STUDY PROTOCOL

A randomized multicenter open-label controlled trial to show that mucous fistula refeeding reduces the time from enterostomy closure to full enteral feeds
(**MUC**ous **FI**stula **RE**feeding ("**MUC-FIRE**") trial)

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LIST OF ABBREVIATIONS

AE	adverse event
ALT	alanine transaminase
AST	aspartate transaminase
CVL	central venous line
DMC	Data Monitoring Committee
eCRF	electronic case report form
FIP	focal intestinal perforation
FTT	failure to thrive
FU	follow-up
GGT	Gamma-GT
HCTC	Hannover Clinical Trial Center
ICD	International Statistical Classification of Diseases and related Health Problems
IMC	intermediate care ward
ITT	intention to treat
IVH	intraventricular hemorrhage
MFR	mucous fistula refeeding
NEC	necrotizing enterocolitis
NG	nasogastric
NICU	neonatal intensive-care units
OR	operation room
PC	phosphatidylcholine
POD	postoperative day
SAE	serious adverse event
SBBO	small bowel bacterial overgrowth
SOP	standard operating procedure
TPN	total parenteral nutrition

STUDY SYNOPSIS

Title of Study	A randomized multicenter open-label controlled trial to show that mucous fistula refeeding reduces the time from enterostomy closure to full enteral feeds (**MUC**ous **FI**stula **RE**feeding ("**MUC-FIRE**") trial)
Short Term	MUC-FIRE
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Study Design	Randomized, multicenter, open-label, controlled, parallel group research study
Patient Population	Infants who underwent creation of an enterostomy receiving postoperative care and awaiting enterostomy closure
Participating Study Sites	Approx. n = 11
Sample Size	To be assessed for eligibility: n = 201 To be assigned to the study: n = 106 To be analysed: n = 106
Objectives	The primary objective of this study is to demonstrate that mucous fistula refeeding between enterostomy creation and enterostomy closure reduces the time to full enteral feeds after enterostomy closure compared to standard of care.
Endpoints	<u>Primary efficacy endpoint:</u> Time to full feeds (hours), defined as time to actual enteral intake of the age-dependent caloric requirements per day (defined as 120kcal/kg/24h)

for at least 24h and a concomitant reduction of parenteral fluids to <20ml/kg/24h
[Nutrition Committee, Canadian Pediatric Society; Committee on Nutrition, American Academy of Pediatrics].

Key secondary endpoints:

1) Reoperation
2) Time to first bowel movement after enterostomy closure (mucous stool is considered a bowel movement)
   Cleaning and changing of infants diapers will be performed according to a fixed schedule in order to uniformly document the time to first bowel movement following enterostomy closure.
3) Postoperative weight gain (g/d) (daily documentations recommended, minimum 2x per week), regular Z-Score (standard deviation score) documentation [WHO - weight-for-age] (daily documentations recommended, minimum 2x per week). This will be carried out according to a fixed schedule during morning rounds prior to feeding in an unclothed status.
4) Days of postoperative total parenteral nutrition (> 20 ml/kg/24h) before and after the 2nd operation (=ostomy takedown) (TPN)
   Days of total parenteral nutrition (TPN) are counted, starting on the day of enterostomy closure and ending on the day of full enteral nutrition. The parenteral nutrition is manufactured by the hospital pharmacy on a daily basis, while considering the simultaneous enteral caloric intake.
5) Laboratory parameters indicating cholestasis (conjugated bilirubin, GGT, ALT, AST,hemoglobin) and sodium resorption (sodium in urine).
   Time points for harvesting of blood samples: Baseline at the time of randomization, then every 2 weeks until enterostomy takedown, at the 3-months follow up and in cases of pathological clinical signs (jaundice, acholic stools)
6) Weight gain during the subsequent 5 days after reaching the primary endpoint
7) Central venous line (CVL) duration (days) and number of CVL infections (definition of infection: Neo-Kiss Guidelines)
8) Length of hospital stay (days)
9) Estimated ratio of the diameter of the two bowel loops which are anastomosed.

Assessment of safety:

Assessment of possible (serious) adverse events (AEs/SAEs) after randomization (e.g. death, sepsis, bowel perforation)

Inclusion and Exclusion Criteria	<u>Key inclusion criteria:</u> Infants < 366 days, Ileostomy / Jejunostomy, double loop enterostomies and split enterostomies (with mucous fistula) Notice: All patients with meconium ileus are included. If later (required) diagnostics verify cystic fibrosis, the diagnostics as well as the diagnosis need to be documented in the eCRF and in further analysis subgroups will be established. Signed written informed consent obtained by parents/legal guardians and willingness of parents/legal guardians to comply with treatment and follow-up procedures of their child <u>Key exclusion criteria:</u> • resection of ileocecal valve, • colostomy, • small bowel atresia, • multiple ostomies (more than just an enterostomy and a mucous fistula), • chromosomal abnormalities (if known at the time of randomization), • Hirschsprung's disease, • participation in another drug-intervention study • Intestinal perforation due to a hemodynamic heart defect Reoperation (e.g. relaparotomy) prior to randomization is not an exclusion criterion, these patients may still be included in the study.
Intervention	All patients will receive standard care with standardized enterostomy creation and closure and will be treated according to a standardized feeding protocol. <u>Experimental intervention:</u> Perioperative mucous fistula refeeding between enterostomy creation and enterostomy closure. <u>Control intervention:</u> No perioperative mucous fistula refeeding between enterostomy creation and enterostomy closure.

	Follow-up per patient: 3 months and 6 months postoperatively, following enterostomy closure (12-month follow-up only applicable for patients that are recruited early enough to complete this follow-up within the 48 month of overall study duration). Duration of intervention per patient of the intervention group: minimum 21 days/3 weeks until patient's weight >2000g (averaged 6 weeks between enterostomy creation and enterostomy closure).
Study Duration	Recruitment: approx. 41 months (176 weeks) Study duration per patient: Maximum 58 weeks to minimum 32 weeks Duration of the entire study (first patient in to last patient out): 48 months (208 weeks)
Statistical Analysis	Efficacy: The type-one error rate is set to 5% (two-sided). Description of the primary efficacy: The primary analysis is performed on the intention to treat population (ITT). The aim of this study is to demonstrate superiority of perioperative mucous fistula refeeding compared to standard care (no mucous fistula refeeding) in reducing the time to full enteral feeds after enterostomy closure. The treatment effect will be estimated with a Cox-regression adjusted for treatment, weight at birth (<1000g / ≥1000g), , study center as well as height of the stoma (jejunostoma/proximal Ileostoma or terminal ileostoma) and will be assessed by the estimated hazard ratio (refeeding vs no refeeding) for reaching full enteral feeds. Superiority of the refeeding procedure will be concluded if the lower bound of the corresponding two-sided 95%-confidence interval for the treatment effect hazard ratio is greater than 1. Safety: (Serious) adverse events (AEs/SAEs) will be compared between treatment groups with a chi-square test and other appropriate tests. P-values will be assessed descriptively. Secondary endpoints: All secondary analyses will be explorative.

RESPONSIBILITIES

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1 SCIENTIFIC BACKGROUND AND STUDY RATIONALE

Enterostomies in infants may be created for different reasons. During the presence of an enterostomy, the regular stool transfer is interrupted since the distal part of the bowel (the part following the enterostomy) does not participate in the processing of stool. Therefore it does not contribute to the resorption of enteral nutrients. As a consequence, these infants need additional parenteral nutrition. Due to the negative side-effects of parenteral nutrition all patients should return to enteral nutrition as soon as possible. Consequently, many pediatric surgical centers worldwide routinely perform mucous fistula refeeding (MFR) into the former unused bowel after enterostomy creation because case reports and retrospective analyses show low complication rates and faster postoperative weight gain. Several providers, however, shy away from this approach because to date there is still no high quality evidence for the benefit of this treatment. The aim of this study is to assess the effects of mucous fistula refeeding in a prospective randomized trial. We hypothesize that MFR between enterostomy creation and enterostomy closure reduces the time to full enteral feeds after enterostomy closure compared to standard of care. Moreover, the side effects of parenteral nutrition may be reduced and the postoperative hospital care of infants undergoing ostomy closure shortened.

