

Original Article

Malnutrition induces gut atrophy and increases hepatic fat infiltration: studies in a pig model of childhood malnutrition

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Abstract: Childhood malnutrition is a problem in developing countries, and pathological changes in digestive organs such as the intestine and liver are poorly understood. An animal model to study the progression of severe acute malnutrition could elucidate pathological changes in the intestine and liver. We sought to characterize growth and clinical changes during malnutrition related to structural and functional indices in the intestine and liver. Newly weaned piglets were given *ad libitum* access to a maize flour diet (MAIZE, $n=9$) or a nutritionally optimized reference diet (REFERENCE, $n=12$) for 7 weeks. Growth, hematology and clinical biochemistry were recorded weekly. After 7 weeks, the MAIZE pigs had lower body weights than the REF pigs (8.3 kg vs. 32.4 kg, $P<0.001$), indicating severe stunting and moderate to severe wasting. This was paralleled by lower values for hematocrit, hemoglobin and mean cell volume in MAIZE vs. REFERENCE ($P<0.01$), indicating anemia. Although the observed temporal changes in MAIZE were associated with atrophy of the small intestinal mucosa ($P<0.001$), digestive enzyme activity was only marginally reduced. Serum alanine aminotransferase, bilirubin and albumin were increased in the MAIZE pigs ($P<0.001$), and the liver had a vacuolated appearance and tendency toward increased triglyceride content ($P=0.054$). We conclude that liver and intestinal indices are compromised during malnutrition and are associated with temporal changes in growth and hematological and biochemical endpoints. The pig model is relevant for malnourished infants and can act as a valuable tool for understanding the pathophysiology of malnutrition.

Keywords: Malnutrition, pig, gut, liver, animal model

Introduction

Malnutrition in developing countries affects 165 million children under 5 years of age [1]. These children exhibit stunted growth, and although the prevalence of stunting is decreasing, the actual number of stunted children in some parts of Africa, for example, is increasing due to population increase [2]. The most complicated form of malnutrition is severe acute malnutrition (SAM), which affects 20 million children under the age of 5 years and is estimated to result in approximately 1 million deaths every year [3].

SAM occurs in one of two forms, marasmus or kwashiorkor. Children with kwashiorkor exhibit

muscle wasting, hair and skin changes, apathy and generalized edema, whereas marasmic children are characterized mainly by severe muscle wasting. The poor understanding of the fundamental biology of severe acute malnutrition in children may stem from the facts that most studies are cross-sectional and that prospective studies are generally not conducted for ethical reasons. In addition, as this particular patient group is extremely vulnerable, rapid advancement of understanding is complicated. Although the classical work by Keys *et al.* [4] remains some of the best information available on temporal changes in adult volunteers subjected to prolonged malnutrition, it may not adequately reflect temporal pathological changes in children.

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Table 1. Diet compositions

| | | REFERENCE | MAIZE |
|-------------------------|-------|-------------|-------------|
| Energy | MJ/kg | 8.71 | 9.37 |
| Protein | g/kg | 219 | 90 |
| Carbohydrate | g/kg | 526 | 715 |
| Fat | g/kg | 61.2 | 43 |
| Starch | g/kg | 366 | 607 |
| Sugar | g/kg | 42.9 | 17.6 |
| Lactose | g/kg | 23.4 | <i>n.d.</i> |
| Vitamin A | IE/kg | 12 | <i>n.d.</i> |
| Vitamin D ₃ | IE/kg | 1 | <i>n.d.</i> |
| Vitamin E | mg/kg | 220 | <i>n.d.</i> |
| Alpha-tocopherol | mg/kg | 200 | <i>n.d.</i> |
| Biotin | mg/kg | 0.24 | <i>n.d.</i> |
| Pantothenic acid | mg/kg | 28 | <i>n.d.</i> |
| Vitamin B ₁ | mg/kg | 2.4 | <i>n.d.</i> |
| Vitamin B ₂ | mg/kg | 10 | <i>n.d.</i> |
| Vitamin B ₆ | mg/kg | 7.2 | <i>n.d.</i> |
| Vitamin B ₁₂ | mg/kg | 0.02 | <i>n.d.</i> |
| Niacin | mg/kg | 24 | <i>n.d.</i> |
| Vitamin K ₃ | mg/kg | 4.4 | <i>n.d.</i> |
| Folic acid | mg/kg | <i>n.d.</i> | <i>n.d.</i> |
| Choline chloride | mg/kg | <i>n.d.</i> | <i>n.d.</i> |
| Calcium | g/kg | 10.1 | 0.43 |
| Phosphorus | g/kg | 7.66 | 3.3 |
| Sodium | g/kg | 1.88 | 0.1 |
| Magnesium | g/kg | 1.63 | 1.15 |
| Potassium | g/kg | 10 | 3.52 |
| Chloride | g/kg | 1.59 | <i>n.d.</i> |
| Iron | mg/kg | 293 | 29.6 |
| Manganese | mg/kg | 66.2 | <i>n.d.</i> |
| Copper | mg/kg | 166 | <i>n.d.</i> |
| Zinc | mg/kg | 155 | <i>n.d.</i> |
| Iodine | mg/kg | 0.3 | <i>n.d.</i> |
| Selenium | mg/kg | 0.3 | <i>n.d.</i> |

n.d. - not determined.

While in malnourished adults, there is evidence of changes in body weight, hematology and clinical biochemistry, very little is known of changes in organ function and morphology. Therefore, to advance our understanding, it is essential to combine growth markers of malnutrition with estimations of organ function. Understanding organ function and morphology following prolonged malnutrition is a prerequisite to identifying optimal refeeding strategies that do not further compromise the clinical situation. This may be especially important to gut and liver function, as they are the keys to prop-

er digestion and metabolism. Several animal models, including rats [5-8], dogs [9], monkeys [10-12] and pigs [13-16], have been used to study malnutrition. However, to the best of our knowledge, none have shown how temporal changes during malnutrition are associated with intestinal and hepatic function and morphology.

We sought to describe the course of malnutrition from a normal physiologic state to a state of SAM, including growth measurements and functional and structural changes in the intestine and liver. Changes in the function and structure of the small intestine and gut microbiota in particular have recently been suggested as causal factors in the development of kwashiorkor [17]. A shift in the gut microbiota induced by malnutrition may favor endotoxin serotypes more likely to cause edema and liver damage [18, 19].

