified their immune suppressive mechanisms [McNab et al., 2015], hence low levels for its negative regulation may support the immune recovery. This suggests that gene regulation in female, but not in male patients, pave the way for better recovery of the adaptive immune system at the later stages.

Limitations and strengths

There are certain limitations of this study. The patients with multiple acute phases that were days apart were not included in the study due to their limited number of such patients and the availability of their transcriptome data. The primary goal of this study was to identify sex-based transcriptomic differences in critically ill patients undergoing MOF, using the study of "Inflammation and the Host Response to Injury (Trauma)" as a case study. To fully understand sex-based differences in the complicated recovery, a different stratification approach with a higher number of time-resolved transcription profiles are required. The strength of the study lies in the fact that it not only highlights the sex-based differences in the critically injured patients but also demonstrates that these differences vary as the disease progresses, which has not been shown before. Also, the identified differences could be replicated in another independent cohort of critically injured patients irrespective of the type of injuries.

5.3 The anti-androgenic and pro-estrogenic treatment potentially enhances tolerance in the liver of septic male mice as a defense strategy

The previous section described the sex-based differences in leukocyte expression of critically ill patients. It demonstrated that the premenopausal female patients show a transcriptomic profile that reason their better molecular response and reduced organ failure scores despite having a similar degree of injury. This third study of the thesis was dedicated to understanding the transcriptomic response of septic male mice upon treatment with drugs that are known to combine anti-androgenic and pro-estrogenic effects. These drugs specifically include Flutamide, an FDA approved synthetic drug prescribed primarily for the treatment of prostate cancer and partly as a component of feminizing hormone therapy for transgender women [Dahl et al., 2015], and a naturally occurring non-steroidal anti-androgenic and potent pro-estrogenic drug called Atraric acid (AA).

The gender dimorphism between septic pre-estrus female mice and septic male mice has been shown in the PCI [Röll, 2015] as well as CLP [Zellweger et al., 1997] sepsis models. As reported by Dr. Röll, in the PCI sepsis model, the difference in mortality was compelling, since 80% of the female mice survived 72 hours post-PCI but, all male mice died at latest as 28 hours. By Dr. Röll, the rescue of septic male mice was shown to be enabled by treatment with either AA or Flu. In Addition, Dr. Röll showed that neither the pure AR inhibition nor the pure ER activation alone was enough for the significant improvement of survival rates in male mice. However, a marginally beneficial effect of solely ER induction has also been shown to improve the sepsis outcome partly [Angele et al., 1997, Raju and Chaudry, 2008]. The manifold improvement has been demonstrated by the treatment of Flu or AA resulting in significantly enhanced survival (20-25%) in males, suggesting that both modes of action together were more effective: the inhibition of AR and the activation of ER. At 10 hours post-PCI, also the time point when gene expression was measured, all animals showed a comparable severity score, thereby indicating that complete prevention of infection was not the reason for the gender dimorphism [Röll, 2015]. However, cytokine levels (IL-6, IL-10, TNF-α, MCP-1, IFN-γ, IL-12p70) measured in blood serum and liver tissue, together with liver function parameters (Lactate

dehydrogenase, Creatinine, Aspartate transaminase, Bilirubin) were failing to provide molecular insights into the survival advantages. Hence, to gain further insights into the influence of AA and Flu on septic male mice and the gender dimorphism at molecular levels, liver transcriptome profiling was carried out.

In this thesis, the analysis of the liver transcriptome profiles was performed. PCA analysis was conducted to study the effect of gender and treatment on a systems view. PCA showed a distinct transcriptomic separation between septic male and female samples. Notably, PCA also revealed a transcriptomic shift toward females upon treatment with AA or Flu, which was further refined by taking the combination of PCs. The marked shift indicates that the treatments have induced female-like transcriptomic changes that may have contributed to the rescue of male septic mice.

The gender dimorphic influence on the transcriptome of the liver was studied using differential gene expression analysis. Anatomically, the liver consists of 70-80% of the hepatocytes, 14-17% of cellular components of extracellular spaces and 5-6% of non-parenchymal cells. The non-parenchymal cells consist of a diverse set of cells, including 45% liver sinusoidal endothelial cells, 33% Kupffer cells, and 22% hepatic stellate cells [Bogdanos et al., 2013]. The liver facilitates a tolerance rather than immunoreactivity, which protects the host from an antigenic overload of dietary components and drugs derived from the gut [Bogdanos et al., 2013]. A disease such as sepsis is known to exaggerate the antigenic overload by bringing a milieu of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and organic/inorganic toxins into the circulation and especially to the liver [Hotchkiss et al., 2016]. During sepsis, the liver of female mice showed overall desensitization to various receptor stimuli by downregulating their response pathways. Receptor desensitization has been known to cause temporary tolerance allowing the cell to protect themselves from prolonged or overstimulation by ligands [Arey, 2014]. In line, the desensitization was observed in female mice for a variety of molecules such as lipids, oxygen-containing compounds and cytokines, which in this context are mainly contributed by the inflammatory cascades and associated damage. Together with other biotic and abiotic stimuli, female mice also repressed the response to immune mediators such as IL-1, and the molecules of bacterial origin such as LPS. Such suppression of immune cascades possibly has resulted in the overall downregulation of the acute phase response in female mice. The inhibition of the IL-1 cascade might have contributed in favor of females, as the therapeutic inhibition of IL-1 by receptor antagonists (anakinra) has been shown to be effective in a subset of sepsis patients [Shakoory et al., 2016].

Female mice have also shown downregulation of energy metabolism. This involved the downregulation of energy synthesis by cellular respiration along with the downregulation of processes involved in the generation of energy precursors such as acetyl-CoA and glucose metabolism. A typical consequence of energy production is the production of Reactive Oxygen Species (ROS) by a series of oxidation-reduction (Redox) processes. In septic conditions, the rate of ROS synthesis has been reported to increase manifold [Galley, 2011]. However, the redox processes were downregulated in female mice suggesting that reduced energy production might have contributed to less oxidative stress by limiting ROS production. As discussed in the previous section several studies have demonstrated that females are less exposed to oxidative stress in healthy conditions. Hence, higher exposure to oxidative stress may be an intrinsic risk factor for male sex. Surprisingly, such reduced energy metabolism in the liver of female mice was sustainable without being driven into apoptosis. On the contrary, cell death mechanisms were downregulated compared to male mice.

