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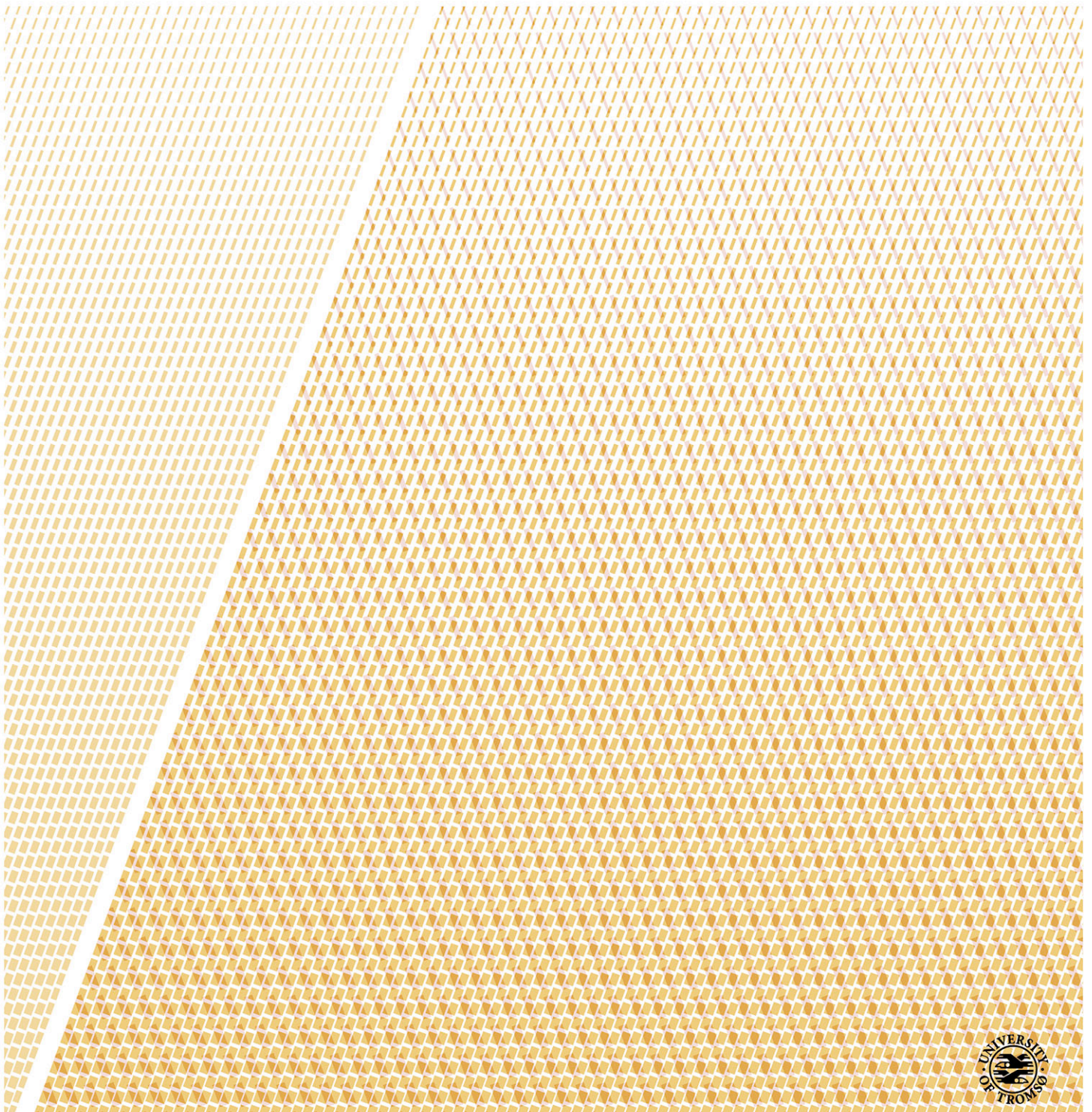
Institute of Clinical Medicine

Antibiotic Therapy for Neonatal Sepsis

Studies on epidemiology, gentamicin safety, and early adverse effects of antibiotics

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Acknowledgements

List of Papers

Paper 1

Fjalstad JW, Stensvold HJ, Bergseng H, Simonsen GS, Salvesen B, Ronnestad AE, Klingenberg C. Early-onset Sepsis and Antibiotic Exposure in Term Infants: A Nationwide Population-based Study in Norway. Pediatr Infect Dis J 2016; **35**: 1-6.[1]

Paper 2

Fjalstad JW, Laukli W, van den Anker JN, Klingenberg C. High-dose gentamicin in newborn infants: is it safe? Eur J Pediatr 2013; **173**: 489-95.[2]

Paper 3

Esaiassen E, Fjalstad JW, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis. J Antimicrob Chemother 2017; **72**: 1858-70.[3]

Paper 4

Fjalstad JW, Esaiassen E, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: a systematic review. J Antimicrob Chemoter 2017 Nov 22 [Epub ahead of print].[4]

Abbreviations

AAP; American Academy of Pediatrics (United States)

AMP; antimicrobial peptides

AUC; area under the plasma drug concentration-time curve

BW; birth weight

CDC; Centers for Disease Control and Prevention

CI; confidence interval

CMV; cytomegalovirus

CoNS; coagulase-negative Staphylococci

CRP; C-reactive protein

EBM; evidence-based medicine

ELBW; extremely low birth weight (< 1000 g)

EOS; early-onset sepsis

ESBL; extended-spectrum beta-lactamase

GA; gestational age

GBS; group B Streptococci

GRADE; Grading of Recommendations Assessment, Development, and Evaluation

IAP; intrapartum antibiotic prophylaxis

IFI; invasive fungal infection

IQR; interquartile range

LB; live-born

LOS; late-onset sepsis

MDR; multi-drug resistant

MIC; minimum inhibitory concentration

MRSA; methicillin-resistant *Staphylococcus aureus*

NEC; necrotizing enterocolitis

NICE; National Institute for Health and Care Excellence (United Kingdom)

NICU; neonatal intensive care unit

NNN; Norwegian Neonatal Network

NNT; number needed to treat

NPV; negative predictive value

OAE; otoacoustic emissions

OR; odds ratio

PCT; procalcitonin

PMA; postmenstrual age

PNA; postnatal age

PPC; peak plasma concentration

PPV; positive predictive value

PRISMA; Preferred Reporting Items for Systematic Reviews and Meta-Analysis

PROM; prolonged rupture of membranes (> 18 hours)

QoE; quality of evidence

RCT; randomized controlled trial

SD; standard deviation

TLR; Toll like receptor

TPC; trough plasma concentration

V_D; volume of distribution

VLBW; very low birth weight (< 1500 g)

Abstract

Background and Objectives: Sepsis is a prominent cause of neonatal mortality and morbidity yet can be very hard to diagnose. The disease is rare, the symptoms are unspecific, the laboratory tests are difficult to interpret, and blood cultures, which can potentially confirm an infection, may take 36-48 hours before they demonstrate any growth. Therefore, antibiotics are the most commonly used medications in neonatal medicine. While antibiotics can be life-saving, they can also have potentially adverse effects. Several early adverse outcomes have been reported from neonatal antibiotic treatment; among these necrotizing enterocolitis (NEC), invasive fungal infection (IFI), death, changes in the gut microbiota, and development of antibiotic resistance. In addition, gentamicin, a commonly used antibiotic in the neonatal period, has ototoxic and nephrotoxic potential, in particular if trough plasma concentrations (TPCs) are elevated or the infant receives prolonged therapy.

The overall aim of this thesis was to investigate different aspects of antibiotic therapy for neonatal sepsis in order to obtain new knowledge that could improve and optimise care. The first aim was to investigate the epidemiology of early onset sepsis (EOS) and exposure to systemic antibiotics during the first week of life in an unselected national cohort of live-born term infants. Secondly, we wished to evaluate a simplified high-dose extended-interval gentamicin dosing regimen with focus on pharmacokinetic safety, potential ototoxicity, and the number of prescription errors. Finally, we aimed to identify, critically appraise, and synthesize evidence from studies reporting different categories of antibiotic exposure in neonates and their subsequent impact on NEC, IFI, death, gut microbiota, and/or antibiotic resistance development.

Material and Methods: The epidemiology of EOS and systemic antibiotic exposure in the first week of life was studied in a nationwide population-based study from the Norwegian Neonatal Network. During the 3-year study period (2009-2011), 20 out of 21 Norwegian neonatal units prospectively collected data. A high-dose extended-interval gentamicin regimen was studied in the neonatal unit in Tromsø from 2004-2012. The main outcome measures were TPCs, ototoxicity, and prescription errors. Early adverse effects of antibiotic therapy were studied in a systematic review. We included observational studies and randomized controlled trials (RCTs) that provided data on different categories of antibiotic therapy and either the risk of NEC, IFI, death, antibiotic resistance development, or changes in the gut microbiota. Risks of bias were assessed according to a modified version of the Cochrane Handbook. When appropriate, data were meta-analysed using the random effect model or a semi-quantitative vote-counting method.

Results: There were 0.54 cases of culture-confirmed EOS per 1000 live-born term infants, and the majority of these cases were caused by Gram-positive bacteria, most commonly group B streptococci. Intravenous antibiotics were administered to 2.3% of all live-born term infants in Norway, and 54% of these infants were not diagnosed with an infection. Empiric treatment consisted of an aminoglycoside and either penicillin or ampicillin in 95% of cases. The EOS-attributable mortality rate was 1%.

In the neonatal unit in Tromsø, gentamicin TPCs were above the threshold of 2 mg/L in 6% of cases, mainly among term infants with renal impairment. Thirty-eight patients failed the neonatal hearing screening, but only five patients had permanent hearing loss. One of these patients had a gentamicin TPC > 2 mg/L. Gentamicin was prescribed correctly in 93% of cases.

The majority of the included studies in our systematic reviews had poor to moderate methodological quality. Prolonged antibiotic exposure was significantly associated with NEC and/or death in preterm infants. Third-generation cephalosporin treatment was associated with a significantly higher risk of IFI than narrow-spectrum antibiotic treatment. Prolonged antibiotic treatment was associated with reduced gut microbial diversity, while antibiotic treatment in general was associated with reduced colonization rates of commensal anaerobic bacteria. All categories of antibiotic exposure were associated with an increased risk of antibiotic resistance development, particularly multi-drug resistant Gram-negative bacteria. Meta-analyses were limited by few RCTs and significant heterogeneity between studies.

Main Conclusions: The incidence of culture-confirmed EOS in Norway was in line with previous international reports, and the mortality was very low. A large proportion of infants were treated with antibiotics without an infection. The extended-interval high-dose gentamicin regimen studied in this thesis seems safe with low numbers of elevated TPCs, few prescription errors, and no evidence for ototoxicity. Prolonged antibiotic exposure in uninfected preterm infants is associated with an increased risk of NEC and/or death, while broad-spectrum antibiotics are associated with an increased risk of IFI. Antibiotic treatment is associated with antibiotic resistance development in neonates and appears to induce potentially disease-promoting changes in the gut microbiota. Measures should be taken to spare neonates of unnecessary antibiotic treatment.

1   Introduction

1.1   Preface

The overarching theme of this thesis are the challenges concerning treatment of neonatal sepsis with antibiotics, and the potentially adverse effects that antibiotic treatment may have in newborn infants. Neonatal sepsis is an important cause of morbidity and mortality world-wide, and antibiotic treatment can be life-saving. Confirmed infections are, however, relatively rare compared to the number of suspected infections, and it is difficult to determine which neonates are truly infected at disease onset. Consequentially, many uninfected neonates are exposed to antibiotics that they, in retrospect, did not need.

In Paper 1, we examined the epidemiology of neonatal sepsis and antibiotic treatment in the first week of life of nearly all term-born neonates in Norway from 2009-2011. In Paper 2, we studied drug concentrations and the rate of ototoxicity in newborn infants who were treated with gentamicin, one of the most commonly used antibiotics in neonatal sepsis treatment. In Paper 3 and 4, we systematically reviewed the literature on early clinical and microbiological adverse effects from antibiotic treatment in the first month of life. In the following introduction, I will present the challenges in correctly diagnosing neonatal sepsis and important considerations regarding antibiotic therapy of this potentially life-threatening condition.

1.2   Host Immunity in the Neonatal Period

The neonatal period, which are the first 28 days of life for term infants and up to 44 weeks postmenstrual age (PMA) for preterm infants, is a particularly vulnerable period in life and neonates are at risk of acquiring infections. The newborn infant is suddenly exposed to a plethora of microorganisms during birth, after a relatively sterile existence in utero.[5, 6] Following a normal, vaginal birth, microorganisms from the maternal vaginal and gastrointestinal tracts, breast feeding, parents' skin, and (if hospitalized) the hospital environment begin to colonize the neonate's gastrointestinal tract, skin, and mucosal surfaces.[7] This eventually develops into a diverse and stable microbiota that largely exists in symbiosis with its host.[8] However, many bacteria are able to cause disease if they enter the blood stream, lungs, central nervous system, urinary tract, or other sterile body parts. Our immune systems monitor and regulate the interactions between microorganism and host and largely enable a peaceful coexistence.[9]

The human immune system can be divided into the innate and adaptive immune systems.[9] The innate immune system is non-specific and serves as a first line of defence with immediate responses against microbial pathogens such as virus, bacteria, and fungi. The adaptive immune system, on the other hand, takes more time to activate, but is more specific and potent. It grants immunity against pathogens with a rapid response upon re-infection. While these two parts of the immune system are discussed separately, it is important to emphasize that they are heavily interlinked and depend on each other for their immune responses.

The innate immune system can largely be divided into two parts. The first part is the surface barrier, which is formed by epithelial cells on skin and mucosal surfaces.[10] The skin protects the host from invading microbes by epithelial cells bound by tight junctions and the stratum corneum layer. This layer is very thin in preterm infants. Additionally, the epidermidis has important immunological functions, such as detecting microbes through pattern recognising receptors and killing bacteria through antimicrobial peptides (AMPs). The mucosal surfaces are protected by epithelial cells linked with tight junctions, but also contain a mucus layer that is secreted by the epithelial cells.[11] Mucus forms a relatively impenetrable gel, in addition to containing bactericidal AMPs. The second part of the innate immune system consists of cells (e.g. granulocytes, monocytes, macrophages, natural killer cells) and the complement system.[9, 12] Neutrophilic granulocytes and macrophages are phagocytes that engulf and destroy microorganisms. Additionally, macrophages and dendritic cells, which are both differentiated from monocytes, are the foremost antigen presenting cells, which is crucial in the activation of an adaptive immune response. The complement system is composed of several plasma and cell surface proteins that are activated through three different pathways; the classical, the alternative, and the lectin pathways.[9] When activated, they promote inflammation, attack the plasma membrane of pathogens, and enhance the abilities of phagocytic cells and antibodies through opsonization.

The adaptive immune response is carried out by lymphocytes of two classes; B cells and T cells.[9, 13] B cells secrete specific antibodies, glycoproteins of the immunoglobin (Ig) family that neutralize pathogens, aid phagocytosis, and activate the complement system. T cells are divided into several subtypes; prominently the cytotoxic T cells, or $CD8^+$ T cells, and the T helper (T_H) cells, or $CD4^+$ T cells. The cytotoxic T cells destroy virus-infected cells and tumour cells, while the T_H cells assist cytotoxic T cells, B cells, and macrophages. Some B and T cells are differentiated into memory cells that enable a rapid response upon reinfection with a previously

encountered pathogen. Additionally, some T cells provide regulatory functions (Tregs) that maintain immunological tolerance.

Toll like receptors (TLRs) are pattern recognising receptors that are important for both the innate and adaptive immune systems to recognize pathogens and separate them from host cells.[12] They are surface receptors expressed on the membranes of leukocytes, particularly dendritic cells and macrophages, and they recognize molecules that are broadly shared by microbes, but not by host molecules. For example, TLR2 recognizes lipoteichoic acid from Gram-positive bacteria and TLR4 recognizes lipopolysaccharides from the outer membranes of Gram-negative bacteria. Upon binding to a pathogen-associated molecular pattern, TLRs recruit adapter proteins that ultimately lead to upregulation or suppression of genes that orchestrate inflammatory responses.

Despite an equal number of TLRs compared to adults, infants have widely different functional responses to TLR stimulation, with lower secretion of pro-inflammatory cytokines, such as IL-6, IFN-γ, and TNF-α, and higher secretion of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10.[13] This increased secretion of anti-inflammatory cytokines and lower secretion of pro-inflammatory cytokines is partially caused by neonates having a skewed T-cell maturation towards T_H2 cells in favour of T_H1 cells.[13] Neonates also have diminished macrophage activation, lower cytotoxic capacity of natural killer cells, and lower levels of complement proteins compared with adults.[12, 14, 15] The severity of these differences in functional response is inversely proportional to gestational age (GA), leaving preterm infants even more exposed to infections than term infants.[16] Preterm infants also have diminished chemotaxis, which is the recruitment of other immune cells, and diminished bactericidal effect from neutrophil granulocytes.[14, 17]

Transplacental transfer of antibiodies (IgG) peaks after 32 weeks' gestation, leaving preterm infants with low levels of circulating IgG.[18] Additionally, the relatively lower rates of breast-feeding in preterm infants compared to term infants may leave them more exposed to infections.[19] Breast milk and colostrum, which is a form of breast milk produced in the first few days after birth, contain beneficial bacteria such as *Bifidobacterium* species and numerous immune factors, including stem cells that help protect the newborn infant. Among these immune factors are IgA, cytokines, AMPs and proteins, for example lactoferrin.[20]

1.3 Neonatal Sepsis

Neonatal sepsis is a clinical manifestation of systemic infection during the first 28 days of life. There is no uniform definition for the disease, and it is varyingly defined by clinical signs, laboratory markers, or isolation of a bacterial pathogen from the blood stream or another sterile site.[21] Many authors and publications only include culture-confirmed sepsis with positive blood cultures and clinical signs of infection as a definite case of neonatal sepsis. However, others include clinical cases not confirmed by a positive blood culture (culture-negative sepsis), which is considered a separate entity causing a large proportion of neonatal sepsis cases.[22-24] Neonatal sepsis is the most common form of severe infection in the neonatal period, and its definition often includes meningitis and pneumonia.[25, 26]

Neonatal sepsis is a major problem world-wide regardless of its definition, and approximately 413 000 neonates died from sepsis in 2015 according to UNICEF.[27] This amounts to 15.3% of the total neonatal deaths world-wide. These deaths are unevenly distributed as the majority of sepsis-related neonatal deaths occur in developing countries.[27, 28] In developed countries, mortality rates from 8-18% have been reported, and mortality is highest among very low birth weight (VLBW) infants (birth weight (BW) < 1500 g).[26, 29-31]

Neonatal sepsis is normally divided into two subtypes, early-onset sepsis (EOS) and late-onset sepsis (LOS). These subtypes require different strategies for treatment and prevention due to different modes of transmission, risk factors, and causative pathogens.[32] EOS is most commonly defined as sepsis with an onset of symptoms in the first 48/72 hours of life, and the neonate is thought to be infected through contaminated amniotic fluid due to bacteria ascending from the birth canal.[32-34] LOS is often defined as sepsis with an onset between 3 and 28 days of life, and is typically nosocomially acquired and closely linked to prematurity and low BW.[29, 35, 36] Determining a precise cut-off in timing of onset between the two subtypes of sepsis is not easy and some authors, particularly those who study EOS caused by group-B Streptococci (GBS), define EOS as having an onset in the first week of life.[37, 38]

1.4 Early-Onset Sepsis

1.4.1 Epidemiology

In developed countries, the incidence of EOS has steadily decreased during the last 30 years to an incidence between 0.5 – 1.0 cases per 1000 live-born (LB) infants.[26, 30, 39-41] The incidence of EOS is inversely correlated to gestational age (GA) and BW, despite the majority of EOS patients having a GA ≥ 30 and BW ≥ 1500 g.[26, 31] EOS generally presents itself with respiratory distress, lethargy, temperature instability, feeding difficulties, and irritability. These symptoms, however, are not specific for EOS, as many uninfected neonates display similar symptoms.[33]

Gram-positive bacteria have been reported to cause between 60-80% of EOS-cases, with Gram-negative bacteria causing the remaining cases.[30, 31, 41] GBS is the most common cause of EOS in industrialised countries, followed by *Escherichia coli*. GBS is reported to cause between 30-58% of EOS cases, with an incidence rate between 0.2-0.5 cases per 1000 LB infants.[26, 30, 37, 38, 41] *E. coli* is reported to cause between 16-38% of EOS cases, with an incidence rate between 0.13-0.28 cases per 1000 LB infants.[26, 30, 31, 41] Other pathogens associated with EOS are *Staphylococcus aureus*, coagulase-negative Staphylococci (CoNS), viridans-group Streptococci, group A Streptococci, and species of *Enterococcus*, *Listeria*, *Bacteriodes*, and *Klebsiella*.[25, 30]

EOS mortality rates have fallen in developed countries, and a single-centre retrospective chart review from a US hospital reported a decrease in sepsis related mortality from 87% in 1928 to 3% in 2003.[40] Antibiotics are likely to be a major reason for the improved survival. Recent studies present EOS-attributable mortality rates between 11-16% when both term and preterm infants are included.[26, 30] Preterm infant have the highest mortality rates, while mortality rates of 2-3% have been reported for term infants.[30, 42] EOS mortality rates vary between causative pathogens, and Gram-negative bacteria reportedly cause higher mortality rates than Gram-positive bacteria.[26, 43] Mortality rates up to 40% have been reported in patients with *E. coli* EOS.[44] Prematurity appears to have a confounding and/or interacting effect on the relationship between the causative pathogen and mortality, as preterm infants are more likely to suffer Gram-negative infections.[30] EOS in VLBW infants is also associated with increased rates of prematurity complications such as bronchopulmonary dysplasia, intraventricular haemorrhage, periventricular leukomalacia, and retinopathy of prematurity.[43, 45]

1.4.2 Risk Factors and Prevention

The most commonly implicated risk factors for EOS are premature birth, prolonged rupture of membranes (PROM; ≥ 18 hours), chorioamnionitis, maternal intrapartum pyrexia (temperature > 38°C), and maternal GBS carriage.[26, 39] A nested case-control study with 350 cases and 1063 controls found that the highest maternal antepartum temperature, the duration of membrane rupture, prematurity, and maternal GBS carrier status were independently correlated with EOS. This study also reported an association between intrapartum antibiotic prophylaxis (IAP) and EOS in univariate analysis, but this effect disappeared when stratifying for treatment indication.[39] Additionally, it is possible that there is some interaction between the risk factors for EOS, as chorioamnionitis can lead to PROM and premature birth.[46]

IAP is preferably commenced at least four hours prior to birth for GBS colonized mothers or mothers with risk factors for having a GBS infected newborn baby. The aim is to prevent transmission of GBS to the infant.[47] IAP is a major cause of the declining EOS rates in developed countries, but there are different opinions on how to identify women that should receive IAP.[48] The British Royal College of Obstetricians and Gynaecologists recommend a risk based screening approach, where they recommend IAP for women with GBS carriage that is incidentally or intentionally detected, GBS bacteriuria, infants with GBS infection after a previous pregnancy, intrapartum pyrexia, known chorioamnionitis, or PROM after 37 weeks' gestation.[49] The American Centers for Disease Control and Prevention (CDC) guidelines, on the other hand, recommend universal rectovaginal screening of all women at 35 to 37 weeks' gestation, and IAP for all GBS-colonized women.[50] Both guidelines recommend benzylpenicillin as the first choice IAP if the mother does not require treatment for suspected infection. The CDC also consider ampicillin as an acceptable alternative to benzylpenicillin.

