# Inflammasome-dependent Alarmins Serve as a Tool for Stratification of Neonatal ICU Patients with Infection

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# List of abbreviations

| Abbreviation | Term |
|---|---|
| A | Adenine |
| AIM2 | Absent in melanoma 2 |
| AIS | Amniotic infection syndrome |
| ALR | Absent in melanoma 2 like receptor |
| APC | Antigen-presenting cell |
| APGAR | Appearance, pulse, grimace, activity, respiration |
| APOBEC1 | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like |
| ASC | Apoptosis-associated speck-like protein containing a CARD |
| ATP | Adenosine triphosphate |
| AWMF | *Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften*<br>(Association of the Scientific Medical Societies in Germany) |
| BE | Base excess |
| BIR | Baculovirus inhibitor repeat |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| BPD | Bronchopulmonary dysplasia |
| C | Cytidine |
| $Ca^{2+}$ | Calcium |
| CARD | Caspase recruitment domain |
| CASP | Caspase |
| Cas9 | CRISPR associated protein 9 |
| CCL4 | Macrophage-inflammatory protein-1ß |
| CCL20 | Macrophage-inflammatory protein-3α |
| CD | Cluster of differentiation |
| CLR | C-type lectin receptor |
| $CO_2$ | Carbon dioxide |
| CoNS | Coagulase-negative staphylococci |
| CPAP | Continuous positive airway pressure |
| CRD | Carbohydrate recognition domain |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CRP | C-reactive protein |
| CRT | Capillary refill time |

| | |
|---|---|
| CSF | Cerebrospinal fluid |
| CXCL8 | Chemokine (C-X-C motif) ligand 8 |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| $ddH_2O$ | Double-distilled water |
| DMEM | Dulbecco´s Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide |
| DPBS | Dulbecco´s Phosphate-Buffered Saline |
| dsDNA | Double-stranded deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EMBL-EBI | European Molecular Biology Laboratory – European Bioinformatics Institute |
| EOS | Early-onset neonatal sepsis |
| ER | Endoplasmic reticulum |
| F | Phenylalanine |
| FCS | Fetal bovine serum |
| FDA-NIH | Food and Drug Administration – National Institutes of Health |
| FIP | Focal intestinal perforation |
| FIRS | Fetal inflammatory response syndrome |
| FSL-1 | Pam2CGDPKHPKSF |
| G | Guanine |
| Gal-1 | Galectin-1 |
| GBP | Guanylate-binding protein |
| GBS | Group B streptococcus *(Streptococcus agalactiae)* |
| GEP | Granulin-epithelin precursor |
| GSDMD | Gasdermin-D |
| G1P | Glycerol-1-phosphat |
| HEK 293 T cell | Human embryonic kidney 293 T cells |
| HELLP | Hemolysis, elevated liver enzymes, low platelet count |
| HMGB1 | High-mobility group box protein 1 |
| HPr | Histidine protein |
| HSP | Heat shock protein |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| $H_2SO_4$ | Sulfuric acid |
| I/T-ratio | Immature to total neutrophil ratio |
| IFN | Interferon |
| IFNAR | Interferon-α/β receptor |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| iLPS | Intracellular LPS |
| IQR | Interquartile range |
| IRAK-1 | IL1-receptor-associated kinase |
| IRDS | Infant respiratory distress syndrome |
| IRF | Interferon regulatory factor |
| IRGB10 | Immunity-related GTPase family member b10 |
| IVH | Intraventricular hemorrhage |
| $K^+$ | Potassium |
| LB | Lysogenic broth |
| Lgt | Phosphatidylglycerol-prolipoprotein diacylglyceryl transferase |
| Lnt | N-acyltransferase |
| LOS | Late-onset neonatal sepsis |
| LPG | Lipophosphoglycan |
| Lpp | Lipoprotein |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| Lsp | Lipoprotein signal peptidase II |
| LTA | Lipoteichoic acid |
| MAPK | Mitogen-activated protein kinase |
| MD2 | Myeloid differentiation factor 2 |
| MHC | Major histocompatibility complex |
| mRNA | Messenger RNA |
| MV | Membrane vesicle |
| MyD88 | Myeloid differentiation primary response 88 |
| n.s. | Not significant |
| NACHT / NBD | Nucleotide binding domain |
| NCBI | National Center for Biotechnology Information |
| NEC | Necrotizing enterocolitis |

| | |
|---|---|
| NEO-KISS | *Surveillance System nosokomialer Infektionen für Frühgeborene auf Intensivstationen*<br>(Nosocomial Infection Surveillance System for Preterm Infants on Neonatology Departments and ICUs) |
| NET | Neutrophil extracellular trap |
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NICU | Neonatal intensive care unit |
| NK cell | Natural killer cell |
| NLR | Nucleotide-binding oligomerization domain like receptor |
| NLRP | NOD-, LRR- and PYD-containing protein |
| NOD | Nucleotide-binding oligomerization domain |
| NRZ | *Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen*<br>(German National Reference Center for Surveillance of Nosocomial Infections) |
| OMV | Outer membrane vesicle |
| oxPAPC | 1-palitoyl-2-arachidonoyl-sn-glyero-3-phosphorylcholine |
| $P2X_7$ | Purinergic P2X7 receptor |
| PAM | Protospacer adjacent motif |
| PAMP | Pattern-associated molecular pattern |
| $Pam_3CSK_4$ | Pam3CysSerLys4 |
| $PaO_2$ | Arterial oxygen partial pressure |
| PCR | Polymerase chain reaction |
| PCT | Procalcitonin |
| PDA | Persistent ductus arteriosus Botalli |
| PG | Peptidoglycan |
| PGRN | Progranulin |
| PMA | Phorbol 12-myristate 13-acetate |
| PROM | Premature rupture of membranes |
| PRR | Pattern recognition receptor |
| PVL | Periventricular leukomalacia |
| PYD | Pyrin domain |
| Q | Glutamine |
| RAGE | Receptor for advanced glycation end products |
| RD | Reagent diluent |
| RIG-I | Retinoic-acid-inducible gene I |
| RLR | Retinoic-acid-inducible gene I like receptor |
| RNA | Ribonucleic acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |
| *S. aureus* / *SA* | *Staphylococcus aureus* |
| S100A8 | S100 calcium-binding protein A8 |
| SARM | Sterile-α and armadillo motif-containing protein |
| SD | Standard deviation |
| SGA | Small for gestational age |
| sgRNA | Synthetic guide RNA |
| SIRS | Systemic inflammatory response syndrome |
| SNP | Single nucleotide polymorphism |
| SOFA score | Sequential organ failure assessment score |
| STAT1 | Signal transducer and activator of transcription |
| TAE buffer | Tris, acetate and EDTA buffer |
| $T_H1$ / 2 / 17 | Type 1 / 2 / 17 helper T cells |
| THP1 cell | Human acute monocytic leukemia cell line THP1 |
| T | Thymidine |
| TIR | Toll/Interleukin-1 receptor |
| TIRAP | TIR-domain containing adaptor protein |
| TLR | Toll-like receptor |
| TMB | Tetramethylbenzidine |
| TNF-α | Tumor necrosis factor alpha |
| TRAM | TRIF-related adaptor molecule |
| TRAF6 | TNFR-associated factor 6 |
| TRIF | TIR-domain containing adaptor protein inducing IFN-ß |
| TSB | Tryptic soy broth |
| U | Uridine |
| UN IGME | United Nations Inter-agency Group for Child Mortality Estimation |
| UV | Ultraviolet |
| VLBW | Very low birth weight |
| vs. | Versus |
| WOP | Weeks of pregnancy |
| WT | Wild typ |
| ∆Lgt | Delta Lgt mutant bacteria |
| Ø | Control |

| Unit | Term |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| $cm^2$ | Square centimeter |
| dl | Deciliter |
| g | Gram |
| *g* | Gravitational force equivalent |
| G | Giga |
| h | *Hora* (hour) |
| IU | International unit |
| kDa | Kilodalton |
| kg | Kilogram |
| l | Liter |
| µg | Microgram |
| µl | Microliter |
| mg | Milligram |
| ml | Milliliter |
| mmol | Millimol |
| min | Minute |
| ng | Nanogram |
| nl | Nanoliter |
| nm | Nanometer |
| pg | Picogram |
| rpm | Revolutions per minute |
| sec | Second |
| U | Unit |

# Summary

**Background/objectives –** Sepsis represents a serious health care burden especially during the neonatal period. Newborns exhibit immature innate immune functions due to their relative inability to trigger proper inflammatory responses. Consequently, they are at increased risk of sepsis-associated mortality and morbidity. As part of the innate defense mechanisms, an inflammasome-based surveillance machinery was discovered that constantly monitors the cytosolic compartment for invading pathogen-encoded products and danger signals. Caspase-11 in mice as well as their human counterparts caspase-4 and caspase-5 scaffold the so-called non-canonical inflammasome. It is activated through the cytosolic presence of lipopolysaccharides of Gram-negative bacteria and induces a lytic cell death, called pyroptosis. We discovered that the protein secretion of the novel alarmins galectin-1 (Gal-1), progranulin (PGRN) and resistin occurs following caspase-4 and caspase-5 activation. Considering Gram-positive bacteria as a potential cause for neonatal sepsis, the molecular mechanisms of bacterial induced non-canonical inflammasome activation are poorly understood. Collectively, the present study aims to elucidate the release of Gal-1, PGRN and resistin during neonatal sepsis and to provide mechanistic insight into how Gram-positive bacteria are involved in the inflammasome-dependent release of alarmins.

**Methods –** For alarmin detection, a total of 78 neonates were included in this study, who were hospitalized between March 2014 and February 2016 in the neonatal intensive care unit (NICU) of the University Hospital in Jena. Blood serum samples were retrospectively analyzed and matched with clinical data. According to study-specific sepsis criteria, neonates were divided into an infected (n = 29) and non-infected group (n = 23). Serum levels of Gal-1, PGRN and resistin were determined via ELISA. To study the role of *S. aureus* in release of PGRN, we created a lipoprotein lipidation-deficient Lgt mutant via CRISPR/Cas9-mediated base editing. THP1 cells were treated with heat-killed *S. aureus* wild type, ΔLgt *S. aureus* and $Pam_3CSK_4$. Finally, the cell supernatant concentrations of PGRN and TNF-α were detected via ELISA.

**Results/discussion –** Serum resistin levels were significantly elevated in infected neonates compared to the non-infected controls (163,5 ± 96,1 vs. 105,7 ± 73,0 ng/ml, p = 0,0182). No such difference was found in the secretion of Gal-1 and PGRN. Gestational age-dependent analysis revealed that among the whole study cohort, PGRN levels were significantly increased, when the infant was born preterm compared to full-term delivery (91,1 ± 44,8 vs. 61,6 ± 17,9 ng/ml, p = 0,0012). Moreover, infected full-term born neonates exhibited higher values of PGRN than non-infected full-term infants (70,9 ± 19,3 vs. 44,4 ± 9,3 ng/ml, p = 0,0173). In turn, resistin showed significant elevated concentrations in infected preterm newborns compared to non-infected preterm newborns (175,2 ± 94,0 vs. 87,1 ± 49,8 ng/ml, p = 0,0007). The release of Gal-1 was not influenced by the gestational age. To investigate the role of alarmins in sepsis diagnosis, alarmin levels were correlated with IL-6 and CRP concentrations of the study population. In infected newborns, only PGRN showed significant association to the infection marker IL-6 (r = 0,3720, p = 0,0469). However, the alarmins did not correlate to CRP. The study further elucidates the role of Gram-positive bacteria in PGRN release. By creating a mutant ∆Lgt *S. aureus* bacteria strain deficient in lipoprotein maturation, surface lipoproteins of *S. aureus* have been demonstrated to induce the release of PGRN without the sufficient stimulation of TLR2.

**Conclusion –** The present thesis outlines the non-canonical inflammasome activation during neonatal sepsis. Caspase-4 and caspase-5-dependent release of the alarmins Gal-1, PGRN and resistin showed different secretion patterns in different patient groups. Stratifying the NICU patients according to infection, the diagnostic potential of the alarmins was investigated by considering the gestational age and infection parameters. Thereby, PGRN emerged as a reliable marker to identify sepsis in neonates. By investigating the *S. aureus*-induced PGRN secretion pathway, the current thesis also contributes to uncovering the pathomechanisms of Gram-positive infection. These findings may help to initiate adequate diagnosis and treatment in response to sepsis.

# Zusammenfassung

**Hintergrund –** Sepsispatienten stellen das Gesundheitssystem vor besondere Herausforderungen. Insbesondere Neugeborene sind einem erhöhten Risiko ausgesetzt, an einer Sepsis zu versterben oder Folgeerkrankungen zu erleiden. Wesentlich daran beteiligt sind ihre noch unreifen Immunfunktionen mit der relativen Unfähigkeit, angemessene, inflammatorische Immunantworten gegen Krankheitserreger zu generieren. Zu den angeborenen Abwehrmechanismen des Immunsystems gehören sogenannte Inflammasomen, die das Zellkompartiment kontinuierlich auf eindringende Pathogene und Gefahrensignale untersuchen. Dabei bilden Caspase-11 in Mäusen, sowie die humanen Gegenstücke Caspase-4 und Caspase-5 das nicht-kanonische Inflammasom. Es wird durch intrazelluläre Lipopolysaccharide Gram-negativer Bakterien aktiviert und induziert eine Art lytischen Zelltod, der als Pyroptose bezeichnet wird. Wir konnten nachweisen, dass die Aktivierung der Caspase-4 und Caspase-5 zur Sezernierung der neuartigen Alarmine Galectin-1 (Gal-1), Progranulin (PGRN) und Resistin führt. Dennoch gibt es wenig Kenntnis darüber, wie Gram-positive Bakterien als potente Sepsis-Erreger bei Neugeborenen das nicht-kanonische Inflammasom aktivieren. Ziel dieser Arbeit ist es nun, die Inflammasom-abhängige Freisetzung der Alarmine Gal-1, PGRN und Resistin während der neonatalen Sepsis zu untersuchen. Des Weiteren erforscht die hier vorliegende Studie, wie Gram-positive Bakterien die Inflammasomen-abhängige Sezernierung der Alarmine beeinflussen.

**Methoden –** In dieser Studie wurden 78 Neugeborene eingeschlossen, die zwischen März 2014 und Februar 2016 auf der Neugeborenen-Intensivstation (NICU) des Universitätsklinikums Jena behandelt wurden. Dabei wurden die Blutseren retrospektiv ausgewertet und mit den klinischen Daten der Patienten verglichen. Anhand studienspezifischer Sepsis-Kriterien wurde die Neugeborenenpopulation als infektiös (n = 29) und nicht-infektiös (n = 23) klassifiziert. Die Serumkonzentrationen von Gal-1, PGRN und Resistin wurden mittels ELISA bestimmt. Um die Rolle von *S. aureus* bei der PGRN-Freisetzung zu erforschen, konnte via CRISPR-Cas9-vermittelter Base Editing Technologie eine ∆Lgt *S. aureus* Mutante kreiert werden. Die somit künstlich erzeugten Punktmutationen im Lgt-Gen verhinderten die Reifung von Lipoproteinen an der Zelloberfläche der Bakterien. Nach der Stimulation von THP1-Zellen mit hitze-

getötetem Wildtyp und Lgt-defizienten *S. aureus*, sowie mit $Pam_3CSK_4$, wurde die Freisetzung von PGRN und TNF-α mit Hilfe der ELISA-Methode analysiert.

**Ergebnisse/Diskussion –** In der hier vorliegenden Arbeit konnte gezeigt werden, dass die Resistin-Konzentrationen in infizierten Neugeborenen im Vergleich zu den nicht-infizierten Säuglingen signifikant erhöht waren (163,5 ± 96,1 vs. 105,7 ± 73,0 ng/ml, p = 0,0182). Kein solcher Unterschied wurde bei der Sekretion von Gal-1 und PGRN beobachtet. Unter Berücksichtigung des Gestationsalters ergaben sich in der gesamten Studienkohorte signifikant erhöhte PGRN-Werte bei Frühgeborenen im Gegensatz zu Reifgeborenen (91,1 ± 44,8 vs. 61,6 ± 17,9 ng/ml, p = 0,0012). Darüber hinaus wiesen infizierte, reifgeborene Säuglinge höhere Werte von PGRN auf als nicht infizierte, reifgeborene Säuglinge (70,9 ± 19,3 vs. 44,4 ± 9,3 ng/ml, p = 0,0173). Resistin wiederum zeigte signifikant erhöhte Konzentrationen bei infizierten Frühgeborenen im Vergleich zu nicht-infizierten Frühgeborenen (175,2 ± 94,0 vs. 87,1 ± 49,8 ng/ml, p = 0,0007). Bei Gal-1 konnte keine Gestationsalter-abhängige Freisetzung beobachtet werden. Um die Rolle der Alarmine in der Sepsisdiagnostik zu untersuchen, wurden die Alarminspiegel mit den IL-6- und CRP-Konzentrationen der Studienpopulation korreliert. Bei infizierten Neugeborenen zeigte nur PGRN eine signifikante Assoziation mit dem Infektionsmarker IL-6 (r = 0,3720, p = 0,0469). Die Alarmine korrelierten nicht mit CRP. Mittels Lipoprotein-defizienten *S. aureus* Mutanten konnte zudem gezeigt werden, dass Oberflächenlipoproteine von *S. aureus* die Freisetzung von PGRN induzieren, ohne TLR2 ausreichend zu stimulieren.

**Schlussfolgerung –** Die hier vorliegende Arbeit skizziert die nicht-kanonische Inflammasomen-Aktivierung während der neonatalen Sepsis. Dabei zeigte die Caspase-abhängige Freisetzung der Alarmine Gal-1, PGRN und Resistin unterschiedliche Sekretionsmuster in verschiedenen Patientengruppen. Die Stratifizierung der Neugeborenen nach Infektion unter Berücksichtigung des Gestationsalters, sowie Infektionsparameter, verdeutlichte das diagnostische Potential der Alarmine. Es kristallisierte sich heraus, dass PGRN als zuverlässiger Marker zur Identifizierung septischer Neugeborener dienen kann. Durch die Untersuchung der *S. aureus*-induzierten PGRN-Sekretion wirkt die Studie zudem wesentlich bei der Aufdeckung pathogener Mechanismen von Grampositiven Infektionen mit. Zusammenfassend tragen diese Erkenntnisse zur weiteren Erforschung von adäquaten Diagnose- und Behandlungsmöglichkeiten der Sepsis bei.

# 1 Introduction

## 1.1 Neonatal sepsis

### 1.1.1 Definition of neonatal sepsis

For the past three decades, the definition of sepsis included the systemic inflammatory response syndrome (SIRS) associated with infection for both adults and children (Bone et al. 1992, Goldstein et al. 2005). The SIRS criteria comprised abnormal core temperature, heart and respiratory rate as well as leukocyte count (Bone et al. 1992, Goldstein et al. 2005). Being criticized for its too broad inclusion criteria (Churpek et al. 2015), the Third International Consensus Definition of Sepsis and Septic Shock (Sepsis-III) in 2016 redefined sepsis as *"life-threatening organ dysfunction caused by dysregulated host response to infection"* (Singer et al. 2016). Thereby, the severity of organ dysfunction is validated by the Sequential Organ Failure Assessment (SOFA) Score which rates respiratory, hepatic, cardiovascular and renal dysfunctions, as well as dysfunctions concerning the coagulation and central nervous system (Vincent et al. 1996). To date, this update in sepsis definition only refers to the adult population. Despite steady progress in neonatal care and increasing research, adequate identification, treatment and outcome of neonatal sepsis is still limited due to several facts:

- There is no consensus definition of neonatal sepsis (Wynn et al. 2014). Neither the Sepsis-III-definition nor the severity grading according to the SOFA score can be applied to neonatal intensive care unit (NICU) patients (Hibbert et al. 2018).
- In newborns, sepsis according to the International Pediatric Sepsis Consensus Conference still bases on the concept of SIRS and lacks the focus on organ dysfunction (Goldstein et al. 2005, McGovern et al. 2020). Furthermore, it excludes preterm born infants and only sparsely suites to term neonates (Goldstein et al. 2005, Hofer et al. 2012b).

Shane et al. (2017) describe neonatal sepsis as "*systemic condition of bacterial, viral, or fungal (yeast) origin that is associated with haemodynamic changes and other clinical manifestations and results in substantial morbidity and mortality."* In conclusion, neonatal sepsis remains a heterogenous disease with complex pathology depending on gestational age, onset and source of infection (Molloy et al. 2020).

### 1.1.2 Classification of neonatal sepsis

Depending on the onset of sepsis, the route of transmission and pathogen spectrum, neonatal sepsis is subdivided into early-onset neonatal sepsis (EOS) and late-onset-neonatal sepsis (LOS) (Shane et al. 2017, Zemlin et al. 2018).

**Early-onset neonatal sepsis:** The clinical manifestation of the early-onset neonatal sepsis occurs within the first 72 hours of life or, if caused by Group B Streptococci (GBS), within 7 days of life (Herting 2016, Shane et al. 2017). It is considered to be acquired vertically from mother to infant in utero, transplacental or during delivery (Shane et al. 2017). In most cases, the causative microorganisms originate from the typical flora of the maternal genitourinary tract and ascend the birth canal until the uterus, from where they are transmitted to the newborn (Rampersaud et al. 2012, Simonsen et al. 2014). Despite intrapartum prophylaxis, GBS remains one of the leading pathogens of EOS in term neonates, whereas *Escherichia coli (E. coli)* is associated with EOS in preterm and very low birth weight infants (Stoll et al. 2011).

**Late-onset neonatal sepsis:** The late-onset neonatal sepsis manifests after 3 days of life or in the time period of 1 week to 3 months after birth (Herting 2016, Shane et al. 2017). In contrast to EOS, pathogens derive from the hospital environment or the community, including contact to hospital staff and family members, nutritional sources, contaminated equipment as well as invasive procedures (Shane et al. 2017). The predominant microorganisms are coagulase-negative staphylococci (CoNS) (Goldstein et al.), which normally colonize the human skin and mucous membranes, but also plastic surfaces due to their capacity to form self-protecting biofilms (Camacho-Gonzalez et al. 2013). Especially premature, low birth weight neonates with an immature immune system and prolonged hospitalization are at high risk for LOS (Camacho-Gonzalez et al. 2013, Dong and Speer 2015, Giannoni et al. 2018).

Thanks to the implementation of maternal intrapartum antimicrobial prophylaxis in the 1990s and universal screening for maternal GBS colonization in 2002, the incidence of early-onset GBS infection decreased between 1997 and 2010, whereas the incidence of late-onset *E. coli* and GBS infection increased during the same period of time (Bauserman et al. 2013, Herting 2016).

### 1.1.3 Risk factors

The incidence of both EOS and LOS is inversely correlated to birth weight and gestational age (Shane et al. 2017). Preterm born infants with low birth weight suffer from infection 3 to 10 times more often than full-term neonates with normal birth weight and experience higher rates of mortality and later neurodevelopmental impairment (Shane et al. 2017, Stoll et al. 2002, Stoll et al. 2004). Besides the newborn´s immaturity, the risk factors for neonatal sepsis further depend on neonatal, maternal and nosocomial factors **(table 1)**.

**Table 1: Risk factors of early- and late-onset neonatal sepsis.** Table modified according to Blatt (2017), Camacho-Gonzalez et al. (2013), Helder et al. (2010) and Simonsen et al. (2014).

| Risk factors of early-onset neonatal sepsis | Risk factors of late-onset neonatal sepsis |
|---|---|
| • Premature or prolonged (> 18 h) rupture of membranes<br>• Maternal GBS colonization<br>• Chorioamnionitis<br>• Maternal peripartum infections, e.g. urinary tract infection<br>• Multiple pregnancies<br>• Low APGAR score<br>• Aspiration/ingestion of contaminated amniotic fluid<br>• Instrument-assisted delivery<br>• Invasive interventions during pregnancy, e.g. cervical cerclage, amniocentesis | • Destruction of physiological neonatal barriers, e.g. skin & mucosa<br>• Long-term use of invasive interventions, e.g. indwelling catheter use, endotracheal intubation<br>• Prolonged use of antibiotics<br>• Necrotizing enterocolitis<br>• Infant´s caregiver |
| **General risk factors** | |
| • Prematurity associated with immature immune functions and decreased transfer of maternal immunoglobulins | |

### 1.1.4 Diagnosis of neonatal sepsis

Early diagnosis of neonatal sepsis aims to identify newborns with actual sepsis to initiate an early and targeted therapy (Cortese et al. 2016, Zemlin et al. 2018). Besides increasing health care costs, delayed and unnecessary treatment carries the risk of neonatal mortality, complications such as cerebral palsy or interventricular hemorrhage, as well as the formation of antibiotic resistance (Cortese et al. 2016, Zemlin et al. 2018). Several obstacles obstruct the postnatal diagnosis, which mainly relies on pathogen detection, clinical examination and laboratory tests (Berger 2019):

- **Blood cultures** – the gold standard of sepsis diagnosis (Wynn and Polin 2018) – are often negative, although clinical and laboratory signs indicate neonatal sepsis (Blackburn et al. 2012, Klingenberg et al. 2018). The small amount of blood taken from the newborn, intermittent bacteremia in the context of e.g. small diagnostic interventions, and the suppression of bacteremia due to pre- and intrapartal antimicrobial treatment complicate the detection of pathogens (Berger 2019, Connell et al. 2007, Polin 2003). Furthermore, slow bacterial growth despite laboratory conditions delays the diagnosis (Markic et al. 2017). Contamination by CoNS must also be considered (Healy et al. 2013, Wynn 2016).
- **Symptoms** of neonatal sepsis range from light to severe disease patterns and are therefore non-specific (Shane et al. 2017). Recent studies revealed that constant monitoring of the heart rate characteristics is a promising method to predict proven or clinical sepsis (Lake et al. 2014).
- **Laboratory tests** lack in consistent specificity and sensitivity among different studies (Iroh Tam and Bendel 2017). The reasons are presumably caused by variable definitions of sepsis, test methods, sampling procedures, inclusion criteria as well as different reference and cut-off values (Iroh Tam and Bendel 2017). Apart from that, they are not able to differentiate sepsis from non-infectious causes of SIRS (Fan et al. 2016). The combination of biomarkers as well as serial measurements have led to improved outcomes in illness prediction (Hofer et al. 2012a).
- **Non-bacterial causes of sepsis,** such as viruses or fungi, as well as non-infectious organ failure can also provoke sepsis-like syndromes (Shane et al. 2017, Wynn 2016). Additionally, infectious and non-infectious causes of sepsis might coexist in the same host (Shane et al. 2017). Symptomatic newborns with negative blood cultures are therefore considered to suffer from clinical sepsis (Wynn 2016).

**Diagnostic criteria according to NEO-KISS:** In 2000, the German National Reference Center for Surveillance of Nosocomial Infections at the Institute for Hygiene and Environmental Medicine of the Charité Berlin, implemented a surveillance system for nosocomial infections of very low birth weight (VLBW) infants < 1500 g, the so-called NEO-KISS (Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen [NRZ] 2017). It was considered to standardize data collection and analysis about sepsis symptoms (NRZ 2017). The developed diagnosis criteria for nosocomial sepsis in VLBW newborns are summarized in **table 2**:

**Table 2: Diagnosis criteria of nosocomial sepsis in VLBW infants according to the NRZ (2017).** BE = base excess; CoNS = coagulase-negative staphylococci; CRP = C-reactive protein; CSF = cerebrospinal fluid; IL = Interleukin; I/T-ratio = immature to total neutrophil ratio.

| Sepsis definition | Diagnostic criteria |
|---|---|
| I. Clinical sepsis | Antimicrobial therapy for ≥ 5 days<br>*and* negative or not performed blood culture[3]<br>*and* no obvious infection<br>*and* two sepsis signs[1] |
| II. Microbiological confirmed sepsis | Pathogen (except CoNS) isolated in the blood or CSF<br>*and* two sepsis signs[1] |
| III. Microbiological confirmed sepsis with CoNS | Only CoNS in blood culture<br>*and* two sepsis signs[1]<br>*and* one laboratory parameter[2] |
| [1] Sepsis signs | • Fever > 38 °C, temperature instability, hypothermia < 36,5 °C<br>• Tachycardia > 200/min or new/increased bradycardia < 80/min<br>• Capillary refill time > 2 sec<br>• New or increased apnea (> 20 sec)<br>• Unclear metabolic acidosis, BE < -10 mmol/l<br>• New hyperglycemia > 140 mg/dl<br>• Other signs:<br>grey complexion, increased oxygen demand (intubation), instable general condition, apathy, I/T-ratio > 0,2, elevated laboratory parameters (CRP, IL-6 or IL-8), central-peripheral temperature difference > 2,0 °C |
| [2] Laboratory parameters | • CRP > 20 mg/l or IL-6/IL-8 increased<br>• I/T-ratio > 0,2<br>• Leukocyte count < 5/nl<br>• Platelet count < 100/nl |
| [3] Diagnosis of clinical sepsis is also possible if CoNS are isolated in blood culture as contamination and further criteria of III.) are not fulfilled. | |

**Laboratory parameters:** Many NICUs use the well-established infection markers Interleukin 6 (IL-6) and C-reactive protein (CRP) to support the diagnosis and monitoring of treatment in case of infection (Zemlin et al. 2018).

