

## **Fecal microbiota transplantation decreases necrotizing enterocolitis but is associated with increased neonatal mortality in preterm pigs**

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*Introduction:* Necrotizing enterocolitis (NEC) remains a common gastrointestinal disease in preterm infants. Inappropriate bacterial colonization of the gut, together with enteral formula feeding, predispose to NEC in both preterm infants and preterm pigs. Regardless, it remains unclear how and when to manipulate the gut microbiota (e.g. by use of pre-, pro- or antibiotics) to improve mucosal immunity and NEC resistance. We hypothesized that fecal microbiota transplantation (FMT) to neonatal preterm pigs would support their initial gut colonization and thereby reduce formula-induced NEC development.

*Material and Methods:* Fifty-eight caesarean-delivered preterm pigs were fed slowly increasing volumes of a preterm formula (3 to 15 mL/kg/3h until 5 days after birth). Pigs were randomly allocated into a control group (CON; n=30) or an FMT group (n=28), receiving homogenized colon contents pooled from five 14 day-old, healthy suckling pigs. The transplant was given as gastric and rectal administration of 10<sup>9</sup> cfu bacteria twice daily on day 1 and 2. Clinical condition, body temperature and diarrhoea (score 1-7) were recorded daily. Pigs were euthanized on day 5 and macroscopic NEC lesions in the stomach, intestine and colon were recorded (score 1-6), together with parameters of intestinal function.

*Results:* Diarrhoea score increased in both groups with age but tended to be lower in FMT pigs (3.9 vs. 5.4 on d 4; p<0.05) and with a lower frequency of bloody stools (0 vs. 5; p=0.08). FMT pigs showed reduced NEC incidence on d 5 (18 vs. 60%; p<0.01), and NEC lesion scores were reduced particularly in colon (1.5 vs. 3.1; p<0.01). FMT pigs showed less gastric residual after a test meal on d 5 (19 vs. 27 g; p<0.05) and lower gastric acidity (pH 4.7 vs. 4.0; p<0.05). Intestinal lactase, maltase and DPPIV activities were increased in the FMT group on d 5 (p<0.05). Spontaneous mortality and euthanasia prior to d 5 (due to poor clinical condition) occurred more often in FMT pigs (11 vs. 4 pigs during first 3 days; p<0.05). The clinical condition and autopsy of these FMT pigs indicated that sepsis was part of the clinical complications observed for preterm pigs during the first days after FMT.

*Conclusion:* Use of FMT just after preterm birth improves NEC resistance and intestinal function in pigs, but may also increase mortality, potentially due to early excessive bacterial colonization and sepsis. Further research on the optimal dosage, route and timing of transplantation is required to balance the proposed benefits and potential harm of enhanced development of gut bacterial colonization, digestive function and immunity in preterm neonates by FMT.

## **Initiation of enteral feeding diet-dependently affects intestinal DNA methylation in preterm pigs**

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**Introduction:** Epigenetics is an important mechanism whereby environmental factors regulate gene expression of various tissues during early development and such epigenetic changes may have life-long consequences. The immature intestine of preterm infants is exposed to dramatic environmental changes immediately after birth, including bacterial colonization and initiation of enteral milk feeding. Using preterm pigs as models for preterm infants, we hypothesized that enteral feeding affects intestinal epigenetics differently than total parenteral nutrition (TPN). In mammalian DNA, gene methylation is a key epigenetic mechanism that takes place mainly for cytosine residues of CpG dinucleotides.

**Materials and Methods:** Preterm newborn piglets were given TPN or slowly advancing volumes (16-64 ml/kg/d) of bovine colostrum (COL) or preterm infant formula (FOR) for five days after birth (n = 13-15). On day 5, pigs were euthanized and the intestine collected for analyses of a series of structural and functional endpoints. From a subsample of pigs from each group (n=2), DNA from the middle intestine was extracted and subjected to reduced representation bisulfite sequencing (RRBS) to assess the genome scale DNA methylation. The BSMAP software was used for sequencing reads alignment and methylation level was calculated for each genomic sites. Chi-square test was used to identify differential methylated regions (DMRs) specific to each of the three feeding protocols.