Materials and methods

Animals and sampling

All animal experimental procedures were approved by the Danish Animal Experiments Inspectorate. Twenty-four female crossbred pigs (Durox x Danish Landrace x Yorkshire) (mean body weight 7.0 ± 0.26 kg) were weaned at 4 weeks of age and given *ad libitum* access to a diet formulated to meet or exceed the nutrient requirements for pigs of this genotype for 5 days to allow acclimatization to their new environment. After 5 days, 12 of the pigs were switched from the formulated diet to a pure maize diet (MAIZE, $n=12$), while the other 12 pigs remained on the formulated diet (REFERENCE, $n=12$). Details on nutrient composition of the diets are presented in **Table 1**. Both groups were given *ad libitum* access to their diets and water for 7 weeks. As some aggression was observed in the MAIZE group, based on experience, a supplement to the diet of 0.2% MgO and a salt lick stone were provided. Growth measurements, including weight, thoracic circumference, crown-rump length (CRL) and length of the metatarsus, were collected weekly. CRL was used to relate linear growth to body weight to determine the degrees of wasting and stunting. To assess the degree of wasting, a reference curve for the relationship between body weight and CRL was established based on the REFERENCE pigs: *weight in*

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$kg \text{ (theoretical)} = 43.38 \times CRL \text{ length (m)}^{2.401}$. The actual body weight of the MAIZE pigs relative to a theoretical weight at a given CRL was used as an estimate of the degree of wasting (weight-for-height, W/H): $100\% \times \text{weight (observed)} / \text{weight (theoretical)}$. Based on this estimate, the degree of wasting was classified as severe (<70%), moderate (70-80%) or mild-normal (>80%) [20]. The degree of stunting was classified as severe (<85%), moderate (85-89%) or mild-normal (90-94%), based on the mean length-for-age of the REFERENCE pigs [21]. Finally, weight-for-age, as an indicator of undernourishment, was determined as a percentage of the mean weight-for-age in the REFERENCE group and was classified as severe (<60%), moderate (60-80%) or mild-normal (>80%) undernourishment [22].

Blood samples were obtained by puncture of the jugular vein at the same time points as growth measurements were collected. EDTA-stabilized blood samples were analyzed on an Advia 120 Hematology System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) to determine hemoglobin, hematocrit and mean cell volume. Serum samples were left to clot at room temperature prior to centrifugation (2500 G, 4°C, 10 min), and serum was isolated and analyzed on an Advia 1800 Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) to determine albumin, alanine aminotransferase (ALAT), aspartate transaminase (ASAT) and bilirubin levels.

Body composition was determined during week 7 using dual-energy X-ray absorptiometry (DXA) (QDR Explorer™, Hologic, Bedford, MA, USA) on pigs anesthetized with a combination of zolazepam/tiletamine (Zoletil 50, Virbac, Kolding, Denmark), xylazine (Narcoxy 20 mg/mL, MSD Animal Health, Ballerup Denmark), ketamine (Ketaminol 100 mg/mL, MSD Animal Health, Ballerup Denmark) and butorphanol (Torbugesic 10 mg/mL, ScanVet, Fredensborg, Denmark). The pigs were placed in a prone position during the scanning process, and data on bone mineral density and fat and lean mass percentages were determined using software provided with the DXA scanner.

At the end of week 7, the pigs were anesthetized with the Zoletil mixture and were then euthanized with an intracardial injection of sodium pentobarbital (60 mg/kg). Intestinal

tissue samples from the proximal jejunum (100 cm from the pylorus) and distal jejunum (100 cm from the ileocecal junction) were rapidly collected. One set of samples was snap-frozen in liquid nitrogen and stored at -80°C, and another set was fixed in 4% neutral buffered paraformaldehyde. Liver samples were also collected and stored in an identical fashion. Finally, the weights of the lungs, kidneys, stomach, colon, liver, small intestine, spleen and heart were recorded.

Histology

After fixation and dehydration, the intestinal samples were embedded in paraffin, and 5- μ m sections were cut and stained with hematoxylin and eosin. Using an Olympus BX 40 microscope, an Olympus Altra 20 camera and the analysis getIT 5.0 software program (Olympus Soft Imaging Solutions GmbH, Münster, Germany), representative pictures of the intestine were digitalized. The lengths of 10 well-defined villi and crypts were measured using the ImageJ version 1.44 software program. Formaldehyde-fixed liver tissue was embedded in paraffin, cut in 5- μ m sections, mounted on glass slides and stained with hematoxylin and eosin. To evaluate fat infiltration of the liver, the cryo-preserved tissue was stained with Sudan red.

Digestive enzyme analysis

Samples from the proximal and distal small intestine were homogenized in ice-cold 1% Triton X-100 and assayed for disaccharidase activity (lactase, maltase and sucrase) and peptidase activity (aminopeptidase A (ApA), aminopeptidase N (ApN) and dipeptidylpeptidase IV (DPP IV)). The tissue homogenate was centrifuged (5000 G, 4°C, 2 min) and placed on ice. A Trisbasebuffer, 50 mmol/L, pH 7.3, was pipetted into a 96-well microtiterplate together with the sample to obtain the correct dilution. After mixing, the substrate was added as follows: a 250- μ L 2 mM ApNA solution (14.7 mg ApNA, 30 ml Trisbasebuffer) to measure amino-peptidase N, a 250- μ L 2 mM Glu-pNA solution (17.1 mg Glu-pNA, 30 ml Trisbase-CaCl₂ buffer) to measure amino-peptidase A, and a 250- μ L 3 mM GpNA solution (41.8 mg GpNA, 30 ml Trisbasebuffer) to measure dipeptidylpeptidase IV. Microtiterplates were run in an ELISA reader at a wavelength of 405 nm for 27.50 min for

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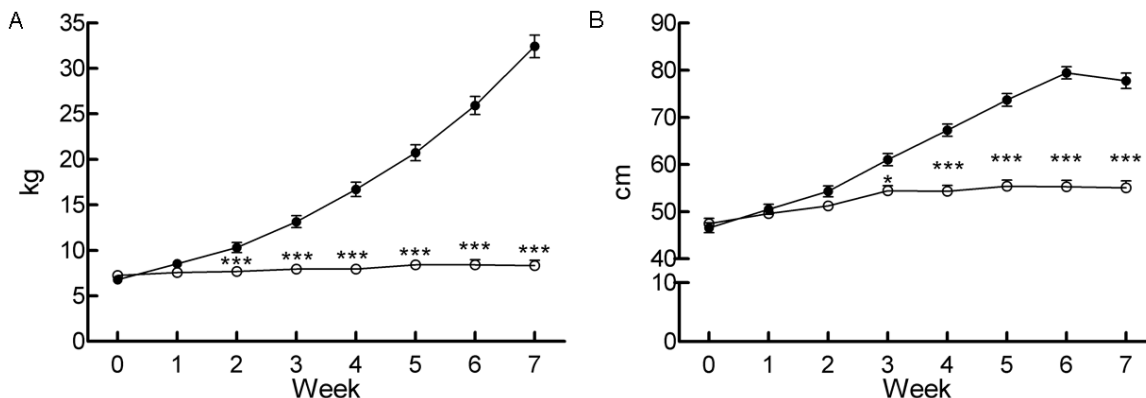


Figure 1. A: Development of body weight during the 7-week period. MAIZE (○); REFERENCE (●). ***Mean values were significantly different from the reference group ($P < 0.001$). B: Development of crown-rump-length during the 7-week period. MAIZE (○); REFERENCE (●). *Mean values were significantly different from the reference group ($P < 0.05$). ***Mean values were significantly different from the reference group ($P < 0.001$).