The transcriptomic response of septic liver of female mice can be correlated to the leukocyte transcriptomic response of female trauma patients during the pre-acute to acute phase to some extent. In both scenarios, processes associated with energy metabolism, generation of ROS, and oxidation-reduction were downregulated. Despite the reduced energy metabolism, the cell death processes were either comparable or downregulated compared to males. However, the immune response was observed to be the opposite. This supports the notion of the immune response being a double-edged sword [Ashida et al., 2011], where, the leukocytes from female patients showed higher innate and adaptive response directed towards pathogen clearance, while liver of female mice showed a repressed innate immune response reducing the organ damage at the site.

In summary, liver of female septic mice showed enhanced tolerance at the transcriptomic level, where the tolerance was defined as protection from antigenic overload by receptor desensitization, reduced immunoreactivity and inhibition of cell death in liver [Bogdanos et al., 2013].

The female-like response of the AA treatment was studied by identifying the significantly expressed co-directional genes between the treatment and female mice compared to solvent treated males. In a female-like manner, AA also induced the desensitization of the receptor response to external stimuli including the response elicited by IL-1, IL-6, and LPS. The role of IL-1 has been extensively studied and its persistent increase during the first seven days from admission is associated with

increased death in septic patients [Mera et al., 2011]. Similarly, IL-6, a pleiotropic cytokine that functions as both pro- and anti-inflammatory has been associated with the development of sepsis [Gouel-Chéron et al., 2012] and with the highest risk of death in patients with sepsis [Kellum et al., 2007]. The reduced response to these cytokines may have delivered the survival benefits. As observed in female mice, the treatment with AA did not induce downregulation of energy metabolism, nor it downregulated the oxidation-reduction processes. However, ROS metabolic processes were present and downregulated. The lower levels of ROS may refrain from accelerating the organ dysfunction. The female-like effects of AA also included downregulation of cell death mechanisms. In summary, as observed in females, the treatment of AA also included protection from antigenic overload by receptor desensitization, reduced immunoreactivity and inhibition of cell death, thereby possibly enhancing the tolerance in the liver.

Despite having a similar degree of survival benefits by AA treatment, female-like effects of Flu treatment were limited to the downregulation of cell death mechanisms, catabolic processes and the upregulation of translation processes. Female-like co-directional genes were similar in both treatments (AA: 157 up, 107 down; Flu: 113 up, 112 down), yet a lower number of significant gene sets were identified in the case of Flu treatment. The limited number of gene sets may indicate broader functional consequences of Flu treatment as opposed to a few highly specific functions being affected. The only commonality between the female-like effects of AA and Flu treatments was the downregulation of cell death mechanisms. The receptor desensitization and reduced immunoreactivity were not observed at the studied time point of 10 hours, however, Kan et al. identified that Flu treatment reduced cytokine/chemokine production (IL-6, TNF-α, MCP-1), oxidative stress, and hepatocyte apoptosis at 2 hours after trauma-hemorrhage [Kan et al., 2008]. Although AA and Flu both induce anti-androgenic and pro-estrogenic effects their kinetics of bringing transcriptomic changes may vary. Hence, time-resolved transcriptome profiles are needed to explore their female-like effects in greater depth. In summary, the observed transcriptomic changes strongly suggest that the survival advantage coming from these treatments could be from the enhanced tolerance as a defense strategy.

Limitations and strengths

The limitation of this study was the lower sample size; hence the observations are needed to be interpreted with caution. Additionally, as pointed out in the previous

section of this thesis that sex-differences vary as the disease progresses, more than one time point may be necessary to decipher the beneficial roles of the treatments in more details. The strength of this study lies in the fact that the effects of the treatments and the gender differences are studied in an animal model where heterogeneity is well controlled as opposed to a patient cohort.

Chapter 6

Conclusions and perspectives

Aim of this thesis was to find clinico-transcriptomics approaches to improve outcome in critically ill patients with cortico-and sex-steroids. Two clinico-transcriptomics approaches were developed, and their findings were validated on unseen datasets. Validation of any new sepsis-related finding is crucial given the heterogeneity in septic patients. Many seemingly promising treatment strategies derived from a single patient cohort could not be replicated. As John Marshall says, "The current challenge for sepsis research lies in a failure of concept and reluctance to abandon a demonstrably ineffectual research model" [Marshall, 2014]. Thus, novel, effective and valid data-driven research models are needed to understand pathomechanisms and maximize the benefits from the existing treatments.

In this thesis, the first clinico-transcriptomics approach was built to identify a subset of patients that might benefit from HC treatment in septic shock patients. Serum ratio of IFNγ and IL-10 was found to be the best marker to make this treatment decision. It was validated further on three independent datasets. High ratio indicated better survival without HC treatment while a benefit was observed for low ratio when HC treatment was initiated. Clinical parameters and transcriptomic evidence suggested that the patients with high ratio might be 'immunologically fit' as these patients survived more often, and their transcriptome reflected the presence of greater extent of activated macrophages and T-cells. Interestingly, the activation of macrophages and T-cells can be switched on in patients with low ratios. This 'switch' would be an interesting research to be followed in the future with more precise immune assays. Currently, the concern about side effects of HC such as infections in patients with less severe septic shock has imposed more restrictive recommendations by the Surviving Sepsis Campaign. Thus, HC application is only recommended for patients not responding to adequate

fluid resuscitation and vasopressor therapy [Dellinger et al., 2008, Dellinger et al., 2013]. It seems intuitively promising to include an immune biomarker, such as IFNγ/IL-10 reflecting the status of the patients' immune system. If confirmed using a randomized clinical trial, IFNγ/IL-10 can be a suitable theranostic marker to guide HC therapy in septic shock patients.