**Results:** Both the COL and FOR feeding protocol increased intestinal mass and some digestive enzyme activities (DPPIV, ApN, ApA) on day 5, relative to TPN. Only the FOR diet was associated with necrotizing enterocolitis (NEC) lesions in the colon, nutrient malabsorption, and increased intestinal permeability and pro-inflammatory cytokine levels (IL-8) on day 5. Compared with TPN (methylation level 56.3%), both the COL and FOR diets increased global CpG methylation in the intestine (to 61.7% and 62.3%, respectively). The CDS regions showed the largest increase (+5.7% for COL and +7.2% for FOR) and the 5'UTR region the smallest increase (+1.3% and +1.1%, respectively). A large number of DMRs was identified between TPN and enteral feeding (COL: 333, FOR: 268), with less difference between the two diets (171 DMRs).

**Conclusion:** Initiation of enteral feeding induces a global methylation increase in the preterm intestine. Despite the marked diet-dependent differences in intestinal structure and function, diet type had less effect on gene methylation than feeding itself. The results suggest that early enteral feeding may be important to mature the intestine short-term, but it may also induce long-term effects on intestinal gene transcription by epigenetic regulation.



## **Persistent hypomethylation of the preterm pig intestine**

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**Introduction:** Epigenetics play an important role in regulation of tissue-specific gene expression during the perinatal period. It is a molecular mechanism whereby environmental factors transiently or permanently alter gene expression and tissue function. Reduced gestational length, coupled with postnatal maladaptation, induce long-term functional deficits in many organs of preterm neonates (e.g. brain, lungs, intestine). Using preterm pigs as models for preterm infants, we hypothesized that the marked age-, diet and microbiota-related intestinal adaptation in the perinatal period would lead to differences in the intestinal epigenome between preterm and term neonates.

**Materials and Methods:** After caesarean delivery, preterm and term piglets were kept in environmentally-controlled incubators, and fed identical diets for 26 d after birth (i.e. transition to enteral milk feeding after 5 days of parenteral nutrition). Pigs from both groups were euthanized at birth (before any feeding), on day 5, or after 26 days (n=8-22) and the middle intestine was collected for analyses. Parameters of intestinal structure and function was assessed (mucosal growth, digestive enzymes, permeability, nutrient absorption), and DNA was extracted for a subfraction of the pigs from each group (n=2). Reduced representation bisulfite sequencing (RRBS) was used to assess the genome scale DNA methylation of cytosines at the CpG dinucleotide sites.

**Results:** The mid intestine showed marked postnatal increase in mucosal mass in both preterm and term pigs but preterm pigs showed a persistent delay in some digestive enzymes until day 26 (sucrase-isomaltase, maltase-glucoamylase). Compared to the term intestine, the preterm intestine was persistently hypomethylated across the entire genome from birth to 26 days. The reduction in global methylation in preterms increased from birth (-3.0%) to 5 days (-4.6%), but then decreased at 26 days (-2.1%). The CDS gene regions showed the greatest difference among all the genic elements from birth to 5 days, but the smallest difference at 26 days. Comparative analyses revealed 261, 519 and 366 DMRs between preterm and term pigs at birth, 5 and 26 days, with ~72% of DMRs located in intergenic regions.

**Conclusion:** The preterm intestine adapts rapidly postnatally and many parameters become similar to those in the term intestine. Regardless, epigenetic aberrancy of certain genomic regions is maintained and this is consistent with a persistent delay in some intestinal functions. If and when these overall developmental delays are modifiable and associated with epigenetic changes in specific genes, remains to be investigated.

## **Early postnatal hydrocortisone fails to prevent against necrotizing enterocolitis in preterm pigs**

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**Introduction:** Postnatal corticosteroids are used in preterm infants to prevent hypotension and chronic lung disease but it may also induce gut maturation and improved necrotizing enterocolitis (NEC) resistance. Potential adverse side effects are impaired growth, brain damage and immunosuppression but low-dose, physiological hydrocortisone (HC) could be less detrimental than high-dose dexamethasone. We hypothesized that early low-dose HC treatment would help organ adaptation, and reduce NEC, without adverse effects.