Interleukin 6 was discovered as pro-inflammatory cytokine (Del Giudice and Gangestad 2018). As an early warning biomarker, the blood levels of IL-6 immediately rise after the onset of bacteremia, 48 hours prior to clinical symptoms (Buck et al. 1994, Kuster et al. 1998). However, due to its short half-life, IL-6 levels rapidly decrease within 24 hours to undetectable baseline concentrations (Buck et al. 1994). Messer et al. (1996) revealed that plasma levels of IL-6 of at least 100 pg/ml were 100 % sensitive for detecting EOS in newborns within the first 12 hours of life.

The acute-phase reactant C-reactive protein is one of the most extensively studied and most frequently used laboratory tests for neonatal sepsis (Hofer et al. 2012a). In the setting of inflammation or tissue injury, CRP is synthesized by hepatocytes after cytokine stimulation with IL-1ß, IL-6 and TNF-α (Steel and Whitehead 1994). Its levels rise within 4 to 6 hours until they reach a peak after 36 hours of infection (Niehues 2017). CRP is considered as a late biomarker for neonatal sepsis, since its detection is delayed by 6 to 8 hours after the onset of clinical symptoms (Ng and Lam 2010). In most studies, the cut-off is set at 10 mg/l (Benitz et al. 1998, Markic et al. 2017). However, the combination of IL-6 and CRP in the first 48 hours provides better diagnostic accuracy than the individual markers alone (Ng et al. 1997).

## 1.2 The innate immune system

Invading microorganisms or toxins are initially warded off by the phylogenetic older innate immune system which represents the first line of immune response (Horner et al. 2004). It comprises skin and mucosal surface barriers, antimicrobial peptides, the complement system and cellular components such as granulocytes, monocytes, macrophages, dendritic as well as natural killer cells (Levy 2007, Wynn and Levy 2010). Since the adaptive immune system in newborns is immature in specificity and immune memory, the newborn host defense mainly relies on the innate immunity (Basha et al. 2014, Yu et al. 2018).

### 1.2.1 Organization of the innate immune system in newborns

At birth, the newborn needs to cope with the change from a sterile intra-uterine surrounding to an environment, which is full of unknown antigens (Levy 2007). This includes the primary colonization of the infant´s skin and mucous membranes by bacteria coming from the maternal anogenital area (Dominguez-Bello et al. 2010, Levy 2007). Thereby, it needs to balance between the acquisition of self-tolerance as well as the differentiated recognition and defense of previously unknown pathogens (Levy 2007). The host defense is additionally assisted by maternal immunoglobulin G (IgG) antibodies, which are passively transferred across the placenta during pregnancy, and immune-effective factors in the breast milk (Hanson and Korotkova 2002). The innate immune system in newborns is rather described as distinct than immature since it is well-equipped but not adapted to the outside world (Schüller et al. 2018). Compared

to older populations, the neonatal innate immunity differs in the capacity, quantity and quality of immune response towards invading pathogens (Wynn and Levy 2010). This contributes to the newborn´s increased susceptibility to infection and immune-mediated diseases, especially in preterm born infants (Goedicke-Fritz et al. 2017, Kollmann et al. 2017). The following **table 3** summarizes the specific anatomic, cellular and humoral components of the neonatal innate immune system and its immature characteristics compared to adults:

**Table 3: Overview of the neonatal innate immune components and their immature characteristics.** Table modified according to Basha et al. (2014), Kollmann et al. (2012), Levy (2007), , Wynn and Levy (2010), Yu et al. (2018). APC = antigen-presenting cell, CD = cluster of differentiation, DC = dentritic cell, IFN = interferon, IL = interleukin, MHC = major histocompatibility complex, NET = neutrophil extracellular trap, NK cell = natural killer cell, $T_H2$ and $T_H17$ = type 2 and type 17 helper T cell, TLR = toll-like receptor, TNF = tumor necrosis factor.

| **Innate immune component** | **Risk factor considering susceptibility to infection** |
|---|---|
| **Anatomic barriers** | |
| Skin & mucosal barrier | • Vernix caseosa is largely absent in preterm newborns < 28 weeks<br>• Increased permeability of stratum corneum<br>• Increased density of hair follicles provides an entry portal for microbes<br>• Impaired respiratory mucosal function (e.g. surfactant deficiency)<br>• Easy destruction of anatomic barriers by invasive medical interventions, antibiotics or hypoxia |
| **Innate immune cells** | |
| Neutrophils | • Diminished in production and storage in bone marrow<br>• Reduced in chemotaxis, cell adhesion, deformation capability and antimicrobial activity (e.g. different granula content, reduced NET formation) |
| Antigen-presenting cell (APCs)<br>Monocytes / Macrophages<br>Dentritic cells (DCs) | • Immaturity of dendritic cells<br>• Increased stimulation required for activation<br>• Inefficient pathogen recognition and antigen presentation due to reduced cell surface receptors and co-stimulatory or adhesion molecules (e.g. MHC-II, CD86, CD80, TLRs)<br>• Tendency towards $T_H2$/$T_H17$ type cytokine response<br>• Reduced in chemotaxis, migration and phagocytosis |
| Natural killer cells (NK cells) | • Increased number of NK cells, but reduced cytotoxic capacity<br>• Diminished recognition of abnormal cells by increased expression of inhibitory cell surface receptors<br>• Reduced cytokine production (e.g. IL-15, IL-12, IFN-γ)<br>• Lower threshold for activation provides some anti-viral protection |
| Mast cells | • Increased release of histamine after stimulation |
| Toll-like receptors (TLRs) | • Well-developed expression patterns and sensor functions<br>• Impaired and biased TLR-mediated production of cytokines |

| **Humoral components** | |
|---|---|
| Complement system | • 30 - 90 % less complement factor concentrations than in adults<br>• Less activation of classical complement pathway due to lack of antibodies<br>• Complement receptor deficiencies hamper chemotaxis and transmigration |
| Acute-phase-reactants | • Rapid activation of the acute-phase response soon after birth<br>• Impaired opsonization capability |
| Cytokines | • Weak cytokine responses after cell stimulation<br>• Impaired production of $T_H1$-mediated cytokines (e.g. TNF-α, IFN-γ, IL-12p70) and increased production of $T_H2$/$T_H17$-mediated cytokines (e.g. IL-6, IL-10, IL-17, IL-23) |

## 1.3 Innate immune signaling pathways

The innate immune signaling pathways fulfill sensor, effector and regulatory functions (Kollmann et al. 2012). In doing so, specific molecular structures called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by various pattern-recognition receptors (PRRs) of professional and non-professional immune cells (Takeuchi and Akira 2010). The ligation between receptor and molecular danger signals subsequently induce downstream signaling cascades resulting in gene expression of various molecules with immunological and regulatory effects (Raymond et al. 2017).

### 1.3.1 PAMPs, DAMPs and alarmins

**Pathogen-associated molecular patterns:** The term pathogen-associated molecular patterns was coined by the immunologist Charles Janeway Jr. in 1989 (Raymond et al. 2017). He suggested the existence of receptors on antigen-presenting cells, which recognize characteristic and highly conserved microbial structures as non-self (Janeway 1989, Pradeu and Cooper 2012). These foreign molecular motifs hardly mutate which indicate their essential role in microbes and make them ideal target structures (Hato and Dagher 2015, Schütt and Bröker 2011). Since that time, the number of PAMPs is continuously rising. Major identified structures include not only bacterial membrane components like lipopolysaccharides (LPS), peptidoglycans (PG) and lipoproteins (Lpp), but also microbial nucleic acids (Raymond et al. 2017).

**Damage-associated molecular patterns and alarmins:** The theory of Janeway was expanded by Polly Matzinger in 1994 (Matzinger 1994, Pradeu and Cooper 2012). She introduced damage-associated molecular patterns, or synonymous alarmins, which represent a heterogenous group of cell-derived molecules including cytoplasmatic, nuclear and extracellular components (Tang et al. 2012). DAMPs or alarmins are actively or passively released by stressed cells following tissue injury (Raymond et al. 2017). Once recognized via PRRs, they induce a downstream signaling cascade resulting in non-infectious inflammatory processes similar to PAMPs (Denning et al. 2019, Yang et al. 2017). Well-known examples for DAMPs are high-mobility group box protein 1 (HMGB1), heat shock proteins (HSP), S100 proteins, histones, hyaluronic acid, complement factors as well as non-protein molecules like adenosine triphosphate (ATP), uric acid, heparin sulfate, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Tang et al. 2012). Recent investigations have demonstrated the association between sepsis severity and the release of DAMPs (Ekaney et al. 2014, Sunden-Cullberg et al. 2005). Therefore, it is discussed whether DAMPs or alarmins can serve as early biomarkers and therapeutic targets in inflammatory disorders (Chan et al. 2012).

### 1.3.2 Pathogen recognition receptors

Endothelial, epithelial and immune cells are constantly scanning the environment in order to detect PAMPs and DAMPs via a diverse family of receptors called pattern recognition receptors. In contrast to the B- and T-cell receptors as part of the adaptive immune system, PRRs are non-clonal, germline-encoded host receptors (Akira et al. 2006). Nowadays, several different classes of PRRs haven been identified: toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), retinoic-acid-inducible gene I (RIG-I) like receptors (RLRs) and scavenger receptors (Kigerl et al. 2014). Their recognition capacity is increased by their strategic location in different compartments of the cell e.g. transmembrane, intracellular or secreted into the blood stream and tissue fluids (Kagan 2012, Raymond et al. 2017). Furthermore, they can be classified according to their binding ligands (Kawai and Akira 2011). The resulting pathogen-specific early immune pathways consist of a similar set of highly conserved response elements, which induce a shared pattern of genes (Nau et al. 2002).

**Toll-like receptors:** The toll-like receptor family is one of the most extensively studied subclass of pattern recognition receptors (Raymond et al. 2017). Depending on the cell type and stimuli, a different set of TLRs is expressed (Akira et al. 2006, Muzio et al. 2000). In humans, ten types of TLRs have been identified with specific and overlapping recognition of PAMPs and DAMPs (Akira et al. 2006). Extracellular danger signals such as microbial membrane components are monitored by TLR1, 2, 4, 5, 6 and 10, which are located on the cell surface (Kawai and Akira 2011). In contrast, TLR3, 7, 8 and 9 are expressed in the intracellular compartments including the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes to sense foreign nucleic acids after the uptake of pathogens (Blasius and Beutler 2010). After ligand attachment, the TLRs dimerize leading to conformational changes and adaptor recruitment (O'Neill and Bowie 2007). In signal transduction, the following adaptor molecules are involved: Myeloid differentiation primary response 88 (MyD88), TIR-domain containing adaptor protein (TIRAP), TIR-domain containing adaptor protein inducing IFN-ß (TRIF), TRIF-related adaptor molecule (TRAM) as well as sterile-α and armadillo motif-containing protein (SARM) (O'Neill and Bowie 2007). The receptor-adaptor protein interaction induces a second messenger cascade which ultimately culminates in the activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IRF) 1, 3, 5 and 7 (Kawai and Akira 2011). They initiate the gene expression of inflammatory cytokines, such as IL-6 and TNF-α, type I interferon, chemokines as well as antimicrobial peptides that target and eliminate invading pathogens via the recruitment and maturation of innate immune cells (Kawai and Akira 2011) **(Fig. 1)**.

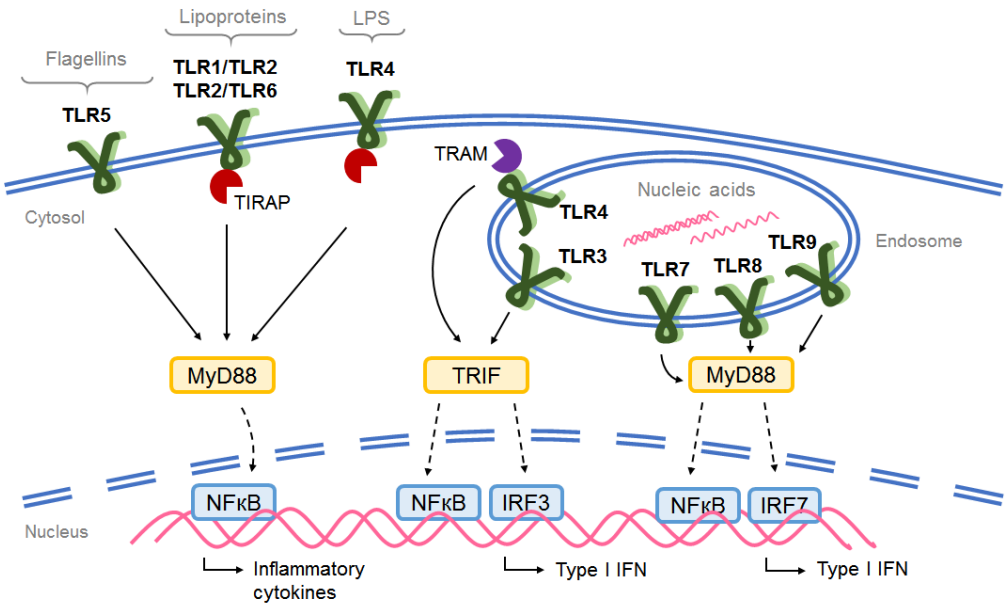



**Figure 1: Toll-like receptor signaling pathways.** After ligand binding, TLRs dimerize and mainly recruit the adaptor proteins MyD88 or TRIF for downstream signaling inducing inflammatory cytokine expression and type I IFN production. IFN = interferon, IRF = interferon regulatory factor, LPS = lipopolysaccharide, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, TIRAP = TIR-domain containing adaptor protein, TRAM = TRIF-related adaptor molecule, TRIF = TIR-domain containing adaptor protein inducing IFN-ß, TLR = toll-like receptor. Figure modified according to Kawai and Akira (2010).

### 1.3.3 Inflammasomes

Some cytosolic pattern recognition receptors, such as the receptors of the NLR family or AIM2-like receptors are able to induce a unique immune response towards infection and endogenous danger signals by assemble into inflammasomes (Hornung et al. 2009, Martinon et al. 2002, Yu et al. 2006). Canonical inflammasomes, first described by Martinon et al. (2002), are large multi-protein complexes that mediate the activation of caspase-1 in immune cells. Caspase-1 was shown to promote interleukin release and cell death (Martinon et al. 2002). 9 years later, Kayagaki et al. (2011) coined the term non-canonical inflammasome by revealing the function of inflammasome-independent activation of immunoregulatory caspases in mice.

### 1.3.3.1 The canonical NLRP3 inflammasome

**Construction:** Many inflammasomes are constructed by members of the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Martinon et al. 2009). In humans, 23 NLRs have been identified, of which 6 participate in inflammasome formation (Broz and Monack 2013). The receptor proteins share a common tripartite structure consisting of a C-terminal leucine-rich repeat (LRR) domain for pathogen detection, an intermediary nucleotide binding domain (NACHT domain, also referred to as NOD or NBD domain) with self-oligomerization capacity, as well as a variable effector binding region at the N-terminus (Chen et al. 2009) **(Fig. 2)**. The latter variably promotes protein-protein interactions via the caspase recruitment domain (CARD), pyrin domain (PYD), baculovirus inhibitor repeat (BIR) domain or acidic transactivating domain (Chen et al. 2009). Depending on their N-terminal domain, NLRs are further classified into four subfamilies (Ting et al. 2008). Among them, the NOD-, LRR- and pyrin domain-containing proteins (NLRPs), represent the largest subfamily (Ting et al. 2008) **(Fig. 2)**.

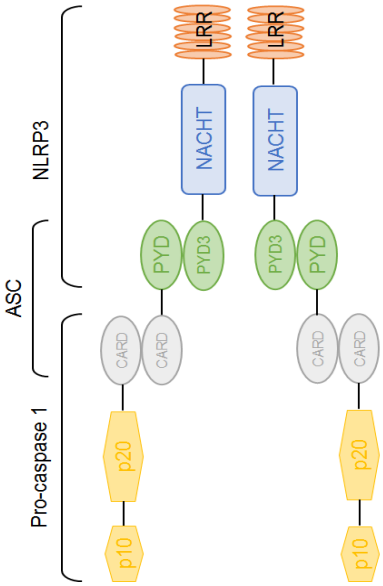



**Figure 2: Structural components of the NLRP3 inflammasome.** ASC = apoptosis-associated speck-like protein containing a CARD, CARD = caspase recruitment domain, LRR = leucine-rich repeat, NACHT = nucleotide binding domain, NLRP3 = NOD-, LRR- and pyrin domain-containing protein, PYD = pyrin domain. Figure modified according to Platnich and Muruve (2019).

Inflammasomes scaffolded by NLRP3 are one of the best studied canonical inflammasomes **(Fig. 2)**. Its architecture combines sensor and effector functions by merging the cytosolic receptor oligomer NLRP3 with the effector protease pro-caspase-1 via the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Schroder and Tschopp 2010). The assembly is mediated by the PYD-PYD interactions between NLRP3 and the adaptor protein ASC as well as the CARD-CARD linkage between ASC and pro-caspase-1 (Lu et al. 2014, Schroder and Tschopp 2010). Pro-caspase-1 consists of the catalytic subunits p20 and p10 (Schroder and Tschopp 2010).

**Activation:** Since the basal expression of NLRP3 is restricted in unstimulated cells, the assembly of the canonical inflammasome undergoes a two-step activation process consisting of a priming signal (signal 1) and an activation or assembly signal (signal 2) (Groslambert and Py 2018, Lamkanfi and Dixit 2014) **(Fig. 3)**:

- Signal 1 is mediated via the stimulation of extracellular TLRs by danger signals and leads to the transcriptional upregulation of the NLRP3 and pro-IL-1ß expression (Bauernfeind et al. 2009). In contrast, pro-caspase-1 as well as pro-IL-18 are constitutively expressed (Groslambert and Py 2018). The priming signal also induces post-translational modifications, such as deubiquitination and phosphorylation steps that stabilize NLRP3 (Py et al. 2013, Song et al. 2017).
- Signal 2 mechanisms are less well understood. A wide range of PAMPs and DAMPs have been confirmed to activate the inflammasome assembly (Liu et al. 2020). Nevertheless, it must be considered that none of the stimuli directly bind and activate the NLRP3 receptor (Rathinam and Fitzgerald 2016). Instead, they engage metabolic dysregulation through potassium ($K^+$) efflux, calcium ($Ca^{2+}$) influx, lysosome destabilization and rupture, mitochondrial reactive oxygen species (ROS) or mitochondrial DNA damage to initiate inflammasome assembly (Liu et al. 2020, Rathinam and Fitzgerald 2016).

**Effector functions:** Upon stimulation, signal 2 promotes the association of the inflammasome components through NLRP3 oligomerization and recruitment of the adaptor protein ASC to build an activation platform for pro-caspase-1 (Schroder and Tschopp 2010). The approximation of pro-caspase-1 triggers the autocatalytic release of its active p10 and p20 subunits, which compose into two heterodimers (Schroder and Tschopp 2010). The mature caspase-1 fulfills two major functions **(Fig. 3)**:

- The cleavage of the progenitor proteins pro-IL-1ß and pro-IL-18 into their biological active forms as pro-inflammatory cytokines (Lamkanfi and Dixit 2014).
- The induction of pyroptosis, an inflammatory and lytic kind of programmed cell death via cleavage of gasdermin-D (GSDMD), a protein with membrane pore-forming capacity (Liu et al. 2016).

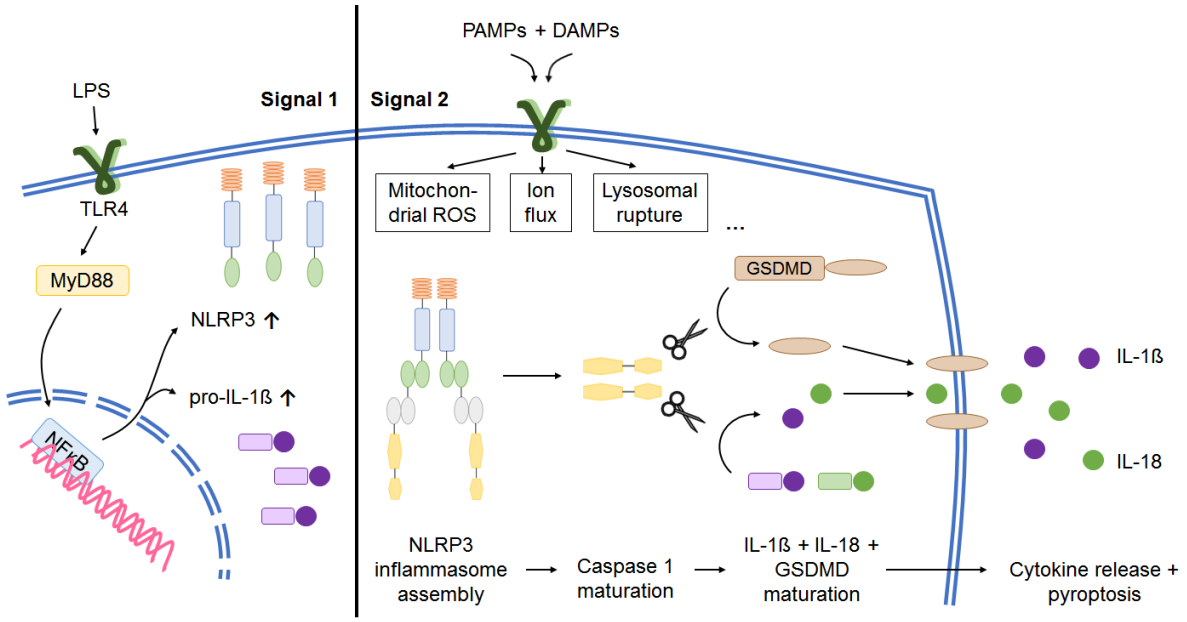



**Figure 3: Canonical inflammasome activation pathways and effector functions.** LPS stimulation (signal 1) mediates the transcriptional upregulation of the NLRP3 components as well as of pro-IL1ß. Several PAMPs and DAMPs (signal 2) engage metabolic dysregulation for NLRP3 assembly and NLRP3 effector functions. DAMPs = damage-associated molecular patterns, GSDMD = gasdermin- D, IL = Interleukin, LPS = lipopolysaccharide, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, NLRP3 = NOD-, LRR- and PYD-containing protein 3, PAMPs = pathogen-associated molecular patterns, ROS = reactive oxygen species, TLR4 = toll-like receptor 4.

### 1.3.3.2 The non-canonical inflammasome

**Construction:** The non-canonical inflammasome was first described by Kayagaki et al. in 2011 and is now central to this study (Kayagaki et al. 2011). Different from the caspase-1-dependent canonical inflammasome, it involves the murine caspase-11 (CASP11) as well as their human counterparts caspase-4 (CASP4) and caspase-5 (CASP5) (Kayagaki et al. 2011, Shi et al. 2014). Together with the closely related caspase-1, the cysteine aspartate-specific proteases are part of the inflammatory caspase group in which all caspases share an N-terminal CARD motif for protein-protein interactions as well as p10 and p20 subunits (Lavrik et al. 2005, Martinon and Tschopp 2004) **(Fig. 4)**. Analogous to the assembly mechanisms of the canonical inflammasomes, an unknown CARD-bearing receptor was hypothesized to mediate pathogen recognition and downstream recruitment of caspase-11, caspase-4 or caspase-5 (Kayagaki et al. 2011). Unexpectedly, it turned out that such multiprotein scaffold is not required. Oligomerization and catalytic activity of the inflammatory

caspases are triggered through direct pathogen binding, suggesting that the caspases are receptors and effectors by themselves (Shi et al. 2014) **(Fig. 4)**.

**Sensing mechanism:** The non-canonical inflammasome plays a crucial role in the host defense against Gram-negative infections (Kayagaki et al. 2013, Rathinam et al. 2012, Stowe et al. 2015). It is activated in response to invading microbes by sensing intracellular LPS, a major cell wall component of Gram-negative bacteria (Hagar et al. 2013, Kayagaki et al. 2013). However, distinct from the canonical inflammasome, caspase-4, -5 and -11 directly bind the lipid A moiety of LPS via their CARD domains (Shi et al. 2014) **(Fig. 4)**. Thereby, sufficient pathogen recognition strongly depends on the hexa-acylation state of the lipid A domain, the same structural component, which binds TLR4 (Hagar et al. 2013, Park et al. 2009, Shi et al. 2014). The bondage to acidic lipid A is facilitated by the CARD domains themselves that exhibit positively charged motifs and a basic isoelectric point (Rathinam et al. 2019, Shi et al. 2014).

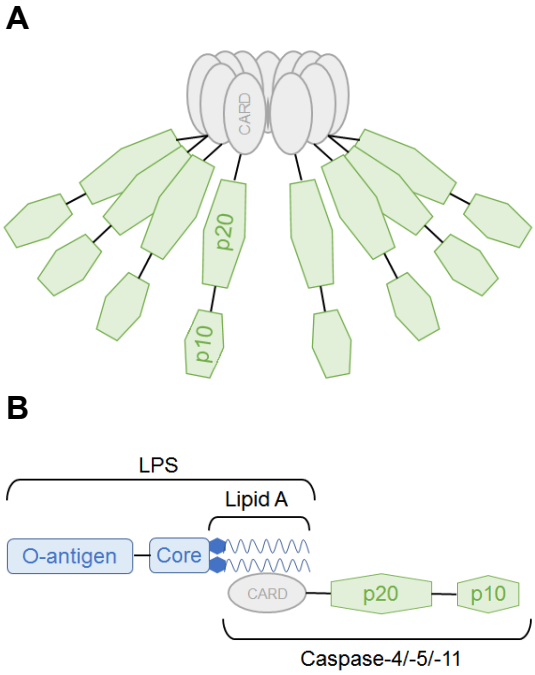



**Figure 4: Structures of the non-canonical inflammasome. (A)** Oligomerization and activation of caspase-4/-5/-11. **(B)** Direct interaction between the lipid A moiety of LPS and the CARD domain of caspase-4/-5/-11. CARD = caspase recruitment domain, LPS = lipopolysaccharide. Figure modified according to Yi (2017).

**Cytosolic access of LPS:** As the primary location of Gram-negative bacteria is the extracellular space, LPS needs to be internalized to be detected by the cytosolic inflammatory caspases of the host (Yi 2020a). Access is provided by TLR4-MD2-CD14-mediated endocytosis or via bacterial outer membrane vesicles (OMVs) of Gram-negative bacteria, which fuse with the host´s cell membrane (Gegner et al. 1995, Vanaja et al. 2016). Furthermore, several cell surface receptors and transport molecules are involved, such as the receptor for advanced glycation end product (RAGE) that associates with HMGB1 for LPS internalization (Ding and Shao 2017). In the host cell, LPS is liberated via the GTPases guanylate-binding proteins (GBPs) and immunity-related GTPase family member b10 (IRGB10) (Man et al. 2016, Meunier et al. 2014, Pilla et

al. 2014). Recent studies identified certain other lipids as well as non-lipid molecules as potential ligands, e.g. the oxidized form of naturally occurring phospholipids, 1-palitoyl-2-arachidonoyl-sn-glyero-3-phosphorylcholine (oxPAPC) or the glycolipid lipophosphogylcan (LPG) on the surface of leishmania parasites (de Carvalho et al. 2019, Yi 2020b, Zanoni et al. 2016).

**Activation:** The initiation process of the non-canonical inflammasome has been intensively studied in mice. Comparable to the canonical inflammasome, the murine caspase-11 activation requires a priming signal (signal 1) and an activation signal (signal 2) to induce the inflammatory response against invading pathogens (Yi 2017):

- Signal 1 provides the transcriptional upregulation of caspase-11 and further components of the non-canonical inflammasome system, including pro-caspase-1 as well as pro-IL-1ß (Yi 2017). It is mediated by LPS, which induces TLR4-TRIF-dependent downstream signaling transduction (Rathinam et al. 2012). The cascade results in the expression of type I interferon (Rathinam et al. 2012). Type I IFN in turn activates the second transcription factor signal transducer and activator of transcription (STAT1) that binds next to NFκB for caspase-11 gene expression (Rathinam et al. 2012, Schauvliege et al. 2002). Interestingly, type I IFNs also induces the expression of the bacterial vacuole lysing GTPases GBPs and IRGB10, which are responsible for LPS release (Man et al. 2016, Meunier et al. 2014).
- Signal 2 involves the direct sensing of intracellular LPS and the subsequent formation of a caspase-11-LPS complex (Hagar et al. 2013, Kayagaki et al. 2013).