1.1 The Medical Problem

After creation of any enterostomy the bowel distal to the enterostomy is not in use. Therefore the physiologic passage of stool, nutrient uptake and growth of the bowel distal of the enterostomy are interrupted. At the time of enteral reanastomosis, the surgeon often sees an enormous discrepancy in diameters of the proximal and the distal loops of bowel. In these cases, the postoperative increase of enteral feeds and the dependence of the infant on parenteral nutrition may be prolonged. Furthermore, it is well known that continuous parenteral nutrition is associated with several side effects including cholestasis and central line infections [1]. The physiological passage of stool through the bowel is important for enterohepatic circulation, resorption of fluids, electrolytes, vitamins, and enteral growth. Moreover, the passage of stool per rectum is important for developing a regular defecation reflex.

1.2 Evidence

Recently Gause et al. presented their results on MFR in neonatal patients [2]. In their retrospective analysis of 28 patients (13 in the MFR group and 15 in the control group) a shorter duration of parenteral nutrition and a faster time to full enteral feeds in the MFR group were reported. In 2006, Richardson et al. performed a systematic review on case reports and small case series of MFR after enterostomy creation [3]. The authors concluded that MFR was safe, as no complications were identified in any of the cited publications. In conclusion, studies published so far showed a faster weight gain in the group of MFR compared to controls [2–6]. These promising results need to be confirmed by a randomized, controlled study, which is the intention of this proposal.

1.3 The need for a study

As suggested by Gause et al. [2] a multicenter study of MFR is warranted in order to address the limitations of retrospective studies carried out so far. The results of this randomized

controlled study may strongly influence the perioperative care of neonates within the pediatric surgical community. If our hypothesis is confirmed, the postoperative hospital stay of infants undergoing ostomy closure will be shortened. The benefits of MFR include a shorter duration and therefore less side effects of parenteral nutrition. Moreover, an economic benefit through lower costs for TPN and a shorter hospital stay may be reached.

1.4 Risk-Benefit-Assessment

Many pediatric surgical centers worldwide routinely perform MFR after enterostomy creation. However, due to a lack of prospective studies the level of evidence showing a benefit of this treatment strategy is low. Although the systematic review by Richardson et al. [3] showed no complications using this technique, MFR into the distal bowel loop may potentially cause complications such as bowel perforation. The risk for possible complications can be minimized by careful and standardized manipulation of the enterostomies. The local condition of the ostomy will be investigated twice daily.

If our hypothesis is confirmed, the postoperative hospital care of infants undergoing ostomy closure will be shortened. The benefits of MFR may include a shorter duration and therefore less side effects of parenteral nutrition. Moreover, an economic benefit through lower costs for TPN and a shorter hospital stay may be reached.

The results of the current study may influence the standard of neonatal intensive care. Therefore the potential benefits of MFR outweigh the possible risks of this study.

Results of data analyses including all data how to perform MFR will be published. If the results of this study will show significant differences between the intervention group and controls, MFR will become the new standard of care for neonates with enterostomies.

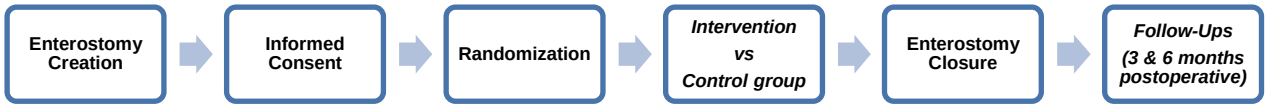
In Germany, the current national guideline for neonatal and surgical treatment of necrotizing enterocolitis (NEC) is currently in revision [Leitlinie 024-009: Nekrotisierende Enterokolitis (NEK)]. One of the principal investigators of the study (Prof. Dr. Martin Lacher) is coauthor of this guideline. If the current study proves the hypothesis that MFR is beneficial for these infants it may not only change the national guideline for the best treatment after enterostomy creation in Germany but in other countries too.

2 STUDY DESIGN, OBJECTIVES AND ENDPOINTS

2.1 Study Design

This is a randomized, multicenter (n=11), open-label, parallel group, controlled research study to demonstrate that mucous fistula refeeding between enterostomy creation and enterostomy closure reduces the time to full enteral feeds after enterostomy closure compared to standard of care.

Intervention scheme/Study flow



2.2 Study Objectives

The primary objective of this study is to demonstrate that mucous fistula refeeding between enterostomy creation and enterostomy closure reduces the time to full enteral feeds after enterostomy closure compared to standard of care.

2.3 Study Endpoints

2.3.1 Outcome measures

Time to full enteral feeds after enterostomy closure (hours) was chosen as the primary outcome parameter because of its clinical relevance representing the influence of MFR on the intestinal autonomy in the course of the disease. The endpoint is highly objective due to the strict and well-defined feeding protocol (see 3.1). In most of the referenced publications postoperative weight gain early after surgery was chosen as the primary outcome parameter.

However, body weight is always affected by the shift of body fluids into the third space. Therefore postoperative weight does not always correlate with enteral/ parenteral caloric supplementation as a sign of enteral resorption. For this reason it was not selected as the primary outcome parameter but will be assessed as secondary outcome measure.

Secondary outcome measures further include the number of days of postoperative total parenteral nutrition (TPN) and the cholestasis parameters (conjugated bilirubin, GGT, ALT, AST) as indicators for hepatotoxicity of parenteral nutrition. The “time to first bowel movement” (hours) which correlates to the postoperative transanastomotic passage of stool, will be another secondary outcome parameter. A bowel movement consisting of only mucous rather than stool is also considered a bowel movement. Finally, all outcome parameters including possible complications will be assessed during the follow-up 3, 6 and 12 months (12-month follow-up only applicable for patients that are recruited early enough to complete this follow-up within the 48 month of overall study duration) after enterostomy closure.

2.3.2 Determination of primary and secondary measures

Primary efficacy endpoint:

Time to full feeds (hours), defined as time to actual enteral intake of the age-dependent caloric requirements per day (defined as 120kcal/kg/24h) for at least 24h and a concomitant reduction of parenteral fluids to < 20ml/kg/24h [Nutrition Committee, Canadian Pediatric Society; Committee on Nutrition, American Academy of Pediatrics].

For determining the time to full enteral feeds, the feeding advancement will be carried out according to the predefined nutritional protocol after 6-8 tolerated feedings in 3-4 hour intervals (24 hours). “Full feeds” is therefore defined as 120kcal/kg/24h actual enteral intake [7,8]. The nurses will document any increase and decrease of nutrition precisely and daily controls will be carried out by the responsible neonatologist and pediatric surgeon.

Secondary endpoints:

1) Reoperation
2) Time to first bowel movement after enterostomy closure (mucous stool is considered a bowel movement),
   Cleaning and changing of infants diapers will be performed according to a fixed schedule in order to uniformly document the time to first bowel movement following enterostomy closure.
3) Postoperative weight gain (g/d) (daily documentations recommended, minimum 2x per week), regular Z-Score (standard deviation score) documentation [WHO - weight-for-age] (daily documentations recommended, minimum 2x per week), This will be carried out according to a fixed schedule during morning rounds prior to feeding in an unclothed status.
4) Days of postoperative total parenteral nutrition (> 20 ml/kg/24h) before and after the 2nd operation (=ostomy takedown) (TPN)
   Days of postoperative total parenteral nutrition (TPN) are counted, starting on the day of enterostomy closure and ending on the day of full enteral nutrition. The parenteral nutrition is manufactured by the hospital pharmacy on a daily basis, while considering the simultaneous enteral caloric intake.
5) Laboratory parameters indicating cholestasis (conjugated bilirubin, GGT, ALT, AST, hemoglobin) and sodium resorption (sodium in urine).
   Time points for harvesting of blood samples during clinical routine blood withdrawal: Baseline at the time of randomization, then every 2 weeks until enterostomy takedown, at the 3-months follow up and in cases of pathologic clinical signs (jaundice, acholic stools)
6) Weight gain during the subsequent 5 days after reaching the primary endpoint
7) Central venous line (CVL) duration (days) and number of CVL infections (definition of infection: Neo-Kiss Guidelines)
8) Length of hospital stay (days)
9) Estimated ratio of the diameter of the two bowel loops which are anastomosed

2.4 Study Duration

Recruitment:
Approximately 41 months (176 weeks)

Study duration per patient:
Maximum 58 weeks to minimum 32 weeks

Duration of the entire study (first patient in to last patient out):
48 months (208 weeks)

3 STUDY POPULATION

3.1 Study Population

Infants who underwent creation of an enterostomy receiving postoperative care and awaiting enterostomy closure:

to be assessed for eligibility: n = 201

to be assigned to the study: n = 106

to be analysed: n = 106

Duration of intervention per patient of the intervention group: minimum 21 days/3 weeksuntil patient's weight >2000g, averaged 6 weeks between enterostomy creation and enterostomy closure

Follow-up per patient: 3 months, 6 months and 12 months following enterostomy closure (12-month follow-up only applicable for patients that are recruited early enough to complete this follow-up within the 48 months of overall study duration).