In Australia, the incidence of GBS EOS dropped from 1.43 per 1000 LB infants in 1993 to 0.25 per 1000 LB infants after implementing universal rectovaginal GBS-screening.[48] After the implementation of risk-based IAP guidelines in the US, GBS EOS incidence rates fell from 1.7 per 1000 LB infants in 1990 to 0.6 per 1000 LB infants in 1998.[51] GBS EOS incidences have fallen to between 0.22 - 0.41 cases per 1000 LB infants in the US after the CDC recommended universal rectovaginal screening in 2002.[26, 30] However, similarly low rates are reported in countries with risk-based approaches to IAP, such as the Netherlands, New Zealand, Sweden, Norway, and the UK. In these countries, GBS EOS rates between 0.19 - 0.49 cases per 1000 LB infants have been reported.[1, 38, 41, 52, 53] There is, however, a concern that opportunities to

administer IAP are missed when using the risk-based approach, and a strict adherence to guidelines is important.[53, 54]

A surveillance study of ten US states found that the percentage of infants exposed to IAP increased from 27% to 32% following the implementation of universal rectovaginal GBS-screening.[55] There are growing concerns that this widespread maternal antibiotic exposure may cause increased rates of *E. coli* infections, as well as leading to increased ampicillin-resistance among *E. coli* strains. US studies on VLBW infants have found unchanged total EOS incidence rates, but increased rates of total LOS and *E. coli* EOS and LOS after formal IAP guidelines were implimented.[43, 56] A potential confounder, however, is that an increasing number of preterm babies are able to survive due to improved health care.[56] IAP has also been linked with increased incidence rates of sepsis caused by ampicillin-resistant *E. coli* strains.[43, 56] Determining the optimal strategy for judicious IAP use is a huge challenge, and an effective GBS vaccine would aid greatly in preventing GBS EOS, as well as reducing antibiotic exposure among neonates.

1.5 Late-Onset Sepsis

1.5.1 Epidemiology

Most LOS cases affect preterm infants, and the total LOS incidence increased after 1990 due to improved survival for this population.[40] More recently, however, incidence rates have fallen in developed countries such as the US and the UK.[41, 57] Among VLBW infants, 15-20% are reported to have culture-confirmed LOS, with an even higher rate of ~35% in extremely low BW (ELBW) infants (BW < 1000 g).[35, 58, 59] There are few studies on LOS that include term born infants, but a recent study from 30 UK NICUs reported 2.2 confirmed LOS cases per 1000 LB infants, regardless of GA.[41] The symptoms and signs are similar to EOS with respiratory distress, pallor/grey skin, lethargy, feeding intolerance, hypoperfusion (capillary refill time > 2 seconds), and temperature instability.[22] The median age of disease onset has been reported between 11-17 days.[60, 61]

Gram-positive bacteria account for 70-83% of LOS cases, while Gram-negative bacteria and fungi cause the remaining cases.[41, 58, 61, 62] CoNS are the most common causative pathogens of LOS and cause between 45-77% of LOS cases.[41, 58, 61, 62] Other reported LOS pathogens are *S. aureus*, *E. coli*, GBS, *Candida albicans*, and species of *Enterococcus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Pseudomonas*, and *Acinetobacter*. Invasive fungal infections (IFIs) are reported to account for

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between 4-12% of LOS cases in VLBW infants, but rates of IFI are declining among neonates, possibly due to the widespread introduction of routine anti-fungal prophylaxis.[58, 59, 61, 63]

LOS is reported to have mortality rates between 12-20% in VLBW infants, and mortality appears to vary between different causative pathogens.[29, 58, 62] Gram-negative infections have an independently higher sepsis-attributable mortality than Gram-positive infections; Gram-negative LOS is reported to have sepsis-attributable mortality rates up to 26% in infants with GA < 32 weeks, while Gram-positive LOS had a sepsis-attributable mortality rate of ~10%.[62] LOS caused by *E. coli* and species of *Pseudomonas*, *Klebsiella*, *Serratia*, and *Candida* are associated with the highest sepsis-attributable mortality rates. CoNS, on the other hand, a group of staphylococci containing species such as *Staphylococcus epidermidis* and *Staphylococcus hominis*, are associated with the lowest sepsis-attributable mortality rates.[59, 64] LOS, and particularly Gram-negative LOS, is also strongly associated with increased rates of prematurity complications such as intraventricular haemorrhage, bronchopulmonary dysplasia, patent ductus arteriosus, NEC, prolonged hospitalization, and prolonged respiratory support.[59, 62] IFIs, most commonly with *Candida* species, are in addition associated with severe complications like endocarditis, meningitis, brain parenchymal infection, and renal abcesses.[65]

1.5.2   Risk Factors and Prevention

The most important risk factors for LOS are prematurity, low BW, and forms of invasive treatment.[35, 62] Indwelling catheters, parenteral nutrition, surgery and mechanical ventilation independently increase the risk of LOS. Prolonged durations of parenteral nutrition, indwelling catheters, and ventilator support are also associated with LOS.[35, 61] Indwelling catheters, such as percutaneous catheters, central venous catheters, and umbilical catheters, provide a passageway past the skin barrier for CoNS and other skin bacteria. These catheters also provide an ideal surface for development of bacterial biofilms, which is one of the most important virulence factors of CoNS as it increases their resilience to antibiotic treatment and host immune responses.[66, 67]

Despite plausible explanations for a cause-effect relationship between invasive treatment and LOS, it is important to note that these treatment variables may be partially confounded by factors that increase the risk of LOS such as prematurity, low BW, and severe disease.[35] Neither EOS nor antibiotic treatment for EOS appear to increase the risk of LOS in general, but prior antibiotic treatment, particularly with broad-spectrum antibiotics like cephalosporins and

carbapenems, increases the risk of fungemia through selection pressure.[60, 68, 69] In addition, IAP appears to increase the incidence rates of *E. coli* LOS in VLBW neonates.[56]

Minimizing the use of catheters and implementing proper hygiene are the primary strategies to prevent LOS. Around 20-35% reductions in LOS rates have been reported after implementing improved catheter care.[36, 70] In a single centre study, something so simple and cheap as adding gloves to a hand hygiene protocol was found to successfully lower the rate of LOS.[71] Probiotics, live microorganisms that provide health benefits to the host, were found to be protective against LOS in a meta-analysis of randomized controlled trials (RCTs) and observational studies.[72] Oral lactoferrin was also found to be protective against LOS in a meta-analysis.[73] A large UK multi-centre RCT (ELFIN study) has recently completed recruitment of 2203 preterm infants below 32 weeks' gestation in order to assess whether enteral lactoferrin supplements reduces the number of late-onset invasive infections. The results are not yet published.[74] Systemic antifungal prophylaxis with fluconazole, and possibly oral nystatin, is effective in preventing IFI in VLBW infants, and is particularly recommended for ELBW infants and VLBW infants who receive broad-spectrum antibiotics.[75, 76]

1.6   Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is a disease characterized by gut inflammation, which typically affects extremely premature (GA < 28 weeks) and VLBW infants with clinical onset in the second or third week of life.[77] It affects approximately 5-7% of VLBW infants and is rare in term born infants.[78-80] The pathogenesis of NEC is multifactorial and not completely understood, but there appears to be an interplay between an immature gut and immune system, unfavourable changes in the gut microbiota, and type of feeding.[81] Important risk factors include prematurity/low BW, prior sepsis, assisted ventilation, and prolonged antibiotic treatment. In a large cohort study of > 5600 VLBW neonates, each additional day of antibiotic treatment was found to increase the risk of NEC.[82] In contrast, probiotics and breast milk have been found to have a protective effect against NEC.[83, 84]

Typical signs of NEC are a distended abdomen, periumbilical erythema, bloody stools, feeding intolerance, and a generally unstable infant. The signs are non-specific, however, and the diagnosis is usually based on radiographic findings such as intramural bowel gas.[77] The severity of NEC can range from mucosal ulceration to transmural necrosis, and NEC is classified according to the modified Bell's staging criteria from stages I to III.[85] Stage I refers to suspected, but

unconfirmed NEC, while stage II is radiographically confirmed NEC requiring medical therapy. This medical therapy includes broad spectrum antibiotics for Gram-positive, Gram-negative, and anaerobic bacteria as well as supportive care.[86] Stage III patients demonstrate clinical signs of bowel necrosis, peritonitis, and septic shock or radiographic findings of gastrointestinal perforation.[85] These patients require surgery in addition to medical therapy. The mortality rate of NEC has been reported between 15-42%, and is highest in infants with a low BW, concurrent sepsis, and/or stage III NEC.[78-80] Those who survive NEC have an increased risk of neurocognitive impairment such as cerebral palsy, blindness, and deafness.[86]

1.7 Diagnostic Challenges in Neonatal Sepsis

Before discussing the diagnostic challenges of neonatal sepsis, it is important to define a few commonly used epidemiological terms. When discussing neonatal sepsis and biomarkers, sensitivity is the proportion of infected neonates with a positive test, while specificity is the proportion of uninfected neonates with a negative test. The positive predictive value (PPV) is the proportion of neonates with a positive test that are truly infected, while the negative predictive value (NPV) is the proportion of neonates with a negative test that are truly uninfected. These predictive values are heavily influenced by prevalence rates, while sensitivity and specificity are not affected by prevalence.

As previously mentioned, symptoms that may cause a suspicion of neonatal sepsis are relatively common and non-specific, while neonatal sepsis is rare.[22] This causes symptoms to have a low PPV for culture-confirmed neonatal sepsis. Additionally, some neonates initially appear asymptomatic despite having an infection.[87] The difficulty in correctly diagnosing neonatal sepsis is further complicated by the lack of sensitive biomarkers in the early stage of the disease and the limitations of blood-cultures in neonates.

1.7.1 Biomarkers

In NICUs, biomarkers such as C-reactive-protein (CRP) and complete blood-counts are very frequently used, while procalcitonin (PCT) is also increasingly used.[88] Other promising biomarkers that are not properly tested clinically are acute-phase proteins such as serum amyloid A and cytokines such as IL-6, IL-10, and TNF-α.[88, 89] In a systematic review of biomarkers for neonatal sepsis, CRP was shown to have relatively decent specificity (0.87-1.00), but variable sensitivity at symptom onset (0.30-0.80).[88] The sensitivity was improved after 24-48

hours, but the PPV (0.77-1.00) and NPV (0.73-0.98) remained variable. It is, however, possible that this high specificity was somewhat overestimated as most of the studies in this review included clinical sepsis, which was partially defined by elevated CRP, as part of their sepsis definition.

PCT rises more rapidly following infection, and had a much higher sensitivity than CRP at symptom onset (0.72-0.79). Therefore, PCT has a moderate NPV (0.88-0.99), and implementation of PCT-guided decision-making has demonstrated a reduction in duration of antibiotic therapy without affecting mortality.[90] In contrast, a study of > 11 000 neonates found no increase in empiric antibiotic prescription rates after reducing the use of CRP and complete blood counts.[91] Additionally a large, prospective before-after study found no difference in outcome whether neonates were evaluated with laboratory tests and physical examination or with physical examination alone.[92]

1.7.2 Detecting Pathogens in Sterile Sites

Neonatal sepsis is confirmed by a combination of clinical symptoms and demonstrable growth of bacteria from a normally sterile site. This usually implies detection of pathogens in blood cultures, but many authors include detection of pathogens in cerebrospinal fluid (CSF) in their definition of neonatal sepsis.[25, 30, 93, 94] Urine cultures are generally not used for neonatal sepsis evaluation.[94] Blood cultures need at least 24-36 hours inoculation before they can demonstrate growth.[95] When samples of ≥ 1 ml are taken, blood cultures are estimated to have a sensitivity approaching 100% for common neonatal pathogens.[96] Despite this, blood cultures have the potential for both type I errors (false positive results) and type II errors (false negative results).

Type I errors can occur due to contamination with bacteria from the patient's skin or health care workers' hands.[97] CoNS are among the most common causes of sepsis in preterm neonates, but they are also a part of the normal skin flora.[25, 61] Because of this, it is difficult to correctly interpret blood cultures with growth of CoNS or other skin bacteria. The Vermont Oxford Network, a non-profit organization of world-wide NICU health care professionals, define CoNS sepsis as a combination of clinical signs of sepsis, a blood culture or CSF sample with growth of CoNS, and antibiotic treatment ≥ 5 days.[98] An alternative definition is two positive blood cultures for CoNS within five days or one positive blood culture with clinical evidence of infection (low white cell count and hypothermia/hyperthermia or hypotension). This definition was tested by expert neonatologists and achieved a sensitivity of 46% and a specificity of 96% in identifying CoNS

sepsis.[97] Some studies on EOS, particularly those that study term infants, classify all CoNS cases as contaminations for the sake of simplicity as CoNS is a rare cause of EOS.[30]

Type II errors can occur due to too small blood culture sample volume, unculturable bacteria, or IAP exposure. Failure to obtain a blood volume ≥ 0.5 ml, which is considered necessary to achieve a sufficient sensitivity, is reported to be frequent, especially in preterm infants.[99] Due to a fear of missed cases "clinical sepsis", also called "culture-negative sepsis", is a commonly used diagnosis. Indeed, clinical sepsis is reported to cause the majority of EOS cases and a significant minority of LOS cases.[22, 23] However, the definition of this diagnosis is highly variable and poorly defined. In 2006, neonatologists in the Norwegian Paediatric Association suggested the following four criteria for the diagnosis of clinical sepsis: i) clinical signs of infection, ii) maximum CRP > 30 mg/L, iii) minimum duration of 5 days antibiotic treatment, and iv) exclusion of other explanations for the clinical picture. Other studies simply define culture-negative sepsis as sepsis in neonates with strong clinical suspicion and slightly elevated haematological markers.[91]

The potential consequences of false negative blood culture results and the delay before results are available leads to a large potential for overtreatment. This caused high hopes for 16s rRNA sequencing as a method with greater sensitivity and faster results than blood cultures. 16s rRNA sequencing is a method where the 16s rRNA gene is amplified using polymerase chain reaction, sequenced, and compared to annotated databases. With this method, the identity of bacterial species, genus, families, or phylum can be inferred. A meta-analysis found that 16s rRNA sequencing achieved a sensitivity of 0.85 (95% confidence interval (CI), 0.81-0.88) and a specificity of 0.96 (95% CI 0.95-0.96) in neonates when compared with blood cultures.[100] In contrast to culture based methods, sequencing based techniques are able to detect unculturable bacteria, dead bacteria, and bacteria that are present in small quantities. However, the clinical relevance of bacteria that are not even able to grow on culture media is considered highly uncertain, and sequencing based techniques are yet to be commonly used in NICUs.[101]

1.7.3 Deciding Who to Treat and How Long

Deciding which neonates should receive empiric antibiotics prior to culture results is a major topic of discussion in neonatology.[102, 103] Most guidelines and authors agree on treating clinically ill infants, but the American Academy of Pediatrics (AAP) also recommend performing laboratory tests on well-appearing neonates whose mothers were diagnosed with chorioamnionitis and treating them for at least 48 hours.[104] The UK National Institute for Health and Care Excellence

(NICE) recommend evaluating and empirically treating neonates who have more than one clinical sign or risk factor indicating EOS. They also recommend treating neonates who have a "red flag sign"; which are respiratory distress >4 hours after birth, seizures, shock, having a twin with infection, or having a mother who was treated for suspected invasive bacterial infection within the 24 hours before or after birth. If the neonate presents with one clinical sign or risk factor, but no red flags, they leave it up to the clinician to decide whether antibiotics should be administered.[94]

Neonatologists world-wide have large differences in opinion on when to initiate treatment for suspected sepsis. In a survey of neonatologists from developed countries, 29% would start treatment in a "low-risk scenario" where the neonate had two maternal risk factors and no clinical signs of infection, while an additional 45% would initiate treatment if laboratory markers were abnormal.[105] In addition, 81% of US neonatologists consider an obstetric diagnosis of chorioamnionitis to be a sufficient reason for empirical antibiotic treatment.[106] Several studies have found a minimal risk of culture-confirmed sepsis among asymptomatic neonates with risk factors.[107-109] Additionally, empirical treatment given for a low suspicion of sepsis is likely to constitute a large amount of neonatal antibiotic exposure. In a 14-month surveillance of antibiotic use in a US NICU, 63% of all antibiotic use was 48-hour treatment for suspected sepsis that was later ruled-out.[110] Recently, consensus has begun to shift towards withholding antibiotic treatment for well-appearing neonates.[102, 111]

Another aspect in the effort to reduce neonatal antibiotic exposure is to reduce treatment length, especially with negative cultures.[101, 103] For culture-confirmed neonatal sepsis or strongly suspected neonatal sepsis, the AAP guidelines recommend treatment for 10 days, while the NICE guidelines recommend treatment for a minimum of 7 days.[94, 104] With negative cultures and a low likelihood of sepsis, both guidelines focus on early cessation of therapy. The NICE guidelines recommend considering stopping antibiotics after 36 hours if blood cultures are negative, the CRP remains low, and the neonate is clinically stable.[94] The AAP guidelines recommend discontinuing antibiotics after 48 hours if the probability of sepsis is low.[104]

Diagnosing neonatal sepsis more rapidly and precisely would greatly reduce the rate and length of antibiotic treatment due to suspected infection. As the current laboratory tests have their limitations regarding sensitivity, specificity, and time until results are available, alternative strategies are needed to decide who to treat with antibiotics. For EOS, risk stratification schemes

have been developed based on maternal risk factors, or a combination of maternal risk factors and clinical data in the first 12 hours of life.[39, 112]

A prediction model developed by Escobar and co-workers used objective maternal data (GA, GBS status, time from rupture of membranes to birth, highest antepartum temperature, and type of IAP) and neonatal data from the first 12 hours of life (Apgar scores, markers of respiratory distress, need for respiratory support, heart rate, respiratory rate, and temperature) to stratify the included neonates into three risk groups: (1) high-risk, should be treated immediately, (2) medium-risk, should be further evaluated, or (3) low-risk, should be observed.[112] When evaluated in a large case-control study, 4% of their population were placed in the high-risk group with a number needed to treat (NNT) of 118, 11% were placed in the medium-risk group with a NNT of 823, and 85% placed in the low-risk group with a NNT of 9370. Theoretically, this approach would reduce the rate of antibiotic treatment in the included NICUs from between 6-10% to 4%.

Taking this approach further, they developed an EOS calculator for neonates with GAs ≥ 35 weeks based on the same maternal risk factors, background incidence in the hospital/region, and clinical signs of infection.[113] The calculator estimates an incidence of EOS per 1000 LB infants. The group behind it recommend obtaining blood cultures if the estimated incidence is ≥ 1 per 1000 LB infants and to institute empirical antibiotics if the estimated incidence is ≥ 3 per 1000 LB infants. The developers evaluated the EOS calculator in a 6-year before-after study of 204 485 neonates. In the first part of the study they followed the CDC guidelines. After applying the EOS calculator, the rate of blood culture sampling declined from 14.5% to 4.9% of the included neonates. Concurrently, the rate of antibiotic use decreased from 5.0% to 2.6% of the included neonates. They also reduced the length of antibiotic treatment from 16.0 to 8.5 days per 100 neonates. Despite this, there were no changes in EOS mortality, signs of complications, or readmissions.[114] A small cohort study retrospectively evaluated the EOS calculator and supported the notion that using it would have reduced the rate of empirical antibiotic therapy.[115]

There are currently no LOS calculators available, but several prediction models exist. In a systematic review of LOS prediction models, the model that performed best required at least two of the following factors; CRP ≥ 14 mg/L, neutrophil fraction > 50%, thrombocytopenia, fever > 38.2°C, or exposure to parenteral nutrition ≥ 14 days to predict LOS.[116] This model achieved a sensitivity of 0.95 (95% CI, 0.86-0.99) and a specificity of 0.43 (95% CI, 0.30-0.56) when tested in the NICU where it was developed. However, it did not perform as well in other NICUs.[117] Another LOS model achieved a sensitivity of 97% and a specificity of 37% by requiring one of

the following four factors to be present; increased respiratory support, capillary refill time ≥ 2 seconds, pallor/grey skin, and/or a central venous catheter.[22]

1.8 Antibiotic Treatment in Neonates

Antibiotics are currently the most commonly used drugs in NICUs, and up to 72% of NICU patients in general and 85% of VLBW infants specifically have been reported to receive antibiotics.[110, 118, 119] Antibiotics are antimicrobial drugs that kill or inhibit the growth of bacteria. They can be classified into several categories based on their mode of action (Table 1). Because treatment is started empirically, e.g. before infection is confirmed, the potential causative pathogen is unknown. This necessitates an initial relatively broad-spectrum treatment that is effective against the organisms that normally cause neonatal sepsis.

Table 1. Classification of Antibiotics Commonly Used in Neonates

Antibiotic Type	Mode of Action	Examples
BETA-LACTAMS	Cell wall synthesis inhibition	
Penicillins		
Beta-lactamase labile		Penicillin, ampicillin
Beta-lactamase stable*		Dicloxacillin, cloxacillin, flucloxacillin
Cephalosporins		
1st generation		Cephalotin
2nd generation		Cefuroxime
3rd generation		Cefotaxime, ceftazidime, ceftriaxone
Carbapenems		Meropenem, imipenem
AMINOGLYCOSIDES	Protein synthesis inhibition	Gentamicin, tobramycin, netilmicin, amikacin
GLYCOPEPTIDES	Cell wall synthesis inhibition	Vancomycin, teicoplanin

Source: www.felleskatalogen.no *Does not include extended-spectrum beta-lactamases

The following segment is going to discuss pharmacokinetic and pharmacodynamic properties of antibiotic classes that are commonly used in neonates. It is therefore important to define a few terms.[120] Minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic drug that prevents visible growth of a bacteria. Time > MIC is the period where the plasma

concentration of the antibiotic drug is higher than the MIC. Peak plasma concentration (PPC) is the maximum plasma concentration of a drug, and it is commonly measured shortly (0.5 - 1 hour) after drug administration when the drug is in steady state. Trough plasma concentration (TPC) is the lowest concentration of a drug during the treatment period, and it is commonly measured shortly before the third dose. The area under the plasma drug concentration-time curve (AUC) represents the total drug exposure over a specific time. It is displayed as an integral in a plot of drug concentration versus time.

1.8.1 Beta-Lactams

Beta-lactams are a major class of antibiotics consisting of several sub-groups such as penicillins, cephalosporins, and carbapenems. Alexander Fleming famously discovered penicillin in 1928, but despite its age, penicillin G (benzylpenicillin), along with ampicillin and cefotaxime, remain among the most commonly used antibiotics in NICUs.[119, 121] Beta-lactams contain a beta-lactam ring and achieve their bactericidal effect through inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall by binding to penicillin-binding proteins.[122] This leads to a futile cycle of peptidoglycan synthesis and degradation that depletes cellular resources and leads to cell death.

