Placebo controlled introduction of prophylactic supplementation of probiotics to decrease the incidence of Necrotizing Enterocolitis (NEC) at Dhulikhel Hospital in Nepal

A. Retrospective data collection and analysis
B. Organization of a prospective study

submitted by
Mag. rer. nat. Daniela S. Klobassa
born on 2nd June 1983

for obtaining the academic degree of
Doctor of Medicine (Dr. med. univ.)
at
Medical University of Graz

Conducted at
Division of Neonatology, Department of Pediatrics and Adolescence Medicine
LKH-Univ. Klinikum Graz
Department of Pediatrics, Dhulikhel Hospital, KUSMS, Nepal

First Supervisor: Ao. Univ.-Prof. Dr. med. univ. Urlesberger Berndt
Second Supervisor: Univ.-Prof. Dr. med. univ. Resch Bernhard

Graz, ..............................
Affidavit

Herewith I, Daniela Klobassa, declare that I have written the present diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, ..............................................
Preface

The idea for this diploma thesis was formed after a clinical elective at Dhulikhel Hospital (DH), Kathmandu University School of Medical Sciences (KUSMS) in Nepal. I was one of the first students from Medical University of Graz (MUG), who got the chance to gain clinical experience at Dhulikhel Hospital within a new started teaching- and research collaboration. I used this chance and pursued the idea of getting a first study project started between DH and MUG. After confirmation from my supervisor and MUG I could start with planning the study project in October 2010.

My tasks involved at one hand, retrospective data collection and data analysis from Necrotizing Enterocolitis (NEC) cases at DH and at the other hand, the planning and organization of a prospective study at DH.

The study organization was a very interesting and diverse task, whereas the retrospective data collection in February 2011 turned out be rather tedious due to a lack of organized documentation system, however, I was also already prepared for this situation in advance.

The roughly scheduled start of the prospective study had a delay of more than half a year. One reason was that I first got the information that no ethical approval from the national research council is needed, but during my stay in February 2011 an ethical approval turned out to be mandatory. After an unexpected long waiting time, the approval of the ethical proposal was finally given in August 2011. Another reason for the delay of the study start was the prolonged construction of a new air filter system at neonatal intensive care unit (NICU).

Finally, the prospective study could start at the end of 2011. The evaluation of the prospective study is not part of the present diploma thesis.
Acknowledgement

First of all, I would like to thank my both supervisors Prof. Berndt Urlesberger and Prof. Bernhard Resch for their effective support. I always got fast and competent answers to all my questions, even when I needed their expertise urgently from far Nepal.

Only with support from MUG through a scholarship for travel costs and from the company Germania Pharmazeutika GesmbH, who sponsored the probiotics, this – the scope of a diploma thesis exceeding – project could be realized.

Special thanks go to the whole pediatric team of Dhulikhel Hospital, in particular to Dr. Srijana Dongol, the consultant in charge of the prospective study at DH, as well as to Dr. Ram Makaju Shrestha, the medical director of the hospital. Dr. Ram welcomed this project highly and always supported and motivated his staff.

Furthermore, I would like to thank the vice rectorate of MUG, who supported my participation at the annual conference of the network “Towards Unity for Health” (TUFH) in September 2011 in Graz. At that international conference I could introduce the present study via a poster presentation in the session „Newborn, Child and Adolescent Health“.

Last but not least I would like to thank my beloved family for their continuous and unconditional support.
Abstract

**Context:** Necrotizing Enterocolitis (NEC) is the most common life-threatening emergency of the gastrointestinal tract in the newborn period, especially affecting preterm newborns. Results from previous studies give evidence that the prophylactic supplementation of probiotics leads to a decrease in the incidence of NEC.

**Objectives:** This study project consists of two parts: a retrospective study, which aims to analyze NEC cases in the past and a prospective controlled study, which aims to evaluate the effect of probiotic supplementation in preterm newborns at Dhulikhel Hospital (DH) in Nepal.

**Design:** Retrospective data analysis focuses on data about NEC at Dhulikhel Hospital from the last two years. Since confirmation through abdominal X-ray has not been a standardized diagnostic tool, only suspected NEC cases are reported here. In this analysis cases were counted as suspected NEC when at least two of the following criteria were fulfilled: abdominal distension, feeding intolerance, occult blood in stool, melena, and Metronidazole treatment, whereby one of these two criteria had to be Metronidazole treatment.

In the prospective study the intervention group receives a single strain probiotic (*lactobacillus casei var. rhamnosus*) solved in expressed breast milk (EBM) whereas the control group receives solely EBM. Evaluation of NEC incidence is planned after a 2-year period.

**Results:** Retrospective analysis identified a total of 91 suspected NEC cases, including 52 % preterm, 46 % term neonates (7 % low birth weight (LBW), 7 % birth weight n.n.). Most neonates fulfilled three (58 %) or four (29 %) criteria accounted for suspected NEC. Mortality rate was high (36 %). Besides neonatal sepsis and respiratory distress the most common comorbidities included hyperbilirubinemia, birth asphyxia, dyselectrolytemia, neonatal meningitis and meconium aspiration. Within preterm neonates 42 % and within LBW neonates 43 % showed symptoms accounted for suspected NEC.

**Conclusion:** Retrospective analysis confirmed that preterm and LBW neonates are at high risk to develop NEC. This work provides detailed information about newborns with suspected NEC in Nepal. Major limitations of this work are that suspected NEC cases were selected solely based on clinical symptoms and treatment, but an analysis of systemic signs or laboratory findings was not included and abdominal X-ray was not available.
Zusammenfassung

**Hintergrund:** Die Nekrotisierende Enterokolitis (NEK) ist der häufigste lebensbedrohliche Notfall des Magen-Darm Trakts des Neugeborenen, wobei vor allem Frühgeborene betroffen sind. Ergebnisse aus früheren Studien zeigen, dass eine prophylaktische Gabe von Probiotika zu einer Reduktion in der Inzidenz der NEK führt.

**Ziel:** Dieses Studienprojekt besteht aus zwei Teilen: einer retrospektiven Studie, welche die Evaluation von NEK PatientInnen der Vergangenheit zum Ziel hat und einer prospektiven kontrollierten Studie, welche den Effekt von Probiotika auf Frühgeborene am Dhulikhel Hospital (DH) in Nepal evaluieren will.

**Methode:** In der retrospektiven Datenanalyse werden NEK Fälle am DH der letzten zwei Jahre beleuchtet. Da zur Diagnosesicherung kein Abdomen-Röntgen durchgeführt wurde, wird hier nur von Verdachtsfällen berichtet. In dieser Analyse wurde die Verdachtsdiagnose NEK gestellt, wenn zwei der folgenden Kriterien erfüllt waren: geblähtes Abdomen, Nahrungsintoleranz, okkultes Blut im Stuhl, Meläna und die Behandlung mit Metronidazol, wobei eines der zwei Kriterien die Behandlung mit Metronidazol sein musste.

In der prospektiven Studie erhält die Interventionsgruppe zur Muttermilch ein Probiotikum, das einen Bakterienstamm enthält (lactobacillus casei var. rhamnosus) während die Kontrollgruppe nur Muttermilch erhält. Eine Evaluation der Inzidenz von NEK ist nach zwei Jahren geplant.

**Ergebnisse:** Die retrospektive Datenanalyse identifizierte 91 Verdachtsfälle von NEK, bestehend aus 52 % Frühgeborenen, 46 % Reifgeborenen (7 % niedriges Geburtsgewicht, 7 % Geburtsgewicht n.n.). Die meisten Neugeborenen erfüllten zwei (58 %) oder drei (29 %) Kriterien. Die Mortalitätsrate war hoch (36 %). Neben Neugeborenen-Sepsis und Atemnot zählten zu den häufigsten Nebendiagnosen Hyperbilirubinämie, perinatale Asphyxie, Elektrolytentgleisungen, Neugeborenen-Meningitis und Mekonium Aspiration. Innerhalb 42 % aller Frühgeborenen und 43 % aller Reifgeborenen mit niedrigem Geburtsgewicht wurde die Verdachtsdiagnose NEK gestellt.

**Fazit:** Die retrospektive Analyse bestätigte, dass Frühgeborene und Neugeborene mit niedrigem Geburtsgewicht ein hohes Risiko haben, eine NEK zu entwickeln. Diese Arbeit liefert detaillierte Informationen über Neugeborene mit der Verdachtsdiagnose NEK in Nepal. Haupteinschränkungen dieser Arbeit sind, dass NEK Verdachtsfälle allein durch klinische Symptome und die Behandlung selektiert wurden und keine Analyse von systemischen Zeichen und Laborresultaten inkludiert wurde und dass Abdomen-Röntgen nicht vorhanden waren.
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<th>Description</th>
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<tbody>
<tr>
<td>AIS</td>
<td>Amniotic Infection Syndrome</td>
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<tr>
<td>APH</td>
<td>Antepartum Hemorrhage</td>
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<tr>
<td>BA</td>
<td>Birth Asphyxia</td>
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<tr>
<td>BF</td>
<td>Breast Feeding</td>
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<tr>
<td>CPAP</td>
<td>Continuous Positive Airway Pressure</td>
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<tr>
<td>CPD</td>
<td>Cephalopelvic Disproportion</td>
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<tr>
<td>DH</td>
<td>Dhulikhel Hospital</td>
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<tr>
<td>DOA</td>
<td>Date of Admission</td>
</tr>
<tr>
<td>DOB</td>
<td>Date of Birth</td>
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<tr>
<td>DOD</td>
<td>Date of Discharge</td>
</tr>
<tr>
<td>EBM</td>
<td>Expressed Breast Milk</td>
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<tr>
<td>EONS</td>
<td>Early Onset Neonatal Sepsis</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United States</td>
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<tr>
<td>GNI</td>
<td>Gross National Income</td>
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<tr>
<td>HMD</td>
<td>Hyaline Membrane Disease</td>
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<tr>
<td>IMR</td>
<td>Infant Mortality Rate</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<tr>
<td>IRDS</td>
<td>Infant Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine Growth Restriction</td>
</tr>
<tr>
<td>IVH</td>
<td>Intraventricular Hemorrhage</td>
</tr>
<tr>
<td>KUSMS</td>
<td>Kathmandu University School of Medical Sciences</td>
</tr>
<tr>
<td>LAMA</td>
<td>Left Against Medical Advice</td>
</tr>
<tr>
<td>LBW</td>
<td>Low Birth Weight</td>
</tr>
<tr>
<td>LCR35</td>
<td>Lactobacillus Casei var. Rhamnosus 35</td>
</tr>
<tr>
<td>LGG</td>
<td>Lactobacillus Gorbach Goldin</td>
</tr>
<tr>
<td>LONS</td>
<td>Late Onset Neonatal Sepsis</td>
</tr>
<tr>
<td>MDG</td>
<td>Millennium Development Goals</td>
</tr>
<tr>
<td>MUG</td>
<td>Medical University Graz</td>
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<tr>
<td>NEC</td>
<td>Necrotizing Enterocolitis</td>
</tr>
<tr>
<td>NEK</td>
<td>Nekrotisierende Enterokolitis</td>
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<tr>
<td>NHRC</td>
<td>Nepal Health Research Council</td>
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<tr>
<td>NICU</td>
<td>Neonatal Intensive Care Unit</td>
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<tr>
<td>NMR</td>
<td>Neonatal Mortality Rate</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NNS</td>
<td>Neonatal Sepsis</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>OPD</td>
<td>Outpatient Department</td>
</tr>
<tr>
<td>PCM</td>
<td>Pressure Controlled Mode</td>
</tr>
<tr>
<td>PDA</td>
<td>Patent Ductus Arteriosus</td>
</tr>
<tr>
<td>PICU</td>
<td>Pediatric Intensive Care Unit</td>
</tr>
<tr>
<td>PIH</td>
<td>Pregnancy Induced Hypertension</td>
</tr>
<tr>
<td>PPP</td>
<td>Purchasing Power Parity</td>
</tr>
<tr>
<td>PROM</td>
<td>Premature Rupture of Membranes</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for Gestational Age</td>
</tr>
<tr>
<td>SIMV</td>
<td>Synchronized Intermittent Mandatory Ventilation</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very Low Birth Weight</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
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1 Introduction

1.1 Health condition of newborns in Nepal

The federal republic Nepal is located in south-east Asia and has a total population of 29,331,000 with 18 % living in urban areas (1). For a rough overview some general statistical data for Nepal are presented in Table 1; to enable referencing and comparison data for Austria are shown as well. Data refer to 2009 (2).

