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Rapid gut growth but persistent delay in digestive function in the postnatal period of preterm pigs

Carl Frederik Hansen,[1] Thomas Thymann,[1] Anders Daniel Andersen,[1] Jens Juul Holst,[2] Bolette Hartmann,[2] Linda Hilsted,[4] Louise Langhorn,[1] Jacob Jelsing,[3] and Per Torp Sangild[1,5]

[1]*Comparative Pediatrics and Nutrition, University of Copenhagen, Copenhagen, Denmark;* [2]*Novo Nordisk Foundation Center for Basic Metabolic Research, and Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;* [3]*Gubra, Hørsholm, Denmark;* [4]*Department of Clinical Biochemistry, Copenhagen University Hospital, Cophenhagen Denmark; and* [5]*Department of Pediatrics and Adolescent Medicine, Copenhagen University Hospital, Denmark*

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Hansen CF, Thymann T, Andersen AD, Holst JJ, Hartmann B, Hilsted L, Langhorn L, Jelsing J, Sangild PT. Rapid gut growth but persistent delay in digestive function in the postnatal period of preterm pigs. *Am J Physiol Gastrointest Liver Physiol* 310: G550–G560, 2016. First published January 28, 2016; doi:10.1152/ajpgi.00221.2015.—Preterm infants often tolerate full enteral nutrition a few weeks after birth but it is not known how this is related to gut maturation. Using pigs as models, we hypothesized that intestinal structure and digestive function are similar in preterm and term individuals at 3–4 wk after birth and that early enteral nutrition promotes maturation. Preterm or term cesarean-delivered pigs were fed total parenteral nutrition, or partial enteral nutrition [Enteral (Ent), 16–64 ml·kg^{-1}·day^{-1} of bovine colostrum] for 5 days, followed by full enteral milk feeding until *day 26*. The intestine was collected for histological and biochemical analyses at *days 0*, *5*, and *26* (n = 8–12 in each of 10 treatment groups). Intestinal weight (relative to body weight) was reduced in preterm pigs at 0–5 days but ENT feeding stimulated the mucosal volume and peptidase activities. Relative to term pigs, mucosal volume remained reduced in preterm pigs until 26 days although plasma glucagon-like peptide 2 (GLP-2) and glucose-dependent insulin-trophic peptide (GIP) levels were increased. Preterm pigs also showed reduced hexose absorptive capacity and brush-border enzyme (sucrase, maltase) activities at 26 days, relative to term pigs. Intestinal structure shows a remarkable growth adaptation in the first week after preterm birth, especially with enteral nutrition, whereas some digestive functions remain immature until at least 3–4 wk. It is important to identify feeding regimens that stimulate intestinal maturation in the postnatal period of preterm infants because some intestinal functions may show long-term developmental delay.

prematurity; gut development; enzymes; digestion; glucagon-like peptide 2

ABOUT 10% OF ALL INFANTS are born preterm (<37 weeks of gestation) and the proportion is increasing in most countries (6). Preterm infants show an increased susceptibility to infections, respiratory distress syndrome, intracranial hemorrhage, bronchopulmonary dysplasia, retinopathy of prematurity, extrauterine growth restriction, neurodevelopmental disturbances, and necrotizing enterocolitis (NEC) (21, 31). The challenges are inversely related to gestational age at birth and the most severe adaptation problems occur during the first few weeks after birth. If preterm infants survive the difficult neonatal period, their body functions often adapt well, although some organ systems may show long-term developmental delay or deficits (18). It remains unknown how different organ systems adapt in preterm infants and when parameters of structure and function become similar to those in term infants. For gastrointestinal (GI) functions, eating disorders and dysregulated appetite have been reported in preterm infants (44) but very little is known about the long-term adaptation of the GI tract in preterm neonates.

Investigations of postnatal GI adaptation in preterm neonates are difficult without an animal model that combines preterm birth, high NEC risk, and clinically relevant feeding interventions, such as parenteral and enteral nutrition. Piglets delivered at 90% gestation suffer from many of the same disorders and physiological problems as moderately immature human infants and they have been widely used to investigate the immediate feeding-, diet-, and microbiota-related gut complications (39, 41, 43). The timing of perinatal GI maturation appears to be intermediate in pigs, relative to the delayed and rapid postnatal GI development in rodents, and the relatively early (partly prenatal) and slower development in humans (39). On the other hand, the high NEC sensitivity in preterm pigs, even after a moderate (10%) reduction in gestational length, suggests that the piglet GI tract is relatively immature at birth. This is an advantage when attempting to model aspects of GI development in very immature infants. Using this model, we have documented that NEC sensitivity is highly diet dependent and that porcine, bovine, and human intact milk diets protect against NEC, relative to infant formulas (5, 26).

Animal studies indicate that early enteral feeding is important for gut maturation and prolonged total parenteral nutrition (TPN) induces intestinal atrophy and dysfunction (8). Early GI maturation may help to prevent later adverse effects of slow growth and inadequate nutrient intake in preterm infants (30). On the other hand, too rapid advancement of enteral feeding is associated with high NEC risk, and delayed advancement of enteral feeding may decrease NEC risk in both preterm infants and pigs (20, 32, 33). Consequently, a gradual introduction of mother's own milk, donor milk, or infant formula ("minimal enteral nutrition," daily increases of 10–20 ml·kg^{-1}·day^{-1}), is often used for 1–2 wk after birth as adjunct to parenteral nutrition (PN), before transition to full enteral feeding at 2–4 wk (e.g., 150–180 ml·kg^{-1}·day^{-1}). Regardless, the optimal feeding time, advancement rate, and diet (especially when

Address for reprint requests and other correspondence: P. T. Sangild, Comparative Pediatrics and Nutrition, Dept. of Clinical Veterinary Medicine and Animal Science, Faculty of Health and Medical Sciences, 68 Dyrlægevej, DK-1870 Frederiksberg, C, Denmark.

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mother's milk is not available) remain unclear. It is also unknown whether enteral feeding during the first week may have both short- and long-term consequences for GI maturation.

The GI tract not only facilitates nutrient digestion and absorption but has major endocrinological, neurological, and immunological functions that are crucial for long-term health. Three gut hormones released in response to feeding and with effects on gut growth and function are gastrin released from gastric G cells, glucose-dependent insulin-trophic hormone (GIP) from (proximal) intestinal K cells and glucagon-like peptide 2 (GLP-2) from (distal) intestinal L cells. Preterm infants show elevated levels of these GI hormones, compared with term infants and adults, and they are sensitive to enteral stimuli (1, 16, 27). During total parenteral nutrition (TPN), in the absence of enteral food, administration of the gut tropic hormone, GLP-2, markedly improves gut growth and adaptation in preterm and term pigs (9, 36, 38), but the role of this and other GI hormones on later GI development remains unknown.

In the present study, we hypothesized that intestinal structure and digestive function are reduced by preterm birth but that gut maturation occur postnatally so that the preterm gut reaches a stage of maturity at 3–4 wk that is similar to that in term animals. Furthermore, we hypothesized that small amounts of enteral food (vs. TPN) during the first week of life would affect gut maturation both within the first week and later (e.g., at 3–4 wk). Bovine colostrum was used as the first enteral diet because it is known to protect against NEC in piglets (9, 36, 38) and has been tested as the first enteral diet in preliminary studies in preterm infants (28). Intestinal morphology and mucosa volume were measured together with key markers of digestive (e.g., hydrolase activities, nutrient absorption) and enteroendocrine cell functions (plasma gastrin, GLP-2, and GIP levels). The results help to understand how the immature GI tract matures after preterm birth until 3–4 wk of age when both preterm pigs and moderately preterm infants tolerate full enteral feeding and are clinically stable enough to leave their intensive care units.