3.2 Inclusion Criteria

1. Only infants younger than 366 days of age with status post ileostomy or jejunostomy creation (double loop enterostomies and split enterostomies (with mucous fistula)) will be included in the study to create a homogenous cohort of patients with similar diseases (e.g. necrotizing enterocolitis [NEC], focal intestinal perforation [FIP]). Also, infants of this age group are unique in several respects such as the response to parenteral nutrition and its hepatic toxicity resulting into neonatal cholestasis.
   The ostomy localization is restricted to the jejunum and ileum. Therefore, the cohort of patients shows a similar bowel length for fluid-, vitamin- and electrolyte resorption
2. All patients with meconium ileus are included into the study. If later (required) diagnostics verify cystic fibrosis, the diagnostics as well as the diagnosis need to be documented in the eCRF and in further analysis subgroups will be established.
3. Signed written informed consent obtained by parents/legal guardians and willingness of parents/legal guardians to comply with treatment and follow-up procedures of their child

3.3 Exclusion Criteria

1. The resection of the ileocecal valve is an exclusion criterion because of its association with extensive bowel resection and therefore prolonged parenteral nutrition [9]
2. Colostomy
3. Patients with small bowel atresia are excluded because of prenatally underdeveloped bowel distal to the atresia
4. Multiple ostomies (more than just an enterostomy and a mucous fistula)
5. Patients with chromosomal abnormalities (if known at the time of randomization) are excluded because of potential malabsorption and malnutrition due to an underlying syndrome.
6. Hirschsprung disease secondary exclusion
7. Participation in another drug-intervention study
8. Prokinetics are not allowed or mean secondary exclusion
9. Intestinal perforation due to a hemodynamic heart defect

Reoperation (e.g. relaparotomy) prior to randomization is not an exclusion criterion, these patients may still be included in the study.

3.4 Feasibility of recruitment

In order to reach a total sample size of 106 patients during 36 months in 11 centers, every center will have to include 3.2 patients per year on average. The participating centers represent institutions treating a large patient volume and are located in different regions of Germany and Austria. All of them are University hospitals with large neonatal intensive-care units (NICU). When analyzing the center data over the last five years, each of the centers treated at least 6.1 infants per year that would fit our inclusion criteria (see 9.).

3.5 Achievability of recruitment rate

The number of participating centers was increased by new partners (university hospitals/ medical providers treating high patient numbers in specialized pediatric intensive care units). All centers have experience in adhering to scientific protocols and have participated in prospective studies. The necessary patient numbers (n=106) calculated by the power analysis will be achieved in the three year period according to patient numbers of the individual centers. The Hannover Medical School and University of Leipzig have participated in several (multi center) prospective studies without encountering problems with patient recruitment after proper counseling on study goals, protocols and the possible complications in relation to estimated benefits [10–24]. The recruitment of patients in this study will occur after enterostomy creation. As the patients should be clinically stable by this time, the parents will have enough time to make their decision on whether they want their infant to participate in the trial.

3.6 Discontinuation Criteria

The following reasons may lead to discontinuation:

1. Death
2. Bowel perforation due to intubation of a catheter into the distal bowel loop during refeeding

Prokinetic drugs are not allowed throughout the study especially after enterostomy closure.

4 STUDY PROCEDURES

No study procedures are allowed to be conducted until parent's written informed consent has been obtained (please also refer to chapter 9.1). The investigator is responsible for obtaining the parent's written informed consent after adequate explanation of the aim, study assessments, potential risks and benefits and consequences of the study as well as alternative treatment options.

4.1 Study Calendar

	Enterostomy Creation	Screening	Pre Treatment Phase at least 1 week up to 6 weeks	Treatment Phase (Refeeding or Control) at least 3 weeks up to 8 weeks	End of Treatment (**Enterostomy Closure**) approx. 6 weeks after Enterostomy Creation	Post Treatment Phase at least 2 weeks up to 8 weeks	FU 1 3 months 3 months after Entero-stomy Closure)	FU 2 6 months 6 months after Entero-stomy Closure)	(FU 3 12 months)* 12 months after Entero-stomy Closure)
Data Assessment			daily	daily		daily	Outpatient clinic	Outpatient clinic	Outpatient clinic
Randomization			x						
Demographic data		x							
Informed consent		x							
In-/ Exclusion criteria		x							
Operation protocol	x				x				
Body weight		x	x	x		x **	x	x	x
Laboratory			x ***	x ***		x ***			
Refeeding protocol				x					
Nutrition protocol				x		x			
Medical history		x							
Adverse events			x	x	x	x	x	x	x
Time to first bowel movement after enterostomy closure [hours]						x			

*****only applicable for patients that are recruited early enough to complete the 12-month follow-up within the 48 months of overall study duration

**weight is measured during the subsequent 5 days after reaching the primary endpoint

***every 2 weeks starting at randomization and in cases of pathologic clinical signs (jaundice, acholic stools); Laboratory analysis: During routine blood withdrawal, laboratory analysis for the blood parameters of GGT, ALT, AST, hemoglobin and conjugated bilirubin will be performed every 2 weeks starting from randomization until enterostomy closure. Additionally, in urine, sodium concentration is determined in the same time interval. No additional sample volume is necessary for this study.

4.2 Standardized protocol for creation of a small bowel enterostomy (*all patients*):

- Exploratory laparotomy (transverse preferred)
- Possible resection of necrotic bowel
- Identification of bowel for the enterostomy
- Proximal and distal limbs of the bowel loop are pulled through the abdominal wall muscles and skin (Loop enterostomy) via the abdominal incision or separate incision (preferred).
- Measurement of the length of small bowel between
  a) the ligament of Treitz (or if malrotation the first mobile part of the duodenum) and the enterostomy and
  b) the enterostomy and the ileocecal valve [cm].
  The measurement should be undertaken at the antimesenteric wall of the bowel.
- Closure of laparotomy:
  - Fascia with continuous suture Polyglactin 2-3/0
  - Subcutaneous interrupted sutures Polyglactin 4/0
  - Intracutaneous interrupted sutures Poliglecaprone 5/0
- Documentation of operative time (OR-Time in minutes).
- Daily documentation of the patient´s weight recommended (minimum 2x per week).

4.3 Standardized protocol on perioperative mucous fistula refeeding (*MFR*):

Definition: Infants are considered capable for MFR after 2 weeks following enterostomy creation if no contraindications for MFR, like sepsis, are present.

