Gaining insight into the determinants of mortality in hospitalized severely malnourished children

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DOI:
10.33612/diss.193369178

CHAPTER 7

General discussion and future perspectives

The rationale for the projects undertaken and described in this thesis derived from the limited insight into factors driving high inpatient mortality in severely malnourished children. Our novel findings add to the larger body of work published in the last few years that identified risk factors for death during inpatient treatment. Moreover, these risk factors support a growing body of evidence that suggests a key role for persistent intestinal dysfunction in mortality. In this thesis, several pathophysiological processes were identified that could be involved in the disruption of intestinal homeostasis in severely malnourished children. Further investigation of these processes could lead to improved treatment as currently no interventions exist that specifically target malnutrition enteropathy.

The main objectives of this thesis were:

1. to study factors that may contribute to diarrhoea and poor clinical outcomes in severely malnourished children in hospital; and
2. to establish and characterize preclinical models of malnutrition enteropathy; and
3. to investigate potential mechanisms involved in disrupted small intestinal homeostasis in preclinical models of severe malnutrition.

Gained insights from the three objectives could aid in optimization of inpatient treatment of severely malnourished children and thereby contribute to reducing mortality in this vulnerable population. The main outcomes of this thesis and the perspectives for future research are discussed in this chapter. Part I focuses on the identified risk factors for inpatient mortality, addresses underlying mechanisms and emphasizes the consequences of both for intestinal health. Part II reviews the potential role of discovered dysregulated processes in malnutrition enteropathy, discusses underlying mechanisms and highlights the implications of dysregulation for intestinal regeneration. In part III, (pre)clinical findings will be translated into potential future therapies for severely malnourished children.

I - Inpatient mortality risk factors, mechanisms and consequences for intestinal health

Severely malnourished children with complications still have a high risk of death during in hospital treatment[1–4]. I aimed to improve our knowledge of factors that are linked to poor clinical outcomes as this may inform new or improved therapeutic interventions. In **chapter 3**, we found that upon hospital admission the presence of diarrhoea, increased

intestinal and systemic inflammation, and low faecal SCFAs were significantly linked to mortality. Systemic inflammation and low faecal SCFAs were directly associated with death, whereas diarrhoea and intestinal inflammation may be linked to mortality through systemic inflammation. These combined results point to the possibility of microbial translocation across an impaired intestinal barrier as a cause of clinical deterioration and death[5]. Gained insight into the interplay of these risk factors for death informs us of severely malnourished children that are at risk of dying. The mechanisms behind each of these risk factors as well as the consequences for intestinal health will be discussed below.

The role of carbohydrate malabsorption and intestinal pathogens in diarrhoea

In **chapter 2** and **4**, we found that diarrhoea often persisted during inpatient treatment. The pathogenesis of diarrhoea is multifactorial and can be considered as outcome of intestinal infections, mucosal damage caused by sequential infections and/or concurrent HIV disease, and other host-related factors (e.g. undernutrition, vitamin deficiency)[6]. Uncertainties remain regarding the optimal inpatient treatment as current management is mostly based on expert opinion and limited observational and intervention trials[7,8]. We investigated if 1) the rehabilitation diets could cause osmotic diarrhoea and if 2) persistent or novel intestinal pathogens could explain diarrhoea during hospital admission.

Intestinal carbohydrate malabsorption and diarrhoea during hospital admission

The original formulations of therapeutic foods used in nutritional rehabilitation were designed before the distinction in treatment was made between complicated and uncomplicated malnutrition[9]. The clinical profile and metabolic changes in severely malnourished children that require hospitalization differ from children without complications that can now be treated in the community. An important question is whether children in hospital can absorb the macronutrients in the provided diets. The consequences of reduced nutrient absorption are twofold. On the one hand the subsequent lower nutrient and energy availability impede their recovery and on the other hand unabsorbed nutrients can lead to osmotic diarrhoea[10]. Although various studies demonstrated that carbohydrate malabsorption is prevalent in severe malnutrition (reviewed in [11]), the majority of calories in F75 and RUTF and a large portion in F100 (65%, 54% and 38% respectively) are derived from carbohydrates (**Figure 1**). We tested

the hypothesis that the carbohydrate content of diets provided during hospitalization exceeds the intestinal absorptive capacity leading to osmotic diarrhoea. We could not detect differences in diarrhoea when comparing transition phase diets with different carbohydrate content, but acidic stool was present during hospital admission in all groups suggesting carbohydrate malabsorption (**chapter 2**). In a larger study a substantial reduction in carbohydrates of almost 30% plus removal of lactose from the diets did not reduce the occurrence of diarrhoea in the stabilization phase either[12]. In line with our findings, stool analysis suggested that the carbohydrate content of the diet may still exceed the threshold for intestinal carbohydrate absorption. The combined findings suggest that malabsorption persists during admission in malnourished children with diarrhoea. The specific capacity of the malnourished intestine to absorb carbohydrates remains mostly unexplored. Also, it is unknown to what extent the condition of the intestine improves during treatment in hospital and how this translates to the absorptive capacity. Furthermore, impaired digestion of fat and protein due to exocrine pancreas insufficiency and hepatobiliary dysfunction plausibly also contribute to diarrhoea through reduction of the nutrient absorptive capacity of the intestine[13,14]. Alternatively, microbiota dysbiosis could favour the growth of lactic acid producing species (e.g. Enterobacteriaceae), which would also lower the faecal pH[15].