Benzylpenicillin is a narrow-spectrum antibiotic that provides coverage against GBS, other streptococci, most listeria strains and penicillin-susceptible staphylococci. The often used empirical combination regimen benzylpenicillin plus an aminoglycoside provides coverage against most EOS pathogens.[123] Ampicillin and other aminopenicillins have relatively similar uses as benzylpenicillin, with an added effect against Gram-negative bacteria due to their amino-group.[124] Both benzylpenicillin and ampicillin are susceptible to the beta-lactamase enzyme commonly found on the cell surface of staphylococci, common causative agents of both EOS and LOS.[25, 125] Cloxacillin and flucloxacillin are stable against some types of beta-lactamases and are consequently used against staphylococci.[123] However, high rates methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* threatens their effectiveness in many countries.[126, 127]

Cephalosporins are broad-spectrum antibiotics often used for treatment of neonatal infections.[128] These antibiotics are grouped into several generations based on their antibacterial spectrums. Cephalotin, a first-generation cephalosporin, is effective against staphylococci, other Gram-positives, and some Gram-negatives, and is therefore a valid part of empiric LOS regimens.[129] The third generation cephalosporins like cefotaxime have a broader antibacterial spectrum than

previous generations with coverage against both Gram-positive and Gram-negative organisms.[123] Moreover, cefotaxime effectively penetrates the blood-brain barrier and is therefore a good option for treatment of neonatal meningitis.[130, 131] As a consequence, cefotaxime is one of the most commonly used medications in NICUs.[128] However, cephalosporins, and in particular third-generation compounds, are associated with an increased selection of antibiotic resistant bacteria.[132]

Amoxicillin and ceftriaxone are suspected of toxicity, despite toxicity being rare among beta-lactams.[133, 134] Ceftriaxone is a competitive inhibitor of bilirubin's binding to albumin, which may place neonates, particularly preterm neonates, at risk of bilirubin encephalopathy.[134] Additionally, co-administration of ceftriaxone and intravenous calcium has been associated with an increased risk of thromboembolism and cardiopulmonary adverse events.[134, 135] There are isolated reports of amoxicillin causing renal toxicity in paediatric patients, but nephrotoxicity was extremely rare in a US nation-wide study of children under 6 years old who received amoxicillin.[133] To avoid toxicity, PPCs < 140 mg/L have been proposed as a target for amoxicillin therapy, despite beta-lactam PPCs rarely being measured and toxicity being too rare to demonstrate a dose-dependent effect.[133, 136]

The bactericidal effect of beta-lactams is dependent on time > MIC, and it is commonly recommended to keep concentrations above the MIC for at least 40-50% of the time for penicillins and 50-60% of the time for cephalosporins.[120, 137, 138] Beta-lactams are water-soluble and have a large volume of distribution (V_D) in neonates than older children and adults.[138] They are eliminated through the kidneys, and half time is increased in neonates, particularly in preterm neonates.[137, 138] To maintain a sufficient time > MIC while avoiding potentially toxic concentrations, small doses are given with 8-12 hour intervals.[120] The British National Formulary for Children recommends beta-lactam dosing intervals of 12 hours for neonates < 7 days of age and 8 hours for neonates ≥ 7 days of age.[139]

1.8.2 Aminoglycosides

Aminoglycosides are a class of antibiotics that consist of tobramycin, gentamicin, netilmicin, and amikacin, among others.[140] Aminoglycosides achieve bactericidal effect through irreversibly binding to the 30S subunit of bacterial ribosomes, thereby inhibiting protein synthesis and altering the integrity of the bacterial cell membrane.[141] They are a mainstay of empiric neonatal sepsis treatment due to their coverage for Gram-negative bacteria.[94, 104] In contrast to beta-

lactams, all aminoglycosides have a very similar antimicrobial spectrum. Gentamicin is currently the most commonly used aminoglycoside in neonates.[119] Despite aminoglycosides effectiveness and relatively low rates of resistance, there has often been some concern about their potential nephrotoxicity and ototoxicity.[142, 143]

Aminoglycosides have a concentration-dependent effect, and achieving a high PPC in relation to the MIC is vital for effective bacterial killing.[144, 145] Aminoglycosides also have a post-antibiotic effect, meaning that bacterial killing continues after the serum concentration has fallen below the MIC.[145] PPCs > 5-10 mg/L is a commonly proposed target for gentamicin, netilmicin, and tobramycin to maintain the bactericidal and post-antibiotic effects,.[146-149] In contrast, aminoglycoside toxicity occurs through saturation of proximal tubule cells (nephrotoxicity) and cochlear cells (ototoxicity).[150, 151] Saturation occurs with prolonged durations of aminoglycoside treatment and high TPCs. Consequently, many authors suggest maintaining TPCs < 2.0 mg/L to prevent potential toxicity.[147, 148]

Aminoglycoside ototoxicity in humans initially affects hearing at the higher frequencies, before progressing to the middle frequencies.[152] The hearing loss is caused by hair cell apoptosis inside the cochlea and is typically irreversible.[153] Hearing loss in early childhood could potentially go undetected until teachers and parents notice delayed language development. Therefore, most developed countries screen neonates for hearing loss with an otoacoustic emissions (OAE) test followed by an auditory brain stem response (ABR) if infants fail the OAE test. Combined, this two-step diagnostic process has been reported to have an estimated sensitivity of 92% and specificity of 98%.[154] Due to the low prevalence of hearing loss in neonates, however, the PPV of this screening is reported to lie between 2-40%.[155]

In general, 2-7% of all tested neonates fail their OAE screening, but sensorineural hearing loss has a reported prevalence of only 0.5-3.6 cases per 1000 LB infants.[156-158] In addition to aminoglycosides, a family history of hearing loss, parental consanguinity, maternal intoxication during pregnancy, medications such as loop diuretics and glycopeptides, cytomegalovirus (CMV) infections, congenital anomalies, prematurity, and respiratory distress are considered risk factors for sensorineural hearing loss in neonates.[158, 159] Moreover, relatively rare mutations in the mitochondrial 12S rRNA gene and some other mitochondrial genes have been associated with aminoglycoside-induced ototoxicity.[160] However, the evidence on aminoglycoside ototoxicity is currently limited, and several studies actually report no associations between aminoglycosides and hearing loss in infants.[158-160] It is possible that there are interactions or additive effects between

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risk factors, as aminoglycosides have been found to cause hearing loss in neonates when used concurrently with other ototoxic drugs.[161]

In contrast to ototoxicity, aminoglycoside nephrotoxicity is largely reversible. Aminoglycosides are excreted through the kidneys, and high concentrations over time may cause apoptosis of renal cells in the proximal tubule.[152] In neonates, aminoglycoside nephrotoxicity is poorly documented.[152] While high TPCs are correlated with high serum creatinine in some studies, the correlation may be a case of reverse causality.[146, 162] An unrelated acute renal injury may cause high gentamicin TPCs through impaired clearance, as aminoglycosides are excreted renally.[140]

Previously, administering small doses multiple times daily was the norm for aminoglycoside treatment in neonates.[147] However, this was irrational for a few reasons. Firstly, aminoglycosides are water-soluble drugs and neonates, particularly VLBW neonates, have proportionally larger V_D than children or adults.[163] Therefore, proportional to body weight, larger doses are needed to achieve therapeutic PPCs. Secondly, aminoglycosides are cleared through the kidneys, and clearance is impaired in neonates shortly after birth, particularly with low BW and postnatal age (PNA).[163] Therefore, neonates need larger time intervals between doses. A Cochrane systematic review reported that multiple doses per day regimens are inferior to one-dose daily regimens in achieving therapeutic PSCs and TSCs in neonates.[147]

Over the last 20 years, larger doses given once daily have become widely established for aminoglycoside treatment in neonates.[149] However, aminoglycoside dosing regimens vary greatly.[139, 147, 148, 164] To achieve satisfactory PPCs and TPCs, a dosing regimen has to account for varying GAs and PNAs. This often leads to complicated dosing-regimens with increased risk of erroneous administration.[165] Additionally, most current neonatal gentamicin dosing regimens recommend 4-5 mg/kg at intervals between 24-48 hours, but dosing regimens for older children beyond the neonatal period recommend larger doses despite these children having proportionately lower V_D.[147, 149, 166] These factors emphasize the need for a simplified high-dose extended-interval dosing regimen in neonates.

1.8.3 Glycopeptides

Glycopeptides are a class of antibiotics that achieve bactericidal effect on Gram-positive bacteria by inhibiting cell wall synthesis.[167] There are concerns regarding empiric vancomycin treatment due to increasing rates of vancomycin-resistant enterococci and staphylococci.[168, 169] In Norway, vancomycin is seldom used empirically as *S. aureus* is largely susceptible to cloxacillin and gentamicin.[125] In some countries, however, high rates of methicillin resistant staphylococci have caused vancomycin to become one of the most commonly used antibiotics in NICUs.[43, 119] Beta-lactams such as cephalotin, however, can be clinically effective against CoNS that are methicillin resistant in vitro.[129]

There are many unexplained factors in vancomycin pharmacokinetics in neonates, but their efficacy seems to be best predicted by the AUC/MIC-ratio.[167] Vancomycin is potentially ototoxic and nephrotoxic, especially with large doses, prolonged treatment, and concurrent use of other ototoxic and nephrotoxic medications. These side-effects are, however, rarely seen in neonates.[158, 170] Vancomycin is, similarly to other antibiotics, water-soluble and cleared through the kidneys. Consequently, neonates have higher V_D and longer clearance of vancomycin compared with older children or adults.[171] V_D and clearance vary greatly among neonates, due to variable protein-binding capacities for vancomycin and variable kidney functions.[158] Consequently, therapeutic drug monitoring is vital to account for this inter-individual variability. Trough concentrations have been found to be predictive of the AUC/MIC ratio, and vancomycin troughs between 10-15 mg/L appear adequate to achieve satisfactory AUC/MIC ratios in neonates.[172]

1.8.4 Empirical Antibiotic Regimens

In many countries, the most commonly used empiric antibiotic regimen for EOS is a combination of an aminoglycoside and either benzylpenicillin or ampicillin.[25, 30, 173] This is supported by the NICE and AAP guidelines. In contrast, third-generation cephalosporins are not recommended as part of empirical sepsis treatment because of their association with increased development of antibiotic resistance.[94, 104, 132] Moreover, in a large retrospective cohort study of ~130 000 neonates, cefotaxime treatment was independently associated with an increased risk of death compared with gentamicin treatment.[174]

While the NICE guidelines recommend benzylpenicillin and gentamicin for suspected EOS, the AAP guidelines recommend ampicillin and gentamicin.[94, 104] Both regimens provide excellent

coverage against common EOS pathogens, with an exception of CoNS, which is more commonly seen in LOS.[123, 175] Ampicillin has traditionally had better Gram-negative coverage than penicillin, but ampicillin-resistance rates among *E. coli* strains are high. According to the Norwegian Surveillance System for Antibiotic Resistance in Microbes, 43.5% of *E. coli* blood culture isolates in Norway were resistant to ampicillin in 2016.[125]

IAP with ampicillin is reported to be a significant risk factor for developing ampicillin resistant *E. coli*.[43, 56] For EOS treatment, however, there is little evidence whether penicillin or ampicillin should be preferred as a part of an empirical regimen. A RCT with treatment failure as the primary outcome compared benzylpenicillin and gentamicin with ampicillin and gentamicin. The rate of treatment failure, defined as the need to change antibiotics within 72 hours or death within seven days, was 14% regardless of empiric antibiotic regimen. In this RCT, with limited number of participants, the authors did not find any significant differences in antibiotic resistance development.[176]

In contrast to EOS treatment, there are few LOS guidelines and the choice of empiric antibiotics is highly variable.[177] However, the British National Formulary for children recommend flucloxacillin and gentamicin for empiric LOS treatment.[139] Except for CoNS, 95% of LOS organisms were susceptible to this combination in a survey of 90% of the hospitals in England and Wales.[123] LOS is usually nosocomially acquired, which causes higher resistance rates among LOS pathogens than EOS pathogens.[175] Variations in empiric LOS regimens are understandable, as LOS pathogens' resistance rates are likely to vary between different countries. In a prospective cohort of suspected LOS cases from five southern- or eastern-European countries, the empiric regimen was meropenem-based in 27% of cases, vancomycin-based in 23% of cases, third-generation cephalosporin-based in 18% of cases, and ampicillin based in 10% of cases.[177] In an American study from 1998 to 2000, 44% of all VLBW infants who survived for at least three days received vancomycin.[59]

1.9 Adverse Effects of Antibiotic Treatment

While antibiotic treatment is potentially life-saving, overuse can lead to adverse effects. In the short-term, prolonged antibiotic therapy in uninfected preterm infants has been implicated as a risk factor for NEC, and broad-spectrum antibiotic therapy has been associated with an increased risk of IFI.[69, 178] Antibiotics may also have long term consequences, such as an increased spread and development of antibiotic resistance. In the last few years, more and more emphasis has been

placed on the gut microbiota and how its composition may affect human health. Antibiotics early in life are thought to disrupt the development of the gut microbiota.[179]

1.9.1 Gut Microbiota and Gut Dysbiosis

The human gut microbiota can be described as the sum of all life living in or on the human body. More practically, it is a complex system of bacteria, virus, fungi, and other microorganisms that colonise the human gut. Bacteria are the most studied part of the gut microbiota, and a common, but poorly documented cliché is that the gut bacteria outnumber the cells of their host by ten to one.[8] In a stable resilient gut microbiota after 2-3 years of age, gut bacteria are estimated to be composed of 1000 species from 40-50 genera.[180] They perform vital functions for the host, including colonisation resistance against potential pathogens and antibiotic resistant bacteria, aiding in digestive functions, and developing and shaping the immune system.[181, 182] In contrast, gut dysbiosis, which can be defined as a microbial imbalance in the gut microbiota, is associated with imbalanced and disease promoting immune responses.[182]

The foetal gut was considered sterile until a unique placental microbiome was discovered using modern sequencing techniques.[6, 183] Some authors, however, consider these findings to be caused by contamination.[5] Nevertheless, during birth the neonate is exposed to a plethora of bacteria from its mother's birth canal, including species of *Bifidobacterium* and *Lactobacillus*. Colonization with maternal bacteria causes a rapid development of the infant's gut microbiota with increasing diversity as the infant encounters bacteria from breast feeding and its environment. The child's microbiota begins to resemble that of an adult one year after birth, and after 2.5 years it is considered stable and adult-like.[184]

In healthy adults, the gut microbiome is highly diverse and is largely comprised of bacteria from three phyla; Bacteriodetes, Firmicutes, and Proteobacteria.[185, 186] The phyla are the major lineages of the bacterial kingdom, and they are further subdivided into classes, orders, families, and genera. Proteobacteria, a phylum of Gram-negative bacteria that includes *E. coli*, *Klebsiella* species, and *Enterobacter* species, only makes up a small proportion of bacteria in the healthy gut.[186] The vast majority of gut bacteria are anaerobes, and *Bacteriodes* is by far the most prevalent genus.[185] Figure 1 displays the hierarchical distribution of relevant gut bacteria.

Figure 1. Hierarchical Distribution of Common Gut Bacteria

This figure was based on http://www.bacterio.net/-classification.html
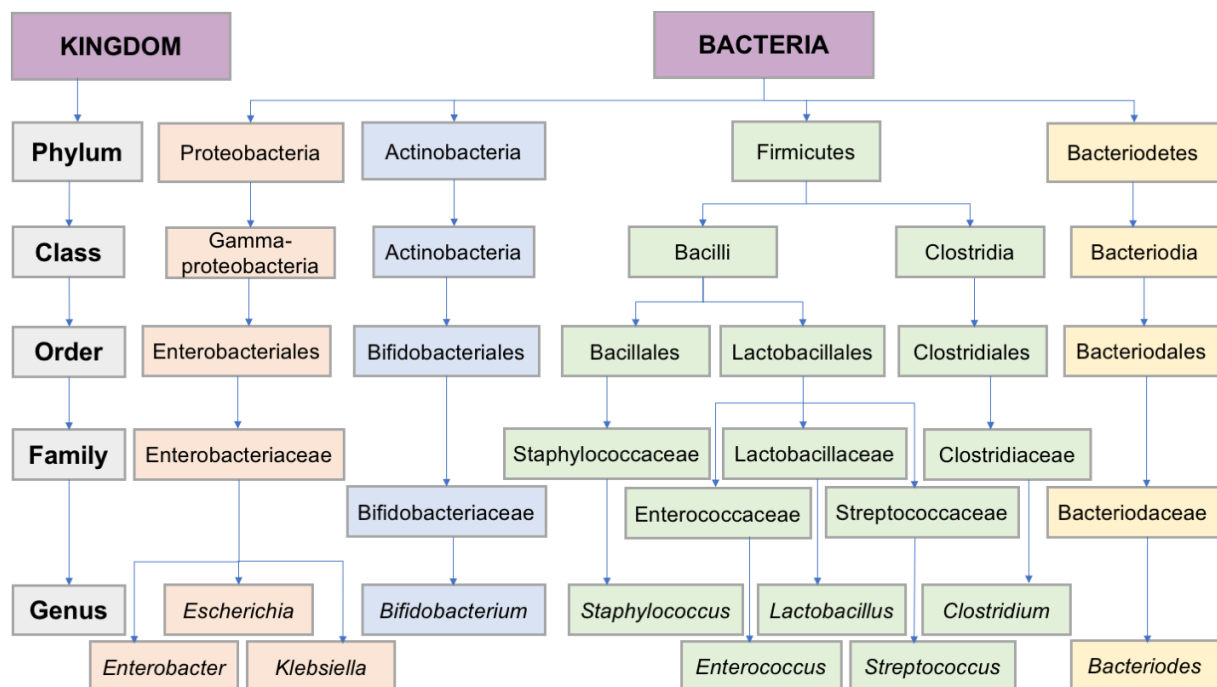
The gut microbiota is highly complex, and high abundances of certain phyla can be protective against some diseases and disease-promoting for others. For instance, obesity and irritable bowel syndrome are associated with an increased abundance of Firmicutes and a decreased abundance of Bacteriodetes.[187, 188] In contrast, a high abundance of Bacteriodetes and a low abundance of lactate and butyrate producing bacteria like *Bifidobacterium* species has been implicated in the development of type I diabetes.[189] Several disease states in childhood and adulthood, such as colorectal cancer, major depressive disorder, and inflammatory bowel disease, are associated with lower diversity, increased abundance of Proteobacteria, and a lower abundance of anaerobic bacteria.[190-192]

While the pathogenesis of NEC is poorly understood, NEC patients have lower gut microbial diversity, increased abundance of Proteobacteria, and lower abundance of Bacteriodetes and obligate anaerobic Firmicutes compared with healthy controls.[193-196] This dysbiosis can alter inflammatory signalling, bacterial detection, and barrier functions, thereby allowing pathogenic bacteria to cross into epithelial cells. TLR4, which detect Gram-negative bacteria, is highly expressed in NEC cases, and this could initiate the inflammation that characterizes NEC.[81] In general, obligate anaerobes such as *Bacteriodes* and *Bifidobacterium* species are considered protective for NEC.[194] Probiotics, largely with species of *Lactobacillus* and *Bifidobacterium*, were also found to reduce the risk of stage II-III NEC in VLBW infants in a meta-analysis.[72]

Several factors may cause dysbiosis in the developing gut microbiota during the neonatal and infant period. An obvious example is the mode of delivery, as infants born via caesarean section are not exposed to commensal bacteria from the maternal vaginal tract. Consequently, newborn babies delivered by caesarean section have lower abundance of *Bifidobacterium* species.[7] Instead, these neonates may be more influenced by bacteria in their environment, such as bacteria from their mother's skin. Neonates delivered by caesarean section that are hospitalized after birth may also be more heavily colonized by bacteria from the NICU environment, including genera from the NICU itself and skin bacteria from caregivers' hands.[197]

Breastfeeding has been associated with an increased diversity of the gut microbiota at one year of age.[198] The introduction of cow milk and a full adult diet causes shifts in the developing microbiota, such as increasing the abundance of Bacteriodetes.[184] Premature infants have a different development of the gut microbiota than term infants with higher abundances of Proteobacteria and Firmicutes, and lower abundances of Bacteriodetes.[199] However, preterm infants also have higher risks of being born via caesarean section, being formula fed and receiving antibiotics, so significant confounding and interaction may occur.

Antibiotic treatment, particularly long-term treatment with broad-spectrum antibiotics, can cause a selection pressure that causes antibiotic susceptible pathogens to die while other pathogens survive.[7] Antibiotic treatment causes an overgrowth of Proteobacteria at the expense of commensal anaerobes.[200] This may be partially due to losing the colonization resistance that obligate anaerobes offer against pathogenic and antibiotic resistant bacteria.[201] Indeed, antibiotics early in life, including IAP and neonatal antibiotic treatment, have been associated with an increased risk of obesity, allergies, inflammatory bowel disease, behavioural difficulties, IFI, and NEC.[69, 178, 202-205]

1.9.2   Antibiotic Resistance

Most antibiotics are derived from antimicrobial substances that are naturally produced by microorganisms. As these substances have existed for millennia, bacteria have naturally occurring resistance mechanisms.[206] However, selection pressure from the wide-spread use of antibiotics in human medicine, veterinary medicine, and agriculture has made antibiotic resistance a developing global health crisis. An estimated 214 500 neonates die yearly due to sepsis with antibiotic resistant bacteria.[207]

Bacteria develop antibiotic resistance primarily through two different pathways; spontaneous mutation and horizontal gene transfer.[208] Spontaneous mutations can develop antibiotic resistance through altering the drug targets, thereby coding for enzymes that change the structure of the antibiotic or up-regulate efflux pumps.[209] A classic example of enzymes changing the structure of antibiotics are the beta-lactamases; enzymes that break down the central beta-lactam ring of beta-lactam antibiotics. Horizontal gene transfer occurs through several different mechanisms, but the transfer of plasmids is perhaps the most important.[208] Antibiotics apply a selection pressure that not only favours bacteria with antibiotic resistance genes, but also induces transfer of resistance genes.[210]

According to a WHO surveillance report from 2014, extended-spectrum beta-lactamase (ESBL)-producing *E. coli* and *Klebsiella pneumoniae* are among the most concerning antibiotic resistant bacteria.[211] In addition to penicillins, ESBL may hydrolyse third-generation cephalosporins and even carbapenems. ESBL-rates are highest in South-East Asia, and 20-61% of *E. coli* isolates and 53-100% of *K. pneumoniae* isolates in this part of the world are resistant to third-generation cephalosporins.[211] In Europe, rates are more variable, and 4.9% of *K. pneumoniae* and 5.8% of *E. coli* were ESBL-producing in Norwegian blood culture isolates in 2016.[125] ESBL-producing Enterobacteriaceae infection have a mortality rate of approximately 31-43% in neonates.[212]

Currently, 73% of Norwegian *S. aureus* isolates produce beta-lactamase and, therefore, cloxacillin is commonly used to treat staphylococcal infections.[125] However, the emergence of methicillin-resistant staphylococci has made glycopeptide treatment necessary in many cases. In Japan, for instance, MRSA causes 88% of *S. aureus* LOS.[213] In contrast, only 11% of *S. aureus* LOS in the UK is caused by MRSA, while 99% of Norwegian *S. aureus* blood-stream isolates are methicillin-sensitive.[25, 125] Other emerging threats are carbapenem-resistant Enterobacteriaceae and vancomycin-resistant enterococci.[214] Broad-spectrum antibiotics have been found to induce more multi-drug resistant (MDR) Gram-negative bacteria in neonatal populations than narrow spectrum antibiotics.[132, 215] Moreover, both antibiotic treatment versus no treatment and prolonged treatment versus shorter treatment have been found to increase the rate of MDR Gram-negative bacteria in neonates.[216]

1.10 Evidence Based Medicine

Evidence-based medicine (EBM) is an approach to medical practice, and the Oxford Dictionary of Epidemiology defines it as "the consistent use of knowledge derived from biological, clinical, and epidemiological research in the management of patients".[217] Clinical epidemiology is one of the foundations of EBM, and it is the study of occurrences and distribution of health related effects in a clinical setting. The highest achievement in epidemiology is to discover and understand the cause-effect relationships behind diseases. Such understanding makes it possible to treat or even prevent disease.

In epidemiology, a cause is something that alters the frequency of a disease or a health status. A necessary step is finding associations between potential causes and the studied outcome, but associations alone do not imply causality. Sometimes an association is erroneously interpreted as causal when it is in fact the result of confounding (a third factor that is the true cause of the association between exposure and outcome), interaction (two or more exposures working together to affect the outcome), or bias (a systematic deviation of results from the truth).[217, 218] To establish causality, certain factors need to be present. A cause needs to precede the disease, show a consistent effect, increase the incidence of the disease, have a dose-response effect (greater effect in greater quantity), and its effects should be consistent across several studies. Bias exists in many forms, and Table 2 explains the kinds of bias that are most relevant for this thesis.

Table 2. Types of Bias Relevant for This Thesis

Type of Bias	Description
Confounding	A variable that causes a spurious association by influencing both the dependent and the independent variable
Selection bias	Choice of study population leads to an uneven distribution of confounding factors
Performance bias	Systematic differences in care provided to members of different study groups that is not the studied exposure
Detection bias	Systematic differences between study groups in assessment, ascertainment, diagnosis, or verification of outcomes
Reporting bias	Selective revelation or concealment of information or results from a study

Source: Porta M. *A Dictionary of Epidemiology*. New York: Oxford University Press, 2014.