- Start 14-42 days after enterostomy creation (modified according to Wong et al. [6])
- Content to be transferred: the infant's own stool
- Intervals of stool transfer: 6-8 hours as a bolus or continuously via a catheter introduced into the distal bowel loop (blocked with 0.5ml of Water)
- Amount of stool transfer: Initiation with 0,5ml/kg/h per day. Increase of 5ml/kg/d or as tolerated
- If the stool is too thick to be transferred, it may be diluted with normal saline 0,9%. (or glucose 5% in case of hypernatremia), no dilution with formula
- Maximum amount of stool transfer (goal): whole amount of own stool
- Documentation of time point and amount
  a) when the maximum amount of feeds are tolerated
  b) if and when the entire amount of stool is transferred
- Duration of refeeding: at least 3 weeks and until the infant's weight exceeds 2000g,

- Probiotics may be given as per protocol of the local institution
- Prokinetic agents are not allowed during the entire trial.
- MFR should at least be performed for 21 days.
- Documentation whether the full amount of stool has been transferred (yes/no)

4.4 Standardized protocol for enterostomy closure (*all patients*):

- Timing of surgery: at least three weeks of MFR or standard treatmentand an infant's body weight of > 2000g
- Preoperative contrast study of the distal loop of the enterostomy to rule out stenosis is only necessary if the infants have not reached MFR of the total stool amounts of the preceding 24h. For all other infants preoperative contrast studies can be performed voluntarily. This study may be performed on the NICU by plain abdominal X-ray with enteral contrast (water-soluble isoosmolar)
- Central line placement if an adequate amount of calories cannot be provided via a peripheral line.
- No preoperative bowel preparation
- Placement of nasogastric (NG) tube in the operation room (OR)
- Size NG tube:
    - Premature infants up to 3 months of age: 6F catheter
    - 3 to 12 months of age: 8F catheter
- Small bowel anastomosis: Interrupted sutures with
    - 5/0 Polyglactin in infants below 6 months of age
    - 4/0 Polyglactin in infants above 6 months of age

- Perioperative antibiotic therapy: type and length based on bacteria profile have to be documented. Suggestion: Perioperative single shot antibiotic treatment. Different antibiotic regimes, adjusted to microbe profiling is possible, but should be documented precisely.

4.5 Standardized protocol on parenteral nutrition during treatment phase (*all patients*):

using the recommendations in "Neugeborenenintensivmedizin" by Rolf Maier and Michael Obladen (9th edition, 2017) on nutrition:

- fluid (ml/kg body weight/ day)	110 – 180
- energy (kcal/kg body weight/ day)	80 – 160
- amino acid (g/kg body weight/ day)	2 – 4
- lipid (g/kg body weight/ day)	2 – 3

4.6 Standardized protocol for management of nutrition after enterostomy closure (*all patients*):

- Calories of the parenteral nutrition [[7,8]] if there is no hyperglycemia (> 200mg/dl), sepsis, hemodynamic instability that require a different caloric intake.
    - Day of surgery, starting 6h post operation: 50 - 90cal/kg/day
    - POD (postoperative day) #1: 80-120kcal/kg/day
    - POD #2: 80-120kcal/kg/day
    - POD #3: 80-120kcal/kg/day
    - POD #4: 80-120kcal/kg/day
- Composition of lipid, amino acid and energy may vary according to the need of the patient and depending on the options (CVL or peripheral catheter)
-Trophic feeding of <3ml x 8 (max 24ml/d) is allowed

Enteral nutrition:
- Initiation: POD #1

- Standardized feeding source
    - In all infant´s age-specific feeding sources will be used
    - Breast milk (if available) as there is a general consensus that breast milk (70kcal/100ml) is the most effective protection against the development of necrotizing enterocolitis ([25]) (document amount used each day)
    - Alternative 1: donor breast milk (document amount used each day)
    - Alternative 2: Formula for preterm infants (name, manufacturer, the kcal/ml and the amount should be documented in the eCRF)
    - condition of the milk needs to be documented (raw or pasteurized)
    - Caloric enhancement of the milk: pure human milk or preterm formula is given until a feeding amount of 100ml/kg/d is tolerated, then the energy content of human milk can be enhanced – type and extent of caloric enhancement should be documented precisely
      Notice: As a large variety of institutional protocols on the fortification of the milk exist, the type of fortification is left to the discretion of the institution but should be documented.
    - Documentation of the selected fortifiers, their amount and the caloric content of the milk

- Prerequisite: continuous measurement of the gastric residual via the nasogastric tube prior to the next feeding

Protocol 1: Gastric residual is below 3ml/kg/nursing-shift or 10ml/kg/day

Feeding protocol (modified protocol of Bohnhorst et al [26])

- Initial amount of enteral nutrition: 20ml/kg/d (in intervals of 3 or 4 hours)
- Increase by 30ml/kg/d, when 8 (or 6, depending on feeding intervals) consecutive feedings were accepted

Example (infant's weight 2000g):

POD # 1: 8 x 5ml	= 40 ml	(20ml/ kg/d)
POD # 2: 8 x 12,5ml	= 100 ml	(50ml/ kg/d)
POD # 3: 8 x 20ml	= 160 ml	(80ml/ kg/ d)
POD # 4: 8 x 27,5ml	= 220 ml	(110ml/ kg/ d)
POD # 5: 8 x 35ml	= 280 ml	(140ml/ kg/ d)

Protocol 2: Gastric residuals prior to the feeding is 20-50% of the previous feeding

For the consecutive feeding, 20% of the preceding feeding volume (=accepted gastric residual) is added to the current volume while the previous gastric residual (>20%) is subtracted of the total volume:

Adapted amount of feeding volume =
current feeding-volume + 20% of the preceding volume – whole amount of previous gastric residuals

1. Example:

Enteral intake 6 x 60ml; gastric residual 21ml (= gastric residual 35%)

Calculation:
60 mL (feeding)+ 12 ml (20% of the previous feeding)
– 21 ml (gastric residual prior to the feeding) → **51 ml**

2. Example:

Enteral intake 6 x 72ml; gastric residual 30ml (= gastric residual 42%

Calculation:
72 ml (feeding) + 14 ml (20% of the previous feeding)
– 30 ml (gastric residual prior to the feeding) → **56 ml**

The next feeding is continued regularly and the feeding volume is then again increased after six consecutive accepted feeds

Protocol 3: Gastric residuals prior to the feeding exceeds 50% of the previous feeding
If gastric residuals exceed 50% of the previous feeding volume or infant´s vomiting, one feeding is skipped

Protocol 4: Gastric residuals prior to the feeding reaches 100% of the previous feeding
If gastric residue reaches 100% of the previous feeding volume or infant´s vomiting, two feedings are skipped

4.7 Further documentations after enterostomy closure (*all patients*):

1. Duration (minutes) of surgery (enterostomy closure)
2. Postoperative duration of assisted respiration (hours)
   prior versus post extubation
3. Daily documentation of morphine use (influencing bowel movement and therefore our primary outcome)
4. Documentation of analgesia type (especially peridural anaesthesia catheters, influencing postoperative bowel motility)

4.8 Additional treatments

The additional treatment of the patient (intervention) group involves the MFR (see 4.3 „standardized protocol on perioperative MFR“) with daily introduction of a catheter into the distal bowel loop followed by stool transfer.
Despite the standardized MFR no additional surgical or drug therapy is planned.

4.9 Control(s)/Comparator(s)

Infants of the control group will receive the current perioperative care.

4.10 Frequency and scope of study visits

All patients will be continuously monitored on the intensive care unit (NICU) or intermediate care ward (IMC) by neonatologists, pediatric surgeons, and nursery staff. Medical records will be analyzed including vital signs, weight, oral intake, and medications.

All participating centers will be visited by the coordinating investigators before the start of the study. In the course of the study investigators of all centers will meet in the local medical institution twice a year and exchange feedback on the feasibility of the protocols, especially on complications and serious adverse events. In addition to these meetings, the study coordinator will be constantly available by email and phone to address questions regarding the study. In the course of the study, medical information will be electronically exchanged monthly via encrypted email, telephone, and Fax).

4.11 Assessment of safety

Assessment of possible (serious) adverse events (AEs/SAEs) after surgery (e.g. intestinal bleeding, bowel perforation) from randomization until reaching the primary endpoint or 12 months post enterostomy closure.

AEs and SAEs have to be reported in the eCRF. Serious adverse events will be reported to the Ethics Committee according to the Declaration of Helsinki.