Table 1: Statistical Data for Nepal and Austria for 2009

<table>
<thead>
<tr>
<th></th>
<th>Nepal</th>
<th>Austria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross national income per capita$^\dagger$ PPP (USD)</td>
<td>1.160</td>
<td>38.410</td>
</tr>
<tr>
<td>Total expenditure on health per capita (USD)</td>
<td>69</td>
<td>4.243</td>
</tr>
<tr>
<td>Life expectancy at birth for males (years)</td>
<td>65</td>
<td>78</td>
</tr>
<tr>
<td>Life expectancy at birth for females (years)</td>
<td>69</td>
<td>83</td>
</tr>
<tr>
<td>Adult mortality rate (per 1000 adults between 15-69 years)</td>
<td>196</td>
<td>76</td>
</tr>
<tr>
<td>Stillbirth rate (per 1000 total births)</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>Under-5 mortality rate (per 1000 live births)$^{**}$</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>Neonatal mortality rate (per 1000 live births)$^{**}$</td>
<td>28</td>
<td>2</td>
</tr>
</tbody>
</table>

$^\dagger$Gross national income (GNI) = Sum of value added by all resident producers plus product taxes not included in the valuation of output plus net receipts of primary income from abroad. GNI per capita is GNI divided by mid-year population (3). Here the GNI based on purchasing power parity (PPP) is listed.

$^{**}$Data refer to 2010

Not only the numbers differ but also the causes of deaths in children under age of five years. In Austria congenital abnormalities (30 %), prematurity (24 %), injuries (7 %), birth asphyxia (5 %) and other causes (33 %) are accounted for death under five years. In Nepal diarrhea (17 %), birth asphyxia (16 %), pneumonia (16 %), prematurity (15 %), neonatal sepsis (13 %), congenital abnormalities (4 %), injuries (3 %) and other (16 %) are the predominate causes (2).

Another common underlying cause of child mortality in Nepal is malnutrition. Data from 2006 (4) indicate that 39 % of under-five children in Nepal are underweight. Recent surveys observed a reduction in the proportion of children underweight;
however, there is a significant rise in the proportion of children having inadequate nutrition (5).

Notable are also inequities in under-5 mortality: 84 deaths per 1000 live births occur in rural areas whereas 47 in urban areas. 98 deaths per 1000 live births happen in 20 % of the poorest whereas 47 in 20 % of the wealthiest (1).

Data from 2006 (1) reveal that only 19 % of births are attended by skilled health personnel. The fact that the majority of children are born at home without any skilled birth attendant is a great challenge towards improving neonatal health and survival. The government has already initiated several child-survival programs, e.g. the Community-Based Integrated Management of Childhood Illness, the Community-Based Newborn Care Package and the National Immunization Program (5).

Reducing child mortality, especially newborn mortality is one of the eight Millennium Development Goals (MDG) set by the United Nations. The MDGs were set at the beginning of the Millennium in 2000 when 189 member states of the United Nations agreed to meet the MDGs with the goal of bringing peace, security and development to all people. The eight MDGs to be achieved by 2015 represent the world’s most urgent development needs (5).

Whereas Nepal has made significant progress in reducing infant mortality (from 113 in 1987 to 50 in 2010, Fig. 1), neonatal mortality did not decrease to the same extent (from 45 in 1987 to 28 in 2010, Fig. 1). Actually, neonatal mortality has increased from 40 % to 68 % as a proportion of infant mortality (Fig. 1, right axis), if we assume that data collection methods did not change. Infant mortality rate (IMR) refers to the probability of dying between birth and the age of 1 whereas neonatal mortality rate (NMR) refers to the probability of dying within the first 28 days of life. WHO is collecting these data once every five years (1).
Figure 1: Infant Mortality Rate (IMR) and Neonatal mortality rate (NMR)

On the left axis mortality rates are presented. A reduction of mortality rates can be seen over the last 23 years. On the right axis neonatal mortality is depicted as proportion of infant mortality, this proportion follows an opposite trend and is increasing.

This means that a child is most vulnerable during neonatal period. Therefore, a reduction of child mortality should focus on reducing neonatal mortality, whereby preterm neonates are at particular high risk.

Preterm birth is defined as gestational age at birth of less than 37 completed gestational weeks. Further, preterm birth can be classified into three categories: mild preterm for 32-36 weeks, very preterm for 28-31 weeks and extremely preterm for less than 28 weeks.

Shrestha et al. (7) investigated risk factors, morbidity and mortality associated with preterm birth at Dhulikhel Hospital (DH) in Nepal. 150 preterm babies admitted to Neonatal Intensive Care Unit (NICU) between January 2007 and December 2009 were enrolled in a retrospective study. Incidence of preterm birth was 19,5 %, mean birth weight was 1670 g (+/- 370 g), and mean gestational age was 30,02 (+/- 0,37) weeks. The most common risk factors for preterm birth were inadequate antenatal checkup (52 %), maternal age below 20 years (34,7 %), ante partum hemorrhage (23,4 %) and pregnancy induced hypertension (13,1 %).

Associated morbidities included clinical sepsis (66,7 %), hyperbilirubinemia (58,8 %), birth asphyxia (26,8 %) and hyaline membrane disease (23,5 %). Overall
survival was 79.4 %. Common causes of death were hyaline membrane disease (64.5 %), followed by sepsis (58.1 %) and necrotizing enterocolitits (25.8 %). Hence, necrotizing enterocolitis is the third common cause of preterm neonatal mortality at DH.

1.2 Necrotizing Enterocolitis (NEC)

Necrotizing Enterocolitis (NEC) is the most common life-threatening emergency of the gastrointestinal tract in the neonatal period, especially affecting preterm newborns (8). Höllwarth (9) describes that the pediatrician Quaiser and the pathologist Schmid from Graz were the first who described NEC in their publication “Enteritis ulcerosa necroticans” in 1952 (10). NEC is a hemorrhagic necrotizing inflammatory bowel disease characterized by various degrees of mucosal or transmural necrosis of the intestine. The cause of NEC is not completely clear yet but is most likely multifactorial (8).

1.2.1 Epidemiology of NEC

Epidemiologic data differ greatly in literature, not only between countries but also between hospitals. The incidence of NEC varies between 1 and 5 % in NICUs and increases with decreasing birth weight and gestational age so that very small preterm neonates are especially susceptible to NEC with highest incidence rates among preterm newborns and newborns with low birth weight (LBW, birth weight below 2500 g) (8). In very low birth weight infants (VLBW, birth weight below 1500 g) incidence varies between 5 and 9 % (11). Kosloske (12) and Noerr (13) review that NEC occurs in one to three in 1000 live births and in 1 to 7.7 % of all admissions to NICUs (14). Actually NEC incidence increased over the last years with improvements in technology and the ability to enable a good outcome to preterm neonates and therefore more of these high-risk neonates survive (15). However, in about 10 % the disease also occurs in term infants (9). NEC occurs equally often in female and male (13).
1.2.2 Pathology and Pathogenesis of NEC

It is assumed that many factors are associated with the development of necrosis in the intestines, a subsequently gas accumulation in the submucosa of the bowel wall (pneumatosis intestinalis) and any progression of the necrotizing process that can lead to perforation, peritonitis, sepsis, and death.

Most often the distal part of the ileum and the proximal segment of the colon are affected, however, in complicated cases, gangrene can reach from the stomach to the rectum.

NEC is a multifactorial disease that is typically associated with the triad of intestinal ischemia (injury), enteral nutrition (metabolic substrate), and pathogenic organisms. NEC may result from an interaction between loss of mucosal integrity due to ischemia, infection or inflammation and the host’s response to that injury (circulatory, immunologic, and inflammatory). In histologic examination of the intestinal specimen characteristically coagulation necrosis is found (8).

Recent studies also discuss a genetic predisposition for an increased risk of intestinal injury, e.g. through a polymorphism of toll-like receptor 4 (TLR4) (16).

One theory for the increased risk of NEC in preterm neonates is that immature intestinal barrier involves increased inflammation and tissue injury. The immaturity of intestinal barrier could be characterized by decreased mucus (gastric acid secretion), decreased IgA, low intercellular junction integrity and increased permeability. Besides the immature barrier function also immaturity of motility, digestion, absorption, immune defenses and circulatory regulation increase the risk of intestinal injury (17).

Hypoxia and ischemia are thought to modulate the balance in microvascular tone and influence the production of vascular regulators (e.g. nitric oxide, endothelin) that might be involved in the pathogenic cascade of NEC (18).

Another theory is that initial microbial colonization in preterm neonates is inappropriate (19). Analyses of fecal samples show that low bacterial diversity and bacterial overgrowth are associated with NEC. In most situations, no pathogen is identified (8). In some clustered cases bacterial (Escherichia coli, Clostridium perfringens, Klebsiella, Staphylococcus epidermidis) and viral agents (Enterovirus, Rotavirus) were found in cultures (11).

Recent animal studies (20) suggest that bacterial overgrowth is rather the consequence than the cause of NEC.
1.2.3 Risk factors of NEC

Several risk factors for NEC could be identified; the most important ones are listed in the following.

The greatest risk factor is prematurity. Since preterm neonates are immunologically immature they are highly sensitive to gut colonizing bacteria. The exact mechanism of gut colonizing remains unclear. The immature bowel function may lead to feeding difficulties, NEC and Sepsis.

In term newborns neonatal risk factors include any perfusion disturbances of the bowel wall due to hypovolemic shock, patent ductus arteriosus (PDA), hypotension, left-sided heart failure (e.g. aortic isthmic stenosis), polycythemia, hypoglycemia, and hypoxemia (21). Another risk factor is a too fast increase in enteral feeding (> 20 ml/kg bodyweight/d), especially hyperosmolar solutions or medications (22). In term newborns also growth retardation increases the risk for NEC (23).

Furthermore pregnancy risk factors and perinatal risk factors can be defined. The most important pregnancy risk factor is amniotic infection syndrome (AIS) or chorioamnionitis. Due to chorioamnionitis intestinal microbial colonization with invasive pathogenic bacteria might happen. In the following inflammatory mediators might be activated and involved in bowel injury and necrosis (23).

Perinatal risk factors include asphyxia, infant respiratory distress syndrome (IRDS) and the duration of use of an umbilical catheter. An umbilical catheter might impede mesenteric flow when catheters are positioned high (25). Guthrie et al. (26) report that neonates with NEC were more likely to have been exposed to invasive procedures as umbilical catheterization and mechanical ventilation. However, Davey et al. (27) report that low positioned umbilical catheter did not increase the risk of NEC.

There are indices that mode of delivery influences the risk of NEC, but this influence is not clear yet. Analyses of fecal samples showed that intestinal flora is determined by its first colonizers and therefore depends on mode of delivery. Hällström et al (28) explain that the intestinal microbial colonization in preterm neonates delivered by cesarean section is different from those delivered by a vaginal birth.
1.2.4 Clinical Manifestations of NEC

NEC becomes usually clinically manifest after starting enteral feeding between the 2\textsuperscript{nd} and 15\textsuperscript{th} day of life. In some cases NEC can present later, around three to six weeks of life, especially in preterm neonates with a birth weight below 1000 g (11). Typically first symptoms are signs of feeding intolerance. These signs might be subtle and include abdominal distension, delayed gastric entering with vomiting or gastric aspiration and a positive result of occult blood (microscopically) in stool. More advanced clinical symptoms may include progressive abdominal distension, evidence of peritonitis, grossly (macroscopically) blood in stool and cardiorespiratory failure (29).

The course of NEC can possible become very progressive, therefore frequent clinical controls are crucial. The severity of illness ranges from mild symptoms to a severe condition with bowel perforation, peritonitis, systemic inflammatory response syndrome, shock and death (8). Some neonates recover from minor symptoms spontaneously over a few days, whereas others develop a severe condition with the need of intubation, fluid resuscitation or immediate surgical intervention over hours (29).

