The central role of mitochondrial health in malnutrition induced enterohepatic dysfunction

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Chapter 5. mTOR inhibition preserves intestinal barrier integrity and improves lactose absorption in a mouse model of severe malnutrition.

Authors
Severely malnourished children with diarrhea are often refractory to treatment and have an increased risk of death. Severe intestinal damage, known as malnutrition enteropathy, is thought to contribute to clinical deterioration and death through bacterial translocation and systemic inflammation. As the pathophysiological mechanism underlying malnutrition enteropathy are unclear, we aimed to investigate the potential involvement of dysregulated mTORC1 and autophagy. Here we show that feeding weanling mice a low-protein diet leads to features of malnutrition enteropathy including reduced villous height, barrier dysfunction and lactose malabsorption. Intestinal dysfunction in LPD-fed mice is accompanied by reduced autophagy and increased mTORC1 activity, which was the opposite of what might be expected under nutrient-deficient conditions. In LPD-fed mice mitochondria were decreased in number and were morphologically different. We further demonstrate that administration of rapamycin, a mTORC1 inhibitor, to LPD-fed mice prevents a decline in both intestinal functions and that functional improvement coincides with decreased mTORC1 activity as well as improved mitochondrial homeostasis. Our findings suggest that interventions targeting mTOR can potentially improve intestinal function in severely malnourished children.
INTRODUCTION

Severe malnutrition in children remains a major public health concern in many developing countries contributing significantly to morbidity and mortality\(^1\). Developments in the therapeutic management of severely malnourished children have improved clinical outcomes\(^1\). However, severely malnourished children presenting with diarrhoea are often refractory to treatment and have an increased risk of death\(^2,3\). This population suffers from severe intestinal mucosal damage, known as malnutrition enteropathy, which encompasses alterations in intestinal architecture including villous blunting\(^4\) intestinal barrier dysfunction\(^4–6\) and nutrient malabsorption\(^7\). The loss of intestinal integrity is thought to contribute to clinical deterioration and death through microbial translocation, leading to systemic inflammation and sepsis\(^4,8,9\). Thus far, the pathophysiological processes involved in the disruption of intestinal homeostasis are unclear and no intervention exist that specifically target malnutrition enteropathy.

The Mechanistic Target of Rapamycin Complex 1 (mTORC1), a protein complex containing the serine/threonine kinase mTOR, can sense fluctuations in nutrients and modulate cellular metabolism and growth accordingly\(^10\). In nutrient-rich conditions, active mTORC1 promotes cell growth by stimulating anabolic processes, such as protein synthesis. In nutrient-deficient conditions, mTORC1 inhibition triggers the catabolic autophagy pathway that degrades damaged or superfluous organelles in lysosomes and maintains nutrient homeostasis via recycling of cytosolic components\(^10\).

It is currently unknown if and how severe malnutrition affects mTORC1 signaling and downstream autophagy in the intestine and if changes in these pathways compromise intestinal structure and function. One would expect that the limited nutrient availability would decrease mTORC1 activity. However, an opposite response has been reported in intestinal diseases which share similarities with malnutrition enteropathy. Enhanced mTORC1 activity has been reported and implicated in the pathogenesis inflammatory bowel disease (IBD)\(^11,12\) and celiac disease (CeD)\(^13\). Accordingly, the use of mTOR inhibitor rapamycin was found to improve mucosal healing in children with severe refractory IBD\(^14\).

Limited insight into the underlying pathology of malnutrition enteropathy is related to challenges in studying the disease at a tissue level, which require invasive endoscopic procedures. Low-protein-diet-induced malnutrition in animal models offers an alternative approach to study pathophysiological mechanisms in the intestine in severe malnutrition\(^15\) and can also be used to study the potential role of dysregulated mTORC1 and autophagy in malnutrition enteropathy.

Here we comprehensively characterize both structural and functional changes in the small intestine in a mouse model of severe malnutrition. Surprisingly, we demonstrate that mTORC1 activity is upregulated and autophagy is downregulated in the small intestine of mice on a low-protein diet. In addition, intestinal barrier function and lactose absorption can be preserved by inhibiting mTORC1 in this model. These results suggest that interventions targeting mTOR can potentially improve intestinal function in severely malnourished children, provided that treatment costs are carefully addressed.
METHOD

Animals
Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were weaned at three weeks postpartum. The 21-day old mice were placed for 2 weeks on isocaloric diets, either a low-protein diet (LPD, 1% of calories from protein) or a normal diet (ND, 18% of calories from protein, as described in Figure 1a and Supplementary Table 1) (Envigo Teklad Diets, Madison, WI, USA). Mice were housed in a light-controlled facility (12 hrs day night cycles) with ad libitum access to water and their respective irradiated diet. Mice were sacrificed through brief exposure to isoflurane and subsequent exsanguination. Animal work in Canada was approved by the Animal Care Committee of The Hospital for Sick Children, Toronto (Animal Use Protocol Number: 1000030900). For animal experiments in the Netherlands, approval was obtained from the Central Authority for Scientific Procedures on Animals (CCD) of the Netherlands and from the University of Groningen Ethical Committee for Animal Experiments (Animal Use Protocol Number: 171504-01-001/3).