4.12 Timeframe complete study

<table>
<tr><td colspan="2">Year 1</td><td>Year 2</td><td>Year 3</td><td>Year 4</td><td>Year 5</td></tr>
<tr><td>Completing all preparations</td><td colspan="4"></td><td>Publication</td></tr>
<tr><td></td><td colspan="3">Recruitment of patients</td><td colspan="2"></td></tr>
<tr><td colspan="2"></td><td colspan="3">„Follow-ups“ 3, 6 (and 12) months following enterostomy closure</td><td></td></tr>
<tr><td></td><td>First pat. in</td><td colspan="2"></td><td>Last pat. in</td><td colspan="2"></td></tr>
<tr><td colspan="2"></td><td>50% pat. recruited</td><td></td><td>100% pat. recruited</td><td colspan="2"></td></tr>
<tr><td colspan="2"></td><td colspan="4">Annual meetings of all recruiting centers at the German Surgical Congress
Annual meetings of all recruiting centers at the German Pediatrician Congress</td></tr>
</table>

5 SUBSTUDY PROCEDURES

Besides the main study, there will be substudies which will be conducted in the following study centers:

Study center / Substudy	Leipzig	Tübingen	Wien	Graz
5.1.1.	X	**X**	X	**X**
5.1.2.	**X**	X	X	X
5.1.3.	**X**	X	-	X
5.1.4.	**X**	X	-	X
5.1.5.	X	**X**	-	X
5.1.6.	-	X	**X**	X

The bold highlights represent the leading study site for each substudy.

The other participating study centers will not take part in any of the substudies.

5.1 Substudies

5.1.1 Effect of MFR on the intestinal microbiome

Responsible Investigators for Substudy 1:

Prof. Dr. Holger Till, Graz, Austria

Dr. Christian Gille, Tübingen, Germany

Background: The composition of the gastrointestinal microbiome differs depending on the location in the gastrointestinal tract, but always serves as a microbial reservoir for the distal part. Functionally the gut microbiota substantially contribute to the host's metabolism by harvesting nutrients. In infants with an ileostomy, the diversion of stool most likely affects the microbial diversity of the large bowel. However, in neonates, it has never been observed, whether stool transfer prior to stoma closure supports repopulation of the microbiome in the large bowel and thus supports early enteral feeding and early energy harvesting.

Hypothesis:

1. Preoperative stool transfer into the distal ileostomy will positively affect the diversity of the fecal microbiome after stoma closure and enhance bile acid content.

Samples:

Specimen / Time point	Enterostomy stool	Rectal stool
Prior to randomization/ start of MFR	3 ml	3 ml or swab
14 days after randomization	3 ml	3 ml or swab
2-3 days before enterostomy closure	3 ml	3 ml or swab
First follow-up	-	3 mL

Methods: The stool will be split into two samples. One sample will be stored for 16S rRNA gene analysis. The other sample specimen will be used for bile acid analysis by the help of tandem mass spectrometry. Each specimen will be stored at the cooperating department/institution according to MUC-Fire regulations.

5.1.2 The effect of MFR on mucosal integrity, thickness and inflammation

Responsible Investigator for Substudy 2:

Dr. Illya Martynov, Leipzig, Germany

Background: Neonates can develop several intraabdominal pathologies that require emergent bowel resection and enterostomy creation. This particular patient group is at risk for fluid losses, electrolyte imbalances, and nutritional deficiencies. Total parenteral nutrition (TPN) is often needed to supplement patient growth and development. The absence of enteral nutrition can lead to mucosal atrophy and decrease of digestive enzymes, even when adequate calories are provided by parenteral nutrition [27]. The parenteral-nutrition-induced mucosal atrophy reverses upon reintroduction of enteral nutrition [28]. Therefore, enteral stimulation is required to preserve the homeostasis and structure of the intestinal mucosa [29]. Mucous fistula refeeding (MFR) may minimize fluid and electrolyte losses and reduce dependence on parenteral nutrition [2]. Several authors have suggested that this technique results in fluid absorption and weight gain [2]. However, mucosal refeeding has been reported to be associated with metabolic acidosis, stomal stenosis and intraabdominal fluid collections [15]. There is little information on the safety and efficacy of this practice.

Moreover, little is known about the structural (crypt depth, villus height, enterocyte hyperplasia, density of goblet cells, tight junction proteins) and functional (expression of TLRs and their effector cytokines, TNF-a, IFN-g, interleukin (IL)-1b, and IL-6) changes that can occur in the distal intestine during postresection adaptation with or without refeeding regime.

Hypothesis:

1. MFR improves the histological and functional status of the distal enteral mucosa due to exposure to luminal nutrition.

2. Enteral feeding prevents intestinal mucosal atrophy. Intestine distal to a point of diversion that did not receive luminal flow will not show signs of adaptation.
3. Mucosa of a bowel segment which is exposed to nutritiants and not diverted will have increased intensity of major tight junction proteins-codinggenes (Occludin, Claudin-4, Cingulin, ZO-1, E-cadherin) and descrease of expression of TLRs, TNF-a, IFN-g, interleukin (IL)-1b, and IL-6.

Samples:

Specimen / **Time point**	**Tissue**
Enterostomy closure	1 cm from each enterostomy site

Methods: During enterostomy closure, 1 cm from each enterostomy site (proximal and distal ends) are routinely discarded during enterostomy closure. These segments of bowel are histologically analyzed by standard staining immunofluorescence microscopy as well as realtime-PCR

5.1.3 Effect of MFR on fat tissue

Responsible Investigators for Substudy 3:

Prof. Dr. Antje Körner, Leipzig, Germany

Background: In this subproject, we aim to identify mechanisms which affect growth and development of children as well as time to full enteral feeding. Therefore, we aim to characterize central and peripheral-acting regulators derived from adipose and

gastrointestinal tissue. In addition, we assess potential differences in composition and function of adipose tissue using expression and immunohistochemical methods.

Hypothesis:

1. Children included in the intervention group differ from children of the control group in the serum profiles of adipokines and gastrointestinal hormones and in adipose tissue composition and function.

Samples:

Specimen / Time point	Blood	Tissue
Enterostomy closure	1 mL serum	fat tissue
First follow up	1 mL serum	-

Methods:

Quantitative expression analysis will be performed for characterization of marker genes for adipocyte differentiation, inflammation, and adipose tissue function. Morphological analyses will allow the comparison of cellularity and size of adipocytes. Macrophages, endothelial cells etc. will be immunohistochemically stained. Serum adiponectin, leptin, ghrelin, GLP-1, phosphatidylcholin, phosphatidylethanolamin, IGF-1 and IGFBP will be quantified by the help of immunoassays and tandem mass spectrometry.

5.1.4 Effects of nutrition during MFR and after closure of enterostomy (retrospective analysis)

Responsible Investigators for Substudy 4:

Prof. Dr. Ulrich Thome, Leipzig, Germany
Dr. Corinna Gebauer, Leipzig, Germany

Background:

Compared to dietary protein and carbohydrate, enteral fats appear to be the most trophic macronutrient to induce intestinal adaptation. Compared to a low-fat diet, a high-fat diet promotes intestinal adaptation. Especially effective are dietary long-chain polyunsaturated

fatty acids (LCPUFA), including n–6 arachidonic acid (AA), n–3 eicosapentaenoic acid (EPA) and n–3 docosahexaenoic acid (DHA). Early enteral fat supplementation with MicrolipidTM (safflower oil) and fish oil increased fat absorption and protein absorption in premature infants with bowel resection and enterostomy [30,31].

Hypotheses:

1. Nutrition with breast milk results in lower viscosity of enterostomy stool than with formula.
2. Nutrition with raw and/or pasteurized breast milk, supplements and formula result in different enterostomy nutrient losses and protein modifications.
3. Nutrition with raw breast milk, pasteurized breast milk and formula result in different enterostomy stool pH.
4. Nutrition with breast milk results in a different small bowel microbiome and different SCFA-profile than formula. (cooperation with C. Gille, Tübingen, Microbiome data will be shared, no further sampling necessary.)
5. Infants who have received MFR have superior neurodevelopment at 24 months corrected age.
6. Infants who have received predominantly human milk have a superior neurodevelopment at 24 months corrected age.