**Material and methods:** Preterm caesarean-delivered pigs were treated for 4 days twice daily with decreasing doses of intra-arterial HC 6 to 2 mg/kg/day (n=19) or an equivalent dose of sterile saline (CON, n=19). Over the same time pigs were fed increasing doses of infant formula (0-80 mL/kg/d) together with parental nutrition. The clinical state, including respiratory function and blood pressure, blood biochemistry and hematology was monitored. On day 5, pigs were euthanized, organs weighed, lung volume measured and the gut was evaluated for macroscopic NEC lesions.

**Results:** More diarrhea and feeding intolerance were observed in CON pigs (P<0.05) while no other of the measured clinical parameters differed between groups. Growth and relative organ weights were similar, except for a smaller spleen (P<0.05) in the HC group. A high incidence of NEC lesions was present in both groups (HC=17/19, 89% vs. CON=15/19, 79%) but small intestinal NEC-like lesions were more frequent in HC pigs (16/19, 84% vs. 9/19, 47%; P<0.05). These HC pigs also showed transient hypoxia shortly after birth (oxygen saturation <90%, up to 6 h after birth), although this disappeared within the first day of life.

**Conclusion:** Early postnatal HC treatment did not improve NEC resistance, respiration or cardiovascular function in formula-fed preterm pigs. Postnatally, HC treatment may be beneficial only following hypotension or chronic lung disease. The HC-related small intestinal NEC-lesions and reduced spleen weight deserve further study but could reflect negative effects of HC on intestinal barrier function and systemic immunity.

## ULTRAVIOLET-C IRRADIATION OF DONOR HUMAN MILK IMPROVES GROWTH, INTESTINAL FUNCTION AND SYSTEMIC IMMUNITY IN PRETERM PIGS

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**Introduction:** Donor human milk (DM) for preterm infants is usually Holder pasteurized (HP, 62.5°C for 30 min) to eliminate transmissible contaminants, but this may also destroy many milk bioactive factors. Ultraviolet-C irradiation (UVC) and HP treatment have shown similar efficacy to remove bacteria from DM, while UVC better preserves bile-salt stimulated lipase and alkaline phosphatase (ALP) activities, and helps to maintain immunoglobulin, lactoferrin (LF) and lysozyme levels in DM compared with HP. We hypothesized that UVC-treated DM stimulates intestinal maturation, diarrhoea resistance and systemic immunity in preterm pigs, used as a model for preterm infants.

**Materials and Methods:** Sixty litres of DM (Danish Donor Milk Bank, Hvidovre Hospital, Denmark) were pooled and divided into three aliquots that received no treatment (raw milk, RM), HP or UVC treatment. Fifty-seven caesarean-delivered preterm pigs received increasing volumes of RM, HP or UVC treated DM (n=19 each, 24-120 mL/kg/d on days 1-5, 144 mL/kg/d on days 6-8). Parenteral nutrition was administered throughout the study period with the same dosage and composition to each group to supplement fluid and nutrient intake. Pigs were euthanized on day 8 for collection of blood and organs. The main outcome parameters were growth, diarrhoea, necrotizing enterocolitis (NEC), intestinal function, and bacteria in blood and bone marrow.

**Results:** HP and UVC reduced the bacterial density (cfu/mL) from  $6 \times 10^5$  in RM to  $9 \times 10^3$  and  $7 \times 10^2$ , respectively. HP abolished milk lipase and ALP activities, and reduced the level of LF by 50%, while UVC maintained levels similar to RM. During the last days of the experiment, weight gain was reduced in HP pigs, relative to RM and UVC pigs ( $P < 0.05$ ). Only on day 5, diarrhoea incidence increased in HP pigs relative to RM ( $P < 0.05$ ), with a similar trend relative to UVC pigs ( $P = 0.10$ ). Osteomyelitis incidence (bacteria present in bone marrow) was higher in HP vs. UVC pigs (68 vs. 28%,  $P < 0.05$ ) while presence of bacteria in blood was similar among groups (33% across all groups). Intestinal NEC, nutrient absorptive capacity and permeability did not differ, while distal intestinal aminopeptidase N activity was higher in UVC pigs than RM pigs ( $P < 0.01$ ), and tended to be higher than HP pigs ( $P = 0.07$ ).