Robust study designs are needed to minimize the risks and impact of bias and confounding. Different study designs have different advantages, but central to EBM is a hierarchy of evidence where meta-analysis, systematic reviews, and RCTs are at the top of the hierarchy.[218] Systematic reviews are critical appraisals of the scientific evidence that apply strategies to limit bias in collection, synthesising, and critically appraising relevant studies.[217] The core premise of this method is to develop a research question and perform systematic searches according to previously established criteria to uncover relevant studies. Studies are included or excluded based on previously established criteria. A meta-analysis is a statistical analysis of results from several studies, which can increase statistical power.[217] Meta-analyses are commonly a part of the systematic review process, but studies with low risks of bias and comparable populations, exposures, and outcomes are required for such methods.[219] RCTs are usually well suited for this, due to their lower risks of bias. Typically, systematic reviews are based on RCTs, but observational studies generally have longer follow-up time and larger population sizes and are therefore well suited to study rare adverse effects.[220]

The Cochrane Collaboration is an esteemed international collaboration of researchers that work to summarise evidence from health research. They have developed the Cochrane Handbook for Systematic Reviews of Interventions; a handbook that aims to improve the methodological quality of systematic reviews.[219] The Cochrane Handbook includes tools for assessing methodological quality in included studies and the quality of evidence. Additionally, it recommends following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement; a checklist for transparent reporting of systematic reviews.[221] Several journals demand that systematic reviews follow this statement, which increases the transparency of systematic reviews.

Publishing the study protocol prior to performing the systematic review is part of the PRISMA checklist.[221] Several databases allow researchers to publish their protocols, and one of the largest and most frequently used is PROSPERO.[222] It is an international database of prospectively registered systematic reviews in several academic fields, among them health care. Prospective registration helps to counter publication bias as systematic reviews are searchable, regardless of whether they were published or not. Additionally, it increases transparency and reduces reporting bias as it allows the reader to compare the finished study with how the review was planned in the protocol.

The Cochrane Handbook recommends using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) system for evaluating the quality of evidence (QoE) in a body of literature.[219] This approach specifies four levels of quality from high to very low. These levels of QoE define the degree to which estimates of effects or associations can be trusted. Findings based on RCTs are initially assigned a high QoE, while findings based on observational studies are initially assigned a low QoE. Several factors could upgrade or downgrade the quality rating. For example, a dose-response effect or large effect estimates could increase the QoE, while a high risk of bias or inconsistent results could decrease the QoE.[223]

An estimated 700-800 articles about antibiotic treatment in neonates are published in the PubMed database every year, which makes it challenging for both clinicians and researchers to stay up to date.[224] Systematic reviews summarize, critically evaluate, and appraise the evidence, and in doing so they can be important and very useful for caregivers. In many cases the evidence is not of a sufficient quality to enable strong conclusions, but thorough and methodologically robust systematic reviews will none the less give a summary of the current evidence in a field and potentially pinpoint the need for further research. To our knowledge, no systematic reviews on the adverse effects of neonatal antibiotic treatment have been published previously.

2 Aims of the Study

The overall aim of this thesis was to investigate different aspects of antibiotic therapy for neonatal sepsis in order to obtain new knowledge that could improve and optimise care.

The specific objectives were:

- To investigate the epidemiology of EOS and exposure to systemic antibiotics during the first week of life in an unselected national cohort of LB term infants.

- To evaluate a simplified high-dose extended-interval gentamicin dosing regimen with focus on pharmacokinetic safety, potential ototoxicity, and the number of prescription errors.

- To identify, critically appraise, and synthesize evidence from studies reporting different categories of antibiotic exposure in neonates and the subsequent risk of developing the following three early adverse outcomes: NEC, IFI, and/or death.

- To identify, critically appraise, and synthesize evidence from studies reporting different categories of antibiotic therapy in neonates and their impact on the gut microbiota and/or antibiotic resistance development.

3 Materials and Methods

3.1 Study Design and Materials

Paper 1

Paper 1 is a registry-based cohort study of LB term infants admitted to neonatal units in Norway during the three-year period from January 1, 2009 to December 31, 2011. Detailed clinical data were prospectively collected by the Norwegian Neonatal Network (NNN), a web based public registry maintained by the Norwegian Institute of Public Health. Twenty of the 21 neonatal units in Norway contributed data during this period. In the NNN, clinical data are entered daily on all infants admitted to each participating neonatal unit. In Norway, all infants receiving intravenous antibiotic therapy are admitted to a neonatal unit situated in one of four regional health-care trusts (South-East, West, Mid and North). Data on the total number of LB infants in Norway were obtained from the Medical Birth Registry of Norway. Supplementary information on systemic GBS infections, a notifiable disease in Norway, was obtained from the Norwegian Surveillance System for Communicable Diseases. Mortality data were compared with data obtained from the Norwegian Cause of Death Registry.

Paper 2

Paper 2 is a retrospective single-centre cohort study of neonates up to 50 weeks PMA that received gentamicin and had ≥1 TPC measured between January 1, 2004 through May 31, 2012. Patients were recruited from the NICU at the University Hospital of Northern Norway in Tromsø. This NICU is the only tertiary neonatal unit in the two northernmost counties in Norway, covering a population of 230 000 with around 3000 births per year.[225] All infants <34 weeks GA and all infants receiving mechanical ventilation in the catchment area are treated in this unit, so for these infants our data are population-based.

Paper 3 and 4

Paper 3 and 4 are systematic reviews of adverse effects following antibiotic treatment in the neonatal period. Both reviews were reported according to the PRISMA statement following a joint, prospectively registered protocol (study protocol registration: PROSPERO CRD42015026743).[226] Our primary research questions were:

- Paper 3: 'Are different types of antibiotic exposure in neonates associated with increased risks of the adverse outcomes NEC, IFI, and/or death in the neonatal period?'

- Paper 4: 'Are different categories of antibiotic treatment in neonates associated with different changes in gut microbiota composition and/or differences in antibiotic resistance development?'

A study was eligible for review if it reported on groups of neonates, preterm or term, with different categories of intravenous antibiotic exposure and examined their impact on either NEC, IFI, or death in the neonatal period or up to discharge from the neonatal unit (Paper 3) or changes in the gut microbiota or antibiotic resistance development (Paper 4). Both RCTs and observational studies such as cohorts, case-control studies, and cross-sectional studies were eligible for inclusion. We excluded case reports and case series, studies with a non-human or non-neonatal population, studies that were not written in English, and studies that investigated antenatal antibiotics, oral antibiotics, or low-dose intravenous vancomycin prophylaxis.

3.2   Gentamicin Dosing Regimen and Monitoring

In Paper 2, we administered gentamicin 6 mg/kg as a 30-min infusion, regardless of GA and PNA. The dosing intervals ranged from 24-48 hours, depending on PNA and GA (Table 3). TPCs were obtained right before the third dose. During the study period, two different immunoassays with a lower limit of detection <0.3 mg/L were used to analyse gentamicin plasma concentration (2004-09: GENT2, Roche, Mannheim, Germany, 2010–2012: CEDIA® Gentamicin II Assay, Microgenics, Passau, Germany). An internal validation showed a good correlation between both methods.

Table 3. Gentamicin Dosing Protocol

	Postnatal age	Gestational age (GA)/ Postmenstrual age (PMA)	Dosage	Dosing Interval
Group A	0-7 days	GA > 36 weeks	6 mg/kg	24 hours
Group B	0-7 days	GA 29-36 weeks	6 mg/kg	36 hours
Group C	0-7 days	GA < 29 weeks	6 mg/kg	48 hours
Group D	>7 days	PMA ≥ 29 weeks	6 mg/kg	24 hours
Group E	>7 days	PMA < 29 weeks	6 mg/kg	36 hours

3.3 Search Strategy in Systematic Reviews

In Paper 3 and 4, we developed our search strategy in consultation with an epidemiologist, a librarian, a paediatric pharmacologist, and a neonatologist. We searched PubMed, Medline, Embase, and the Cochrane database using MeSH-terms and free-text searches with no time restrictions (last search December 22, 2016). The first search was conducted using MeSH terms. The search strategy in PubMed, Medline and the Cochrane Database was to combine 'Infant, Newborn' and 'Anti-Bacterial Agents' with either 'Enterocolitis, Necrotizing', 'Fungemia', 'Candidiasis, Invasive', 'Meningitis, Fungal', or 'Mortality' (Paper 3) or 'Drug Resistance, Bacterial' or 'Microbiota' (Paper 4). The Embase database uses its own key words, and we combined 'Newborn' and 'Antibiotic Agent' with either 'Necrotising Enterocolitis', 'Fungemia', 'Invasive Candidiasis', 'Fungal Meningitis', or 'Mortality' (Paper 3) or 'Antibiotic Resistance' or 'Microbiome' (Paper 4).

The second search was conducted using free text in PubMed, Medline and Embase by combining the keywords 'Infant, Low Birth Weight', 'Infant, Postmature', 'Infant, Premature' or 'Infant, Newborn' with 'Anti-Bacterial Agents' or 'Antibiotics' and one of the following outcome terms: 'Necrotizing Enterocolitis', 'Fungaemia', 'Fungemias', 'Candidemia', 'Invasive Candidiasis', 'Fungal Meningitis', or 'Mortality' (Paper 3) or 'Antibiotic Resistance', 'Antibacterial Drug Resistance', 'Microbiota', 'Microbiome', 'Microbiomes', or 'Gut Flora' (Paper 4). We examined reference lists of included studies and relevant reviews to identify additional eligible studies. We then combined all citations and excluded duplicates or triplicates. We did not contact authors for supplementary information and we did not perform searches in the "grey literature", e.g. materials and research produced by organizations outside of the traditional commercial or academic publishing and distribution channels; thus not controlled by commercial publishers.

3.4 Variables and Definitions

Paper 1

Information on GA, birth weight by 500 g groups, Apgar scores, blood culture results and information on treatment and clinical diagnoses were included in the analysis. We did not have information on maternal fever or chorioamnionitis. Clinical diagnoses registered in NNN were defined according to the International Classification of Diseases, 10th Revision. Bacterial sepsis in the newborn (P36.0–P36.8) is defined as growth of bacteria in blood cultures together with clinical signs and symptoms compatible with infection. Unspecified bacterial sepsis (P36.9) is

applied when there are clinical and biochemical signs of sepsis without growth of bacteria in blood cultures or when blood cultures are not obtained.

For infants with EOS, infection onset was defined as the day antibiotic treatment began. We defined EOS as infection onset in the first week of life. Infants diagnosed with sepsis (P36) who did not receive intravenous antibiotics were considered misclassified. We ascertained all cases of P36.0 - P36.8 by evaluating blood culture results and requested the neonatal units to register blood culture results if they were missing. Cases of unspecified bacterial sepsis (P36.9) with antibiotic treatment <5 days were not defined as EOS. Coagulase-negative staphylococci, micrococci, *Propionibacterium* and *Corynebacterium*/diphtheroids in a single blood culture were classified as contaminants, in line with suggestions by Stoll *et al.*[30] Data on culture-confirmed EOS in preterm infants were also collected to present incidence rates for all infants, irrespective of GA.

Paper 2

Two of the authors (Jon W. Fjalstad and Claus Klingenberg) reviewed the medical records of all eligible patients. We registered background data (sex, age, weight, diagnoses and complications including acute renal failure) and gentamicin TPCs. Gentamicin TPCs <0.3 mg/L were assigned a value of 0.2 mg/L. We took extra care to assess medical staff prescription and to evaluate whether dosing (mg/kg) and dosing intervals were in line with the dosing protocol (Table 3). We evaluated nursing staff administration and we defined a dose given >3 h earlier or later than scheduled as an administration error.

Paper 3 and 4

Two reviewers (Jon W. Fjalstad and Eirin Esaiassen) independently screened search results and assessed each potentially eligible study per our predetermined inclusion and exclusion criteria. We only excluded studies that we agreed were irrelevant according to our predefined criteria. A third researcher (Claus Klingenberg) had the deciding vote in cases of disagreement. We extracted the following information from included studies: author, year, country, study design, study population, including GA and BW, comparison of outcomes between groups with different categories of antibiotic treatment, and, if available, risk estimates with 95% confidence intervals (CI) for the specific outcome.

We compared three different categories of antibiotic therapy: (i) antibiotics yes versus no; (ii) antibiotics long versus short duration; and (iii) broad-spectrum versus narrow-spectrum antibiotic

regimens. For category (ii), we suggested in advance that 'prolonged' antibiotic exposure was either ≥3 days or the longest of two antibiotic regimens. For category (iii), we always defined regimens including third-generation cephalosporins or carbapenems as a broad-spectrum regimen when compared with regimens containing aminoglycosides for coverage against Gram-negative bacteria. This definition was based on previous reports indicating that empirical treatment using a third-generation cephalosporin for Gram-negative coverage induces significantly more antibiotic resistance than regimens containing an aminoglycoside.[132] If two similar regimens were compared, the regimen with the broadest spectrum was labelled broad-spectrum.

We defined the neonatal period as up to 44 weeks PMA if the neonate was born prematurely. NEC was defined as Bell's stage 2–3.[227] IFI was defined as fungaemia or detection of fungi in otherwise sterile body sites. Death as an adverse outcome was defined as any cause of death, including death attributed to infection during antibiotic therapy in the neonatal period or up to discharge from the neonatal unit. Gut microbiota analyses were based on faecal samples using both standard culture-based methods and culture-independent methods relying on DNA amplification and sequencing. We decided to present data on the gut microbiota in three main categories acknowledging some clear overlap; i) microbial load, ii) microbial diversity, and iii) microbial composition. We defined microbial load as the total number of bacteria in a sample, microbial diversity as the number of different bacterial genus or species in a sample, and microbial composition as the taxonomic composition in a sample.

Antibiotic resistance development was based on detection of antibiotic susceptibility patterns in bacteria isolated from blood, urine, CSF, faeces, tracheal aspirates, or the skin surface. We defined MDR bacteria as bacteria resistant to either ≥ 2 unrelated classes of antibiotics or broad-spectrum antibiotics. Included in this category were carbapenem resistant *Acinetobacter baumannii*, ESBL-producing Gram-negative bacteria, and other third-generation cephalosporin resistant Gram-negative bacteria. Antibiotic-resistant bacteria that did not meet any of these criteria were defined as 'other antibiotic resistant bacteria'.

3.5   Audiology Assessment

In Paper 2, all infants were screened for ototoxicity with a transient-evoked OAE test (Madsen, AccuScreen, GN Otometrics, Denmark) before discharge. Prior to 2007, a risk based screening approach was used, including all neonates treated with gentamicin. Since January 2007, OAE has been implemented as a universal screening test for all newborn infants. Patients who failed OAE screening had an automatic ABR test as the first follow-up test. Further follow-up was then individualised in the audiology unit. We carefully reviewed hearing data for all patients referred for follow-up. An experienced audiologist reassessed all cases with possible persistent hearing problems. To ensure that no patients with severe ototoxicity were missed, the audiologist also identified all children who went on to have hearing aids or cochlear implants and were born during the audit period. Furthermore, all patient at risk for neurological sequelae (GA <32 weeks, VLBW, or severe perinatal asphyxia) were seen at regular intervals in the outpatient clinic up to 2 years of age, and sensory impairment was recorded.

3.6   Assessment of Methodological Quality

In Paper 3 and 4, the methodological quality of included studies was assessed by using the Cochrane Handbook of Systematic Reviews of Interventions and recently published recommendations on how to assess risk of bias and confounding in observational studies.[219, 228] Five domains related to risk of bias were assessed for each study included: selection bias, performance bias, detection bias, reporting bias, and confounding. Risks of bias were judged as low, high or unclear for each domain (Appendix 9.1). The risk of reporting bias was considered unclear in studies that did not have a previously published protocol. The risk of detection bias was considered high in studies that examined the gut microbiota with culture-based methods, unclear in studies that applied 16S rRNA sequencing techniques, and low in studies that applied shotgun metagenome sequencing techniques. Two reviewers (CK and either EE or JWF) assessed the risks of bias for each study. In Paper 4, we applied the GRADE approach to evaluate the QoE for each relevant outcome category.[223]

3.7   Statistical Analyses

Paper 1 and 2

Data were analysed using IBM-SPSS (IBM, Armonk, NY) statistical software, versions 20 (Paper 2) and 22 (Paper 1). Continuous variables are expressed as mean (standard deviation (SD)) if variables were normally distributed or median (interquartile range (IQR)) if variables were not

normally distributed. Categorical variables are displayed as frequency (%). Paper 2 is purely descriptive, and we did not test any variables for statistical significance.

In Paper 1, interval data were tested for normality using the Shapiro–Wilks test. Paired t-tests were used to compare continuous data, and proportions were compared using χ^2 test. Correlation was calculated using Spearman correlation. We used Kruskal–Wallis to test differences between multiple groups. A post hoc analysis with Tamhanes T2 test, catering for unequal variances, was used to test differences between individual groups. We calculated the number of antibiotics that accounted for 90% of the total volume used. P values < 0.05 were considered statistically significant.

Paper 3

We classified studies according to their outcome categories, including comparisons of different categories of antibiotic therapy. In each outcome category, we combined adverse outcomes of interest from studies we considered sufficiently homogeneous to provide a meaningful summary and calculated combined effect estimates. Data entry and meta-analysis were performed using RevMan version 5.3 (The Nordic Cochrane Centre, Copenhagen, Denmark). In the meta-analyses, we pooled RCTs and non-randomized studies, the latter only if clinical baseline characteristics of patient groups that experienced different antibiotic exposures (categories i–iii) were similar and the studies reported dichotomous outcomes. Subgroup analysis was performed for RCTs and observational studies.

We quantified inconsistency between the results of the studies by using the I2 test. Interpretation of thresholds for statistical heterogeneity was as follows: I2 values between 0% and 40% might not be important, whereas higher I2 values may represent moderate (30%–60%), substantial (50%–90%) or considerable heterogeneity (75%–100%).[219] We calculated odds ratios (ORs) with 95% CIs for the outcomes of interest. We present the effect estimates by using the random-effect model due to assumption of clinical and methodological diversity among the studies, subsequently often leading to statistical heterogeneity. Most non-randomized studies are reported separately and were not pooled for meta-analysis because of marked clinical and methodological diversity regarding interventions, antibiotics used, study design, and reported outcomes.

Paper 4

The large heterogeneity in study designs, comparisons, and outcomes made it impossible to perform traditional meta-analysis of the included studies. Vote-counting methods can be used for studies that do not contain enough information to compute an effect size estimate but do contain information about the direction and the statistical significance of results, or that contain just the direction of results.[229] We therefore applied a vote-counting method to meta-analyse and investigate whether the different categories of antibiotic therapy had any effect on the outcomes of interest. Studies were classified based on whether they showed a reduction in the outcome measure, no effect, or an increase in the outcome measure. When appropriate, outcomes were presented in vote-count figures. The size of the squares in the vote-count figures were proportional to the relative number of infants included in that study.

3.8 Ethical Approval

The regional ethical committee approved the study leading to Paper 1 (2013/358/REK nord). The regional ethical committee also considered the retrospective study leading to Paper 2, but characterized this study as a "quality assurance project" (2013/713/REK nord). The study was consequently approved by the hospital institutional review board. Paper 3 and 4 did not require ethical approval as they were systematic reviews with no patient interactions and did not contain any confidential data.

4 Main Results

4.1 Paper 1

A total of 168 877 LB infants were born with GA ≥37 weeks in the catchment areas of the 20 units reporting data to the NNN during the three-year study period, and 10 175 of these (6.0%) were hospitalized in their first week of life. There were 91 cases of culture-confirmed EOS (0.54 per 1000 term LB infants) and 1447 cases classified as culture-negative EOS (8.57 per 1000 term LB infants). Among preterm infants (GA < 37 weeks), there were 50 cases of culture-confirmed EOS among 11 649 infants (4.29 per 1000 preterm LB infants). This gave a total incidence rate of 0.78 culture-confirmed EOS cases per 1000 LB in all infants, irrespective of GA.

Gram-positive bacteria caused 83 of 91 (91%) culture-confirmed EOS cases among term infants. Gram-negative bacteria caused 8 cases (9%). Figure 2 shows the distribution of EOS pathogens in blood cultures. GBS was the most frequently isolated pathogen, with an incidence of 0.31 GBS-EOS cases per 1000 term LB infants. Seven preterm infants also had GBS-EOS; the total incidence rate of GBS-EOS was 0.33 cases per 1000 LB infants in all infants, irrespective of GA. There was one single EOS-attributable death (GBS-sepsis) among of 91 cases of culture-confirmed EOS in term infants. Three patients with culture-negative EOS died; however, the primary cause of death was a non-infectious condition for all three.

Intravenous antibiotic treatment was commenced during the first week of life in 3964 out of 10 175 (39.0%) infants included in the study, corresponding to an incidence of 2.3% of term LB infants in Norway. Of these, 3725 (94.0%) commenced treatment within the first 72 hours of life. Among 3964 neonates receiving antibiotic therapy, 2128 (53.7%) were never diagnosed with a bacterial infection, but still received antibiotic therapy for a median (IQR) duration of 4 (3–5) days. Table 4 shows the regional variations in antibiotic consumption and EOS incidence, as well as differences in treatment depending on blood culture results and EOS diagnosis.

Empiric therapy consisted of an aminoglycoside and either benzylpenicillin or ampicillin in 3746 of 3964 cases (94.5%) (Table 4). Change of antibiotic regimen during the course of therapy was more frequent in the patients receiving benzylpenicillin with an aminoglycoside (66/724; 9.1%) compared with patients receiving ampicillin with an aminoglycoside (160/3022; 5.3%) (P < 0.001), but we observed no difference in mortality between these groups (aminoglycoside and benzylpenicillin: 9/724=1.2% versus ampicillin and aminoglycoside: 29/3022=1.0%; p=0.41).

Figure 2. Distribution of Bacteria in Blood Culture-confirmed EOS
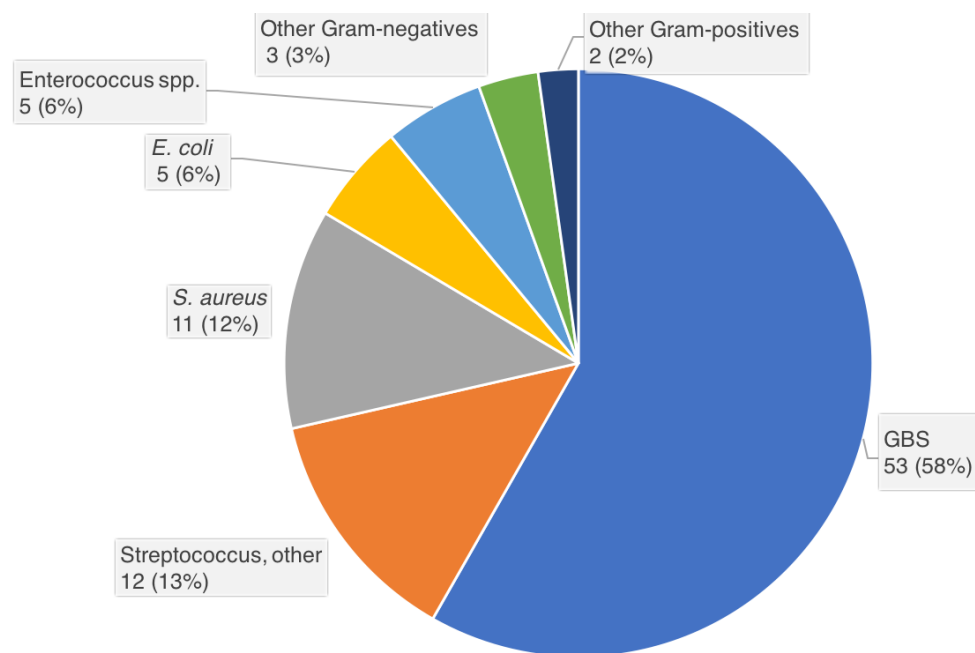
GBS; group B streptococci, *S. aureus*; *Staphylococcus aureus*, *E. coli*; *Escherichia coli*. Created using Microsoft Excel (version 15.40).