Samples:

Specimen / Time point	Enterostomy stool	Rectal stool	Milk
Prior to randomization/ start of MFR	10 mL	-	1 mL
14 days after randomization	10 mL	5 mL	1 mL
2-3 days before enterostomy closure	10 mL	5 mL	1 mL
First follow up	-	3 mL	-

Methods:

Samples will be analyzed for their viscosity (cooperation with Prof. Mihatsch, Pforzheim) and for their pH by using a pH indicator paper with a pH range from 1 to 14. Protein content is analysed with “DC Protein Assay” (BIO-RAD). Dietary protein absorption (g/kg/d and % of dietary protein) are calculated as described by Yang [31]. Iron content is analysed with “Iron

Assay Kit" (Sigma-Aldrich). Gas-chromatography coupled to mass spectrometry is used to quantify fecal SCFAs (acetate, formate, propionate, butyrate, and isobutyrate), amino acids, mono- and disaccharides, lipids and fatty acids are analysed by GC-MS (gas-chromatography-mass spectrosmetry). Milk (formula and human milk) samples are analyzed for non-enzymatic protein modifications.

All infants have to be reevaluated by a neurodevelopmental exam at 24 months corrected age, which is mandatory by current German neonatal care guidelines. Infants will receive a structured exam according to Bayley version III. Data of follow up results will be collected from all infants and evaluated according to the above hypotheses.

5.1.5 Effect of MFR on phosphatidyl choline loss and subsequent choline deficiency in preterm infants with enterostomy

Responsible Investigators for Substudy 5:

Prof. Dr. Axel Franz, Tübingen, Germany

Prof. Dr. Dr. Wolfgang Bernhard, Tübingen, Germany

Background:

While the fetus is supported in utero by an active trans-placental transfer of choline, achieving plasma concentrations of choline in the fetus that are more than twice the plasma concentration of the mother, the preterm infant is dependent on enteral supply of choline and adequate choline uptake from nutritional sources.

An important (but not the only) nutritional source of choline is phosphatidylcholine (PC) which is taken up after being cleaved by phospholipases. In addition to enterally administered, nutritional PC, large amounts of PC are also secreted into the duodenum with the bile a) to emulsify lipid-soluble components of the bile and b) to support intraluminal micelle formation and fat digestion. The secreted PC is usually taken-up again in the ileum as part of the enterohepatic cycle.

Both processes a) digestion and uptake of nutritional PC and b) re-uptake of secreted biliary PC, may be impaired following placement of an enterostomy – and this alteration of PC reabsorption may be compensated in part by MFR. Consequently, patients with enterostomy without MFR may be particularly prone to choline and PC deficiency potentially contributing to intestinal failure associated liver disease, whereas MFR following enterostomy may prevent (or alleviate) such choline deficiency (and subsequent liver disease).

Hypotheses:

1. (Primary hypothesis) Patients with MFR maintain higher plasma levels of choline and PC
2. Patients with signs of cholestasis (as an indicator of IFALD) show lower values of choline and/or PC in plasma
3. The enteral loss of PC (in mmol/kg/d) negatively correlates with the plasma choline concentration
4. MFR may change the molecular composition of neutral lipids and PC in fatty tissue

Samples:

Specimen / Time point	Blood	Enterostomy stool	Tissue
Prior to randomization/ start of MFR	100 µL EDTA-plasma 100 µL Ery-pellet	-	-
14 days after randomization	100 µL EDTA-plasma 100 µL Ery-pellet	1 mL	-
2-3 days before enterostomy closure	100 µL EDTA-plasma 100 µL Ery-pellet	1 mL	fat tissue

Methods:

EDTA blood will be sampled, immediately (i.e. within 30min) centrifuged at 2000g for 10min and 100 µl EDTA plasma and 100 µl erythrocyte pellet will be frozen for subsequent analysis of choline, phosphatidylcholine and phosphatidylethanolamine concentrations by GC/ESI-MS/MS. The exact sampling procedure is detailed in a separate SOP.

Furthermore, 1ml of enterostomy–secretion/stool will be collected at the same time points and frozen until subsequent analysis. Additionally, the total volume of enterostomy losses (in ml) during the relevant preceding 24h period will be recorded in the study database. The exact sampling procedure is detailed in a separate SOP.

Samples of plasma, erythrocyte pellet and enterostomy secretion/stool are transported frozen on dry ice after prior agreement on the day of transport.

In Prof. Bernhards lab, the samples will be analyzed as follows:
In plasma the following concentrations will be determined: choline, betaine, DMG, total PC, total phosphatidylenthanolamine (PE), molecular subtypes of PC and PE by GC/ESI-MS/MS.
In erythrocyte pellets: total PC, total phosphatidylenthanolamine (PE), molecular subtypes of PC and PE will be determined by GC/ESI-MS/MS.
In stool: total PC, total phosphatidylenthanolamine (PE), molecular subtypes of PC and PE will be determined by GC/ESI-MS/MS
In fatty tissue: molecular composition of PC and PE will be determined by GC/ESI-MS/MS, molecular composition of neutral lipids will be determined by GC/MS.

The primary outcome variables to test hypothesis 1 will be the plasma concentration of choline and total PC at enterostomy closure, which will be compared between both treatment groups by Wilcoxon test (assuming a non-normal distribution).
Secondary outcome variables will be plasma concentrations of the other water soluble derivatives of choline and of PE.

To evaluate hypothesis 2, the plasma concentrations of choline and total PC will be compared between infants with cholestasis (defined by a direct bilirubin plasma concentration > 2mg/dl at any time between randomization and enterostomy closure) and infants without cholestasis (irrespective of treatment group assignment).

To evaluate hypothesis 3, the plasma concentration of choline and PC will be correlated to the daily loss of total PC in stool (calculated as mg/kg/d from the concentration of total PC in enterostomy stool (mg/ml) and the total amount of stool output (ml/kg/d) during the relevant 24h period) according to Spearman.

To evaluate hypothesis 4: molecular composition of PC, PE and neutral lipids will be compared between treatment groups.

5.1.6 Effect of MFR on growth and body composition

Responsible Investigators for Substudy 6:

Dr. Christoph Binder, Wien, Austria
Dr. Christian Gille, Tübingen, Germany
Prof. Dr. Holger Till, Graz, Austria

Background:

Preterm infants who developed NEC are at high risk for malnutrition due to long-term parenteral nutrition and malabsorption [32,33]. The major nutritional goal in these infants is to achieve adequate nutritional intake to avoid growth restriction, which is the major problem in survivors and associated with long-term neurodevelopmental impairment [34]. However, the optimal nutritional management in these infants is still unknown and the evaluation of growth and body composition after NEC surgery of interest.

Growth is usually monitored by anthropometric parameters and gives us information about the quantity, but not about the quality of growth (fat mass or lean mass). Body composition provides additional information on the nutritional status of the infants and especially on fat-free mass (FFM - lean mass) [35]. Gain in lean mass represents linear growth, which is a proxy for protein accretion [36]. Sufficient protein supply is very important during critical illness [37]. It provides essential amino acids required for body protein synthesis, which is substantially for linear growth and lean mass gain [36]. Studies showed that deficits in lean mass growth are associated with neurodevelopmental impairment [38,39]. Furthermore, previous study indicated that a higher ratio of fat to lean body mass (fat-free mass) at term-equivalent age is a predictor for obesity, hyperlipidemia, hypertension and type II diabetes in later life [40,41]. Consequently, knowledge of the body composition in preterm infants with NEC is of major interest to evaluate the nutritional management.