**Conclusion:** UVC treatment effectively reduced the bacterial contamination of RM and preserved bioactive factors in DM, relative to HP. UVC-treated DM improved weight gain and peptidase activity, and reduced systemic infections in preterm pigs. UVC may be used as a novel technology to pasteurize DM and limit the breakdown of bioactive milk factors that may be important for intestinal maturation, bacterial resistance and systemic immunity in preterm infants.

## DECREASED PHYSICAL ACTIVITY PRECEDES ONSET OF NECROTIZING ENTEROCOLITIS IN PRETERM PIGS

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**Background and aims:** Necrotizing enterocolitis (NEC) is a serious feeding-related gut inflammatory disease with high mortality. Early clinical markers of NEC are of great importance for preventive interventions. Using preterm pigs as models, we hypothesized that the postnatal onset of NEC could be predicted by decreased physical activity level in the first few days after birth.

**Methods:** Caesarean-delivered preterm pigs were fed parenteral nutrition (PN) plus enteral milk nutrition for 8 (Exp 1), 5 (Exp 2), or 5 (Exp 3) days after birth by caesarean section. Pigs were scored for macroscopic NEC lesions (score 1-6) in stomach, intestine and colon after euthanasia on day 9 (Exp 1), 5 (Exp 2) or 5 (Exp 3). Physical activity was recorded by continuous camera surveillance for 8 (Exp 1), 5 (Exp 2) or 3 (Exp 3) days after birth with automatic activity detection quantified as total activity counts and proportion of active time during these time periods.

**Results:** The incidence of clear NEC lesions (score 4-6) was 38, 61 and 32%, respectively, in the three experiments. For the mean daily activity values, there was no difference between NEC and control pigs in Exp 1 (7 d recordings,  $P>0.05$ ) while the mean daily activity over the first 3-5 days were reduced in pigs developing NEC lesions on day 5 in Exp.2 and Exp. 3 ( $P<0.05$ ).

**Conclusions:** The early postnatal onset of NEC lesions is associated with decreased physical activity level during the first 3-5 days after birth, maybe due to some initial abdominal discomfort. As NEC develops, activity counts may increase above normal in response to more severe abdominal pain, explaining that activity levels recorded over the entire period from birth to euthanasia failed to show NEC effect (Exp 1). Decreased physical movement may be an clinical sign of NEC.

## NEOMUNE-NEONUTRINET: NUTRITION FOR PRETERM INFANTS AROUND THE WORLD DURING THE FIRST WEEKS AFTER BIRTH

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*Introduction:* Poor nutrition and impaired growth is associated with adverse long-term consequences in very low birth weight (VLBW) infants. However, feeding these preterm infants is a challenge and the optimal timing, volume and diet remain controversial. The aim of the database is to give an overview of differences in feeding practice for VLBW infants around the world. This will help to identify optimal feeding regimens and design appropriate intervention studies.

*Patients and Methods:* Fourteen hospitals in 'Western' (USA, Denmark, Netherlands, UK, Australia, New Zealand) and 'non-Western' (Mainland China, Taiwan, Nigeria) regions participated. Infants with a birth weight of 1500g or less were included. Collected data included timing and composition of (par)enteral nutrition and use of anti-/probiotics, anthropometrics and clinical outcomes from birth until a post-menstrual age of 37 weeks or discharge from hospital. Here we present preliminary results from all hospitals, 8 non-Western (A-H) and 6 Western (I-N).

*Results:* A total of 2905 infants were included. Gestational age (mean 29.6 wks), birth weight (median 1210g), gender distribution, and mortality differed significantly among hospitals. Additionally nutritional regimes and outcomes, including time to full enteral feeding (150 ml/kg/day), incidence of necrotizing enterocolitis, growth velocity and probiotics use differed markedly. Infants from Western hospitals had lowest birth weight, but reached full enteral feeding earlier (median 14 vs. 31 days,  $p < 0.0001$ ).