Table 4. Regional Variations in Incidence of EOS and Antibiotic Consumption

	South-East	West	Mid	North	*P* Value	Total
Patients, N	5444	2886	970	875		10,175
EOS, culture-confirmed n (%)	48 (0.9)	23 (0.8)	8 (0.8)	12 (1.4)	0.45	91 (0.9)
Intravenous antibiotics (d)	8 (6–10)	8 (7–10)	9 (7–13)	9 (7–12)	0.38	8 (7–10)
Prescription change, n (%)	22 (46)	11 (48)	4 (50)	6 (50)	0.99	43 (47)
Age at discharge (d)	8 (7–11)	8 (7–10)	9 (12–16)	12 (9–14)	0.01*	9 (7–13)
EOS, culture-negative, n (%)	867 (15.9)	282 (9.8)	162 (16.7)	136 (15.5)	<0.001†	1447 (14.2)
Intravenous antibiotics (d)	6 (5–7)	6 (5–7)	7 (7–8)	6 (5–7)	<0.001‡	6 (5–7)
Prescription change, n (%)	58 (6.7)	24 (8.5)	5 (3.1)	10 (7.4)	0.18	97 (6.7)
Age at discharge (d)	7 (6–8)	6 (5–8)	8 (7–8)	7 (6–8)	<0.001§	7 (6–8)
Not EOS, n (%)	4531 (83.2)	2581 (89.4)	800 (82.5)	727 (83.1)	<0.001†	8637 (84.9)
Intravenous antibiotics, n (%)	1291 (28.5)	728 (28.2)	215 (26.9)	192 (26.4)	0.55	2426 (28.1)
Intravenous antibiotics (d)	4 (3–5)	4 (3–5)	4 (3–7)	4 (3–5)	0.053	4 (3–5)
Prescription change, n (%)	71 (5.5)	37 (5.1)	10 (4.7)	14 (7.3)	0.49	132 (5.4)
Age at discharge (d)	4 (2–6)	4 (2–6)	5 (2–7)	4 (2–7)	<0.001¶	4 (2–6)
Empiric antibiotic treatment, n (%)	2206 (40.5)	1033 (35.8)	385 (39.7)	350 (38.9)	<0.001‖	3964 (39.0)
Ampicillin/aminoglycoside	2089 (94.7)	390 (37.8)	350 (90.9)	193 (56.8)	<0.001**	3022 (76.2)
Benzylpenicillin/aminoglycoside	8 (0.4)	601 (58.2)	5 (1.3)	110 (32.4)	<0.001**	724 (18.3)
Containing cephalosporin	72 (3.3)	21 (2.0)	8 (2.1)	21 (6.2)	0.002††	122 (3.1)
Days of antibiotic use per 100 admissions	233.0	195.4	287.1	247.2	<0.001‡‡	228.7

Interval data presented as median (interquartile range), nominal data presented as frequency (%). *P* values were calculated using Kruskal–Wallis test. We used Tamhane T2 test for post hoc analysis. Days of antibiotic use per 100 admissions were calculated as the mean days with antibiotic administration, multiplied by 100.
*Significant with Kruskal–Wallis test but not with post hoc analysis.
†Significant difference between west and the other regions.
‡Significant difference between south-east and west, south-east and north, west and mid, and mid and north.
§Significant difference between west and mid.
¶Significant difference between mid and west, and mid and south-east.
‖Significant difference between south-east and west.
**Significant difference between west and the other regions, and north and the other regions.
††Significant difference between mid and north.
‡‡Significant difference between west and the other regions, and south-east and mid.

4.2 Paper 2

We identified 546 treatment episodes from 457 neonates who had one or more gentamicin TPC registered during the 8-year study period. 37 episodes (37/546; 6.7 %) were excluded from final analyses on TPC and ototoxicity due to incorrect medical staff prescriptions. We included a total of 509 treatment episodes (≥ three doses gentamicin) belonging to 440 patients. For the whole study population, the mean (SD) GA was 36.4 (5.3) weeks and the mean (SD) BW was 2739 (1326) gram. There were 85 (19 %) patients with a very low birth weight (<1500 g) and 61 patients (14 %) with GA <29 weeks. Table 5 shows population and outcome data among the five different treatment groups.

The mean (SD) gentamicin TPC for all treatment episodes during the first week of life was 1.1 (0.5) mg/L and after first week of life 0.8 (0.6) mg/L. Figure 3 shows pharmacokinetic data on all 509 treatment episodes, divided by the five treatment groups. We observed a potential toxic TPC (≥2.0 mg/L) in 31/509 (6.1 %) treatment episodes. Of these, 22 were observed in group A and predominantly in children with perinatal asphyxia (n =13) or acute renal injury for other reasons, including congenital renal malformations (n =4).

Thirty-eight of 440 patients (8.6 %) failed the OAE screening before discharge and were referred for follow-up in the audiology unit. Four patients who failed their OAE test were suspected to have permanent sensorineural hearing loss, and one additional patient who passed the OAE test later received a cochlear implant. Two of these five patients probably have small unilateral hearing losses, two received hearing aids and one received a cochlear implant. Only one out of 31 patients with a TPC ≥ 2.0 mg/L suffered a permanent hearing loss, but this patient was also diagnosed with a congenital CMV infection.

Thirty-one of 37 treatment episodes with medical staff prescription errors involved ordering a 12-h too long interval. Mean (SD) TPC among these was 0.6 (0.4) mg/L, and none had a TPC ≥2.0 mg/L. Six treatment episodes were prescribed with too short intervals (12 h). Mean (SD) among these was 1.5 (0.9) mg/L, and in two episodes, TPC was ≥2.0 mg/L (33 %). We identified 81/509 (16 %) episodes with nursing staff errors regarding timing of administration. Gentamicin was administered too late in 59 episodes (mean (SD) TPC, 0.9 (0.4) mg/L) and too early in 22 episodes (mean (SD) TPC, 1.0 (0.5) mg/L).

Table 5. Treatment Groups, Population Data, and Audiology Assessment

	Group A PNA 0–7 days GA >36 weeks	Group B PNA 0–7 days GA 29–36 weeks	Group C PNA 0–7 days GA <29 weeks	Group D PNA >7 days CA ≥29 weeks	Group E PNA >7 days CA <29 weeks
Dosing intervals	Every 24 h	Every 36 h	Every 48 h	Every 24 h	Every 36 h
Treatment episodes (*n*)	250	61	48	138	12
First treatment in this treatment group	247	60	48	107	11
Patients (*n*)	247	60	48	83	2
GA (weeks)	39.9 (1.3)	32.6 (2.3)	26.0 (1.5)	33.9 (5.7)	24.1 (1.0)
CA (weeks)	–	–	–	38.0 (5.6)	26.5 (1.2)
BW (g)	3668 (630)	2087 (751)	790 (196)	2403 (1301)	661 (140)
Failed OAE	7 (3 %)	8 (13 %)	11 (23 %)	12 (15 %)	
Confirmed hearing impairment	1 (0 %)	2 (3 %)	0 (0 %)	2 (3 %)	

Values are presented as mean (SD)

GA gestational age, *PNA* postnatal age, *CA* corrected age (PNA + GA), *BW* birth weight, *OAE* otoacoustic emission test

Figure 3. Gentamicin Trough Plasma Concentrations in Treatment Groups
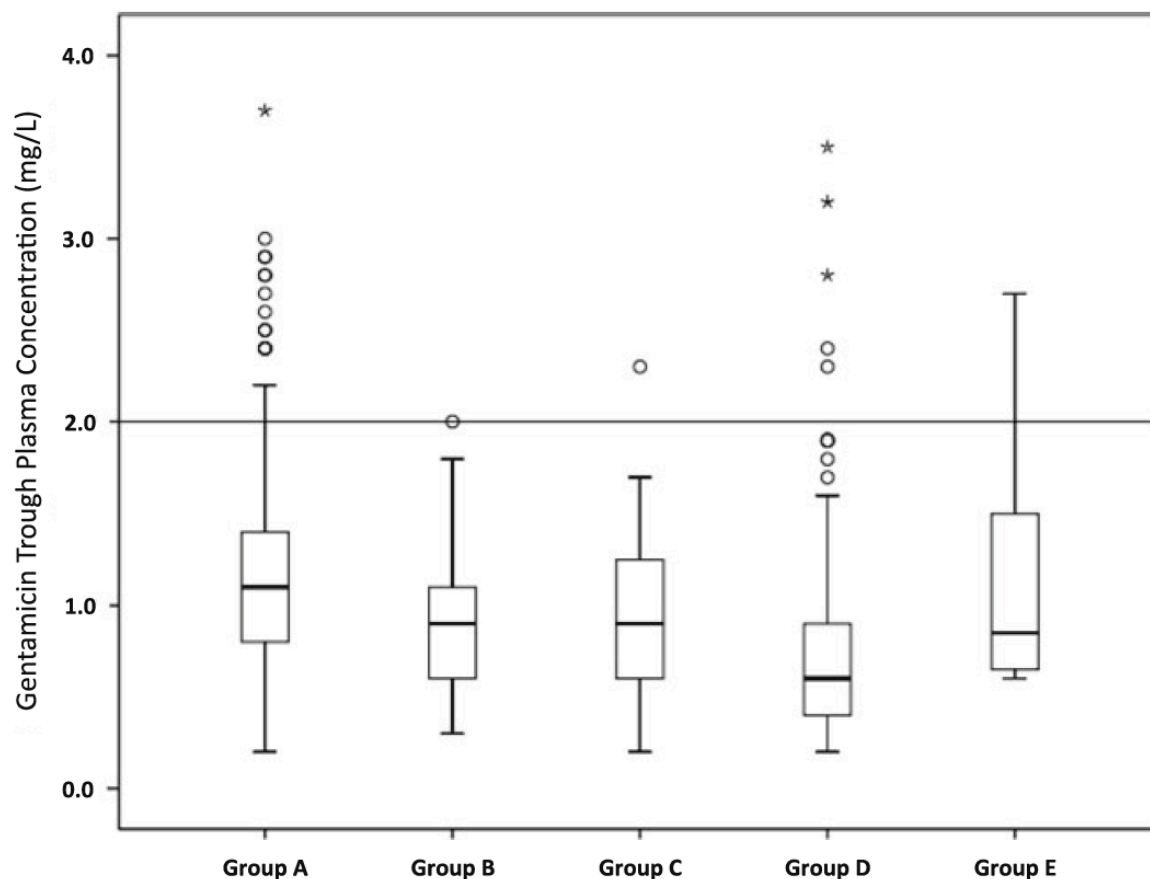
Box plots show median values (solid bar), interquartile ranges (margins of box), and 5 and 95 percentile (whiskers).

Created using IBM SPSS (version 20.0)

4.3 Paper 3

47 studies met our inclusion criteria: 9 RCTs[176, 230-237] and 38 observational non-randomized studies (Appendix 9.2).[1, 82, 118, 174, 238-271] There was a large diversity between the studies regarding antibiotics used, as well as onset and duration of antibiotic exposure after birth (Appendix 9.3 a-c). The majority of the included studies were judged to be of moderate to poor quality due to many risks of bias (Appendix 9.4 a-c).

In the NEC category, there were highly divergent results in the six studies comparing antibiotic therapy yes versus no,[82, 230, 234, 260-262] and between the seven studies comparing broad- versus narrow-spectrum antibiotic regimens.[174, 176, 236, 237, 243, 264, 268] There was no significant difference between antibiotics broad versus narrow regarding risk for NEC in the pooled analysis (Figure 4a). However, five studies comprising more than 5000 preterm infants showed significant associations between duration of antibiotic exposure and NEC or the composite outcome of NEC, LOS, or death.[82, 238, 241, 261, 262] In contrast, five studies did not show a significant difference in NEC rates.[242, 260, 263, 268, 271] However, one of these five studies (2502 neonates total) predominantly contained infants with GAs >34 weeks.[271] Moreover, three of these five studies (448 neonates total) showed a trend towards higher NEC rates in patients with prolonged antibiotic therapy, but all these studies were too small to detect significant differences.[242, 260, 268]

In the IFI category, twelve out of 15 studies reported an increased risk of IFI after broad-spectrum antibiotic treatment, mainly third-generation cephalosporins or carbapenems, compared with narrow spectrum treatment.[232, 233, 240, 246, 248, 253-257, 266-270] Five studies reported an increased risk of IFI following prolonged antibiotic therapy,[240, 247, 249, 251, 252] while eight studies found no significant difference.[231, 246, 248, 250, 253, 265, 268, 270]

In the mortality category, two studies, one of them extremely large (128 914 neonates), found an increased risk of death after broad-spectrum antibiotic treatment.[174, 243] However, seven studies found no difference between antibiotics broad versus narrow and there was no significant difference in the pooled analysis (Figure 4b).[1, 176, 233, 235, 236, 264, 268] Four studies containing 12 832 preterm infants reported an increase in mortality following prolonged antibiotic therapy,[82, 118, 241, 268] while seven studies containing 7506 neonates found no significant difference.[231, 238-240, 242, 263, 271] However, one of the larger studies (2502 neonates) showing no difference included predominantly term infants with a low risk of death.[271]

Figure 4. Forest Plots Stratified by Outcomes

(a) Pooled results of six studies comparing risk of NEC between neonates who received broad- versus narrow-spectrum antibiotic regimens
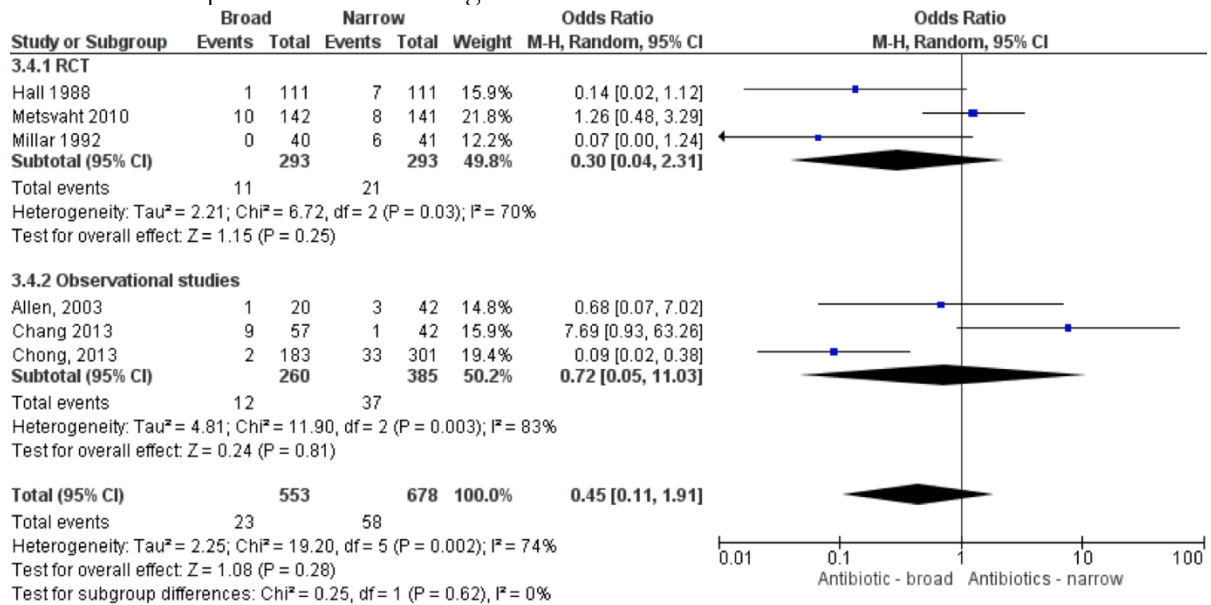
(b) Pooled results of eight studies comparing risk of death between neonates who received broader- versus narrower-spectrum antibiotic regimens
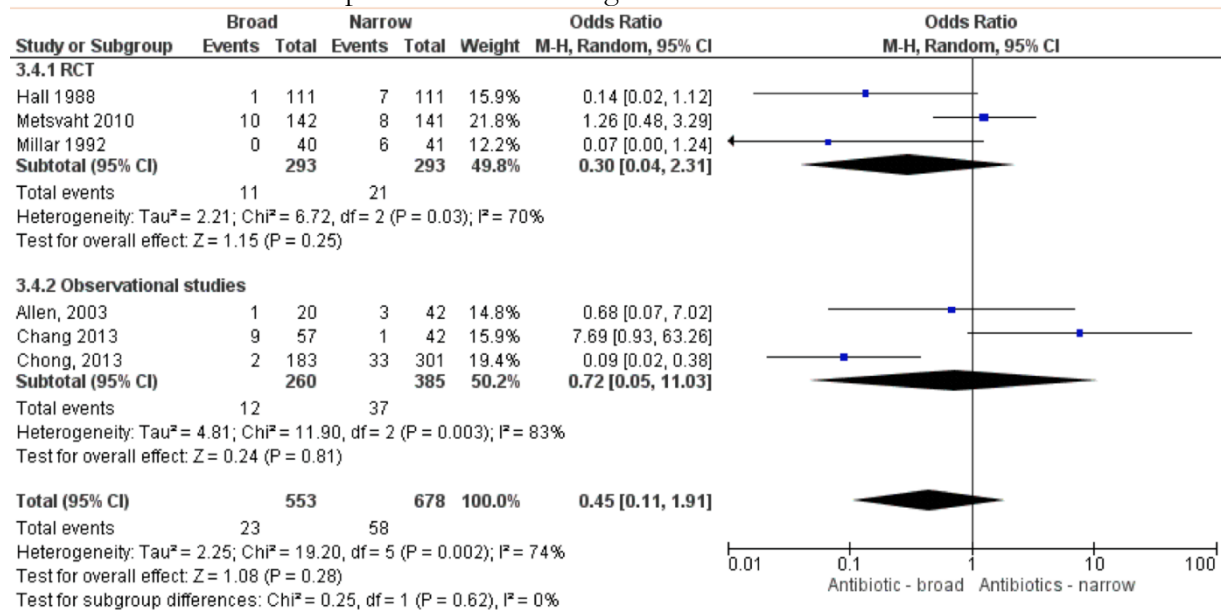
Subgroup analysis of RCTs and observational studies. The sizes of the squares are proportional to study weights.

Diamond markers indicate pooled effect sizes.

4.4 Paper 4

48 studies met our inclusion criteria: 3 RCTs[132, 272, 273] and 45 observational studies (Appendix 9.2).[179, 196, 215, 216, 263, 271, 274-313] The included studies were highly heterogeneous in both exposures and outcomes (Appendix 9.3 d & e). Moreover, a large proportion of studies had a high risk of bias, particularly selection bias, reporting bias, and confounding (Appendix 9.4 d & e).

Four studies examined the impact of antibiotic therapy on microbial loads with inconclusive results.[273, 279, 285, 286] Two out of four studies that compared antibiotic treatment yes versus no found reduced microbial diversity following antibiotic treatment.[196, 263, 285, 288] Three studies examined the impact of antibiotic therapy duration (long versus short) on microbial diversity and all three found decreased diversity following prolonged therapy.[286, 288, 291] Nine studies focused on Enterobacteriaceae; four reported an increase and five studies reported unchanged composition after antibiotic treatment (yes versus no), mainly ampicillin plus an aminoglycoside (Figure 5a).[179, 263, 278, 279, 281, 282, 285, 287, 290] Five studies focused on different commensal obligate anaerobes, showing a clear trend towards reduced colonization rates following antibiotic treatment.[280, 281, 283, 285, 287] Two studies found lower colonization rates of Enterobacteriaceae after treatment with third-generation cephalosporin compared with narrow-spectrum antibiotics.[282, 290] We graded the QoE as very low for outcomes in the gut microbiota category due to inclusion of observational studies with serious risk of bias and/or inconsistent results.

In the antibiotic resistance category, 20 out of 31 studies focused on MDR Gram-negative bacteria.[132, 215, 216, 271, 272, 274-277, 282, 292-312] Nine studies reported data after antibiotic treatment yes versus no, and seven of them reported increased rates of MDR Gram-negative bacteria following treatment.[216, 276, 296, 297, 299, 303, 307, 309, 310] Thirteen studies reported data after treatment with broad-versus narrow-spectrum antibiotics, and the overwhelming majority reported higher rates of MDR Gram-negative bacteria following treatment with broad-spectrum antibiotics (Figure 5b).[132, 215, 274-277, 292, 296, 298, 304, 305, 307, 311] Five studies reported data after long versus shorter duration of treatment, and four of them found significantly more MDR Gram-negative bacteria after prolonged treatment.[216, 271, 275, 297, 304] We graded the QoE as moderate for the outcomes relating to antibiotic resistance development due to inclusion of observational studies that either had large effect sizes or a dose–response effect.

Figure 5. Vote-Counts on Selected Outcomes Following Antibiotic Therapy

(a) Impact of antibiotic treatment (yes versus no) on Enterobacteriaceae

Study	Abundance and/or colonization rates			Specific outcome	Abundance or colonization rates
	Lower	Unchanged	Higher		
Arboleya, 2015		■		Enterobacteriaceae	Abundance
Bennet, 1986 & 1987			■	*Klebsiella/Enterobacter* spp.	Colonization rates
Blakey, 1982*		■		Enterobacteriaceae	Colonization rates
Bonnemaison, 2003*		■		Enterobacteriaceae	Colonization rates
Fouhy, 2012			■	Enterobacteriaceae	Colonization rates
Greenwood, 2014			■	*Enterobacter* spp.	Colonization rates
Hall, 1990		■		Coliforms	Colonization rates
La Rosa, 2014			■	Gammaproteobacteria	Abundance
Tullus, 1988	■			Enterobacteriaceae	Colonization rates

(b) Impact of broad-spectrum treatment (versus narrow) on MDR Gram-negative bacteria

Study	Infection and/or colonization rates			Risk estimates	Colonization or infection
	Lower	Unchanged	Higher		
Abdel-Hady, 2008			■	OR 4.9, 95% CI 1.1-21.5†	Infection
Acolet, 1994			■	NDA	Colonization
Calil, 2001			■	NDA	Colonization
De Araujo, 2007			■	NDA	Colonization
De Champs, 1994			■	NDA	Colonization
De Man, 2000			■	RR 3.14, 95% CI 1.76-5.56	Colonization
Le, 2008			■	OR 33.73, 95% CI 1.02-1136.20	Infection
Linkin, 2004			■	NDA	Infection
Mammina, 2007			■	NDA	Colonization
Millar, 2008		■		NDA	Colonization
Pessoa-Silva, 2003			■	OR 4.60, 95% 1.48-14.31	Colonization
Thatrimontrichai, 2013			■	NDA	Infection
Thatrimontrichai, 2016			■	OR 4.4; 95% CI 1.2-15.6	Infection

The sizes of squares are proportional to study populations. An asterisk symbolizes a lack of testing for statistical significance. A dagger symbolizes multivariate regression analysis.

5   Discussion

The studies included in this thesis focused on the epidemiology of EOS and antibiotic use in the first week of life in term born infants (Paper 1), the pharmacokinetics and potential toxicity of an extended-interval gentamicin dosing regimen in neonates (Paper 2), and clinical (Paper 3) and microbiological (Paper 4) adverse effects following neonatal antibiotic treatment.

We used different study designs in the different papers included in this thesis. Paper 1 is a registry-based study with clinical and demographic data from a Norwegian cohort of term infants. In Paper 2, we retrospectively collected clinical and pharmacokinetic data related to gentamicin therapy in term and preterm infants from a single NICU. Paper 3 and 4 were systematic reviews of RCTs and observational studies reporting adverse effects of antibiotic therapy in the neonatal period, and the reviews followed a previously published protocol.

5.1   Epidemiology of Early Onset Sepsis

Using data that included all Norwegian neonates born during a three-year period (Paper 1), we found an incidence rate of culture-confirmed EOS of 0.78 per 1000 LB infants. For term born infants, the incidence was 0.54 culture-confirmed EOS per 1000 LB infants. This rate is in line with data published from both England and the US. A UK multi-centre study reported an incidence rate of 0.70 culture-confirmed EOS cases (0-48 hours) per 1000 LB infants, regardless of GA.[41] In the US, incidence rates between 0.78-0.98 culture-confirmed EOS cases (0-48/72 hours) per 1000 LB infants have recently been reported, with an incidence of 0.58 cases per 1000 LB infants for neonates with GAs $\geq$ 34 weeks.[26, 30, 39] We applied a wider definition of EOS (0–6 days) than comparable studies, but the overwhelming majority of our EOS cases received treatment within the first 3 days of life.