Hypothesis:

1. MFR (due to a shorter time interval to full enteral feeding) will result in higher fat-free mass in comparison to the control group

Substudy-specific inclusion criterion

All preterm infants born <37 weeks gestational age

Substudy-specific exclusion criterion

Genetic or metabolic diseases with the primary effect on growth

Study visits

Study visits	Measurements at each study visit
At term-equivalent age (37-42 weeks postmenstrual age)	• Body composition • Weight, height and head circumference • Evaluation of the nutritional intake
3 months after enterostomy closure (First follow-up)	

Methods: The „Pea Pod Body Composition System" (Pea Pod®) is measuring the **body composition** through a non-invasive air displacement plethysmography. It is based on a two-compartment model of body composition: fat mass (FM) and fat-free mass (FFM) and uses the inverse relationship between pressure and volume to derive body volume for a subject. It is a safe non-invasive method measuring the body composition by air displacement. The test time takes about 7 minutes and parents are welcome to be present during the measurement.

Anthropometric measurements will also be expressed as z-scores relative to the growth standards of the World Health Organization for breastfed children (WHO Multicenter Growth Reference Study Group. WHO Child Growth Standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development (Geneva, World Health Organization 2006). Z-scores will be calculated using WHO programs (http://www.who.int/childgrowth/software/en).

5.2. Summarized additional sample amounts

Blood samples will be taken during routine blood withdrawal.

The collection of enterostomy stool samples does not harm the child. The amounts of stool needed for the substudies are not disadvantageous for those children randomized into the MFR group. Enterostomy stool of children who do not receive MFR as well as rectal stool from all included children will be either disposed or used for routine analysis and could therefore be used for the substudies.

During enterostomy closure a small piece of both, the afferent and efferent, bowel loops is resected with its attached fatty tissue prior to re-anastomosis. These tissue samples will be stored and used for any further investigations, described precisely in the study protocol. We

guarantee, that this procedures and the following analysis do not include any additional traumatization of the children or any modifications of the routine treatment.

The amounts of milk will be taken only if it is left over and is therefore not disadvantageous for the child.

Summarized, blood samples are retrieved during routine withdrawel; sampling of stool, tissue and milk does not mean an additional intervention for the child.

Specimen / Time point	Blood	Enterostomy stool	Rectal stool	Tissue	Milk	Anthropometric measures
Prior to randomi-zation/start of MFR	100 µL EDTA-plasma 100 µL Ery-pellet	13 mL (2)	3 mL		1 mL	
14 days after randomization	100 µL EDTA-plasma 100 µL Ery-pellet	14 mL (3)	8 mL (2)		1 mL	
2-3 days before Enterostomy closure		14 mL (3)	3 mL 5 mL (only Leipzig!)		1 mL	
Enterostomy closure	1 mL serum 100 µL EDTA-plasma 100 µL Ery-pellet			1 cm from each enterostomy site (2) fat tissue (2)		
At term-equivalent age (37-42 weeks postmenstrual age)						x
First follow up	1 mL serum		6 mL (2)			x

The number in parenthesis represents the amount of aliquots which will be prepared at the study centers prior to storage of the samples.

The anthropometric measurements will be performed via the „Pea Pod Body Composition System" which is non invasive.

5.3. Standardized sample treatment

Samples will be collected, aliquoted and stored at each study center until the end of the study.

Specimen	Sample treatment	Storage
Serum	Centrifuge 10 min at 2500g	-80°C
EDTA Plasma	Centrifuge 10 min at 2000g	-80°C
Erythrocyte pellet	Prepaired from EDTA-plasma	-80°C
Stool	-	-80°C
tissue	Immediately stored in cryo tubes in liquid nitrogen	-80°C
milk	-	-20°C

Shipment of samples for analysis will be performed batchwise after informed consent of both study sites. Stored samples will be shipped to the responsible substudy investigator.

5.4. Substudy specific data assessment and statistical analysis

Different from the main study, data assessment and statistical analysis of the substudies will take place at the end of the study in each responsible substudy leading center, not at the Hannover Medical School, Institute for Biostatistics. The data of the substudies will be analyzed in the study site of the responsible substudy investigator. Substudy data monitoring is not included in contrast to the main study. Each substudy investigator is responsible for data correctness. With the exception of study 5.1.6, no substudy data will be entered into the eCRF. The substudies will be descriptively analysed with t-tests for continuous data and chi-squared-tests for categorical data, unless stated differently.

6 ADVERSE EVENTS

Current data suggest a low complication rate in mucous fistula refeeding. Lau et al. [28], with the up to date largest study population (n=77), documented no major complications. However, a retrospective analysis by Haddock et al. [15]with an inhomogenous population reported on the risk of bowel perforation, bleeding and death associated with mucous fistula refeeding. Therefore, these criteria are adverse events during the study period.

Postoperative complications are classified, using the Clavien-Dindo classification and assessment of complications on daily basis. However, when not associated to MFR these complications are not considered adverse events [12,22].

7 STATISTICAL ANALYSIS

The primary analysis will be performed on the ITT population, i.e. all randomized patients will be analyzed in the treatment group to which they have been initially allocated. The treatment effect will be assessed by the Hazard Ratio for reaching full enteral feeds estimated with a Cox regression adjusted for center, weight at birth, height of stomata and treatment, and the respective 95% confidence interval. Superiority of the refeeding procedure will be concluded if the lower bound of the two-sided 95%-confidence interval for the Hazard Ratio (refeeding vs no refeeding) is greater than 1. In case of missing information on the time to full feeds, patients will be censored at the last known status before full feeds. All secondary analyses will be exploratory and will be conducted on the ITT population.

7.1 Methods against bias

This is an open-label study. Blinding is not possible because active refeeding of stool in the intervention group is obvious to any person participating in the medical care of the patient. Randomization will be performed centrally (with variable block length) and stratified by study center, height of stomata [3,42] and ,weight at birth (<1000g / ≥1000g), as this is an important prognostic factor for primary endpoint. Randomization will take place after enterostomy creation in order to reduce the amount of missing values due to patient exclusion after surgery (e.g. due to unforeseen need for resection of ileocecal valve). The primary analysis will be performed on the ITT population as this is an open study and parents may have preferences not outspoken before randomization. A per protocol analysis will be conducted as a sensitivity analysis. Consistency between the findings in the ITT population and the per protocol population will be examined as it is an important pre-requisite for a successful interpretation of the study. Drop-Outs are not expected because all patients will constantly undergo neonatal intensive care and will therefore not be lost to follow-up. If parents withdraw their infant from study participation they will be asked to allow data collection at a final analysis in order to avoid that information would be wasted. Nonetheless, if missing values should occur (e.g due to death, or parents' refusal of data collection) observations will be censored at the last timepoint with known enteral feeding status. Since this censoring may be informative, missing values for time to full feeds will be replaced by the worst observation in each group in a sensitivity analysis in order to check how censoring may have influenced the results. If any death should occur before the respective patient reaches full enteral feeds a sensitivity analysis will be performed on all surviving patients.

7.2 Proposed sample size/Power calculations

The literature of MFR is scarce and information on the primary endpoint “time to full enteral feeds” is limited[3]. A recently published retrospective analysis of 24 patients [2] of which 13 received refeeding of stool to the mucus fistula and 11 did not receive refeeding of stool showed a median time from reanastomosis to enteral feeds of 7 days in the control group and 4 days in the refeeding group. The data presented for the control group is in line with retrospective data of 42 patients collected at Hannover Medical School. These 42 patients are all patients fulfilling the inclusion criteria who were treated at Hannover Medical School between 2005 and 2015. They did not receive refeeding of stool and had a median time to full enteral feeds of 7 days. According to Gause et al. [2] a survival analysis is appropriate. In their respective publication, median times are reported corresponding to a hazard ratio of 1.751 for time to enteral feeds (4 days vs 7 days), 2.331 for parenteral nutrition discontinuation (6 days vs 14 days) and 2.667 for goal feeds (7.5 days vs 20 days). Because time to enteral feeds in this publication is in line with our retrospective data of time to full feeds, a hazard ratio of 1.751 is assumed for the treatment effect. In order to show a treatment effect with a power of 80% and a two-sided type I error probability of 5 % with a logrank test a total of 100 events (full enteral feeds) is required, if the hazard ratio for the treatment effect is 1.751. Since patients will be in neonatal intensive care, every patient is expected to reach full enteral feeds. Nonetheless, to account for possible deaths, the sample size was increased by 6 patients, resulting in a total of 106 patients. Sample size was estimated in nQuery Advisor 7.