Table 2. Numbers of MAIZE pigs classified as mild-normal, moderately or severely wasted and stunted¹

| | MAIZE (n=9) |
|-------------|-------------|
| Wasted | |
| mild-normal | 6 |
| moderate | 2 |
| severe | 1 |
| Stunted | |
| mild-normal | 0 |
| moderate | 0 |
| severe | 9 |

¹Wasting defined by weight-for height (W/H) and stunting defined by length-for-age (L/A).

aminopeptidase A and N and 24.42 min for dipeptidylpeptidase IV. For disaccharides, the following substrates were mixed with the sample: lactose (0.12 mol/L), sucrose (0.01 mol/L) and maltose (0.0112 mol/L) to the correct dilutions. The microtiterplate was incubated for 30 min in the ELISA reader. After incubating, a 250- μ L PGO color solution was added, and the plate was run for 30 min at 37°C at 450 nm.

Liver triglyceride concentration

A liver sample weighing approximately 25 mg was placed in a tube with 0.33 mL chloroform and 0.67 mL methanol and homogenized using a TissueLyser (Qiagen Retsch, Haan, Germany). The supernatant was collected after centrifugation (2000 G, 20°C, 15 min) and was mixed with 1 mL of chloroform and 0.5 mL of salt water (0.04% CaCl_2 , 0.034% MgCl_2 and 0.58 NaCl) and centrifuged (2000 G, 20°C, 15 min). The upper phase was removed with a pipette,

and 0.25 mL of methanol was added to the lower phase. The lipid extract was dried under constant N_2 airflow at 50°C for 30 min using a ZipVap Evaporator (Glas-Col LLC, Terre Haute, IN) and saponified in KOH-ethanol (1.2 mL of 0.5-M stock) at 60°C for 30 min. The hydrolyzed extract was neutralized with MgSO_4 (2.8 mL of 0.15-M stock) and used for analysis of triglyceride. The concentration of triglyceride was measured according to standard procedures (Siemens Diagnostics, Tarrytown, NY) [23].

Statistics

The data are presented as means \pm standard errors. Organ weights relative to body weight, digestive enzyme activity, body composition, liver triglyceride content and histomorphometry were tested using the xtmixed procedure. For repeated measurements, including weekly recording of blood values and growth measurements, the xtmixed procedure was used, including the pig as a random variable. Stata 12 was used for all statistical analyses (StataCorp LP, College Station, Texas, USA). Probability values less than 0.05 were considered significant.

Results

Growth measurements

During the study period, 2 MAIZE pigs were prematurely euthanized due to ear biting by littermates, and 1 MAIZE pig died for unknown reasons. Relative to REFERENCE, the remaining MAIZE pigs displayed clinical signs of malnutri-

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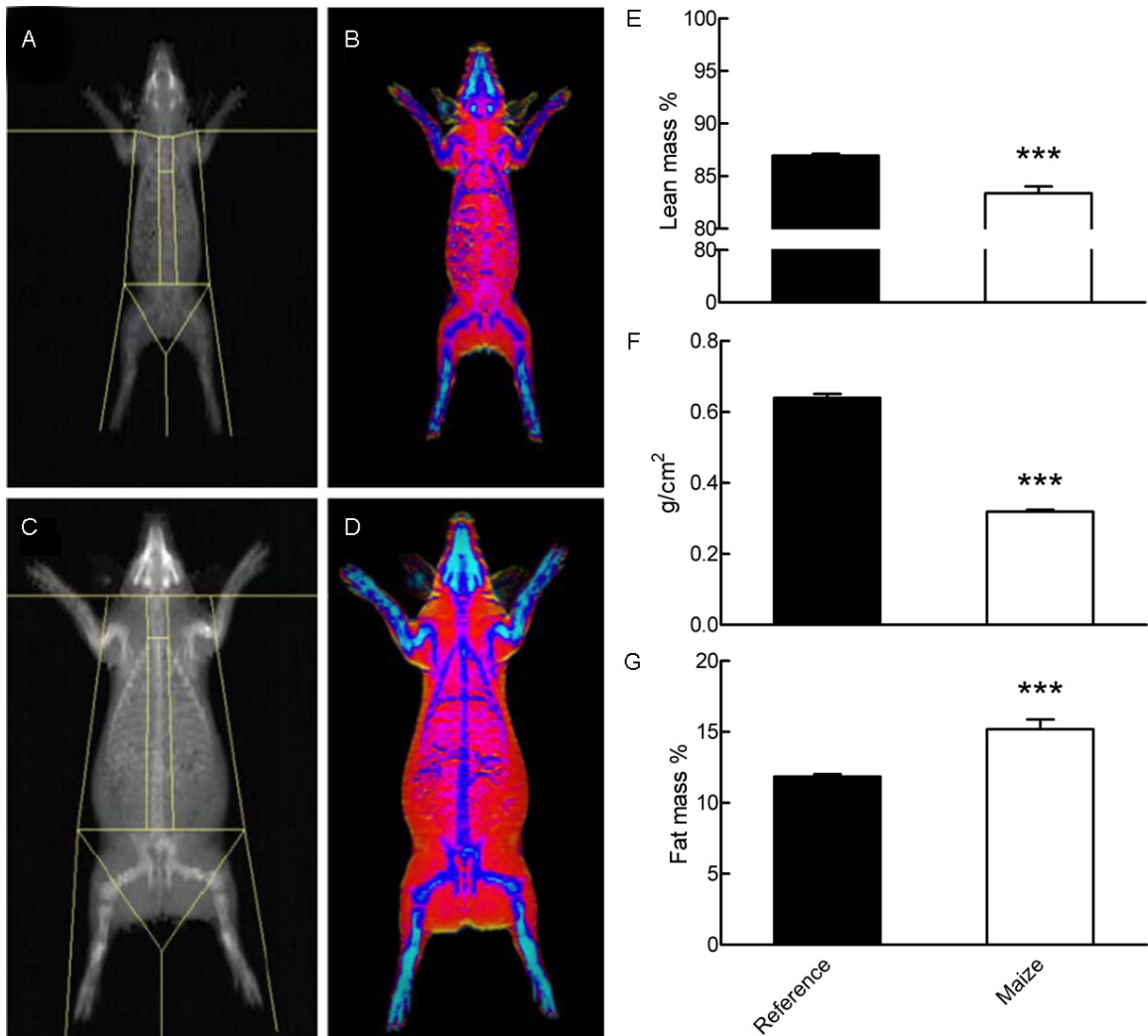


Figure 2. DXA scanning of a MAIZE pig (A and B) and a REFERENCE pig (C and D) after 7 weeks. E: Lean mass percentage. F: Bone mineral density, expressed as g/cm². G: Fat mass percentage. ***Mean values were significantly different from the reference group ($P < 0.001$).

tion, including slow growth, anemia and long coarse hair coating. The dietary intake increased in REFERENCE pigs from 330 g/d in week 1 to 770 g/d in week 7, whereas the dietary intake decreased in MAIZE pigs from 250 g/d to 170 g/d. As the data were calculated from recordings of feed consumption on a pen basis, variation and significant differences between means could not be estimated. A marked difference in body weight accretion between MAIZE and REFERENCE was evident from week 2 onward. Whereas the REFERENCE pigs gained an average of 25 kg (from 6.79 ± 0.38 to 32.41 ± 1.23 kg), the MAIZE pigs gained only 1 kg over the 7-week study period (from 7.25 ± 0.36 kg to 8.31 ± 0.60 kg) (Figure 1A), indicating that they were severely underweight.