1.2.5 Diagnosis of NEC

In Table 2 the golden standard of diagnosing NEC using modified bell’s staging criteria for NEC is presented. Important is a continuous clinical control of the abdomen with laboratory tests including coagulation profile, acid-base and electrolyte balance (8).
Table 2: Modified Bell’s Staging Criteria for Necrotizing Enterocolitis

<table>
<thead>
<tr>
<th>Stage</th>
<th>Systemic signs</th>
<th>Abdominal signs</th>
<th>Radiographic signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A Suspected</td>
<td>Temperature instability Apnea Bradycardia Lethargy</td>
<td>Gastric retention Abdominal distention Emesis Heme-positive stool</td>
<td>Normal or intestinal dilation Mild ileus</td>
</tr>
<tr>
<td>1B Suspected</td>
<td>Temperature instability Apnea Bradycardia Lethargy</td>
<td>Grossly bloody stool</td>
<td>Normal or intestinal dilation Mild ileus</td>
</tr>
<tr>
<td>2A Definite Mildly ill</td>
<td>Temperature instability Apnea Bradycardia Lethargy</td>
<td>Grossly bloody stool Absent bowel sounds Possible abdominal tenderness</td>
<td>Intestinal dilation ileus Pneumatosis intestinalis</td>
</tr>
<tr>
<td>2B Definite Moderately ill</td>
<td>Temperature instability Apnea Bradycardia Lethargy Mild metabolic acidosis Thrombocytopenia</td>
<td>Grossly bloody stool Absent bowel sounds Definite abdominal tenderness Possible abdominal cellulitis or right lower quadrant mass</td>
<td>Intestinal dilation ileus Pneumatosis intestinalis Ascites</td>
</tr>
<tr>
<td>3A Severe ill Intact bowel</td>
<td>Temperature instability Apnea Bradycardia Lethargy Hypotension Combined respiratory + metabolic acidosis DIC* Neutropenia</td>
<td>Grossly bloody stool Absent bowel sounds Possible abdominal cellulitis or right lower quadrant mass Marked abdominal tenderness Signs of peritonitis Abdominal distention</td>
<td>Intestinal dilation ileus Pneumatosis intestinalis Ascites</td>
</tr>
<tr>
<td>3B Advanced Severe ill Perforated bowel</td>
<td>Temperature instability Apnea Bradycardia Lethargy Hypotension Combined respiratory + metabolic acidosis DIC* Neutropenia</td>
<td>Grossly bloody stool Absent bowel sounds Possible abdominal cellulitis or right lower quadrant mass Marked abdominal tenderness Signs of peritonitis Abdominal distention</td>
<td>Intestinal dilation ileus Pneumatosis intestinalis Ascites Pneumoperitoneum</td>
</tr>
</tbody>
</table>

*DIC: disseminated intravascular coagulation

The method of choice for diagnosing NEC is abdominal X-ray. As reported in Table 2 (30) radiographic findings are nonspecific during early stages (1A, 1B), they include dilated bowel loops, generalized bowel distention, and bowel-wall thickening.

A more specific and important radiographic sign in diagnosing NEC is pneumatosis intestinalis or gas in the bowel wall. When pneumatosis intestinalis extends into the portal circulation the course of NEC is often severe. When signs
of pneumoperitoneum are diagnosed frequently intestinal perforation occurred. Usually, pneumatosis alone is treated medically and pneumoperitoneum is considered as indication for surgery (30).

Another option is to support NEC diagnoses by sonography. Like abdominal radiography, sonography can depict intramural gas, venous gas and free intraperitoneal gas. Advantages of abdominal sonography are the possibility to detect intra-abdominal fluid, bowel wall thickness and bowel wall perfusion. Thinning of the bowel wall and a reduction of perfusion may be seen via sonography before visualization of pneumoperitoneum at plain abdominal radiography. Since mortality is higher after perforation, early detection of ischemic or necrotic bowel before perforation is essential (31).

1.2.6 Treatment of NEC

Treatment is based on the clinical signs and the stage of NEC (Table 2).

In Stage 1A and 1B all enteral feeding should be stopped (nil per os, NPO) and antibiotics should be given for three days. In Stage 2A NPO continues and antibiotics should be given for seven to 10 days, whereas in stage 2B antibiotics should be administered for 14 days. In stage 3A besides NPO, antibiotics for 14 days, fluid resuscitation and inotropic support, ventilator therapy are recommended. In stage 3B in addition to management done in stage 3A surgery is needed (30).

Surgical treatment may include drain placement, exploratory laparotomy with resection of necrotic bowel and creation of an enterostoma (16).

Actually, therapy is focused on supportive care and preventing further injury. Blood cultures should be drawn before immediately starting with systemic antibiotics with broad coverage. Possible umbilical catheters should be removed and intravenous access maintained. In the presence of apnea or hypercapnia ventilator therapy should be started. For further stabilization intravascular volume replacement with crystalloids or blood products, for cardiovascular support, administration of volume and inotropes and correction of hematologic, metabolic and electrolyte abnormalities maybe required (8).

Surgery is indicated if perforation is present (pneumoperitoneum in abdominal X-ray). Relative indications for exploratory laparotomy are failure of medical
treatment, a single fixed bowel loop (persistently dilated bowel loop in X-ray), abdominal wall erythema or a palpable mass. In best case surgery should be done after any necrosis developed and before any perforation or peritonitis follows. For infants who are too unstable to undergo surgery peritoneal drainage can be an alternative treatment (8).

At Dhulikhel Hospital in case of suspected NEC Metronidazole is given as antibiotic treatment according to the dosage scheme in Nelsons Pediatrics (8) as shown in Table 3, usually for 7 to 10 days. Metronidazole belongs to the antibiotic group of nitroimidazole and can be used as antibiotic, amebicide and antiprotozoal.

### Table 3: Weight and age dependent dosage Scheme for Metronidazole

<table>
<thead>
<tr>
<th>Weight</th>
<th>Age</th>
<th>Metronidazole (mg/kg) iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1200 g</td>
<td>0-4 weeks</td>
<td>7.5q*48h after loading dose**</td>
</tr>
<tr>
<td>1200-2000 g</td>
<td>0-7 days</td>
<td>7.5q24h after loading dose</td>
</tr>
<tr>
<td>1200-2000 g</td>
<td>&gt; 7 days</td>
<td>7.5q12h after loading dose</td>
</tr>
<tr>
<td>&gt; 2000 g</td>
<td>0-7 days</td>
<td>7.5q12h after loading dose</td>
</tr>
<tr>
<td>&gt; 2000 g</td>
<td>&gt; 7 days</td>
<td>15q12h after loading dose</td>
</tr>
</tbody>
</table>

*q means ‘every’, e.g. 7.5 mg/kg every 48h

**Loading iv dose: 15 mg/kg

A more general scheme does not orientate on weight and uses 15 mg/kg loading dose and 24 h later (in term infants) or 48 h later (in preterm infants) 7.5 mg/kg every 12 hours.

### 1.2.7 Outcome of NEC

In about 20-40 % of patients with pneumatosis intestinalis at diagnoses medical treatment is unsuccessful, 10-30 % of these patients die (8). Overall mortality of NEC has been cited as 10-50 % of all affected infants (32).

In case of needed surgery early postoperative complications comprise wound infection, dehiscence and stoma related complications like prolapse and necrosis. In about 10 % of NEC patients intestinal strictures at the site of the necrotizing lesion can be a late postoperative complication but also a late complication after
medical management. However, these strictures that may be cause of any obstruction can be resected curatively.
Radical surgery may result in short-bowel syndrome involving malabsorption, growth failure or malnutrition, complications related to central venous catheters (sepsis, thrombosis) and cholestatic jaundice.
In preterm neonates with NEC and surgical intervention or bacteremia increased risk for adverse growth and neurodevelopmental outcome has been observed (8).

1.2.8 Prevention of NEC

As reported above NEC can follow a fatal course and in progressed stages only supportive care and avoiding of further injury can be done. Therefore the main focus should lie on prevention of this disease.
Despite the pathogenesis of NEC is unclear (see subchapter 1.2.2), prevention strategies have been developed based on clinical observations and studies. Approaches for NEC prevention reported so far include withholding enteral feeding, breast milk feeding and the administration of probiotics or prebiotics.
Also glucocorticoids are in discussion, whereby safety is questionable because of side effects. Anticytokine agents or growth factors seem also to be preventive for NEC but are only examined in animal models and not in humans yet (16).
Another approach focusing on using antibiotics is discussed diverse since often resistant bacteria may be the consequence.

1.2.8.1 Withholding enteral feeding

In the subchapter risk factors (1.2.3) it was already stated that a rapid increase in enteral feeding increases the probability of NEC. However, a complete withholding of enteral feedings can lead to long use of parenteral nutrition, intestinal atrophy, increased permeability and inflammation (33). Therefore, complete withholding is not suggested, but avoiding fast increase of amount in enteral feeding (not more than 20 ml/kg bodyweight/d) (22), (34).
1.2.8.2 Breast milk feeding

Some studies suppose a protective factor through breast milk. Lucas and Cole (25) showed that the incidence of NEC for newborns, who got breast fed, was significantly lower than for newborns, who got formula milk. The authors concluded their results from a prospective multicenter study with a sample of 926 infants. Confirmed NEC was six to ten times as common in neonates who were only fed formula milk than in those only fed breast milk. Also, the risk of NEC was 3.5 times higher in neonates only fed formula milk than in those fed breast milk and formula combined. The findings suggest that breast milk is supportive even when intermixed with formula. Sullivan et al. (35) also report that breast milk is correlated with lower incidence of NEC.

Breast milk contains besides lactose, whey and casein, bioactive components like IgA, lactoferrin and β-lactoglobulin and live bacteria dominated by bifidobacteria. Cilieborg et al. (36) conclude from their review that diet has limited and variable effects on neonatal gut colonization. However, there are consistent findings about a decreased prevalence and number of clostridia in breast fed neonates.

Fituch et al. (37) conclude that breast milk can reduce the incidence of NEC but not eradicate NEC, possible because of interleukin 10 (IL-10) deficiency. Besides IL-10 several other factors in breast milk are hypothesized to prevent the development of NEC, including immunoglobulins, erythropoietin, epidermal growth factor (EGF) and platelet-activating factor (PAF)-acetylhydrolase (38).

1.2.8.3 Administration of Prebiotics and Probiotics

Prebiotics are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus in effect improve host health” (39).

Prebiotics include oligosaccharides like inulin, galactose, fructose, lactulose, and combinations of these nutrients. Little information is known about the benefit in the prevention of NEC (16), (40).
Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by definition of the WHO and FAO, Food and Agriculture Organization of the United States (41). Probiotics may prevent NEC by promoting colonization of the gut with beneficial organisms, preventing colonization by pathogens, improving the maturity and function of gut mucosal barrier, and modulating the immune system to the advantage of the host (42), (43).

In the following a short overview will be given on several studies that showed a decreased incidence of NEC after probiotic supplementation.

Hoyos (44) compared 1237 newborns admitted to a NICU in Colombia during one year with a historical control group of 1282 newborns. The intervention group received Lactobacillus acidophilus and Bifidobacterium infantis until discharge and showed significant lower NEC incidence (3 %) than in the control group (7 %).

In Taiwan Lin et al. (45) investigated NEC in very low birth infants (below 1500 g) who survived the first week of life. They were randomly assigned to one of the two study groups. The intervention group with 180 neonates received breast milk with Lactobacillus acidophilus and Bifidobacterium infantis, whereas the control group with 187 neonates received breast milk alone. NEC incidence in the intervention group was significantly lower (1 %) than in the control group (5 %).

2008 Lin et al. (46) conducted a prospective, blinded, randomized, multicenter controlled trial at 7 NICUs in Taiwan. The intervention group with 217 very low birth weight (below 1500 g) neonates receiving L. acidophilus and B. infantis was compared to a control group with 217 VLBW neonates also. NEC incidence in the intervention group (2 %) was significantly lower than in the control group (7 %).