Rapamycin Intervention
mTOR was pharmacologically modulated in one experimental group of animals kept on LPD. Rapamycin (Sigma-Aldrich, MO, USA) was dissolved in 100% ethanol at 50 mg ml\(^{-1}\) and diluted in 10% Tween-80 (Sigma-Aldrich) before being administered by intraperitoneal injection. Rapamycin administration was started at weaning, simultaneously with the LPD intervention, and was given once daily for 14 consecutive days at a concentration of 6 mg/kg of body weight\(^{16}\). A comparative control group received daily injections with the diluent.

FITC-Dextran Assay
To assess intestinal permeability, mice were fasted overnight and then administered 100mgml\(^{-1}\) of 4kDa Fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich) through oral gavage, at a volume of 150 µL per 10g of bodyweight. One-and-a-half-hours post gavage, the mice were anesthetized and whole blood was collected via cardiac puncture. Serum was separated and FITC was measured using a fluorescence plate reader with the excitation of 485 nm and emission at 528 nm. FITC concentration in serum was calculated against a serially diluted standard curve.

Intestinal glucose and lactose absorption using tracers labeled with stable isotopes
After a 4 hour fast (6.00h-10.00h), bodyweight and basal blood glucose were assessed. At time point zero, a bolus of [6-6\(^2\)H\(_2\)]-glucose (~1000umol per kg, Cambridge Isotope Laboratories, MA, USA) was given intravenously via retro-orbital injection, immediately followed by oral administration of a mixed bolus containing D-glucose (~2000 umol per kg) and lactose (~4000 umol per kg) of which ~3% was labeled with tracers, i.e. [U\(^\text{13}\text{-C}\)]-glucose (Cambridge Isotope Laboratories) and [1\(^\text{13}\text{-C}\)]-lactose
Blood glucose levels were measured from the tail-tip at the start of the experiment and after 10, 20, 30, 40, 50, 60 and 90 minutes. At the same time points blood spots were collected on filter paper to measure the fractional contributions of glucose tracers in the blood. Absorption of [U-13C]-glucose and lactose-derived [1-13C]-glucose over time was calculated using non-steady equations of Steele et al.\textsuperscript{17} as modified by Debodo et al.\textsuperscript{18}. The volume of distribution of glucose was considered 200 ml/kg and the pool fraction 0.75\textsuperscript{19}. We used an approach suggested by Radziuk et al.\textsuperscript{20}, including the assumption that the clearance rates of all glucose isotopologues, i.e. tracers and tracee, are metabolized identically. In short, after extraction of glucose from the blood spots, the fractional contributions of [6-6-2H\textsubscript{2}]-glucose, [U-13C]-glucose and lactose-derived [1-13C]-glucose enrichments in blood glucose was measured by Gas Chromatography-Mass Spectrometry (GCMS) (Agilent 9575C series GC/MSD, Agilent Technologies, Amstelveen, The Netherlands) according to Van Dijk et al.\textsuperscript{21}. The measured fractional distributions of glucose isotopologues (m\textsubscript{0}-m\textsubscript{6}) were adjusted for natural abundance of 13C-atoms according to Lee (M\textsubscript{0}-M\textsubscript{6})\textsuperscript{22}. Next, blood glucose concentrations and the fractional contribution M\textsubscript{2} due to the intravenous administration of [6-6-2H\textsubscript{2}] glucose tracer were used to calculate its concentrations and subsequently to estimate glucose clearance rates. Furthermore, blood glucose concentrations and the fractional contributions M\textsubscript{6} and M\textsubscript{1}, due to the administered glucose bolus ([U-13C]-glucose) and lactose bolus ([1-13C]-glucose)-derived glucose were used to calculate their concentrations. All equations are listed in Table 1. The curve described by equation A was fitted to the calculated curves of the IV-administered tracer (SAAM II, v2.3, The Epsilon Group, Charlottesville, VA, USA). The retrieved parameters were used to calculate the area under the curve (AUC) according to equation B. Next, the glucose clearance rate was estimated from the administered IV-bolus and the AUC of this tracer curve, equation C. From the individual tracer concentrations, and the glucose clearance rates (GCR), disposal rates (Rd\textsubscript{t}) of the orally administered tracers can be determined by equation D. Using non-steady state equations (Equation E), the appearances rates (Ra\textsubscript{t}) of both orally administered bolus were estimated for each time point. The cumulative appearances were calculated from Ra\textsubscript{t} and the time (Equation F) after which the fractional absorption can be calculated (Equation G).