A similar pattern was observed for CRL (Figure 1B), thoracic circumference and length of metatarsus, with the MAIZE pigs showing decreases from week 3 onward. From these growth measurements, the degree of stunting was categorized as severe, as the mean CRL in MAIZE pigs was only 70.8% of the mean CRL in REFERENCE pigs. All of the MAIZE pigs were stunted, as they were all below the cut-off value of 85% of the mean CRL of the REFERENCE pigs after 7 weeks. Finally, degree of wasting assessments of indicated that one-third of the MAIZE pigs were either moderately or severely wasted (Table 2). The marked difference in body weight accretion was also associated with a discrepancy in body composition, as determined by DXA (see Figure 2). Bone mineral den-

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Table 3. Organ weights relative to body weight, g/kg

| | REFERENCE (n=12) | | MAIZE (n=9) | |
|-----------------|------------------|------|-------------|------|
| | Mean | SEM | Mean | SEM |
| Heart | 5.52 | 0.17 | 6.17 | 0.38 |
| Lung | 9.55 | 0.24 | 12.96* | 0.59 |
| Liver | 29.73 | 0.67 | 30.52 | 1.72 |
| Spleen | 5.33 | 0.37 | 4.26* | 0.41 |
| Kidney | 5.15 | 0.15 | 5.50 | 0.25 |
| Stomach | 8.14 | 0.21 | 10.17* | 0.47 |
| Small intestine | 39.91 | 0.74 | 39.51 | 1.39 |
| Colon | 17.61 | 0.89 | 22.21* | 1.68 |

*Mean values within a row differ significantly ($P<0.05$).

sity (0.32 ± 0.01 vs. 0.64 ± 0.01 g/cm², $P<0.001$) and lean body mass percentage (83.37 ± 0.66 vs. $86.95 \pm 0.18\%$, $P<0.001$) were lower in the MAIZE pigs than in the REFERENCE pigs. In contrast, the fat mass percentage was higher in the MAIZE pigs than in the REFERENCE pigs (15.19 ± 0.69 vs. $11.85 \pm 0.17\%$, $P<0.001$). In general, the weights of the abdominal and thoracic organs were proportional to body weight, except the weights of the lung, stomach and colon, which were higher relative to body weight in the MAIZE pigs than in the REFERENCE pigs ($P<0.05$). In contrast, the relative weight of the spleen was lower in the MAIZE pigs ($P<0.05$). Details of the organ weights are given in **Table 3**.

Blood parameters

After 3 weeks, ALAT was markedly higher in the MAIZE pigs ($P<0.001$), indicating hepatocyte or myocyte disruption. Both groups exhibited an initial increase in ALAT at the beginning of the intervention, which may reflect acute adaptation to the weaning process. Clinical biochemistry revealed increases in ASAT (63.33 ± 9.06 vs. 37.33 ± 2.36 U/L, $P<0.001$) in the REFERENCE pigs at week 1 and again from week 5 onward ($P<0.05$). As a further indication of liver function, bilirubin levels were increased in the MAIZE pigs from week 1 to week 7 ($P<0.001$). Bilirubin and ALAT levels were within the normal physiological ranges for both groups. Plasma albumin was markedly lower in the MAIZE pigs than in the REFERENCE pigs from week 1 onward, and the difference increased throughout the study period ($P<0.001$). During the last 3 weeks, the albumin concentration in the MAIZE pigs approached the lower limit for normal albumin levels (22.6

g/L). No edema was observed in the groups.

Hematocrit and hemoglobin in the MAIZE pigs decreased rapidly and were lower than the values observed in the REFERENCE pigs from week 3 onward (23 ± 0.8 vs. $30 \pm 0.7\%$ for hematocrit and 5.01 ± 0.39 vs. 6.06 ± 0.12 mmol/L for hemoglobin in week 7, both $P<0.01$), which was consistent with clinical signs of anemia. The mean cell volume decreased in the MAIZE pigs from week 3 onward (45.56 ± 0.65 vs. 54.53 ± 0.69 fL in week 7, $P<0.05$), indicating iron deficiency anemia.

The reference interval for clinical biochemistry and hematology was taken from tabulated values in the Merck Veterinary Manual, 8th edition, provided by the clinical laboratory.

Tissue morphology and function

The histomorphology of the intestine revealed lower villi numbers in both the proximal and distal parts in the MAIZE pigs than in the REFERENCE pigs ($P<0.001$, **Figure 3**). Lactase and ApA activity in the distal small intestine were decreased in the MAIZE pigs (lactase: 0.84 ± 0.21 tissue vs. 0.99 ± 0.11 U/g tissue, $P<0.05$; ApA: 2.29 ± 0.21 vs. 3.18 ± 0.27 U/g tissue, $P<0.01$). There were no differences between the two groups for other enzymes in either the distal or the proximal part of the small intestine.

Histologic evaluation of hematoxylin/eosin-stained liver tissue revealed vacuolization (**Figure 4**) of hepatocytes. Sudan red staining, which stains triglycerides and lipids, revealed the presence of positively stained droplets (red). The triglyceride content of the liver tended to be higher in the MAIZE pigs than in the REFERENCE pigs (34.38 ± 4.11 vs. 24.45 ± 3.10 $\mu\text{mol/g}$, $P=0.054$); see **Figure 5**. This was consistent with the increased serum bilirubin and ALAT levels.