Intestinal pathogens and diarrhoea during hospital admission

Next, we aimed to improve our understanding of the contribution of intestinal pathogens to diarrhoea in severely malnourished children during hospitalization. We found that many intestinal pathogens were cleared while the children received broad-spectrum antibiotics[16], but the decrease in detected pathogens did not lead to a reduction in diarrhoea prevalence during admission (**chapter 4**).

Notably, 55% of the children still harboured at least one pathogen before being discharged back to the community. Children are discharged from hospital when clinical improvement is seen, but when the identified pathogens lead to an intestinal infection this could contribute to the ongoing high risk of mortality and relapse that has been reported in children recovering from severe malnutrition[17,18]. This brings into question if the current antibiotic treatment is optimally targeting intestinal pathogens in this specific patient population. Bacterial resistance to the first-line antibiotics may occur and explain the persistence of intestinal pathogens[19]. Furthermore, parasitic infections, which are not covered by broad-spectrum antibiotics, could contribute to the continuance of diarrhoea during admission. *Giardia lamblia* tended to persist in our cohort (**chapter 4**). This could

indicate suboptimal use of antiparasitic drugs (e.g. metronidazole) that are currently only prescribed when the malnourished child has persistent or worsening diarrhoea. Yet, assigning diarrhoea aetiology to this parasite is controversial given the frequent asymptomatic carriage of *Giardia lamblia* in children living in low-income countries[20,21]. Alternatively, or in addition, antibiotics could lead to diarrhoea. Although antibiotics are invaluable for fighting bacterial infections that pose a threat to hospitalized severely malnourished children, antibiotics can negatively impact the microbial community in the gut, referred to as gut microbiota[22]. Microbiota disruption and resilience to perturbation by antibiotics are determined by a multitude of factors including the state of the microbiome, used antibiotics (e.g. spectrum, duration), diet and comorbidities[23,24]. Antibiotics-induced dysbiosis can encompass loss of diversity, loss of beneficial commensals (e.g. SCFA-producing bacteria) and blooming of potentially pathogenic and/or antibiotic-resistant microbes (e.g. Enterobacteriaceae)(reviewed in [22]). The use of broad-spectrum antibiotics ampicillin/amoxicillin and gentamicin in severely malnourished children greatly increased the relative abundance of pathogenic Enterobacteriaceae[25]. It is a reasonable possibility that antibiotics, through dysbiosis of gut microbiota, contribute to diarrhoea during hospitalization. Despite these unfavourable effects, the use of antibiotics can be defended in light of the high susceptibility of severely malnourished children to life-threatening systemic infections[26].

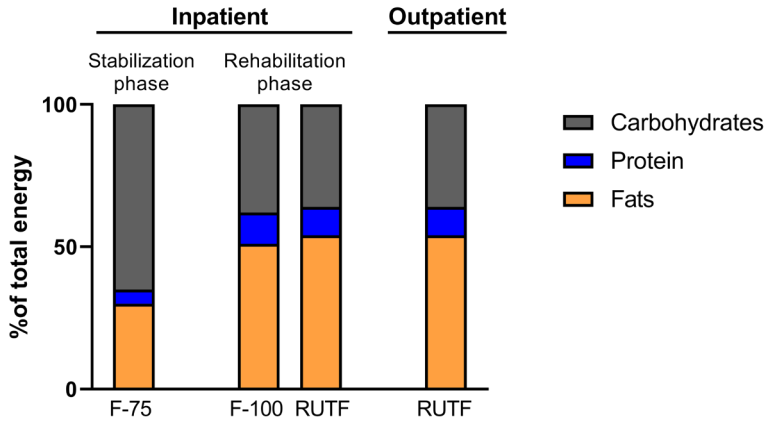

Figure 1. Carbohydrate composition of nutritional rehabilitation diets. The estimated percentage of the total energy for the different macronutrients are shown for the different diets.