The second clinico-transcriptomics approach was developed to understand the sex-based pathomechanisms in the trauma patients. The findings from this approach were validated using another dataset of patients with burn injuries. The approach included the statistical analysis pipeline that synchronized the time lapse of the profiles based on the temporal severity score of each patient. After critical trauma, female and male patients showed distinct transcriptomic differences that might have worked in support of female patients. For example, a stronger innate immune response at the very early phase of the disease may support early clearance of the pathogen and its associated molecular patterns. Upregulation of wound healing processes may explain reduced multiple organ failure during the acute phase. Downregulated energy metabolism during the acute phase may make female patients less susceptible to oxidative stress, and the upregulated adaptive immune system reflects an earlier recovery and rebuilding of the adaptive immunity that may protect them from secondary infections. These results support our understanding of the better clinical course in female patients after trauma and may encourage investigations into gender-based medicine in the future.

Although the survival benefits of pre-estrus females and sex-hormone modulation in males have been successfully demonstrated in animal septic models [Röll, 2015], there has not been any clinical trials testing the benefits of the sex-hormone modulation in septic patients. The transcriptomic evidence gathered in this thesis argued that such modulation by the treatment of Flutamide or Atraric acid might have enhanced the tolerance in the liver of septic male mice. Flutamide is an FDA-approved drug currently being used in the treatment of prostate cancer [Akaza, 2011]. Hence, if proven effective in the randomized clinical trial of septic patients, Flutamide can serve as a therapy which is ready for adoption for the treatment of septic patients, especially males.

The insights provided by the clinico-transcriptomics approaches may significantly contribute to improving the outcome in critically ill patients with cortico- and sex-steroids.

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Appendix

Table A.1: Detailed inclusion criteria of CORTICUS, HSSG, SISPCT and the Crossover study

Dataset	Inclusion criteria
CORTICUS	1. Clinical evidence of infection within the previous 72 hours (may be present longer than 72 hours), only one of a, b, c, or d required: a) Presence of polymorphonuclear cells in a normally sterile body fluid (excluding blood); b) Positive culture or Gram staining of blood, sputum, urine or normal sterile body fluid; c) Focus of infection identified by visual inspection (e.g. ruptured bowel with the presence of free air or bowel contents in the abdomen found at the time of surgery, wound with purulent drainage); d) Other clinical evidence of infection - treated community acquired pneumonia, purpura fulminans, necrotising fascitis, etc.
	2. Evidence of a systemic response to infection as defined by the presence of two or more of the following signs within the previous 24 hours (these signs may be present longer than 72 hours): a) Fever (temperature $>$ 38.3°C) or hypothermia (rectal temperature $<$ 35.6°C); b) Tachycardia (heart rate of $>$ 90 beat/min); c) Tachypnea (respiratory rate $>$ 20 breaths/min, $PaCO_2 <$ 32 mmHg) or patient requires invasive mechanical ventilation; d) Alteration of the WBC count: $>$ 12,000 cells/mm^3, $<$ 4,000 cells/mm^3 or $>$ 10% immature neutrophils (bands).
	3. Evidence of shock defined by (a and b both required within the previous 72 hours (may NOT be present longer than 72 hours). a) A systolic blood pressure $<$ 90 mmHg or a decrease in SBP of more than 50 mmHg from baseline in previous hypertensive patients (for at least one hour) despite adequate fluid replacement OR need for vasopressors for at least one hour (infusion of dopamine $\geq$ 5 mcg/kg/min or any dose of adrenaline, noradrenaline, phenylephrine or vasopressin) to maintain a SBP $\geq$ 90 mmHg; b) Hypoperfusion or organ dysfunction which is not the result of underlying diseases or drugs, but is attributable to sepsis, including one of the following: 1. Sustained oliguria (urine output $<$ 0.5 ml/kg/h for a minimum of 1 hour) 2. Metabolic acidosis [pH of $<$ 7.3, or a base deficit of $\geq$ 5.0 mmol/L, or an increased lactic acid concentration ($>$ 2 mmol/L)].

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Table A.1 – *Continued from previous page*

Dataset	Inclusion criteria
	3. Arterial hypoxemia ($PaO_2/FiO_2 < 280$ in the absence of pneumonia)($PaO_2/FiO_2 < 200$ in the presence of pneumonia). 4. Thrombocytopenia - platelet count $\leq$ 100,000 cells/mm3. 5. Acute altered mental status (Glasgow Coma Scale < 14 or acute change from baseline).
	4. Age $\geq$ 18 years
	5. Informed Consent
	6. Measured cortisol level at baseline and 60 minutes after 0.25 mg cosyntropin stimulation
HSSG	1. Patients reclassified into infection and sepsis using the Sepsis-3 classification criteria [Singer et al., 2016, Giamarellos-Bourboulis et al., 2017]
	2. Age $\geq$ 18 years
	3. Informed Consent
	4. Community-acquired pneumonia and intraabdominal infections
SISPCT	1. Onset of severe sepsis (A and B both required) or septic shock (C) $<$ 24 h. Severe sepsis was defined as the presence of microbiologically proven, clinically proven, or suspected infection; presence of Systemic Inflammatory Response Syndrome (A); and development of at least one organ dysfunction (B) within the last 24 hours. A) Diagnosis of SIRS required the fulfilment of at least two of the following criteria: hypo- ($\leq$ 36°C) or hyperthermia ($\geq$ 38°C), tachycardia ($\geq$ 90 bpm); tachypnea ($\geq$ 20 breaths/min) and/or an arterial $pCO_2 \leq$ 4.3 kPa (32 mmHg) and/or mechanical ventilation; leukocytosis $\geq 12000/\mu$l or leukopenia $\leq 4000/\mu$l and/or a left shift in the differential white blood cell count $\geq$ 10% B) For the diagnosis of organ dysfunction one of the following criteria had to be fulfilled: i. Presence of acute encephalopathy with reduced vigilance, agitation, disorientation, delirium not explained by psychotropic medication, ii. Thrombocytopenia $\leq 100.000/\mu$l or a drop in the thrombocyte count $>$ 30% within 24 hours not explained by hemorrhage, iii. Arterial hypoxemia with an arterial $pO_2 <$ 10 kPa (75 mmHg) when breathing normally or an oxygenation index ($paO_2/FiO_2 \leq$33kPa (250 mmHg) not explained by presence of a pulmonary or cardiac disease,