In Paper 1, GBS was the most commonly isolated pathogen, with an incidence rate of 0.31 GBS-EOS per 1000 term LB infants. Including preterm infants, the Norwegian incidence rate was 0.33 GBS-EOS per 1000 LB infants. This is comparable with rates reported in US multi-centre studies (0.41 per 1000 LB infants and 0.22 per 1000 LB infants), a UK study (0.30 per 1000 LB infants), a Dutch nation-wide study (0.19 per 1000 LB infants), data from Sweden in 2009-2011(0.30 per 1000 LB infants) and data from a meta-analysis spanning several countries (0.43 per 1000 LB infants).[26, 30, 37, 38, 41] In accordance with guidelines from the Royal College of Obstetrics and Gynaecology in United Kingdom, Norwegian health authorities recommend a risk-based

approach to identify women who may benefit from IAP for prevention of GBS EOS.[49, 314] This is in contrast to the Centers for Disease Control and Prevention's guidelines who recommend universal rectovaginal GBS-screening and IAP for all colonized women.[50]

Studies from the US and Australia indicate that universal swab-based screening programs have lowered the rate of GBS EOS.[30, 48, 315] However, some authors report an unchanged overall rate of EOS with an increase in EOS caused by Gram-negative bacteria associated with higher mortality.[56, 316] In our study, the prevalence of *E. coli* and other Gram-negative EOS cases was very low and the rate of GBS EOS was similar to or lower than that reported in countries using universal swab-based screening programs. It is however, worth noting that our study consisted of term neonates that have a lower risk of Gram-negative EOS.[30] Additionally, the incidence rates of GBS EOS may also be affected by clones with increased virulence or epidemic potential.[38]

We found a low EOS attributable mortality among Norwegian term infants. Only one neonate (1%) died from culture-confirmed EOS after suffering from GBS sepsis. An additional three infants with culture negative EOS died, but none of these deaths were attributable to infection according to the Norwegian Cause of Death Registry. Other studies have found much higher mortality rates from EOS (11-16%), but these studies also included preterm infants, which is likely to be one major reason for the discrepancy.[26, 30] Indeed, other studies on term-born infants have reported mortality rates between 2-3% among EOS patients.[30, 42]

5.2 Antibiotic Consumption and Potential Implications

Overall, approximately 39% of hospitalized term infants in Norway received intravenous antibiotics at some point during first week of life, with regional variations ranging from 36% to 41% (Paper I). We have no explanation for the regional differences in antibiotic use. However, we have reasons to believe that regional differences may reflect differences in antibiotic policy, including the use of CRP to guide treatment, as it is not likely that these differences reflect disease severity in such a large, homogenous population-based study. In total, 2.3% of all term infants in Norway received intravenous antibiotics in the first week of life.

There are few other population-based studies examining antibiotic consumption in the neonatal population. In a selected population of newborns delivered at ≥34 weeks' gestation at the Kaiser Permanente Northern California network of hospitals, almost 6% of all infants received systemic antibiotics in the neonatal period, and an even larger proportion receive antibiotics in other US

hospitals.[112] There are no national Norwegian guidelines on when to start antibiotics in the newborn infant at risk of or with clinical suspicion of EOS. In contrast, the guidelines from the British National Institute for Health and Care Excellence (NICE) and the American Academy of Pediatrics (AAP) specifically address these issues.[94, 104] However, guidelines are often non-dynamic, challenging to follow and may lead to overtreatment.[317] Indeed, a study from the US reported that when using the Centers for Disease Control and Prevention's 2010 guidelines, 13% of all infants were evaluated for EOS and 11% were treated empirically with antibiotics, although only 0.04% of the cohort of infants had blood culture-confirmed infection.[87]

In retrospect, it is worth noting that 54% of the neonates who received antibiotics were not diagnosed with an infection. Only 91 neonates had an infection with demonstrable growth in blood-cultures, while ~1400 neonates were treated for an infection with negative blood-cultures. Considering that blood cultures with samples above 1 ml have been reported to have a sensitivity approaching 100% and that all included neonates in our study were term born, it is unlikely that a large proportion of these culture-negative cases were severe infections with false-negative blood cultures.[96] Overall, ~3 term neonates were exposed to intravenous antibiotics for each case of diagnosed, but unconfirmed infection, while ~44 neonates were exposed to antibiotics for every case of confirmed EOS that was treated. Escobar *et al.* used a stratification scheme based on maternal risk factors and objective neonatal clinical data to reduce the NNT to 118 per proven EOS case, while a study from 18 North American and European hospitals reported a NNT of 63 per proven EOS case.[90, 112] These findings therefore imply that Norwegian neonatologists are relatively judicious in their antibiotic use.

It is important to consider the potential side-effects of antibiotic treatment in light of the high rate of antibiotic exposure in neonates. Based on findings in Paper 4 we are moderately confident that neonatal antibiotic therapy increases the risk of antibiotic resistance development, in particular ESBL-producing Gram-negative bacteria and other MDR bacteria.[216, 276, 296, 297, 299, 303, 307, 309, 310] Antibiotics overuse may lead to increased antibiotic resistance through several mechanisms. Antibiotic resistance genes exist even in the absence of antimicrobial drugs, but antibiotics apply a direct selection pressure that gives significant advantages to bacteria expressing resistance genes.[318-320] A recent study reported that only a fraction of the enriched antibiotic resistance genes following antibiotic therapy are specific to the particular antibiotics given.[321]

Antibiotic treatment also contributes to changes in the human gut-associated resistome, which comprises numerous functional antibiotic resistance genes in the gut microbiota.[322] An antibiotic

induced increase in the gut resistome and decrease in colonization resistance could theoretically increase horizontal transfer of antibiotic resistance genes from commensals to potential pathogens.[323] Although in vivo horizontal transfer between commensals and pathogens in the gut microbiota remains to be proven, there is evidence of exchange of antibiotic resistance genes between environmental bacteria and human pathogens.[324]

We are less confident about our findings related to antibiotic therapy and changes in the gut microbiota (Paper 4). Neonatal antibiotic treatment was associated with an increased abundance and/or colonization rates of Enterobacteriaceae in four out of nine included studies, whereas none of the studies reported reduced abundance.[179, 263, 278, 279, 281, 282, 285, 287, 290] Neonatal antibiotic treatment was also associated with reduced colonization rates of protective commensal anaerobic bacteria such as bifidobacteria, lactobacilli, or bacteriodes in four out of five included studies.[280, 281, 283, 285, 287] It is possible that neonatal antibiotic therapy, regardless of treatment length, leads to reduced microbial diversity, but the studies included in this category were small and two out of four studies did not detect a significant difference.[196, 263, 285, 288]

All included studies in our systematic review (Paper 4) published prior to 2007 used culture-based techniques to examine the gut microbiota composition. It has been estimated that <20% of environmental bacteria can be grown in defined growth media.[325] However, sequencing-based techniques also have limitations. Studies relying on 16S rRNA analysis allow only a coarse sorting of bacteria, mainly at phylum level. Deep shotgun metagenome sequencing allows for finer distinction at the genus or species level, but it is of crucial importance to standardize sampling and temperature control during the pipeline up to DNA extraction in order to obtain valid results.[326] Moreover, bioinformatic presentations are often challenging to understand and interpret.

The frequent use of culture-based techniques added a significant detection bias for many of the included studies (Paper 4), and the high risk of bias in the included studies was often the reason for the very low QoE in the gut microbiota category. Nonetheless, our results are in line with findings in adult populations showing decreased diversity, reduced colonization rates of obligate anaerobes and increased colonization rates of Enterobacteriaceae following antibiotic exposure.[327-329] In contrast with the adult gut microbiota, the early-life gut microbiota is thought to be less resilient and more susceptible to antibiotic treatment, causing larger shifts in the microbial composition if antibiotics are administered in the neonatal period rather than later in life.[202]

5.3 Choice of Antibiotic Regimen

We found that approximately 95% of term infants in Norway received an aminoglycoside combined with either ampicillin or benzylpenicillin as their initial antibiotic therapy (Paper 1). We found no difference in overall mortality between the regimens, but because of low mortality the study was not powered for this comparison. It was slightly more common to change antibiotic regimen during the course of therapy for neonates that were started on penicillin and gentamicin. This was, however, a soft endpoint, and it may reflect differences in attitude and culture between neonatal units, as the choice of empiric antibiotic regimens in neonates are based on local policy in Norway.

To minimize harmful ecological effects of antibiotic therapy, some experts recommend using empiric therapy with the narrow-spectrum combination of benzylpenicillin plus gentamicin for suspected EOS.[94] Others, including the AAP, recommend ampicillin and gentamicin.[104] We included two papers based on the same RCT comparing ampicillin with benzylpenicillin in our systematic reviews (Paper 3 and 4).[176, 272] In this RCT the researchers found no differences in mortality, dysbiotic changes in gut microbiota, or development of MDR bacteria between the two regimens.[176, 272] However, this RCT was underpowered to detect clinical differences, and gut flora analysis was performed with conventional culture-based methods.

In Norway, GBS isolates are uniformly susceptible to both benzylpenicillin and ampicillin.[125] Neonatal listeria infection, a notifiable disease, is extremely rare in Norway, but listeria strains are often susceptible to benzylpenicillin. However, a steady rise in gentamicin resistance among *E. coli* blood culture strains in Norway (~6% in 2016) is of great concern.[125] Furthermore, 96% of gentamicin resistant *E. coli* isolates are also resistant to ampicillin. The prevalence of *E. coli* sepsis was low in our term infant population, but it is more frequent in preterm infants.[26] A further increase in gentamicin resistance could potentially threaten the value of gentamicin as Gram-negative back-bone coverage in the traditional empiric regimens.

The alternative to gentamicin-based regimens would be to use a more broad-spectrum antibiotic such as a third-generation cephalosporin, piperacillin-tazobactam, or a carbapenem. Norwegian *E. coli* blood culture isolates have similar resistance rates to cefotaxime (6%) as gentamicin, but in 2016 they were all susceptible to meropenem.[125] There are, however, findings in our systematic reviews (Paper 3 and 4) that indicate an increase in adverse effects following treatment with broad-spectrum antibiotics. First, there is evidence from ten observational studies that previous

exposure to third-generation cephalosporins or carbapenems is associated with an increased risk of developing IFI.[240, 246, 253-256, 266-269] Preterm infants are more prone to early colonization of fungi than term infants due to an immature immune system and impaired skin and mucosal integrity.[330] Broad-spectrum antibiotics may foster IFIs by suppressing normal flora and allowing fungi to occupy muco-epithelial niches that facilitate invasion and dissemination.[331] Cephalosporin use has been associated with intestinal colonization with Candida among neonates, and colonization is a risk factor for IFIs.[247, 250] Moreover, twelve out of 13 studies found a higher chance of infection or colonization with MDR Gram-negative bacteria in neonates who were treated with broad-spectrum antibiotics rather than narrow-spectrum antibiotics (Paper 4).[132, 215, 274-277, 292, 296, 298, 304, 305, 307, 311] Taken together, the results from Paper 3 and 4 imply that there are substantial data indicating that broad-spectrum antibiotics may pave the way for IFI and development of MDR Gram-negative bacteria. In light of these findings, it is reassuring that cefotaxime appears to be less commonly used for empirical EOS treatment than ten years ago.[119, 128]

5.4 Gentamicin Pharmacokinetics and Toxicity

Potential ototoxicity and nephrotoxicity has traditionally been a concern with aminoglycoside based regimens.[143] In neonates, this toxicity has never been proven, and aminoglycosides are not associated with increased rates of hearing loss with high-dose extended interval dosing regimens.[332] In our evaluation of a simplified high-dose extended-interval gentamicin regimen (Paper 2), we found that 6 % of all treatment episodes had a TPC ≥2 mg/l. This proportion is similar or lower than in most comparable studies,[148, 149, 162, 333, 334] but two studies reported even lower rates of potential toxic TPCs.[335, 336] In one of these studies, gentamicin was administered every 24 hours with 4 mg/kg to infants with a GA ≥35 weeks and 3 mg/kg to infants with a GA <35 weeks. All patients had TPCs < 2.0 mg/L, but 20 of the preterm infants with GA <35 weeks had PPCs < 6mg/L.[335] A dosing protocol from Christchurch, New-Zealand has complex dosing equations based on birth weight, leading to higher dose (mg/kg) and longer intervals (up to 60 h) for infants with the lowest body weight. In their evaluation of more than 1,000 TPCs, they reported high PPCs and low TPCs, but 87 % of all patients had only received one dose of gentamicin.[336]

Impaired renal function and high plasma creatinine values are well-known risk factors for high aminoglycoside TPCs.[146, 162] Accordingly, we found that most term infants in the first week of life with a TPC >2 mg/L had perinatal asphyxia and renal impairment (Paper 2). When renal failure is likely, it may be advisable to either check TPCs already before the second dose of gentamicin,

to routinely increase dosing intervals to 36 hours, or to use a different empiric antibiotic until renal function is clarified. In the NICU in Tromsø cefotaxime is routinely used for empiric treatment of infants with severe perinatal asphyxia, in particular infants undergoing hypothermia who are already at high risk for later hearing impairment.[146]

The gentamicin dosing regimen in this thesis (Paper 2) has a higher dosage (mg/kg) than what is commonly recommended for neonates. Higher peak levels most likely optimise the efficacy of gentamicin treatment. In contrast, there is little support in the literature for an association between high peak levels and toxicity in neonates.[161, 337, 338] A lack of data on peak gentamicin levels diminished our ability to fully assess the pharmacokinetic efficacy of our dosage regimen. However, in the NICU in Tromsø we felt it was unnecessary to continue measuring peak levels in this high-dose regimen after already having evaluated peak levels in a previous study.[146] Repeated blood tests for therapeutic drug monitoring increases the patient's pain and may cause clinically important blood loss. Furthermore, 75 % of the cost of gentamicin therapy is due to therapeutic drug monitoring.[339] Based on previous results from a study in Tromsø using the same dose (mg/kg) for netilmicin, and other studies using gentamicin 4–5 mg/kg, we would expect that the majority of peak levels with the current dosing regimen (Paper 2) are >10 mg/L.[146, 148, 149]

Newborn infants treated with aminoglycosides are at risk of developing hearing impairment. However, there are many other potential risk factors for hearing impairment including perinatal asphyxia, CMV infections, intracranial complications, congenital malformations, prematurity and treatment with loop diuretics.[161, 340, 341] A combination of more than one risk factor is often found in children who later develop hearing impairment. In one study, gentamicin did not seem to induce any ototoxicity, and in fact, a protective effect against ototoxicity was proposed.[158]

OAE is considered an effective screening test for detecting aminoglycoside-induced cochlear ototoxicity, but PPV is low due to low prevalence.[155] In Paper 2, 38 (8.6%) infants failed the OAE test. Only 4 out of 38 patients who failed the OAE tests were later diagnosed having permanent hearing impairment, and all four had TPCs < 2 mg/L. The only child who had a TPC ≥2 mg/L and acquired a hearing impairment passed the OAE test, but gradually evolved hearing impairment due to a congenital CMV infection. The low rate of hearing impairment among our high-risk intensive care infants, and in particular among patients with potential toxic TPCs, is a strong indication that gentamicin treatment is safe. Long-term follow-up studies with detailed hearing evaluation are still needed to confirm this.

We did not perform serial creatinine measurement or analyse urinary biomarkers for detailed assessment of potential gentamicin nephrotoxicity. Gentamicin nephrotoxicity, however, is challenging to assess in the first week of life when plasma creatinine values are unstable and influenced by renal maturity and changes in systemic circulation of sick neonates.[342] Furthermore, it seems that in neonates, aminoglycosides rarely induce clinically relevant renal injury in a normal course of treatment when TPC is in a safe range.[147] In contrast, when infants have high TPCs gentamicin is often discontinued as these infants usually already have an impaired renal function and one does not want to further exaggerate this with gentamicin.

Gentamicin is one of the drugs most commonly associated with prescription errors in the paediatric setting, increasing the risk of high TPCs.[343] Simpler dosing protocols are associated with less prescription errors.[165] In Paper 2 we found that 93 % of all treatment episodes were correctly prescribed. Among the cases where we detected prescription errors, almost 2/3 were made in preterm infants after the first week of life, leading to a too large dosing interval and less potential toxicity. It is likely that medical staff only considered the low GA and failed to recognise and assess the PNA. Improvements in education of medical staff may reduce such errors.

5.5 Prolonged Antibiotic Therapy

In our epidemiological study of Norwegian term infants (Paper 1) median treatment duration was 8 (7–10) days for culture-confirmed EOS and 6 (5–7) days for culture-negative EOS. In contrast, a study from Switzerland reported a substantially longer duration of antibiotic treatment (mean 13 days) for infants with confirmed infection.[344] The AAP guidelines recommend a minimum of 10 days treatment for culture-confirmed sepsis, while the NICE guidelines recommend a minimum of 7 days for culture-confirmed sepsis and culture-negative neonates with a strong clinical suspicion of sepsis.[94, 104] We believe that the low mortality among term infants in Paper 1 indicates that most infants with culture-confirmed EOS can be treated safely with 7–10 days systemic antibiotics, and that a shorter course may be appropriate for culture-negative EOS with rapid clinical improvement.

Recent guidelines on neonatal sepsis emphasize the importance of stopping antibiotics after 36–48 hours if there is no longer suspicion of sepsis.[94, 104] In Paper 1, 26% of all admitted infants received a median of 4 days antibiotics without being diagnosed with an infection. Furthermore, it is likely that among the infants in our study diagnosed with a culture-negative EOS there were a substantial number of infants not being truly infected, but still treated with a 5–7-day course of

antibiotic therapy. In many of these cases it is therefore likely that treatment could have been safely stopped several days earlier.

Stopping antibiotics some days earlier would shorten the average length of stay in the neonatal unit, leading to a significant reduction in hospital expenditures. Further advantages are reductions in maternal–infant separation and the pain for the infants associated with frequent blood samples and insertion of intravenous lines. However, in spite of guidelines emphasizing early cessation of antibiotics if sepsis is ruled out, the effects of guidelines may be different. A recent report showed that after implementing NICE guidelines, more investigations and increased length of stay were observed in newborns with suspected EOS when following the new guidelines.[317]

Prolonged antibiotic treatment was associated with several adverse effects in our systematic reviews (Paper 3 and 4). First, five observational studies including around 5000 infants showed that prolonged duration of antibiotic exposure for uninfected preterm infants is associated with an increased risk of developing NEC later in the neonatal period (Paper 3).[82, 238, 241, 261, 262] NEC has previously been associated with dysbiotic changes in the gut microbiota such as low diversity, overgrowth of Proteobacteria and decreased abundance of obligate anaerobic bacteria from the Bacteriodetes and Firmicute phyla.[193, 196] In Paper 4, prolonged antibiotic therapy seemed to reduce gut microbial diversity, but QoE according to GRADE evaluations was very low.[286, 288, 291] We did not find any conclusive evidence that prolonged antibiotic treatment caused more changes in the abundance of specific gut bacteria than shorter treatment durations, but very few studies examined this.[263, 284, 291, 313] However, shorter courses of antibiotic therapy are associated with a more rapid recovery from suppression of the gut microbiota.[263, 345]

Several biological mechanisms have been proposed to explain the association between gut dysbiosis and the massive gut inflammatory response seen in NEC. NEC cases have been reported to have an overexpression and dysregulation of TLR4.[81] An increased abundance of Enterobacteriaceae could lead to overexpression and increased activation of TLR4, resulting in the excessive inflammation that characterizes NEC. Antibiotic-induced killing of obligate anaerobes can potentially also lead to an increased abundance of Enterobacteriaceae due to a loss of colonization resistance.[201] It is also well known that bifidobacteria may reduce expression of inflammatory response genes and stimulate genes promoting the integrity of the mucosal barrier.[346] Moreover, certain lactobacilli appear to lower the inflammatory response from LPS stimulation, and these factors might explain why probiotics are associated with lower risks of

NEC.[72, 347] There were, however, few studies included in paper 4 that examined the impact of prolonged antibiotic therapy specifically on Enterobacteriace or commensal anaerobes.

Prolonged antibiotic treatment was also associated with an increased risk of colonization or infection with MDR Gram-negative bacteria, and this outcome had a moderate QoE (Paper 4).[216, 271, 275, 297, 304] We also found an association between prolonged antibiotic therapy and the risk of death in four studies including very preterm infants (Paper 3).[82, 118, 241, 268] Two of these studies were extremely large retrospective cohorts with a total population of 12 863 VLBW infants. They specifically examined the impact of antibiotic treatment for uninfected neonates.[82, 118] In contrast, seven studies found no significant difference, but many of these studies were small or largely contained term infants with a lower risk of death. It is possible that the associations between prolonged treatment and mortality were statistical anomalies, as even small differences can produce p-values <0.05 if the study population is large enough. On the other hand, it is possible that the studies that did not find a significant difference were underpowered to detect an actual difference. If it happens to be real, there are several possible explanations for an association between prolonged antibiotic treatment and mortality in uninfected neonates, including higher risk of NEC, LOS, IFI, infection with MDR bacteria, or immune-related diseases secondary to a certain degree of immune suppression.[182]

We did not study the impact of prolonged gentamicin treatment on potential hearing loss in Paper 2. This was due to both the very low incidence of permanent hearing loss in the study population and also the low rate of prolonged gentamicin therapy (≥ 5 days). Other studies have examined the relationship between prolonged gentamicin treatment and hearing loss, and a recent cohort study detected a non-significant trend for increased rates of hearing loss following gentamicin treatment ≥ 5 days compared with shorter durations of treatment.[332]

5.6 Methodological and Ethical Considerations

5.6.1 Registry-Based Cohort Studies

Norway has several nationwide medical registries that cover practically the entire population.[348] The NNN is one of the newer nationwide medical registries in Norway, and has covered all Norwegian neonatal units since 2011. Nationwide registries enable medical research on large cohorts over long time periods, which is especially useful when studying rare diseases such as neonatal sepsis. Indeed, the main strength of Paper 1 was the population-based design that

captured approximately 97% of all term LB infants admitted to a neonatal unit in Norway during the 3-year study period. This large and unselected study population minimizes the risk of selection bias.

The main limitation of registry-based studies is that data has already been collected when the study is planned. This could potentially increase the risk of detection bias as the researcher depends on the judgments of multiple clinicians for the accuracy of outcomes, as well as their zealousness in reporting exposures. In Paper 1, we relied on a substantial number of clinicians performing the daily web-based registration in the NNN and concluding with diagnoses at discharge. However, the data in the NNN was registered prospectively and the data on antibiotic therapy was registered on a daily basis in the NNN. This makes underestimation of treatment length unlikely. We also took steps to verify the outcome data we collected from the NNN by comparing it to data from other Norwegian public registries. In fact, the NNN managed to capture all cases of GBS EOS in term infants according to data from the Norwegian Surveillance System for Communicable Diseases. We also confirmed diagnoses of culture-confirmed EOS by examining blood culture results.

The diagnosis of culture-negative sepsis (P36.9) is particularly controversial, and the definition proposed by Norwegian neonatologists was not universally followed in NNN. Data on CRP levels that could have supported or refuted a clinical sepsis diagnosis were not included in the NNN during the study period. In addition, it was difficult to determine whether skin flora isolates in blood cultures were causes of actual infection or contaminants in a registry based study. We chose to define all skin isolates as blood culture contaminants, in line with a comparable US study.[30] It is possible, however, that some cases of CoNS bacteraemia represented true infections, despite our entire population being term born. We also lacked information on maternal risk factors for EOS, such as maternal fever, rupture of membranes, and chorioamnionitis, which we could have added in a truly prospective study.

5.6.2 Retrospective Cohort Studies

Retrospective cohorts are possible to perform when medical records allow accurate assessment of both exposures and outcomes without any additional data collection.[218] Retrospective cohorts are, similarly to registry-based cohort studies, cheap and data can be collected rapidly. Paper 2 was a retrospective cohort study. Paper 2 was, to our knowledge, the largest study ever to analyse an extended-interval gentamicin dosing regimen in neonates that included infants with all GAs

and a large number of infants with PNAs of at least one week. These data were population based for infants born in the two northernmost counties in Norway with GA < 34 weeks or requirement of mechanical ventilation. Again, this minimized the risk for selection bias.