**Effector functions:** The LPS-caspase interaction induces oligomerization of the monomeric enzymes through the homotypic interactions of their CARD motifs (Yi 2017). The approximation of the caspases ultimately activates their cysteine protease function (Ding and Shao 2017, Shi et al. 2014). The activation of the non-canonical inflammasome has two major consequences:

- The induction of pyroptosis via the cleavage of GSDMD, similar to caspase-1 (Kayagaki et al. 2015, Shi et al. 2015).
- The activation of the canonical NLRP3 inflammasome leading to caspase-1-dependent secretion of IL-1ß and IL-18 (Baker et al. 2015, Kayagaki et al. 2011).

**Non-canonical inflammasome in humans:** The human caspase-4 and caspase-5 share around 60 % and 54 % amino acid sequence identity to murine caspase-11, respectively (Lamkanfi et al. 2002). Thereby, caspase-4 exhibits a higher similarity in sequence, size and function to caspase-11 than caspase-5 (Baker et al. 2015, Downs et al. 2020, Viganò et al. 2015). However, different from the murine caspase-11, caspase-4 is constitutively expressed (Lin et al. 2000, Yang et al. 2015b). It is broadly represented in various cell types including myeloid as well as non-myeloid cells, such as keratinocytes and epithelial cells (Knodler et al. 2014, Shi et al. 2014). The wide tissue expression of caspase-4 compared to caspase-5 suggests cell- or tissue-specific functions of the caspases (Awad et al. 2018, Lin et al. 2000, Salskov-Iversen et al. 2011). Additionally, the recognition capacity of caspase-4 seems more comprehensive, as it is able to respond to tetra-acylated LPS from *Francisella novicida* as well as the synthetic lipid IVa (Lagrange et al. 2018). Whether caspase-5 is similarly required for pathogen detection is still unclear. Its contribution to the NLRP1 inflammasome led to the implication that it might mainly participate in the induction of apoptosis (Martinon et al. 2002). In contrast to caspase-4 and similar to caspase-11, caspase-5 expression can be induced by LPS as well as IFN-γ (Bian et al. 2011, Lin et al. 2000). Recent studies uncovered the crucial role of caspase-5 in LPS recognition by processing of their catalytic domain (Baker et al. 2015, Viganò et al. 2015). Further investigations are required to identify the specific and overlapping functions of caspase-4 and caspase-5, as well as their involvement in inflammasome activation.

#### 1.3.3.3 Functional crosstalk between the canonical and non-canonical inflammasome

The activation of the non-canonical inflammasome results in the NLRP3-ASC-caspase-1-dependent release of IL-1ß and IL-18 (Baker et al. 2015, Kayagaki et al. 2011). Most likely, the link between the two inflammasomes is mediated by the drop of intracellular potassium levels (Ruhl and Broz 2015). Several mechanisms were identified to trigger the ion efflux including via the activation of the ATP-dependent ion channel $P2X_7$ or via GSDMD-mediated membrane pores (Yang et al. 2015a, Yi 2020b) **(Fig. 5)**. The indirect activation of the canonical inflammasome is only present in a certain subset of cells as many non-monocytic cells with caspase-4, -5 or -11 expression do not possess the NLRP3 inflammasome (Shi et al. 2017).

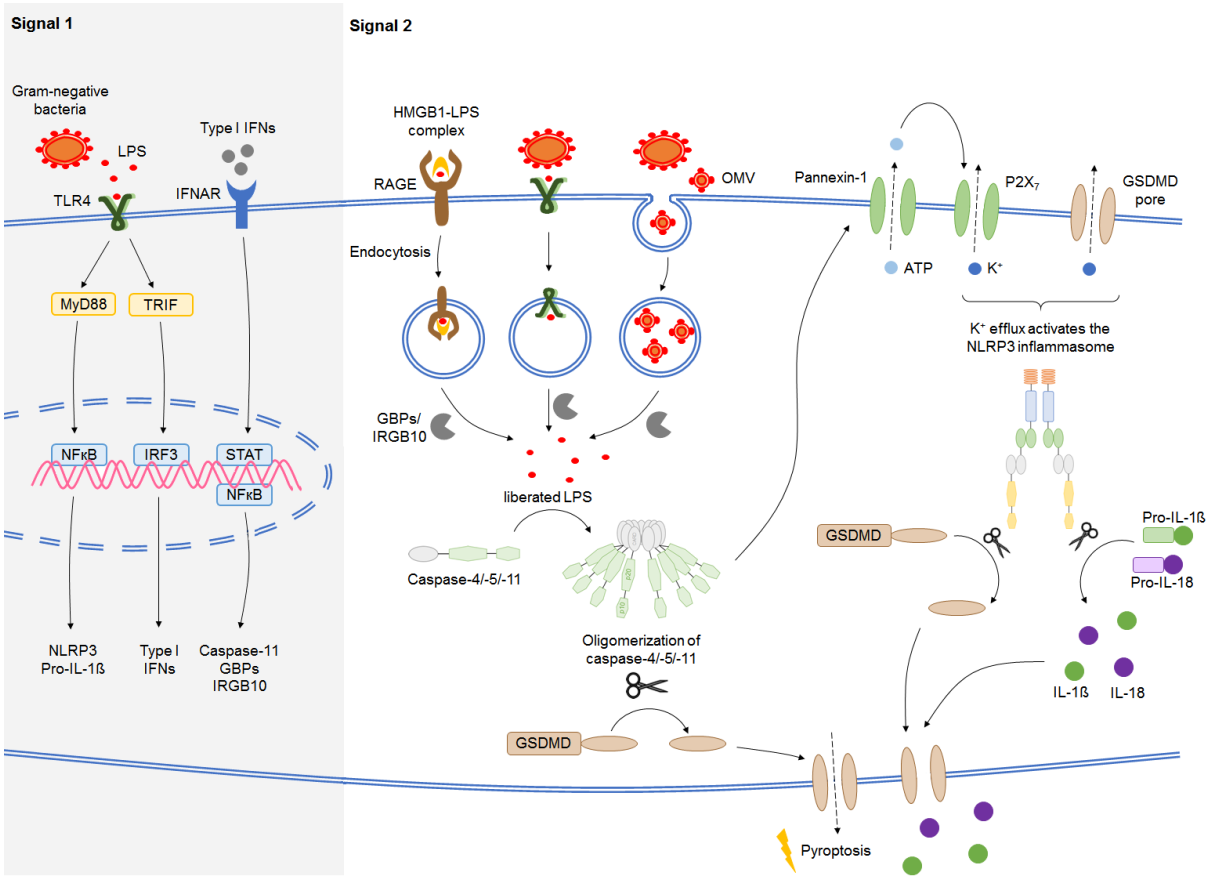



**Figure 5: Functional crosstalk between the canonical and non-canonical inflammasome.** Signal 1 is mediated via LPS of Gram-negative bacteria and Type I IFNs which bind to TLR4 and IFNAR receptors, respectively. The receptor activation leads to the transcriptional upregulation of inflammasome components and cytokines. Signal 2 comprises the activation of the non-canonical inflammasome via cytosolic LPS. It is internalized through receptor-mediated endocytosis, involving RAGE or TLR4 as well as the uptake of bacterial OMVs. The pathogens are released from the membrane vacuole by GBPs and IRGB10 and are directly recognized by caspase-4, -5 or -11. After autoactivation, the caspases cleave GSDMD and induce the NLRP3 inflammasome for cytokine release and pyroptosis. The NLRP3 activation is mediated via $K^+$-efflux through the $P2X_7$ ion channel as well as GSDMD pores. ATP = Adenosine triphosphate, GBPs = guanylate-binding proteins, GSDMD = gasdermin-D, HMGB1 = high-mobility group box protein 1, IFN = interferon, IFNAR = interferon-α/β receptor, IL = interleukin, IRF3 = interferon regulatory factor 3, IRGB10 = immunity-related GTPase family member b10, LPS = lipopolysaccharide, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, NLRP3 = NOD-, LRR- and PYD-containing protein 3, OMV = outer membrane vesicle, $P2X_7$ = Purinergic P2X7 receptor, RAGE = receptor for advanced glycation end-product, STAT1 = signal transducer and activator of transcription 1, TLR4 = toll-like receptor 4. Figure modified according to Downs et al. (2020), Matikainen et al. (2020) and Pfalzgraff and Weindl (2019).

## 1.3.4 Inflammasome-dependent effector functions

### 1.3.4.1 Pyroptosis

The activation of both the canonical as well as the non-canonical inflammasome leads to pyroptosis, a special type of necrotic cell death. It is defined by the induction through inflammatory caspases, plasma membrane perforation, cell swelling as well as osmotic lysis, DNA damage and the subsequent release of cytosolic content (Jorgensen and Miao 2015). Two independent studies identified the protein gasdermin-D as the key substrate of the caspases-1, -4, -5, and -11 (Kayagaki et al. 2015, Shi et al. 2015). GSDMD consists of an N-terminal GSDMD-N domain and a C-terminal GSDMD-C domain, which are connected by a linker loop (Shi et al. 2017). The caspase-dependent processing within the linker loop dissociates the N-terminal domain from its inhibitory C-terminal counterpart (Liu et al. 2016). The liberated and activated N-terminal fragment translocates into the inner plasma membrane through the binding with glycerophospholipids (Ding et al. 2016). Similar to bacterial pore-forming toxins, it oligomerizes and generates membrane pores with an inner diameter of approximately 10 – 14 nm (Ding et al. 2016).

### 1.3.4.2 Unconventional protein secretion

Most secretory proteins contain an amino-terminal signal peptide which guides them out of the cell using the traditional secretory pathway from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane (Nickel and Rabouille 2009). The cytokines IL-1ß and IL-18 were among the first proteins discovered to be released independently of the ER-Golgi complex (Monteleone et al. 2015). These proteins are characterized by absent signal sequences and follow a poorly defined unconventional secretory route (Monteleone et al. 2015). Besides the release via vesicle- and non-vesicle-based mechanisms, it is assumed that they are secreted through the membrane pores formed by GSDMD after inflammasome activation (Monteleone et al. 2015, Shi et al. 2014). Recently, Lorey et al. (2017) characterized the protein secretion following human non-canonical inflammasome activation. These findings were extended by our laboratory, which discovered the novel alarmins galectin-1 (Gal-1), progranulin (PGRN) and resistin to be released involving the non-canonical inflammasome.

**Galectin-1:** The 15 kDa-protein was the first discovered member of the galectin family, which is characterized by the affinity for ß-galactosides and a conserved carbohydrate recognition domain (CRD) sequence motif (Liu and Rabinovich 2010). To date, 15 galectins have been discovered in mammals (Liu and Rabinovich 2010). Galectin-1 is synthesized in the cytoplasm, from where it is able to translocate into the nucleus, the extracellular compartment as well as the inner and outer compartment of the cell membranes (Camby et al. 2006). However, literature lacks the detailed mechanisms of Gal-1 release. Whereas its extracellular functions depend on the carbohydrate-binding properties, their intracellular effects emerged as carbohydrate-independent involving protein-protein interactions (Camby et al. 2006). Recent research reveals that galectin-1 does not only bind to endogenous molecules, but also senses glycans on the surface of pathogens and therefore acts as pathogen recognition receptor (Vasta et al. 2012). The alarmin is expressed in a wide range of cells and contributes to inflammatory conditions, infections, autoimmunity, allergy, cancer, reproductive disorders or neurodegenerative disease (Sundblad et al. 2017). Its role in sepsis was first discovered by Russo et al. (2021).

**Progranulin**: Also known as granulin-epithelin precursor (GEP), progranulin is a cysteine-rich multifunctional secretory protein with the molecular weight of 68,5 kDa (Tian et al. 2020). It consists of seven and a half granulin repeats, which are separated by linker sequences (Tian et al. 2020). Various proteolytic enzymes, such as matrix metalloproteinases, neutrophil elastase and proteinase 3 further digest progranulin by the cleavage of the linker regions to produce single or connected granulins (Tian et al. 2020). The glycoprotein is expressed in the cytosol of a wide range of cells and tissues including macrophages, T cells and DCs, epithelial cells, fibroblasts, hematopoietic cells, neurons, chondrocytes, adipose as well as skeletal muscle tissue (Tian et al. 2020). Progranulin takes part in multiple physiological and pathophysiological processes. As such, PGRN is upregulated in several cancer types and involved in cancer cell proliferation and migration (Arechavaleta-Velasco et al. 2017). Mutations in the PGRN gene were identified to cause frontotemporal lobular dementia (Baker et al. 2006, Jian et al. 2018). Apart from neurodegenerative diseases, PGRN has been extensively studied in the field of inflammatory and infectious diseases. Previous research revealed elevated levels in septic patients compared to healthy control groups (Rao et al. 2020, Song et al. 2016, Yang et al. 2020, Yu et al. 2016).

**Resistin:** Resistin is a cysteine-rich peptide hormone, which mainly exists as oligomer (660 kDa) or trimer (45 kDa) (Acquarone et al. 2019). It was first discovered in white adipose tissue in mice indicating an important role in insulin resistance and obesity in diabetic mice models (Steppan et al. 2001). In humans, resistin was found to be rather secreted by mononuclear cells, macrophages, bone marrow and neutrophils than by adipose tissue (Johansson et al. 2009, Patel et al. 2003, Savage et al. 2001). The peptide hormone is involved in many acute and chronic inflammatory processes, such as diabetes, atherosclerosis and rheumatoid arthritis as well as sepsis (Acquarone et al. 2019, Koch et al. 2009, Sundén-Cullberg et al. 2007). Resistin is reported to be expressed in response to stimulation with the pro-inflammatory mediators as well as with LPS (Kaser et al. 2003). In turn, it upregulates the expression of IL-1ß, IL-6, IL-12 and TNF-α as well as several cell adhesion molecules (Acquarone et al. 2019, Bokarewa et al. 2005, Silswal et al. 2005). In contrast to its pro-inflammatory profile, the hormone is able to block LPS-TLR4 interactions (Jang et al. 2017, Tarkowski et al. 2010). Recently, resistin was found to diminish neutrophil bacterial killing and to imitate immunosuppressive effects (Miller et al. 2019).

## 1.4 *Staphylococcus aureus*

*Staphylococcus aureus (S. aureus)* is a gram-positive microorganism and belongs to the group of coagulase-positive bacteria (Aryee and Edgeworth 2017). In contrast to coagulase-negative staphylococci, they are characterized by their plasma-coagulating effects (Aryee and Edgeworth 2017). *S. aureus* is a typical commensal of the human skin and mucosa, but also a common cause for severe infections with high mortality and morbidity (Brown et al. 2014, Tong et al. 2015). Experiments in this thesis were performed on the *S. aureus* strain RN4220. RN4220 is a typical laboratory strain, which originates from a derivate of NCTC8325 after UV modification and chemical mutagenesis (Kreiswirth et al. 1983, Nair et al. 2011). It is deficient in DNA restriction and therefore a common cloning host (Nair et al. 2011).

### 1.4.1 Colonization in newborns

*S. aureus* belongs to the developing microflora of the newborn which is acquired peri- or postnatal (Popoola and Milstone 2014). Among healthy neonates, carriage rates vary between 40 and 50 % in the first 8 weeks and decrease to 21 % within the first 6

months of life (Peacock et al. 2003). It was shown that *S. aureus* is transmitted from mother to infant, since newborns and their mothers exhibited commonly identical strains (Bourgeois-Nicolaos et al. 2010, Chatzakis et al. 2011, Leshem et al. 2012). Thereby, horizontal transfer of *S. aureus* from mother to child via close contact appears to be more common than vertical transmission, for example through vaginal delivery. (Bourgeois-Nicolaos et al. 2010, Chatzakis et al. 2011, Leshem et al. 2012). External sources, including health care workers, parents and visitors are discussed to contribute to *S. aureus* colonization in neonates (Bourgeois-Nicolaos et al. 2010, Popoola and Milstone 2014). Within the first 12 hours of life the infant´s nose, ears, umbilicus and rectum are equally colonized (Maayan-Metzger et al. 2017). Afterwards, *S. aureus* is mainly distributed in the mucus organs, including the nose and rectum, and less detectable on the skin surface, such as the ears and umbilicus (Maayan-Metzger et al. 2017). However, the comparison of *S. aureus* nasopharyngeal carriage among adults and infants revealed lowest colonization rates in newborns that might even decrease through enhanced hygiene standards during delivery or maternal breast-feeding behavior (Deinhardt-Emmer et al. 2018). Due to higher rates of cell deaths, biofilm formation and hemolytic activity in newborns compared to other older populations, *S. aureus* strains are discussed to be more virulent in neonates (Deinhardt-Emmer et al. 2018). Especially in the setting of LOS, vascular-access catheters and ventilation tubes are common portals of entry in preterm newborns (Shane et al. 2017, Zemlin et al. 2018).

### 1.4.2 Lipoprotein expression

Like other Gram-positive bacteria, *S. aureus* possess an arsenal of cell surface proteins, which serve as pathogen-associated molecular pattern (Askarian et al. 2018a). These include lipoteichoic acid (LTA), peptidoglycan (PG), as well as lipoproteins (Lpp), which are sensed by immune cell receptors of the host (Askarian et al. 2018a). Among them, bacterial lipoproteins have been established as major class of membrane proteins considering that the *S. aureus* strain N315 encodes about 55 potential Lpps (Stoll et al. 2005), which represent at least 2 % of the staphylococcal proteome (Babu et al. 2006). Due to the absence of an outer membrane in Gram-positive bacteria, lipoproteins are localized on the outer leaflet of the plasma membrane (Nguyen and Gotz 2016). Membrane anchorage is mediated by the lipid moiety at the N-terminus of the

molecule via a di- or triacylglycerol linkage (Nguyen and Gotz 2016). Lipoproteins are expressed as precursors and are further modified into their mature form after insertion into the cytoplasmatic membrane (Nguyen and Gotz 2016). First observed in *E. coli*, synthesis is realized by three membrane-attached enzymes (Hantke and Braun 1973, Nakayama et al. 2012, Tokunaga et al. 1982):

1. The phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt) transfers a diacylglyceryl domain from a membrane phospholipid on the type II N-terminal signal peptide of the pre-prolipoprotein (Sankaran and Wu 1994) **(Fig. 6)**.
2. The lipoprotein signal peptidase II (Lsp) cleaves the signal peptide of the prolipoprotein to generate a diacylated lipoprotein (Hussain et al. 1982) **(Fig. 6)**.
3. The apolipoprotein N-acyltransferase (Lnt) generates a triacylated lipoprotein by transferring an acyl group (Sankaran and Wu 1994). Despite the absence of Lnt-analogue genes in *S. aureus*, Gram-positive bacteria are able to produce di- as well as triacylated Lpps through an unknown mechanism (Kurokawa et al. 2009, Nguyen and Gotz 2016).

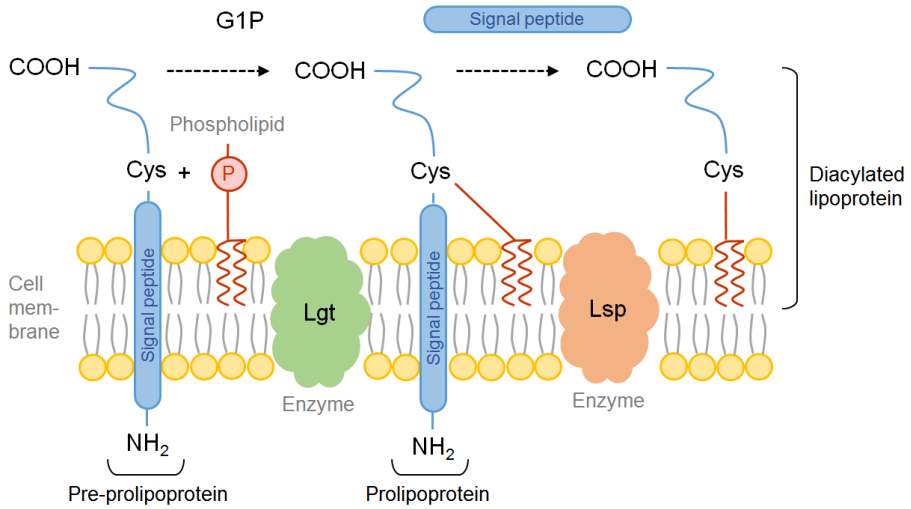



**Figure 6: Lipoprotein maturation in Gram-positive bacteria**. First, the Lgt enzyme promotes the lipidation of the pre-prolipoprotein. Second, the Lsp enzyme cleaves the signal peptide to generate the mature diacylated lipoprotein. G1P = glycerol-1-phosphat, Lgt = phosphatidylglycerol-prolipoprotein diacylglyceryl transferase, Lsp = lipoprotein signal peptidase II. Figure modified according to Bubeck Wardenburg et al. (2006).

The metabolic effects of the lipoproteins are restricted to the layer between the membrane and the cell wall (Schmaler et al. 2010). However, lipoproteins take part in many processes including the uptake of nutrients, acquisition of ions, bacterial signal transduction, transport, antibiotic resistance and adhesion to host tissues during infection

(Kovacs-Simon et al. 2011, Shahmirzadi et al. 2016). Furthermore, the lipid domain of Lpps plays a crucial role in the host-pathogen interaction by serving as pathogen-associated molecular pattern (Raymond et al. 2017).

### 1.4.3 Lipoprotein-induced immune activation

Lipoproteins are the predominant ligands of toll-like receptor 2 (Hashimoto et al. 2006a). Thereby, diacylated Lpp promote the dimerization of the TLR2/TLR6 complex (Takeuchi et al. 2001) and triacylated Lpp are recognized by TLR2/TLR1 heterodimers (Takeuchi et al. 2002). In this thesis, the synthetic lipoproteins FSL-1 (diacylated) and $Pam_3CSK_4$ (triacylated) were used as cell stimulants. The subsequent MyD88-dependent downstream signaling pathway results in the translocation of NFκB into the nucleus and in the expression of proinflammatory cytokines and chemokines, e.g. IL-8 (Kang et al. 2011, Schmaler et al. 2009) **(Fig. 7)**.

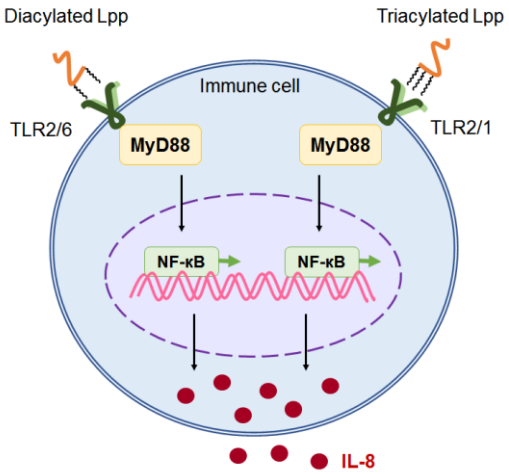



**Figure 7: Lipoprotein-induced activation of the TLR2-MyD88-dependent signaling pathway.** The synthetic Lpps bind to TLR2 and initiate the MyD88- and NFκB-dependent expression of IL-8. IL = Interleukin, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, TLR = toll-like receptor. Figure modified according to Nguyen and Gotz (2016).

In Gram-positive bacteria, the lipid modification during the lipoprotein biosynthesis is not considered vital, compared to their Gram-negative relatives (Gupta et al. 1993, Nguyen and Gotz 2016). However, the relevance of Lpp processing for immune signaling has been demonstrated by creating ΔLgt *S. aureus* bacteria that lack the Lgt enzyme. By the deletion of the Lgt gene, the bacteria fails to modify the precursor lipoproteins (Stoll et al. 2005). Subsequently, the molecule is only weakly attached to the cytoplasmatic membrane and elevated secretion of unprocessed prelipoproteins has been reported (Stoll et al. 2005). Besides growth defects, the mutant exhibits impaired pathogenicity by reduced secretion of pro-inflammatory cytokines (Kang et al. 2011, Schmaler et al. 2009, Stoll et al. 2005).

# 2 Aims of the study

The non-canonical inflammasome supports the innate immune system in the defense against Gram-negative bacteria that escape the phagosome and enter the cytosol of the host cell (Aachoui et al. 2013). Through the secretion of cytokines, DAMPs and alarmins, respectively, it alerts neighboring cells and initiates the elimination of the infected cell (Downs et al. 2020). However, if the initial inflammatory response fails to fight the infection, the primarily localized infection may result in sepsis by generating systemic and overstimulated immune activation (Yadav and Cartin-Ceba 2016). Through an unbiased screen, our laboratory recently contributed to the exploration of the novel alarmins galectin-1 (Gal-1) (Russo et al. 2021), progranulin (PGRN) (Duduskar 2020) and resistin (Appendix **Fig. 23**) involving the caspase-4 and caspase-5-dependent non-canonical inflammasome. Further investigations in adult sepsis patients revealed significant elevated levels of Gal-1 (Russo et al. 2021), PGRN (Song et al. 2016) and resistin (Koch et al. 2009, Sundén-Cullberg et al. 2007) compared to a healthy control group. Despite all these efforts, little is known about the contribution of cytosolic sensing and inflammasome activation in innate immunity of newborns. Currently, no study links the non-canonical inflammasome activation with Gram-positive infection as commonly identified microorganisms associated with neonatal sepsis. Thus, the thesis focuses on elucidating the inflammasome-dependent alarmin secretion in newborns with sepsis and clarifies the mechanism by which Gram-positive bacteria activate the non-canonical inflammasome for the release of PGRN. Therefore, the following hypothesis were addressed:

**Hypothesis 1** Gal-1, PGRN and resistin are increasingly released in infected, compared to non-infected newborn infants.