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Table A.1 – *Continued from previous page*

Dataset	Inclusion criteria
	iv. Arterial hypotension with a systolic blood pressure $\leq$ 90 mmHg or mean arterial blood pressure $\leq$ 70 mmHg for at least one hour despite adequate fluid loading not explained by other causes of shock, v. Renal dysfunction with an urine output $\leq$ 0.5 ml/kg/h for at least one hour despite adequate fluid loading and/or increase of serum creatinine more than twofold above the reference range of the local laboratory, vi. Metabolic acidosis with a base deficit $\geq$ 5.0 mmol/l or a serum lactate $\geq$1.5 fold above the reference range of the local laboratory.
	2. Age $\geq$ 18 years
	3. Informed consent
Crossover	1. Presence of septic shock including, a) Proven or strongly suspected infection b) Three or more of these conditions: mechanical ventilation, heart rate of more than 90 beats per minute, temperature of more than 38°C or less than 36°C, a white blood cell count of more than 12,000 cells/μl or less than 4,000 cells/μl, or more than 10% immature cells c) Sepsis-induced hypotension (systolic blood pressure of less than 90 mmHg or a reduction of more than 40 mmHg from baseline in the absence of other causes of hypotension)
	2. Patients requiring norepinephrine to maintain a mean arterial pressure of more than 70 mmHg despite adequate fluid resuscitation.
	3. Age $\geq$ 18 years
	4. Informed Consent

Table A.2: Detailed exclusion criteria of CORTICUS, HSSG, SISPCT and the Crossover study

Dataset	Exclusion criteria
CORTICUS	1. Pregnancy,
	2. Age less than 18,
	3. Underlying disease with a prognosis for survival of less than 3 months,
	4. Cardiopulmonary resuscitation within 72 hours before study,
	5. Drug-induced immunosuppression, including chemotherapy or radiation therapy within 4 weeks before the study,
	6. Administration of chronic corticosteroids in the last 6 months or acute steroid therapy (any dose) within 4 weeks (including inhaled steroids). Topical steroids are not exclusions,
	7. HIV positivity,
	8. Presence of an advanced directive to withhold or withdraw life sustaining treatment (i.e. DNR),
	9. Advanced cancer with a life expectancy less than 3 months,
	10. Acute myocardial infarction or pulmonary embolus,
	11. Another experimental drug study within the last 30 days,
	12. Moribund patients likely to die within 24 hours,
	13. Patients in the ICU for more than 2 months at the time of the start of septic shock,
HSSG	1. Infection by the human immunodeficiency virus,
	2. $<$ 1,000 neutrophils/mm3
	3. Systemic intake of more than 0.3mg/kg of equivalent prednisolone the last 15 days
SISPCT	1. Pregnant or breast-feeding women,
	2. Fertile female women without effective contraception,
	3. Participation in interventional clinical trial within the last 30 days,
	4. Current participation in any study,
	5. Former participation in this trial,
	6. Selenium intoxication,
	7. No commitment to full patient support (i.e. DNR order),
	8. Patient's death is considered imminent due to coexisting disease,

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Table A.2 – *Continued from previous page*

Dataset	**Exclusion criteria**
	9. Relationship of the patient to study team member (i.e. colleague, relative),
	10. Infection where guidelines recommend a longer duration of antimicrobial therapy (i.e. endocarditis, tuberculosis, malaria etc),
	11. Immunocompromised patients.
Crossover	1. Pregnancy,
	2. Glucocorticoid medication within the last 3 months,
	3. Ongoing immunosuppressive therapy,
	4. Hematologic diseases,
	5. Moribund state.

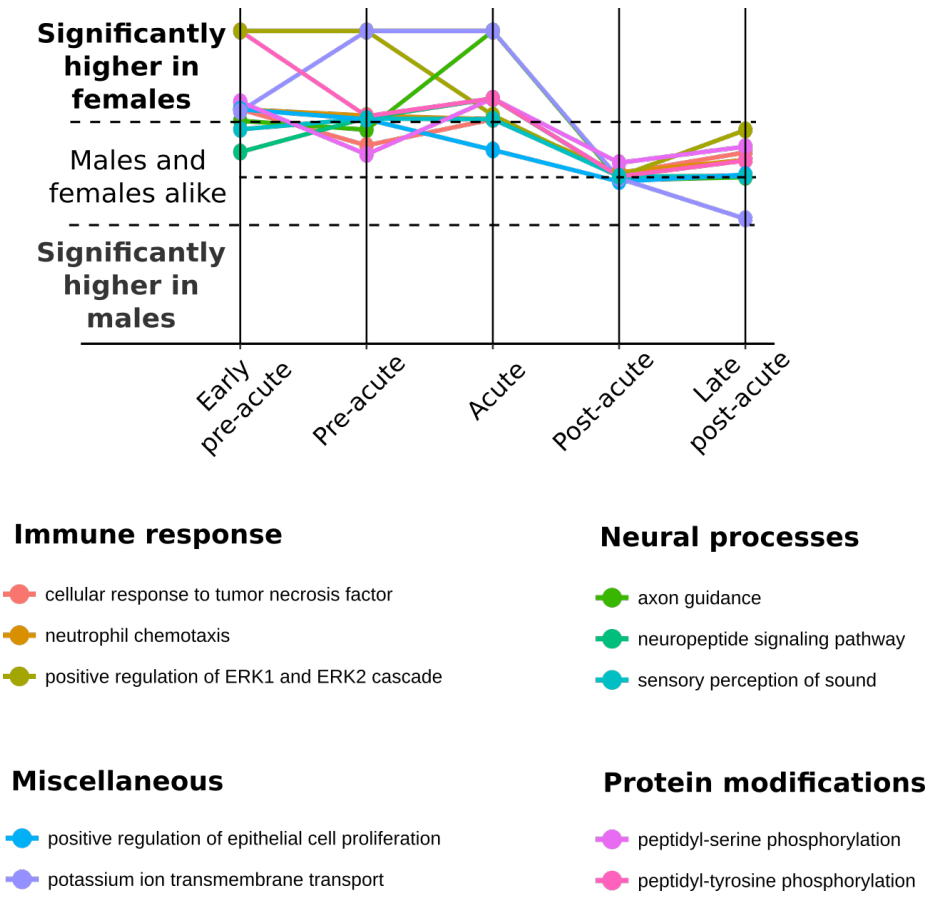


Figure A.1: Cluster of all gene sets being downregulated in female patients in at least two phases among the early pre-acute, pre-acute and acute phase.