Table 1. Formulas for calculation of intestinal absorption of carbohydrates using stable labeled isotopes

<table>
<thead>
<tr>
<th>Measure</th>
<th>Equation</th>
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| Blood concentrations of glucose tracers (umol.mL\textsuperscript{-1}) | \[\text{H}^{2}\text{glc} \text{t} = (M_{2}) \times \text{[glc]} \text{t} \]
| | \[\text{C}^{13}_{6}\text{glc} \text{t} = (M_{6}) \times \text{[glc]} \text{t} \]
| | \[\text{C}^{13}_{1}\text{glc} \text{t} = (M_{1}) \times \text{[glc]} \text{t} \]
| Equation A. Concentration curve of IV-administered glucose tracer | \[\text{H}^{2}\text{glc} = C_{0}e^{-k_{el}t} - C_{0}^{ab}e^{-k_{ab}t} \]
| Equation B. Area under the curve | \[\text{AUC} = \frac{C_{0}}{k_{el}} - \frac{C_{0}^{ab}}{k_{ab}} \]
| Equation C. Glucose Clearance Rate (GCR) (ml.kg\textsuperscript{-1}.min\textsuperscript{-1}) | \[\text{GCR} = \frac{\text{H}^{2}\text{glc}}{\text{AUC}} \]
Equation D. Rate of disposal (RD)
(umol.kg⁻¹.min⁻¹)
\[ Rd_t = GCR \times [A_{x,glc}'] \]

Equation E. Rate of appearance (Ra)
(umol.kg⁻¹.min⁻¹)
\[ Ra_t = Rd_t + \frac{d[A_{x,mic}']}{dt} \times pV \]

Equation F. Total rate of appearance
(umol.kg⁻¹)
\[ Ra = Ra_t \times t \]

Equation G. Fractional absorption (%)
\[ F = \frac{Ra}{bolus} \]

**Plasma amino acids**

Plasma was mixed with equal volumes of internal standard (Norleucine), centrifuged at 14000 rpm for 5 minutes and subsequently measured on Biochrom 30+ Amino Acid Analyzer (Biochrom, Cambridge, UK).

**Histology, electron microscopy and immunofluorescence**

For light microscopy, intestinal sections from the proximal end of the jejunum and ileum (1cm) were fixed in 10% buffered formalin, or Methcarn fixative (60% methanol, 30% chloroform, 10% Glacial Acetic Acid) for Alcian Blue-Periodic Acidic Schiff (PAS) staining. Samples were paraffin embedded, sectioned (4 um) and stained with hematoxylin and eosin (H&E) using standard techniques. Villous height was enumerated using Aperio ImageScope (Leica Biosystems, version 12.4). For each sample approximately 15 fully intact and well-oriented villi were measured from the tip of the villus to villus-crypt junction. Number of goblet cells were counted in sections stained with Periodic Acid-Schiff (PAS)/Alcian Blue as previously described²³. Intestinal tissues collected for electron microscopy were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1M sodium cacodylate and postfixed in 1% osmiumtetroxide/1.5% potassiumferrocyanide in 0.1M sodium cacodylate. Subsequently samples were dehydrated using ethanol and embedded in EPON epoxy resin, sectioned at 60-nm, collected on formvar coated single slot grids and contrasted using 2% uranyl acetate in water followed by Reynolds lead citrate. Images were taken with a Zeiss Supra55 in STEM mode 29kV using ATLAS-5 (Fibics, Ottawa, ON, Canada) yielding mosaics of large-area scans at 2,5 nm pixel resolution. These large-scale TIFF images were stitched and converted to html files using ATLAS-5 (Fibics).

**RNA isolation, reverse transcription and quantitative real-time qPCR**

Total RNA was extracted from the small intestine using a commercially available kit (Direct-zol™ RNA MiniPrep (ZYMO research Inc, Irvine, CA, USA)). RNA quality and quantity was determined with NanoDrop (NaoDrop Technologies, Wilmington, DE, USA). Reverse transcription was completed with the qScript cDNA synthesis kit (Quantabio, Beverly, ME, USA) according to the manufacturer’s instructions. Real-time qPCR analysis for the gene expression of Muc2 and TJ proteins (cldn-3, cldn-4, cldn-7, ZO-1, Ocld) was performed in 10 ul reaction volumes utilizing advanced qPCR Master mix (Wisent Inc, Saint-Jean-Baptiste, QC, Canada). All primers sequences (Integrated DNA technologies
Inc., Coralville, IA, USA) are listed in Supplementary Table 2. PCR reaction were carried out on a CFX384 Touch Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA). Ribosomal protein L3A (Rpl3a) served as endogenous control, as expression was stable between experimental groups and was used for normalization. Relative expression was calculated using the ΔΔC(t) method relative to the control mice (ND).