*Conclusions:* Nutritional practices and associated clinical outcomes in VLBW infants showed marked differences among hospitals. The variations may relate to differences in clinical practice, traditions or national recommendations among hospitals. This is important to clarify because early enteral feeding is suggested to influence both short- and long-term outcomes. Results from the NeoNutriNet database will be a valuable tool to help design future intervention studies.



## NEONATAL HYDROCORTISONE REDUCES CEREBELLAR GROWTH AND PHYSICAL ACTIVITY IN PRETERM PIGS

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**Introduction:** Preterm infants are frequently administered glucocorticoids (GC) to prevent bronchopulmonary dysplasia after prolonged periods of mechanical ventilation. Early treatment may benefit initial respiratory function but especially high-dose, long-acting GC treatment is suspected to induce adverse neurological and intestinal effects (e.g. cerebral palsy, intestinal perforation). We hypothesized that short-acting neonatal hydrocortisone (HC) treatment is not associated with such negative effects, using preterm pigs as a model for preterm infants.

**Methods:** We delivered thirty-five piglets from two sows by cesarean section at 90% gestation. Piglets were randomized to receive clinically relevant, diminishing doses of HC (6 → 2 mg/kg/d, n=18) or equivalent saline doses (CTRL, n=17) for four days beginning immediately after birth. We quantified home-cage activity by continuous video recordings and registered eye-lid opening and first stand. We euthanized the piglets at postnatal day 5, macroscopically assessed gastrointestinal pathology, and collected the brains for regional weights (cerebrum, cerebellum and stem) and hydration level.

**Results:** HC cerebella were smaller than CTRL ( $2.60 \pm 0.20$  vs.  $2.74 \pm 0.17$ g,  $p < 0.05$ ) and cerebellar fraction of whole brain was reduced ( $p < 0.01$ ). Body weight, brain weight and brain water content did not differ. HC piglets were slower to acquire the ability to stand after birth ( $45 \pm 26$  vs.  $28 \pm 9$  h,  $p < 0.05$ ), with no difference in eye lid opening. The HC piglets had higher frequency of NEC-like lesions in the mid intestine ( $p < 0.01$ ) with no differences in other regions (stomach, proximal and distal intestine, colon). Piglets with jejunal lesions had a significantly lower home cage activity level ( $p < 0.05$ , Figure).

**Conclusions:** Just four days of HC treatment impaired the rapidly growing cerebellum brain region, delayed neonatal arousal and was associated with more NEC-like lesions in the mid small intestine. Reduced physical activity from day 1 was associated with increased incidence of NEC lesions on day 5. We conclude that neonatal HC treatment is associated with adverse brain and gut effects.

## Immature systemic immunity during the first two weeks of life in preterm pigs

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**Introduction:** Immature systemic and mucosal immunity predispose preterm infants and preterm pigs to infections and necrotizing enterocolitis (NEC) during the first weeks of life. Correspondingly, antibiotics are commonly used to prevent or treat these conditions. By many clinical variables, preterm pigs delivered at 90% gestation can be considered similar to infants born at 70-75% gestation. We used this clinically-relevant pig model to better characterize how and when key components of the systemic immune system develop postnatally.

**Materials and Methods:** Preterm pigs were delivered by caesarean section (n = 34, 90% gestation) and gradually transitioned from parenteral nutrition to enteral nutrition with bovine milk. Maternal plasma (20 mL/kg) was provided at birth as passive immunity, and antibiotics (intramuscular enrofloxacin and oral gentamicin) were given if required according to clinical symptoms of infections, sepsis or severe diarrhea. Venous blood was collected at birth and during week 1, 2, 3 and 4 for cell counting, analysis of plasma C-reactive protein (CRP), and various blood assays including analyses of NK cells (CD172a<sup>-</sup> CD16<sup>+</sup> lymphocytes), progenitor cells, immature (CD172a<sup>+</sup> 6D10<sup>+</sup> 2B2<sup>-</sup>) and mature neutrophils (CD172a<sup>+</sup> 6D10<sup>+</sup> 2B2<sup>+</sup>), phagocytosis capacity against *E.coli*, and cytokine responses to TLR and NOD agonists.