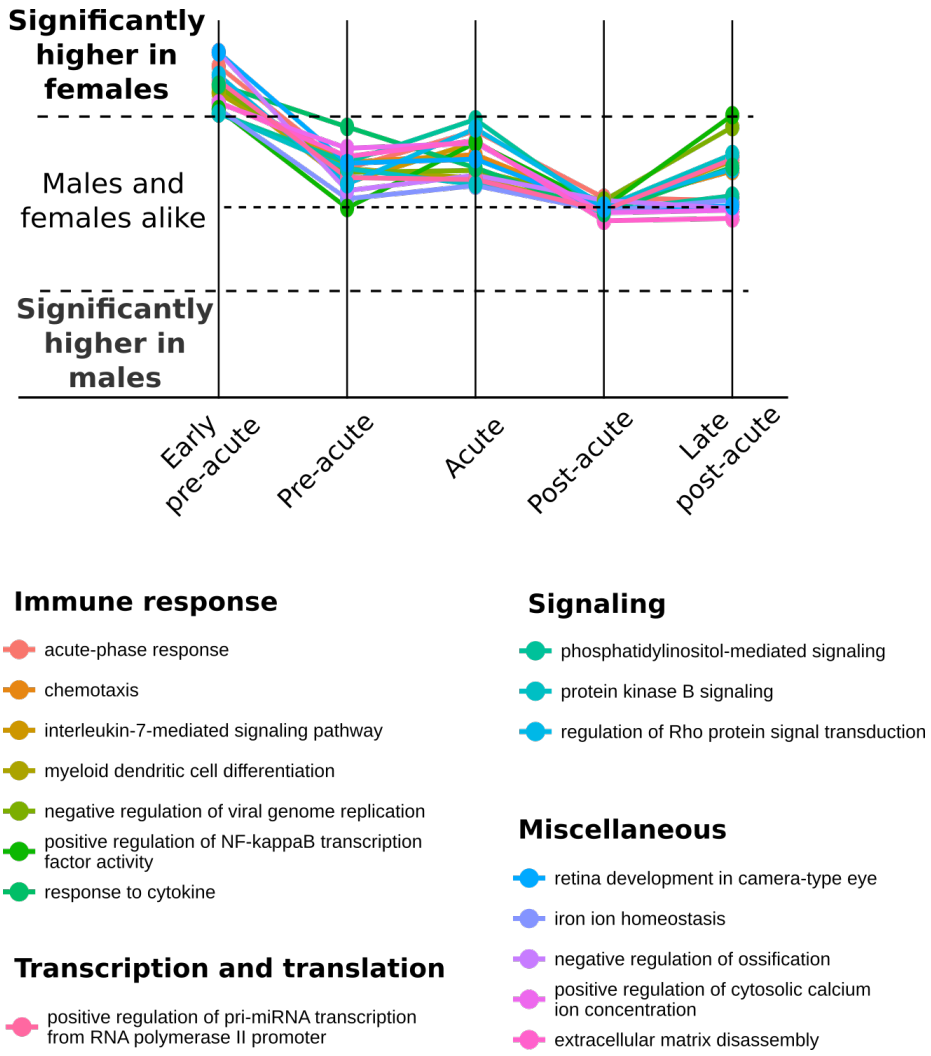


Figure A.2: Cluster of all gene sets being upregulated in female patients in the early pre-acute phase.