**Hypothesis 2** The secretion of Gal-1, PGRN and resistin is influenced by the gestational age of the newborn.

**Hypothesis 3** The alarmin levels of Gal-1, PGRN and resistin correlate to the infection parameters IL-6 and CRP.

**Hypothesis 4** Bacterial lipoproteins trigger PGRN release independent of TLR2.

# 3 Materials and methods

## 3.1 Materials

**Table 4: List of cell lines**

| Cell line | Source |
|---|---|
| HEK 293T wild type | DSMZ (ACC 635) |
| THP1 wild type | DSMZ (ACC 16) |

**Table 5: List of bacteria**

| Bacteria | Source |
|---|---|
| *E. coli* XL10-Gold Ultracompetent cells | Agilent Technologies, Inc. |
| *S. aureus* RN4220 | Dr. Lorena Tuchscherr de Hauschopp, Institute for Medical Microbiology, University Hospital Jena |
| ΔLgt *S. aureus* | This study |

**Table 6: Consumables for cell culturing and stimulation**

| Materials | Company |
|---|---|
| CELLSTAR® cell culture flask | Greiner Bio-One GmbH |
| Ciprofloxacin Kabi 200 mg/100 ml Infusion | Fresenius Kabi Deutschland GmbH |
| DMEM (Dulbecco´s Modified Eagle Medium), high glucose, GlutaMAX™ supplement | Thermo Fisher Scientific Inc. |
| DPBS (Dulbecco´s Phosphate-Buffered Saline), no calcium, no magnesium | Thermo Fisher Scientific Inc. |
| FCS (Fetal Bovine Serum) | Sigma-Aldrich Chemie GmbH |
| FSL-1 | InvivoGen |
| Lipofectamine™ 2000 Transfection Reagent | Thermo Fisher Scientific Inc. |
| Neubauer counting chamber, 0,1 mm | Paul Marienfeld GmbH & Co. KG |
| Opti-MEM™ I Reduced Serum Medium, GlutaMAX™ Supplement | Thermo Fisher Scientific Inc. |
| $Pam_3CSK_4$ | InvivoGen |
| Penicillin-Streptomycin | Thermo Fisher Scientific Inc. |
| PMA (phorbol 12-myristate 13-acetate) | InvivoGen |
| RPMI (Roswell Park Memorial Institute) 1640 Medium, GlutaMAX™ Supplement | Thermo Fisher Scientific Inc. |

| | |
|---|---|
| Trypsin-EDTA (0.25 %), phenol red | Thermo Fisher Scientific Inc. |
| 96 Well Polystyrene Cell Culture Microplates, transparent | Greiner Bio-One GmbH |

**Table 7: Consumables for bacteria culturing, storage and stimulation**

| Materials | Company |
|---|---|
| Ampicillin Sodium Salt Biochemica SC | AppliChem GmbH |
| Columbia Agar with 5 % Sheep Blood | Becton Dickinson GmbH |
| Cryogenic tubes, CryoTubes™, Nunc™ | VWR International GmbH |
| LB-Medium (Luria/Miller) | Carl Roth GmbH + Co. KG |
| NucleoSpin® Plasmid Kit, 250 preps | Macherey-Nagel |
| hTLR2 flag plasmid | Addgene |
| TSB (Tryptic Soy Broth) | Sigma-Aldrich Chemie GmbH |

**Table 8: Consumables for ELISA**

| Materials | Company |
|---|---|
| DuoSet® ELISA Kit<br>• Human Progranulin<br>• Human Galectin-1<br>• Human Resistin<br>• Human IL-8/CXCL8<br>• Human TNF-alpha | R&D Systems, Bio-Techne GmbH |
| Reagent Diluent Concentrate 2 (10x) | R&D Systems, Bio-Techne GmbH |
| TMB Substrate set (Substrate A & Substrate B) | BioLegend® |
| Tween® 20 | Sigma-Aldrich Chemie GmbH |
| 96 Well ELISA Microplates, transparent | Greiner Bio-One GmbH |

**Table 9: Consumables for sequencing**

| Materials | Company |
|---|---|
| Biozym LE Agarose | Biozym Scientific GmbH |
| CoralLoad PCR Buffer | QIAGEN |
| Deoxynucleotide (dNTP) Solution Mix | New England Biolabs GmbH |
| Ethidium bromide solution 1 %, 15 ml, dropp. bottle | Carl Roth GmbH + Co. KG |
| Forward primer SA_LGT_PCR_F | Sequence 5´→ 3´: ACGTGCACTAG-TTAAAG<br>PCR Primer Design Tool by Eurofins Genomics LLC |
| GeneRuler DNA Ladder Mix | Thermo Fisher Scientific Inc. |
| NucleoSpin® Gel and PCR Clean-up Kit | Macherey-Nagel |

| | |
|---|---|
| Oxoid™ Chloramphenicol Selective Supplement | Thermo Fisher Scientific Inc. |
| Q5® High GC Enhancer | New England Biolabs GmbH |
| Reverse primer SA_LGT_PCR_F | Sequence 5´→ 3´: GCCAC-CTATTAAACCAC<br>PCR Primer Design Tool by Eurofins Genomics LLC |
| RNeasy® Mini Kit | QIAGEN |
| Taq DNA Polymerase, recombinant (5 U/µL) | Thermo Fisher Scientific Inc. |
| 5x Q5® Reaction Buffer | New England Biolabs GmbH |

**Table 10: Technical devices**

| Materials | Company |
|---|---|
| Centrifuge 5418 R | Eppendorf AG |
| Centrifuge 5804 R | Eppendorf AG |
| Centrifuge UNIVERSAL 320 R | Andreas Hettich GmbH & Co. KG |
| DeNovix DS-11 FX Spectrophotometer/Fluorometer | DeNovix Inc. |
| GBOX-Chemi-XRQ gel documentation system | Syngene |
| Incubation shaker Ecotron | Infors AG |
| Incubator New Brunswick™ Galaxy® 170 S | Eppendorf AG |
| Incubator New Brunswick™ S41i | Eppendorf AG |
| Microscope Primovert | Carl Zeiss Microscopy GmbH |
| neoVortex® shaker D-6012 | neoLab Migge GmbH |
| Spark® Multimode Microplate Reader | Tecan AG |
| Veriti™ 96-Well Thermal Cycler | Thermo Fisher Scientific Inc. |
| VORTEX Genius 3 | IKA®-Werke GmbH & CO. KG |

**Table 11: Expendable materials**

| Materials | Company |
|---|---|
| Disposable cup 100 ml, PP | SARSTEDTAG & Co. KG |
| Multi-channel Research® plus pipette<br>(10 – 100 µl; 30 – 300 µl) | Eppendorf AG |
| Petri Dish, without Vents, 94 x 16 mm, heavy design, sterile | Greiner Bio-One GmbH |
| Safe-Lock Tubes (0,5 ml; 1,5 ml; 2,0 ml) | Eppendorf AG |
| Single channel Research® plus pipette<br>(0,1 – 2,5 µl; 0,5 – 10 µl; 2 – 20 µl; 10 – 100 µl;<br>20 – 200 µl; 100 – 1000 µl) | Eppendorf AG |
| 15 & 50 ml CELLSTAR® Polypropylene Tubes | Greiner Bio-One GmbH |

# 3.2 Methods

## 3.2.1 Study subjects

The examination of alarmin levels in infected neonates is based on the retrospective analysis of blood samples and medical files from 78 newborns, who were hospitalized between March 2014 and February 2016 in the neonatal intensive care unit (NICU) at the University Hospital in Jena. The sample collection was approved by the ethics committee of the University Hospital Jena (reference number 4055-004/14).

### 3.2.1.1 Blood sampling

The newborn blood samples were obtained upon admission to the NICU (0 – 24 h) and in the following 24 hours (24 – 48 h). It was of venous as well as capillary origin and collected in serum monovetts. The samples were immediately centrifuged on NICU. The resulting serum was stored in sterile tubes at -70 °C until analysis. The current study refers to serum samples, which were collected on the second day of hospitalization (24 – 48 h).

### 3.2.1.2 Clinical data

The medical files of the newborns included data about birth, vital signs, blood count, infection parameters, blood gas analysis, microbiological testing, neonatal morbidity, medical treatment and information about the mother **(table 12)**. Clinical and paraclinical parameters, which describe the current condition of the newborns, were collected on admission date (0 – 24 h) and in the following 24 hours (24 – 48 h) simultaneously to the blood sampling. They include vital signs as complexion, capillary refill time, heart rate, lung function, neurological and intestinal symptoms, blood count, infection parameters as well as the blood gas analysis with pH, base excess and lactate.

Measurements of the routine laboratory parameters were performed by the central laboratory of the University Hospital Jena. For the concentrations of the infection markers CRP and IL-6, the lower detection limits were 2,0 mg/l and 7,5 pg/ml, respectively. Values below the detection limit were considered as 1 pg/ml or 1 mg/l, respectively. According to the laboratory standards of the NICU of the University Hospital Jena, the cut-off values to predict infection were set at ≥ 100 pg/ml for IL-6 and ≥ 10 mg/l for CRP. The bacteriological testing was evaluated by the Institute of Medical Microbiology

of the University Hospital Jena. This trial refers to clinical and laboratory data, which were collected on the second day of hospitalization.

**Table 12: Summary of the medical files of the patient cohort.** BE = base excess, BPD = bronchopulmonary dysplasia, CPAP = continuous positive airway pressure, CRP = C-reactive protein, FIP = focal intestinal perforation, IL = interleukin, IRDS = infant respiratory distress syndrome, IVH = intraventricular hemorrhage, NEC = necrotizing enterocolitis, $PaO_2$ = arterial oxygen partial pressure, PDA = persistent ductus arteriosus Botalli, PVL = periventricular leukomalacia, SGA = small for gestational age.

| Category | Collected data |
|---|---|
| Birth | Date of birth, week of gestation, birth weight, sex, SGA, delivery route, APGAR score (1 min, 5 min, 10 min) |
| Vital signs | Temperature, mean arterial blood pressure, urinary extraction, cerebral seizures, complexion[1], capillary refill time[1], heart rate[1], lung function[1], neurological symptoms[1], intestinal symptoms[1] |
| Blood count | White blood cell count[1], platelet count[1], hematocrit[1] |
| Infection parameters | CRP[1], IL-6[1] |
| Blood gas analysis | $PaO_2$, pH of arterial and venous umbilical cord blood, pH[1], BE[1], lactat[1] |
| Microbiological testing | Pharyngeal smear, rectal smear, blood culture |
| Neonatal morbidity | NEC, FIP, IVH, PVL, PDA, BPD, IRDS, hyperbilirubinemia, duration of hospitalization & transfer |
| Medical treatment | Intubation in delivery room, surfactant, duration of ventilation, amount and duration of oxygen demand, CPAP in delivery room and on ward, duration of CPAP, antibiotics and duration of antibiotic treatment, catecholamines, immunoglobulins, steroids, erythrocytes, platelets, fresh frozen plasma, parenteral nutrition |
| Data about the mother | Age, gravida and para, in vitro fertilization, birth history, GBS smear, infection, preeclampsia, HELLP syndrome, gestational diabetes, diseases during pregnancy |

[1] Labeled data were collected on admission date (0 – 24 h) and 24 – 48 h afterwards

### 3.2.1.3 Diagnosis of sepsis

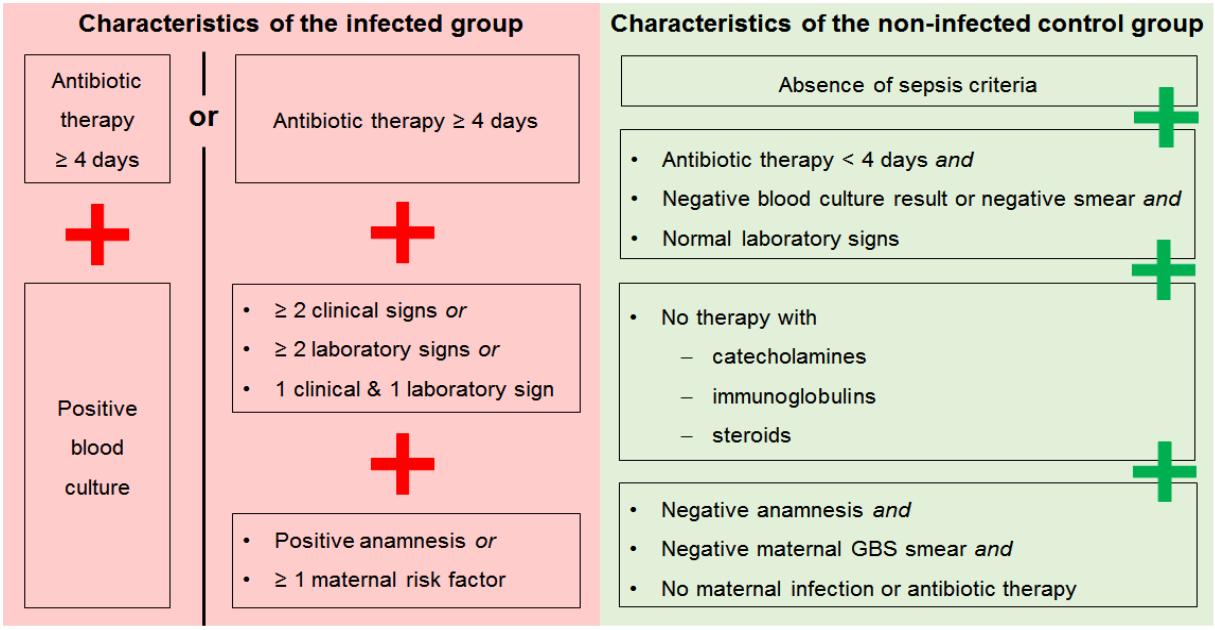
The patient cohort was divided into an infected group, also considered as sepsis group, and a non-infected or non-septic control group. By means of the medical files of the newborns, sepsis was diagnosed retrospectively according to clinical, laboratory, microbiological and anamnestic criteria as well as maternal risk factors **(table 13)**.

**Table 13: Clinical, laboratory, microbiological and anamnestic criteria for neonatal sepsis diagnosis**

| Criterion | Clinical data |
|---|---|
| Clinical criteria | • Tachycardia (> 160/min) or bradycardia (< 120/min)<br>• Pathological respiratory frequency (full-term: 60 – 70/min, preterm: 40 – 50/min)<br>• Suspicious state of awareness (hypotension, lethargy)<br>• Suspicious intestinal symptoms (abdominal distension, sucking weakness, feeding intolerance, vomiting)<br>• Capillary refill time ≥ 3 sec<br>• Pale complexion |
| Laboratory criteria | • CRP ≥ 10 mg/l<br>• IL-6 ≥ 100 pg/ml |
| Microbiological criterion | • Positive blood culture |
| Anamnestic criteria | • Suspected or manifest infection, chorioamnionitis or sepsis diagnosed by the doctor |
| Maternal risk factors | • Premature rupture of membranes<br>• Maternal infection or antibiotic treatment<br>• Positive GBS smear |

The stratification based on the surveillance protocol of the NRZ (2017) and was further adapted to the standards of the NICU of the University Hospital Jena. The diagnosis of sepsis refers to data, which were surveilled 24 – 48 hours after admission to the hospital. The patient cohort was stratified according to the following criteria **(Fig. 8)**:

**Figure 8: Flow chart of the neonatal sepsis diagnosis and control group classification**



### 3.2.2 Cytokine and alarmin analysis via enzyme-linked immunosorbent assay (ELISA)

The concentrations of the alarmins Gal-1, PGRN and resistin in the neonatal blood sera as well as the concentrations of PGRN, IL-8 and TNF-α in the cell culture supernatants were determined using the enzyme-linked immunosorbent assay (ELISA). The ELISA method relies on an antigen-antibody interaction: The provided microtiter plates are coated with analyte-specific antibodies (capture antibody), which bind the alarmins and cytokines (antigen) of the sample. A biotinylated secondary antibody (detection antibody) detects the antibody-antigen complexes by initializing a color reaction via the streptavidin-linked horseradish peroxidase. The enzyme catalyzes the redox reaction of the added tetramethylbenzidine (TMB) substrate solution and $H_2O_2$, which is visualized by a blue color change. The color development is stopped by adding sulfuric acid stop solution (2 N $H_2SO_4$). The color intensities are determined via photometer and directly correlate to the number of bound antigens. Final concentrations of the alarmins and cytokines were calculated in relation to a standard curve.

The assays were performed according to the manufacturer´s instructions with antibodies, standard solution and streptavidin provided by the DuoSet® ELISA-kit: First, a 96-well microplate was coated with 50 µl/well of prediluted capture antibody and incubated overnight at 4 °C in the dark. The next day, unbound antibodies were removed by carefully eroding each well with washing buffer (0,05 % Tween-20 in 500 ml DPBS) three times. The washing buffer was removed by spilling the microplate over a sink. The coated wells were blocked with 100 µl/well of reagent diluent (10 % Reagent Diluent concentrate 2 diluted to 1 % in sterile water) for 1 h at room temperature while shaking. Blocking buffer was removed in three washing steps as described above. Standard solutions and prediluted samples were added (50 µl/well) and incubated for 2 h at room temperature while shaking. Again, unbound standards and samples were discarded by three washing steps. 50 µl/well of prediluted detection antibody solution was added and the microplate was incubated for 2 h at room temperature while shaking. After removal of unbound detection antibodies via three washes, 50 µl/well of prediluted horseradish peroxidase-linked streptavidin was added followed by incubation for 20 min at room temperature while shaking. Finally, the wells were washed out three times and subsequently filled with 50 µl/well of TMB substrate solution. After 5 – 10 min

incubation time, 25 µl/well of sulfuric acid was added to stop the color development of the samples. Photometrical analysis of the optical density of each well was performed by the Spark® Multimode Microplate Reader at the absorbance of 450 nm. Cytokine levels were analyzed using the standard curve.

**Table 14: Dilutions of antibodies, samples and streptavidin-HRP in the PGRN-, Gal-1, resistin-, TNF-α- and IL8-ELISA kits.** RD = reagent diluent.

| | Capture antibody dilution in DPBS | Detection antibody dilution in RD | Sample dilution in RD | Streptavidin-HRP dilution in RD |
|---|---|---|---|---|
| Gal-1 | 1:125 | 1:60 | 1:25 | 1:40 |
| PGRN | 1:180 | 1:180 | 1:50 and 1:20[1] | 1:200 |
| Resistin | 1:180 | 1:180 | 1:100 | 1:200 |
| TNF-α | 1:120 | 1:60 | 1:10 | 1:40 |
| IL-8 | 1:120 | 1:60 | 1:5 | 1:40 |

[1] Blood sera were diluted 1:50, cell supernatants were diluted 1:20

## 3.2.3 Cell culture and bacteria culture

### 3.2.3.1 Maintenance of immortalized HEK 293T cell line

The HEK 293T cells were cultured in DMEM modified with 10 % FCS and 0,5 % ciprofloxacin in 75 $cm^2$ cell culture flasks. The flasks were incubated at 37 °C in a humidified incubator with 5 % $CO_2$. After reaching a confluence of approximately 70 %, the cells were passaged to avoid overgrowth.

### 3.2.3.2 Maintenance of immortalized THP1 cell line

THP1 cells were grown in 25 or 75 $cm^2$ cell culture flasks under incubation conditions at 37 °C and 5 % $CO_2$. The culture medium was RPMI 1640 supplemented with 10 % FCS as well as penicillin (100 IU/ml) and streptomycin (100 µ/ml). The cells were split every 3 days in fresh medium to avoid overgrowth.

### 3.2.3.3 Culturing and storage of *S. aureus* RN4220

The *Staphylococcus aureus* wild type and ΔLgt were cultured on blood agar plates in a $CO_2$-incubator at 37 °C overnight. For liquid cultures, the bacteria were grown in falcon tubes with 10 ml TSB medium at 36 °C while shaking at 160 rpm overnight. For storage, the wild type and ΔLgt *S. aureus* were frozen in 50 % glycerol at -80 °C.

### 3.2.4 ΔLgt *S. aureus* detection assay

The ΔLgt mutant bacteria was created with the kind help of Ph.D. student Shivalee Duduskar, from the University Hospital Jena. Based on the CRISPR/Cas9 technology, the gene manipulation of S. aureus RN4220 was performed by using the base-editing plasmid pnCasSA-BEC first engineered by Gu et al. (2018). The modified pnCasSA-BEC-lgt plasmid was transformed into S. aureus via electroporation. After the incorporation, it expresses APOBEC1-nCas9, a fusion protein consisting of a Cas9nickase, a synthetic guide RNA (sgRNA) and a cytidine deaminase (Gu et al. 2018) **(Fig. 9)**. For base editing, the complex binds sgRNA-guided on the Lgt gene. After detecting the protospacer adjacent motif (PAM) as the essential binding site on the target gene, the Cas9 enzyme nicks the non-edited strand of the target DNA. The linked cytidine deaminase subsequently converts the nucleoside cytidine (C) into uridine (U) (Gu et al. 2018). Through DNA replication mechanisms, the generated uridine-guanine (U-G)-mismatch is first repaired into an uridine-adenine (U-A) base pair, before it is correctly transformed into a thymidine-adenine (T-A) base pair (Eid et al. 2018) **(Fig. 9)**. The artificially generated C → T conversions result in premature stop codons that interrupt the Lgt gene transcription.

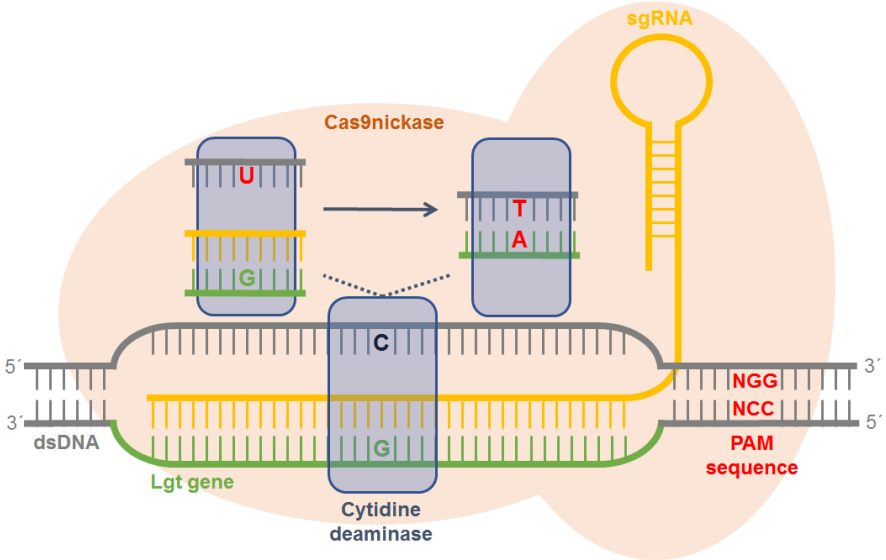



**Figure 9: CRISPR/Cas9 mediated creation of ΔLgt *S. aureus*.** Mechanism of CRISPR/Cas9 mediated base editing via the cytidine deaminase. The synthetic guide RNA (sgRNA) directs the Cas9nickase to the genomic location of Lgt after binding on the protospacer adjacent motif (PAM) sequence. The fused cytidine deaminase creates a point mutation in the Lgt gene of S. aureus strain RN4220 by changing the base cytidine into thymidine. A = adenine, C = cytidine, dsDNA = double-stranded desoxyribonucleic acid, G = guanine, Lgt = phosphatidylglycerol-prolipoprotein diacylglyceryl transferase, PAM = protospacer adjacent motif, sgRNA = synthetic guide RNA, T = thymidine, U = uridine.

The successful creation of point mutations in the *S. aureus* Lgt gene was confirmed via sequencing before and after the colonies were processed for plasmid curing. Permissions on genetic modifications using CRISPR technologies have been acquired according to biological security and gene technology guidelines.

#### 3.2.4.1 Sequencing preparation

In order to detect the Lgt mutation in the genome of the *S. aureus* bacteria, the prokaryotic DNA of the wild type and Lgt mutated strains were amplified via polymerase chain reaction (PCR). The successful gene amplification was detected via gel electrophoresis. Mutation in the Lgt gene was ultimately confirmed by Sanger sequencing (GATC, Biotech, Germany).

1. **Polymerase chain reaction:** The PCR was performed using the RNeasy® Mini Kit by QIAGEN. Primer design for PCR cloning of wild type and mutated Lgt gene was constructed by the Ph.D. student Shivalee Duduskar according to Gu et al. (2018). The samples were prepared as described in the kit instructions:

| Components | Volume (25 µl reaction) |
|---|---|
| 5x Q5 Reaction Buffer | 5 µl |
| 10 mM Deoxynucleotide (dNTP) Solution Mix | 0,5 µl |
| 10 µM forward primer (5´– ACGTGCACTAGTTAAAG – 3´) | 1,25 µl |
| 10 µM reverse primer (5´– GCCACCTATTAAACCAC – 3´) | 1,25 µl |
| 5 U/µl Taq DNA Polymerase (recombinant) | 0,25 µl |
| 5x Q5 High GC Enhancer | 5 µl |
| Template | 2 µl |
| Nuclease free water | 9,75 µl |

   The cyclic amplification steps of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec) and extension (72 °C for 30 sec) took place in the Veriti 96-Well Thermal Cycler. The reaction recurred 30 times. The first cycle was initiated by heating the samples for 10 min at 94 °C and the last cycle ended at 72 °C for 7 min.

2. **Gel electrophoresis:** To visualize the successful gene amplification, the PCR end products were fragmented via gel electrophoresis. The gel was prepared by heating 57 ml 1x TAE buffer (Tris, acetate and EDTA), supplemented with 0,75 g agarose and 3 µl ethidium bromide. After gel hardening, the pockets of the gel were

filled with 5 µl of amplified products, which were mixed with CoralLoad PCR Buffer. GeneRuler DNA Ladder Mix was used as a reference to estimate the size of the DNA molecules that were separated through the gel. The electrophoresis ran for 45 min at 90 volts.

3. **PCR clean-up:** The PCR samples were purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel) according to the manufacturer´s protocol: 1 volume of sample was mixed with 2 volumes of NTI buffer in a provided column with collection tube to improve the DNA binding conditions. After centrifugation (10 000 *g* for 30 sec), the flow-through in the collection tube was removed. The PCR product was washed with 700 µl of NT3 buffer followed by centrifugation (10 000 *g* for 30 sec) and removal of the flow-through. In order to eliminate the residual NT3 buffer and to dry the silica membrane, the columns were centrifuged again (10 000 *g* for 1 min), followed by the removal of the flow-through. The gene products were resolved by adding 15 µl of NE buffer and subsequent incubation at room temperature for 1 min. After centrifugation (11 000 *g* for 1 min), the gene products were collected in a new tube.

4. **Sequencing:** The isolated DNA of the wild type and ΔLgt mutated *S. aureus* were measured spectrophotometrically before sending for sequencing. The sanger sequencing was performed by the Eurofins GATC Sequencing Department in Cologne. To locate the desired mutation, the sequenced nucleotides were first translated into amino acid sequences by using the alignment tool blastx from BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) of the U.S. National Library of Medicine. Second, the nucleotide and amino acid sequences of the sent wild type and ΔLgt *S. aureus* strains were compared via the multiple (https://www.ebi.ac.uk/Tools/msa/clustalo/) and pairwise (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) sequence alignment tools provided by the EMBOSS Needle and Clustal Omega database from EMBL-EBI. The genomic location of the wild type Lgt gene in *S. aureus* was further analyzed via the BLAST databank by using a shotgun sequence of *S. aureus* strain RN4220.

### 3.2.4.2 Plasmid curing

The bacteria colonies containing the desired mutation were further treated to segregate the pnCasSA-BEC-lgt plasmid. Therefore, the Lgt mutated *S. aureus* strains were cultured in 10 ml TSB medium, supplemented with 10 µg/ml chloramphenicol, followed by overnight incubation at 30 °C. On the next day, the cells were diluted 1:1000 in TSB without antibiotics and again cultured at 42 °C for 12 h. Subsequently, the bacteria were transferred on an antibiotic-free TSB plate. After overnight incubation at 37 °C, some colonies were arbitrarily selected and separately dissolved in 10 µl of $ddH_2O$. Each of the dissolved bacteria culture was transferred on a TSB plate in presence of chloramphenicol (5 µg/ml) **(Fig. 10 A)** and on a TSB plate in absence of chloramphenicol **(Fig. 10 B)**. The successful plasmid elimination was proofed by the lack of bacterial growth on the medium supplemented with chloramphenicol, indicating the removal of the plasmid-associated chloramphenicol resistance marker **(Fig. 10 A)**.

**A** **B**

**Figure 10: Verification of plasmid curing**. After plasmid curing, bacteria were cultured on TSB medium in presence **(A)** and absence **(B)** of chloramphenicol. No growth in presence of antibiotics confirmed the successful elimination of the pnCasSA-BEC plasmid from the cells.

## 3.2.5 ΔLgt *S. aureus* testing assay

HEK 293T cells were transfected with TLR2 plasmids to express TLR2 receptors for lipoprotein binding. Untransfected cells served as a control. The cells were subsequently stimulated with ΔLgt *S. aureus*, *S. aureus* wild type and $Pam_3CSK_4$. The lack of lipoprotein production was verified with the lack of IL-8 production detected via ELISA.

#### 3.2.5.1 TLR2 plasmid amplification

TLR2 plasmids were amplified in competent *E. coli* bacteria. Therefore, 100 µl of bacteria were supplemented with 2 µl of plasmids and incubated for 1 h on ice. Subsequently, the *E. coli* cells were incubated for 3 min on 42 °C while shaking and directly placed on ice again. After being washed in LB-Medium once, the bacteria were resuspended in 100 µl of LB-Medium and streaked out on a LB-plate in presence of ampicillin. The plate was incubated at 37 °C overnight. The next day, the grown colonies were transferred into liquid LB-Medium supplemented with 100 µg/ml Ampicillin and incubated at 37 °C overnight.

TLR2 plasmids were isolated using the NucleoSpin plasmid kit from Macherey-Nagel according to the manufacturer´s instructions: The *E. coli* culture was centrifuged (11 000 *g* for 30 sec) and carefully resuspended in 500 µl A1 buffer and 500 µl A2 buffer for 5 min in room temperature. By adding 600 µl A3 buffer, the samples turned blue. Subsequently, they were gently mixed until they turned colorless. The lysate was clarified via centrifugation (11 000 *g* for 10 min). The supernatant was transferred into a provided column with collection tube. After centrifugation (11 000 *g* for 1 min), the flow-through was removed. The samples were washed by adding 600 µl of washing buffer A4. Again, the samples were centrifuged (11 000 *g* for 1 min) and the flow-through discarded. In order to dry the silica membrane, the column was centrifuged (11 000 *g* for 2 min), before the collection tube was removed. The gene products were resolved by adding 50 µl of preheated AE buffer. After incubation at 70 °C for 2 min, the gene products were collected by centrifugation (11 000 *g* for 1 min) in a new collection tube. The amplified TLR2 plasmids were stored at -20 °C.

#### 3.2.5.2 Heat-fixation of *S. aureus* RN4220

After culturing the liquid bacteria of the wild type and the ΔLgt mutated strain in TSB medium overnight as previously described, the cells were centrifuged for 5 min at 4000 rpm. The pellets were washed two times with DPBS and dissolved in 100 µl of DPBS. The cells were heated in the water bath at 70 °C for 30 min. According to the optical density measured by 600 nm wavelength, the bacteria number was concentrated up to $10^8$ and $10^7$ cells/ml. The heat-killed bacteria were stored at -20 °C.

#### 3.2.5.3 HEK cell stimulation and IL-8 ELISA

1. **Preparation of HEK TLR2 cells:** The HEK 293T cells were seeded in a 96-well plate at a density of 80 000 cells/well and incubated for 4 h at 37 °C. Cells were counted with the help of the Neubauer counting chamber and the cell suspension was adjusted to the desired concentration. Before plasmid transfection, the TLR2 gene products were incubated with lipofectamine (10 µl/well) in Opti-MEM for 10 min at 37 °C. One half of the plate (48 wells) was transfected with 200 ng of TLR2-FLAG-plasmids per well. The other half of the plate served as a control and was transfected with lipofectamine in Opti-MEM without TLR2 plasmid. The cells were incubated overnight under already described conditions.

