

ALLOMETRIC SCALING OF BRAIN GROWTH IN PRETERM INFANTS AND IN PIGLETS

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**Background:** The relationship between brain size (measured as head circumference) and body weight in human infants is allometric. This means that the relative growth rates of the body and the brain stays in a constant relation during infancy. We are in the process of developing a preterm piglet model to study nutritional interventions on the brain. Here we present an analysis of the growth pattern during the first weeks of life.

**Materials and methods:** Piglets (n=146) were delivered by planned C-section at 90% and 100% gestation. All piglets were part of nutritional intervention studies in which daily body weight gain and body and brain weight upon euthanasia (d0-26) were obtained.

**Results:** An allometric scaling model was established by linear regression using the log-transformed values of brain and body weight for piglets at 4 different ages at euthanasia: -10d (preterm at birth), 0d (term at birth), 5d and 26d for term piglets. Preterm piglets aged 4-26 days at euthanasia (n=52) gained less weight after birth compared to term (12 vs. 26g/(kg\*d), (p<0.01), but the relation between body and brain weight did not deviate from the allometric scaling model (mean Z-score 0.014, p=0.94).

**Conclusion:** As in humans, the relationship between the piglet brain and body weight appears to follow allometric scaling regardless of gestational age at birth. Preterm piglets were extra-uterinely growth-restricted but the relationship between the brain and body growth did not deviate from the normal scaling relation.

STEREOLOGIC QUANTIFICATION OF BRAIN VOLUME DEVELOPMENT IN PRETERM PIGS IN THE PERINATAL PERIOD

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**Background and aims.** Preterm birth is associated with an increased risk of brain injury, smaller brain volume and cognitive deficits. To gain insight into how premature birth affects brain development in a pig model of preterm birth, we evaluated the growth of the neocortex and cerebellum using designed based stereology.

**Methods.** Piglets born preterm or at term (postconceptional age (PA) 106 and 118, respectively) were euthanized on postnatal day 0, 5 or 26 (n=10-22). The left cerebral and cerebellar hemispheres were fixed in formalin, embedded in agar, and sectioned coronally. The grey and white matter volumes were estimated using the Cavalieri method. Data were analysed by ANCOVA including PA, postnatal age, weight, litter, and gender as covariates.

**Results.** Cerebral and cerebellar grey and white matter volumes increased significantly with PA and postnatal age (p<0.05). Interestingly, the cerebral white matter volume increased by 127% during the last 12 days of fetal life (p<0.001) and by 37% (p<0.001) from birth to postnatal day 26 in term piglets. The preterm piglets had smaller cerebral white matter and cerebellar grey and white matter volumes compared to term piglets of same

postnatal age ( $p < 0.05$ ).

**Conclusions.** The large increase in white matter volume during the last 12 days of fetal life suggests that this is a very sensitive period for brain growth in the piglet. These data are in agreement with human studies and thus supports the use of the preterm pig as a model for brain development in premature human infants.

#### NEONATAL AROUSAL AND HOME-CAGE ACTIVITY ARE FEEDING-DEPENDENT IN PRETERM PIGS

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**Background and aims:** Preterm infants exhibit delayed neonatal arousal and impaired motor function. Impaired neuromuscular development, probably interacting with gut and metabolic dysfunctions, may explain this. Using preterm pigs as models, we hypothesized that early initiation of enteral feeding stimulates both gut growth and neonatal arousal and physical activity.

**Methods:** Experiment 1: Caesarean-delivered preterm and term pigs were fed parenteral nutrition (PN) or PN plus enteral bovine colostrum (BC) for five days. Other preterm pigs were fed PN with or without BC or formula for five days (Experiment 2), or increasing doses of BC, formula or human milk (HM) for 10 days (Experiment 3). Daily energy intake was matched among the groups in each experiment and home cage activity (HCA) was recorded by continuous camera surveillance.

**Results:** Prematurity at birth delayed eye lid opening, first stand and walk, and reduced relative intestinal weight and HCA (Experiment 1, all  $P < 0.01$ ). Supplementing PN with BC or formula increased intestinal weight and HCA values (Experiment 2,  $P < 0.05$ ). Enteral BC feeding increased HCA and intestinal weights, relative to formula or HM (Experiment 3,  $P < 0.05$ ).