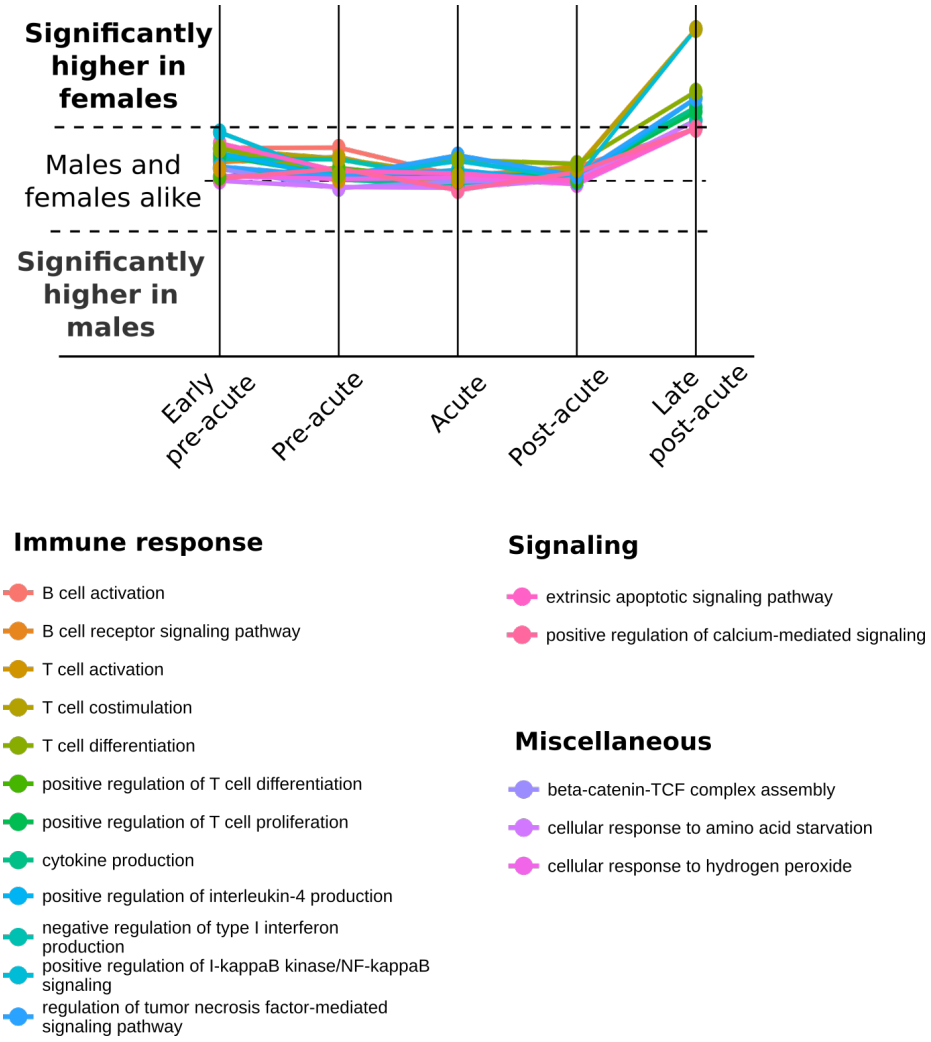


Figure A.3: Cluster of all gene sets being upregulated in female patients in the late post-acute phase.

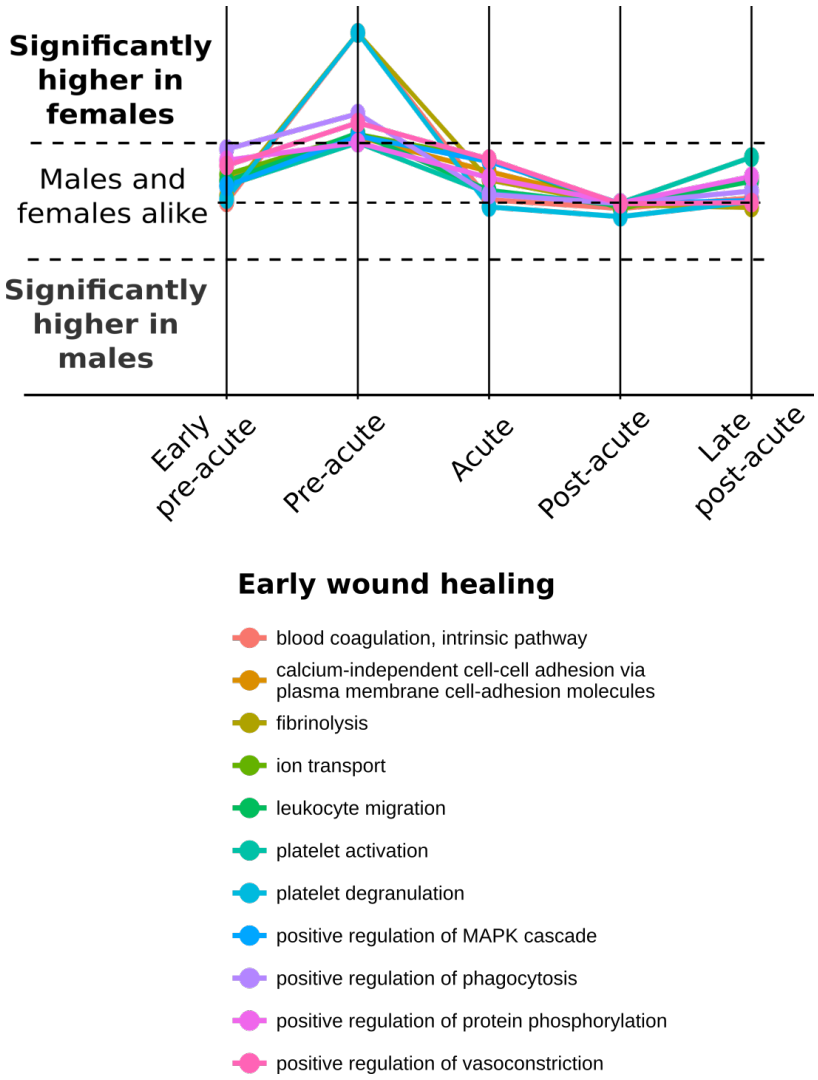


Figure A.4: Cluster of all gene sets being upregulated in female patients in the pre-acute phase.