METHODS

Animals and nutrition. One hundred and sixty-eight piglets from eight sows (Danish Landrace × Large White × Duroc) were caesarean delivered, either preterm (*day 106* or 90% of gestation; $n = 112$ pigs from five sows) or at full term (*day 118* or 100% gestation, $n = 56$ pigs from three sows) according to preestablished protocols (43). All piglets were immediately transferred to a piglet neonatal intensive care unit and reared in temperature-, moisture-, and oxygen-regulated incubators. All preterm and term pigs were fitted with orogastric (6 Fr; Pharmaplast, Roskilde, Denmark) and umbilical arterial catheters (4 Fr; Portex, Kent, UK) and block-randomized based on birth weight in two groups receiving total parenteral nutrition (TPN) or PN plus minimal enteral nutrition with bovine colostrum (ENT) for the first 5 days. Within the first 24 h they received 12 ml/kg of their mother's plasma intra-arterially to obtain immunological protection. Throughout the 5 days of catheterization we provided continuous infusion of heparin via the PN solution to avoid thrombosis.

For both preterm and term TPN groups, PN (modified combination of Kabiven, Vitalipid, Soluvit, and Vamin; Fresenius Kabi, Bad Homburg, Germany) (43) was given via umbilical catheterization at 96 $ml \cdot kg^{-1} \cdot day^{-1}$ on *day 1*, gradually increasing to 144 $ml \cdot kg^{-1} \cdot day^{-1}$ on *day 5* when the catheters were removed from all animals. The nutrient composition of the TPN was (in g/l) carbohydrates (71.4), amino acids (44.6), and fat (30.9) with a total energy density of 745 kcal/l. For the ENT group, enteral nutrition with bovine colostrum (BC) was provided as a solution of 170 g colostrum powder, obtained from Biofiber Damino, Vejen, Denmark, mixed in 1 liter of sterile water. The BC was given in boluses every 3 h, starting at 16 $ml \cdot kg^{-1} \cdot day^{-1}$ on *day 1* and increasing to 64 $ml \cdot kg^{-1} \cdot day^{-1}$ on *day 5*. The ENT group was provided with supplemental PN such that the two dietary regimens were isoenergetic (74–110 $kcal \cdot kg^{-1} \cdot day^{-1}$ over the first 5 days) as described in a previous study using the same initial diet regimen (45). On *day 5*, the PN was discontinued and all piglets were given increasing amounts of raw bovine milk (64–150 $ml \cdot kg^{-1} \cdot day^{-1}$) to *day 9* and subsequently transferred to reconstituted whole milk powder at 150–200 $ml \cdot kg^{-1} \cdot day^{-1}$ (Arla Foods Ingredients, Viby, Denmark) until *day 26*. The dosing strategies for both TPN and enteral feeding are comparable with the feeding advancement rates used for moderately preterm infants (4, 13, 17, 25, 53). See Fig. 1.

To prevent infections and sepsis, amoxicillin trihydrate (Paracillin 70%, MSD, Animal Health, Ballerup, Denmark) was administered prophylactically in the feed (20 mg/l) on *days 5-15*. Animals were euthanized with pentobarbital (50) and tissues collected at *days 0*, *5*, and *26*, resulting in a total of 10 treatment groups (newborn 0 days, 5-day-old TPN and ENT, 26-day-old TPN and ENT after either preterm or term delivery). All animal experiments were approved by the Danish Committee for Animal Research (license no. 2012-15-2934-00-193).

Plasma hormone measurements. Plasma samples for gastrin, GLP-2, and GIP measurements were collected at euthanasia. For gastrin, samples were analyzed as described previously, with an antibody validated for pigs (code no. 2604) (40). For GLP-2, samples were extracted in a final concentration of 75% ethanol and GLP-2 was measured with an NH_2-terminal specific antiserum (code no. 92160), measuring only GLP-2 with an intact NH_2 terminus, as previously described (24). For standards, recombinant human GLP-2 and rat GLP-2 tracer with an $Asp^{33} \rightarrow Tyr^{33}$ substitution was used. After plasma extraction (70% ethanol), total GIP measurements were performed by using an antiserum recognizing the COOH-terminal part (code no. 80867), as previously described (29).

Lactulose-mannitol test. To measure in vivo intestinal permeability, piglets were dosed intragastrically via an orogastric tube with a combined 5% lactulose and 5% mannitol solution. A bolus of 15 ml/kg body wt was given 3 h before euthanasia after withdrawal of feeds for 3 h. At the time of euthanasia, urine was collected by intra-abdominal cystocentesis. The urine samples were stored at −20°C and analyzed spectrophotometrically by an end-point assay, as previously described (5, 7, 34).

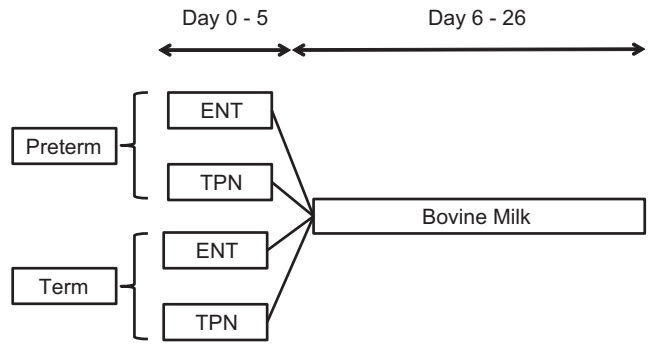

Fig. 1. Study outline. Animals were caesarean delivered, either preterm (90% gestation) or at full term, and randomized into 1 of 2 groups: total parenteral nutrition (TPN) or parenteral nutrition plus minimal enteral nutrition with bovine colostrum (ENT) for the first 5 days. On *day 5*, the parenteral nutrition was discontinued and all piglets were given increasing amounts of bovine milk until *day 26*.

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Galactose test. The test was performed by administering a 15 ml/kg oral bolus of a 5% galactose solution via an orogastric tube 2–3 h after the last bolus feeding on *day 4* or *25*, respectively. Blood samples were collected either via the umbilical catheter, or via jugular vein puncture, at 0 or 20 min after the galactose bolus. Plasma was isolated and analysis of galactose concentration was performed as previously described (48).

Tissue and blood sampling. Following induction of anesthesia, blood was drawn by cardiac puncture and the pigs were then euthanized with pentobarbital (50). The final blood sample was taken 2–4 h after birth for newborn pigs and exactly 1.5 h after the last meal for the 5- and 26-day-old pigs. Blood samples were centrifuged and the plasma fraction was rapidly frozen. The small intestine, from the pyloric sphincter to the cecum, was rapidly excised and placed on an ice-cold metal plate in a relaxed state. The total length and weight were measured, and the intestine was sampled for histology and stereology with systematic uniform random sampling. A total of nine sections were taken throughout the intestine starting from a predetermined random start site and with a set sampling distance (total length/9). At each site, a 15-mm transverse biopsy was taken and fixed in 4% neutral buffered paraformaldehyde.