**Immunoblotting**

The mucosa of the jejunum was scraped on ice immediately after sacrifice, snap frozen in liquid nitrogen and stored at -80°C. Next, jejunal mucosa scraping were mixed with ice cold radio immunoprecipitation assay buffer (1% IGEPAL CA-630, 0.1% SDS, and 0.5% sodium deoxycholate in PBS) supplemented with Complete Protease Inhibitor Cocktail (Cat. No. 11836145001, Sigma-Aldrich), Phosphatase Inhibitor Cocktail 2 (Cat. No. P5726, Sigma-Aldrich) and Phosphatase Inhibitor Cocktail 3 (Cat. No. P0044, Sigma- Aldrich). Samples were homogenized (Precellys 24 Homogenizer, Bertin, MD, USA) and centrifuged for 15 minutes at 15000 g at 4°C. Protein concentration in the supernatant was determined with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). For each lane, 30mg protein was loaded and separated on 10/14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to PVDF membranes (Cat. No. IPVH00010; Merck). Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for at least 20 minutes. The membranes were incubated overnight at 4°C with polyclonal primary antibody against LC3B (1:2000, Cat no. 2775, Cell Signaling), S6 Ribosomal Protein (1:1000, Cat no 2317, Cell Signaling) and Phospho-S6 Ribosomal Protein (1:1000, Cat no. 4856, Cell Signaling). Membranes were washed and incubated with a horse-radish peroxidase conjugated secondary antibody (goat-anti-mouse and goat-anti-rabbit) for 2 hours at room temperature. Pierce enhanced chemiluminescence western blotting substrate (Cat no. 32209, Thermo Fisher Scientific) or SuperSignal West Femto (Cat no 34095, Thermo Fisher Scientific) was used to detect chemiluminescence using a LAS-4000 mini camera system (GE Healthcare). The subtraction of the background was performed using the rolling ball method. The pixel value of all the signals were normalized to the average intensity of all the signals belonging to the same readout. Afterwards, the signals were normalized to total protein (Simply Blue™ SafeStain, Thermo Fisher Scientific), which was achieved by measuring the intensity of the entire lane. Raw images were exported as red green blue (RGB) color TIFF files using ImageJ and further processed in Adobe Photopshop CC version 19.1. Quantification of raw image files was performed using ImageQuant TL Version 8.1 GE Healthcare.

**Statistical analysis**

All results are expressed as mean ± standard deviation (SD), mean ± standard error of mean (SEM) or median and inter quartile range (IQR), as indicated. Normality was tested using the Shapiro-Wilk test. Group differences were assessed with an either an unpaired two-tailed Student’s T-test, Mann-Whitney
U-test for non-parametric data, repeated measures ANOVA for multiple day body weight measurements, or ordinary two-way ANOVA with Tukey’s post hoc multiple comparisons analyses. Statistical analysis was performed with GraphPad Prism Software Version 9.02 (GraphPad Software, San Diego, CA, USA). Statistical significance was given as ***p value <0.001, **p values <0.01 and *p values <0.05; NS (not significant).

RESULTS

Impact of malnutrition on growth, plasma amino acids and the intestine
We first characterized the impact of the LPD (Figure 1a, Supplementary Table 1) on body growth, plasma amino acids, intestinal architecture, intestinal barrier function and intestinal carbohydrate absorption in 5-week-old mice. Mice fed the LPD diet lost body weight, whilst mice fed the isocaloric normal diet (ND) gained body weight (Figure 1b,c). At the end of the experiment, LPD-fed mice had a significantly shorter body length indicating stunted growth (Figure 1b,d). In LPD-fed mice, we found reduced levels of most essential amino acids (EAA) whereas the levels of EAA histidine and non-essential amino acids (NEAA) glycine and serine were increased (Figure 1e). Histologic visualization of the small intestine revealed reduced villous height without evidence of histological inflammation in LPD-fed mice (Figure 1f, g). To measure whether these structural changes had an impact on intestinal barrier function, we administered FITC conjugated-dextran via oral gavage and measured levels in the serum. There was a 2.4-fold increase in LPD-fed mice compared to ND-fed mice, indicating increased intestinal permeability (Figure 1h). Next, we assessed intestinal carbohydrate absorption using $^{13}$C labeled carbohydrates and found that LPD reduced intestinal absorption of lactose and glucose (Figure 1i). These data show that LPD affected body growth, altered plasma amino acids and disrupted intestinal homeostasis with reduced villous height, barrier dysfunction and lactose malabsorption. Apart from the lack of signs of intestinal inflammation, the findings are in line with observations in severely malnourished children supporting the use of this model for the study of intestinal homeostasis in severe malnutrition.$^{4,6,7}$

LPD reduced autophagy and increased mTOR activity in the small intestine
As autophagy is involved in maintaining intestinal epithelial barrier integrity$^{25}$, we investigated the effects of LPD on autophagy in the small intestinal mucosa. The ratio of LC3-II (lipidated form) to LC3-I (non-lipidated form), indicative of autophagy activity$^{26}$, was reduced in the jejunum of LPD-fed mice (Figure 2a,d). This suggests that protein starvation leads to a reduction in autophagy. This finding was surprising as amino acid or protein deprivation is widely considered to enhance autophagy$^{27,28}$. In an effort to identify cues that might lead to aberrant suppression of autophagy, we quantified the phosphorylation of downstream target of the key autophagy suppressor mTORC1 in the jejunum of
Figure 1. Assessment of body growth, plasma amino acids, villous height, intestinal barrier function and carbohydrate absorption in weanling mice fed the low-protein diet or isocaloric normal diet.