**Conclusions:** Prematurity decreased physical activity and relative gut weight within the first week after birth. Small volumes of enteral feeds increased the activity. This may result from general metabolic effects of enteral feeding but could also reflect a direct diet-dependent, gut-neuromuscular maturation in preterm neonates fed enterally. The results support the importance of early enteral feeding of preterm infants with adequate amounts of an optimal diet.

#### BIOMARKERS OF DELAYED BRAIN DEVELOPMENT IN A PIG MODEL OF PRETERM BIRTH

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**Background and aims:** Neurodevelopmental sequelae of premature birth involve cognitive and motor deficits, often persisting into adult life. The molecular mechanisms involved remain yet to be elucidated, but certain regions e.g. cerebellum and neocortex appear particularly sensitive. The current study aimed to evaluate the relevance of Brain derived neurotrophic factor (BDNF), involved in the formation of synaptic connections, and Sonic Hedgehog (SHH), important for perinatal neuronal differentiation, as potential biomarkers of brain development. **Methods:** Piglets were born via planned C-section either at full term (gestational age 118d) or 12 days preterm. Euthanization and brain dissection was performed at postnatal day 5 ( $n=11$ ,  $n=33$ ) and day 26 ( $n=22$ ,  $n=18$ ), for terms and preterms respectively. BDNF and SHH levels were analyzed by ELISA in pig cerebellar homogenates. Western blotting (WB) of downstream targets for BDNF (TrkB) and SHH (Patched, Smoothed, Gli-1) were included together with qPCR-array of 84 neurogenesis pathway related genes (including *Bdnf* and *Shh*) on cerebellar and prefrontal cortical tissue. **Results:** Overall BDNF analysis showed no

differences between term and preterm brains but levels were significantly different between day 5 and 26 in preterms only. SHH appeared to be lower in preterms compared to terms, but only significantly on Day 26 ( $P < 0.01$ ). WB and qPCR analyses will be presented at the meeting. **Conclusions:** The reduced levels of SHH, specifically at day 26, suggest that SSH may be a useful biomarker for delayed brain development and indicate that the pig may provide a relevant model to study the premature brain.

#### PRETERM AND TERM PIGLETS SHOW SIMILAR POSTNATAL ELECTROENCEPHALOGRAPHY (EEG)

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**Background** Electroencephalography (EEG) changes rapidly with maturation of the brain in preterm infants. Amplitude-integrated EEG (aEEG) becomes more continuous, bandwidth narrows, and frequencies switch towards faster rhythms, as the child grows older. In preterm infants with brain damage this maturation is typically delayed. We are in the process of developing a preterm piglet brain model. Here we present the results of the EEG as a measurement of brain development in 1-11-day-old preterm and term piglets.

**Methods** One hour of EEG was recorded in 31 preterm piglets aged 1, 2, 4 and 11 days and in 10 term piglets aged 2 and 11 days. All piglets were delivered by C-section at either 90% or 100% gestation. Upper and lower margins of the aEEG band were visually identified and bandwidth calculated as the difference between the two values. Spectral analysis of the raw EEG was used to determine the relative power in the delta-(0.5-3Hz), theta-(4-7.5Hz), alfa-(8-12.5Hz), and beta-(13-30Hz) bands. General linear models were used with term vs. preterm, and age as predictors.

**Results** All aEEGs were continuous. The overall means(SD) of upper and lower margin, and bandwidth were 7mcV(1.7), 15mcV(5.7), and 8mcV(4.3). Upper-, lower margin, bandwidth, alpha, beta and delta bands were unaffected by the predictors whereas the theta band was negatively correlated to age.

**Conclusions** The preterm piglet EEG was continuous already 10 days prior to term. Thus, maturation of EEG was neither seen in preterm nor term piglets and EEG may not be useful for studying perinatal brain maturation.