Ex vivo brush border enzyme activities. Activities of brush border enzymes, lactase, maltase, sucrase, aminopeptidase N (ApN), aminopeptidase A (ApA), and dipeptidyl peptidase 4 (DPP4) were analyzed in homogenates of proximal, middle, and distal intestinal tissues by spectrophotometry, as described previously (42). Enzyme activities were expressed as units per gram of wet tissue. Tissue homogenates were obtained by homogenizing the tissue sample in 1% Triton X-100 water solution (10 ml/g tissue).

Enteroendocrine cells detected by immunohistochemistry. Enteroendocrine cells were identified by immunohistochemistry using the following procedure. Sections were deparaffinized in xylene and rehydrated. Sections were then subjected to antigen retrieval in Tris-EGTA buffer and blocked for endogenous peroxidase activity and nonspecific binding, before being incubated with a GLP-1 antibody diluted 1:16,000 (GLPa-1F5 0P009, gift from Novo Nordic, Copenhagen, Denmark) overnight. Sections were subsequently visualized with Ultravision One (TL-015-HDJ Thermo Scientific) and finally developed by using DAB as chromogen. Slides were counterstained with hematoxylin, dehydrated, coverslipped with Pertex (Sakura), and finally digitalized on an Aperio Scanscope AT slide scanner (Aperio). The localization and shape of the positively stained cells confirmed that these were indeed enteroendocrine cells. The same antibody has previously been validated for visualizing GLP-1-positive L cells in pigs (3).

Mucosal structure and stereological estimation of mucosa volume. To evaluate mucosal morphology, fixed samples from the proximal, middle, and distal regions of the small intestine were embedded in paraffin, sectioned, and stained with hematoxylin and eosin before measurement of villus height and crypt depth with light microscopy images via the ImageJ processing and analysis software program, as described previously (23).

The intestinal mucosa volumes were estimated by using newCAST (Visiopharm) on digital slides. For each of the nine intestinal sections, fields of views were sampled in a random systematic manner by use of the newCAST software. The volumes of the mucosal layer, as well as the submucosa/muscularis/serosa layer, were estimated by use of a 16-point grid at ×10 magnification. The number of points hitting the structure of interest was converted into volume by using the principle of Cavalieri: $Vol_{ref} = \sum p \times A(p) \times t$, where $\sum p$ is the total number of points hitting the structure of interest, $A(p)$ is the area associated with each grid point, and t is the distance between sections (23).

Estimation of the total number of enteroendocrine cells. The total number of immunoreactive GLP-1-positive L cells in the small intestine was estimated by the principle of the physical dissector (22, 47). For this purpose 5-μm-thick tissue sections were sampled as two consecutive levels. The slides were processed for immunoreactivity, digitized, and finally analyzed on a computer running newCAST (Visiopharm) software at ×20 magnification. The total number of stained cells in a defined sampling volume was counted and the particle density N_v was calculated as $N_v = [\sum Q/a(\text{frame})] \times h \times \sum p$, where $\sum Q$ is the total number of uniquely counted cells, a(frame) is the area of the counting frame, h is the distance between the two sections, and $\sum p$ is the total number of points hitting the reference space. The total number of enteroendocrine cells was finally determined by multiplying N_v with the total reference volume.

Statistics and data presentation. Results are presented as means ± SE unless otherwise stated. All data were analyzed with pig and litter as random variables and time, treatment and intestinal region as fixed variables in the mixed procedure of the SAS statistical software program (SAS version 4.3; SAS Institute, Cary, NC). Post hoc comparison was carried out without correction and only within same gestational age (preterm or term). Specifically for enzymatic activities, treatment comparisons (ENT, TPN) were carried out for each intestinal region (proximal, mid, distal) at each of the postnatal ages (0, 5, 26 days). Probability levels below 0.05 were considered significant.

RESULTS

Body weight gain and clinical condition. Relative to term, preterm piglets had a lower body weight at birth ($P = 0.06$), with the difference increasing further to 5 days ($P < 0.01$) and 26 days ($P < 0.001$, Fig. 2*A*). This led to an overall reduced body weight gain in preterm vs. term pigs (25.5 ± 1.5 vs. 31.0 ± 0.5 $g \cdot kg^{-1} \cdot day^{-1}$, $P < 0.01$), despite their identical nutrient intakes per kilogram body weight. The slower growth may be partly explained by lowered nutrient digestion in the preterm pigs since these showed more days with diarrhea, relative to term piglets ($P < 0.01$, as analyzed across diet groups, Fig. 2*B*). TPN-fed preterm pigs displayed the highest number of days with diarrhea and ENT term pigs the fewest days with diarrhea (Fig. 2*B*). A detailed description of the clinical condition, blood chemistry values, organ weights, body composition, and behavioral characteristics of 0- to 26-day-old preterm and term pigs is published as a separate report (2).

Gut morphology, mucosa volume, and gut weight. At all three ages, preterm pigs showed lowered absolute values for intestinal length ($P < 0.001$, Fig. 2*C*), intestinal weight ($P < 0.05$, data not shown), and intestinal mucosa volume ($P < 0.05$, Fig. 2*D*), compared with term pigs. When expressed relative to body weight, the intestinal weight and mucosa volume were significantly lower in preterm pigs, but only at birth ($P < 0.001$, data not shown). At *day 5*, both term and preterm ENT animals displayed a higher mean mucosa volume, compared with TPN, but the increase did not reach statistical significance. On the other hand, the tendency to increased absolute intestinal weight in the 5-day-old ENT pigs (Fig. 2*D*) became highly significant when expressed relative to body weight (28.7 ± 0.6 vs. 24.5 ± 0.7 g/kg, $P < 0.001$ for ENT vs. TPN across preterm and term pigs).

Villus height and crypt depth. Villus height tended to decrease with age for both preterm and term animals and were generally lower for preterm than for term pigs ($P < 0.001$, Fig. 3*A*). With age, crypt depths increased markedly for both preterm and term animals (Fig. 3, *B* and *D*), likely reflecting increased mitotic activity with advancing age ($P < 0.05$, Fig. 3*C*). Across gut regions and gestational ages, ENT feeding tended to lower the villus/crypt ratio, relative to TPN (Fig. 3*C*, $P = 0.07$), but the differences within each postnatal age group