(a) Macronutrient composition of each diet, expressed as percent of total calories. (b) Representative images of mice in each treatment group. (c) Body weight over the period of 14 days. Data points represent mean and error bars indicate the SD, n=6 mice per group (*** S<0.001, repeated measures analysis of variance). After 2 weeks of being fed the respective diet, the total body length (d) was measured. Individual data points are shown with the line representing the mean and error bars indicate the SD, n=6 mice per group (*** S<0.001, two-tailed Student’s T-test). (e) Amino acids were measured in plasma of mice fed the ND (n=10) and LPD (n=12) on day 14. Individual data points are shown with mean and error bars that indicate the SD (* p<0.05, ** p<0.01, *** p<0.001, two-tailed Student’s T-test on log transformed data). (f) The average measured villous height in the jejunum and ileum (g) with representative H&E-stained images of jejunum from mice exposed to each diet, n=8 mice per group. Scale bar, 100 um. Bar graph indicate the mean with SD (Mann-Whitney U test, *** p<0.001). (h) Concentration of FITC in the serum was measured 1.5 h post oral administration, after mice were fed each diet for 14 days. Bars indicate the mean with SEM, n= 8 per group (* p<0.05, two tailed Student’s T-test with Welch correction. (i) Fractional absorption of glucose and lactose after mice were fed the normal diet (n=9) or low-protein diet (n=11) for 14 days. Individual data points are shown with mean and error bars that indicate the SD (** p<0.01, two-tail Students’s T-test with Welch correction). LPD, low-protein diet; ND, normal diet.
LPD-fed mice\textsuperscript{39}. mTORC1 activation enhances phosphorylation of the translational regulator ribosomal protein S6 kinase 1 (RPS6K1) and of its substrate ribosomal protein S6 (RPS6)\textsuperscript{30}. We found that phosphorylated RPS6 at serine 235/236 increased (Figure 2b,c) indicative of mTORC1 activation. Thus, enhanced mTORC1 activity might represent a plausible explanation for decreased autophagy in LPD fed mice. We assessed if mTORC1 inhibition with rapamycin could increase autophagy. Rapamycin reduced phosphorylated S6 at serine 235/236 to total S6 (Figure 2b,c), indicating reduced mTORC1 activity. Yet, we did not observe a significant increase in autophagy (Figure 2a,b). Taken together, feeding a low-protein diet to young mice leads to reduced autophagy and hyperphosphorylation of mTORC1 targets in the jejunum.

![Figure 2. Reduced autophagy in LPD-fed mice can be partially restored with rapamycin.](image)

Protein levels of LC3B-I and -II (a), RPS6 and RPS6-pS235/236 (b) were measured in the mucosa of the jejunum in the different experimental groups, n=4-6 mice per group (** S<0.01, *** S<0.001 Two-way ANOVA with Tukey’s post-hoc test). (c) Relative intensity of protein measured in each entire lane (details of total protein normalization in methods section). Individual data points are shown with mean and error bars that indicate the SD. (d) Representative blots of the detected proteins are shown for 3 independent animals. LPD, low-protein diet; ND, normal diet; RAP, rapamycin.

**Rapamycin prevents intestinal barrier dysfunction and lactose malabsorption in LPD-fed mice**

We examined if rapamycin prevents disruption in intestinal homeostasis. Rapamycin did not impact body weight but did decrease body length (Figure 3a, b). Rapamycin increased plasma levels of several amino acids including alanine and serine with arginine, proline and tryptophan attaining similar levels as in ND-fed mice (Figure 3c). However, this response was heterogeneous between animals (Supplementary Figure 1). Rapamycin did not prevent reduction in villous height in the jejunum or ileum (Figure 3d,e). Importantly, rapamycin did prevent increased paracellular permeability to FITC-
dextran in LPD-fed mice with levels similar to ND-fed mice (Figure 3f). Likewise, LPD-fed mice receiving rapamycin showed higher intestinal lactose absorption than LPD-fed mice, albeit still lower than ND-fed mice (Figure 3g). In contrast, rapamycin did not increase intestinal glucose absorption (Figure 3g). Taken together, rapamycin maintained intestinal barrier integrity and partially preserved capacity for lactose absorption in this mouse model of malnutrition.

Figure 3. Rapamycin treatment does not prevent weight loss or stunting but improves intestinal function.
Post weaning mice were given a normal diet (ND), low-protein diet (LPD) or low-protein diet combined with daily intraperitoneal injections of rapamycin (RAP). (a) Body weight over the period of 14 days. Individual data points are shown with mean and error bars that indicate the SD, n=6 mice per group (**p<0.01, ***p<0.001, repeated measures analysis of variance). After 2 weeks, the total body length (b) was measured. Individual data points are shown with mean and error bars that indicate the SD, n=6 (** p<0.01, ***p<0.001, Two-way ANOVA with Tukey’s post-hoc analysis). Amino acids (c) were measured in plasma on day 14. Individual data points are shown with mean and error bars that indicate the SD, n=6-12 mice per group (*p<0.05, ** p<0.01, *** p<0.001, Two-way ANOVA with Tukey’s post-hoc analysis on log transformed data). (d) The average measured villous height in the jejunum and ileum with representative H&E-stained images of jejunum (e) from each experimental group. Scale bar, 100 um. Bar graph indicated the mean with SD, n=5 mice per group (***p<0.001, Two-way ANOVA with Tukey’s post-hoc analysis). (f) Concentration of FITC in the serum measured 1.5h post oral administration after 14 days. Bars indicate the mean with SEM, n=8 per group (*p<0.05, Two-way ANOVA with Tukey’s post-hoc analysis). (g) Fractional absorption of glucose and lactose after mice were fed the normal diet (n=9) or low-protein diet (n=11) or low-protein with daily rapamycin injections (n=7) for 14 days. Individual data points are shown with mean and error bars that indicate the SD (* p<0.05, *** p<0.001, Two-way ANOVA with Tukey’s post-hoc analysis). LPD, low-protein diet; ND, normal diet; RAP, rapamycin.