In Paper 2, the retrospective nature of the study made it difficult to fully assess all levels of ototoxicity. Infants with severe hearing impairment were identified, but we may have missed less severe ototoxicity in the neonates who were born towards the end of our study with less than 21 months of observation. While OAE is an effective screening tool for detecting hearing loss, it is possible that high-frequency hearing loss, which was not clinically apparent may have been missed.[155] These issues increased the risk of detection bias. We are currently performing a prospective long-term follow-up with a complete audiological assessment of the same cohort now in the age between 5-15 years in order to get an even more reliable assessment of whether this high-dose and extended interval regimen has an ototoxic potential (ClinicalTrials.gov identifier: NCT03253614).[349]

5.6.3 Systematic Review Methodology

The primary strengths of our systematic review (Paper 3 and 4) were our rigorous and sensitive search strategy. The fact that we published our study protocol in advance of the reviews themselves increased transparency and shows that our research questions and methodology were decided a priori. We also used two to three authors to decide whether to include or exclude studies based on our protocol, and to evaluate the methodological quality of included studies based on a modified version of the Cochrane Handbook.[219, 228] This reduced the risk of mistakes causing deviations from protocol.

The main challenges for both reviews were the low number of RCTs, and the heterogeneity in study designs, sample sizes, outcomes, categories of antibiotic treatment and methodological quality. These challenges meant that traditional meta-analysis was only possible for a small subset of studies in Paper 3 and that we had to use the vote-counting method in Paper 4 to assess the effect of neonatal antibiotic treatment on relevant outcomes. The vote-counting method has limitations as it usually fails to account for the population size and methodological quality of pooled studies. Nevertheless, vote-counting may be an effective method to assess the ranking of outcomes.[350] Moreover, we attempted to improve the method by presenting the differential weight of each study with squares corresponding to sample size.

Observational studies are prone to biases and confounding, and many of the included studies attempted to adjust for confounders, such as risk factors and illness severity, through multivariable regression analysis. This reduced the risk of random findings in our reviews, but we cannot rule out residual confounding and confounding by indication: sicker neonates receive more antibiotics, but antibiotic exposure does not make them sicker.[118] According to the GRADE approach, evidence from observational studies is usually considered to be of low quality.[223] However, well-designed observational studies have been shown to provide similar results to RCTs and they can therefore be useful for detecting rare adverse outcomes by allowing larger sample sizes and longer lengths of follow-up than RCTs for lower costs.[351] We included observational studies due to our intention to collect as much evidence related to our research questions as possible.

The evidence of a significant association between prolonged duration of antibiotic therapy and increased risk of NEC and/or death is mainly supported by retrospective studies in preterm infants, and we cannot conclude that there is a causal relationship. This also applies to the association between broad-spectrum antibiotics and increased risk of IFI. However, antibiotic exposure was identified before the outcomes and cohort studies potentially have a temporal framework to assess causality. We decided a priori to include studies with both term and preterm infants as we anticipated that some studies would include a mix of both, and we did not want to exclude these. Term infants, however, rarely develop NEC and IFI, and have a low mortality in general. The differences in study populations therefore need careful consideration when interpreting the results of our systematic review. Based on studies in Paper 3, we believe that it is possible to draw conclusions about the association between antibiotic exposure and early adverse outcomes in preterm infants, whereas data on NEC, IFI, and death are more limited in term infants and do not justify clear conclusions. We feel more able to draw conclusions in term infants regarding changes in gut microbiota and antibiotic resistance development (Paper 4), as these changes are not exclusive to preterm infants.

In Paper 4, we used the GRADE approach to assess the QoE. Overall, we graded the QoE as very low for all outcomes presented in the gut microbiota category. In contrast, we considered the QoE to be moderate in the antibiotic resistance category owing to large effect sizes and a dose–response effect. Based on current evidence we are therefore moderately confident that all types of antibiotic treatment lead to increased rates of antibiotic resistance. We felt that the GRADE approach strengthened our interpretations in Paper 4, and the fact that we did not use this method in Paper 3 is a limitation.

We also acknowledge that our definition of broad-spectrum and narrow-spectrum antibiotics is somewhat arbitrary as most of the narrow-spectrum regimens covered both Gram-negative and Gram-positive bacteria. However, Paper 4 confirms previous findings, clearly suggesting that antibiotic regimens containing third-generation cephalosporins or carbapenems are more frequently associated with antibiotic resistance development than regimens with aminoglycosides for Gram-negative coverage.[132, 215, 274-277, 292, 296, 298, 304, 305, 307, 311] Finally, we decided to exclude studies from Paper 3 and 4 that only examined antenatal antibiotic treatment, despite the frequent use of IAP for prevention of neonatal infections and its reported effects on the infant gut microbiota and carriage of antibiotic resistance genes.[352] The focus of these reviews was neonatal antibiotic treatment given for suspected neonatal infection, and the isolated effects of antenatal antibiotics given to infants who did not receive antibiotics after birth were beyond the scope of these studies.

5.6.4 Ethical Considerations

None of the studies that formed this thesis were ethically controversial. Papers 3 and 4 were systematic reviews of already published studies, and as such there were no ethical aspects to consider. Paper 1 was based on the NNN, and all the information in this registry was anonymized. We chose to contact neonatal units for blood culture results for patients with a diagnosis of culture-confirmed EOS when blood culture results were missing in the NNN, but we did not directly access confidential information. This study was approved by the regional ethical committee.

Paper 2 was based on medical records, and there was no contact with study subjects. We did, however, need to access to confidential information to collect data for the study. Access to confidential patient information is regulated by the Health Personnel Law in Norway. However, the ability to grant dispensation to access confidential information for medical research is delegated to the Regional Ethical Committees. The Regional Ethical Committee considered in their feedback to the study protocol that our study was a "quality assurance project", and they suggested that we only needed approval from the institutional review board. The institutional review board granted us access to this data. Information that could be traced back to individual patients was stored separately and safely and was not part of the published study.

6 Conclusions

- The incidence of culture-confirmed EOS in term born infants was low in Norway (0.54 per 1000 live-born term infants), and in line with comparable reports from other developed countries. Gram-positive bacteria caused 90% of culture-confirmed EOS, and GBS was the most common causative pathogen. The EOS-attributable mortality rate was very low (1%).

- Of all Norwegian term infants, 2.3 % were treated with antibiotics in the first week of life, primarily with an aminoglycoside and either penicillin or ampicillin. Over half of these were never diagnosed with an infection. Guidelines commonly recommend ending treatment if blood cultures are negative after 36-48 hours, but the median treatment length was 4 days for neonates that received antibiotics without infection and 6 days for infants with culture-negative EOS.

- We found no evidence for ototoxicity from gentamicin treatment following a high-dose extended interval regimen. Only 6% of trough plasma concentrations were above the commonly recommended 2 mg/L threshold. Our simplified dosing regimen resulted in a low number of prescription errors.

- Prolonged antibiotic therapy was associated with an increased risk of NEC and/or death in preterm infants and broad-spectrum antibiotics were associated with an increased risk of invasive fungal infections.

- All types of increased antibiotic exposure in the neonatal period, whether it was antibiotics versus no antibiotics, prolonged treatment versus shorter treatment, or broader-spectrum antibiotics versus narrower-spectrum antibiotics, increased the rates of colonization and/or infection with MDR Gram-negative bacteria (moderate quality of evidence.

- Neonatal antibiotic therapy, in general, appeared to induce various potentially disease promoting alterations in the gut microbiota, in particular a reduced microbial diversity and a reduction in "protective" commensal obligate anaerobes (very low quality of evidence).

7   Future Perspectives

While antibiotic can be life-saving, our findings strongly emphasize the need to reduce unnecessary antibiotic treatment in neonates. In addition, they illustrate that while Norwegian neonatologists are relatively judicious in their use of antibiotics, there remains further potential for reducing neonatal antibiotic exposure. Preventing infections, antibiotic stewardship, and knowledge-based use of today's antibiotics are central principles to avoid overuse and adverse outcomes related to antibiotic exposure in the neonatal period, and to maintain safe and effective treatment for those who need it.

In general, it is better to prevent rather than treat disease. Development of a GBS vaccine could potentially reduce rates of EOS and the amount of antibiotics neonates are exposed to. Until such a vaccine is developed however, the debate on whether to use a universal screening approach or a risk-based approach for IAP would be greatly informed by studies that directly compare their effectiveness. It is possible that a large amount of IAP exposure causes more harm than benefit for neonates, and a systematic review of the potential adverse effects from IAP treatment would be an important step in determining this.

Development of new diagnostic tools could lead to a faster and more precise diagnosis of neonatal sepsis, which in turn would reduce antibiotic exposure for healthy neonates. As it remains difficult to decide early on whether a neonate is truly infected or not with current diagnostic tools, it is vital to find safe ways to reduce unnecessary antibiotic exposure for neonates. Strategies that separate neonates into different risk categories for EOS appear to be promising in reducing the proportion of antibiotic treated neonates in a safe manner. Moreover, further studies could determine whether it is safe to withhold treatment for well-appearing neonates with maternal risk factors for EOS. Measures should also be taken to discontinue antibiotic treatment early (36-48 hours) if a clinically suspected infection is not confirmed.

It is important to restrict the empirical use of broad-spectrum antibiotic treatment. Aminoglycoside-based regimens cause less resistance than cephalosporin- or carbapenem-based regimens, but have often been thought to cause hearing loss and renal failure. While gentamicin in the neonatal period appears to be safe regarding ototoxicity in retrospective studies, prospective follow-up studies with audiometry testing could help to determine whether aminoglycosides cause subclinical hearing loss. Development of new antibiotics and new ways to

combat antibiotic resistance could ensure effective treatment for neonatal infections in the future as increasing resistance rates threaten the effectiveness of aminoglycoside-based regiments.

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9 Appendix

9.1 Risk of Bias Evaluation Charts

Antibiotic Systematic Review Data extraction sheet

- Manuscript Title:

- Authors:

- Year of Publication:

- Study design:

	Controlled study	**Observational study**
Randomised		
Non-randomised		
Prospective cohort		
Retrospective cohort		
Interrupted time series		
Nested case control study		
Case control study		
Before-after study		
Cross-sectional study		

- Number of patients enrolled:

- PICO (tick off relevant comparisons and selected outcomes, there may be > 1 outcome)

Population (P)	Intervention (I)	Comparison (C)	Outcomes (O)
Neonate	Antibiotic exposure Yes Long term Broad spectrum	Antibiotic exposure No Short term Narrow spectrum	Death in the neonatal period Neonatal fungemia Necrotizing enterocolitis Changes in gut microbiome composition Changes in development of antibiotic resistance

Risk of bias assessment

For each study, a risk of bias assessment was performed by one investigator using a tool based on the Cochrane handbook (Cochrane), which we adapted and clarified to also assess observational studies (Viswanathan M, 2013).

We categorised for each study the risks of bias as high, low or unclear

Selection bias:	High	Low	Unclear
Performance bias:	High	Low	Unclear
Detection bias:	High	Low	Unclear
Reporting bias:	High	Low	Unclear
Confounding :	High	Low	Unclear

Selection bias:
Controlled studies:
Low risk if random sequence generation and allocation concealment
Uncontrolled studies:
Low or high risk if patients had been enrolled or not enrolled as consecutively observed based on a pre-existent study protocol and if numbers and reasons for possible exclusions were reported or not reported specifically.
High risk when the association between exposure and outcome is different for those who participate compared with those who do not participate in a study (i.e., all those who are theoretically eligible). This includes inappropriate selection of controls in a case-control study, differential loss to follow-up for groups being compared (attrition bias), incidence-prevalence bias, nonresponse bias, and in- or exclusion of specific groups for study.

Performance bias
Controlled studies:
High risk if not blinding of the study personnel as to which intervention a neonate had received.
Uncontrolled studies:
High risk if systematic differences in the care provided to participants and protocol deviation. Examples include contamination of the control group with the exposure or intervention, unbalanced provision of additional interventions or co-interventions, difference in co-interventions, and inadequate blinding of providers and participants.

Detection bias
Controlled studies:
High risk if not blinding of personnel evaluating outcomes
Uncontrolled studies:
High risk if systematic differences in outcomes assessment among groups being compared, including misclassification of the exposure or intervention, covariates, or outcomes because of variable definitions and timings, diagnostic thresholds, recall from memory, inadequate assessor blinding, and faulty measurement techniques. Erroneous statistical analysis might also affect the validity of effect estimates.

Reporting bias
Controlled studies:
High risk if not reporting of the study's prespecified or expected outcomes of interest to the review. Including attrition bias; high risk if not completeness of reporting data, reason and balance across groups of missing data.
Uncontrolled studies:
High risk if systematic differences between reported and unreported findings (e.g., differential reporting of outcomes or harms, incomplete reporting of study findings, potential for bias in reporting through source of funding).

Confounding
Low risk if any attempt to (if necessary) to balance the design or allocation between the groups or match groups (e.g., through stratification, matching, propensity scores or other statistical adjustment such as instrumental variables) are done (When selection bias produces imbalances in prognostic factors associated with the outcome of interest then 'confounding' is said to occur. Statistical methods are sometimes used to counter bias introduced from confounding by producing 'adjusted' estimates of intervention effects, and part of the assessment of study quality may involve making judgements about the appropriateness of the analysis as well as the design and execution of the study)

Important confounding factors that should be similar between groups
- Age
- Feeding
- Disease severity
- Same/different environment (hospital, country)
- Antifungal prophylaxis used

9.2 Flowcharts detailing Study Selection Process

Paper 3:

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Flow diagram
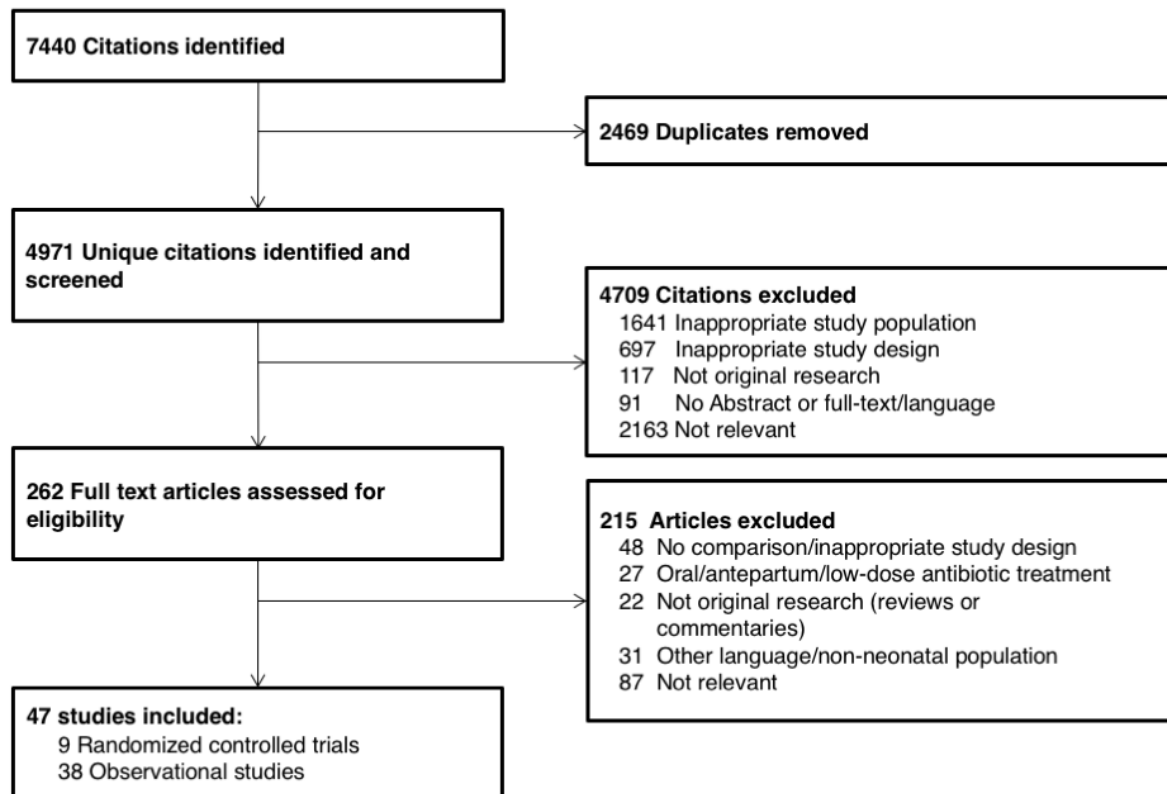
Paper 4

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Flow diagram

7379 Citations identified

3999 Duplicates removed

3380 Unique citations identified and screened

3243 Citations excluded
1626 Inappropriate study population
388 Inappropriate study design
69   Not original research
126 No Abstract or full-text/language
1034 Not relevant

137 Full text articles assessed for eligibility

89 Articles excluded
4   No comparison/inappropriate study design
9   Inappropriate study population
17 Not original research (reviews, commentaries or already included study populations)
1   Other language
58 Not relevant

48 studies included:
3   Randomized controlled trials
45 Observational studies

9.3 Tables Summarizing Main Characteristics and Results from Studies Reporting Early Adverse Outcome Following Neonatal Antibiotic Therapy

(a) Necrotizing Enterocolitis

Source	Design	N	GA and BW	Antibiotic exposure and risk of NEC		
				No vs Yes	Short vs Prolonged	Narrow vs Broader spectrum
Cantey et al., 2016 (USA)	Before-after	2502	All GAs	NDA	No difference	NDA
Greenwood et al., 2014 (USA)	Prospective cohort	74	GA ≤ 32 w	NDA	No difference	NDA
Chang et al., 2013 (S-Korea)	Retrospective cohort	99	BW < 1.5 kg	NDA	Prolonged use: ↑ risk of NEC	Broader spectrum: ↑ risk of NEC
Chong et al., 2013 (USA)	Retrospective matched cohort	484	BW 0.5-1.5 kg	NDA	NDA	Broader spectrum: ↓ risk of NEC
Shah et al., 2013 (Australia)	Retrospective cohort	216	GA < 28 w, survival > 3 d	NDA	No difference	NDA
Ghany et al., 2012 (Egypt)	Retrospective cohort	207	BW < 1.5 kg, survival > 5 d	NDA	Prolonged use: ↑ risk of NEC and/or death	NDA
Alexander et al., 2011 (USA)	Case-control	372	Preterm, mean GA 28 w	NDA	Prolonged use: ↑ risk of NEC	NDA
Kuppala et al., 2011 (USA)	Retrospective cohort	365	GA ≤ 32 w BW ≤ 1.5 kg	No difference	Prolonged use: No difference NEC (alone) ↑ risk of NEC, LOS or Death	NDA
Metsvaht et al., 2010 (Estonia)	RCT	283	All GAs	NDA	NDA	No difference
Tagare et al., 2010 (India)	RCT	140	Preterm, GA < 37 w	No difference	NDA	NDA
Cotten et al., 2009 (USA)	Retrospective cohort	4039	BW ≤ 1 kg, survival > 5 d	NDA	Prolonged use: ↑ risk of NEC and/or death	NDA
Wang et al., 2009 (USA)	Case-control	20	GA 25-32 w	NDA	Prolonged use: ↑ risk of NEC	NDA
Clark et al., 2006 (USA)	Retrospective cohort	128 914	All GAs (median GA 29 w)	NDA	NDA	Broader spectrum: ↓ risk of NEC
Allen et al., 2003	Retrospective cohort	62	BW < 1 kg, survival >4 d	NDA	NDA	No difference

(Canada)						
Krediet et al., 2003(Netherlands)	Case-control	208	All GAs, median GA 29 w	Early use: ↓ risk NEC	NDA	NDA
Harms et al., 1995 (Germany)	RCT	148	Preterm, mean GA 29 w	No difference	NDA	NDA
Millar et al., 1992 (England)	RCT	81	GA < 33 w	NDA	NDA	No difference
Mufti et al., 1992 (Pakistan)	Case-control	39	BW ≤ 2 kg	No difference	NDA	NDA
Hall et al., 1988 (England)	RCT	222	All GAs	NDA	NDA	No difference
Stoll et al., 1980 (USA)	Case-control	133	All GAs	No difference	NDA	No difference

GA; Gestational age, BW; birth weight, d; days, w; weeks, kg; kilogram, LOS; late-onset sepsis, NEC; necrotizing enterocolitis, NDA; no data available

(b) Invasive Fungal Infection

Source	Design	N	GA and BW	Antibiotic exposure and risk of IFI		
				No vs Yes	**Short vs Prolonged**	**Narrow vs Broader spectrum**
Fu et al., 2016 (China)	Case-control	96	BW < 1.5 kg	NDA	Prolonged use: ↑ risk of IFI	Broader spectrum: ↑ risk of IFI
Tewari et al., 2014 (India)	RCT	187	GA ≥ 28 w, BW ≥ 1 kg	NDA	NDA	No difference
Aliaga et al., 2013 (USA)	Retrospective cohort	709 325	All GAs	NDA	NDA	Broader spectrum: ↑ risk of IFI
Chang et al., 2013 (S-Korea)	Retrospective cohort	99	Preterm, BW < 1.5 kg	NDA	No difference	Broader spectrum: ↑ risk of IFI
Lee et al., 2013 (USA)	Retrospective cohort	530 162	BW > 1.5 kg	NDA	NDA	Broader spectrum: ↑ risk of IFI
Yu et al., 2013 (China)	Case-control	135	All GAs	NDA	No difference	Broader spectrum: ↑ risk of IFI
Ariff et al., 2011 (Pakistan)	Case-control	81	All GAs	NDA	No difference	Broader spectrum: ↑ risk of IFI
Benjamin et al., 2010 (USA)	Retrospective cohort	1515	BW ≤ 1 kg, survival > 3 d	NDA	NDA	Broader spectrum: ↑ risk of IFI
Benjamin et al., 2006 (USA)	Retrospective cohort	4579	BW ≤ 1 kg, survival > 3 d	NDA	NDA	Broader spectrum: ↑ risk of IFI
Cotten et al., 2006 (USA)	Retrospective cohort	3702	BW ≤ 1 kg, survival > 3 d	NDA	Prolonged use: ↑ risk of IFI	Broader spectrum: ↑ risk of IFI
Manzoni et al., 2006 (Italy)	Nested case-control	201	Preterm, BW < 1.5 kg	NDA	No difference	No difference
Feja et al., 2005 (USA)	Case-control	180	Preterm, mean GA 30 w	No difference	NDA	NDA
Linder et al., 2004 (Israel)	Case-control	112	Preterm, mean GA 28-29 w	NDA	NDA	No difference
Auriti et al., 2003 (Italy)	RCT	130	GA < 32 w	NDA	No difference	NDA
Benjamin et al., 2003 (USA)	Retrospective cohort	6172	BW < 1.25 kg, survival ≥ 3 d	NDA	NDA	Broader spectrum: ↑ risk of IFI
Pera et al., 2002 (USA)	Case-control	334	Preterm, BW < 1.25 kg	NDA	Prolonged use: ↑ risk of IFI	NDA
Warris et al.,	Case-control	24	GA ≤ 33 w	NDA	Prolonged use:	NDA

2001(Netherlands)					↑ risk of IFI	
Benjamin et al., 2000 (USA)	Case-control	51	Preterm, mean GA 28 w and BW 1.1 kg	NDA	NDA	Broader spectrum: ↑ risk of IFI
Saiman et al., 2000 (USA)	Prospective cohort	2847	All GAs, hospitalization ≥ 3 d	NDA	No difference	NDA
Singh et al., 1999 (India)	Prospective cohort	70	Preterm	Antibiotic use: ↑ risk of IFI	NDA	NDA
Lin et al., 1998 (Taiwan)	Case-control		BW < 1.5 kg, GA ≤ 33 w	NDA	No difference	NDA
Faix et al., 1989 (USA)	Prospective cohort	358	BW ≤ 1.5 kg	NDA	Prolonged use: ↑ risk of IFI	NDA
Weese-Mayer et al., 1987 (USA)	Case-control	41	All GAs, mean BW 1.9 kg and mean GA 32-33 w	NDA	Prolonged use: ↑ risk of IFI	NDA
Snelling et al., 1983 (England)	RCT	55	All GAs, mean BW 1.7 kg and mean GA 33 w	NDA	NDA	No difference

GA; Gestational age, BW; birth weight, d; days, w; weeks, kg; kilogram, LOS; late-onset sepsis, IFI; Invasive fungal infection, NDA; no data available