Discussion

We established a pig model of child malnutrition with clinical signs similar to children with SAM. Using a classification system of growth similar to that used for children, this study showed severe stunting in malnourished pigs, with a CRL of 70.8% relative to the CRL in the

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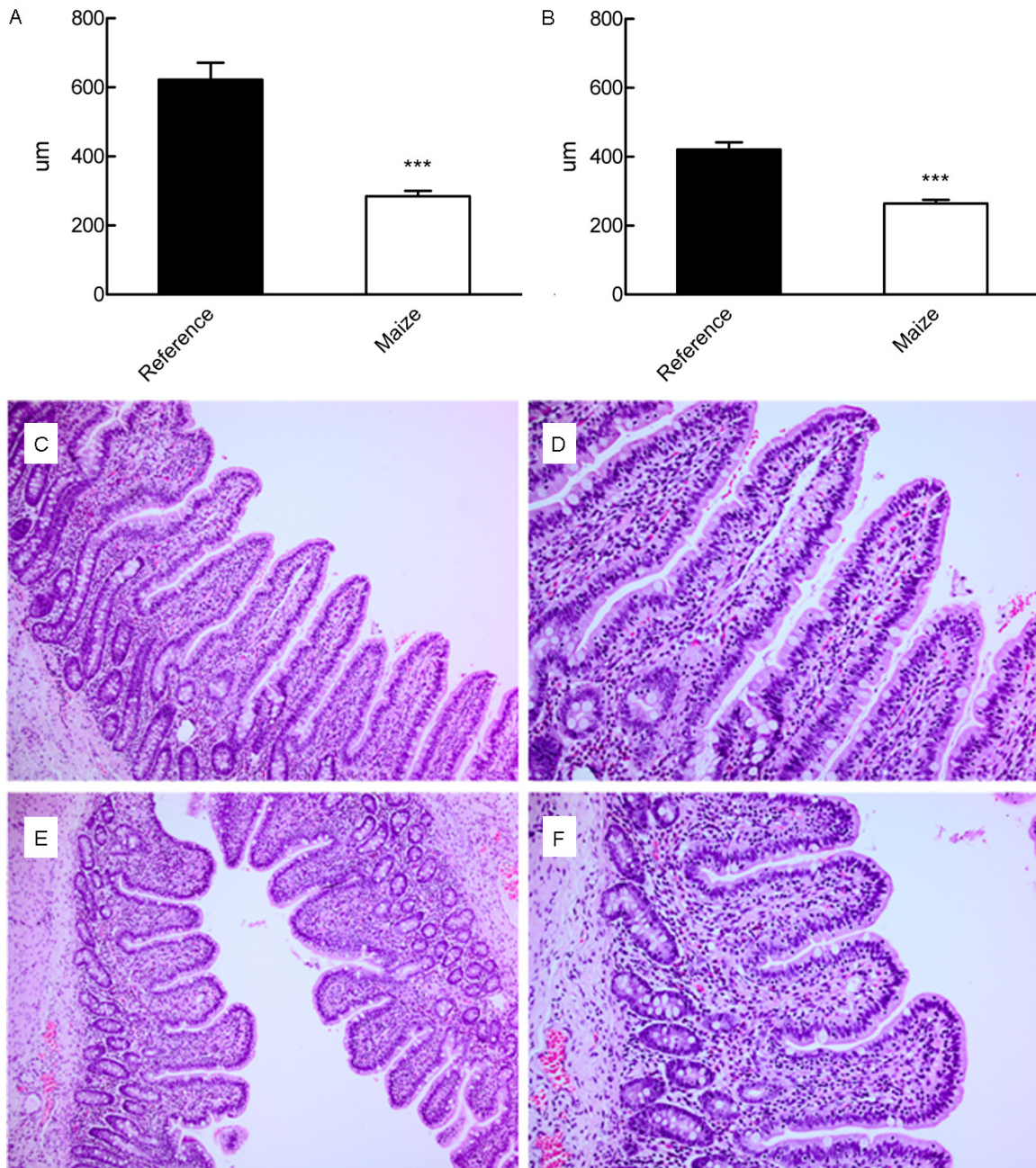


Figure 3. Length of the villi in the proximal (A) and distal (B) parts of the small intestine. C and D: Distal part of the small intestine of a REFERENCE pig at magnifications of 10X and 20X, respectively. E and F: Distal part of the small intestine of a MAIZE pig at magnifications of 10X and 20X, respectively. ***Mean values were significantly different from the reference group ($P < 0.001$).

REFERENCE pigs. As the malnutrition in this particular study affected both body weight accretion and linear growth, the degree of wasting (i.e., body weight relative to CRL) indicated that two-thirds of the MAIZE pigs were normal to mildly wasted, while one-third was moderately to severely wasted, as severe wasting was defined as having a weight-for-height

70% of that of the REFERENCE pigs (**Table 2**). A weight-for-height between 70% and 80% was classified as moderate wasting, and a weight-for-height above 80% was considered normal [24]. Although two-thirds of the MAIZE pigs still had a weight-for-height above 80% after 7 weeks, data from a separate study indicated that the weight-for-height continues to decrease

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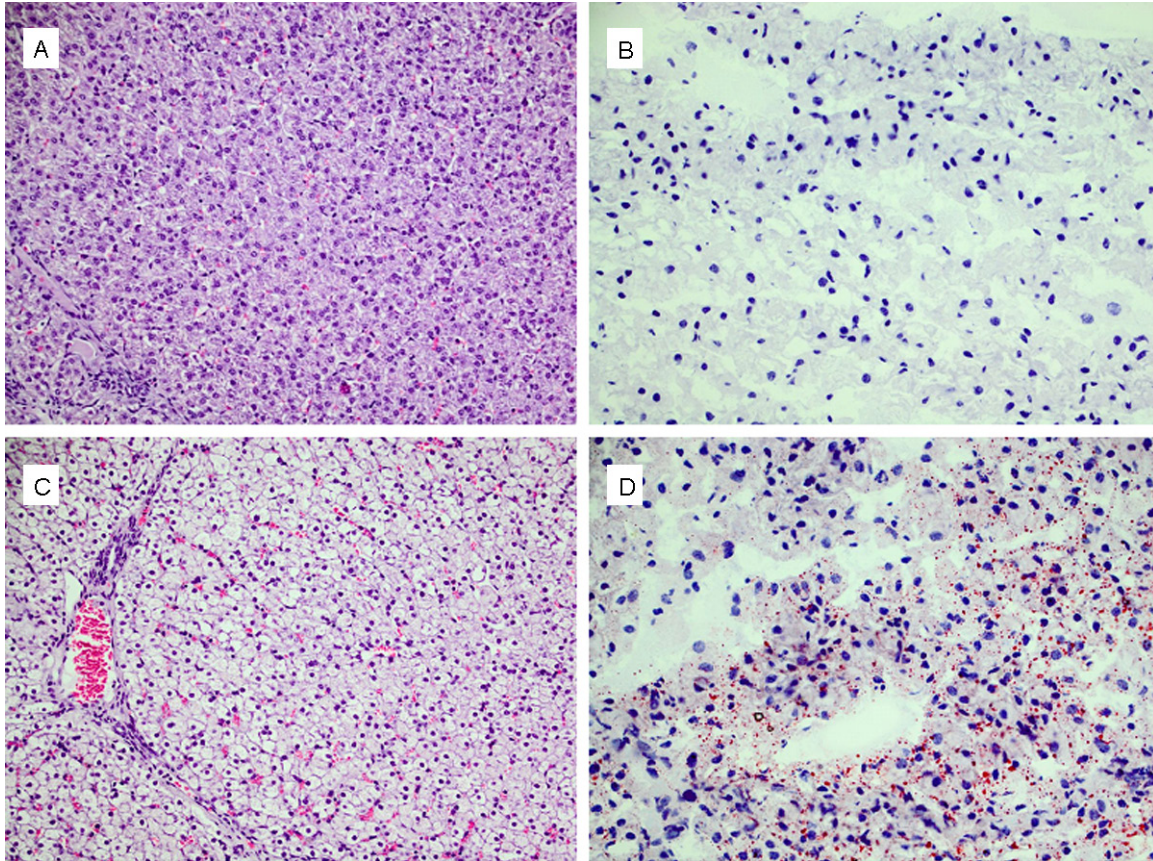