Rapamycin preserves tight junction proteins and number of goblet cells
Tight junction (TJ) proteins and the mucus layer are important for intestinal barrier function. Thus, we investigated the expression of several tight junction proteins and features of the mucus layer in the small intestine. As illustrated in Figure 4a, mRNA levels of claudin-3, claudin-4 and occludin were reduced in LPD-fed mice as compared to ND-fed mice. In rapamycin-administered mice, mRNA levels of the TJ proteins, except for claudin-7, were markedly increased towards levels of ND-fed mice (Figure 4a). This could explain, at least in part, the preservation of barrier function by rapamycin. Furthermore, we observed a significant reduction in the number of goblet cells normalized to villous height in the LPD group compared to the ND group (Figure 4b, d). Yet, the mRNA expression of MUC2, the primary component of the protective mucus layer, in the small intestine was not significantly lower in LPD-fed mice (Figure 4c). Rapamycin preserved goblet cells in LPD-fed mice with similar numbers in both the jejunum and the ileum as ND fed mice (Figure 4b,d). Also, MUC-2 expression was similar to ND-fed mice in LPD-fed mice receiving rapamycin (Figure 4c). These data suggest that rapamycin prevents LPD-induced reduction in specific tight junction proteins and protects the mucus layer.

Rapamycin prevents low-protein-diet induced mitochondrial changes
Recently mitochondrial dysfunction has been linked to disrupted intestinal barrier integrity\textsuperscript{31,32}. The reduction in autophagy in LPD-fed mice could compromise the selective removal of damaged or superfluous mitochondria, known as mitophagy, with negative consequences for mitochondrial function\textsuperscript{33}. Moreover, in the liver mitochondrial morphology and function are compromised by a LPD\textsuperscript{34}. Therefore, we assessed whether intestinal mitochondria were also affected by prolonged LPD. Upon
EM analysis, the number of mitochondria per cell was decreased (Figure 5a). In addition, mitochondrial morphology differed in LPD-fed mice: the mitochondria were wider but did not change in length (Figure 5b,c). Cristae appeared deformed or absent in the LPD-fed mice. Electron dense granules in the mitochondrial matrix, most likely calcium phosphate granules, were reduced in LPD-fed mice, indicative of altered mitochondrial Ca\(^{2+}\) sequestration (Figure 5d, e). Of note, rapamycin normalized the number of mitochondria per cell and mitochondrial morphology to that in ND-fed mice (Figure 5a-e).

Figure 4. Rapamycin prevents low-protein diet-induced disruption of tight junction proteins and mucus layer resulting in improved barrier function.

(a) mRNA levels were measured of tight junction proteins claudin-3, claudin-4, claudin-7, ZO-1 and occludin. Individual data points are shown with mean and error bars that indicate the SEM, n=4-5 mice per group (*p<0.05, **p<0.01, *** p<0.001, Ordinary One-way ANOVA with Tukey’s post-hoc analysis). Next, we counted the number of goblet cells per 50 µm of villus (b). Individual data points are shown with mean and error bars that indicate the SD, n=5 per group (*p<0.05, **p<0.01, Two-way ANOVA with Tukey’s post-hoc analysis). (c) mRNA levels of mucin-2 in the jejunum were assessed. Individual data points are shown with mean and error bars that indicate the SEM, n=4-5 mice per group (NS, Two-way ANOVA with Tukey’s post-hoc analysis). (d) Representative images of Alcian Blue/PAS staining for goblet cells in the ileum of the different experimental groups.
Figure 5. Rapamycin improves LPD-induced mitochondrial changes.

Using large area electron microscopy datasets (full datasets available on www.nanotomy.org), we counted the number of mitochondria per cell (a) and measured the length (b) and width (c) of the mitochondria in the experimental groups, n=9 cells per experimental group (*p<0.05, **p<0.01, Two-way ANOVA with Tukey’s post-hoc test). (d) In addition, we visualized the morphology of mitochondria. Electron dense granules (white arrows) are indicated, which are absent in the LPD samples. Scale bar, 500 nm. (e) Zoomed in images of representative mitochondria demonstrating absence of cristae in LPD. Scale bar, 500 nm.