2. **Stimulation of HEK wild type and HEK TLR2 cells:** The next day, the wild type and TLR2-transfected HEK 293T cells were stimulated with $Pam_3CSK_4$ (50 ng/ml and 200 ng/ml, respectively), heat-killed strains of *S. aureus* wild type and ∆Lgt mutant. The heat-killed bacteria were concentrated up to $10^8$ cells/ml. The stimulants were diluted in DMEM and given to the cells (100 µl/well), after they have been washed in DMEM once. Cells were incubated overnight at 37 °C.

3. **Determination of IL-8 release by ELISA:** On the next day, the supernatant of the HEK 293T stimulated cells was isolated after centrifugation (400 rpm, 5 min). The cytokine secretion was analyzed via ELISA Kit from R&D Systems according to the manufacturer´s instructions as described in chapter 3.2.2.

### 3.2.6 ∆Lgt *S. aureus* stimulation assay

THP1 cells were stimulated with $Pam_3CSK_4$, FSL-1 and heat-killed wild type and ∆Lgt *S. aureus* bacteria. The subsequent release of PGRN and TNF-α was detected via ELISA (see chapter 3.2.2).

1. **Bacterial stimulation:** The THP1 cell lines were seeded in a concentration of 80 000 cells/well on a 96-well plate. Differentiation into macrophages was induced by overnight cultivating with 100 ng/ml phorbol 12-myristate 13-acetate (PMA). Afterwards, THP1 macrophages were washed with DPBS and stimulated with $Pam_3CSK_4$ (100 ng/well), FSL-1 (100 ng/well) as well as heat-killed wild type and ∆Lgt *S. aureus* bacteria ($10^8$ cells/ml each). Cells were kept overnight in Opti-MEM.

2. **Determination of PGRN and TNF-α release by ELISA:** The following day, the microtiter plate was centrifuged (400 rpm, 5 min) to isolate the supernatant from the THP1 cells. Excessive supernatant was frozen at -20 °C for later use. The release of the alarmins Progranulin and TNF-α were detected by ELISA as previously described in chapter 3.2.2.

### 3.2.7 Statistical analysis

Data were analyzed and illustrated using GraphPad Prism version 6.01 (2012) by Graphpad software Inc., IBM® SPSS® Statistics version 25 (2017) and Microsoft Excel 2019.

Descriptive statistics about the patient cohort were reported as mean ± standard deviation (SD), median with interquartile range (IQR) as well as frequencies and percentages. The significance between two groups was evaluated with Mann-Whitney-U-test and Fisher´s exact test for binary variables. The alarmin levels of the blood samples were expressed as mean ± SD. The normality was checked using the Shapiro-Wilk test. Comparisons between two groups or conditions were performed using Man-Whitney-U test. The difference between multiple groups were assessed with the Kruskal-Wallis test and the Dunn´s multiple comparison test as post hoc test. The Spearman´s rank correlation analysis was used to detect associations between the alarmin levels and infection parameters. Data of the cell experiments were presented as mean ± SD (n = 3) and statistically analyzed with unpaired t-test.

All statistical tests were two-sided. P values of $p < 0,05$ were considered significant. Asteriks indicate the statistically significant differences (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$, **** $p < 0,0001$).

# 4 Results

## 4.1 Description of the study population

In this study, 78 neonates were included. 29 NICU patients were assigned to the infected group, and 23 to the non-infected control group. The remaining 26 neonates were excluded, as they did not meet the inclusion criteria to be grouped as infected or non-infected. In the whole study cohort, 5 infants died during their hospital stay. Among them, 4 newborns were categorized as infected. The clinical characteristics of the infected and non-infected cohorts are summarized in **table 15** on page 44.

The mean gestational age of the newborns was 33,1 ± 4,9 weeks. The infected group includes 6 full-term (> 36 + 6 weeks of gestation) born and 23 preterm (≤ 36 + 6 weeks of gestation) born children, whereas the non-infected group consists of 6 full-term and 17 preterm neonates. Compared with the non-infected group, the infected group had a significantly lower gestational age (30,3 ± 5,8 vs. 35,1 ± 2,2 weeks, p = 0,001). Similarly, the birth weight was significantly lower in the infected group than in the non-infected group (1612,5 ± 1083,4 vs. 2448,2 ± 644,6 g, p = 0,000). There was no statistical significance between the groups regarding small for gestational age newborns (p = 0,686), gender distribution (p = 0,263) and delivery route (p = 0,734).

The medians of the APGAR score after 1 min, 5 min and 10 min were significantly lower in the infected cohort than the non-infected cohort (p = 0,000, p = 0,000 and p = 0,006 respectively). Further examination of the vital signs during the hospital stay revealed significantly higher incidences of pathological respiration (p = 0,000), complexion (p = 0,034) and neurological symptoms (p = 0,034) in the infected group compared to the non-infected control group. No significant difference was found in the comparison of heart rate (p = 0,379), capillary refill time (p = 0,497) and intestinal symptoms (p = 0,110).

The cut-off value of CRP to predict infection was set at 10 mg/l. As the CRP analysis of 48 neonates of the total study cohort was below the detection limit (< 2 mg/l), the newborns were classified according to CRP levels below or above 10 mg/l. Out of 29 infected patients, the CRP values were above the cut-off concentration in 8 cases

(27,6 %) and below in 21 cases (72,4 %). CRP levels of 13 infected neonates (44,8 %) were below the measurable limit. In the non-infected group, CRP ≤ 10 mg/l was a precondition and applied to all group members (n = 23). Among them, only one sample (4,3 %) had a detectable CRP value. The difference between the two groups regarding the infection parameter CRP was statistically proven (p = 0,006). Compared to the non-infected group, the mean IL-6 levels were significantly higher in the infected cohort (423,5 ± 1205,3 vs. 22,0 ± 10,3, p = 0,002). In the non-infected cohort (n = 22), one patient was excluded, because the concentration of IL-6 was below the detection limit (< 7,5 pg/ml). The leukocyte counts in the sepsis population were similar to those in the control group (16,1 ± 9,3 G/l vs. 14,9 ± 4,5 G/l, p = 0,689). Further evaluation of the blood gas analysis revealed significant differences in pH levels (p = 0,008), BE (p = 0,003) and lactate concentrations (p = 0,037).

Among the 78 patients, 8 neonates (21,1 %) had a positive blood culture. Microbiological testing showed predominantly Gram-positive infections including one case of *Staphylococcus epidermidis*, one double infection with *Staphylococcus epidermidis* and *Staphylococcus aureus* as well as one case each of *Staphylococcus haemolyticus, Staphylococcus capitis, Granulicatella adiacens,* and *Propionibacteria*. Additionally, *Gram-negative rods* and *Paenibacteria macerans* were detected. The latter is described as Gram-variable (Grady et al. 2016). For the analysis, only patients with microbiological testing were included (n = 38). In the infected group (n = 22), 5 newborns (22,7 %) were blood culture positive and 17 neonates (77,3 %) were tested negative. 7 patients were not tested at all and therefore excluded from the evaluation. Only 4 neonates of the non-infected control group received a microbiological testing, whose results were negative. In summary, there was no statistical difference between the infected and non-infected patients (p = 0,555) regarding microbiological testing.

Both the infected and non-infected groups can be distinguished significantly in the antibiotic therapy. Whereas all 29 patients of the infected cohort received an antimicrobial treatment with an average duration of 6,7 ± 2,4 days, only 5 (21,7 %) of the non-infected patients received antibiotics for 0,4 ± 0,9 days on average (p = 0,000 and p = 0,000, respectively). In contrast to the infected newborns, the non-infected control group were not treated with catecholamines (0 vs. 9 cases, p = 0,003), immunoglobulins (0 vs. 6 cases, p = 0,028) and steroids (0 vs. 4 cases, p = 0,120). Except for the

treatment with steroids, the difference between the groups turned out significant when comparing the medicinal therapy. Summing up, the infected neonates were also significantly longer in hospital care than the non-infected patients (47,9 ± 44,0 vs. 11,8 ± 9,2 days, p = 0,000).

The two study groups did not exhibit any significant influence of comorbidities such as genetic syndromes (1 vs. 0 cases, p = 1,000) and congenital malformations (7 vs. 6 cases, p = 1,000). However, the infected group had significantly more cases of hyperbilirubinemia (19 vs. 7 patients, p = 0,025) and IRDS (18 vs. 1 patient, p = 0,000) than the non-infected newborns. Graduated from level 1 to 3/4 according to the medical diagnosis, most infected neonates suffered from IRDS level 2 (11 out of 18 patients with IRDS). The IRDS patient of the non-infected control group is diagnosed with level 1.

Because the total cohort included four pairs of twins, the maternal data analysis refers to 28 mothers of the infected newborns and 21 mothers of the non-infected newborns. The maternal impact on developing infection in newborns was higher in the sepsis cohort compared to the non-infected cohort. Mothers of infected neonates exhibited more premature rupture of membranes (PROM, 9 vs. 5 cases) and premature labor (9 vs. 2 cases) at birth. Nevertheless, the comparison between the two study groups revealed no significant difference (p = 0,750 and p = 0,087, respectively). Among the infected patients, 13 mothers were tested for GBS colonization and were considered for data analysis. Similarly, the control group only included 12 mothers with performed GBS smears. There was no statistical significance between the two cohorts (1 vs. 0 cases, p = 1,000). The sepsis patients showed a significantly higher rate of maternal infection (6 vs. 0 mothers) and maternal antibiotic treatment (6 vs. 0 mothers) than the non-infected newborns (p = 0,000).

**Table 15: Clinical characteristics of the study population.** Data were represented as mean ± standard deviation, median with IQR or number with percentage in parenthesis. P values from Mann-Whitney-U-test or Fisher´s exact test. CRT = capillary refill time, GBS = Group B Streptococci, IQR = interquartile range, IRDS = infant respiratory distress syndrome, n.s.= not significant, PROM = premature rupture of membranes, SGA = small for gestational age.

| **Clinical characteristics** | **Total (n = 78)** | **Infected (n = 29)** | **Non-infected (n = 23)** | **p*** |
|---|---|---|---|---|
| **Demographics** | | | | |
| Gestational age (weeks) | 33,1 ± 4,9 | 30,3 ± 5,8 | 35,1 ± 2,2 | 0,001 |
| Birth weight (g) | 2112,3 ± 1034,3 | 1612,5 ± 1083,4 | 2448,2 ± 644,6 | 0,000 |
| SGA | 12 (15,4) | 3 (10,3) | 4 (17,4) | n.s. |
| **Gender** | | | | |
| Male | 44 (56,4) | 18 (62,1) | 10 (43,5) | n.s. |
| Female | 34 (43,6) | 11 (37,9) | 13 (56,5) | |
| **Delivery route** | | | | |
| Vaginal delivery | 17 (21,8) | 5 (17,2) | 5 (21,7) | n.s. |
| Caesarean section | 61 (78,2) | 24 (82,8) | 18 (78,3) | |
| **APGAR** | | | | |
| 1 min | 7 (5 – 8) | 6 (4 – 7,5) | 8 (7 – 9) | 0,000 |
| 5 min | 8 (7 – 9) | 7 (6 – 8) | 9 (8 – 9) | 0,000 |
| 10 min | 9 (8 – 10) | 8 (8 – 9) | 9 (8 – 10) | 0,006 |
| **Vital signs** | | | | |
| Pathologic heart rate | 19 (24,3) | 12 (41,4) | 6 (26,1) | n.s. |
| Pathologic respiration | 46 (59,0) | 26 (89,7) | 9 (39,1) | 0,000 |
| Pathologic complexion | 13 (16,7) | 8 (27,6) | 1 (4,3) | 0,034 |
| CRT ≥ 3 sec | 4 (5,1) | 2 (6,9) | 0 (0,0) | n.s. |
| Neurological symptoms | 12 (15,4) | 8 (27,6) | 1 (4,3) | 0,034 |
| Intestinal symptoms | 17 (21,8) | 10 (34,5) | 3 (13,0) | n.s. |
| **Laboratory data** (n, see footnote) | | | | |
| CRP < 10 mg/l[1] | 64 (82,1) | 21 (72,4) | 23 (100,0) | 0,006 |
| CRP ≥ 10 mg/l[1] | 14 (17,9) | 8 (27,6) | 0 (0,0) | |
| IL-6 (pg/ml)[2] | 188,6 ± 772,8 | 423,5 ± 1205,3 | 22,0 ± 10,3 | 0,002 |
| Leukocytes (G/l)[3] | 15,0 ± 7,1 | 16,1 ± 9,3 | 14,9 ± 4,5 | n.s. |
| pH[4] | 7,34 ± 0,06 | 7,31 ± 0,07 | 7,36 ± 0,03 | 0,008 |
| BE (mmol/l) | -3,1 ± 2,9 | -4,4 ± 3,4 | -1,8 ± 2,0 | 0,037 |
| Lactate (mmol/l)[5] | 1,7 ± 1,0 | 2,0 ± 1,4 | 1,4 ± 0,4 | 0,003 |
| Positive blood culture[6] | 8 (21,1) | 5 (22,7) | 0 (0,0) | n.s. |

| | | | | |
|---|---|---|---|---|
| **Therapy** | | | | |
| Antibiotics | 55 (70,5) | 29 (100,0) | 5 (21,7) | 0,000 |
| Antibiotic therapy (days) | 3,8 ± 3,3 | 6,7 ± 2,4 | 0,4 ± 0,9 | 0,000 |
| Catecholamines | 13 (16,7) | 9 (31,0) | 0 (0,0) | 0,003 |
| Immunoglobulins | 6 (7,7) | 6 (20,7) | 0 (0,0) | 0,028 |
| Steroids | 7 (9,0) | 4 (13,8) | 0 (0,0) | n.s. |
| Hospitalization (days) | 28,8 ± 33,5 | 47,9 ± 44,0 | 11,8 ± 9,2 | 0,000 |
| **Comorbidities** | | | | |
| IRDS | 25 (32,1) | 18 (62,1) | 1 (4,3) | 0,000 |
| Hyperbilirubinemia | 34 (43,6) | 19 (65,5) | 7 (30,4) | 0,025 |
| Genetic syndrome | 1 (1,3) | 1 (3,4) | 0 (0,0) | n.s. |
| Congenital malformation | 23 (29,5) | 7 (24,1) | 6 (26,1) | n.s. |
| **Maternal risk factors** (n, see footnote) | | | | |
| PROM[7] | 20 (27,0) | 9 (32,1) | 5 (23,8) | n.s. |
| Premature labor[7] | 16 (21,6) | 9 (32,1) | 2 (9,5) | n.s. |
| Positive GBS smear[8] | 4 (9,3) | 1 (7,7) | 0 (0,0) | n.s. |
| Infection[7] | 9 (12,2) | 6 (21,4) | 0 (0,0) | 0,000 |
| Antibiotic therapy[7] | 12,8 (13,5) | 6 (21,4) | 0 (0,0) | |

* Comparison between infected and non-infected cohort

[1] CRP values under the detection limit (< 2 mg/l) were considered as 1 mg/l

[2] IL-6 values under the detection limit (< 7,5 mg/l) were excluded.
Total n = 74; infected n = 29; non-infected n = 22

[3] Total n = 76; infected n = 29, non-infected n = 22

[4] Total n = 77; infected n = 28; non- infected n = 23

[5] Total n = 74; infected n = 28; non-infected n = 21

[6] Neonates without microbiological testing were excluded.
Total n = 38; infected n = 22; non-infected n = 4

[7] Total cohort contains 4 pairs of twins: n = 74; infected group contains 1 pair of twins: n = 28; non-infected group contains 2 pairs of twins: n = 21

[8] Mothers without microbiological testing were excluded. Total n = 43; infected n = 13; non-infected n = 12

## 4.2 Serum levels of alarmins in NICU patients

The study examines the relevance of Gal-1, PGRN and resistin release as a result of non-canonical inflammasome activation and unconventional protein secretion in newborn patients with sepsis. Therefore, the following results are based on the stratification of NICU patients according to infection. In order to elucidate the role of potential confounders, the neonatal patients were further classified according to their gestational age. At last, alarmin levels were correlated with inflammasome-independent inflammatory markers including IL-6 and CRP.

### 4.2.1 Serum levels of alarmins in infected versus non-infected neonatal blood samples

The resistin secretion was significantly elevated in infected newborns compared to the non-infected control group (163,5 ± 96,1 ng/ml vs. 105,7 ± 73,0 ng/ml, p = 0,0182). However, there was no statistical significance between the infected and non-infected cohort regarding the secretion of Gal-1 (72,9 ± 71,3 ng/ml vs. 70,0 ± 63,1 ng/ml, p = 0,9389) and PGRN (94,4 ± 54,9 ng/ml vs. 72,1 ± 27,8 ng/ml, p = 0,1687). Nevertheless, the mean PGRN release was higher in case of infection. The results are summarized in **table 16** and illustrated in **Fig. 11 A – C**.

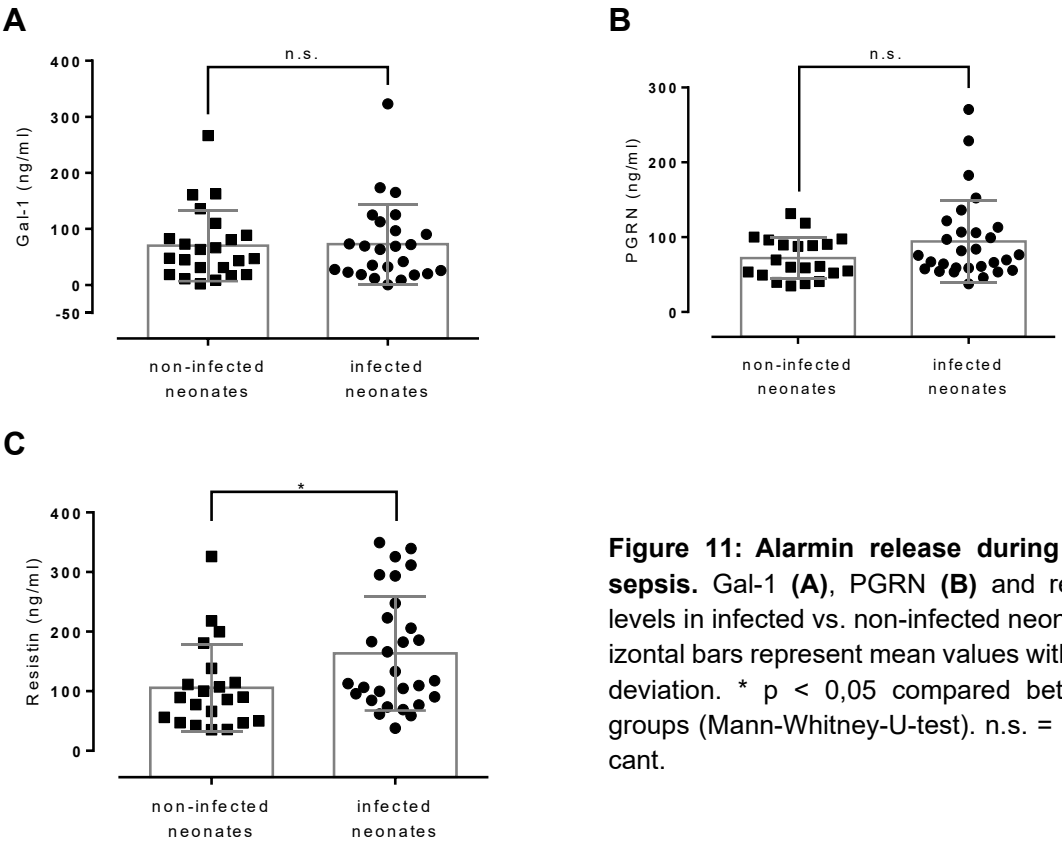



**Figure 11: Alarmin release during neonatal sepsis.** Gal-1 **(A)**, PGRN **(B)** and resistin **(C)** levels in infected vs. non-infected neonates. Horizontal bars represent mean values with standard deviation. * p < 0,05 compared between two groups (Mann-Whitney-U-test). n.s. = not significant.

**Table 16: Summary of alarmin levels in infected vs. non-infected neonates.** Values are shown in mean ± standard deviation (Mann-Whitney-U-test). n.s. = not significant.

| | Gal-1 (ng/ml) | PGRN (ng/ml) | Resistin (ng/ml) |
|---|---|---|---|
| Non-infected neonates | 70,0 ± 63,1 (n = 23) | 72,1 ± 27,8 (n = 21) | 105,7 ± 73,0 (n = 21) |
| Infected neonates | 72,9 ± 71,3 (n = 25) | 94,4 ± 54,9 (n = 29) | 163,5 ± 96,1 (n = 29) |
| P value | 0,9389 (n.s.) | 0,1687 (n.s.) | 0,0182 |

### 4.2.2 Serum levels of alarmins in full-term versus preterm neonatal blood samples

Since prematurity is one of the greatest risk factors for neonatal sepsis (Born et al. 2021, Shane et al. 2017), we further investigated the influence of the gestational age on the secretion of Gal-1, PGRN and resistin in newborns. Full-term born neonates were born after 36 + 6 weeks of pregnancy, whereas preterm born infants were born before 36 + 6 weeks of pregnancy. The study population of 78 patients was next stratified according to their premature and full-term time of birth **(Fig. 12 A – C)** and further divided according to sepsis diagnosis **(Fig. 12 D – F)**. The results of the alarmin levels are summarized in **table 17**

Out of the whole study cohort, the mean concentrations were 85,0 ± 186,7 ng/ml for Gal-1, 82,9 ± 41,3 ng/ml for PGRN and 127,8 ± 86,3 ng/ml for resistin. Classification into full-term and preterm born infants revealed no significant difference in the levels of Gal-1 (141,7 ± 340,4 ng/ml vs. 62,7 ± 54,7 ng/ml, p = 0,9016, **Fig. 12 A**) and resistin (126,1 ± 88,8 ng/ml vs. 128,5 ± 86,2 ng/ml, p = 0,9682, **Fig. 12 C**). Interestingly, the PGRN concentrations were significantly higher in premature born neonates compared to the PGRN levels in full-term born infants (91,1 ± 44,8 vs. 61,6 ± 17,9, p = 0,0012, **Fig. 12 B**).

In case of infection, the secretion of the alarmins Gal-1 (66,4 ± 57,4 ng/ml vs. 105,4 ± 106,9 ng/ml, **Fig. 12 D**) and resistin (118,8 ± 98,9 ng/ml vs. 165,3 ± 107,3 ng/ml, **Fig. 12 F**) did not significantly differentiate between full-term infected and full-term non-infected newborns (p = 0,8528 and p = 0,4242, respectively). Notably, PGRN levels in full-term newborns were significantly increased during infection compared to the alarmin levels observed in the absence of sepsis (70,9 ± 19,3 ng/ml vs. 44,4 ± 9,3 ng/ml, p = 0,0173, **Fig. 12 E**). The comparison between preterm infected and preterm non-infected infants exhibited no significant difference in the serum concentrations of Gal-1 (74,9 ± 76,5 ng/ml vs. 57,5 ± 35,5 ng/ml, p = 0,8447, **Fig. 12 D**) as well as PGRN (100,5 ± 59,7 ng/ml vs. 80,7 ± 26,0 ng/ml, p = 0,5552, **Fig. 12 E**). Otherwise, preterm newborns exhibited higher levels of resistin in the case of infection compared to the alarmin secretion in preterm non-infected neonates (175,2 ± 94,0 ng/ml vs. 87,1 ± 49,8 ng/ml, p = 0,0007, **Fig. 12 F**).

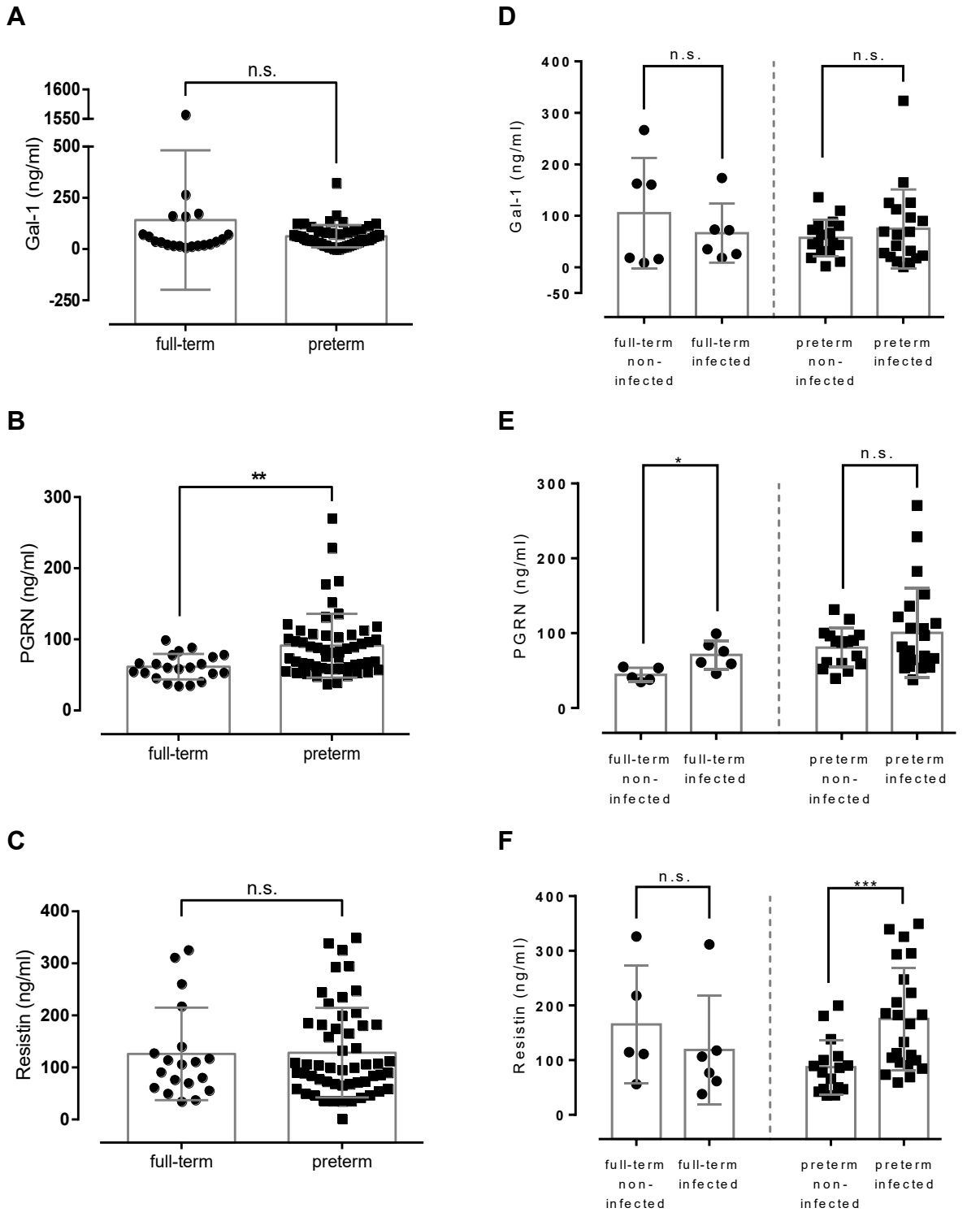



**Figure 12: Alarmin release in the context of gestational age.** Gal-1 **(A)**, PGRN **(B)** and resistin **(C)** release in full-term (> 36 + 6 WOP) vs. preterm (≤ 36 + 6 WOP) born neonates included in the whole study cohort. Gal-1 **(D)**, PGRN **(E)** and resistin **(F)** secretion in infected vs. non-infected full-term born neonates and infected vs. non-infected preterm born neonates. Horizontal bars represent mean values with standard deviation. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ compared between two groups (Mann-Whitney-U-test). n.s. = not significant, WOP = weeks of pregnancy.

**Table 17: Summary of alarmin levels in full-term and preterm born neonates as well as infected vs. non-infected full-term and preterm born neonates.** Values are shown in mean ± standard deviation (Mann-Whitney-U-test). n.s. = not significant.

| | Gal-1 (ng/ml) | PGRN (ng/ml) | Resistin (ng/ml) |
|---|---|---|---|
| Full-term born neonates | 141,7 ± 340,4 (n = 20) | 61,6 ± 17,9 (n = 21) | 126,1 ± 88,8 (n = 19) |
| Preterm born neonates | 62,7 ± 54,7 (n = 51) | 91,1 ± 44,8 (n = 54) | 128,5 ± 86,2 (n = 54) |
| P value | 0,9016 (n.s.) | 0,0012 | 0,9682 (n.s.) |
| **Full-term neonates** | | | |
| Non-infected neonates | 105,4 ± 106,9 (n = 6) | 44,4 ± 9,3 (n = 5) | 165,3 ± 107,3 (n = 5) |
| Infected neonates | 66,4 ± 57,4 (n = 6) | 70,9 ± 19,3 (n = 6) | 118,8 ± 98,9 (n = 6) |
| P value | 0,8528 (n.s.) | 0,0173 | 0,4242 (n.s.) |
| **Preterm neonates** | | | |
| Non-infected neonates | 57,5 ± 35,5 (n = 17) | 80,7 ± 26,0 (n = 16) | 87,1 ± 49,8 (n = 16) |
| Infected neonates | 74,9 ± 76,5 (n = 19) | 100,5 ± 59,7 (n = 23) | 175,2 ± 94,0 (n = 23) |
| P value | 0,8447 (n.s.) | 0,5552 (n.s.) | 0,0007 |

### 4.2.3 Correlation between alarmin levels and IL-6

In contrast to the resistin secretion, PGRN and Gal-1 showed no significant difference in the release during neonatal sepsis. We further investigated the correlation between the alarmins and the well-established infection parameters IL-6 and CRP.