7.3 Compliance/Rate of loss to follow up

Multiple retrospective data analyses show low complication rates related to MFR. During 14-years of MFR, a group of the University of Hong Kong observed no major complications associated to the refeeding in 77 patients with necrotizing enterocolitis [43].
All centers participating in the current study have experience on MFR and recorded no major complications in any of the centers. This observation is well in line with data on 13 patients undergoing MFR at the Department of Pediatric Surgery at Johns Hopkins University School of Medicine in Baltimore. The authors documented no major complications associated to refeeding but observed benefits of the intervention [2].

We are very confident that there will be almost no loss of follow-up in this study. Due to the severe course of the diseases, parents of patients with neonatal surgical conditions have an intense emotional relationship with the treating surgeons and neonatologists. Almost all parents prefer follow-up appointments at the treating hospital after their infants have been discharged from the hospital. We therefore do not expect any loss of follow-up. However, as a precaution, the patient recruitment was increased to 11 centers with 201 expected patients.

8 DATA MANAGEMENT

All study data will be collected by the investigator and/or other study personnel. A validated clinical trial data base (electronic case report form) is provided in which the data are entered. These data includes further relevant diagnosis, using the International Statistical Classification of Diseases and Related Health Problems (ICD 10 coding system). In particular, because of the risk of comorbidities in preterm infants (e.g. bronchopulmonary dysplasia, retinopathy of prematurity, intraventricular hemorrhage (IVH),). Authorized and trained staff of the study sites will enter the data in the eCRF in a timely manner. Only SAEs will be documented and reported on paper forms. Verification of the data in the eCRF occurs by risk-based monitoring as well as via range, validity and consistency checks programmed in the system. Additionally, manual queries can be raised in the system if discrepancies are detected. Based on the queries, the investigator can review the data and resolve the discrepancy or justify the entered data directly in the system. All changes of data entered in the eCRF are documented in an audit trail. A quality control will be performed before the database is closed. This procedure is documented. Finally, data transfer takes place for statistical evaluation.

The data management plan contains further details about data management processes.

9 QUALITY ASSURANCE AND MONITORING

All initiation visits, onsite monitoring visits, close-out visits and in-house monitoring will be conducted by monitors of Hannover Clinical Trial Center (HCTC). HCTC SOPs will be utilized. Prior to the start of the study, pre-study visits by the primary investigators will be conducted to be able to instruct the local investigators in how to follow the study protocol and documentation of data. Initiation visits will be done in each study center prior to patient recruitment to ensure adherence with all study procedures by the monitor of HCTC and the study coordinators. To assure high data quality and patients safety, regular on-site monitoring visits will be performed by HCTC monitors. Checking of signed informed consents and source data verification will be carried out according to a risk adapted approach. At the end of the study, close out visits will be performed at all study sites. Project managers, monitors, study coordinators and PIs will be in close and regular contact throughout the study and with all study sites.

Monitoring details will be summarized in a monitoring plan which will be prepared by the project manager (HCTC). The monitoring plan will be reconciled with the coordinating investigator and members of the clinical project management. It will serve as guiding document for all monitors and will contain details on monitoring activities, responsibilities and interfaces between study team, data management, source data and adverse events. In-house monitoring will assure high data quality. Data capture will be achieved by electronic data capture (electronic CRF). On-site source data verification will be done according to a risk adapted monitoring afterwards. In total, 3 monitoring visits are planned per study site.

9.1 Data Monitoring Committee

An independent Data Monitoring Committee (DMC) will be implemented to detect possible harms and to assure continuous risk/benefit assessment. A DMC is a group of independent

experts external to the study assessing the progress, safety data and, if needed, critical efficacy endpoints. Details of the definition of DMC, its composition and its roles and responsibilities can be found in the separate DMC charter.

10 ETHICAL AND LEGAL CONSIDERATIONS, ADMINISTRATION

The study will be conducted in accordance with the principles of ICH-GCP (as far as possible for this kind of study) and the Declaration of Helsinki.
Study protocol and patient consent form will be submitted to ethics committees before start of the study. No amendment to the protocol may be made without consideration by the Ethics Committee.

10.1 Patient Information and Informed consent

The investigator is responsible for obtaining the parent's written informed consent after adequate explanation of the aim, study assessments, potential risks and benefits and consequences of the study as well as alternative treatment options. Parents will have sufficient time to ask questions before deciding on whether to participate in the study or not. The patient information/informed consent form has to be signed in duplicate by the patient's parents and the investigator. One document will be given to the parents, the other one will be kept at the participating study sites. No study procedures are allowed to be conducted until parent's written informed consent has been obtained.
The patient information/informed consent form has to be revised whenever important new information becomes available that may be relevant to the parent's consent.
In case of the infants transfer into another clinic, the investigator obtained the informed consent from the parents to release the physicians in the external clinic from their medical confidentiality to retrieve the data for the study.
Participation in this clinical trial is voluntary. Withdrawal from the study at any time and for any reason is without any disadvantages to the patient's further treatment.

10.2 Patient Insurance

The trial will be covered by a participant insurance in case the trial site (clinic) does not cover the study by its liability insurance (Haftpflichtversicherung). All subjects (parents) will be informed about their rights and obligations in regard to insurance policies before participating in the study. A copy of the insurance policies will be handed out to each patient (parents).

10.3 Data Protection

Data will be collected, handled, stored and analysed in accordance with national regulations. All study staff have to give due consideration to data protection and medical confidentiality.
If the participant withdraws the previously given informed consent, the participant has the right to demand the deletion of all data collected so far. If the participant withdraws and does not demand the deletion of data, these so far collected data will be anonymised and used for the statistical analysis.

10.4 Registration

The study will be registered at a public study register (ClinicalTrials.gov) prior to the start of recruitment.

10.5 Record Retention

The original study documents wil be stored in an archive of the participating study site for at least 10 years after the final study report.

10.6 Financing

The clinical trial is funded by public funds through the German Research Foundation.

11 HANDLING OF BIOMATERIAL

Biomaterials in the main study include the analyses of sera, plasma and urine and the use of enterostomy stool for MFR. Sera, plasma and urine will be collected and analyzed using the current concepts of each department. Therefore, no additional trauma will be present.
Enterostomy losses will be collected in strict intervals [1x (continuous refeeding) – 3x (separated refeeding every 8 hours) daily] for the refeeding. Stool will not be stored for the MFR. The necessary amount will be transferred and the surplus will be thrown away.

12 PUBLICATION

After completion of the trial, data analyses will be performed by the Institute of Biostatistics (MHH). Results will be published and the study protocol including all data how to perform MFR. If the results of this study will show significant differences between the intervention group and controls MFR will become the new standard of care for neonates with enterostomies.

In Germany, the current national guideline for neonatal and surgical treatment of necrotizing enterocolitis (NEC) is currently in revision [Leitlinie 024-009: Nekrotisierende Enterokolitis (NEK)]. One of the principal investigators of the trial (Prof. Dr. Martin Lacher) is coauthor of this guideline (Delphi method). If the current trial proves the hypothesis that MFR is beneficial for these infants, it may not only change the national guideline for the best treatment after enterostomy creation in Germany but in other countries too.

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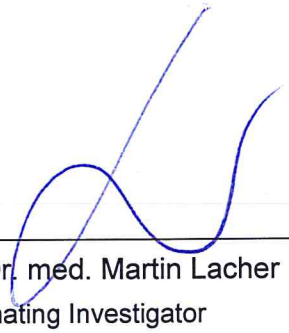
14 SIGNATURES

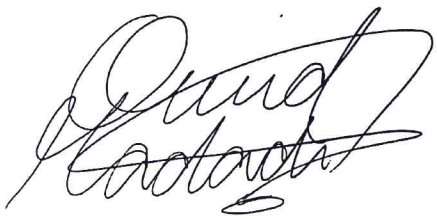
This document has been approved by the following persons. The following signatures document their approval.



10.09.2019

Prof. Dr. med. Martin Lacher
Coordinating Investigator

Date



10.09.2019

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