DISCUSSION

We report that feeding weanling mice a very low-protein diet profoundly disrupts the structure and function of the small intestine. Surprisingly, we demonstrate that these changes coincide with a reduction in autophagy, hyperactivation of mTORC1 and changes in mitochondrial number and morphology. We are the first to demonstrate that rapamycin preserves intestinal barrier integrity and improves intestinal carbohydrate absorption in a mouse model of severe malnutrition.

We found the LPD impaired the intestinal barrier function, which is in line with data from severely malnourished children.\textsuperscript{5,37,38} We examined two components of the intestinal barrier: TJ proteins that regulate permeability by sealing the paracellular space\textsuperscript{39}, and goblet cells that produce a mucus layer...
on top of the intestinal epithelium. Reduced expression of mRNA’s coding for TJ proteins (claudin-3, claudin-4, claudin-7, ZO-1 and occludin) is likely to contribute to increased intestinal permeability, provided that this translates to changes at the protein level. Due to limitations in obtaining intestinal biopsies, data on TJ proteins in severely malnourished children is limited to one study, indeed showing a reduction and altered localization of claudin-4 and E-cadherin in duodenal biopsies. Variable changes in intestinal barrier in the small intestine have been reported in low-protein fed animals with reductions in TJ proteins occludin, ZO-1 and claudin-3 and 7. The role of amino acids in maintaining barrier function is further supported by studies that showed that in vitro supplementation with glutamine and 5 tryptophan enhanced protein levels of TJ proteins. Besides changes in TJ proteins, LPD decreased the number of goblet cells in the small intestine, which can negatively impact the protective mucous layer. This has also been observed in other low-protein diet animal studies, but not in the small intestine of severely malnourished children where goblet cells appeared normal in number and size. Our results suggest that differentiation of transit amplifying cells into the secretory epithelial lineage (e.g. goblet cells, Paneth cells) is reduced. The impact of protein restriction on factors that control intestinal epithelial cell differentiation (e.g. Wnt signaling, Notch pathway) deserves further investigation.

We are the first to demonstrate intestinal carbohydrate malabsorption in a malnutrition mouse model. Lactose malabsorption and milder effects on glucose absorption correlate with reported carbohydrate malabsorption in general and lactose specifically in severely malnourished children (reviewed in). The decrease in villous height in LPD-fed mice could through reduced absorptive surface lead to carbohydrate malabsorption. The marked impact on lactose absorption points toward a deficiency in disaccharidases, which are present at the tip of villi and through a hydrolysis step convert disaccharides (e.g. lactose) to monosaccharides (e.g. glucose) to allow transport across the apical membrane by specific transporters. This has also been observed in other diseases associated with villous blunting.

Our results suggest that decreased autophagy contributes to intestinal dysfunction in LPD-fed mice since autophagy has key roles in maintaining intestinal barrier integrity and epithelial cellular function. The reduction in autophagy was surprising as amino acid or protein deprivation is considered to enhance autophagy. We found that autophagy suppressor mTORC1 was activated, which represents a plausible explanation for reduced autophagy in the small intestine of LPD-fed mice. Upon mTORC1 inhibition with rapamycin, however, the ratio of LC3-II to LC3-I as a measure of autophagy did not significantly increase. As we did not measure autophagic flux, we cannot exclude the possibility that autophagy was actually increased. mTORC1 activation in LPD-fed mice was also an unexpected finding considering that the exogenous protein supply was low. Endogenous amino acids, such as from the breakdown of muscle proteins, can also stimulate mTORC1. We found increased plasma levels of glycin, histidine and serine in LPD-fed mice and these amino acids are able to stimulate mTORC1. It is also possible that autophagy itself reactivates mTORC1 via a feedback
mechanism that avoids excess autophagy, such as has been shown in prolonged amino acid starvation in vitro. Alternatively, stimuli other than amino acids can activate mTORC1, including oxidative stress. Damaged or dysfunctional mitochondria can increase reactive oxygen species (ROS) production, especially when not adequately removed by autophagy. Although we did not measure mitochondrial function, loss of cristae structure has been linked to mitochondrial dysfunction and could be responsible for stimulating mTORC1 in LPD-fed mice. Further studies should aim to elucidate the underlying mechanism of mTORC1 hyperactivation.

Pharmacological suppression of mTORC1 with rapamycin preserved intestinal barrier function and improved lactose absorption in LPD-fed mice. Both in vitro and in vivo studies in other disease models have demonstrated that rapamycin could partially rescue intestinal barrier dysfunction. Similar to our findings, improved barrier function with rapamycin aligned with preservation of TJ proteins (claudin-1, ZO-1 and occluding). Preservation of the number of goblet cells and MUC2 expression in rapamycin-administered mice can also strengthen the intestinal barrier. It was shown earlier that rapamycin could reverse reduced differentiation of goblet cells and Paneth cells, caused by high mTORC1 activity, and thereby increased the number of these cell types. Furthermore, rapamycin can restore MUC2 expression. The beneficial results of rapamycin on intestinal lactose absorption that we observed, have not been described in the literature. Given that there was no effect on villous height and mainly lactose absorption was improved, the data suggests that rapamycin can positively affect the mucosal disaccharidase lactase, leading to preservation of lactose absorption although lower than ND-fed mice. However, to our knowledge effects of rapamycin on the disaccharidase lactase have not been reported.