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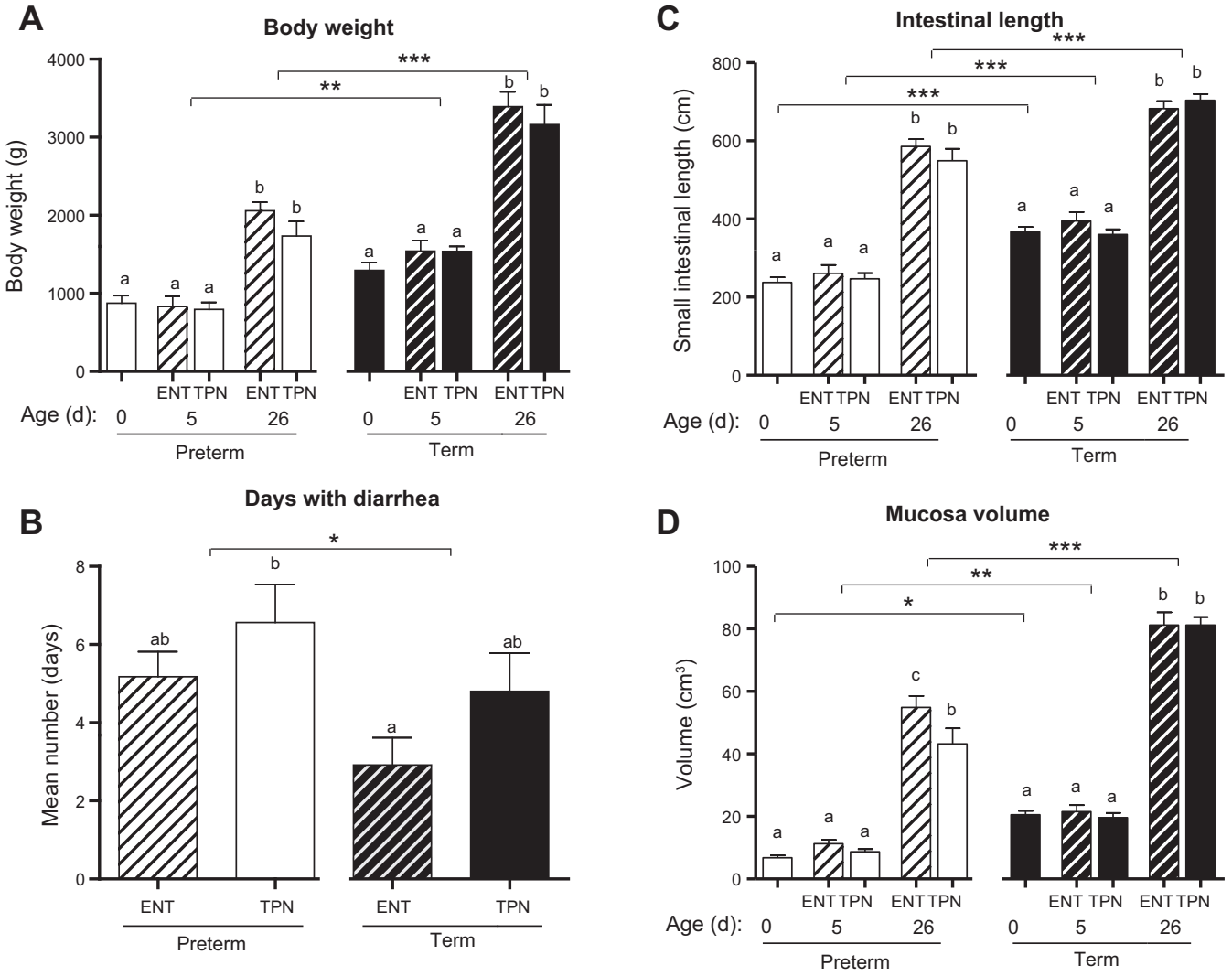

Fig. 2. Body weight (*A*), cumulative days with diarrhea (*B*), intestinal length (*C*), and mucosal volume (*D*) in 0-, 5-, and 26-day-old preterm or term pigs fed total parenteral nutrition (TPN) or supplemented with enteral nutrition (ENT) for the first 5 days (means ± SE). Columns within the preterm or term groups not sharing the same letter are significantly different ($P < 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences between preterm and term piglets for each postnatal age group (0, 5, 26 days), analyzed across diets (ENT, TPN). d, Days.

remained marginal. Figure 3*D* shows representative micrographs of sections from the middle intestine of preterm and term pigs at the three different ages. The panels indicate the decreasing villus height per crypt depth ratio with advancing postnatal age, a relatively open villus structure in preterm newborn pigs (Fig. 3*D*, *top left*), and a much higher density of immature vacuolated cells in preterm 5-day-old pigs (open white areas on villus), relative to the term 5-day-old pigs (arrows in Fig. 3*D*, *middle*).

Gut peptide levels in plasma and the number of enteroendocrine cells in the small intestine. Gastrin, GLP-2, and GIP plasma levels were measured in all pigs at the time of tissue collection, i.e., 2–4 h after birth for newborn pigs and 1.5 h after the last meal for 5- and 26-day-old pigs. At birth, plasma gastrin levels were significantly higher in preterm pigs, relative to values in term pigs (63 ± 7 vs. 28 ± 3 pmol/l, $P < 0.001$), but at 5 and 26 days values were similar across all delivery and diet groups (20–30 pmol/l, data not shown). Only at 5 days was there a tendency to increased values in ENT vs. TPN pigs (26 ± 3 vs. 20 ± 2 pmol/l across delivery groups, $P = 0.09$).

Analyzed across all postnatal age groups, GLP-2, secreted mainly from L cells in the distal intestine, showed elevated levels in plasma of preterm vs. term animals, most clearly at 26 days ($P < 0.05$, Fig. 4*A*). The ENT treatment stimulated plasma GLP-2 levels at 5 days in term pigs (~60% increase, $P < 0.05$) whereas the effect in preterm pigs was less pronounced ($P = 0.11$, ~30% increase). For both groups, the levels increased with age ($P < 0.001$), with the highest GLP-2 levels detected in 26-day-old preterm pigs fed ENT from birth (Fig. 4*A*). GIP, secreted mainly by proximally located K cells, also increased in concentration in plasma with advancing age ($P < 0.001$ analyzed across preterm and term pigs, Fig. 4*B*). The ENT treatment was associated with a very marked increase in preterm pigs (~200% increase, $P < 0.001$) whereas the effect in term pigs was less pronounced (~50% increase, $P = 0.07$). Importantly, these