(c) Mortality

Source	Design	N	GA and BW	Antibiotic exposure and risk of death		
				No vs Yes	Short vs Prolonged	Narrow vs Broader spectrum
Cantey et al., 2016 (USA)	Before-after	2502	All GAs	NDA	No difference	NDA
Fjalstad et al., 2016 (Norway)	Retrospective cohort	10 175	GA ≥ 37 weeks	NDA	NDA	No difference
Ting et al., 2016 (Canada)	Retrospective cohort	8824	BW < 1.5 kg	NDA	Prolonged use: ↑ risk of death	NDA
Greenwood et al., 2014 (USA)	Prospective cohort	74	GA ≤ 32 w	NDA	No difference	NDA
Tewari et al., 2014 (India)	RCT	187	GA ≥ 28 w, BW ≥ 1 kg	NDA	NDA	No difference
Chang et al., 2013 (S-Korea)	Retrospective cohort	99	BW < 1.5 kg	NDA	Prolonged use: ↑ risk of death	Broader spectrum: ↑ risk of death
Chong et al., 2013 (USA)	Retrospective matched cohort	484	BW 0.5-1.5 kg	NDA	NDA	No difference
Shah et al., 2013 (Australia)	Retrospective cohort	216	GA < 28 w, survival > 3 d	NDA	No difference	NDA
Ghany et al., 2012 (Egypt)	Retrospective cohort	207	BW < 1.5 kg, survival > 5 d	NDA	Prolonged use: ↑ risk of death	NDA
Kuppala et al., 2011 (USA)	Retrospective cohort	365	GA ≤ 32 w, BW ≤ 1.5 kg	No difference	Prolonged use: No difference death (alone) ↑ risk of NEC, LOS or Death	NDA
Metsvaht et al., 2010 (Estonia)	RCT	283	All GAs	NDA	NDA	No difference
Tagare et al., 2010 (India)	RCT	140	Preterm, GA < 37 w	No difference	NDA	NDA
Cotten et al., 2009 (USA)	Retrospective cohort	4039	BW ≤ 1 kg, survival > 5 d	NDA	Prolonged use: ↑ risk of death	NDA
Clark et al., 2006 (USA)	Retrospective cohort	128 914	All GAs (median GA 29 w)	NDA	NDA	Broader spectrum: ↑ risk of death
Cotten et al., 2006 (USA)	Retrospective cohort	3702	BW ≤ 1 kg, survival > 3 d	NDA	No difference	NDA
Allen et al., 2003 (Canada)	Retrospective cohort	62	BW < 1 kg, survival >4 d	NDA	NDA	No difference

Auriti et al., 2003 (Italy)	RCT	130	GA < 32 w	NDA	No difference	NDA
Cordero et al., 2003 (USA)	Retrospective matched cohort	517	BW < 1 kg	NDA	No difference	NDA
Harms et al., 1995 (Germany)	RCT	148	Preterm, mean GA 29 w	No difference	NDA	NDA
De Louvois et al., 1992 (Europe)	RCT	1316	All GAs	NDA	NDA	No difference
Millar et al., 1992 (England)	RCT	81	GA < 33 w	NDA	NDA	No difference

GA; Gestational age, BW; birth weight, d; days, w; weeks, kg; kilogram, LOS; late-onset sepsis, NDA; no data available

(d) Gut Microbiota

Study	Design	N	GA and BW	Empiric regimen	Categories of antibiotic exposure and changes in gut microbiota
Arboleya et al., 2015 (Spain)	Prospective cohort	40	All GAs	EOS: AMP + GEN, LOS: VAN + AMK	Yes vs. no: <u>Composition</u>: ↓ *Staphylococcus* spp. & *Comamonadaceae*
Bennet et al., 1986 & 1987 (Sweden)	Prospective cohort	164	All GAs	NDA	Yes vs. no: <u>Load</u>: ↑; <u>Composition</u>: ↑ *Klebsiella/Enterobacter* spp., ↓ Anaerobes, ↓*Bifidobacterium* spp., ↓*Lactobacillus* spp., ↓*Bacteriodes* spp. Broad vs. narrow: <u>Composition</u>: ↑ *Enterococcus* spp., ↑ *S. faecalis*
Blakey et al., 1982 (Australia)	Prospective cohort	28	GA ≤ 36 weeks	EOS: PEN + GEN	Yes vs. no: <u>Composition</u>: No difference*
Bonnemaison et al., 2003 (France)*	Prospective cohort	30	All GAs	EOS: AMX + NET ± CTX	Yes vs. no: <u>Composition</u>: No difference Broad vs. narrow: <u>Composition</u>: No difference*
Butel et al., 2007 (France)	Prospective case-control	52	GA 30 - 35 weeks	NDA	Yes vs. no: <u>Composition</u>: No significant difference
Ferraris et al., 2012 (France)	Retrospective cohort	76	GA < 36 weeks	NDA	Yes vs. no: <u>Composition</u>: ↑ *C. butyricum* Long vs. short: <u>Composition</u>: ↓ *Clostridium* spp.
Fouhy et al., 2012 (Ireland)	Prospective cohort	18	GA ≥ 37 weeks	AMP + GEN	Yes vs. no: <u>Composition</u>: ↑ Enterobacteriaceae, ↑Gammaproteobacteriae, ↑ Peptostreptococcaceae, ↑ *Enterococcus* spp., ↑ *Clostridium* spp., ↓ *Lactobacillus* spp., ↓ *Bifidobacterium* spp., ↓ *Bacteriodetes*
Gewolb et al., 1999 (USA)	Prospective cohort	29	BW < 1000 g	EOS: AMP + GEN, LOS: VAN + CTX	Long vs. short: <u>Load</u>: ↓; <u>Diversity</u>: ↓
Goldmann et al., 1978 (USA)	Prospective cohort	63	All GAs	NDA	Long vs. short: <u>Composition</u>: ↑ *Klebsiella* spp., ↑ *Enterobacter* spp., and/or ↑ *Citrobacter* spp.
Greenwood et al., 2014 (USA)	Prospective cohort	74	GA ≤ 32 weeks	EOS: AMP + GEN	Yes vs. no: <u>Diversity</u>: ↓; <u>Composition</u>: ↑ *Enterobacter* spp. Long vs. short: <u>Composition</u>: ↑ *Enterobacter* spp., ↓ *Staphylococcus* spp.
Hall et al., 1990 (UK)	Prospective cohort	42	GA ≤ 33 weeks	NDA	Broad vs. narrow: <u>Composition</u>: ↓ *Lactobacillus* spp.
Jacquot et al., 2011 (France)	Prospective cohort	29	GA ≤ 30 weeks	EOS: AMK + (1) PEN or (2) AMP or (3) CTX, LOS: VAN + AMK	Yes vs. no: <u>Diversity</u>: No significant effect Long vs. short: <u>Diversity</u>: ↓
Jenke et al., 2013 (Germany)	Prospective cohort	68	GA < 27 weeks	NDA	Yes vs. no: <u>Composition</u>: ↑ *C. difficile*
La Rosa et al., 2014 (USA)	Prospective cohort	58	BW ≤ 1500 g	NDA	Yes vs. no: <u>Composition</u>: ↑ Gammaproteobacteria (GA ≥ 26 weeks), ↓ *Clostridium* spp. (GA ≤ 28 weeks)
Parm et al., 2010 (Estonia)	RCT	276	All GAs	EOS: (1) PEN + GEN or (2) AMP + GEN	Broad vs. narrow: <u>Composition</u>: ↑ *S. haemolyticus*, ↑ *S. hominis*, ↑ *K. pneumonia*, ↓ *Enterococcus* spp. ↑ *S. aureus*
Tullus et al., 1988	Retrospective	953	All GAs	AMP + GEN	Yes vs. no: <u>Composition</u>: ↓ *E. coli* Broad vs. narrow: <u>Composition</u>: No

(Sweden)	cohort				significant difference
Ward et al., 2016 (USA)	Case-control	166	All GAs	EOS: AMP + GEN	Long vs. short: <u>Diversity:</u> ↓
Westerbeek et al., 2013 (Netherlands)	RCT	113	GA < 32 weeks ± BW < 1500 g	NDA	Yes vs. no: <u>Load:</u> ↓
Zhou et al., 2015 (USA)	Case-control	38	GA < 32 weeks	NDA	Yes vs. no: <u>Diversity:</u> ↓

Outcomes: <u>Load;</u> the total number of bacteria in a sample, <u>Diversity;</u> the number of bacterial genus or species in a sample, and <u>Composition;</u> the taxonomical composition in a sample. Categories: **Yes vs. no** compares neonates exposed to antibiotics with non-exposed neonates, **Long vs. short** compares long and short treatment durations, **Broad vs. narrow** compares broad spectrum antibiotic treatment to narrow spectrum treatment. *; did not test for statistical significance, RCT; randomized controlled trial, GA; gestational age, PNA; post-natal age, BW; birth weight, g; gram, EOS; early onset sepsis, AMP; ampicillin, GEN; gentamicin, LOS; late onset sepsis, VAN; vancomycin, AMK; amikacin, NDA; no data available, PEN; penicillin, AMX; amoxicillin, NET; netilmicin, CTX; cefotaxime

(e) Antibacterial Resistance

Study	Design	N	Empiric regimen	Categories of antibiotic exposure and changes in antibacterial resistance
Abdel-Hady et al., 2008 (Egypt)	Prospective cohort	380	NDA	Broad vs. narrow: ↑ESBL producing *K. pneumoniae* infection
Acolet et al., 1994 (UK)	Case-control	60	EOS: AMX + CTX, LOS: CTX	Broad vs. narrow: ↑CREC colonization
Bergin et al., 2015 (USA)	Case-control	258	NDA	Broad vs. narrow: No significant difference
Bonnemaison et al, 2003 (France)	Prospective cohorts	30	EOS: AMX + NET ± CTX	Yes vs. no: Did not assess significance Broad vs. narrow: Did not assess significance
Burman et al., 1992 (Sweden)	Retrospective cohort	953	EOS: (1) AMP + GEN or (2) CTX	Yes vs. no: ↑TEM-1 in *E. coli* Broad vs. narrow: No significant difference
Burman et al., 1993 (Sweden)	Retrospective cohort	46	EOS: (1) AMP + GEN or (2) CTX	Yes vs. no: *E. cloacae*: ↑MIC to ampicillin, cephalotin, cephalexin
Calil et al., 2001 (Brazil)	Prospective cohort	342	EOS: AMX + (1) GEN or (2) CRO, LOS: OXA + (1) GEN or (2) CRO	Yes vs. no: ↑MDR *E. cloacae* colonization Broad vs. narrow: ↑MDR *E. cloacae* colonization
Cantey et al., 2016 (USA)	Before-after study	2502	EOS: AMX + GEN, LOS: OXA + GEN	Long vs. short: No significant difference
Crivaro et al., 2007 (Italy)	Case-control	167	AMP + GEN	Yes vs. no: ↑ESBL-producing *S. marcescens and K. pneumoniae* Long vs. short: ↑ESBL-producing *S. marcescens and K. pneumoniae*
De Araujo et al., 2007 (Brazil)	Before-after study	995	PEN & GEN	Broad vs. narrow: ↑MDR GNB
De Champs et al., 1994 (France)	Before-after study	636	(1) AMP + GEN or (2) AMP + AMK	Broad vs. narrow: ↑Gentamicin-resistant, cephalosporin-resistant, and MDR *E. cloacae*, ↑Amikacin-resistant *P. aerunginosa*; ↓Gentamicin & amikacin-resistant GNB, MRSE
De Man et al., 2000 (Netherlands)	RCT	436	EOS: (1) PEN + TOB or (2) AMX + CTX, LOS: FLU + (1) TOB or (2) CTX	Broad vs. narrow: ↑Colonization with cefotaxime-resistant *Enterobacter* spp. & GNB
Duman et al., 2005 (Turkey)	Prospective cohort	118	NDA	Yes vs. no: ↑ESBL-producing Enterobacteriaceae colonization
Gaynes et al., 1984 (USA)	Case-control	32	(1) PEN or (2) AMP + (1) GEN or (2) KAN	Yes vs. no: ↑Aminoglycoside-resistant *E. coli*
Giuffrè et al., 2016 (Italy)	Prospective cohort	1152	SAM + GEN	Yes vs. no: ↑MDR GNB colonization Long vs. short: ↑MDR & ESBL-producing GNB colonization
Isaacs et al., 1988 (UK)	Before-after study	NDA	EOS: PEN + (1) NET or (2) GEN, LOS: FLU + (1) NET or (2) GEN	Long vs. short: No significant difference

Kalenic et al., 1993 (Croatia)	Before-after study	440	(1) AMP + GEN or (2) CXM + GEN	Broad vs. narrow: ↓ Ampicillin-resistant GNB, cefuroxime-resistant GNB & cefuroxime-resistant *K. pneumoniae*
Kumar et al., 2014 (India)	Case-control	65	NDA	Yes vs. no: ↑ CRAB blood stream infections
Le et al., 2008 (USA)	Before-after study	250	EOS: AMP + GEN, LOS: VAN + (1) CTX or (2) TOB	Long vs. short: ↑ ESBL-producing Enterobacteriaceae infection Broad vs. narrow: ↑ ESBL-producing Enterobacteriaceae infection
Linkin et al., 2004 (USA)	Case-control	10	NDA	Yes vs. no: ↑ ESBL-producing Enterobacteriaceae
Mammina et al., 2007 (Italy)	Prospective cohort	210	EOS: SAM + GEN	Long vs. short: ↑ MDR GNB colonization Broad vs. narrow: ↑ MDR GNB colonization
Millar et al., 2008 (UK)	Prospective cohort	124	EOS: PEN + GEN, LOS: (1) TZP + VAN or (2) FLU + GEN	Yes vs. no: No significant difference Broad vs. narrow: ↑ MDR Enterobacteriaceae colonization
Noy et al., 1974 (UK)	Prospective cohort	584	NDA	Yes vs. no: ↑ Antibiotic-resistant *E. coli* & *Klebsiella* spp. colonization
Parm et al., 2010 (Estonia)	RCT	276	EOS: (1) PEN + GEN or (2) AMP + GEN	Broad vs. narrow: ↓ Ampicillin-resistant *Acinetobacter* spp. colonization
Pessoa-Silva et al., 2003 (Brazil)	Prospective cohort	379	EOS: AMP + GEN, LOS: Varying antibiotics	Yes vs. no: ↑ ESBL-producing *K. pneumoniae* colonization
Raz et al., 1987 (Israel)	Before-after study	118	(1) AMP + GEN or (2) AMP + AMK	Broad vs. narrow: ↑ Gentamicin-resistant GNB and *E. cloacae*
Rettedal et al., 2013 (Norway)	Case-control	99	NDA	Yes vs. no: ↑ ESBL-producing *K. pneumoniae* colonization
Sehgal et al., 2007 (India)	Case-control	63	EOS: AMP + GEN, LOS: 3rd gen. cephalosporin + AMK	Yes vs. no: ↑ ESBL-producing GNB blood stream infection
Thatrimontrichai et al., 2013 (Thailand)	Case-control	96	EOS: AMP + GEN, LOS: 3rd gen. cephalosporin + AMK	Broad vs. narrow: ↑ CRAB blood stream infection
Thatrimontrichai et al., 2016 (Thailand)	Case-control	101	EOS: AMP + GEN, LOS: varying antibiotics	Broad vs. narrow: ↑ odds of CRAB ventilator associated pneumonia
Toltzis et al., 2001 (USA)	Prospective cohort	1180	NDA	Long vs. short: ↑ antibiotic resistant GNB colonization

Categories: **Yes vs. no;** compares neonates exposed to antibiotics with non-exposed neonates, **Long vs. short;** compares long and short treatment durations, and **Broad vs. narrow;** compares broad spectrum antibiotic treatment to narrow spectrum treatment. RCT; randomized controlled trial, NDA; no data available, EOS; early onset sepsis, AMX; amoxicillin, CTX; cefotaxime, LOS; late onset sepsis, NET; netilmicin, AMP; ampicillin, GEN; gentamicin, CRO; ceftriaxone, OXA; oxacillin, TOB; tobramycin, FLU; flucloxacillin, KAN; kanamycin, SAM; ampicillin/sulbactam, CXM; cefuroxime, TZP; piperacillin/tazobactam, CREC; cephalosporin-resistant *Enterobacter cloacae*, GNB; Gram-negative bacteria, CRAB; carbapenem-resistant *Acinetobacter baumannii*

9.4 Risk of Bias Assessments in the Systematic Reviews of Early Adverse Effects

Risk of bias graph: review of authors' judgements about each risk of bias item for each included study and the five outcomes. (a) Studies reporting on risk of necrotizing enterocolitis (n=20). (b) Studies reporting on risk of invasive fungal infection (n=24). (c) Studies reporting on risk of death (n=21). (d) Studies reporting on changes in gut microbiota (n=20). (e) Studies reporting on changes in antibiotic resistance development (n=31).
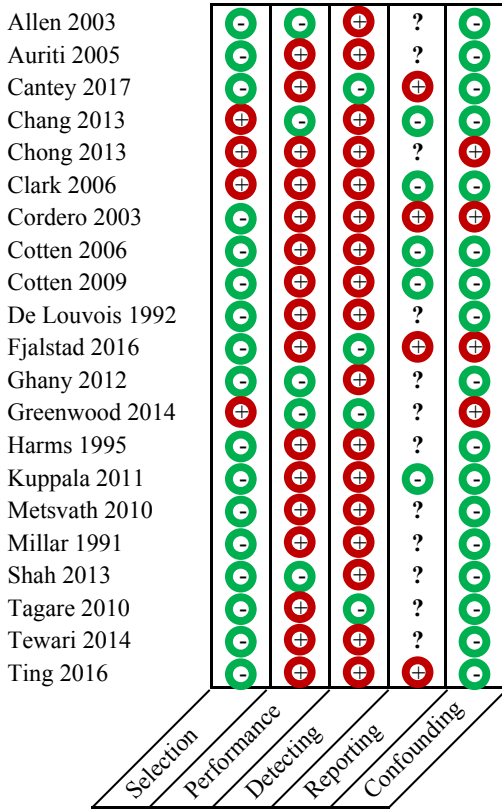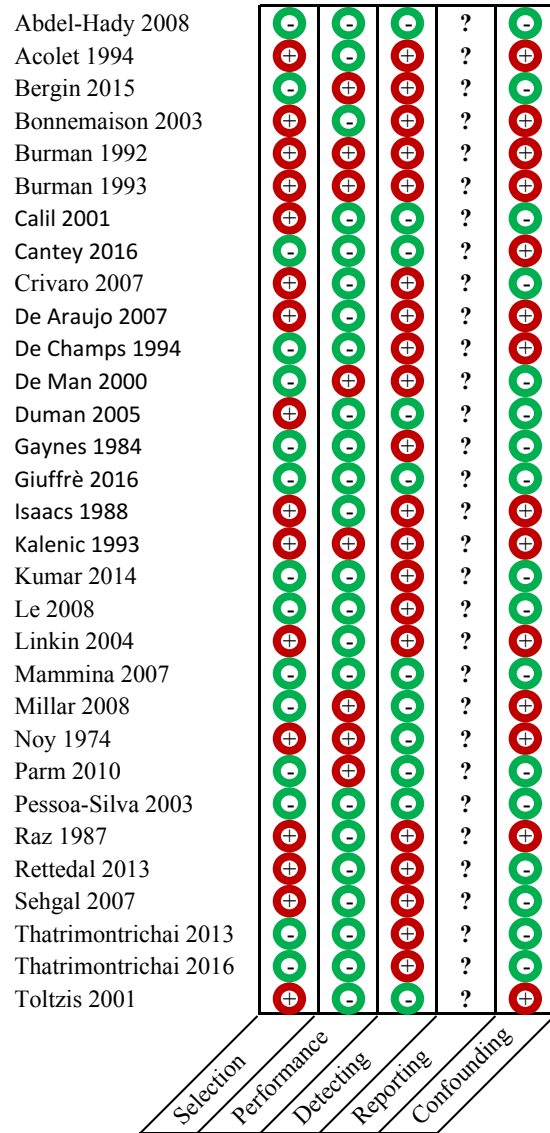
(c)

Legend: G− = green (−), G+ = green (+), R− = red (−), R+ = red (+), ? = unclear

Study	Selection	Performance	Detecting	Reporting	Confounding
Allen 2003	G−	G−	R+	?	G−
Auriti 2005	G−	R+	R+	?	G−
Cantey 2017	G−	R+	G−	R+	G−
Chang 2013	R+	G−	R+	G−	G−
Chong 2013	R+	R+	R+	?	R+
Clark 2006	R+	R+	R+	G−	G−
Cordero 2003	G−	R+	R+	G−	R+
Cotten 2006	G−	G−	R+	G−	G−
Cotten 2009	G−	R+	R+	G−	G−
De Louvois 1992	G−	R+	R+	?	G−
Fjalstad 2016	G−	R+	G−	R+	R+
Ghany 2012	G−	G−	R+	?	G−
Greenwood 2014	R+	G−	G−	?	R+
Harms 1995	R+	R+	R+	?	G−
Kuppala 2011	G−	R+	R+	G−	G−
Metsvath 2010	G−	R+	R+	?	G−
Millar 1991	G−	R+	R+	?	G−
Shah 2013	G−	G−	R+	?	G−
Tagare 2010	G−	R+	G−	?	G−
Tewari 2014	G−	R+	R+	?	G−
Ting 2016	G−	R+	R+	R+	G−

(d)

Study	Selection	Performance	Detecting	Reporting	Confounding
Abdel-Hady 2008	G−	G−	R−	?	R−
Acolet 1994	R+	G−	R+	?	R+
Bergin 2015	G−	R+	R+	?	G−
Bonnemaison 2003	R+	G−	R+	?	R+
Burman 1992	R+	R+	R+	?	R+
Burman 1993	R+	R+	R+	?	R+
Calil 2001	R+	R+	G−	?	G−
Cantey 2016	G−	G−	G−	?	R+
Crivaro 2007	R+	G−	R+	?	G−
De Araujo 2007	R+	G−	R+	?	R+
De Champs 1994	G−	G−	R+	?	R+
De Man 2000	G−	R+	R+	?	G−
Duman 2005	R+	R+	G−	?	G−
Gaynes 1984	G−	G−	R−	?	G−
Giuffrè 2016	G−	G−	G−	?	G−
Isaacs 1988	R+	G−	R+	?	R+
Kalenic 1993	R+	R+	R+	?	R+
Kumar 2014	G−	G−	R+	?	G−
Le 2008	G−	G−	R+	?	G−
Linkin 2004	R+	R+	R+	?	R+
Mammina 2007	G−	G−	G−	?	R−
Millar 2008	G−	R+	G−	?	R+
Noy 1974	R+	R+	G−	?	R+
Parm 2010	G−	R+	G−	?	G−
Pessoa-Silva 2003	G−	G−	G−	?	G−
Raz 1987	R+	G−	R+	?	R+
Rettedal 2013	R+	G−	R+	?	R−
Sehgal 2007	R+	G−	R+	?	R−
Thatrimontrichai 2013	G−	G−	R+	?	G−
Thatrimontrichai 2016	G−	G−	R+	?	G−
Toltzis 2001	R+	G−	G−	?	R+

(e)

Study	Selection	Performance	Detecting	Reporting	Confounding
Arboleya 2014	R+	G−	?	?	R+
Bennet 1986 & 1987	R+	R+	R+	?	R+
Blakey 1982	R+	R+	R+	?	R+
Bonnemaison 2003	R+	G−	R+	?	R+
Butel 2007	R+	R+	R+	?	R+
Ferraris 2012	R+	R+	R+	?	G−
Fouhy 2012	R+	G−	?	?	R+
Gewolb 1999	G−	?	R+	?	G−
Goldman 1978	G−	G−	R+	?	R+
Greenwood 2014	R+	G−	?	?	R+
Hall 1990	G−	G−	R+	?	R+
Jacquot 2011	G−	G−	?	?	R+
Jenke 2013	R+	R+	?	?	R+
La Rosa 2014	G−	G−	G−	?	G−
Parm 2010	R+	R+	R+	?	G−
Tullus 1988	R+	R+	R+	?	R+
Ward 2016	G−	R+	G−	?	G−
Westerbeek 2013	G−	G−	?	?	G−
Zhou 2015	G−	G−	?	?	G−

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