The correlation analysis between the alarmin levels and the proinflammatory cytokine IL-6 is shown in **Fig. 13 A – C**. It refers to the paraclinical data of the infected cohort and includes 25 neonates for the Gal-1 analysis, 29 newborns for the PGRN analysis and 29 newborns for the resistin analysis. Patient samples with IL-6 levels below the detection limit were not included. **Fig. 13 D – F** compares the alarmin levels and IL-6 concentrations in infected and non-infected infants depending on the cytokine release. Therefore, septic neonates were grouped according to their IL-6 values above or below the cut-off concentration of 100 pg/ml. Non-infected neonates served as a control group, in which all patients exhibit IL-6 levels below 100 pg/ml. The concentrations of the alarmin levels are summarized in **table 18** on page 52.

In septic newborns, Gal-1 exhibited no correlation with IL-6 ($r = -0{,}0400$ and $p = 0{,}8494$, **Fig. 13 A**). Similarly, resistin showed no association to the infection parameter IL-6 ($r = 0{,}0005$ and $p = 0{,}9980$, **Fig. 13 C**). PGRN was the only alarmin, which was significantly positively correlated to IL-6 ($r = 0{,}3720$ and $p = 0{,}0469$, **Fig. 13 B**).

The comparison of alarmin levels between infected newborns with IL-6 values below and above the cut-off limit and non-infected neonates revealed no significant difference in Gal-1 release (80,3 ± 77,4 ng/ml vs. 53,7 ± 52,6 ng/ml vs. 70,1 ± 63,1 ng/ml, $p = 0{,}7332$, **Fig. 13 D**) as well as in resistin secretion (170,0 ± 100,0 ng/ml vs. 146,7 ± 89,1 ng/ml vs. 105,7 ± 73,0 ng/ml, $p = 0{,}0530$, **Fig. 13 F**). Performing Dunn´s multiple comparison test, resistin levels were significantly higher in the serum of septic infants exhibiting IL-6 < 100 pg/ml than in the control group (170,0 ± 100,0 ng/ml vs. 105,7 ± 73,0 ng/ml, **Fig. 13 F**). In contrast, the comparison of the three cohorts in the release of PGRN was substantially different (85,8 ± 53,8 ng/ml vs. 116,9 ± 54,9 ng/ml vs. 72,1 ± 27,8, $p = 0{,}0451$, **Fig. 13 E**). More precisely, PGRN levels in infected newborns with IL-6 ≥ 100 pg/ml were significantly elevated compared to the alarmin concentration secreted by the non-infected infants (116,9 ± 54,9 ng/ml vs. 72,1 ± 27,8, **Fig. 13 E**).

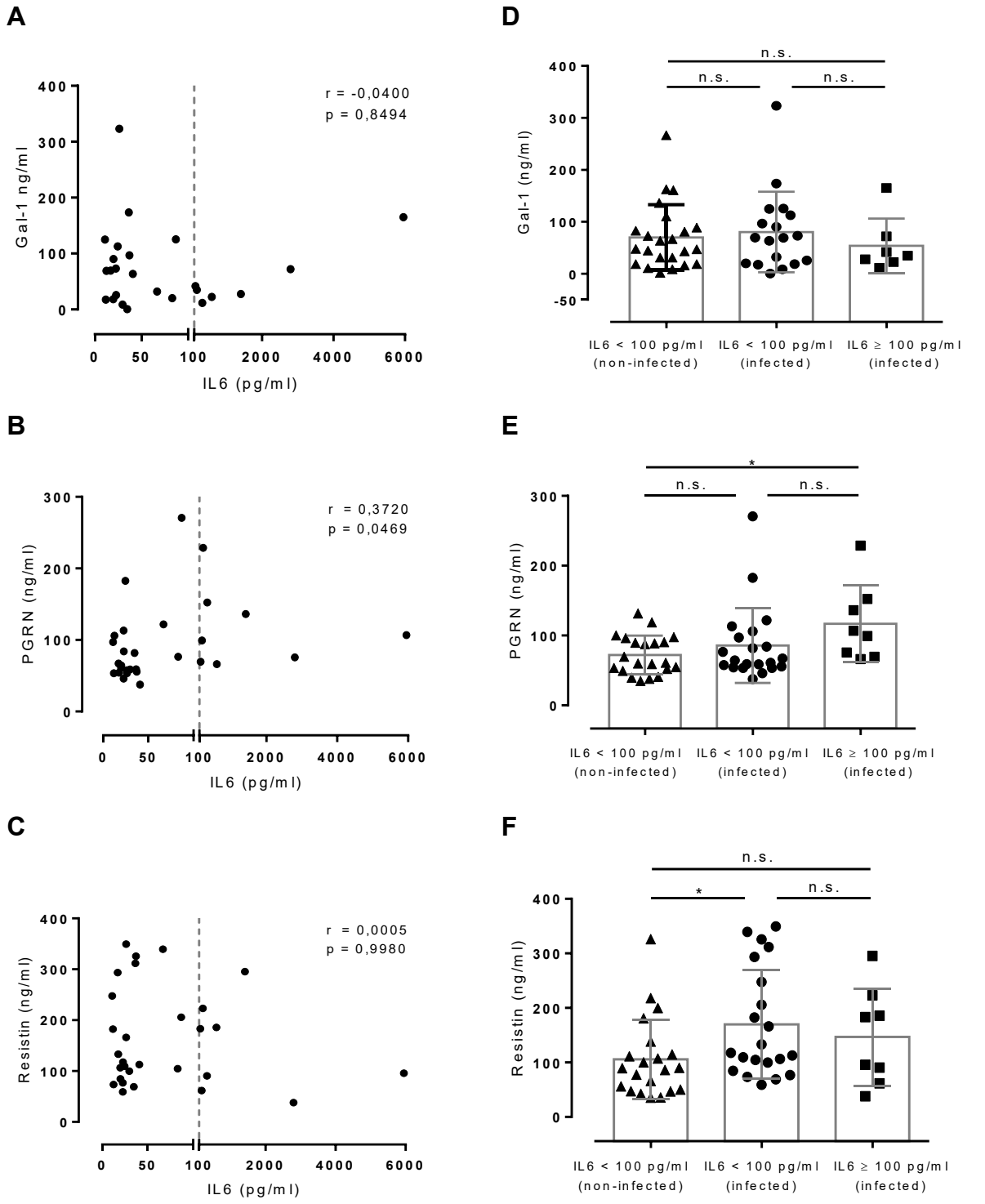



**Figure 13: Correlation of the alarmin levels with IL-6.** Correlation of Gal-1 **(A)**, PGRN **(B)** and resistin **(C)** with IL-6. Results refer to the infected cohort. Alarmin levels of Gal-1 **(D)**, PGRN **(E)** and resistin **(F)** in infected vs. non-infected neonates separated according to IL-6 values ≥ or < 100 pg/ml. Horizontal bars represent mean values with standard deviation. Dashed lines illustrate the IL-6 cut-off level. * $p < 0,05$ compared between three groups (Kruskal-Wallis-test & Dunn´s multiple comparisons test). n.s. = not significant.

**Table 18: Summary of alarmin levels in infected vs. non-infected neonates, separated according to IL-6 values ≥ or < 100 pg/ml.** Data are shown in mean ± standard deviation (Kruskal-Wallis-test). n.s. = not significant.

| | Gal-1 (ng/ml) | PGRN (ng/ml) | Resistin (ng/ml) |
|---|---|---|---|
| IL-6 < 100 pg/ml (Non-infected neonates) | 70,1 ± 63,1 (n = 23) | 72,1 ± 27,8 (n = 21) | 105,7 ± 73,0 (n = 21) |
| IL-6 < 100 pg/ml (Infected neonates) | 80,3 ± 77,4 (n = 18) | 85,8 ± 53,8 (n = 21) | 170,0 ± 100,0 (n = 21) |
| IL-6 ≥ 100 pg/ml (Infected neonates) | 53,7 ± 52,6 (n = 7) | 116,9 ± 54,9 (n = 8) | 146,7 ± 89,1 (n = 8) |
| P value | 0,7332 (n.s.) | 0,0451 | 0,0530 (n.s.) |

### 4.2.4 Correlation between alarmin levels and CRP

We next investigated the correlation between the alarmin release and the late infection marker CRP. **Fig. 14 A – C** illustrates the Gal-1, PGRN and resistin levels in 14, 16 and 16 infected neonates, respectively. Patient samples with CRP levels below the detection limit were excluded. Similar to the IL-6 analysis, **Fig. 14 D – E** presents the alarmin distribution in infected and non-infected neonates with regards to CRP levels. Whereas the non-infected control group necessarily exhibited CRP levels below the 10 mg/l cut-off value, the septic neonates were separated according to the CRP concentrations below or above 10 mg/l. Here, patients with CRP levels below the detection limit were included.

According to Spearman´s rank correlation coefficient, no relationship was detected between CRP levels and the alarmins Gal-1 (r = 0,1429 and p = 0,6266), PGRN (r = -0,0118 and p = 0,9694) as well as resistin (r = -0,1294 and p = 0,6327) in septic neonates **(Fig. 14 A – C)**. Moreover, there was no significant difference in the alarmin release between the three cohorts of infected newborns with CRP values below and above the cut-off limit as well as the non-infected control group (Gal-1: p = 0,4727; PGRN: p = 0,1534; resistin = 0,0520, **Fig. 14 D – F**). Results are summarized in **table 19** on page 54.

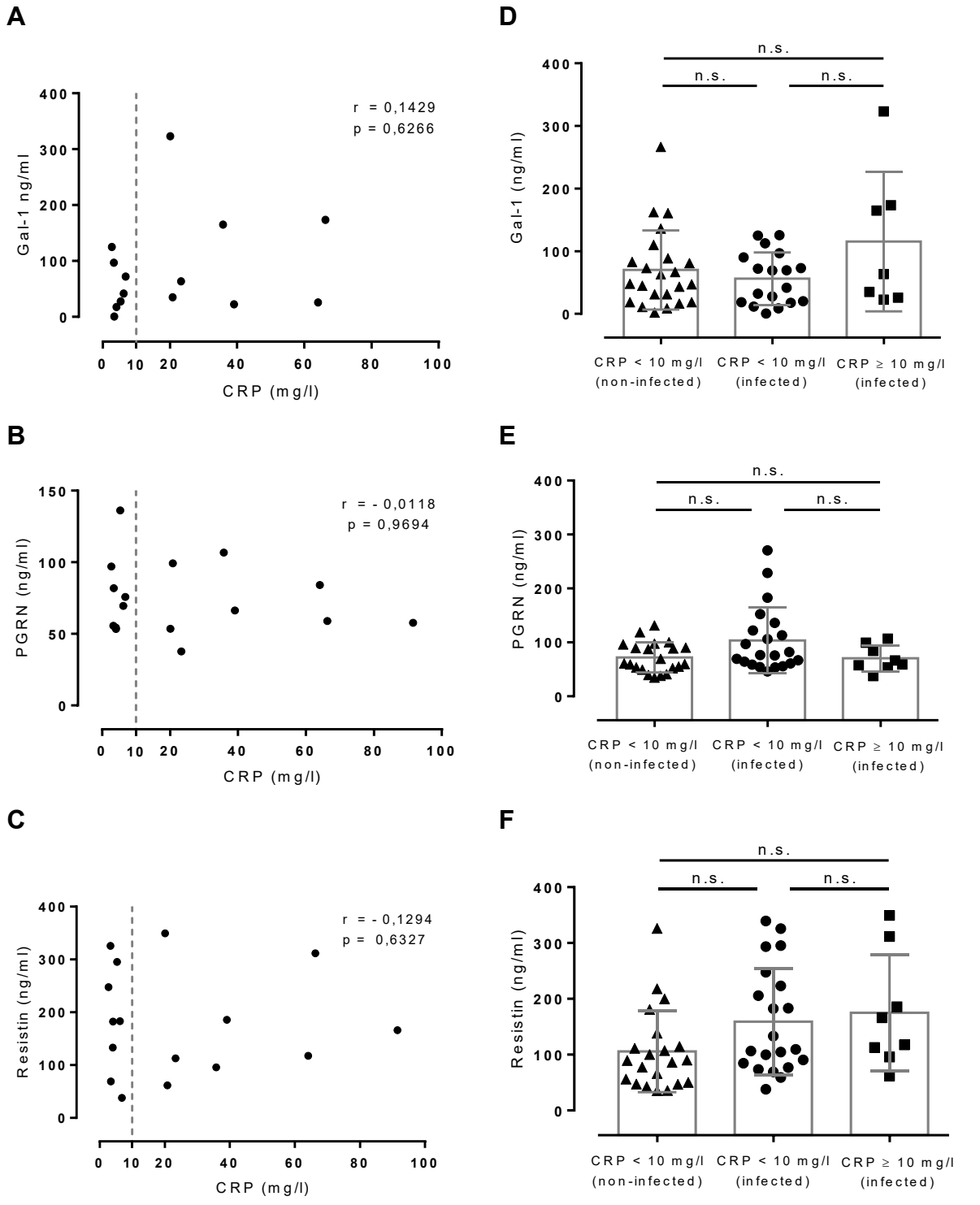



**Figure 14: Correlation of the alarmin levels with CRP.** Correlation of Gal-1 **(A)**, PGRN **(B)** and resistin **(C)** with CRP. Results refer to the infected cohort. Alarmin levels of Gal-1 **(D)**, PGRN **(E)** and resistin **(F)** in infected vs. non-infected neonates separated according to the CRP value ≥ or < 10 mg/l. Horizontal bars represent mean values with standard deviation. Dashed lines illustrate the CRP cut-off level. Comparison between three groups (Kruskal-Wallis-test & Dunn´s multiple comparisons test). n.s. = not significant.

**Table 19: Summary of alarmin levels in infected vs. non-infected neonates, separated according to CRP values ≥ or < 10 mg/l.** Data are shown in mean ± standard deviation (Kruskal-Wallis-test). n.s. = not significant.

| | Gal-1 (ng/ml) | PGRN (ng/ml) | Resistin (ng/ml) |
|---|---|---|---|
| CRP < 10 mg/l (Non-infected neonates) | 70,0 ± 63,1 (n = 23) | 72,1 ± 27,8 (n = 21) | 105,7 ± 73,0 (n = 21) |
| CRP < 10 mg/l (Infected neonates) | 56,3 ± 41,7 (n = 18) | 103,5 ± 60,1 (n = 21) | 159,1 ± 95,3 (n = 21) |
| CRP ≥ 10 mg/l (Infected neonates) | 115,5 ±111,7 (n = 7) | 70,6 ± 23,9 (n = 8) | 175,1 ± 103,9 (n = 8) |
| P value | 0,4727 (n.s.) | 0,1534 (n.s.) | 0,0520 |

## 4.3 Mechanism of PGRN release in response to *S. aureus* infection

The previous results highlight the role of PGRN. The alarmin tends to distinguish between infected and non-infected newborns dependent on their gestational age (**Fig. 12 E**, page 48) and correlates with the infection marker IL-6 (**Fig. 13 B & E,** page 51). As our laboratory has found its release to be associated with caspase-5 in response to Gram-positive infection (Duduskar 2020), the investigation of the trigger factors of PGRN secretion is an important aspect to expand the understanding of its release.

### 4.3.1 CRISPR/Cas9 mediated creation of ΔLgt *S. aureus*

The innate immune defense against *S. aureus* infection mainly depends on the recognition of bacterial lipoproteins via TLR2 (Aliprantis et al. 1999, Bubeck Wardenburg et al. 2006). In *S. aureus*, the phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt) encoded in the Lgt gene is responsible for the lipoprotein maturation (Stoll et al. 2005). In order to investigate the mechanistic role of lipoproteins in PGRN secretion, we created the mutant ΔLgt *S. aureus*, which is deficient in surface lipoproteins. Via CRISPR/Cas9-mediated genome editing, the expression of lipoproteins was inactivated by generating point mutations in the Lgt gene of *S. aureus* strain RN4220 (**Fig. 9**, page 34).

The wild type Lgt gene and protein sequence of *S. aureus* strain RN4220 consists of 840 base pairs (bp) and 279 amino acids, respectively (NCBI Reference Sequence: NZ_AFGU01000056.1, https://www.ncbi.nlm.nih.gov/nuccore/NZ_AFGU01000056.1?report=graph). The screening of the sequenced and genome-edited Lgt genes revealed two point mutations at nucleotide position 16 and 17 upstream of the PAM sequence 3´ CGG 5´ **(Fig. 15)**. Translated into the amino acid sequence, the C → T conversion at position 74 (TTC → TTT) had no consequence on the expression of the amino acid phenylalanine (F) **(Fig. 15, 16)**. Differently, the C → T conversion at position 75 changed the amino acid glutamine (Q) into an N-terminal stop codon (CAA → TAA) which resulted in the creation of *S. aureus* strain SA RN4220 ΔLgt Q75X **(Fig. 15, 16)**. The location of the wild type Lgt gene in *S. aureus* was further analyzed in a genome fragment of *S. aureus* strain RN4220 generated via shotgun sequencing. The lgt gene was found to be embedded between the genes for HPr kinase/phosphatase upstream and acetyltransferase downstream, which is followed by a tetratricopeptide repeat protein **(Fig. 16)**.

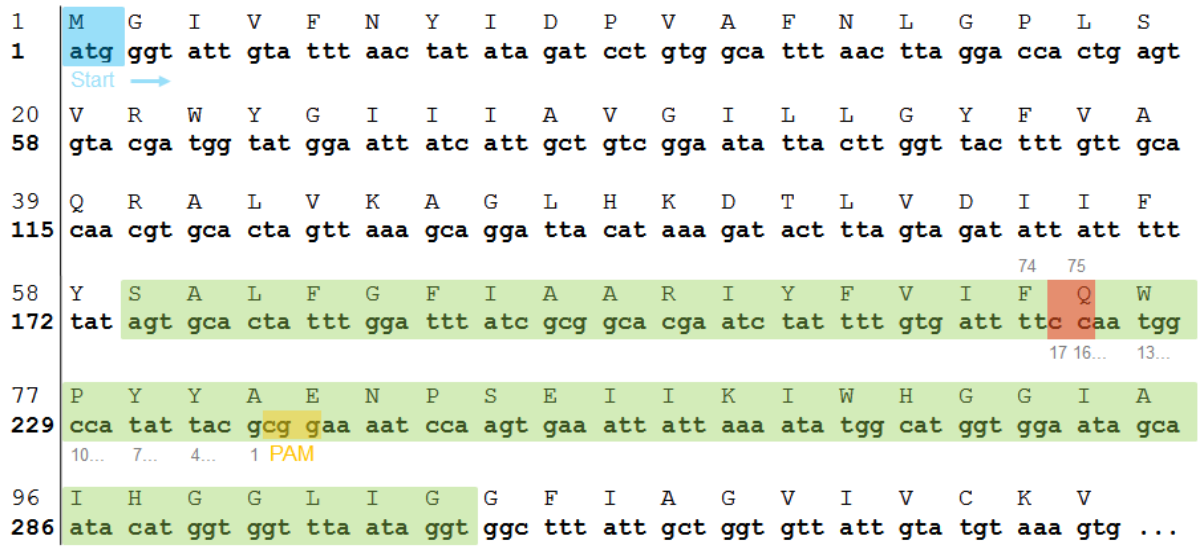



**Figure 15: Extract from the Lgt nucleotide and amino acid sequence of the wild type genome of *S. aureus* RN4220.** Blue: start codon. Green: sequenced gene segment. Red: location of point mutation. Yellow: PAM sequence. F = phenylalanine, PAM = protospacer adjacent motif, Q = glutamine.

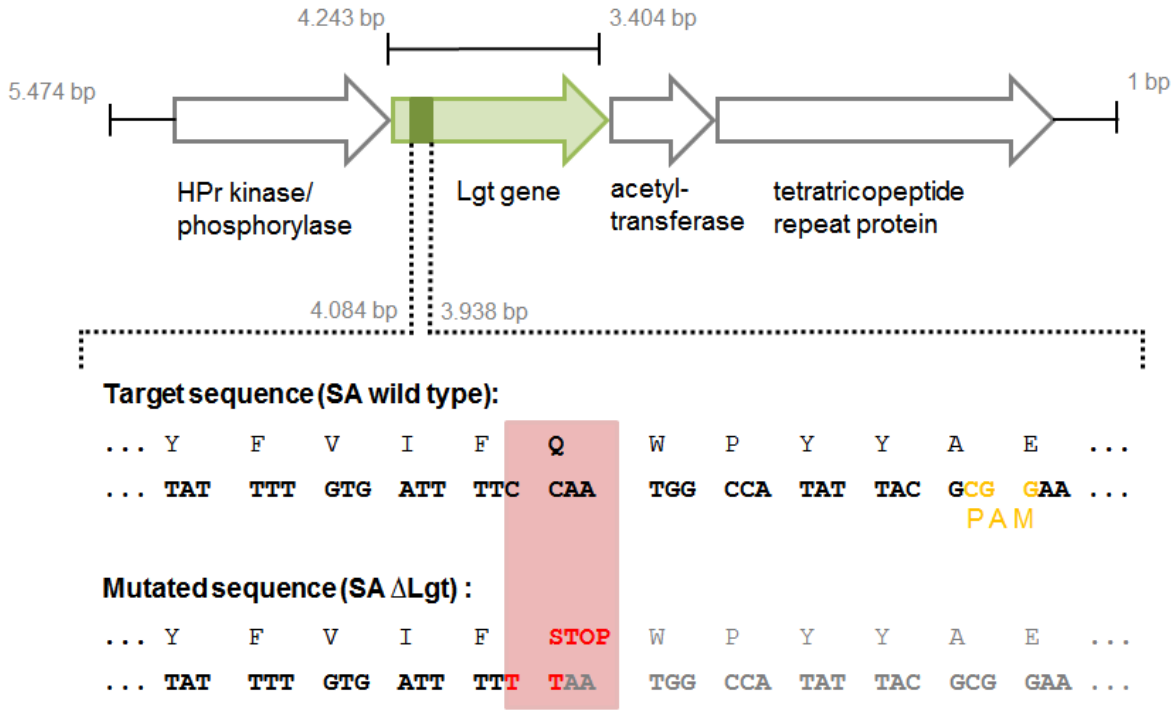



**Figure 16: Location of the Lgt gene in the wild type genome of *S. aureus* RN4220 (whole genome shotgun sequence).** The Lgt gene is embedded between an HPr kinase/phosphorylase and an acetyl-transferase. Through a C → T conversion by the cytidine deaminase, the amino acid glutamine (Q) changed into a stop codon (CAA → TAA). Lgt = phosphatidylglycerol-prolipoprotein diacylglyceryl transferase, PAM = protospacer adjacent motif.

### 4.3.2 Functional analysis of lipoprotein-deficient ΔLgt *S. aureus*

For validating the lack of lipoprotein production in *S. aureus* due to Lgt mutation, HEK 293T cells were stimulated with the synthetic lipoprotein $Pam_3CSK_4$ as well as heat-killed wild type and ΔLgt *S. aureus*. A part of the HEK 293T cells were previously processed to express the lipoprotein binding receptor TLR2. $Pam_3CSK_4$ was used as positive control for TLR2 activation.

As shown in **Fig. 17**, the IL-8 release after stimulation with $Pam_3CSK_4$ and heat-killed *S. aureus* wild type was significantly higher in TLR2-transfected cells compared to the cells lacking on TLR2 expression. Furthermore, the IL-8 secretion of HEK TLR2 cells after the treatment with heat-killed ΔLgt *S. aureus* was significantly lower than after the treatment with wild type bacteria **(Fig. 17 B)**. These results confirm the deactivation of the Lgt gene via CRISPR/Cas9 mediated base editing. The signaling pathways of IL-8 release after stimulation with $Pam_3CSK_4$ and *S. aureus* are illustrated in **Fig. 18**.

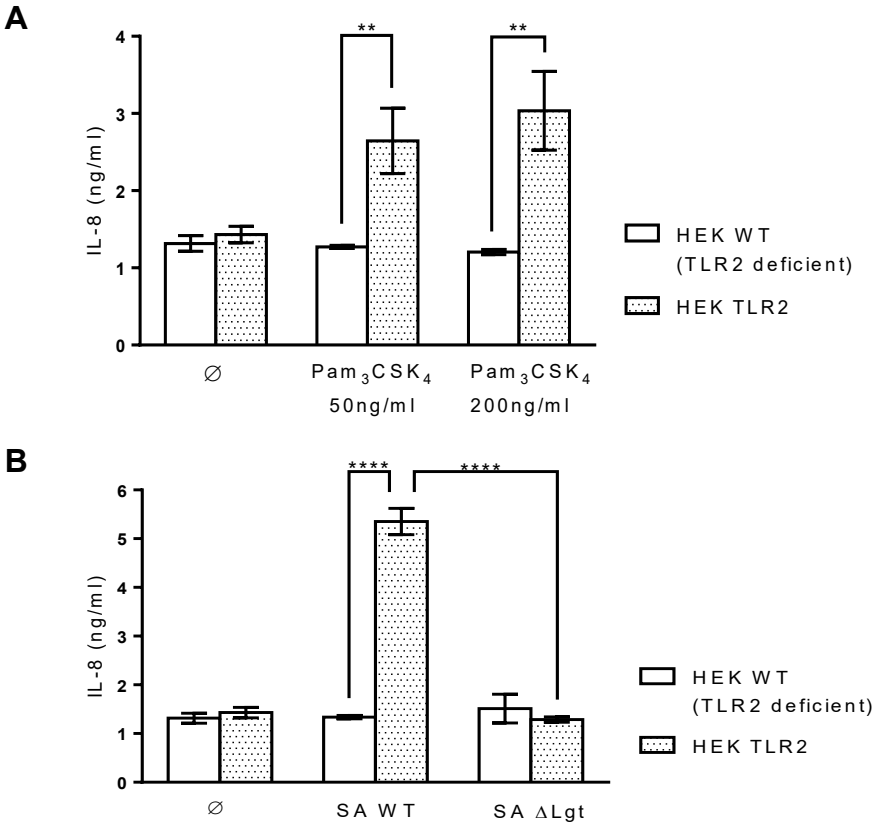



**Figure 17: Functional analysis of lipoprotein-deficient ΔLgt *S. aureus*.** IL-8 release in wild type HEK cells and HEK TLR2 cells after stimulation with $Pam_3CSK_4$ **(A)** and heat-killed *S. aureus* wild type and ΔLgt **(B)**. The bar charts represent mean values with standard deviation. ** $p < 0,05$, **** $p < 0,0001$ compared between two groups (unpaired t-test). SA WT = *S. aureus* wild type, SA ΔLgt = *S. aureus* Lgt mutant, WT = wild type, Ø = control.

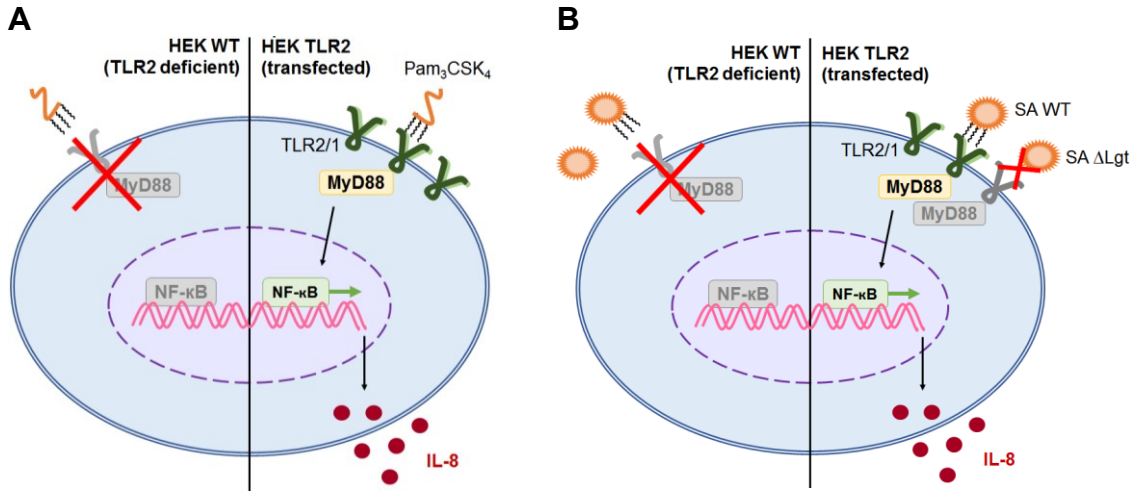



**Figure 18: Simplified signaling pathways of IL-8 release in wild type HEK cells and TLR2 transfected HEK cells after stimulation with $Pam_3CSK_4$ (A) and heat-killed bacteria (B).** IL = interleukin, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, SA WT = *S. aureus* wild type, SA ΔLgt = *S. aureus* ΔLgt, TLR2/1 = toll-like receptor 2/1, WT = wild type.