**Results:** Preterm pigs showed poor growth and some diarrhea throughout the experiment. Antibiotics were used for 5-7 days but no pigs developed NEC. Newborn preterm pigs had low leukocytes vs. term and adult pigs (0.5 vs. 3.0-5.0×10<sup>9</sup> neutrophils/L, P<0.001), and marginal cytokine responses to TLR1/2/5/7, and NOD1/2 agonists. The postnatal systemic immunity gradually matured by increasing number (5-10 fold) and phagocytic capacity (2 fold) of neutrophils and monocytes, and numbers of NK cells, immature and mature neutrophils at week 2-4 (P<0.05). At week 3-4, the ratio of immature to total neutrophils was greater than 0.2, the cut-off value for suspected sepsis although CRP levels remained low (<10 mg/L). TLR2/4 agonist-induced IL-6 and TNF-α secretion elevated at week 2 with no increase thereafter. Neutrophil counts at week 2 (7×10<sup>9</sup>/L) were close to those obtained from term pigs at birth.

**Conclusion:** Systemic immunity is immature in newborn preterm pigs but reaches a degree of maturity that is similar to that in newborn term pigs within 2 weeks. This immune immaturity may result in a pro-inflammatory state, slow growth and need for antibiotics, reflecting short term postnatal conditions in preterm infants. The results underline the importance of optimal hygiene and protective milk diets to avoid excessive antibiotics use during the first postnatal weeks.

## Growth and development of pigs fed infant formulas varying in protein quantity and quality

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**Introduction:** High protein intake during infancy associates with later life obesity prompting an incentive to reduce protein levels in infant formulas. We designed novel infant formulas with a reduced total N content but with an optimized AA composition based on a recent series of studies on the specific AA requirements for infants. We hypothesized that a formula with a reduced N level and an optimized AA composition would not limit growth, intestinal health and clinical characteristics compared to a control product. We further anticipated that the form in which the AAs are provided, i.e. as either free AA or as intact protein, would affect the clinical outcomes.

**Materials and Methods:** Seven-day old piglets were randomly allocated to one of four iso-energetic infant formulas (n=14-19 per group), and were fed individually for 20 days thereafter. Three of the diet groups (ST75, O75, and O75AA) had 25% reduced total N intake (protein equivalents) relative to the ST100 group (7.0 vs 9.4 g protein equivalent/kg/d). The source of N in the standard (ST) and optimized (O) groups was a combination of intact proteins (70%) and added specific free AAs (30%), whereas for the O75AA diet group it was free AAs only. The optimized diets (O) contained an adapted BCAA ratio with reduced leucine levels compared to ST. Growth, selected clinical biochemical parameters, and intestinal morphometry and enzyme activities were analyzed in this protein- and growth-restricted model.

**Results:** Weight gain (in g) was highest in the ST100 piglets. Weight gain in the O75 group was numerically higher than in ST75 piglets, but did not reach statistical significance. The gain-to-nitrogen ratio did not significantly differ between the ST75 and O75 group. The O75 piglets had higher total bilirubin, ASAT, BASP and total cholesterol levels than ST75. Also, higher blood urea nitrogen (BUN) levels (+124%, P<0.001), isoleucine and valine levels were present in the O75 piglets (+223% and +233% respectively, P<0.001). The O75AA piglets showed lowest growth, and a much lower gain-to-nitrogen ratio than the O75 piglets (P<0.001). This was accompanied by lower DPP-IV and lactase activities in the proximal intestine (P<0.05), and a tendency to lower sucrase, ApN and ApA activities (P<0.10). There were no signs of mucosal damage, and morphology was similar among the four diet groups. (888/900)

**Conclusion:** A formula with optimized but reduced total N, mainly provided as intact protein, did not negatively affect growth. The reduced growth observed in the O75AA diet group confirms that a formula entirely based on free AAs requires a higher N level to ensure adequate growth compared to an iso-nitrogenous protein-based formula. There were some indications that the AA composition designed to be optimal for children was not optimal for piglets.