In Israel Bin-Nun et al. (47) randomly assigned VLBW neonates (less than 1500 g) either to the intervention group with 72 neonates administered B. infantis, Streptococcus thermophiles and Bifidobacteria bifidus or to the control group with 73 neonates. NEC incidence was significantly lower in the intervention group (4 %) than in the control group (16 %).
Samanta et al. (48) conducted a randomized double-blind controlled trial in India. The intervention group with 91 very low birth weight neonates (< 1500 g) received Bifidobacterium bifidus, Bifidobacterium lactis, Bifidobacterium infantis and Lactobacillus acidophilus. NEC incidence was significantly lower in the intervention group (6 %) in comparison to the control group (16 %).

Braga et al. (49) also investigated VLBW neonates in a randomized double-blind controlled trial in Brasilia. 119 neonates received human milk supplemented with Bifidobacterium breve and Lactobacillus casei whereas 112 neonates received human milk alone. NEC incidence was significantly lower in the intervention group (0 %) than in the control group (4 %).

Guthmann et al. (50) performed a meta-analysis with 2193 study participants of which 1117 received probiotics. In six studies with 542 neonates in the intervention group only one probiotic strain was used (B. lactis, B. breve, L. rhamnosus GG, L. acidophilus, S. boulardii) with a relative risk of NEC incidence of 0,58. Four studies with 560 neonates used two to four probiotic strains and could halve NEC incidence in comparison to single probiotic trials and showed relative risk of 0,25 compared to placebo. The authors conclude that multi-strain probiotics seem to reveal better results than single-strain probiotics. The most used combination was Bifidusbacterium spp. and Lactobacillus acidophilus in three trials with 488 neonates with a relative risk in NEC incidence of 0,29 and an open label study (not included in the meta-analysis) with 1237 preterm neonates.

In no case of the neonates administered with probiotics adverse effects were reported. Results from Guthmann et al. (50) suggest that probiotics seem to be safe and effective in reducing NEC rates and mortality in preterm neonates with a birth weight below 1500 g and suggest using more than one probiotic strain.

In 2010 Deshpande et al. (51) updated their meta-analysis from 2007. Eleven studies (seven from the meta-analyses from 2007 plus four new) from the period 1997 to 2009 with a total of 2176 preterm newborns (< 34 weeks) resp. newborns below 1500 g were analyzed. The authors conclude that the risk for NEC and death was significantly lower in newborns with probiotic supplementation.
However, the risk for sepsis did not differ significantly. Since no significant adverse effects were reported the authors suggest that probiotics should be offered as routine therapy for preterm neonates.

Another meta-analysis was done by AlFaleh et al. (52) who included nine studies from the period between 1986 and 2005. Six of these studies are also included in the meta-analysis of Desphande et al. (51). In 1425 preterm neonates (< 37 weeks) or neonates with low birth weight (< 2500 g) probiotics significantly reduced the incidence of NEC and mortality, but not of sepsis.

Despite the positive results of studies on probiotics, one should also be aware that the food and drug administration has not approved the administration of a microorganism in preterm neonates yet. Introducing live microorganisms into the gastrointestinal tract of premature infants need to be handled with caution and safety. Until now, no cases of bacterial sepsis or other adverse effects have been observed after supplementation of probiotics in preterm neonates; however, little is known about any possible long-term effects of probiotics on the development of the immune system. Chou et al. (53) reported about the long-term neurodevelopmental outcome of preterm VLBW neonates who received probiotics. Growth measures (weight, length, head circumference), neurologic and sensory performance as well as neurodevelopmental status (Bayley Scales of Infant Development II) were tested at three years corrected age. No significant differences were found in any outcome between the probiotic group with 153 neonates and the control group with 148 neonates.
1.3 Study purpose

The incidence of necrotizing enterocolitis (NEC) is highest among preterm newborns and newborns with low birth weight (8). Results from several studies over the last 15 years give evidence that the prophylactic supplementation of probiotics lead to a decrease in the incidence of NEC (15), (45-51).

At Dhulikhel Hospital NEC incidence is with estimated 10,7 % (estimation done by Dr. Srijana Dongol for July 2009 to July 2010) higher than the average international incidence of NEC with 6 % (26), (54-58).

Furthermore Shrestha et al. (7) identified NEC with 25,8 % as the third common cause of preterm neonatal mortality at DH after hyaline membrane disease (64,5 %) and sepsis (58,1 %).

One more reason that shows the importance of prevention of NEC is that no pediatric surgery is available at DH. If later stages of NEC occur and surgery is needed either the adult surgeons would do the surgery or the neonate would need to be transferred to a specialized center in Kathmandu, which in turn is related to the factors time and money.

Therefore, a placebo controlled introduction of prophylactic supplementation of probiotics to preterm neonates (< 37 gestational weeks) is planned in order to reduce incidence of NEC and to reduce mortality.
2 Methods

2.1 Study design

The study comprises two parts, a first part collecting secondary data in a retrospective data analysis about NEC and a second part collecting primary data about NEC in a prospective study.

2.1.1 Retrospective Study

2.1.1.1 Study plan

The retrospective study aims the acquisition of NEC data (including incidence) from the last two years at Dhulikhel Hospital so far available. All available archived data files at pediatric duty room were screened by the three variables “preterm”, “low birth weight”, and “suspected NEC”.

Preterm was defined as gestational age below 37 weeks. Low birth weight was defined as birth weight below 2000 g. The variables “preterm” and “low birth weight” were selected since these two depict major risk factors for NEC.

Note that usually low birth weight is defined by < 2500 g. Based on a small sample of birth weights of termed newborns with an average weight below 3000 g (collected in July 2010), we assumed that Nepalese newborns might have a lower birth weight than the average European neonate and therefore defined low birth weight by ≤ 2000 g for the purpose of this data analysis.

Suspected NEC was defined by at least two of the following criteria:

- Abdominal distension: girth increase (measured by a measuring tape)
- Gastric aspiration (as sign of feeding intolerance)
- Positive test for occult blood in stool (detected by guaiac method)
- Melena
- Metronidazole treatment

One of these two criteria had to be Metronidazole treatment.
If at least two criteria were fulfilled the data files were analyzed in more detail (described in 2.1.1.3 Data collection).

2.1.1.2 Study population

All preterm neonates (less than 37 gestational weeks), low birth weight neonates (below 2000 g) or neonates with suspected NEC admitted to NICU between May 2009 and end of January 2011 were included in the study. Neonate period is defined by 28 days of life; therefore, maximum age at admission was 28 days of life.

However, it is important to note, there is no evidence of completeness of archived data files. Data archiving at pediatric duty room started in 2009. The screened files include admissions between May 2009 and end of January 2011.

2.1.1.3 Data collection

Information from the data files were collected in Excel files. In one Excel file information about preterm or low birth weight neonates and in another file information about neonates with suspected NEC was gathered. A further file was created to note daily changes in some parameters. In Table 4 the collected variables are listed.

These variables also include the outcome of the neonates. There are two special terms of discharge at Dhulikhel Hospital that need further explanation.

“Discharge on request” means that the neonate is improving but clinicians would not recommend discharge yet. However, parents decide to take their newborns home from hospital.

“Left against medical advice (LAMA)” means that the neonate is not improving, maybe the diagnosis has not been made yet and the child is deteriorating. In this case clinicians are strictly against discharge. When prognosis of the newborn is poor, families with low income need to calculate if they can afford spending money on the newborn with the poor prognosis or if they better keep the money for the survival of the other family members. Each day in the hospital means unexpected costs for the families (see subchapter 2.2.2). If poor families have to struggle to
survive and have a newborn with poor prognosis they often leave against medical advice with the baby.

Table 4: Recorded Variables
In total 28 variables were recorded for suspected NEC cases and 18 variables (in bold) for preterm and LBW neonates

<table>
<thead>
<tr>
<th>QUANTITATIVE VARIABLES</th>
<th>QUALITATIVE VARIABLES</th>
<th>DAILY REC. VARIABLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of Admission (DOA)</td>
<td>Sex</td>
<td>Weight</td>
</tr>
<tr>
<td>Date of Birth (DOB)</td>
<td>Birth place (inborn/outborn)</td>
<td>Ventilation (yes/no/method)</td>
</tr>
<tr>
<td>Age at Admission</td>
<td>Type of delivery</td>
<td>Feeding (parenteral, breast milk, formula)</td>
</tr>
<tr>
<td>Gestational age at birth</td>
<td>Discharge (on request, LAMA*)</td>
<td></td>
</tr>
<tr>
<td>Birth weight</td>
<td>NEC (yes/no)</td>
<td></td>
</tr>
<tr>
<td>APGAR score (1min/ 5min)</td>
<td>Diagnoses</td>
<td></td>
</tr>
<tr>
<td>Date of Discharge (DOD)</td>
<td>Antibiotics (yes/no)</td>
<td></td>
</tr>
<tr>
<td>Date of death</td>
<td>Birth complications</td>
<td></td>
</tr>
<tr>
<td>Date of start of treatment</td>
<td>NEC symptoms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abd. X-ray (yes/no)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other antibiotic treatment (yes/no)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnancy risk factors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Perinatal risk factors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neonatal risk factors</td>
<td></td>
</tr>
</tbody>
</table>

DEVELOPED VARIABLES

<table>
<thead>
<tr>
<th>Duration of hospitalization</th>
<th>Duration of ventilation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of oral feeding</td>
</tr>
<tr>
<td></td>
<td>Weight development</td>
</tr>
</tbody>
</table>

*LAMA = left against medical advice

2.1.2 Prospective Study

2.1.2.1 Study plan

The prospective study aims to evaluate if prophylactic probiotic supplementation brings a clear benefit or not. For this reason a comparison between the intervention group and the control group will be made.
Subsequently we have one independent variable, treatment (probiotic supplementation vs. placebo) and one dependent variable, NEC incidence. The probiotic supplementation will be started for preterm neonates at admission to NICU and will last until discharge or the diagnosis of NEC. To evaluate the effect of probiotic supplementation a statistical comparison of NEC incidence between the intervention group and the placebo controlled group will be conducted. The null hypothesis says that there will be no significant difference in the incidence of NEC between the intervention group and the placebo group. The alternative hypothesis says that prophylactic supplementation of probiotics will lead to a decreased incidence of NEC in comparison to the placebo control group. A decreased incidence will count as clear benefit of probiotic supplementation.

2.1.2.2 Study population

All preterm neonates (less than 37 gestational weeks) admitted to NICU are included in the study. Neonate period is defined by 28 days of life; therefore, maximum age at admission is 28 days of life. Start of the intervention is at admission and end of the intervention is at discharge, diagnosis of NEC or death of the neonate. Depending on the study group, the neonate will either get a probiotic solved in EBM or solely EBM per oral (described in subchapter 2.1.2.3). The allocation to one of the two groups is randomized. The study is single-blinded, i.e. the patient (neonate and parents) is unaware of the treatment. Required sample size was estimated by the following formula (59):

\[ n = K[(R+1) - p_2(R^2+1)]/p_2 (1 - R)^2 \]

The parameters in the formula are:
n = required sample number for both groups
K = factor (in case of alpha-error 0,05 and power 0,8 = 7,85; see Table 5)
p_1 = incidence rate in the intervention group, here: 3,20 %
p_2 = incidence rate in the control group, here: 10,70 %
R = relative risk (p_1/p_2), here: 0,30
Table 5: Factor K dependent on alpha-error and power

Source: Kranke et al., 2008 (59)

<table>
<thead>
<tr>
<th>$\alpha$-Fehler</th>
<th>0.8</th>
<th>0.9</th>
<th>0.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>7.85</td>
<td>10.51</td>
<td>13.00</td>
</tr>
<tr>
<td>0.01</td>
<td>11.68</td>
<td>14.88</td>
<td>17.82</td>
</tr>
</tbody>
</table>

Relative Risk was estimated to be 0.30 in reference to previous studies (Lin et al., 2005 (45): $R = 0.21$; Lin et al., 2008 (46): $R = 0.28$; Bin-Nun et al., 2005 (47): 0.24; Samanta et al., 2009 (48): $R = 0.35$; Deshpande et al., 2010 (51): $R = 0.35$; AlFaleh et al., 2010 (52): $R = 0.32$).