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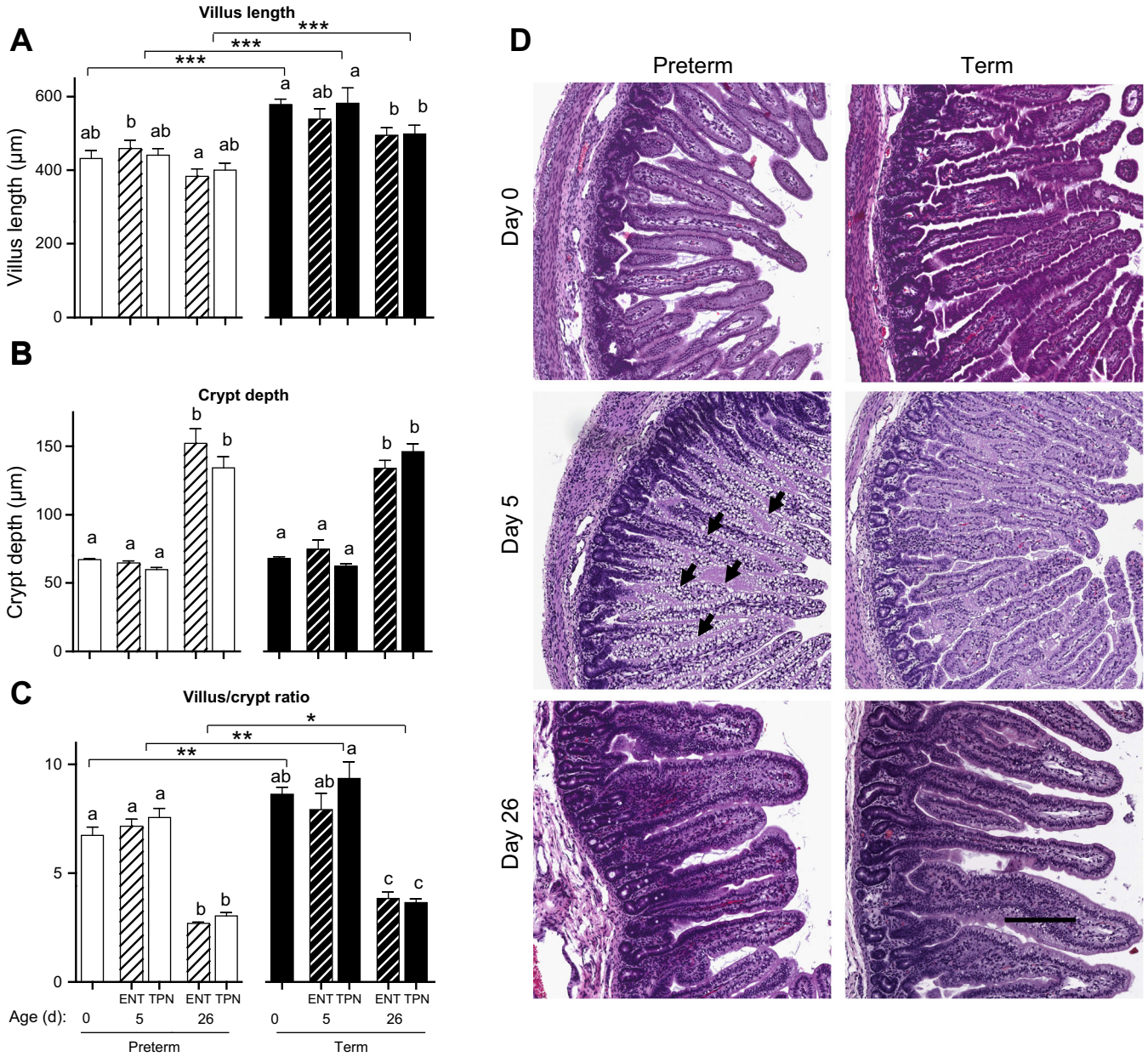

Fig. 3. Villus height (*A*), crypt depth (*B*), and villus/crypt ratio (*C*) in 0-, 5- and 26-day-old preterm or term pigs fed total parenteral nutrition (TPN) or supplemented with enteral nutrition (ENT) for the first 5 days (means ± SE). Representative photomicrographs (*D*) that indicate intestinal morphology in term and preterm animals throughout the study period are shown for the middle intestine of pigs supplemented with ENT. Arrows indicate immature vacuolated cells in the preterm intestine. Columns within the preterm or term groups not sharing the same letter are significantly different ($P < 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences between preterm and term piglets for each postnatal age group (0, 5, 26 days), analyzed across diets (ENT, TPN).

effects were transient since they were no longer evident at *day 26* (Fig. 4*B*).

To specifically investigate whether the high plasma GLP-2 levels in ENT 26-day-old pigs (Fig. 4*A*) reflected a higher number of L cells, their number and density was estimated by stereological methods in the preterm group of animals at *days 0* and *26* following ENT and TPN nutrition. The number of GLP-1 immunoreactive L cells were distributed across the crypt and basal part of the villi (Fig. 4*E*) and were significantly higher at *day 26* than at birth (Fig. 4*C*), with a tendency to the highest number in ENT pigs. However, the density was similar between newborn and 26-day-old ENT and TPN pigs (Fig. 4*D*).

Gut permeability and hexose absorptive capacity. Figure 5*A* shows the urine lactulose/mannitol ratio as a measure for the overall gut leakiness. Mean values for permeability increased after birth in both term and preterm animals with the highest mean values in preterm pigs ($P = 0.12$ at 26 days). Five days of ENT tended to lower the permeability in preterm animals ($P = 0.05$), and subsequent milk feeding for 21 days was not able to decrease the leakiness of the preterm gut down to the level in corresponding term animals. Large variations in the data set potentially masked an overall effect of prematurity ($P = 0.11$) and diet intervention ($P = 0.18$). Plasma galactose increment at 20 min in response to an oral bolus of galactose was measured as a marker of hexose absorptive capacity. The

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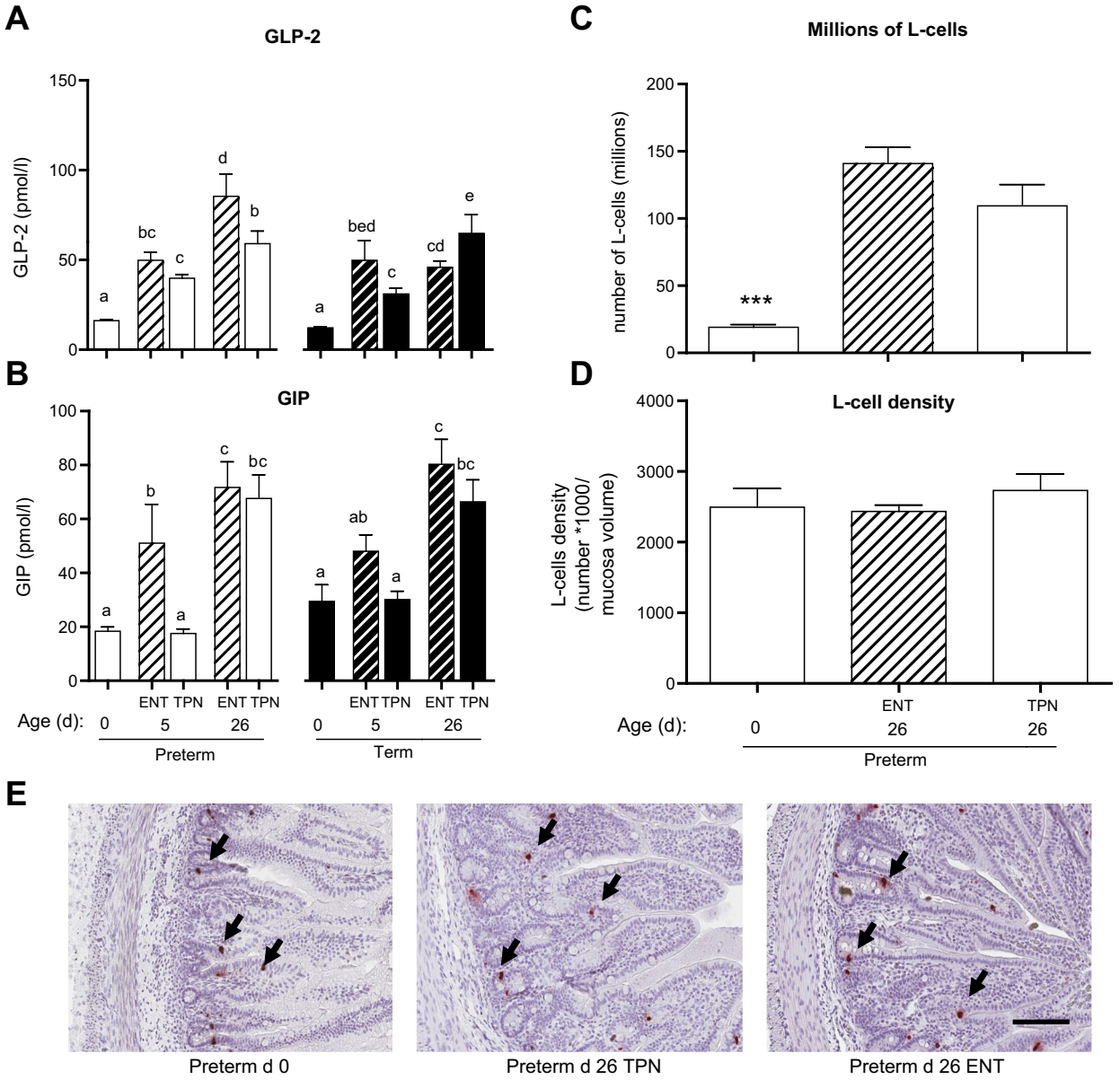