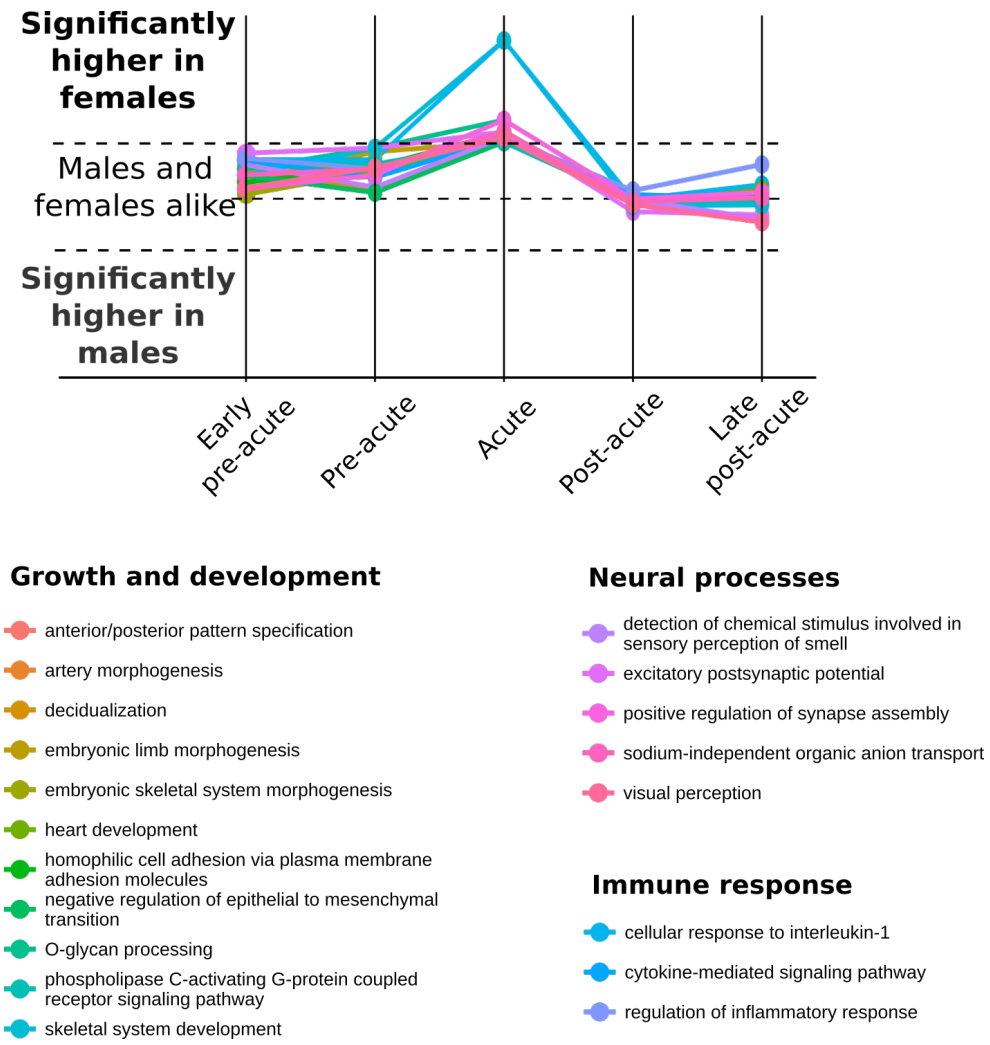


Figure A.5: Cluster of all gene sets being upregulated in female patients during the acute phase.

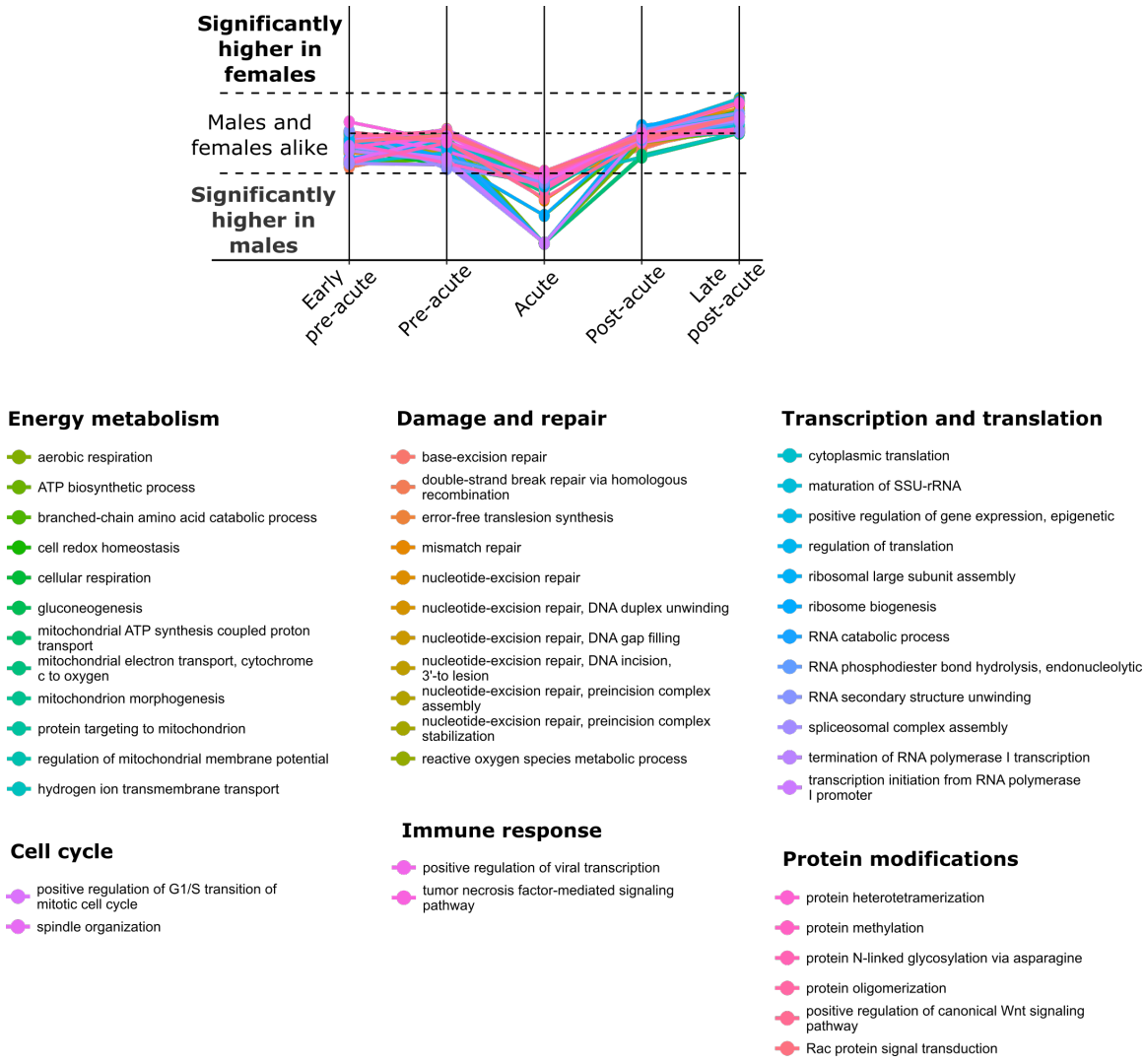
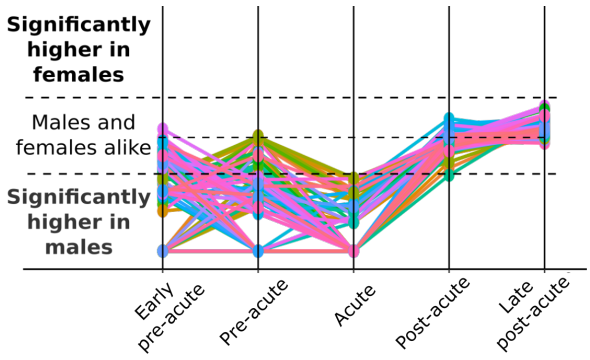


Figure A.6: Cluster of all gene sets being downregulated in female patients during the acute phase.