Figure 4. A: Liver from a REFERENCE pig. B: Sudan-red staining of liver from a REFERENCE pig. C: Liver from a MAIZE pig; notice the vacuolated appearance. D: Sudanred staining of liver from a MAIZE pig; notice the presence of positively stained droplets (red) for lipid and triglyceride compared to (B). A and C: Were taken at a magnification of 20X. B and D: Were taken at a magnification of 40X.

if maize feeding is continued up to 10 weeks (Hother *et al.* unpublished results). In that study, all MAIZE pigs became moderately or severely wasted, as the weight-for-height for some was lower than 60%. However, in the present study, the piglets displayed characteristics of severe undernourishment and stunting with various degrees of wasting, classifying the model as moderately acute malnutrition with severe stunting.

In the malnourished group, there were clinical signs of anemia, and the hair coating was typical of animals that are not thriving, with long coarse hairs. The clinical signs of anemia were confirmed by decreased levels of hemoglobin, hematocrit and mean cell volume. Anemia is also commonly observed in children suffering from SAM [25]. The cause of microcytic anemia, as observed in the MAIZE pigs, was most likely iron deficiency, which is consistent with

the low iron content of the diet (**Table 1**). However, anemia in kwashiorkor is usually normocytic and may reflect an anemia due to a lack of not only iron but also B vitamins [26-28]. Collectively, the clinical and hematological indices from the MAIZE pigs were more like children with marasmus than children with kwashiorkor, although some elements of kwashiorkor were present, such as hypoalbuminemia, wasting, stunting, anemia and fatty liver. We did not observe any signs of edema in the MAIZE pigs, which would have been a key feature of kwashiorkor.

Having validated the degree of malnutrition in this pig model via growth, biochemical and hematologic measurements, we were then interested in studying the association with functional and structural pathological changes in the intestine and liver. There was clear mucosal atrophy in both the proximal and distal small

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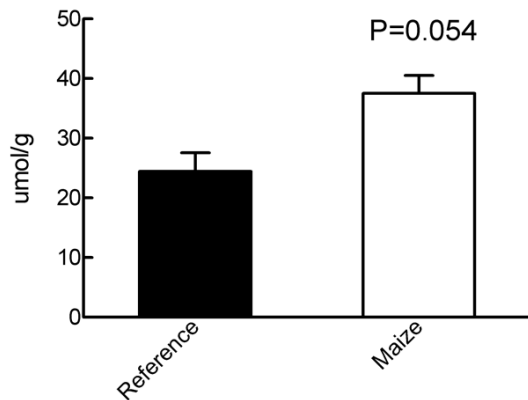


Figure 5. Triglyceride content of the liver.

intestine, with blunt villi, as is observed in malnourished infants with tropical enteropathy. It was somewhat surprising that this clear difference in mucosal morphology was not paralleled by functional endpoints such as specific digestive enzyme activity. In general, no differences were observed in brush border enzyme activity between the MAIZE and REFERENCE pigs, except for decreased lactase and ApA activity in the distal part of the small intestine in the MAIZE group. However, the trend for the MAIZE pigs was to have lower levels of peptidase activity in both the proximal and distal parts of the small intestine. The notion that malnourished pigs may have reduced protein digestive capacity was indirectly supported by *in vivo* measurements of lower protein digestibility of the maize (68.2%) relative to table values (80-88%) (data not shown). Reduced enzyme activity and protein digestive capacity is a well-known phenomenon in anorexic post-weaning piglets and is thought to play an important role in post-weaning diarrhea, as undigested nutrients pass through the small intestine and enter the colon, where they become substrate for fermentation by resident microorganisms. A large amount of fermentative activity in the colon may cause accumulation of small organic acids, including lactic acid, which may exacerbate diarrhea symptoms. To put this observation in perspective, feeding of malnourished children with mucosal atrophy and lower digestive capacity may be a contributing factor to malabsorption and subsequent diarrhea development [29]. However, this may be more pronounced when gut atrophy is accompanied by intestinal infection and inflammation, as is observed in anorexic weanling pigs. For standardization reasons, we chose pigs that were free of specific patho-

gens (SPF) and observed them in a high-hygiene environment to study the isolated effect of malnutrition without any bias introduced by infection. However, malnourished children often have infectious comorbidity, which may exacerbate malnutrition symptoms [30, 31]. In pigs, it has been shown that reducing infectious pressure by treating anorexic weanlings with mucosal atrophy with antibiotics improves digestive enzyme activity [32], which suggests that being malnourished *per se* only marginally decreases specific digestive enzyme activity.

The intestine represents an organ system that has a high need for an adequate supply of dietary nutrients provided as enteral nutrition. This is evident when examining the first-pass metabolism in the intestinal mucosa of several amino acids, which can be up to 80% of enterally supplied amino acids [33-35]. Together with first-pass metabolism, several other factors are important in maintaining optimal gut health, including a stable microbial flora, optimal barrier function and adequate motility. Low food intake, which was observed in the MAIZE group, may result in inadequate enteral stimuli, leading to decreased gastrointestinal motility and stasis of luminal content. Decreased motility has been associated with small intestinal bacterial overgrowth in a number of diseases [36-38] and may also be an important factor in malnutrition. It is of particular interest that small intestinal bacterial overgrowth can increase the production of bacterial lipopolysaccharides and endotoxins, which are associated with pathological changes, including the development of fatty liver [19].