Fig. 4. Plasma glucagon-like peptide 2 (GLP-2, *A*) and glucose-dependent insulin-tropic peptide (GIP, *B*) levels, number of intestinal L cells (*C*), and density of intestinal L cells (*D* and *E*; arrows indicate the GLP-1 stained cells) in 0-, 5-, and 26-day-old preterm or term pigs fed total parenteral nutrition (TPN) or supplemented with enteral nutrition (ENT) for the first 5 days (means ± SE). Columns within the preterm or term groups not sharing the same letter are significantly different ($P < 0.05$). ***Significant difference ($P < 0.001$) between newborn and 26-day-old preterm pigs, as analyzed across diets (ENT, TPN).

ENT treatment tended to increase values for both delivery groups within the first week, but the differences were not significant ($P = 0.16–0.18$). By 4 wk, the term animals had increased hexose absorptive capacity, relative to preterm pigs ($P < 0.01$, Fig. 5*B*).

Ex vivo digestive enzyme activities. Activity of six different brush border enzymes were analyzed for preterm and term pigs at all three ages and across three intestinal regions (Fig. 6, *A–F*). Previous studies show that these enzyme activities are diet dependent and region specific and show differential regulation by pre- and postnatal age. Sucrase activity (resulting from the sucrase-isomaltase enzyme) and maltase activity (resulting from two enzymes, sucrase-isomaltase and maltase-glucoamylase) were low at birth and there were no differences between preterm and term pigs (*left*, Figs. 6*A* and 5*B*). These enzyme activities matured with advancing postnatal age in both delivery groups, especially in the proximal and middle intestinal regions, but preterm pigs were persistently associated with lower activities. In the proximal intestine of 5-day-old preterm animals, sucrase and maltase activities were less than 15% of the values in term animals (Fig. 6, *A* and *B*, *middle*). At this time, ENT slightly lowered the proximal sucrase and maltase activities in term pigs, relative to values for TPN pigs ($P < 0.05$), without any effects for preterm pigs. At 26 days, these enzyme activities showed large increases in both proximal and middle regions but remained markedly lowered in preterm vs. term animals (less than 30%, *right*, Fig. 6, *A* and *B*). At 26 days, ENT treatment for 5 days after birth increased

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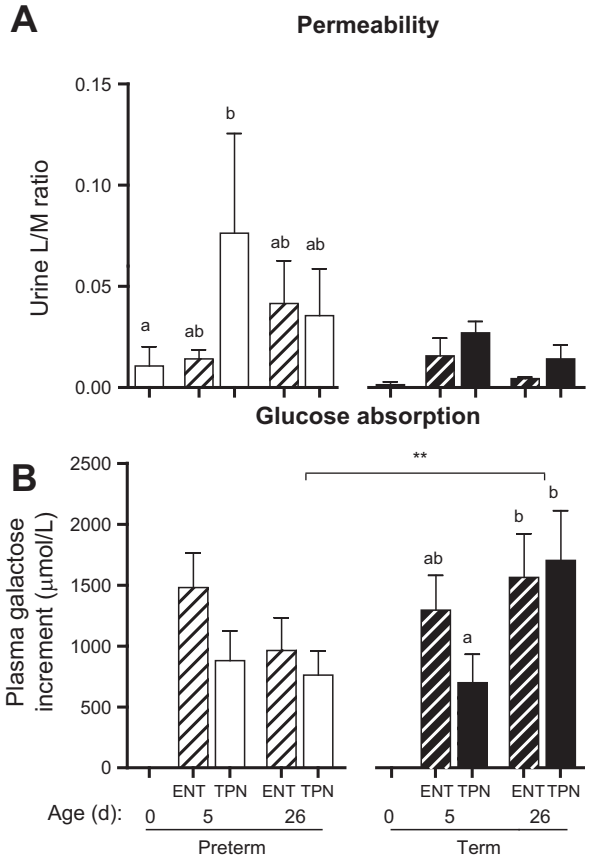

Fig. 5. Intestinal permeability [*A*, urinary lactulose/mannitol (L/M) ratio] and hexose absorptive capacity (*B*, galactose increment after a galactose bolus) in 0-, 5-, and 26-day-old preterm or term pigs fed total parenteral nutrition (TPN) or supplemented with enteral nutrition (ENT) for the first 5 days (means ± SE). Columns within the preterm or term groups not sharing the same letter are significantly different ($P < 0.05$). **Significant difference ($P < 0.01$) between preterm and term piglets for 26-day-old pigs, analyzed across diets (ENT, TPN).

the sucrase activity in the proximal region for term pigs and in the middle intestine for preterm pigs (both $P < 0.05$).

In contrast to sucrase and maltase activities, the activity of lactase was high at birth, but more in term vs. preterm pigs, and especially in the proximal and middle regions (Fig. 6*C*, *left*). Lactase activity decreased markedly in the postnatal period of term pigs but remained stable in preterm pigs (Fig. 6*C*, *right*, 26 days). The ENT treatment lowered the proximal lactase activity for both preterm and term pigs on *day 5* ($P < 0.01$), but this effect disappeared by *day 26*.

Activity of all three peptidase enzymes increased with postnatal age ($P < 0.001$), especially after *day 5*, and was most pronounced in the middle and distal intestine (Fig. 6, *D–F*; ~100% increases from 0–5 to 26 days of age). The activity of ApN on *day 5* was increased by ENT vs. TPN treatment in these intestinal regions ($P < 0.01$), with no differences between preterm and term pigs. On *day 26*, the highest ApN activity was found in ENT preterm pigs and the lowest values in term pigs fed only TPN for the first 5 days ($P < 0.05$, Fig. 6*D*, *right*). Activity of ApA (Fig. 6*E*) was lowered in preterm vs. term pigs for all three postnatal age groups ($P < 0.01$), mostly for newborn pigs (−50%, $P < 0.01$). At 5 days, but not at 26 days, the ENT treatment increased the ApA activity ($P <$