Cell cycle

- anaphase-promoting complex-dependent catabolic process
- CENP-A containing nucleosome assembly
- centrosome cycle
- chromosome segregation
- DNA replication
- DNA replication initiation
- G1/S transition of mitotic cell cycle
- G2/M transition of mitotic cell cycle
- mitotic cell cycle
- mitotic nuclear envelope disassembly
- mitotic spindle assembly checkpoint
- negative regulation of G2/M transition of mitotic cell cycle
- nucleic acid phosphodiester bond hydrolysis
- positive regulation of telomere maintenance via telomerase
- positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition
- regulation of G2/M transition of mitotic cell cycle
- regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
- sister chromatid cohesion
- telomere maintenance
- telomere maintenance via semi-conservative replication
- negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle

Protein modifications

- chaperone-mediated protein folding
- 'de novo' protein folding
- protein folding
- protein N-linked glycosylation
- protein peptidyl-prolyl isomerization
- protein sumoylation
- SCF-dependent proteasomal ubiquitin-dependent protein catabolic process

Damage and repair

- DNA damage response, detection of DNA damage
- error-prone translesion synthesis
- global genome nucleotide-excision repair
- interstrand cross-link repair
- nucleotide-excision repair, DNA damage recognition
- nucleotide-excision repair, DNA incision
- nucleotide-excision repair, DNA incision, 5'-to lesion
- peroxisome organization
- transcription-coupled nucleotide-excision repair

Energy metabolism

- fatty acid beta-oxidation
- mitochondrial calcium ion transmembrane transport
- mitochondrial electron transport, NADH to ubiquinone
- mitochondrial respiratory chain complex I assembly
- mitochondrial respiratory chain complex IV assembly
- mitochondrial translation
- mitochondrial translational elongation
- mitochondrion organization
- tricarboxylic acid cycle
- cristae formation
- regulation of glycolytic process

Miscellaneous

- ciliary basal body-plasma membrane docking
- heme biosynthetic process
- nuclear import
- regulation of cellular amino acid metabolic process
- regulation of cellular response to heat
- Wnt signaling pathway, planar cell polarity pathway

Transcription and translation

- regulation of translational initiation
- ribosomal large subunit biogenesis
- 7-methylguanosine mRNA capping
- mRNA 3'-end processing
- mRNA export from nucleus
- mRNA processing
- regulation of mRNA stability
- RNA export from nucleus
- RNA metabolic process
- RNA splicing
- RNA splicing, via transesterification reactions
- snRNA transcription from RNA polymerase II promoter
- spliceosomal snRNP assembly
- tRNA aminoacylation for protein translation
- tRNA export from nucleus
- tRNA modification
- tRNA processing
- exonucleolytic nuclear-transcribed mRNA catabolic process involved in deadenylation-dependent decay
- nucleobase-containing small molecule interconversion
- methylation
- regulation of gene silencing by miRNA
- regulation of transcription from RNA polymerase II promoter in response to hypoxia
- termination of RNA polymerase II transcription
- transcription elongation from RNA polymerase II promoter
- transcription elongation from RNA polymerase I promoter
- transcription from RNA polymerase III promoter

Immune response

- antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent
- Fc-epsilon receptor signaling pathway
- intracellular transport of virus
- NIK/NF-kappaB signaling
- regulation of hematopoietic stem cell differentiation

Figure A.7: Cluster of all gene sets being upregulated in female patients in at least two phases among the early pre-acute, pre-acute and acute phase.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Rainer König und Prof. Michael Bauer.

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Ort, Datum Unterschrift des Verfassers

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May 2014 - Present **Research employee**, Universitätsklinikum, Jena, Germany

- Stratification of corticosteroids therapy in septic shock patients.
  - Use of medical data from clinical trials, machine learning, transcriptomic data and statistics
- Deciphering sex-dependent transcriptome modulation in critically ill patients after trauma.
  - Use of medical data, transcriptomic data and statistics
- Understanding the mechanisms behind sex-based hormone replacement therapy in sepsis.
  - Use of transcriptomic data and statistics

Dec 2012 - Mar 2014 **Senior research fellow**, Indian Institute of Science Education and Research (IISER), Pune, India

- Hydra genome and transcriptome analysis
  - Use of Next generation sequencing (NGS) data, setting up analyses pipeline
- SanGeniX: A comprehensive Next Generation Sequence Analysis Solution.
  - Setting up NGS data analysis pipeline
- Teaching practical courses, supervision and mentoring students

EDUCATION:

May 2014 – Present **Doctor rerum naturalium**, Friedrich-Schiller-Universität, Jena, Germany

Thesis project

- Improving the outcome of the critically ill with cortico- and sex- steroids employing clinico-transcriptomic approaches

June 2007 – July 2012 **Integrated Bachelor and Master of Science in Biotechnology**, Institute of Bioinformatics and Biotechnology, University of Pune, India

Thesis projects

- JePhCort: A genotype-phenotype correlation tool based on phylogenetic analysis
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- Poos A, Kordaß T, **Kolte A**, Ast V, Oswald M, Rippe K, König R (2019) Modelling TERT regulation across 19 different cancer types based on the MIPRIP 2.0 gene regulatory network approach, ***bioRxiv***, page 513259; doi: https://doi.org/10.1101/513259

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