We did not study through which mechanisms rapamycin exerts its beneficial effects on the function of the small intestine. Preserved mitochondrial number and morphology could indicate improved mitochondrial function, which can in turn preserve the intestinal barrier. Alternatively, or in addition, rapamycin may remove the negative effects of constitutive mTORC1 activation on the small intestine such as intestinal epithelial cell dedifferentiation. This is supported by the finding of preservation of goblet cell number and expression of MUC-2, as marker of goblet cell differentiation, in rapamycin-administered mice suggesting improved differentiation. The exact mechanism of action of rapamycin in this model warrants further investigation.

In conclusion, this study is unique in its comprehensive characterization of malnutrition enteropathy in a mouse model. It is the first study to demonstrate that rapamycin can improve intestinal barrier function and lactose absorption in malnutrition. Further studies are needed to confirm the role of mTOR inhibition and mitochondria preservation in maintaining intestinal function. Improvement of both functions could aid in the recovery of malnourished children. In early stages of in-hospital treatment of severely malnourished children, swift restoration of the intestinal barrier could have the potential to prevent mortality that is related to bacterial translocation (e.g. sepsis). The immune suppressive effect of rapamycin, which may be dos-dependent, in this patient population with a high
burden of infections deserves further investigation\textsuperscript{45}. Another possible application of rapamycin could be in the treatment of stunted children, who also suffer from a comparable form of enteropathy that hardly improves with nutritional interventions alone\textsuperscript{66}. This murine model can be used to further study this, investigate underlying mechanisms and test other potential interventions that could improve intestinal homeostasis in severely malnourished children.

\textbf{Acknowledgements}

The financial support from the UMCG Junior Scientific Masterclass to CJV is gratefully acknowledged. Part of the work has been performed in the UMCG Microscopy and Imagining Center (UMIC), specifically in the lab of Ben Giepmans, sponsored by ZonMW grant 91111.006 and the Netherlands Electron Microscopy Infrastructure (NEMI), NOW National Roadmap for Large-Scale Research Infrastructure of the Dutch Research Council (1:2 184.034.014).
REFERENCES


Supplementary Table 1. Compositions of the low-protein diet (LPD, 1% of calories from protein) and normal diet (ND, 18% of calories from protein).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>LPD (g/kg)</th>
<th>ND (g/kg)</th>
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<tbody>
<tr>
<td>Casein</td>
<td>11.5</td>
<td>207.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.36</td>
<td>2.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>350.0</td>
<td>350.0</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>426.54</td>
<td>251.3</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>54.2</td>
<td>52.6</td>
</tr>
<tr>
<td>Cellulose</td>
<td>60.6</td>
<td>41.06</td>
</tr>
<tr>
<td>Vitamin Mix</td>
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<td>10.0</td>
</tr>
<tr>
<td>Ethoxyquin</td>
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<td>0.01</td>
</tr>
<tr>
<td>Mineral Mix</td>
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<td>13.37</td>
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<tr>
<td>Calcium Phosphate</td>
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<td>17.36</td>
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<tr>
<td>Calcium Carbonate</td>
<td>1.1</td>
<td>4.6</td>
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</table>

Supplementary Table 2. List of primer sequences used in RT-qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward and reverse primer sequence (5’-3’)</th>
</tr>
</thead>
</table>
| Claudin-3 (Cldn3)  | Fwd: CGCGACAAGTATGCACCCAC
                  | Rev: AGACGTAGTCCTTGCGGTCG                   |
| Claudin-4 (Cldn4)  | Fwd: GGCAAGTGCACCAACTGCAT
                  | Rev: CCAGGACACGGGCCACCATAA                  |
| Claudin-7 (Cldn7)  | Fwd: GGCCATGTACAAGGGGCTCT
                  | Rev: CACTTCATGCCCATGCTGCG                   |
| Occludin (Ocln)    | Fwd: CACTGCACCCCTGAGAAGCAT
                  | Rev: CGAGCCTCCTTAGCTCGTAG                   |
| Zonula Occludin-1 (ZO-1) | Fwd: GGAGATGTTTATGCCGACGG
                            | Rev: CCATTGCTGTGCTCTTAGCG                   |
| Mucin-2 (Muc2)     | Fwd: GTAGTGGAGATTGTGCCGCT
                  | Rev: CAGGAACACGCACAGGTGG                    |
| Ribosomal protein L3a (Rpl3a) | Fwd: TCCCTCCACCCCTATGACAAG
                                | Rev: GTCACTGCTGGTACTTCC                    |

Fwd, forward primer; Rev, reverse primer.
Supplementary Figure 1. Plasma levels of individual amino acids in the different experimental groups.

Individual data points are shown with mean and error bars that indicate the SD, n=6-12 mice per group (*p<0.05, **p<0.01, ***p<0.001, Two-way ANOVA with Tukey’s post-hoc analysis). ND, normal diet; LPD, low-protein diet; RAP, rapamycin.