Assuming a relative risk of 0.30 a study population of 178 neonates per group will be required to allow drawing conclusions from statistical analyses.

Per year around 150 preterm neonates are admitted to NICU at DH, giving 75 for each study group. Aiming a study population of 178 per group will probably require a minimum study period of 2 years and 3 months.

We expect to get a statistical result which indicates if there is a difference between the intervention group and the placebo group. This result will lead to further implications.

2.1.2.3 Material

The intervention group gets prophylactic probiotic supplementation.

The trade name of this product is “Antibiophilus” and its manufacturer is Laboratoires Lyocentre in Aurillac, France. Sales and distribution is conducted by Germania Pharmaceuticals in Vienna, Austria. In sum we estimated to need around 2000 bags of Antibiophilus for this study and this amount is provided by Germania Pharmaceuticals.

Antibiophilus was chosen because it is used since more than 20 years at Neonatology in Graz with positive experience (15).

Antibiophilus is a single strain probiotic and contains Lactobacillus casei var. rhamnosus 35 (LCR35). Originally the bacterium lactobacillus rhamnosus was considered to be a subspecies of Lactobacillus casei, but recent molecular genetic
research revealed that rhamnosus is a species of its own (60), (61), so Lactobacillus rhamnosus 35 would be a more reasonable nomenclature. Today it is known that LCR35 and Lactobacillus Gorbach Goldin (LGG) are very similar (similarity in about 96% regarding to Germania resp. Laboratoires Lyocentre).

The dosage of Lactobacillus casei var. rhamnosus is two times daily 0.80 mg Antibiophilus (1/2 package) solved in 2 ml expressed breast milk for neonates weighing more than 1500 g and two times daily 0.40 mg Antibiophilus (1/4 package) solved in 1 ml expressed breast milk for neonates weighing less than 1500 g.

Probiotics are given for prophylaxis. If NEC occurs despite probiotic prophylaxis, the probiotic supplementation will be stopped immediately and NEC will be treated with the standard antibiotic treatment at Dhulikhel Hospital (see 1.2.6 Treatment of NEC).

The exact composition of Antibiophilus contains a minimum of 1000 Mio/g of lyophilisate of Lactobacillus casei var. rhamnosus (LCR35), potato starch, 0.38 g lactose and maltodextrine. One bag contains 1.5 g powder of the above mentioned composition.

The placebo group receives solely expressed breast milk, two times daily 2 ml for neonates weighing more than 1500 g and two times daily 1 ml for neonates weighing less than 1500 g.

2.1.2.4 Data collection

Data are collected via three standardized forms (see chapter 6 Appendix 4-6); one form for all included neonates, one for those who developed NEC and one daily protocol for variables that change during the study period (e.g. weight) is included. The variables are the same as collected in the retrospective study (Table 4 in subchapter 2.1.1.3). For all preterm neonates included in the study the following variables are collected:

Admission date, date of birth, age at admission, sex, admission (inborn/outborn), birth place (DH, other hospital, health post, home), gestational age at birth, birth weight, type of delivery (elective/emergency Cesarean section, vaginal), APGAR (1 min/ 5 min), diagnosis, any antibiotic treatment, type of feeding (enteral vs.
parenteral), type of enteral feeding (breast feeding (BF), expressed breast milk (EBM), lactogen), daily weight, date of discharge/death/LAMA.

For those neonates who developed NEC in addition the following variables are collected: Staging after abdominal X-ray (classification by Bell > stage 2, see subchapter 1.2.5), comorbidities, ventilation, pregnancy risk factors (amniotic infection syndrome (AIS), perinatal risk factors (asphyxia, umbilical catheter, patent ductus arteriosus (PDA), infant respiratory distress syndrome (IRDS), neonatal risk factors (intensive care, congenital heart defect with low output, state of shock, omphalitis, sepsis).

From the recorded data, NEC incidence, mortality, weight development, duration of ventilation and duration of hospitalization can be calculated.

Data are collected in an Excel file. At the end of intervention statistical comparison between intervention group (with probiotic supplementation) and the placebo controlled group will be conducted. However, evaluation of the NEC incidence is also planned during the intervention.

A potential bias could be treatment that neonates received before admission to Dhulikhel Hospital. Dhulikhel Hospital not only treats neonates born inside but also from other health centers or babies delivered at home. To detect this bias, parents will be asked if their neonates got any treatment outside of Dhulikhel Hospital (asked in Form 1, chapter 6 Appendix).

2.2 Setting

2.2.1 Dhulikhel Hospital

Dhulikhel Hospital (DH) is a university hospital in collaboration with Kathmandu University. The non-governmental and non-profit hospital was established in 1996 and follows the principles of social equity, sustainable development and harmony with nature. DH provides cost-effective high-quality health care services to a population of around 1,9 Mio. The hospital also puts great focus on public health projects and community programs and runs 12 outreach clinics that are a first contact point for the population in rural and remote areas.
Dhulikhel is located 1650 m above sea level in Bagmati, 30 km east of Kathmandu and is headquarter of Kavre district. Dhulikhel has a population of about 14,000, Kavre district of about 1.6 Mio. Moreover, people from surrounding districts consult health care services at DH (62).

![Figure 2: Dhulikhel Hospital (Source: left own image, right (63)).]

### 2.2.2 Department of Pediatrics

The hospital is providing pediatric services since its establishment in 1996 for children from birth up to 14 years.

In mid-2002 the department was divided into an outpatient (pediatric OPD, outpatient department) and an inpatient division (pediatric ward). Two years later (mid 2004) the pediatric ward was extended with four neonatology beds. The next notable change took place at the end of 2006 when the department moved into a separate building and expanded to 45 beds (30 general, 5 Pediatric Intensive Care Unit, PICU and 10 Neonatal Intensive Care Unit, NICU) (62).

At the time of data collection the pediatric staff comprised four consultants, one resident, three medical offers, four interns and 20 staff nurses.

The hospital is providing routine service six days a week from Sunday to Friday.

The outpatient department rooms are equipped with a computer with internet access, a weighing machine, a stadiometer (scale for measuring children), an infantometer (scale for measuring babies) and diagnostic standard sets. In 2009 the highest numbers of pediatric patients were seen in May with 680 patients and
November with 702 patients. On average 516 patients per month and 17 patients per day were serviced in 2009 (62).

Figure 3: Department of Pediatrics (Sources: Upper left (64), other own images)

Inpatient services include the general pediatric ward, the postnatal ward, NICU and PICU.

The facilities of the general pediatric ward comprise a television, a Video-CD Player, books, drawing and painting materials, toys and dolls for patients. In 2009 1032 patients were admitted in the pediatric ward. The highest admission rate was in October with 121 admissions. On average there were 94 patients per month and three patients per day hospitalized. Most of the patients were less than one year, followed by those aged one to five years.

The most frequent diseases were in decreasing order, respiratory diseases, gastrointestinal diseases, central-nervous-system diseases, renal diseases, infectious diseases, hematological diseases, failure to thrive, heart diseases and rheumatic diseases.
The pediatric team is also responsible for examining all the neonates delivered in DH every day in the postnatal ward. For each neonate a standard neonatal record is completed with all clinical details, also including perinatal information. All neonates are screened for congenital hypothyroidism.

Figure 4: First postnatal investigation (Sources: own images)

Neonatal intensive care unit (NICU) is equipped with two incubators, three radiant warmers, seven baby cots, three patient monitors, two ventilators, four phototherapy units, portably X-ray, two infusion pump-sets, two syringe infusion pumps, a pulse oxymeter, a transcutaneous bilirubinometer, a glucometer and a resuscitation trolley. A separate lab with arterial blood gas analysis is available. 2009 a total of 282 babies were admitted to NICU, the admissions per month ranged from 17 to 33. The major causes of admissions were neonatal sepsis and hyperbilirubinemia. Most of the babies were of low birth weight. The total number of deaths in NICU was 31 giving a mortality rate of 11 %. The causes of death included preterm birth, preterm with hyaline membrane disease (HMD = IRDS), birth asphyxia (BA) and neonatal sepsis (NNS).

In 2009 NICU reported about improved survival with neonatal ventilators and successful management of very low birth weight babies (VLBW), i.e. successful management of a baby with a birth weight of 820 g and gestational age of 28 weeks.
In Pediatric intensive care unit (PICU) a total of 84 patients were admitted in 2009, 14 of those died, giving a mortality rate of 17%.

Patients have to cover the costs for diagnostic approach and treatment by themselves, whereby the prices are distinct lower than in other hospitals since DH aims to provide its health care services to the whole population, also to the poor from rural parts. Examples for prices at the Department of Pediatrics at DH are presented in Table 6 (Cashier, DH, Feb 2011).
### Table 6: Examples for hospital costs at Pediatrics

<table>
<thead>
<tr>
<th>Investigation/Treatment</th>
<th>Charge (NPR)</th>
<th>Charge (Euro*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NICU or PICU Admission Charge (per day)</td>
<td>1000,-</td>
<td>9,-</td>
</tr>
<tr>
<td>Oxygen therapy without Ventilation (per day)</td>
<td>1500,-</td>
<td>13,50</td>
</tr>
<tr>
<td>Ventilation (per day)</td>
<td>2500,-</td>
<td>22,80</td>
</tr>
<tr>
<td>Phototherapy (per day)</td>
<td>500,-</td>
<td>4,50</td>
</tr>
<tr>
<td>Test for occult blood in stool</td>
<td>25,-</td>
<td>0,23</td>
</tr>
<tr>
<td>Metronidazole 500 mg iv</td>
<td>30,-</td>
<td>0,27</td>
</tr>
<tr>
<td>X-ray for neonates (8x10)</td>
<td>120,-</td>
<td>1,09</td>
</tr>
<tr>
<td>CT abdomen full</td>
<td>5000,-</td>
<td>45,60</td>
</tr>
<tr>
<td>Arterial blood gas analysis</td>
<td>500,-</td>
<td>4,50</td>
</tr>
<tr>
<td>Blood or Urine or Stool culture</td>
<td>200,-</td>
<td>1,80</td>
</tr>
<tr>
<td>CRP</td>
<td>200,-</td>
<td>1,80</td>
</tr>
<tr>
<td>WBC or Differential Count or Platelets</td>
<td>40,-</td>
<td>0,36</td>
</tr>
<tr>
<td>Potassium or Sodium</td>
<td>120,-</td>
<td>1,09</td>
</tr>
<tr>
<td>Calcium</td>
<td>140,-</td>
<td>1,27</td>
</tr>
</tbody>
</table>

*Currency conversion from Nepalese Rupees (NPR) to Euros on 20.04.2012

At this side it has to be mentioned that the gross national income (GNI) per capita is 490 USD giving 41 USD per month. Fifty-five percent of Nepal’s population is below international poverty line of 1,25 USD per day (65). Albeit DH aims to keep costs for patients as low as possible, for the poor ones treatment is not always affordable. In Nepal no health insurance system is provided by the government.

### 2.3 Ethical Approval

For the retrospective data analysis approval by the Institutional Review Board (IRB) of Kathmandu University School of Medical Sciences was sufficient.

The prospective placebo-controlled study needed approval by the national ethical review committee “Nepal Health Research Council” (NHRC) in Kathmandu. An ethics proposal was submitted in February 2011. In March 2011 we got a first response with suggestions for revision. The final approval was given in August 2011 (see chapter 6 Appendix 3).

For the prospective study parents get extensive information and have to sign a consent form (see chapter 6 Appendix 1 English Consent Form and Appendix 2 Nepali Consent Form).
3 Results of the retrospective data analysis

According to NICU admission books 499 neonates were hospitalized between May 2009 and end of January 2011. All available archived NICU data files were screened for the three variables “preterm”, “low birth weight”, and “suspected NEC”. If one of these variables was present, files were selected and analyzed in detail.

At first, results of the analysis of all preterm neonates and low birth weight neonates are described (subchapter 3.1) and afterwards, results of the analysis of all suspected NEC cases (subchapter 3.2).