#### INTESTINO-TROPHIC EFFECTS OF MINIMAL ENTERAL NUTRITION IN PRETERM AND TERM PIGLETS

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**Background and aims:** In preterm infants, slow advancement of minimal enteral nutrition (MEN), combined with parenteral nutrition (PN), may be important to increase food tolerance and minimize the risk of necrotizing enterocolitis (NEC). We hypothesized that MEN for five days would increase gut growth and gut hormone release relative to total parenteral nutrition (TPN). **Methods:** Preterm and term piglets were delivered by caesarean section and fed TPN or PN+MEN (MEN: 0-64 mL bovine colostrum /kg/d) for five days. From day 5-26 all pigs were fed total enteral nutrition with milk-replacer. Pigs were euthanized at 0, 5 or 26 days of age, and gut weight, mucosal volume, L-cell density and plasma levels of GIP and GLP-2 were measured. **Results:** Body weight gain was markedly reduced in preterm vs. term ( $P < 0.01$ ). Relative to TPN feeding, MEN for 5 days increased relative gut weight, mucosa volume and plasma GLP-2 and GIP in both preterm and term pigs (all  $P < 0.05$ ). At 26 days of age, mucosa volume tended to be higher in preterm MEN versus preterm TPN ( $P = 0.11$ ),

whereas relative gut weight, L-cell density and plasma GLP-2 and GIP levels were similar between term/preterm and MEN/TPN. **Conclusion:** Despite the compromised growth in preterm pigs, the intestine is highly growth-responsive to MEN just after birth in both preterm and term pigs. The effects of MEN on gut dimensions and gut peptide release are minimal after few weeks of full enteral nutrition. MEN provision may be important for short term gut maturation in preterm infants.

#### PRETERM PIGLETS DISPLAY IMPAIRED PHYSICAL ACTIVITY AND ALTERED BEHAVIOR DURING THE FIRST WEEKS OF LIFE

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**Background and aims:** Premature birth interrupts normal growth and may affect postnatal brain development. We hypothesized that prematurity in pigs would affect neuromuscular control and behavior also beyond the neonatal period. **Methods:** Caesarean-delivered preterm (n=44, 90% gestation) and term (n=33, 100% gestation) piglets were fed parenterally for five days and then enterally with milk-replacer until d26. Time until basic motor skill (BMS) acquisition (eye lid opening, first walk and stand) were recorded, coordination assessed, and locomotion and general exploration were tracked from open field video recordings on d4, d9, d16 and d23. A novel-object recognition test was performed on d24 (assessing both specific exploration and short-term memory), and learning ability was assessed with a clicker-based poke-reward test from d18-d25 in a subset of piglets. **Results:** BMS acquisition was delayed in preterm piglets (all  $P<0.001$ ). Coordination scores were lower in preterm piglets at all ages whereas locomotion and exploration were reduced only on d4 (all  $P<0.05$ ). Preterm piglets explored novel objects less ( $P<0.001$ ) but short-term memory assessments were not different. Poke-reward performance improved over time in both preterm and term pigs but did not differ significantly between groups, which partly reflects that only clinically healthy preterm piglets could be tested. In preterm piglets, locomotion on d23 was increased ( $P<0.01$ ) when parenteral nutrition had been supplemented with enteral nutrition the first five days after birth. **Conclusion:** Acquisition of neuromuscular control, locomotion and exploration are quantifiable functional neurological endpoints in preterm piglets that may be used to characterize developmental disturbances and nutritional interventions.

#### NECROTIZING ENTEROCOLITIS IS ASSOCIATED WITH HIPPOCAMPAL NEURON LOSS, MICROGLIAL ACTIVATION AND INCREASED IL-8 LEVELS IN PRETERM PIGS

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**Background and aims:** Preterm birth predisposes to neurological sequelae. Necrotizing enterocolitis (NEC) may further increase the susceptibility to neurological damage, possibly via gut-derived inflammatory signals. To investigate this, we test if NEC severity and intestinal permeability in formula-fed preterm pigs is associated with histopathology, microglial activation, and increased proinflammatory cytokine levels in the hippocampus.

**Methods:** Forty-four preterm piglets were fed increasing doses of formula and euthanized on day five. Macroscopic NEC lesions were scored in five regions of the gut (stomach, proximal, middle, and distal small intestine, colon). Intestinal permeability was assessed by urinary lactulose-mannitol-ratio. Hippocampal IL-1 $\beta$  and IL-8 levels were determined by ELISA. Histopathology, neurodegeneration, and microglia were investigated by analyses of hematoxylin-eosin, Fluoro-jade B (FJB), and Iba-1 stained coronal sections, respectively.