### 4.3.3 Lipoprotein-dependent release of PGRN

Lipoproteins of *S. aureus* are potent activators of the immune system by initiating the TLR2 signaling cascade (Hashimoto et al. 2006b). In order to evaluate the effect of bacterial lipoproteins on cytokine production and PGRN release, THP1 macrophages were stimulated with heat-killed wild type and ΔLgt *S. aureus*. As HEK 293T cells do not express the core inflammasome components, we used THP1 macrophages, which serve as a well-known model for studying the alarmin release in response to inflammasome activators (Shi et al. 2014). **Fig. 20** illustrates the release of PGRN and TNF-α in response to the wild type *S. aureus* and ΔLgt mutant. In both cases, the wild type bacteria induced significantly higher levels of PGRN and TNF-α in contrast to ΔLgt **(Fig. 19)**. These results confirm that lipoproteins act as potential ligands for the secretion of cytokines as well as the release of PGRN.

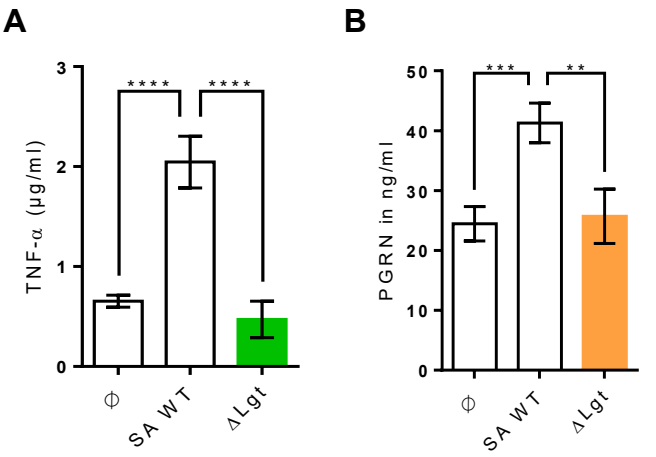



**Figure 19: Lipoprotein-dependent release of PGRN.** TNF-α **(A)** and PGRN **(B)** release after the stimulation of THP1 cells with *S. aureus* wild type (SA WT) and Lgt mutant (SA ΔLgt). The bar charts represent mean values with standard deviation. ** p < 0,01, *** p < 0,001, **** p < 0,0001 compared between two groups (unpaired t-test). n.s. = not significant, SA = *S. aureus* wild type, SA ΔLgt = *S. aureus* Lgt mutant, Ø = control.

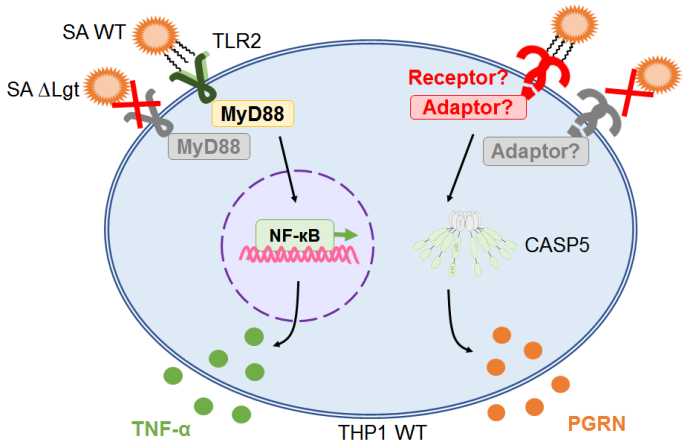



**Figure 20: Simplified signaling pathways of TNF-α and PGRN release after stimulation of THP1 cells with heat-killed bacteria.** SA WT but not SA ΔLgt induce MyD88- and NFκB-dependent release of TNF-α. PGRN is secreted upon SA WT lipoprotein stimulation through an unknown receptor and adaptor protein, which activate CASP5. CASP5 = caspase-5, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, SA ΔLgt = *S. aureus* Lgt mutant, SA WT = *S. aureus* wild type, TLR2 = toll-like receptor 2, WT = wild type.

### 4.3.4 Insufficient TLR2 activation for PGRN release

After identifying bacterial lipoproteins as ligands for PGRN release, the role of the cell surface receptor TLR2 in sensing *S. aureus* was further investigated. Therefore, THP1 cells were stimulated with the synthetic di- and triacylated lipoproteins $Pam_3CSK_4$ and FSL-1, respectively, which serve as TLR2-agonists. TLR2 activation was evaluated via the release of TNF-α and PGRN.

The cell stimulation with synthetic lipoproteins led to a significant increase in the secretion of TNF-α **(Fig. 21 A)**. In contrast, the release of PGRN remained unaltered in response to $Pam_3CSK_4$ and FSL-1 **(Fig. 21 B)**. The intense secretion of TNF-α indicates the activation of the TLR2 signaling cascade. Interestingly, the PGRN release remained unaffected despite TLR2 activation, which indicates that TLR2 activation alone is not sufficient for the secretion of PGRN in human macrophages **(Fig. 22)**.

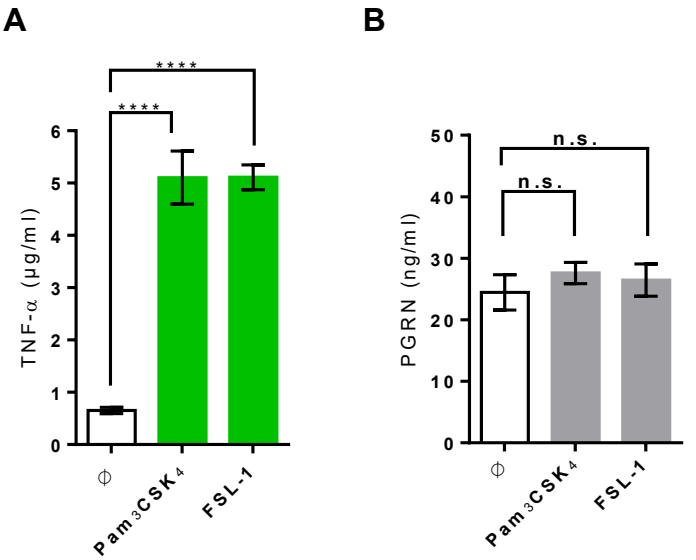



**Figure 21: Insufficient TLR2 activation for PGRN release.** TNF-α **(A)** and PGRN **(B)** release after stimulation of THP1 cells with the synthetic lipoproteins $Pam_3CSK_4$ (triacylated) and FSL-1 (diacylated). The bar charts represent mean values with standard deviation. **** $p < 0{,}0001$ compared between two groups (unpaired t-test). n.s. = not significant, Ø = control.

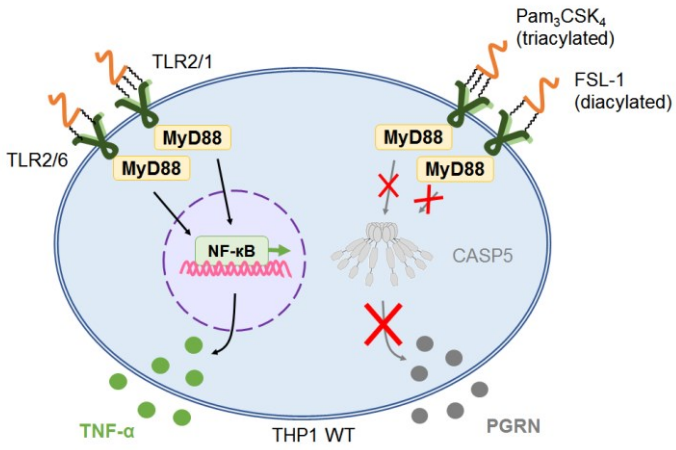



**Figure 22: Simplified signaling pathways of TNF-α and PGRN release after stimulation of THP1 cells with $Pam_3CSK_4$ and FSL-1.** $PAM_3CSK_4$ and FSL-1 induce TLR2-, MyD88- and NFκB-dependent release of TNF-α. TLR2 stimulation alone is not sufficient for PGRN secretion. CASP5 = caspase-5, MyD88 = myeloid differentiation primary response 88, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, TLR = toll-like receptor, WT = wild type.

# 5 Discussion

In newborns, pathogen defense primarily relies on the innate immune system involving the induction of PRR signaling (Basha et al. 2014, Yu et al. 2018). This is particularly well understood for Gram-negative bacteria, which engage TLR4 and subsequently initiate the production of inflammatory cytokines, such as IL-6, which in turn activates the synthesis of acute-phase proteins like CRP (Kawai and Akira 2011, Steel and Whitehead 1994). Besides surface TLR signaling, various studies have demonstrated the importance of inflammasomes for the clearance of intracellular bacteria. The activation of the cytosolic caspases is responsible for the induction of pyroptosis that culminates in the release of cytokines and danger signals, such as the potential alarmins Gal-1, PGRN and resistin, into the extracellular milieu.

Major advances and technological progress in the field of neonatal medicine have led to a significant improvement in perinatal survival, especially in the industrialized countries (Lawn et al. 2005, United Nations Inter-agency Group for Child Mortality Estimation [UN IGME] 2020). In Germany, only 1,006 newborns per 100 000 live births suffer neonatal sepsis and the case fatality due to neonatal sepsis is around 4 % (Born et al. 2021). In comparison, it is estimated that globally nearly 22 of 1000 live births develop neonatal sepsis with a mortality rate between 11 and 19 % (Fleischmann-Struzek et al. 2018). The incidence varies depending on the accessibility of medical resources across different health-care settings (Fleischmann-Struzek et al. 2018). Owing to the high mortality, the risk of diverse physical complications as well as the socioeconomic costs, sepsis is identified as high-priority research topic for clinicians and scientists worldwide (World Health Organization 2020). Against this background, the knowledge of neonatal immune defense mechanisms in response to invading pathogens plays a pivotal role in developing efficient diagnostic methods for early sepsis recognition.

## 5.1 Lack of consistent definition of neonatal sepsis

Within the past 15 years, the understanding of sepsis changed. Several studies concluded that sepsis in neonates cannot simply be ruled out by age-adapted SIRS criteria (Hornik et al. 2012a, Hornik et al. 2012b, Leante-Castellanos et al. 2017, Ussat et al. 2015, Wynn et al. 2014). Considering the difficulties of sepsis diagnosis in newborns, the study population in this thesis was stratified following the sepsis criteria of the NEO-KISS protocol provided by the German National Reference Center for Surveillance of Nosocomial Infections (NRZ 2017). In contrast to the pediatric sepsis definition of Goldstein et al. (2005), the NEO-KISS protocol includes a broader range of clinical as well as laboratory parameters, which might enable the clinician to register various organ dysfunction in septic newborns. Furthermore, the NEO-KISS surveillance protocol precisely distinguishes between culture-negative, that is clinical sepsis, and culture-positive sepsis in the presence or absence of coagulase-negative staphylococci, since CoNS are suspected to act as contaminating germ, but also emerge as predominant causative agent for LOS (Dong and Speer 2015, Hall and Lyman 2006, Wagstaff et al. 2019). Another advantage of the NEO-KISS sepsis definition is the continuous update and adaption to the conditions of German NICUs. As part of the Association of the Scientific Medical Societies in Germany (AWMF e.V.), it is well established in the guideline about bacterial infections in newborn infants (Zemlin et al. 2018).

However, the NEO-KISS sepsis criteria possess some limitations and were further modified to be applicable to the current study population. First, they only refer to preterm born neonates weighing less than 1500 g at birth. Instead, the NEO-KISS could have paid more attention to the age-specific demands on preterm and term born neonates. This includes the developmental physiological changes, risks for invasive infections as well as the treatment policy. However, in this study, the sepsis cohort exhibited a mean gestational age of 30,3 ± 5,8 weeks and a mean birth weight of 1612,5 ± 1083,4 g. Additionally, the non-septic control group was 35,1 ± 2,2 weeks old and weighed 2448,2 ± 644,6 g. As such, the study population predominantly requires sepsis criteria adapted to preterm infants that were partially covered by the NEO-KISS.

Second, the NEO-KISS sepsis criteria solely correspond to nosocomial sepsis. According to the NEO-KISS, it is defined as non-transplacental-acquired infection, which

develops after admission on the NICU (NRZ 2017). As clinical signs are not able to distinguish the origin of sepsis, infections manifested before 72 h are considered as vertical and after 72 h are considered as nosocomial (NRZ 2017). The time interval corresponds to the traditional classification of neonatal sepsis into early onset and late onset sepsis. However, the sepsis criteria of this study lack the differentiation into early, late or nosocomial acquired infection, respectively, because information about the newborn´s age at the onset of infection and NICU admission was not available. An EOS was presumed by evaluating maternal risk factors such as PROM, maternal infection or antibiotic treatment and positive smear for GBS.

The third modification refers to the evaluation of clinical symptoms and laboratory signs. For the assessment of organ failure, the sepsis criteria defined in this study failed to consider the broad spectrum of clinical and laboratory parameters provided by the NEO-KISS. On the one hand, reasons were the missing collection of data, for example about the blood sugar and I/T ratio. On the other hand, some laboratory parameters were consistently within the physiological range throughout the study population so that they did not have an impact on the evaluation of sepsis disease (e.g. leukocyte count, platelet count, BE). Interestingly, Modi et al. (2009) investigated the impact of clinical signs to predict a positive blood culture result. In contrast to the NEO-KISS sepsis definition and the sepsis criteria in this thesis, the presentation of ≥ 3 predefined clinical signs had the best predictive accuracy for a positive blood culture in infants with bloodstream infection (Modi et al. 2009).

For comprehensive sepsis diagnosis, the clinical criteria in this study were accompanied by laboratory signs, the documentation of suspected or proven infection by the doctor and maternal risk factors. Through the establishment of cut-off values, clinical and laboratory markers were further objectified. Thereby, the cut-off levels defined in this study refer to the laboratory reference values of the NICU of the University Hospital Jena. For example, whereas the NEO-KISS tolerated a heart rate between 80 – 200 beats per minute, the heart rate interval of the University Hospital Jena was restricted to 160 – 120 beats per minute. Similarly, the cut-off limit for CRP was adapted to ≥ 10 mg/l, a commonly used cut-off concentration of CRP (Benitz et al. 1998, Markic et al. 2017).

Since the evaluation of bacteremia in septic patients was hindered, as only 38 of 78 neonates were microbiologically tested, the sepsis definition in the current thesis was simplified into culture-positive and culture-negative sepsis. The presence or absence of CoNS was not considered in contrast to the NEO-KISS sepsis definition. However, to ensure the exclusion from contamination, sepsis diagnosis was only assigned to infants who already received antibiotic therapy for at least 4 days. Despite the recommendation for the immediate onset of empiric antimicrobial treatment in case of clinically suspected infection, evidence about the ideal duration of antibiotic treatment is missing (Zemlin et al. 2018). The AWMF protocol suggests the termination within 36 – 48 h after negative blood culture results and negative clinical as well as laboratory signs of infection were confirmed (Zemlin et al. 2018).

In conclusion, the sepsis criteria in this study were broadly defined which harbors the danger of forming a heterogenous study group. The comparison between the NEO-KISS sepsis criteria and the sepsis criteria defined in this study demonstrated the complex demands on the neonatal sepsis definition. The diagnosis should be applicable across different gestational ages, to both EOS and LOS and based on objective criteria (Wynn and Polin 2020).

## 5.2 Alarmin release during neonatal sepsis

The recognition of septic newborns on NICUs remains elusive and requires clear guidance on the diagnosis of neonatal sepsis as well as valid screening tools to detect organ dysfunctions in newborns. Since there is no proper system to stratify sepsis in newborns, we hypothesized, if the new signaling pathway involving the innate non-canonical inflammasome could support the conventional sepsis diagnosis in newborns as previously described. In adults, cytosolic inflammasomes, involving human caspase-4 and caspase-5, are known to assist the innate immune system in removing pathogens and injured cells. In cases of sepsis, dysregulated activation of pyroptosis associated with the release of IL-1ß, IL-18 and various other DAMPs, might contribute to endotoxic shock and microcirculatory failure (Steinhagen et al. 2020). Our laboratory investigated the non-canonical inflammasome and discovered the caspase-4- and caspase-5 dependent release of the novel alarmins galectin-1 (Gal-1), progranulin (PGRN) and resistin. Upon stimulation with cytosolic LPS, Gal-1 was found to be

secreted downstream of caspase-4 activation through pyroptosis in a GSDMD-dependent manner (Russo et al. 2021). Similarly, progranulin was discovered to be released using the caspase-5- and GSDMD-dependent inflammasome pathway (Duduskar 2020). Resistin was found to be secreted by both the human capase-4 and caspase-5 (Appendix **Fig. 24,** page 102). Interestingly, these alarmins were observed to be elevated during adult sepsis, indicating their potential as biomarker in sepsis diagnosis (Koch et al. 2009, Russo et al. 2021, Song et al. 2016, Sundén-Cullberg et al. 2007).

Despite advanced research in the field of sepsis, little is known about non-canonical inflammasome signaling and caspase activity in newborns with sepsis. In neonates, the immune system is already arranged in a dampened state within the first days of life to prevent excessive inflammation in response to perinatal stimuli (Levy 2007, Wynn and Levy 2010). Age-specific investigations of the transcriptomic response of children with septic shock revealed the significant downregulation of genes related to key pathways of both the innate and adaptive immunity in neonates compared to toddlers and older children (Wynn et al. 2011). Neonates seem to be unable to generate a proper inflammatory response and actively initiate immunosuppression (Hibbert et al. 2018, Maddux and Douglas 2015). It must be taken into account that knowledge about sepsis immunopathology is still limited and sepsis mechanisms are much more complex than the simple division into a hyper- as well as hypoinflammatory state (Rubio et al. 2019). Against this background, this thesis focuses on investigating the release of Gal-1, PGRN and resistin in septic newborns. To date, this is the first study that investigates the non-canonical inflammasome-dependent secretion of the above mentioned alarmins during neonatal sepsis. It is estimated to draw conclusions from caspase-4 and caspase-5 activation as well as to identify the diagnostic value of alarmin levels in infected compared to non-infected neonates.

**Galectin-1:** In this study, the analysis of the galectin-1 secretion in the neonatal circulation revealed no difference between infected and non-infected patients (**Fig. 11 A,** page 46). Compared to adults, Gal-1 levels failed to indicate sepsis in newborns. Considering the immune suppressive state of the neonates during infection, it is likely that the expression of caspase-4 might be downregulated and that the non-canonical effector functions like pyroptosis and alarmin release are cancelled. Similarly, the

investigation of monocytes derived from adult sepsis patients undergoing endotoxin tolerance showed impaired caspase-4 expression compared to non-tolerized patients (Ghait 2021). However, several discoveries indicate that newborns might benefit from the suppression of caspase-4 synthesis. The expression of caspase-4 in transgenic mice was observed to be associated with LPS-induced hyperinflammation and mortality (Kajiwara et al. 2014). Gentile et al. (2015) showed the beneficial effect of caspase-11 depletion in neonatal mice on surviving neonatal sepsis. Moreover, investigations in galectin-1-deficient mice revealed increased survival during LPS-induced septic shock (Russo et al. 2021). It is assumed that the increased release of Gal-1 following caspase-4 activation might worsen the sepsis outcome in already immune deficient neonates and seem to hinder the newborn in establishing a proper inflammatory response. On the contrary, low Gal-1 levels due to caspase-4 impairment might be protective in the fight against sepsis by limiting inflammation in case of neonatal sepsis.

**Progranulin:** Similar to Gal-1, PGRN release in septic newborns did not significantly differ compared to the non-infected control cohort (**Fig. 11 B**, page 46). Nevertheless, mean concentrations of PGRN were elevated in the infected group. Increased levels of the alarmin in the context of sepsis were first described by Song et al. (2016), who examined the PGRN secretion in adult and pediatric patients. However, in contrast to the current study, the pediatric population did not refer to infants in the neonatal period. Instead, children from 1 to 9 years were included, who might already possess a fully shaped immune repertoire (Simon et al. 2015). Recently, two independent studies reassessed the meaning of PGRN release in newborns with early-onset neonatal sepsis. Consistent with the results found in adult patients (Song et al. 2016), septic neonates exhibited higher PGRN levels compared to the uninfected control group (Rao et al. 2020, Yang et al. 2020). PGRN was even identified as a significant predictive marker for neonatal EOS within 72 hours after birth (Yang et al. 2020). Nevertheless, there is no knowledge about the non-canonical inflammasome involvement in PGRN release. Following the studies previously mentioned, caspase-5 activation might be initiated during neonatal sepsis for the secretion of PGRN. Moreover, the expression of caspase-5 was found to be upregulated in adult sepsis patients independent of endotoxin tolerance (Ghait 2021). However, in the current thesis, the release of this alarmin in infected newborns was not significantly different compared to non-infected infants. Still, the results suggest a tendency towards sepsis-dependent PGRN secretion. It is

possible that the heterogenous study group contributes to the indistinct study results, since the newborns were not differentiated according to their sepsis onset, that is LOS or EOS. As such, PGRN levels were mapped during various stages of sepsis and various activation levels of caspase-5. However, an elevated alarmin secretion may contribute to recovery during neonatal or adult sepsis since progranulin has been identified to possess protective roles in the clearance of bacterial infection or LPS-induced endotoxic shock in mice models (Song et al. 2016, Yin et al. 2010, Yu et al. 2016).

**Resistin:** Unlike the previously mentioned alarmins, serum concentrations of resistin were significantly elevated in newborns with sepsis compared to the neonates of the non-septic control group (**Fig. 11 C**, page 46). The sepsis-dependent release of resistin as well as its association to sepsis severity, organ damage and correlation to other inflammatory parameters, such as IL-6, IL-10, TNF-α and CRP, was primarily investigated in adult patients (Koch et al. 2009, Sundén-Cullberg et al. 2007). Our results were consistent with several studies that evaluated the efficacy of resistin in diagnosing EOS. By comparing the resistin concentrations in septic and non-septic newborns, alarmin levels in case of neonatal infection were observed to be increased released (Ahmed et al. 2018, Aliefendioglu et al. 2014, Cekmez et al. 2011, Gokmen et al. 2013, Ozdemir and Elgormus 2017). Being mainly expressed in immune cells in humans (Johansson et al. 2009, Patel et al. 2003, Savage et al. 2001), resistin was identified to be associated with inflammatory processes (Acquarone et al. 2019, Koch et al. 2009, Sundén-Cullberg et al. 2007). However, knowledge about the secretory pathways in the context of sepsis is sparse. In our laboratory, resistin was found to be released in a manner requiring both, caspases-4 and caspase-5 (Appendix **Fig. 24**, page 102). Considering the immune deficient milieu in newborns associated with limited activation of the non-canonical inflammasome, heightened levels of resistin may indicate additional mechanisms of secretion. Accordingly, it is still unclear, if the alarmin exits the cell during GSDMD-mediated pyroptosis. These observations prompt further experiments on the mechanism of resistin release.

Overall, these results demonstrate that only resistin is able to predict infection in critically ill neonates. The non-canonical inflammasome activation and subsequent alarmin release of Gal-1, PGRN and resistin during neonatal sepsis is determined by the distinct and suppressive immune system of the newborns. Further investigations are

needed to shed some light on the mechanisms of caspase-4 and caspase-5 activation as well as on their complex interplay in septic newborns.

### 5.2.1 Influence of the gestational age

Following the investigation of alarmin release in case of neonatal sepsis, the study further aimed to identify possible confounders, which might influence the non-canonical inflammasome effector functions and sepsis prediction capacity of alarmin release. Therefore, the secretion of galectin-1, progranulin and resistin was examined with regards to the gestational age. In this study, childbirths after the completion of 36 + 6 weeks were considered as (full-)term, whereas events prior to the completion of 37 weeks of gestation were considered to be preterm, as the average human pregnancy period is estimated to last between 37 and 42 weeks (Kiserud 2012). It has been demonstrated that microbial-mediated preterm delivery is initiated by the activation of the innate immune system resulting in the release of stimulating pro-inflammatory cytokines, e.g. TNF-α, IL-1ß and IL-8 (Casey et al. 1989, Keelan et al. 1999, Romero et al. 2007). Moreover, amniotic fluid contaminated with bacteria or pro-inflammatory agents might be transferred to the fetus to induce a fetal inflammatory response syndrome (FIRS) (Jung et al. 2020). It is well known that preterm newborns are characterized by a more comprised immature immune system compared to full-term neonates (Melville and Moss 2013). Additionally, premature born neonates miss the protection through the transfer of maternal IgG, which is mainly acquired during the last 4 weeks of gestation (Palmeira et al. 2012, van den Berg et al. 2010). Together with the increased demand for pivotal medical interventions, premature born neonates are extremely vulnerable to infection accompanied by the increased risk of various short- and long-term diseases as well as mortality considering prematurity as a main cause of child death under the age of 5 years (Romero et al. 2014, UN IGME 2020). As such, the identification of age-specific biomarkers could accelerate early sepsis diagnosis and treatment.

**Galectin-1:** Our results demonstrate that Gal-1 release during infection is independent of the gestational age. The comparison between Gal-1 levels in full-term and preterm born neonates of the whole study cohort showed no significant difference (**Fig. 12 A**, page 48). Furthermore, infection among full-term and preterm infants did not induce elevated secretion of Gal-1 compared to the non-infected control group (**Fig. 12 D**,

page 48). Interestingly, these results were refuted by a recent study by Faust et al. (2020). The group investigated the postnatal dynamics of Gal-1 concentrations in preterm newborns between the first and 28$^{th}$ day of life and further analyzed the alarmin release during an inflammatory setting. At day one, galectin-1 release was observed to be increased in neonates suffering from severe amniotic infection syndrome (AIS) and early-onset sepsis. Noteworthy, the alarmin levels rapidly declined after birth and were inversely correlated with the gestational age. The authors concluded that Gal-1 might be associated with early inflammation. Nevertheless, Faust et al. (2020) missed to investigate the maternal impact on neonatal Gal-1 levels. As such, it has been demonstrated that Gal-1 has an important role in the fetal-maternal interface by dampening inflammatory responses and therefore promoting maternal immune adaption to the semi-allogenic fetus (Blois et al. 2007, Gomez-Chavez et al. 2015, Ramhorst et al. 2012). Furthermore, maternal circulating concentrations of Gal-1 showed a significant increase during the course of pregnancy (Tirado-Gonzalez et al. 2013). Inversely, low levels of Gal-1 are discussed to be associated with miscarriage, recurrent fetal loss and fetal growth restriction (Jin et al. 2021, Ramhorst et al. 2012, Tirado-Gonzalez et al. 2013), whereas an upregulated expression of choriodecidual Gal-1 was detected during preterm labor and chorioamnionitis (Shankar et al. 2010, Than et al. 2008). With regards to these findings, it may be possible that maternal Gal-1 levels may affect Gal-1 concentrations in the fetal circulation. Consequently, rapid decline of alarmin concentrations in newborns after birth can be explained by the reduction of maternal galectins on the one hand, and the manifestation of immune suppression with caspase-4 downregulation on the other hand. Further experiments need to be done to reveal the maternal influence on neonatal Gal-1 expression. Additionally, the different study results by Faust et al. (2020) might also be explained by the large study cohort including 170 patients that allows the stratification of sepsis disease into AIS, EOS and LOS. In this thesis, the neonatal study cohort was not differentiated according to the beginning of sepsis, since the onset of infection remained unknown. Consequently, the measured concentrations of Gal-1 rather represent a mixture of early and late sepsis phases.