Microscopic evaluation of the hepatic tissue revealed lesions consistent with fatty liver. Hepatocytes had a vacuolated appearance but with no clear distinction or presence of vacuolated cells around the central veins or in the periportal area. Livers from children with kwashiorkor are also infiltrated with fat that has a periportal distribution [39], and in other experiments with animals with SAM, the fat distribution of the liver has been shown to be periportal [40]. The periportal area is where processes related to fatty acid oxidation and oxidative energy metabolism occur [41, 42]. The liver triglyceride contents were 2.8% in the MAIZE pigs versus 2.0% in the REFERENCE pigs. Although the difference is significant, it would not qualify as clinical hepatic steatosis,

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which would require a triglyceride content of at least 5% [43]. The liver enzymes ALAT and ASAT, although not specific to the liver, presented a varied picture. ALAT was increased in the MAIZE pigs, but the concentration was above the normal levels for both the MAIZE and the REFERENCE pigs. ASAT levels were increased in the REFERENCE pigs at some time points, but the levels were within the normal range throughout the intervention for both groups. Bilirubin levels, which are indicative of cholestasis, were also increased in the MAIZE pigs but were still within the normal range. Albumin, which is synthesized by the liver, was markedly decreased in the MAIZE pigs during the intervention; however, this may have been due to the restricted dietary protein intake, which affected *de novo* synthesis of albumin in the liver. After 7 weeks on a maize diet, the plasma concentration of albumin was 2.01 g / 100 ml, while others have reported concentrations as low as 0.32 g / 100 ml [44], 0.7 g / 100 ml [16] and 1.14 g / 100 ml [45] for periods of protein restriction ranging from 8 to 28 weeks. Collectively, the histopathologic lesions and the increased triglyceride contents, along with the differences in concentrations of hepatic enzymes, bilirubin and albumin, all indicate that the livers of the MAIZE pigs were affected by malnutrition in a manner similar to that observed in malnourished children.

Animal models of SAM and more specifically kwashiorkor have been conducted with various degrees of success and various clinical outcomes [14-16, 45]. In studies investigating low-protein diets as a way to induce kwashiorkor, lack of growth or even cessation of growth has commonly been observed in pigs fed low-protein diets [44, 46]. In our study, the MAIZE pigs fed a diet containing 9% protein (**Table 1**) increased their body weight by only 9.8% during the 7-week study period. In another study, feeding a diet containing 3% protein for 8 weeks resulted in an increase in weight of 50%, from 7.5 kg to 11.3 kg, whereas the weight of controls increased by a factor of 3.5. Body composition was not determined in that study, but for the low-protein group, protein synthesis was reduced in the skin, jejunal mucosa and liver [14]. In another study, a gradual decrease in the dietary protein content from 25% to 2.2% over a period of 24 weeks showed that it was not until week 16 that growth ceased and the pigs started to lose weight [45]. Pigs fed diets

with either 4.5% or 6.5% protein also ceased to grow, and after a period of 12-16 weeks, signs of kwashiorkor became evident [15]. However, observed growth rates in these studies are difficult to compare because of differences in protein source and quality, the genetic potential of the animals for growth and the methods of handling and housing. One major reason for the very low growth rate observed in the current study, compared to growth rates observed in other studies, is that we did not fortify the low-protein maize diet with vitamins or minerals, so the diet was inadequate in not only protein but also a wide range of other type II nutrients, resulting in growth failure [47]. Feeding with only maize flour was conducted to mimic real-life situations in which young children are often fed a cereal-based diet with limited variety. The aim was not to evaluate how single nutrients in the maize diet would give rise to specific symptoms but rather to test the effect of a uniform diet over a period of time. Although maize flour is commonly ingested among infants, other staples such as sorghum or cassava could also have been chosen.

In conclusion, pigs fed pure maize flour for 7 weeks developed moderate acute malnutrition resembling marasmus based on assessment criteria similar to what is used for malnourished infants. Having validated the degree of malnutrition, we demonstrate that the digestive system, including the small intestine and liver, are primarily affected in their structure, whereas functional endpoints were affected to a lesser extent at this stage of malnutrition.

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Disclosure of conflict of interest

None.

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References

- [1] United Nations Children's Fund, World Health Organization, The World Bank. UNICEF-WHO-World Bank Joint Malnutrition Estimates. UNICEF, New York; WHO, Geneva; The World Bank, Washington, DC; 2012.
- [2] de Onis M, Frongillo EA, Blossner M. Is malnutrition declining? An analysis of changes in levels of child malnutrition since 1980. *Bull World Health Organ* 2000; 78: 1222-1233.
- [3] World Health Organization, World Food Programme & United Nations System Standing Committee on Nutrition and United Nations Children's Fund. Community-based management of severe acute malnutrition. 2007; available at http://www.who.int/nutrition/topics/Statement_community_based_man_sev_acute_mal_eng.pdf.
- [4] Keys A, Brozek J, Henschel A, Mickelsen O, Taylor HL. *The Biology of Human Starvation*. vol. 1. St. Paul, MN: University of Minnesota Press; 1950.
- [5] Frisch RA, Mendel LB, Peters JP. The production of edema and serum protein deficiency in white rats by low protein diets. *J Biol Chem* 1929; 84: 167-177.
- [6] Gillman J. Effects on rats of prolonged feeding with the staple African diet. *BMJ* 1944; 1: 149-150.
- [7] Lago ES, Teodósio NR, Araújo CR, Azevedo MC, Pessoa DC, Campos FA, Zucas SM, Flores H. Rat Models of Protein and Protein-Energy Malnutrition. *Int J Vitam Nutr Res* 1993; 63: 52-56.
- [8] Mcguire EA, Young VR. Nutritional Edema in A Rat Model of Protein-Deficiency - Significance of the Dietary Potassium and Sodium Content. *J Nutr* 1986; 116: 1209-1224.
- [9] Kamalu BP. Pathological-Changes in Growing Dogs Fed on A Balanced Cassava (Manihot-Esculenta Crantz) Diet. *Br J Nutr* 1993; 69: 921-934.
- [10] Rana S, Gupta D, Malik A, Katyal R, Mehta SK. Mild-To-Moderate Malnutrition and Small-Intestine of Young Rhesus-Monkeys. *Nutrition* 1995; 11: 292-295.
- [11] Coward DG, Whitehead RG. Experimental Protein-Energy Malnutrition in Baby Baboons - Attempts to Reproduce Pathological Features of Kwashiorkor As Seen in Uganda. *Br J Nutr* 1972; 28: 223-237.
- [12] Follis RH. A Kwashiorkor-Like Syndrome Observed in Monkeys Fed Maize. *Proc Soc Exp Biol Med* 1957; 96: 523-528.
- [13] Pond WG, Ellis KJ, Schoknecht P. Response of Blood-Serum Constituents to Production of and Recovery from A Kwashiorkor-Like Syndrome in the Young-Pig. *Proc Soc Exp Biol Med* 1992; 200: 555-561.
- [14] Wykes LJ, Fiorotto M, Burrin DG, Del Rosario M, Frazer ME, Pond WG, Jahoor F. Chronic low protein intake reduces tissue protein synthesis in a pig model of protein malnutrition. *J Nutr* 1996; 126: 1481-1488.
- [15] Knowles CB. Protein Malnutrition in the Pig. *Proc Nutr Soc* 1957; 16: R9-R10.
- [16] Heard CRC, Platt BS, Stewart RJC. The Effects on Pigs of A Low-Protein Diet with and Without Additional Carbohydrate. *Proc Nutr Soc* 1958; 17: R41-R42.
- [17] Smith MI, Yatsunenkov T, Manary MJ, Trehan I, Mkakosya R, Cheng J, Kau AL, Rich SS, Concannon P, Mychaleckyj JC, Liu J, Houpt E, Li JV, Holmes E, Nicholson J, Knights D, Ursell LK, Knight R, Gordon JI. Gut Microbiomes of Malawian Twin Pairs Discordant for Kwashiorkor. *Science* 2013; 339: 548-554.
- [18] Nedrebø T, Reed RK. Different serotypes of endotoxin (lipopolysaccharide) cause different increases in albumin extravasation in rats. *Shock* 2002; 18: 138-141.
- [19] Reddy JK, Rao MS. Lipid Metabolism and Liver Inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol* 2006; 290: G852-G858.
- [20] Waterlow JC. Note on the assessment and the classification of protein-energy malnutrition in children. *Lancet* 1973; 302: 87-89.
- [21] Waterlow JC. Classification and definition of protein-calorie malnutrition. *Br Med J* 1972; 3: 566-569.
- [22] Gómez F, Ramos GR, Frenk S. Mortality in second and third degree malnutrition 1956. *Bull World Health Organ* 2000; 78: 1275-1280.
- [23] Sejersen H, Sørensen MT, Larsen T, Bendixen E, Ingvarsen KL. Liver protein expression in dairy cows with high liver triglycerides in early lactation. *J Dairy Sci* 2012; 95: 2409-2421.
- [24] World Food Programme & Centers for Disease Control and Prevention. A manual: Measuring and interpreting malnutrition and mortality. 2005; Available at: <http://www.unhcr.org/45f6abc92.html>.
- [25] Allen DM, Dean RFA. The anaemia of kwashiorkor in Uganda. *Trans R Soc Trop Med Hyg* 1965; 59: 326-341.
- [26] Adams EB. Anemia Associated with Kwashiorkor. *Am J Clin Nutr* 1969; 22: 1634-1638.
- [27] Fondu P, Hariga-Muller C, Mozes N, Neve J, Van Steirteghem A, Mandelbaum IM. Protein-energy malnutrition and anemia in Kivu. *Am J Clin Nutr* 1978; 31: 46-56.