**Results:** Proximal, middle, and distal small intestinal NEC score, and intestinal permeability correlated positively

with IL-8 levels (all  $p < 0.05$ ) but not with IL-1 $\beta$ . In preterm piglets with severe NEC lesions, numerous shrunken, hyperchromatic neurons were observed. Neurodegeneration was confirmed by positive FJB staining. Iba-1 positive cells with a morphology resembling activated microglia populated the area in which neurons had disappeared.

**Conclusions:** Acute development of NEC is associated with neuron loss, microglial activation, and increased IL-8 levels in the hippocampus of preterm pigs. Gut inflammatory disorders and increased intestinal permeability may affect the immature brain and contribute to long term neurological disorders.

## NEONUTRINET - INTERNATIONAL DATABASE ON NEONATAL NUTRITION IN VERY LOW BIRTH WEIGHT INFANTS

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**Background and aims:** Nutrition for VLBW infants varies widely among countries. To better understand differences in nutritional practices internationally, we compare data from fourteen hospitals from eight countries (Guangzhou, Shenzhen, Foshan, Amsterdam, Auckland, Copenhagen, Chennai, Chicago, Perth, Newcastle). **Methods:** Infants with birth weights <1500g are included and data include time, type, and amount of (par)enteral nutrition, anti-/pro-biotics, anthropometric measurements and clinical complications from birth to 37w corrected gestational age, or discharge. **Results:** Data collection is ongoing, but preliminary data are reported for two selected hospitals (2011-2012,  $n=96+107=203$ ) with similar demographic data (e.g. birth weight, median 1335g; gestational age, median 30.1w; gender, 59% boys). In hospital A, the growth velocity and proportion of infants reaching 120 mL/kg/d enteral feeding at 5 weeks was higher (median 14.5 vs. 9.1 g/kg/d and 84 vs. 69%,  $P < 0.05$ ), and the decrease in weight Z-score was lower vs. B (median -0.55 vs. -0.94,  $P < 0.05$ ). Neither of the units reached the protein intake recommended by ESPGHAN (3.5 g/kg/d, JPGN, 50, 89-95, 2010) within the first month although the average daily deficit was less in hospital A vs. B (median -0.8 vs. -1.2 g/kg/d,  $P < 0.05$ ). NEC incidence was lower in hospital A vs. B (1 vs. 9%,  $P < 0.05$ ). There were no differences in total days on antibiotics (~50% hospitalization day). **Conclusion:** Large differences in nutrition and growth outcomes were evident between the two units. The NeoNutriNet cohort will show how differences in nutrition may relate to feeding guidelines, clinical traditions, and use of anti-/pro-biotics around the world.

## DEVELOPMENT OF A NEONATE PIGLET MODEL TO UNDERSTAND BLOOD-BRAIN BARRIER PHYSIOLOGY IN EARLY PRETERM BABIES

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**Introduction:** The instant developmental switch of nutritional and oxygen supply from the umbilical cord to the lungs and the intestinal canal are necessary and dramatic changes for the offspring in order to adapt to life outside the uterus. This period is also characterized by the exposure and colonization by live bacteria, an assumed important step towards physiological programming of the newborn. The perinatal period is associated with establishment of a functional blood-brain barrier (BBB), essential for the brain development and protection from adverse systemic influences, which in rodents, has been suggested to be regulated by intestinal microbiome. To investigate whether the pig could be used as a model for preterm infant brain maturation, we studied the BBB in preterm and term newborn piglets.

**Methods:** The integrity of the BBB was evaluated in caesarean-delivered preterm (90% gestation) and term-born neonate pigs immediately after birth (n= 10). The expression of main tight junctions proteins (TJPs) controlling the BBB, and the glucose transporter-1 (Glut-1) in the hippocampus and striatum were determined by western blot technique.

**Results:** Alterations of TJPs expression in brain tissue were observed in hippocampus and striatum of preterm piglets compared to full-term controls. In addition, Glut-1 expression in the brain endothelial cells exhibited changes in a region-specific manner.

**Conclusion:** This pilot study demonstrate altered expression patterns of TJPs and Glut-1 in hippocampus and striatum of preterm piglets compared to full term piglets which support that the BBB impairment observed in rodents may also extend to the BBB in preterm piglets.