3.1 Preterm and low birth weight neonates

All screened files comprised 113 preterm neonates (< 37 GW) and 14 term (> 37 GW) but low birth weight (≤ 2000 g) neonates. According to the admission books the screened period comprises 499 neonates, giving an incidence of 23 % for preterm birth; however, one should consider that there is no evidence of data completeness when referring to this incidence data.

Descriptive results for preterm and low birth weight neonates are shown in Table 7.

Table 7: Mean values for gestational age and weight for preterm and LBW neonates

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm (65 male, 48 female)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational Age (weeks)</td>
<td>33</td>
<td>3</td>
<td>25 to 36</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>1781</td>
<td>511</td>
<td>840 – 3500</td>
</tr>
</tbody>
</table>

| Term, Low Birth Weight (5 male, 9 female) |       |                    |            |
| Weight (g)                             | 1661  | 209                | 1440 – 1990 |
In Figure 6 a distribution of gestational age among the included preterm neonates is presented. Most preterm neonates were born at a gestational age of 32, 33 or 34 weeks. Gestational age was defined by Modified Ballard Score.

![Preterms: Gestational Age](image)

**Figure 6: Gestational age of 113 preterm neonates**

In this data collection five extremely preterm neonates between 25 and 27 gestational weeks were recorded. All of those five were born at DH by vaginal delivery; however, all of them left against medical advice (LAMA). Two of them (twins) were taken by their parents against medical advice on the second day of life, whereas the other three left between the first and fourth day of life. None of them was suspected for NEC.

The most interesting question in this first part of the analysis is: How many preterm neonates and term neonates with low birth weight developed NEC? In 47 out of 113 preterm neonates (42 %) and in 6 out of 14 low birth weight neonates (43 %) NEC was suspected (Fig. 7). A more detailed analysis of these suspected NEC cases is described in the next subchapter.
3.2 Suspected NEC cases

3.2.1 Case characteristics

No case of NEC was confirmed by abdominal X-ray (international gold standard), thus staging according to the modified Bell’s criteria for NEC (30) was not possible.

In this analysis cases were counted as suspected NEC when at least two of the following criteria were fulfilled:

1.) Abdominal distension
2.) Gastric aspiration (as sign of feeding intolerance)
3.) Positive test for occult blood in stool
4.) Melena
5.) Metronidazole treatment

One of these two criteria had to be Metronidazole treatment.

As presented in Figure 8, from all screened patient files 93 neonates with suspected NEC were identified. For two neonates only discharge files but no complete patient records were available, thus those two neonates were excluded from further analysis. Finally 91 neonates with suspected NEC remained for detailed analysis. According to the admission books the screened period comprises 499 neonates, giving an incidence of 18 % for the suspected NEC cases; however, one should consider that there is no evidence of data completeness when referring to this incidence data.
As described before (subchapter 3.1), within all preterm and low birth weight neonates, 47 preterm neonates and 6 term LBW neonates were identified as suspected NEC cases. Looking at all suspected NEC cases, these 47 preterm neonates account for 52 % and the 6 term LBW neonates for 7 % of all suspected NEC cases. In Figure 9 the population of suspected NEC cases is split up and can be analysed in more detail: 46 % were born at term, 52 % preterm and for 2 % information about gestational age at birth is missing. Within term neonates 72 % had a birth weight above 2000 g, whereas 14 % had a birth weight less than or equal to 2000 g (small for gestational age) and for 14 % the birth weight was not known. Within preterm neonates 15 % had a birth weight above 2000 g and 85 % had a birth weight less than or equal to 2000 g.
In Table 8 mean values are depicted for weight, Apgar-score, duration of hospitalization and duration of ventilation for all 91 suspected NEC cases.

Table 8: Mean values for weight, Apgar score, hospitalization and ventilation

<table>
<thead>
<tr>
<th>NEC (50 male, 41 female)</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g) (7 missing)</td>
<td>2080</td>
<td>597</td>
<td>1000 – 3400</td>
</tr>
<tr>
<td>Apgar 1 min (16 missing)</td>
<td>6</td>
<td>2</td>
<td>3 – 9</td>
</tr>
<tr>
<td>Apgar 5 min (16 missing)</td>
<td>8</td>
<td>1</td>
<td>5 – 10</td>
</tr>
<tr>
<td>Hospitalization (days) (16 missing)</td>
<td>12</td>
<td>7</td>
<td>1 – 43</td>
</tr>
<tr>
<td>Ventilation* (days) (2 missing)</td>
<td>3</td>
<td>5</td>
<td>0 – 20</td>
</tr>
</tbody>
</table>

*Ventilation included SIMV (Synchronized Intermittent Mandatory Ventilation), PCM (Pressure Controlled Mode) and CPAP (Continuous Positive Airway Pressure) Mode.

In Figure 10 a detailed analysis of gestational age in neonates with suspected NEC is presented. There is one peak during preterm period (32 and 33 weeks), and a further peak in term infants.

Figure 10: Gestational Age and suspected NEC
In total 81 neonates were inborn (admission within first 24 hours of life) and 10 neonates were outborn (admission after first 24 hours of life). From 81 inborn neonates 69 were delivered at DH, seven were delivered at home, four were delivered at a health post and one was born at another hospital. From 10 outborn neonates three were delivered at home, three were delivered at another hospital and one was born at DH but admitted to NICU at 8th day of life. For three outborn neonates site of delivery was not documented.

In Figure 11 the mode of delivery is described. Most often neonates were delivered by emergency Cesarean section and vaginal delivery.

![Mode of Delivery in Suspected NEC Cases](image)

LSCS = Lower Segment Cesarean Section

**Figure 11: Mode of delivery in suspected NEC cases**

Risk factors were divided in pregnancy risk factors, perinatal risk factors and neonatal risk factors.

Pregnancy risk factors and complications were documented for 22 neonates (24%). One risk factor was intrauterine growth restriction (IUGR) occurring in 11 patients, whereas in three of them also oligohydramnion was documented and in one of them antepartum hemorrhage (APH). Antepartum hemorrhage was also present in one patient alone and in one patient combined with placenta previa.
Other pregnancy complications included HELLP syndrome in one case, eclampsia in two cases, cephalopelvic disproportion (CPD) in one case, one oblique breech with cord presentation, one case of pregnancy induced hypertension (PIH), one case of placenta previa and cord prolapse and two cases of premature rupture of membranes (PROM).

For 55 of 91 suspected NEC cases (60,5 %) no pregnancy risk factor was documented. For 14 neonates (15,5 %) detailed information about pregnancy was missing.

Perinatal risk factors were documented for 70 neonates (77 %). The first big risk factor was respiratory distress (RD) documented in 64 neonates in total, whereby for 20 neonates respiratory distress and birth asphyxia (BA) occurred at the same time and for two besides RD also an umbilical catheter was documented. The second big risk factor was birth asphyxia (BA) documented in 26 neonates in total. For 14 neonates (15,5 %) no perinatal risk factors were recorded, for 7 neonates (7,5 %) detailed data associated with birth were missing.

Neonatal risk factors were present in all suspected NEC neonates (100 %). All had neonatal sepsis, 83 neonates with early onset neonatal sepsis (EONS) and 8 with late onset neonatal sepsis (LONS).

Mechanical ventilation is also considered as neonatal risk factor (see subchapter 1.2.3). The different ventilation modes used at DH are face mask (< 4 litres of oxygen), head box (4-10 litres of oxygen), Continuous Positive Airway Pressure (CPAP), Pressure Controlled Mode (PCM) and Synchronized Intermittent Mandatory Ventilation (SIMV) for weaning. In the present analysis ventilation includes CPAP, PCM and SIMV.

Nearly one half of all suspected NEC cases was ventilated (48,5 %), the other half was not ventilated (49,5 %) and for 2 % data about ventilation are missing. The mean duration of ventilation was 3 days, whereby variation was large (STD = 5 days). 30 neonates were ventilated between 1 and 7 days, 10 neonates were ventilated between 8 and 14 days, and 4 neonates between 15 and 20 days.
In most of the cases comorbidity was high. In Table 9 the most common diseases diagnosed in suspected NEC cases are listed.

Table 9: Comorbidity

<table>
<thead>
<tr>
<th>Most common diseases in 91 suspected NEC cases:</th>
</tr>
</thead>
<tbody>
<tr>
<td>91 % Neonatal Sepsis (Early Onset) 11 % Pneumonia</td>
</tr>
<tr>
<td>70 % Respiratory Distress 9 % Neonatal Sepsis (Late Onset)</td>
</tr>
<tr>
<td>50 % Hyperbilirubinemia 9 % Cardiopulmonary Arrest</td>
</tr>
<tr>
<td>20 % Dyselectrolytemia 9 % Acute Renal Failure</td>
</tr>
<tr>
<td>19 % Birth Asphyxia HIE¹ I 6 % Birth Asphyxia HIE¹ III</td>
</tr>
<tr>
<td>17 % Neonatal Meningitis 4 % Neonatal Seizure</td>
</tr>
<tr>
<td>15 % Meconium aspiration 4 % Birth Asphyxia HIE¹ II</td>
</tr>
<tr>
<td>12 % IUGR² 4 % Hyaline Membrane Disease</td>
</tr>
<tr>
<td>12 % DIC³ 2 % Hemipneumothorax</td>
</tr>
</tbody>
</table>

¹HIE = Hypoxic Ischemic Encephalopathy  
²IUGR = Intrauterine Growth Restriction  
³DIC = Disseminated Intravascular Coagulopathy

3.2.2 Criteria

As already mentioned before, in this analysis cases were counted as suspected NEC when at least two of the following criteria were fulfilled: abdominal distension, gastric aspiration, occult blood in stool, melena, and Metronidazole treatment, whereby one of these two criteria had to be Metronidazole treatment. Gastric aspiration was present in different forms: curdy (milky) aspiration, yellowish/brownish aspiration, brownish aspiration, brownish/blood stained aspiration, brownish/greenish, greenish aspiration or blood aspiration.

Ten neonates (11 %) fulfilled two of the above listed criteria, 53 neonates (58 %) fulfilled three criteria, 26 neonates (29 %) fulfilled four criteria and two neonates (2 %) fulfilled all five criteria (Fig. 12).
Figure 12: Suspected NEC cases and number of criteria

From ten neonates with two fulfilled criteria, seven presented with abdominal distension and Metronidazole treatment, two presented with greenish or brownish aspiration and Metronidazole treatment and one presented with a positive test result for occult blood in stool and Metronidazole treatment.

From 53 neonates with three fulfilled criteria, all 53 presented with abdominal distension and Metronidazole treatment. As third criterion, 51 neonates presented any kind of aspiration and two neonates showed a positive result for occult blood in stool.

From 26 neonates with four fulfilled criteria, all 26 neonates presented with abdominal distension, any kind of aspiration and Metronidazole treatment. Additionally, in 22 neonates stool test for occult blood was positive and in four neonates melena was present.

Two neonates fulfilled all five criteria, abdominal distension, aspiration (brownish in both neonates), occult blood in stool, melena, and Metronidazole treatment.
In Figure 13 the relative frequency of each criterion within all 91 suspected NEC cases is presented.

![Graph showing relative frequency of criteria for suspected NEC](image)

**Figure 13: Relative frequency of criteria accounted for suspected NEC**

### 3.2.3 Outcome

All in all, out of 91 suspected NEC cases, 37 got discharged, 33 died, 18 left against medical advice, two were discharged on request and for one discharge data are missing (Fig. 14).

![Outcome tree diagram](image)

**Figure 14: Outcome of all suspected NEC cases**
As already described in subchapter 2.1.1, “discharge on request” means that the neonate is improving but clinicians would not recommend discharge yet. “Left against medical advice (LAMA)” means that the neonate is not improving, maybe the diagnosis has not been made yet and the child is deteriorating. In this case clinicians are strictly against discharge.

In Figure 15 outcome is analysed regarding term and preterm birth.
Out of all survived neonates 46 % were preterm and 54 % were term, whereby 3 % were term LBW neonates and for 5 % birth weight is unknown so that 46 % remain within term neonates.
Out of all neonates with unknown outcome 33 % were preterm and 67 % were term, whereby 14 % were term LBW neonates and for 10 % birth weight is unknown so that 43 % remain with term neonates.
Out of all mortality cases 70 % were preterm and 24 % were term, whereby 6 % were term LBW neonates and for 6 % birth weight is unknown so that 12 % remain within term neonates.