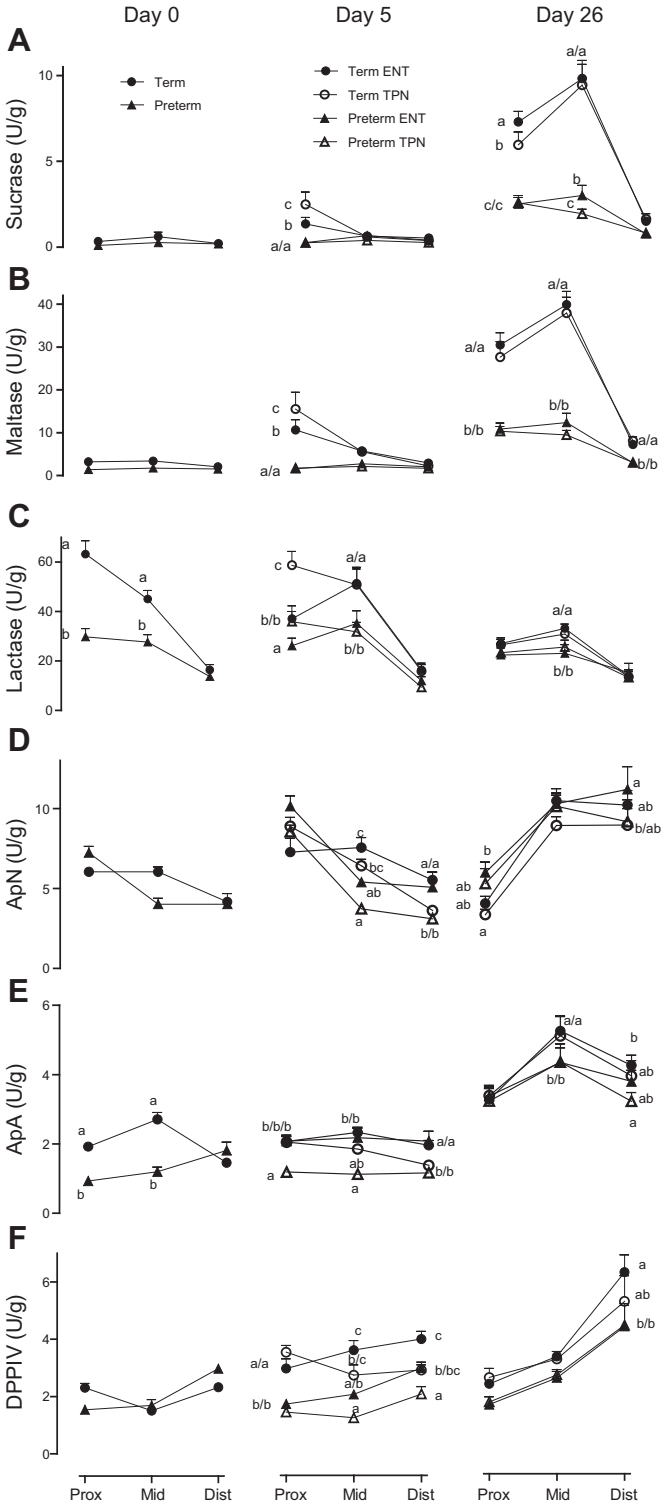

Fig. 6. Activity of sucrase (*A*), maltase (*B*), lactase (*C*), aminopeptidase N (ApN, *D*), aminopeptidase A (ApA, *E*) and dipeptidylpeptidase-4 (DPPIV, *F*) in 0-, 5-, and 26-day-old preterm or term pigs fed total parenteral nutrition (TPN) or supplemented with enteral nutrition (ENT) for the first 5 days (means ± SE). In the figure, mean values for each intestinal region [proximal, middle, distal (dist)] and age after birth (0, 5, 26 days) are different when they do not share the same superscript letter (*a*, *b*, *c*; days, $P < 0.05$).

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0.01), and most for the preterm pigs. Activity of DPP4 (Fig. 6*F*) was significantly lowered in preterm vs. term pigs at 5 and 26 days. At 5 days, ENT increased the DPP4 activity for both preterm and term pigs, especially in the distal region ($P < 0.05$). Across all the enzymes at 26 days, ENT treatment for the first 5 days was associated with similar or higher mean activity of disaccharidases in the proximal and middle intestine (Fig. 6, *A–C*) and similar or higher activity of peptidases in the middle and distal intestine (Fig. 6, *D* and *E*).

DISCUSSION

Several decades of observational studies in preterm infants have provided valuable information about the functional adaptation of many organs (e.g., lungs, liver, gut, and brain) to preterm birth. Regardless, studies in preterm infants do not provide any knowledge about the timing, extent, and mechanisms of postnatal organ adaptation, and any direct comparison to adaptation in term infants is usually not possible. It is important to understand the consequences of preterm birth and how best to alleviate the short- and long-term deficits in organ function in preterm neonates. The preterm pig is the only animal model of preterm infants that not only shows the common respiratory and metabolic deficits of preterm birth but also spontaneously develops many of the immediate GI problems of prematurity, e.g., nutrient maldigestion, dysmotility, and high sensitivity to NEC (43). Here, we show how intestinal structure and function develop beyond the immediate neonatal period in preterm pigs and how early introduction of enteral feeding may influence this development. We show that the intestinal morphology of the immature intestine adapts surprisingly well and becomes similar to that in term pigs within 4 wk after birth. In contrast, digestive functions such as sucrase and maltase activities, barrier function, and hexose absorptive capacity remain compromised at this age. Early and gradual introduction of enteral milk (ENT treatment) benefited many of the structural and functional indexes within the first week, especially in preterm pigs, but most effects of ENT disappeared by 4 wk. We conclude that the preterm intestine adapts rapidly postnatally in pigs, partly facilitated by early enteral food introduction, but still, some developmentally dependent digestive functions remain immature until at least 4 wk. It is not possible to say exactly what this postnatal age in preterm pigs corresponds to in preterm infants. On the other hand, preterm pigs are likely to be more physiologically mature at 4 wk than preterm infants, also regarding the GI tract, as pigs would normally enter into the weaning transition period at this time.

Together with lowered body weight at birth, preterm pigs also showed lowered intestine-to-body weight ratio during the first week after birth. During the following 3 wk, weight gain was lowered in preterm pigs, despite identical rearing and feeding conditions, probably in part explained by lowered nutrient digestive function, as indicated by more days with diarrhea. Although there was a persistent reduction in villus heights in preterm pigs, there was no overall reduction in intestinal mass beyond the first week, and our detailed stereological measurements along the entire small intestine confirmed that total intestinal mucosa volume was similarly responsive in preterm and term pigs to enteral food introduction and advancing postnatal age. In fact, the postnatal increase in relative intestinal mass at 4 wk was greatest in preterm pigs (+200% from birth) and this impressive trophic response may depend not only on postnatal age and enteral food stimulation, but also on the specific effect of enteroendocrine signals such as GLP-2. The GLP-2 release in preterm pigs was similar to, or even higher than that in term pigs (26 days), despite that L cell density per mucosa volume remained constant from birth to 4 wk in the preterm pigs and between ENT and TPN fed animals. Postnatally, the number of L cells closely followed the increase in mucosal mass, despite that other cell markers, such as the high density of vacuolated enterocytes, documented that the intestine was indeed immature within the first week after preterm birth. With increasing age, higher crypt depths were observed in both preterm and term animals. Although proliferation and apoptosis were not quantified in this study, this could potentially reflect increased enterocyte turnover with advancing postnatal age (10).