**Progranulin:** Stratification of the study cohort according to the gestational age revealed that infected full-term infants exhibited significantly higher levels of PGRN compared to non-infected full-term infants (**Fig. 12 E**, page 48). On the contrary, the alarmin

failed to discriminate neonatal sepsis in preterm born infants (**Fig. 12 E**, page 48). These results were supported by the findings of Rao et al. (2020) and Yang et al. (2020), who investigated the PGRN release in EOS patients ≥ 34 weeks of age. Nevertheless, literature about PGRN secretion in preterm infants is scarce. The current analysis demonstrates the influence of the gestational age on the accuracy of sepsis diagnosis according to PGRN levels. Interestingly, the alarmin is only able to distinguish between septic and non-septic neonates after they completed 37 weeks of gestation. These findings indicate a maturation process in caspase-5 activity, since the PGRN secretion in preterm infants failed to distinguish sepsis in preterm newborns (**Fig. 12 E**, page 48). On the contrary, looking at the whole patient cohort, PGRN was increasingly released in preterm born neonates compared to term infants (**Fig. 12 B**, page 48). This result may be explained by the fact that the preterm cohort comprises more neonates, who are known to be more susceptible to infection and more likely to suffer inflammation due to their impaired immune system (Melville and Moss 2013).

**Resistin:** Full-term and preterm neonates were observed to secret similar amounts of resistin. There was no significant difference between the two cohorts (**Fig. 12 C**, page°48). Prior to that, Ng et al. (2005) investigated the release of resistin in non-infected term and preterm neonates. The workgroup found significant correlations between the alarmin level and the gestational age as well as the anthropometric indices like birth weight, body height and body mass index at birth (Ng et al. 2005). Resistin levels were strikingly higher in term than in preterm infants and assumed to prevent hypoglycemia by culminating in the fetal fat mass as metabolic hormone (Ng et al. 2005). Otherwise, the neonatal alarmin levels were observed to be significantly elevated after vaginal delivery than after caesarean section suggesting inflammation or stress triggered by vaginal birth (Ng et al. 2005). Thereby, the correlation between mode of delivery and gestational age was not taken into account. Unlike Ng et al. (2005), the birth mode was ingnored in this study as only 21,8 % of the infants of the whole study population were born naturally. However, considering the release of resistin being independent of the gestational age, there was a significant difference in age-dependent alarmin secretion in the case of infection. We further evaluated the gestational age-dependent release of resistin in infected versus non-infected neonates. Notably, the alarmin was identified to predict sepsis only in preterm born infants, whereas infection did not change resistin levels in term newborns (**Fig. 12 F**, page 48). Several

studies confirmed resistin as potential diagnostic marker for sepsis in premature, but also in term and near-term neonates (Ahmed et al. 2018, Aliefendioglu et al. 2014, Cekmez et al. 2011, Gokmen et al. 2013, Ozdemir and Elgormus 2017). It may be possible that the small sample size contributes to the non-significant results in the current thesis, since the full-term cohort only comprises 6 infected and 5 non-infected patients. Additional term neonates need to be included in future studies to clarify the role of resistin release in infected term infants.

Concluding, the release of the alarmins PGRN and resistin during neonatal sepsis was identified to be influenced by the gestational age. Whereas elevated PGRN levels indicate infection in full-term newborns, neonatal sepsis in preterm infants is associated with increased secretion of resistin. Gal-1 was unsuitable to predict infection irrespective of the gestational age.

### 5.2.2 Correlation to the infection parameters IL-6 and CRP

The early and precise identification of neonates suffering infection is still a major challenge in neonatal intensive care units. Biomarkers act as measurable indicators for the presence of a disease (FDA-NIH Biomarker Working Group 2016), but their diagnostic accuracy remains limited due to high clinical as well as laboratory demands (Ng and Lam 2010). In the neonatal ICU, IL-6 and CRP have emerged as valid infection parameters (Zemlin et al. 2018). Whereas IL-6 is released during early infection within the onset of bacteremia and subsequently declines after 24 hours (Buck et al. 1994, Kuster et al. 1998, Zemlin et al. 2018), CRP is considered as late diagnostic biomarker, since it is delayed by 6 to 8 hours after onset of clinical symptoms (Ng and Lam 2010). In the neonatal setting, the early cytokine production is influenced by the initial contact to the unsterile environment as well as by the metabolic state of the neonate (Levy 2007). Consequently, fetal IL-6 has been found to rise during labor-related events independent from the presence of chorioamnionitis (Chan et al. 2013, Jokic et al. 2000, Malamitsi-Puchner et al. 2005). However, TLR signaling pathways for IL-6 production and subsequent CRP synthesis are generally diminished in neonates due to their unadapted immune system (Wynn et al. 2009). Despite well-developed sensor functions of toll-like receptors in newborns and their adequate upregulation during bacterial sepsis, TLR-dependent expression of innate immune effector molecules is remarkable reduced (Kollmann et al. 2012). Thus, the current thesis identifies the supportive role of

non-canonical inflammasome signaling in early and late sepsis prediction. In order to correlate the alarmin levels with IL-6 and CRP, the study cohort of infected newborns was stratified according to IL-6 and CRP values below or above the cut-off concentrations of 100 pg/ml and 10 mg/l, respectively. The non-infected cohort was characterized by infection parameter levels below the cut-off values and served as a control group.

**Galectin-1:** The current analysis revealed no significant association between the infection marker IL-6 and the studied alarmin in the blood circulation of septic newborns (**Fig. 13 A**, page 51). Compared with the non-infectious control group, the infected groups with IL-6 values below and above 100 pg/ml revealed no significant difference in Gal-1 secretion (**Fig. 13 D**, page 51). There was also no association to the acute-phase reactant CRP (**Fig. 14 C**, page 53) and no significant difference in the comparison of septic and non-septic neonates, which were classified according to CRP values below or above the cut-off of 10 mg/l (**Fig. 14 D**, page 53). Lakshmanan and Porter (2007) investigated the role of the non-canonical inflammasome in human THP1 monocytic cell lines. By silencing caspase-4, the cells secreted significant impaired levels of IL-8, IL-1ß, macrophage-inflammatory protein-1ß (CCL4), as well as macrophage-inflammatory protein-3α (CCL20) (Lakshmanan and Porter 2007). It was shown that caspase-4 interacts with TNFR-associated factor 6 (TRAF6) to mediate the NFκB-dependent transcriptional upregulation of their messenger RNAs (mRNAs) in response to LPS (Lakshmanan and Porter 2007). Consistent with these findings, Viganò et al. (2015) observed the diminished release of IL-6 in caspase-4 deficient mice in response to LPS and concluded that the non-canonical inflammasome may also contribute to NF-κB-mediated gene expression of IL-6. These results imply the simultaneous requirements of caspase-4 for Gal-1 as well as for IL-6 secretion. This finding led to the following assumptions: 1) Neither IL-6 nor Gal-1 might be released involving the inflammatory caspase, since caspase-4 is considered to be downregulated in neonates as previously described, and 2) caspase-4 effector functions are rather shifted to support the formation of pro-inflammatory responses instead of contributing to Gal-1 release during neonatal sepsis. However, since the alarmin showed no correlation to IL-6 as well as CRP, it may not support early and late sepsis diagnosis. As such, the role of Gal-1 as DAMP and prognostic sepsis marker needs to be further scrutinized.

**Progranulin:** The comparison of PGRN levels in infected neonates with the non-infected group in the context of IL-6 levels below or above the cut-off value (100 pg/ml) showed significant difference (**Fig. 13 E**, page 51). Compared to the non-septic control group, PGRN values were substantially elevated in infected newborns with IL-6 concentrations over 100 pg/ml (**Fig. 13 E**, page 51). In contrast, the difference to septic neonates exhibiting IL-6 levels under the cut-off was not significant (**Fig. 13 E**, page 51). Moreover, progranulin did not induce an acute-phase response demonstrated in the absent effect on elevated CRP secretion in infected newborns compared to the control group (**Fig. 14 E**, page 53). The convincing role of PGRN in neonatal sepsis has been intensely studied before. Yang et al. (2020) compared the secretion of progranulin and conventional inflammatory markers in neonates with early-onset sepsis. The group found elevated levels of PGRN, IL-6 and CRP, as well as IL-17a, IL-23, IL-33 and procalcitonin (PCT) in septic newborns compared to non-septic neonates with significant predictive value for EOS (Yang et al. 2020). Instead of IL-6 and CRP, they identified PGRN as an independent predictor for early-onset sepsis (Yang et al. 2020). According to the study by Rao et al. (2020), an increased release of PGRN highly corresponds to EOS. The alarmin was even found more adequate than CRP and PCT to distinguish between infected and non-infected neonates within two days after birth (Rao et al. 2020). However, unlike caspase-4, which enhances the NFκB-mediated gene transcription of IL-6, caspase-5 is not involved in IL-6 production (Viganò et al. 2015). Nevertheless, the synergistic secretion of PGRN and IL-6 in the current study reveals their similar responsiveness during an early inflammatory state. Since PGRN alone was not able to distinguish between infected and non-infected infants (**Fig. 11 B**, page 46), its diagnostic value may be increased, when measured in combination with IL-6 (**Fig. 13 B & E**, page 51). The supportive role of PGRN may become evident in case of severe sepsis or sepsis progression, as PGRN levels were significantly elevated in infected neonates who exhibited IL-6 values above the cut-off (**Fig. 13 E**, page 51). Apart from that, considering the rapid decline of IL-6 during sepsis progression, the alarmin detection during sepsis may be beneficial due to its stable characteristics. As such, reverse transcription-PCR analysis of progranulin mRNA in wound tissues of mice detected transcriptional upregulation of the PGRN gene up to 10 days after injury (He et al. 2003). Compared to Rao et al. (2020), Yang et al. (2020) demonstrated a time-dependent increase of PGRN secretion in septic newborns within

the first three days of life. In other words, the alarmin emerges as novel dynamic as well as static infection parameter. Nevertheless, in the current study PGRN was not observed to be associated with late infection, since there was no association to CRP release (**Fig. 14 B**, page 53).

**Resistin:** The resistin secretion of septic newborns showed no correlation to the release of the infection parameters IL-6 (**Fig. 13 C**, page 51) and CRP (**Fig. 14 C**, page 53). Alarmin concentrations in infected newborns with IL-6 levels under 100 pg/ml were significantly increased compared to the neonates of the non-infected control group (**Fig. 13 F**, page 51). By observing the secretion patterns of resistin in the septic infants, who exhibited CRP values below or above the cut-off threshold, there was no significant difference compared to the resistin release in non-infected neonates (**Fig. 14 F**, page 53). Different from these results, several studies reported the association of resistin with inflammatory biomarkers. Aliefendioglu et al. (2014) and Gokmen et al. (2013) investigated the alarmin release in septic newborns who were born premature, and found significant correlation with IL-6, CRP as well as PCT levels. Similar results were demonstrated in term neonates by Cekmez et al. (2011) and Ozdemir and Elgormus (2017). Remarkably, there was no consistent conclusion about the diagnostic value of resistin compared to the infection parameters. Aliefendioglu et al. (2014) as well as Ozdemir and Elgormus (2017) deduced that the alarmin has only limited value in diagnosis and follow-up of neonatal sepsis. On the contrary, Cekmez et al. (2011) and Gokmen et al. (2013) found resistin at least equivalent if not superior to CRP, PCT and IL-6 in the diagnosis of neonatal sepsis. In adult sepsis, serum resistin values were found to be associated to inflammatory markers including CRP, leukocytes, PCT and cytokines, such as IL-6, IL-8, as well as TNF-α (Koch et al. 2009, Sundén-Cullberg et al. 2007). Comparing the expression profile of resistin with other cytokines, the alarmin release seemed to be delayed, but persistently elevated during sepsis (Sundén-Cullberg et al. 2007). Supported by experiments, which demonstrate the resistin-induced production of pro-inflammatory cytokines, such as IL-6, resistin is suggested to be an important promotor of inflammation (Bokarewa et al. 2005, Sundén-Cullberg et al. 2007). This may be one of the reasons, why Sundén-Cullberg et al. (2007) and Koch et al. (2009) evaluated resistin as an acute-phase protein. The delayed but prolonged elevation of serum resistin levels may also be present during neonatal sepsis. In this study, the alarmin levels seem to rise, while IL-6 is already in

depletion process and under the cut-off limit (**Fig. 13 F**, page 51)**.** These results imply that resistin may predict ongoing sepsis in newborns, when the infection marker IL-6 decreases and loses its diagnostic accuracy. On the contrary, there was no substantial difference in resistin secretion of septic infants with IL-6 values above 100 pg/ml compared to the non-septic neonates (**Fig. 13 F**, page 51). As such, resistin seems to be inaccurate to indicate neonatal sepsis onset, which is distinguished by early elevated IL-6 concentrations (**Fig. 13 F**, page 51). However, by correlating CRP with resistin, resistin failed to predict late stages of sepsis (**Fig. 14 C**, page 53). Still, the alarmin levels were slightly increasing dependent on CRP below or above 10 mg/l (**Fig. 14 F**, page 53). Therefore, resistin may fill the time gap between IL-6- and CRP-dependent, that is early and late sepsis diagnosis.

To sum up, the analysis of alarmin levels compared to the infection parameters IL-6 and CRP in septic and non-septic neonates mainly revealed an association to the early infection marker IL-6. Whereas PGRN was identified to assist the cytokine in severe sepsis and sepsis progression, resistin secretion seemed to bypass the diagnostic gap between IL-6 and CRP release during infection. Gal-1 showed no association with the infection parameters.

## 5.3 Lipoproteins of *S. aureus* induce PGRN release without sufficient TLR2 activation

Gram-negative bacteria have been identified to activate the non-canonical inflammasome via the cytosolic delivery of LPS (Shi et al. 2014). Consequently, stimulation of the target cell is associated with pyroptosis and the unconventional release of proteins, such as S100A8 and HMGB1, which are known to serve as alarmins (Andersson et al. 2018, Donato et al. 2013, Lorey et al. 2017). Our laboratory clarified the unconventional secretion of progranulin upon inflammasome activation. Besides its contribution to embryogenesis, wound healing as well as many diseases including cancer and neurodegenerative diseases, the glycoprotein is also considered a novel alarmin through its participation in acute and chronic inflammatory processes (Bateman et al. 2018). However, little is known about the secretory pathway leading to PGRN release. The current thesis aims to investigate this mechanism in response to Gram-positive infection with *S. aureus*. Next to GBS, these bacteria are potent sources for neonatal

sepsis and increase the risk of aggressive treatment through their Methicillin-resistant characteristics (Nelson and Gallagher 2012). Research in the field of *S. aureus* triggering PGRN release may help to establish the basis for new therapy options.

It has been demonstrated that the inflammatory response towards *S. aureus* infection is initiated by staphylococcal surface lipoproteins, which are recognized via TLR2 heterodimers on macrophages (Aliprantis et al. 1999, Schmaler et al. 2009, Takeuchi et al. 2001, Takeuchi et al. 2002). Furthermore, bacterial Lpps seem to be the sole ligand responsible for TLR2 signaling (Hashimoto et al. 2006b). However, cell damage and the release of alarmins in response to pathogenic microbes may further aggravate the secretion of inflammatory cytokines and harbor the risk of uncontrolled systemic infection (Schmaler et al. 2010). As such, our laboratory investigated the role of bacterial lipoproteins in PGRN secretion through the creation of a mutant *S. aureus* strain that lacks surface lipoproteins. We interfered with the process of Lpp maturation by deactivating the Lgt gene, which expresses the phosphatidylglycerol-prolipoprotein diacylglyceryl transferase. The enzyme catalyzes the first step of lipoprotein anchorage on the bacterial membrane (Sankaran and Wu 1994).

In the most bacterial genomes, Lgt is represented by a single gene (Hutchings et al. 2009). Lgt gene manipulation was mediated via CRISPR RNA-guided cytidine deaminase that generated premature stop codons through C → T conversions (**Fig. 15**, page 55 and **Fig. 16**, page 56). The technology of base editing established by Gu et al. (2018) is reported to be more efficient and accurate than traditional genome editing systems by avoiding DNA double-strand breaks (Komor et al. 2016). The lipoprotein-deficient *S. aureus* was formerly constructed by Stoll et al. (2005), who investigated lipoprotein expression, growth behavior and immune activating capacity in Lgt mutant *S. aureus* strain SA113. The group disrupted the Lgt gene via allelic replacement based on a shuttle vector that exchanged the Lgt gene with an erythromycin resistance cassette (Stoll et al. 2005). Consistent with this study, they found the Lgt gene embedded between genes encoding the HPr kinase and an O-acetyltransferase-like protein (Stoll et al. 2005). They further highlighted the conserved gene organization throughout different staphylococci strains and suggested the co-transcription of the three genes (Stoll et al. 2005). Nevertheless, they could not identify their role in lipoprotein modification (Stoll et al. 2005).

In the current study, the absence of surface lipoproteins in ΔLgt *S. aureus* through CRISPR/Cas9 mediated Lgt gene inactivation was confirmed by the lack of TLR2-mediated IL-8 release (**Fig. 17**, page 56). The testing of the successful gene manipulation is based on the research of Kang et al. (2011), who previously investigated the lipoprotein-triggered and TLR2-dependent IL-8 secretion in the context of phagocytosis-coupled cytokine production. Different from Kang et al. (2011), experiments were performed on HEK cells that naturally lack the lipoprotein binding receptor TLR2. However, TLR2 expression was induced by TLR2 plasmid transfection for specific Lpp-TLR2 interaction.

Due to the Lgt gene interruption, the bacteria lost their ability to induce PGRN secretion in macrophages suggesting the lipoprotein-dependent release of the alarmin (**Fig. 19, 20**, page 58). In order to rule out multi-receptor stimulation of numerous immune stimulating PAMPs beside Lpp of *S. aureus*, we examined whether direct TLR2 activity itself was able to initiate alarmin release. In contrast, the surface stimulation with synthetic lipoproteins abrogated the secretion of progranulin, while it revealed robust release of TNF-α (**Fig. 21, 22**, page 59). Despite TLR2 activation in response to Lpp ligation, the TLR2 involvement alone does not appear to be sufficient for PGRN release (**Fig. 20**, page 58). Whether PGRN secretion requires supplementary stimulation or is released independently of TLR2 needs to be further scrutinized. In conclusion, these results point to the pivotal role of Lpp in alarmin release and challenge the involvement of the TLR2 receptor for PGRN secretion. We rather hypothesize that bacterial lipoproteins may enter the cytosol for non-canonical inflammasome activation similar to LPS and subsequently induce caspase-5-dependent secretion of PGRN (Duduskar 2020). Whereas Gram-negative bacteria have been reported to secrete outer membrane vesicles (OMVs) as vehicles that smuggle LPS into the host cell cytosol for murine caspase-11 activation (Vanaja et al. 2016), little is known about how lipoproteins of Gram-positive bacteria enter the cytosol for non-canonical inflammasome activation. Various proteomic analysis studies revealed that Gram-positive bacteria including *S. aureus* are able to release heterogenous lipoprotein-rich membrane vesicles (MVs) (Nagakubo et al. 2019). They have been demonstrated to promote immune modulation through the delivery of intracellular virulence factors (Askarian et al. 2018b, Gurung et al. 2011). Further research needs to be done to answer the question whether these vesicles are also able to infiltrate the host cell with lipoproteins.

## 5.4 Limitations and future perspectives

The current work faces several limitations in answering the question about non-canonical inflammasome activation and alarmin release in newborns with sepsis. First, no healthy control group was included in this study. The venipuncture of healthy newborns, who did not require interventions, was not considered ethical. Instead, the alarmin levels were determined in the blood samples of hospitalized neonates, who already exhibit a primary disease and the subliminal activation of the immune system. As such, it remains unclear if the release of the alarmins Gal-1, PGRN and resistin was triggered by other immunodeficiencies than sepsis. Furthermore, we did not describe any exclusion criteria for the study population, and potential influencing factors of alarmin release remained unknown. For example, Rao et al. (2020) and Yang et al. (2020) did not include patients with congenital malformations, genetic metabolic diseases, severe ischemic hypoxic encephalopathy, severe trauma, hemolytic anemia, confirmed intrauterine viral infection or treatment with antibiotics, hormones and immunomodulators for the detection of PGRN. Also, Cekmez et al. (2011), Gokmen et al. (2013) as well as Ozdemir and Elgormus (2017) excluded patients with antibiotic therapy at admission and hospitalization after the first four days of life for the detection of resistin. Moreover, infants born by mothers with chorioamnionitis, infection, pregestational or gestational diabetes mellitus, parathyroid, bone, renal, and gastrointestinal disorders were not considered (Cekmez et al. 2011, Gokmen et al. 2013, Ozdemir and Elgormus 2017). Apart from that, it must be mentioned that the neonatal blood samples were from capillary as well as venous origin, which might differ in alarmin concentrations. Furthermore, the samples have already been used for previous study purposes. Repeated thawing processes of the samples may have contributed to altered alarmin levels through the denaturation and degradation of proteins.

The second part of this thesis refers to the question how PGRN is released in response to gram-positive infection. Experiments were performed with heat-killed *S. aureus*, since they have been demonstrated to activate THP1 cells for the production of proinflammatory cytokines (Kang et al. 2011). In order to simulate physiological conditions, infections with live bacteria may be helpful for studying PGRN secretion. Additionally, it would be of interest to analyze the PGRN release in TLR2-deficient THP1 cells after ∆Lgt *S. aureus* infection to rule out TLR2 involvement for PGRN secretion.

Nevertheless, this is the first study examining the role of non-canonical inflammasome signaling in neonates. It provides the basis for further research about neonatal sepsis diagnosis, despite limited knowledge in the field of inflammasomes, alarmins and sepsis. One of the long-standing issues is the definition of neonatal sepsis itself. Recently, the Pediatric Sepsis Definition Taskforce of the Society of Critical Care Medicine published a systematic review about risk factors, clinical criteria and disease severity scores to detect infected children who are at high risk of sepsis and who progress to sepsis exacerbation, such as multiple organ failure and death (Menon et al. 2020, Menon et al. 2022). The taskforce analyzed randomized trials and cohort studies published between January 2004 and November 2020 (Menon et al. 2022). Despite the exclusion of preterm newborns, this represents a crucial step towards the implementation of revised criteria of sepsis in children (Menon et al. 2020, Menon et al. 2022).

Besides the definition of neonatal sepsis, the current state of the art in neonatal sepsis diagnosis includes cytokines like IL-6 and IL-8, as well as acute-phase proteins, such as CRP or the prohormone PCT (Polic et al. 2017). Considering the limitations of this study mentioned above, further experiments are required to consolidate the role of the alarmins Gal-1, PGRN and resistin in sepsis prediction. It may be of interest to investigate their kinetic profile to draw conclusions from the regulation of the non-canonical inflammasome involving caspase-4 and caspase-5. Furthermore, longer observation periods are required to investigate the alarmin secretion patterns during late infection. Future studies aiming to uncover the expression patterns of the inflammatory caspases for the release of the alarmins during neonatal sepsis will provide new perspectives in the understanding of sepsis. Moreover, they open the door for new diagnostic possibilities.

Finally, the current thesis sheds some light on the induction of infection by Gram-positive bacteria through the activation of the non-canonical inflammasome. We demonstrated that the release of PGRN is triggered by *S. aureus*. Further research is required to uncover receptor and adaptor molecules that initiate caspase-5, since TLR2 signaling alone was not sufficient for PGRN secretion. The knowledge about the cascades leading to alarmin release involving the non-canonical inflammasome is not only of diagnostic importance, but also has therapeutic relevance in the context of sepsis.

# 6 Conclusion

The current study illustrates the importance of non-canonical inflammasome signaling in newborns and provides new insights in terms of neonatal sepsis diagnosis. The caspase-dependent alarmins Gal-1, PGRN and resistin are used as surrogate parameters to predict non-canonical inflammasome activation and sepsis activity. Whereas the release of Gal-1 shows no association to infection in newborns and no correlation to the established early and late infection markers IL-6 and CRP, respectively, PGRN and resistin emerge as potential sepsis markers in newborns considering their age-dependent secretion. We report that PGRN secretion in full-term newborns has a superior potential in the diagnosis of sepsis compared to the PGRN release in preterm neonates. The study also emphasizes that the alarmin serves as an early biomarker for neonatal sepsis since PGRN correlates to IL-6. On the contrary, resistin differentiates sepsis in infants who are born preterm. Resistin rather bridges the gap between early and late infection marker, as it does not directly correlate to the infection markers IL-6 and CRP. Altogether, the analysis of alarmin levels in newborns reveals a quite oppositional regulation of caspase-4 and caspase-5 during neonatal sepsis. In this regard, the study highlights the relevance of thorough patient stratification in order to facilitate appropriate diagnostic and therapeutic care in case of neonatal sepsis. Besides the diagnostic potential of the novel alarmins Gal-1, PGRN and resistin, the study further provides mechanistic insight into how the non-canonical inflammasome is involved in the release of PGRN after infection with gram-positive bacteria. However, further research is needed to identify the receptor and adaptor molecules, which are responsible for caspase-5 initiation.

Concluding, the study contributes to unraveling the activation steps and effector functions of the non-canonical inflammasome pathway during neonatal sepsis. It lays the foundation for research of further biomarkers, which are linked to the non-canonical inflammasome. Together with the obtained knowledge about the pathogenicity of Gram-positive bacterial infection, the current work provides new perspectives on targeted diagnosis and treatment of sepsis.

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# 8 Appendix

## List of Figures

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# Supplementary material

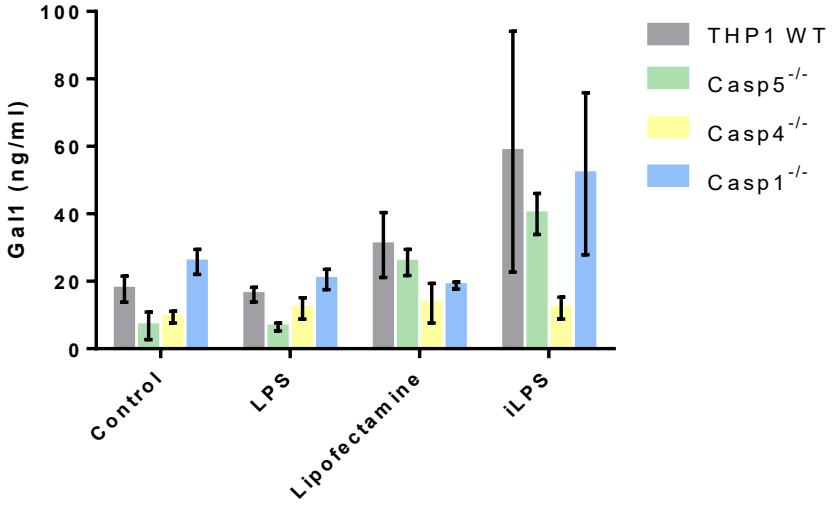


**Figure 23: Caspase-4-dependent release of Gal-1.** Gal-1 release measured in THP1 wild type cells and THP1 knock out cell lines deficient in CASP1, CASP4 and CASP5 expression. Gal-1 levels were detected after cell stimulation with extracellular LPS, lipofectamine and intracellular LPS (iLPS).

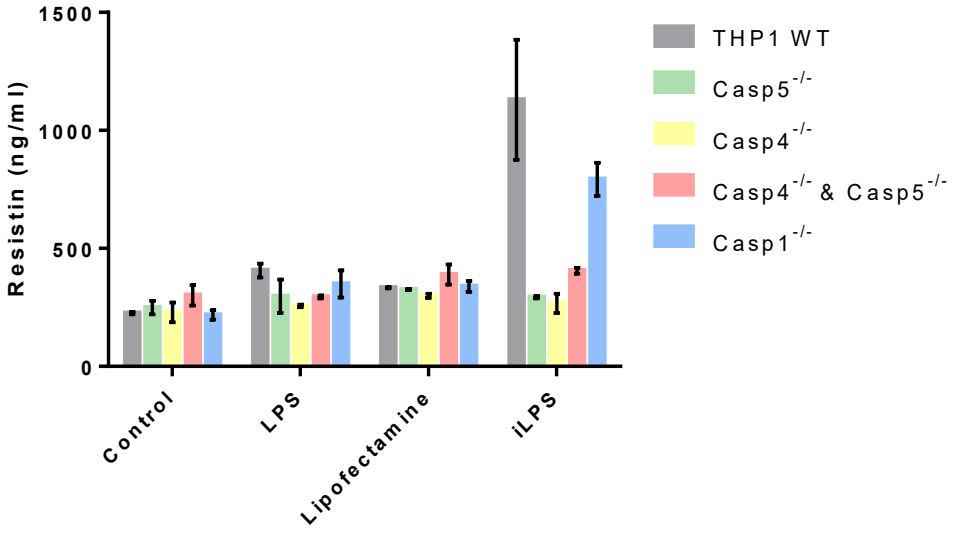


**Figure 24: Caspase-4- and caspase-5-dependent release of resistin.** Resistin release measured in THP1 wild type cells and THP1 knock out cell lines deficient in CASP1, CASP4 and CASP5 expression. Resistin levels were detected after stimulation with extracellular LPS, lipofectamine and intracellular LPS (iLPS).

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# Ehrenwörtliche Erklärung

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

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Prof. Dr. Hans Proquitté, Prof. Dr. Ignacio Rubio, Dr. Sachin Deshmukh,
Shivalee Duduskar, Mohamed Ghait, Michael Rooney.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

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