Increased intestinal inflammation may hamper nutritional and intestinal recovery

In **chapter 4**, we showed that increased intestinal inflammation persisted during hospitalization. Albeit inflammation as an adaptive immune response to harmful conditions (e.g. infection) is essential for survival and tissue homeostasis[27], uncontrolled inflammation can damage the intestine with consequences for the intestinal barrier and epithelium regeneration[28]. The key factors that initiate and perpetuate high intestinal inflammation in severely malnourished children are not known. The factors that may participate in this process include the high intestinal pathogen burden (**chapter 4**), antibiotic use[29], gut microbiota dysbiosis[30], reduced microbial metabolites (e.g. SCFAs)[31], immune system dysregulation and mucosal damage/impaired barrier function[28]. Jones et al. showed that treating severely malnourished children with immunomodulatory drug mesalazine modestly reduced markers of enteric inflammation without signs of uncontrolled pathogen response[32]. This suggests that intestinal inflammation in severely malnourished children could be (in part) a maladaptive immune response. Provided that this observation is valid, uncontrolled inflammation can hamper nutritional recovery (e.g. through reduced nutrient absorption) and recovery of the damaged intestine in severely malnourished children. Intestinal inflammation can be a promising therapeutic target to improve nutritional and intestinal recovery. Yet, more insight is needed into factors driving and sustaining intestinal inflammation in severely malnourished children.

Gut microbiota dysbiosis as contributing factor in poor clinical outcomes in hospital

The novel finding of lower faecal SCFAs in severely malnourished children that died, could reflect specific or more severe gut microbiota dysbiosis[31]. It is known that severely malnourished children have impaired development of their gut microbiota leaving them with immature and less diverse gut communities compared to those of chronologically age-matched healthy children[25,33,34]. The gut microbiota diversity, in particular anaerobic species, decreased from controls to children with severe wasting and to children with oedematous malnutrition[35]. Thus far, no studies have directly compared microbiota composition of severely malnourished children that died with children that survived during inpatient treatment. Also, studies have not compared microbiota composition and SCFAs levels in hospitalized severely malnourished children. It can be hypothesized that gut microbiota dysbiosis is more severe in children that died compared to children that survived. A recent study demonstrated that volatile organic compounds (VOCs)

profiles, which reflect microbiota composition and their metabolic activity, were markedly different between severely malnourished children that died compared to survivors[36]. This supports the hypothesis that the microbiota composition is related to the susceptibility to mortality, but the specific microbiota changes and particular metabolites linked to mortality were not identified.

Severe gut dysbiosis could increase the risk of death directly or indirectly through the concomitant reduction in microbial metabolites (e.g. SCFAs, vitamins). Gut dysbiosis can cause or sustain intestinal inflammation[30] and thereby contribute to intestinal barrier dysfunction in severely malnourished children[5,37,38]. Subsequently, intestinal barrier dysfunction is thought to lead to microbial translocation and cause systemic inflammation and sepsis[5,39,40], which was directly associated with inpatient mortality (**chapter 3**). Along similar lines, gut dysbiosis can reduce the production of beneficial microbial metabolites, which are required for the maintenance of the intestinal epithelium[41], and could eventually contribute to systemic inflammation. The consequences and potential underlying mechanisms of reduced SCFAs in severe malnutrition will be discussed next.

Reduced faecal short-chain fatty acids are associated with death and can negatively impact intestinal homeostasis

We showed that faecal SCFAs were lower in severely malnourished children that died compared to those that survived (**chapter 3**). Reduced faecal SCFAs have also been reported in IBD patients with active disease[42], whereas SCFAs levels were higher in IBD patients in remission[43]. This suggests that lower faecal SCFAs in IBD, and possibly also in severely malnourished children, may impact disease severity.

SCFAs have a variety of function including serving as primary energy sources for intestinal epithelial cells[44], reducing local inflammation, promoting intestinal barrier function (e.g. TJ proteins, production of antimicrobial peptides) and regulating epithelial cell turnover[31]. Decreased levels of SCFAs in the intestine can have important consequences for immune functions in the intestinal mucosa. SCFAs exert anti-inflammatory effects on the intestinal mucosa through inhibition of histone deacetylases and activation of G-protein coupled receptors (GPCRs) in intestinal epithelial cells and immune cells (reviewed in [31]). The reduction in SCFAs levels may contribute to intestinal inflammation and could compromise epithelial repair in severely malnourished children. At the same time, SCFAs are also involved in mounting normal immune responses to microbes, including regulating the innate- and adaptive immune responses (reviewed in [45]). Thus,

low SCFAs levels could also compromise pathogen defence in the intestine of severely malnourished children.