Despite that mass and volume of the mucosa grew similarly in preterm and term pigs from birth to 4 wk, our study showed that several aspects of mucosal function failed to adapt and showed more persistent developmental delay. Consistent with indications from preterm infants (46), intestinal lactose digestive capacity and the absorption of glucose and galactose by the sodium-coupled glucose transporter 1 (SGLT-1) were lowered in 26-day-old preterm pigs, although lactase activity (by the lactase-phloridzin hydrolase enzyme) was most reduced at birth (to ~50% of values in term pigs). When sucrase and maltase enzymatic activities matured after birth, values in 26-day-old preterm pigs reached only ~30% of those in corresponding term pigs, and values were similar to those in 5-day-old term pigs. This marked developmental delay at 26 days was more than expected from the 12-day difference in gestational age at birth and was the most pronounced delay in intestinal function that we observed in preterm pigs. Although digestion of sucrose- and maltose-containing supplementary foods is of limited importance for preterm pigs and infants at this age, the lacking postnatal adaptation is important, since these enzymes are highly developmentally dependent and they are often used as key intestinal maturation markers across many species (39). Our results show that the developmental expression of these enzymes is relatively independent of environmental factors, such as diet (which was the same for preterm and term pigs), and that intrinsic mechanisms related to ontogenetic age and genetic control could be more important. We recently documented that both preterm birth and enteral food introduction induce epigenetic effects on some immune-related genes in the immature pig intestine (19, 53). It will be important to know whether preterm birth also induces epigenetic modifications to the sucrase-isomaltase and maltase-glucoamylase genes because this may help to explain why their corresponding enzyme activities were affected more long term whereas other gene functions tended to adapt more rapidly after preterm birth.

Enteral feeding promotes intestinal maturation and growth after birth (39), but specifically for preterm neonates it is important to introduce enteral feeds gradually to minimize the risk of NEC (12, 52). We therefore used an early and slow clinically relevant feeding regimen for preterm pigs, comparing ENT with TPN pigs. We used bovine colostrum because this enteral diet induces intestinal maturation and NEC protection in preterm pigs (39) and has recently been tested as the

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first enteral diet for preterm infants (28). The intestinal trophic effects of the ENT treatment for the first 5 days generally disappeared by 26 days in both preterm and term pigs. Nevertheless, several lines of evidence suggest that early and slow introduction of trophic milk diets may be beneficial for preterm infants more long term (15). In our study, the ENT diet did not affect villus heights and mucosa volumes at *day 5* but it increased relative intestinal weight, peptidase activities, and the endocrine GLP-2 and GIP release, and it tended to improve mucosal barrier and glucose absorptive capacity. Interestingly, these effects were as high, or higher, in preterm vs. term pigs, and this may help adaptation of the immature intestine to later full enteral feeding. We did not in this study make a detailed record of feeding intolerance and intestinal dysmotility, but, like in preterm infants, gastric residuals and vomiting were occasionally observed in preterm pigs during the first 3 wk after birth. During this critical period, intestinal permeability was also higher and our results indicate that even small amounts of enteral diet help to mature the intestinal barrier. In preterm infants, intestinal permeability is high during the first weeks after birth followed by a decrease, probably in part mediated by increasing milk intake (46) and GLP-2 release (35). Although such trophic effects of the first enteral feeds help to increase mucosal growth and mature some intestinal functions, others may remain compromised for a longer period, as we demonstrated in this study on piglets for sucrase and maltase activities in the proximal and middle intestine.

Exogenous GLP-2 administration stimulates intestinal growth and disaccharidase activities in TPN-fed preterm pigs with or without intestinal resection (37, 49, 51). Preterm piglets normally show increased basal circulating levels of GLP-2, compared with pigs born at term, indicating a role for GLP-2 in the maturational process around birth (38). In the present study, preterm pigs displayed low sucrase and maltase activities despite relatively high postnatal GLP-2 levels, and the ENT treatment tended to affect GIP more than GLP-2 release within the first 5 days whereas gastrin was little affected. Probably the small volumes of enteral milk exposed mainly the proximal part of the intestine, and thereby the GIP-producing K cells, whereas the gastrin-producing stomach G cells and GLP-2 secreting L cells in the distal small intestine were less affected. Such enteroendocrine cells may be present as a fixed proportion of the total number of cells in the mucosa, regardless of time of birth and increasing mucosal mass during the postnatal period. Hence, we observed that the L cell density was constant from birth to 26 days in preterm pigs and we did therefore not to investigate this parameter in the remaining groups. The enteroendocrine cells have a relatively slow cell turnover [around 20 days (11)], making their total number less sensitive to environmental stimuli. Thus, from an enteroendocrine perspective, preterm pigs appeared relatively mature already at birth.

As part of perinatal maturation, the small intestine undergoes changes in its regional distribution of specific digestive functions (42). Consistent with this, the proximal-to-distal decrease in villus height and lactase activities within the first week, and of sucrase and maltase activities at 4 wk, were much less pronounced in preterm vs. term pigs. Relative to disaccharidases, peptidase activities showed a less distinct age-related postnatal maturation although they were more sensitive to enteral food stimulation. Accordingly, in both preterm and term pigs, peptidase activities were found mainly in the middle and distal intestine at 26 days, and ENT nutrition mainly stimulated peptidase activities in these regions on *day 5*. Previous studies in pre- and postnatal pigs confirm that the intestine matures in a proximal to distal direction and that the enzyme activities are most sensitive to age and hormonal stimuli (e.g., cortisol, GLP-2) proximally, whereas enteral milk diets affect enzyme activities (e.g., peptidases) most in the distal parts (39, 42). We conclude that postnatal adaptation of intestinal structure and function differs among different intestinal regions and takes place in a complex interplay among ontogenetic age, gestational age at birth, and diet. Although intestinal morphology and structure adapt rapidly within the first 4 wk in preterm pigs, selected gut functions remain compromised and below the levels in corresponding term pigs. Even small amounts of enteral milk feeds improve intestinal morphology and function within the first week after birth but have limited effects 3–4 wk later.

Perspectives

It is important to know whether the apparent deficits observed following preterm birth are temporary or more long lasting, and whether they are affected by the early enteral diet. A lasting functional deficit of the intestine may contribute to the commonly observed extrauterine growth restriction in preterm infants (14). Our study suggests that the immature intestinal morphology in newborn preterm pigs is largely normalized within the first 4 wk after birth, while several aspects of intestinal function remain immature. Caution is required when extrapolating intestinal prematurity in pigs at 90% gestation to the highly variable group of preterm infants born at 60–90% gestation, especially since the development of each organ system is temporally different between humans and pigs. Although pigs delivered at 90% gestation show severe GI, respiratory, and metabolic immaturities, some other functions (e.g., neurodevelopment) appear more mature in preterm pigs than in most preterm infants (14). Regardless, it is important to identify the GI functions that show lasting deficits following preterm birth in each species because this may help to design optimal supportive and preventive care.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.F.H., T.T., A.D.A., J.J.H., B.H., L.H., and L.L. performed experiments; C.F.H., T.T., and L.L. analyzed data; C.F.H., T.T., L.L., and P.T.S. interpreted results of experiments; C.F.H. prepared figures; C.F.H. and P.T.S. drafted manuscript; C.F.H., T.T., A.D.A., J.J.H., B.H., L.L., J.J., and P.T.S. edited and revised manuscript; C.F.H., T.T., A.D.A., and P.T.S. approved final version of manuscript; T.T. and P.T.S. conception and design of research.

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