A piglet model of pediatric malnutrition

- [28] Macdougall LG, Moodley G, Eyberg C, Quirk M. Mechanisms of anemia in protein-energy malnutrition in Johannesburg. *Am J Clin Nutr* 1982; 35: 229-235.
- [29] Haghghi P, Wolf PL. Tropical sprue and sub-clinical enteropathy: a vision for the nineties. *Crit Rev Clin Lab Sci* 1997; 34: 313-341.
- [30] Lunn PG, Northrop-Clewes CA, Downes RM. Intestinal permeability, mucosal injury, and growth faltering in Gambian infants. *Lancet* 1991; 338: 907-910.
- [31] Stephensen CB. Burden of Infection on Growth Failure. *J Nutr* 1999; 129: 534.
- [32] Thymann T, Sørensen KU, Hedemann MS, Elnif J, Jensen BB, Banga-Mboko H, Lesser TD, Sangild PT. Antimicrobial treatment reduces intestinal microflora and improves protein digestive capacity without changes in villous structure in weanling pigs. *Br J Nutr* 2007; 97: 1128-1137.
- [33] van der Schoor SR, Wattimena DL, Huijmans J, Vermes A, van Goudoever JB. The gut takes nearly all: threonine kinetics in infants. *Am J Clin Nutr* 2007; 86: 1132-1138.
- [34] Stoll B, Burrin DG. Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. *J Anim Sci* 2006; 84: E60-E72.
- [35] Riedijk MA, Stoll B, Chacko S, Schierbeek H, Sunehag AL, van Goudoever JB, Burrin DG. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. *Proc Natl Acad Sci* 2007; 104: 3408-3413.
- [36] Chang CS, Chen GH, Lien HC, Yeh HZ. Small intestine dysmotility and bacterial overgrowth in cirrhotic patients with spontaneous bacterial peritonitis. *Hepatology* 1998; 28: 1187-1190.
- [37] Vantrappen G, Janssens J, Hellemans J, Ghoois Y. The Interdigestive Motor Complex of Normal Subjects and Patients with Bacterial Overgrowth of the Small Intestine. *J Clin Invest* 1977; 59: 1158-1166.
- [38] Pimentel M, Soffer EE, Chow EJ, Kong Y, Lin HC. Lower Frequency of MMC Is Found in IBS Subjects with Abnormal Lactulose Breath Test, Suggesting Bacterial Overgrowth. *Dig Dis Sci* 2002; 47: 2639-2643.
- [39] World Health Organization Joint FAO/WHO expert committee on nutrition, third report. 1953; Available at: http://apps.who.int/iris/bitstream/10665/40219/1/WHO_TRS_72.pdf.
- [40] Shiels ME, Stewart WB. Development of Portal Fatty Liver in Rats on Corn Diets - Response to Lipotropic Agents. *Proc Soc Exp Biol Med* 1954; 85: 298-303.
- [41] Jungermann K. Functional heterogeneity of periportal and perivenous hepatocytes. *Enzyme* 1986; 35: 161-180.
- [42] Thurman RG, Kauffman FC. Sublobular compartmentation of pharmacologic events (SCOPE): metabolic fluxes in periportal and pericentral regions of the liver lobule. *Hepatology* 1985; 5: 144-151.
- [43] Hoyumpa AM Jr, Greene HL, Dunn GD, Schenker S. Fatty liver: Biochemical and clinical considerations. *Digest Dis Sci* 1975; 20: 1142-1170.
- [44] Lowrey RS, Pond WG, Barnes RH, Krook L, Loosli JK. Influence of Caloric Level and Protein Quality on Manifestations of Protein Deficiency in Young Pig. *J Nutr* 1962; 78: 245-253.
- [45] Grimble RF, Whitehead RG. Relationship Between An Elevated Serum Amino Acid Ratio and Development of Other Biological Abnormalities in Experimentally Malnourished Pig. *Br J Nutr* 1969; 23: 791-804.
- [46] Pond WG, Barnes RH, Bradfield RB, Kwong E, Krook L. Effect of Dietary Energy Intake on Protein Deficiency Symptoms and Body Composition of Baby Pigs Fed Equalized But Suboptimal Amounts of Protein. *J Nutr* 1965; 85: 57-66.
- [47] Golden MHN. The role of individual nutrient deficiencies in growth retardation of children as exemplified by zinc and protein. In: Waterlow JC, editor. *Linear growth retardation in less developed countries*. New York: J. C. Raven Press; 1988. pp: 143-163.