Figure 15: Outcome of term and preterm neonates
As described in subchapter 3.2.2 the number of criteria for suspected NEC differed among all cases, some neonates fulfilled two, many fulfilled three or four criteria and only few fulfilled all five criteria.

Since the number of cases within each group was different, and two groups comprise only a small number of cases, no comparison between these groups is possible, only outcome within each group can be analyzed (Fig. 16).

Within the group with two fulfilled criteria the majority died, within the group with three fulfilled criteria more neonates survived than died. In the group of neonates with four fulfilled criteria survival rate is also higher than mortality rate, however, the difference is only small. In the group with all fulfilled criteria, no general assumption can be made because of the small number of two neonates – one died and from one neonate outcome is unknown.

Highest survival rates were observed in the group with three fulfilled criteria and the group with four fulfilled criteria.

---

**Figure 16: Detailed outcome of all suspected NEC cases**
4 Discussion

This work comprises the organization of a prospective controlled trial on the basis of a retrospective data analysis.

4.1 Retrospective study

Analysis of retrospective data has shown a high incidence of suspected NEC. Since confirmation of NEC with abdominal X-ray was not done in any case, we can only refer to suspected NEC cases. 91 cases fulfilled at least two criteria accounted for NEC. Most of the neonates fulfilled three (58 %) or four (29 %) criteria. For the two neonates fulfilling all five criteria, NEC can be assumed with highest certainty, but still it was not confirmed via radiographic examination. Having no abdominal X-rays is one of the major limitations of this study.

The most frequently presented symptoms were abdominal distension and gastric aspiration as sign of feeding intolerance. These initial symptoms are typical for NEC (16). Macroscopic blood in stool is usually detected in 25 % of NEC patients (8). In our sample only 7 % showed melena but 30 % had a positive result for occult blood in stool.

Clinical symptoms accounted for NEC could also occur in specific intestinal or systemic infections, gastrointestinal obstruction, volvulus or isolated intestinal perforation. Another differential diagnosis is idiopathic focal intestinal perforation that can occur spontaneously or after the early use of postnatal steroids and indomethacin. Indomethacin is a Non-Steroidal Anti-inflammatory Drug (NSAID), used for preventing intraventricular hemorrhage (IVH) and patent ductus arteriosus (PDA). However, indomethacin is not used at DH. These patients also show pneumoperitoneum, but usually they are less ill than those with NEC (8).

Another limitation of this study is that the analysis of systemic signs (temperature instability, apnea, bradycardia, lethargy, hypotension) and laboratory results (acidosis, blood count, differential blood count) could not be included into this retrospective analysis.

Regarding laboratory results in this study, 20 % of the suspected NEC cases had the diagnosis dyselectrolytemia, but no details for laboratory parameters were collected.
Because of having only suspected NEC cases in this study, comparison with other studies can only be done with restriction. The present incidence of suspected NEC cases with 18 % is high. A similar NEC incidence (16,4 %) was reported by Bin-Nun et al. (47) in Israel. However, only VLBW neonates (less than 1500 g) were included in their control group of 73 neonates. Likewise, Samanta et al. (48) recorded a NEC incidence of 15,8 % in their control group of 95 preterm (below 32 weeks) and VLBW neonates in India. Hoyos (44) report about an incidence of 6,6 % in their historical control group of 1282 newborns in Colombia. In Taiwan Lin et al. (46) report about an incidence of 6,5 % in their control group of 217 very low birth infants (below 1500 g).

From 91 suspected NEC cases 52 % were preterm neonates, 46 % were born at term, and for 2 % gestational age at birth was missing. The high number (46 %) of term neonates is unusual. Literature reports a NEC incidence of 10 % in term neonates (9). However, 7 % were term but low birth weight neonates and can also be classified as small for gestational age (SGA) indicating growth retardation. Growth retardation in turn is a risk factor for NEC (23). For another 7 % birth weight is not known so that 29 % term neonates with suspected NEC remain, still a high incidence.

Mortality rate was very high, 33 out of 91 suspected NEC cases (36 %). In comparison Samanta et al. (48) report a mortality rate of 14,7 % in their control group in India or Schmölzer et al. (15) had 5,6 % mortality in their control group in Austria.

It also needs to be considered that there is a high number of neonates with unknown outcome (23 %). The 18 neonates who left against medical advice (LAMA) had poor prognosis and it can be assumed that they most likely died. The two neonates who were discharged on request had a better prognosis and thus a higher likelihood to survive. So the real mortality rate is not known.

When prognosis of the newborn is poor, families with low income need to calculate if they can afford spending money on the newborn with the poor prognosis or if they better keep the money for the survival of the other family members. Each day in the hospital means unexpected costs for the families. If poor families have to
struggle to survive and have a newborn with poor prognosis they often leave against medical advice with the baby.

Also, in one mortality case the note was added that further resuscitation was refused due to parents’ request.

From this handling one could assume that some families might not even seek for medical care for their newborn, especially when they think their preterm newborn has poor prognosis. This thought is also underlined by the fact that all of the five extremely preterm neonates were given birth at the hospital. So there might be extremely preterm neonates delivered at home or minor health centres but these cases might not all reach the hospital and higher medical care.

Most of the mortality cases (70%) were preterm and 30% were term. This goes in line with the hospital report from 2009 (62) where preterm birth is listed first among the most frequent death causes at NICU.

The cause of death is only known in a few cases (9% cardiopulmonary arrest); however, since in fact all 91 suspected NEC cases also had the diagnosis of neonatal sepsis high mortality might also be related to sepsis.

In Kliegman (8) it is mentioned that due to nonspecific sings, sepsis may be suspected before NEC. This fact may also explain the high correlation between NEC and sepsis. At Dhulikhel Hospital blood cultures are done routinely, but the results of blood cultures were not included in this study, since the focus was not on sepsis.

Another reason for the high mortality rate might be the high number of neonates with respiratory distress (70%) and the fact that surfactant is not routinely available as therapy for preterm neonates.

Shrestha (7) also mentioned that intracranial hemorrhage and congenital heart diseases are possible underdiagnosed, since cranial ultrasound and echocardiography are not performed routinely.

Besides neonatal sepsis and respiratory distress other common comorbidities included hyperbilirubinemia, birth asphyxia, dyselectrolytemia, neonatal meningitis, meconium aspiration and pneumonia.

From all screened files 114 included preterm neonates. According to the admission books the screened period comprised 499 neonates giving an incidence
of 23% for preterm birth, whereby this number might be biased by missing data files. However, analyses of Shrestha et al. (7) revealed a similar incidence (19.5%) of preterm birth at DH between 2007 and 2009. Preterm birth and low birth weight could be confirmed as high risk factor. Within preterm neonates around 42% and within low birth weight neonates around 43% showed symptoms accounted for suspected NEC.

Several further risk factors were confirmed. The most common pregnancy risk factor was IGUR occurring in 12% of all suspected NEC cases. Perinatal risk factors were documented for 70 of 91 neonates, whereby respiratory distress (70%) and birth asphyxia (29%) were diagnosed most often. Respiratory distress and birth asphyxia might have played a prominent role in the development of NEC. Neonatal risk factors were present in all suspected NEC cases, since all suffered from neonatal sepsis (like already discussed above). Mechanical ventilation is also considered as risk factor. Here, half of the neonates were ventilated.

It has to be mentioned that not for all neonates detailed information about pregnancy, birth or first days of life were available.

In general the fact of missing data is also a limitation of this study. Furthermore it cannot be excluded that also files are missing. The screened period comprised a total number of 499 cases according to the admission books; however, no prove was done, if all 499 files were complete. Therefore the number of 91 suspected NEC cases out of 499 cases could be biased by missing files.

However, this work offers a first detailed analysis of cases suspected for NEC at DH. Several parameters were documented and analyzed and provide important information about the situation of a newborn in Nepal.

Important conclusions from this retrospective study include that NEC diagnosis needs to be improved by abdominal X-ray according to international standards, that incidence of suspected NEC cases is high and associated with a high mortality rate, and that therapeutic options in case of NEC are limited (no pediatric surgery at DH, NICU stay and treatment means high and often unaffordable costs for the families).

Thus, prophylaxis from NEC by probiotic supplementation could be a means of improvement at DH and therefore the prospective study is of high relevance.
4.2 Prospective study

Since this work does not include any evaluation of the prospective study only the study design can be discussed.

One might argue that a randomized control like the prospective study planned here is not justified. Desphande et al. (51) argue that a clear benefit of probiotics has been shown over the last two decades and therefore a placebo group is not needed anymore. However, recently Mihatsch et al. (66) point out that not all randomized controlled trials on probiotics are of high level of evidence and therefore recommend large, multi-center randomized controlled trials.

Originally the present study design was different. The original aim of the retrospective analysis was the acquisition of a historical control group. However, this aim could not be met, mainly because proper diagnosis of NEC (abdominal X-ray or sonography) was missing at DH. All NEC cases in the screened period of 2009 and 2010 were only suspected NEC cases, not a single case was confirmed radiologically.

In the prospective study abdominal X-rays will be a standard diagnostic tool in case of suspected NEC. This discrepancy in diagnosis of NEC does not allow any comparison between the historical group and the intervention group. Additionally, there are steadily changes at NICU (e.g. installation of an air filter system) to improve infection rate and mortality in general. These changes might have also coincided when comparing a historical control group with the intervention group. In a prospective study these steadily changes will be balanced between the two study groups.

Thus, a comparison with a historical control group was not an option anymore and a placebo group was planned for control.

Ensuring that abdominal X-ray will be a standard diagnostic tool at NICU also shows that research and specifically this study aims to improve the quality of medical care in general. The costs for an abdominal X-ray are very low, so that everyone can afford. Despite, in case patients cannot afford, it was arranged that these costs will be covered by the hospital.

Further, there are no studies about probiotics used in preterm neonates in Nepal so far and therefore we do not know the risk of intervention even when we can
deduce the risk from studies in other countries. Scientific evaluation of a possible effect of the probiotics needs a comparison between the intervention group and a control. For the above explained reasons (diagnosis with abdominal X-ray, comparable control group, first use of probiotics in Nepal) a placebo control group was included in the study.

Another critic could be the use of a single strain probiotic albeit literature showed an advantage in NEC risk reduction when more than one probiotic is used (50). However, when starting the intervention with two probiotics at the same time, it is not possible to conclude to which extend both probiotics are involved in any possible effect. Therefore the decision was taken to start with one probiotic (lactobacillus that is used at NICU in Graz) and maybe add another one later in a second study. A second probiotic strain suggested in literature could be Bibidobacterium.

Further discussion might be related to sample size of the study population that was estimated based on the expected relative risk. The estimation of relative risk resp. risk reduction is very optimistic, but literature indicated a relative risk of 0.30 by probiotic prophylaxis (45-48), (51), (52).

During the study close monitoring of the intervention group and intermediate data analysis will be done to observe the trend of the data. If probiotic supplementation will not show the expected effect resp. be adverse, supplementation will be stopped. If risk reduction is not as large as assumed, more data and a longer study period might be necessary to get stable statistics. If probiotics show any preventive effect, it might also be interesting to calculate cost reduction. Especially in a health system like Nepal where the patients have to pay everything by themselves and the majority of Nepalese is poor, costs do matter. If a clear benefit in costs can be shown, acceptance from patients would be higher. Probiotics are a simple method for prevention of NEC whereas treatment of NEC can involve antibiotics, hospitalization at NICU, ventilation or in worst case even surgery resp. transfer in a higher hospital center.

Related future research will focus on the evaluation of prospective probiotics study.
5 References


(64) Dhulikhel Hospital Picture [image on the internet]. 2009 [cited 2012 May 7]. Available from: http://www.flickr.com/photos/38678049@N08/3651417700/in/photostream/


6 Appendix

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Appendix 1: Consent Form in English

CONSENT FORM for the study
“Placebo controlled introduction of prophylactic supplementation of probiotics to decrease the incidence of Necrotizing Enterocolitis (NEC) at Dhulikhel Hospital in Nepal”

Dear Parent,
The aim of this study is to evaluate the effect of prophylactic supplementation of probiotics to reduce the incidence of Necrotizing Enterocolitis. Necrotizing Enterocolitis is a common and serious disease of the intestines in newborns, especially in preterm newborns. Usually Necrotizing Enterocolitis is treated by antibiotics. Now we want to find out if we can prevent this disease by prophylactic supplementation of probiotics. Several studies over the last 15 years already showed this positive effect. In this study one group of newborns will get prophylactic supplementation of probiotics and one group will get a placebo. The allocation to the groups will be randomized, that means that it is random if your newborn will get probiotics or a placebo.

The supplementation of the probiotics or the placebo will be orally and does not cause any harm to your newborn. Despite the prophylactic supplementation of probiotics or placebo your newborn may still develop Necrotizing Enterocolitis. In this case prophylactic supplementation of probiotics or placebo will be stopped immediately and antibiotic treatment will be started.

Please note, that you can withdraw your newborn from this study at any time without giving reason and without fear. In this case, you just let to know the doctor on duty about your decision. You can be sure that all data are highly confidentially. We will use an anonymous subject code for your newborn in our database and for further analysis.

Statement:
I thoroughly read the consent form above and fully understood everything.
I agree that my newborn will participate in this study.
Name of parent:

Date:  
Signature:  

Name of witness:

Date:  
Signature:
Appendix 2: Consent Form in Nepali

शोधको लागि मन्त्रीरीत्या

"Placebo controlled introduction of prophylactic supplementation of probiotics to decrease the incidence of necrotizing enterocolitis at Dhulikhel Hospital in Nepal."

आदरणीय अभिमानक बयां,

यस शोधको प्रमुख आविष्कार नेपालमा इन्टररोकोलाइटि्स भनेर रोग लागू भएको अगाडि नै प्रोबाकोटिसको प्रयोगले प्रदान गर्न सक्ने पाइदानक असर हेतु हो। नेपालमा नवजात शिशुहरूको आन्द्रामा लागूँ एक फायदा रोग हो र यसको लागि एन्टीबायोटिकसको प्रयोग गरिस्। अहिल्ये यस शोधको हामी प्रोबाकोटिसको प्रयोग गरर यस रोग लागू बचाव भएको सकिने समावेशका बौझमा खोजी गरिएक्छौं।

१५ वर्षको अन्तरालमा गरिएका विभिन्न शोधले प्रोबाकोटिसको फाइडाजनक असर पनि देखाएका छन्।

यो शोधमा हामी बल्कहरूलाई दुईवटा समुहमा बाँटिएका छ। एउटा समुहले Placebo पाउँछ भने अर्को समुहले प्रोबायोटिक पाउँछ। समुहले वर्गीकरण भने विना कुनै होम नियमले गरिएका र यसले प्रोबायोटिक शिशुहरूलाई कुरा तपाईलाई बाह्य हुने छ।

प्रोबायोटिक र placebo दुवै मुख्यत दिइने हुँदैर र यसले बल्कहरूलाई कुनै किसिमको प्राप्तिको भविष्यको छ।

यो placebo अथवा प्रोबायोटिकको प्रयोग गर्दा-गर्दा पनि तपाईलाई शिशुलाई नेऐ हुन सक्छ। यसले अवस्थामा थिइ, एकै हरुले तपाईलाई प्रोबायोटिक सुरु गरिएका छ।

तपाईलाई यो कुरा सुचित गरिएका र तपाईलाई आफ्नो नवजात शिशुलाई कुनै पनि समयमा यो अनुसार विना निर्देशन गर्न सक्छौं हुनुहुनै र कुनै पनि स्पष्टिकरणको आवश्यकता पनि छैन। त्यसतो अवस्थामा तपाईले आफ्नो Duty भएका डाक्टरलाई जानकारी गराउन पनि छ।

हामी यो कुरा रोधको पनि जानकारी गराउन भएको र विना नवजात शिशुहरूको नामांची कतै प्रकाशित गरिन्छ। त्यसैलाई यो आफ्नो जानकारी गराउन भएको कारण हामी संग भएको निति र रहेको एन्टीबायोटिक भनेर र तपाईले शिशुलाई एउटा कोइले मान्त्र्य प्रयोग गरिएका र त्यसको आधारमा शोध गरिएका हुन।

बयां:

मैले यो मन्त्रीरीत्या विस्तारमा पढेदै र यसमा स्पष्ट भएको देखिएको हुनेछ। आफ्नो शिशुलाई यस शोधमा सहभागी गराउन मन्त्रु हुँ छ।

अभिमानको नाम:

भित्रः हस्ताक्षरः

साझीको नाम:

भित्रः हस्ताक्षरः
Appendix 3: Ethical Approval

Nepal Health Research Council
Estd. 1991

Ref. No. 133

Executive Committee

Executive Chairman
Prof. Dr. Chop Lal Bhusal

Vice - Chairman
Dr. Rishi Ram Koirala

Member-Secretary
Dr. Shanker Pratap Singh

Members
Dr. Narendra Kumar Singh
Dr. Meeta Singh
Dr. Suman Rijal
Dr. Samjana Dhakal
Dr. Devi Gurung

Representative
Ministry of Finance
National Planning Commission
Ministry of Health & Population
Chief, Research Committee, IOM
Chairman, Nepal Medical Council

16 August 2011

Dr. Srijana Dongol
Principal Investigator
Kathmandu University,
School of Medical Sciences,
Dhulikel Hospital

Ref: Approval of Research Proposal entitled Placebo Controlled Introduction of
Prophylactic Supplementation of Probiotics to Decrease the Incidence of
Necrotizing Enterocolitis (NEC) at Dhulikel Hospital in Nepal

Dear Dr. Dongol,

It is my pleasure to inform you that the above-mentioned proposal submitted on 24
Feb 2011 (Reg. no. 17/2011 please use this Reg. No. during further correspondence)
has been approved by NHRC Ethical Review Board on 14 Aug 2011 (2008-04-29). Further, the board directs you to ensure that the Probiotics be used only for the above-
mentioned research purpose.

As per NHRC rules and regulations, the investigator has to strictly follow the
protocol stipulated in the proposal. Any change in objective(s), problem statement,
research question or hypothesis, methodology, implementation procedure, data
management and budget that may be necessary in course of the implementation of the
research proposal can only be made so and implemented after prior approval
from this council. Thus, it is compulsory to submit the detail of such changes
intended or desired with justification prior to actual change in the protocol.

If the researcher requires transfer of the bio samples to other countries, the
investigator should apply to the NHRC for the permission.

Further, the researchers are directed to strictly abide by the National Ethical
Guidelines published by NHRC during the implementation of their research
proposal and submit progress report and full or summary report upon completion.

As per your research proposal, your research is self-funded and NHRC processing fee
is US$ 100.00.

If you have any questions, please contact the research section of NHRC

Thanking you.

Sincerely Yours,

[Signature]

Dr. Shanker Pratap Singh
Member Secretary
Appendix 4: Data Collection Form 1

Form 1: For all preterms (< 37 weeks gestation)

<table>
<thead>
<tr>
<th>Placebo □</th>
<th>Antibophilus</th>
<th>NICU □</th>
<th>Paediatric Ward □</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission date (dd/mm/yy) [international calendar]</td>
<td>Date of Birth (dd/mm/yy) [international calendar]</td>
<td>Age at admission [max. 28th DOL for study inclusion]</td>
<td>Name of Neonate</td>
</tr>
<tr>
<td>Hospital Nb.</td>
<td>Sex Female □</td>
<td>Male □</td>
<td>Admission Inborn □ or Outborn □ (Admission within 24 h of life) (Admission ≥ 2nd DOL)</td>
</tr>
<tr>
<td>Birth place DH □, Other hospital □, Health post □, Home □</td>
<td>Weeks of gestation at birth</td>
<td>Birth weight (g)</td>
<td>Type of delivery Emergency LSCS-Section □ Elective LSCS-Section □ Vaginal Delivery □</td>
</tr>
<tr>
<td>APGAR Score (1 min; 5 min)</td>
<td>Diagnosis</td>
<td>Antibiotic treatment yes □ no □</td>
<td>Feeding Enteral □ Parenteral □</td>
</tr>
<tr>
<td>Type of enteral feeding EBM/BF □ Lactogen □</td>
<td>Date of discharge (dd/mm/yy) [international calendar]</td>
<td>Discharge □ LAMA □ Death □</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 5: Data Collection Form 2

Form 2: In addition to Form 1 when NEC is diagnosed

<table>
<thead>
<tr>
<th>Placebo □</th>
<th>Antibophilus □</th>
<th>NICU □</th>
<th>Paediatric Ward □</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission date (dd/mm/yy)</td>
<td>Date of Birth (dd/mm/yy)</td>
<td>Age at admission</td>
<td>Name of Neonate</td>
</tr>
<tr>
<td>[international calendar]</td>
<td>[international calendar]</td>
<td>[max. 28th DOL for study inclusion]</td>
<td></td>
</tr>
<tr>
<td>Hospital Nb.</td>
<td>Sex</td>
<td>Admission</td>
<td>Birth place</td>
</tr>
<tr>
<td></td>
<td>Female □</td>
<td>Inborn □ (Admission within 24 h of life)</td>
<td>DH □, Other hospital □, Health post □, Home □</td>
</tr>
<tr>
<td></td>
<td>Male □</td>
<td>Outborn □ (Admission ≥ 2nd)</td>
<td></td>
</tr>
<tr>
<td>Weeks of gestation at birth</td>
<td>Birth weight (g)</td>
<td>Type of delivery</td>
<td>APGAR Score (1 min; 5 min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emergency LSCS-Section □</td>
<td>Enteral □</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elective LSCS-Section □</td>
<td>Parenteral □</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vaginal Delivery □</td>
<td></td>
</tr>
<tr>
<td>Birth complications</td>
<td>Feeding</td>
<td>Type of enteral feeding</td>
<td>Comorbidities</td>
</tr>
<tr>
<td></td>
<td>Enteral □</td>
<td>EBM/BF □</td>
<td>Abdominal X-ray (&gt; Bell stage 2a)</td>
</tr>
<tr>
<td></td>
<td>Parenteral □</td>
<td>Lactogen □</td>
<td>Stage 2b □, Stage 3a □, Stage 3b □</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Abdominal girth increase □, Gastric aspiration □, Stool occult blood test pos. □, Melena □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Date of NEC diagnosis/Start of Treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>Metronidazole □  Other □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other antibiotic treatment</strong></td>
<td>yes □  no □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Days on Ventilator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pregnancy risk factors</strong></td>
<td>Amniotic infection syndrome □  Other: □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Perinatal risk factors</strong></td>
<td>Asphyxia □  Umbilical catheter □  PDA □  RD □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neonatal risk factors</strong></td>
<td>ICU □  Congenital heart defect □  State of Shock □  Omphalitis □  Early Onset Sepsis □  Late Onset Sepsis □</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Date of discharge (dd/mm/yy)</strong></td>
<td>Discharge □  LAMA □  Death □</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[International calendar]
# Appendix 6: Daily Protocol Form

<table>
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<tr>
<th>Name of Neonate</th>
<th>DOB (dd/mm/yy)</th>
<th>Day of Admission (DOA) (dd/mm/yy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date (dd/mm/yy)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>½ bag □ □ □</td>
<td>¼ bag □ □ □</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>g</th>
<th>g</th>
<th>g</th>
<th>g</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Feeding (ml)</th>
<th>i.v. ml</th>
<th>EBM</th>
<th>Lactogen</th>
<th>ml</th>
<th>BF</th>
<th>BF</th>
<th>BF</th>
<th>BF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ventilation</th>
<th>CPAP □</th>
<th>PC □</th>
<th>SIMV □</th>
<th>CPAP □</th>
<th>PC □</th>
<th>SIMV □</th>
<th>CPAP □</th>
<th>PC □</th>
<th>SIMV □</th>
<th>CPAP □</th>
<th>PC □</th>
<th>SIMV □</th>
</tr>
</thead>
</table>