Given the important role of SCFAs in intestinal health and immune regulation, it is of importance to elucidate the underlying cause of lower SCFAs levels. This can be attributed to various causes that will be discussed below and include reduced nutrient intake, reduced production by an altered microbiome, increased use by colonocytes and/or increased leakage to the blood through an impaired intestinal barrier. Firstly, SCFAs are the end product of bacterial anaerobic formation of ingested non-digestible carbohydrates (NDC)[46]. If food intake is limited, this could have deprived the microbiome of NDC leading to lower SCFAs concentrations. However, in our study reported anorexia before admission did not differ between children who recovered or died (**chapter 3**). The role of NDC intake in low faecal SCFAs could be further explored by studying changes in faecal SCFAs during admission when fibre or NDC dietary content is still low (e.g. RUTF <5%), but this data is not available. Secondly, changes in the microbiome can affect the SCFA production. The main SCFAs producers in the human intestine belong to the phyla Bacteroidetes and Firmicutes. Bacteroidetes mainly produce acetate and propionate, whereas Firmicutes primarily produce butyrate[47,48]. In severely malnourished children, depletion of obligate anaerobic species including Firmicutes and Bacteroidetes have been demonstrated[25,33,34,49] with differences between severe wasting and oedematous malnutrition[35]. Although the loss of obligate anaerobes is a plausible explanation for decreased SCFA production, it is unknown if the depletion of SCFA producing bacteria is more severe in children that died compared to children that recovered. Alternatively, colonocytes may consume all available SCFAs to meet their energy requirements. Yet, intestinal inflammation has been linked to decreased SCFAs uptake[50]. Moreover, IBD studies showed that inflammation is tightly linked to repression of genes related to uptake and metabolism of SCFAs in colonic mucosa[51,52]. Reduced mucosal perfusion in the most severely-ill malnourished children may also reduce intestinal metabolic activity including SCFA metabolism. Finally, in paediatric IBD patients increased gut-to-blood penetration of SCFAs was found compared to healthy controls[53]. This was positively correlated with intestinal inflammation. Additionally, the increased ratio aligned with increased intestinal permeability in mice. Increased leakage of SCFA across an impaired intestinal barrier could also occur in hospitalized severely malnourished children that often experience increased intestinal inflammation (**chapter 4**).

Future clinical studies should aim to elucidate the cause of the reduction in faecal SCFAs. To date, most of the knowledge about SCFAs and microbial fermentation in the human gut is

derived from analysis of faecal samples. Yet, this may not be an adequate representation of the luminal content[54]. Developments of human gastrointestinal capsules, which often allows sampling of the luminal content, offer a non-invasive approach to study diet-microbiota-host interactions[5455] . Rios-Morales et al. recently published a toolbox of procedures that allow adequate analysis of small volume samples obtained by gastrointestinal sampling capsules[56]. Although the gastrointestinal sampling capsules are high-priced, this method allows the simultaneous collection of information about fibres, SCFAs and gut microbiota composition, and could give us important mechanistic insights in severe malnutrition.

Systemic inflammation

We found that markers of systemic inflammation at hospital admission were higher in children that died and in children with diarrhoea compared to survivors and children without diarrhoea, respectively (**chapter 3**). The link between systemic inflammation and mortality was extended to post-discharge from hospital: severely malnourished children that were deemed nutritionally stable and died within 60 days after discharge had increased markers of systemic inflammation[57]. The underlying mechanisms are not entirely clear. Systemic inflammation may result from more severe and unresolved infections and/or microbial translocation across an impaired intestinal barrier[5,39,40]. Ongoing lack of nutrients can also lead to systemic inflammation as studies have reported that nutrient deprivation, even without cell death, can stimulate the synthesis and secretion of certain pro-inflammatory cytokines[58,59].

Intestinal dysfunction and inpatient mortality

The combined clinical findings (**chapter 2-4**) support a growing body of evidence that suggests an important role for the intestine in mortality in severe malnutrition. Loss of intestinal integrity in malnutrition enteropathy could contribute to death through microbial translocation leading to systemic inflammation and sepsis[5,40]. Our understanding of mechanisms leading to disrupted intestinal epithelial integrity is limited due to the limited availability of non-invasive techniques to study intestinal function in this vulnerable patient population living in low-resource settings. Preclinical models offer an alternative approach to overcome these constraints and to study pathophysiological mechanisms in the intestine. The preclinical models of malnutrition enteropathy that were developed in this thesis and the findings will be discussed in the next part.

II - Dysregulation of cellular pathways possibly involved in malnutrition enteropathy

As discussed and presented in **Table 1** of the general introduction, different animal malnutrition models have been developed but evaluation of intestinal changes in these models is fragmentary. This hinders a more in-depth understanding of the mechanisms underlying malnutrition enteropathy. Therefore, I developed a murine malnutrition model using protein restriction and comprehensively characterized both structural and functional changes in the intestine. While barrier dysfunction has been measured and reported in some other malnutrition mouse models, we are the first to also measure nutrient absorption with ^{13}C stable labelled isotopes and to report carbohydrate malabsorption in malnourished mice. To further investigate the impact of severe malnutrition on the intestinal epithelium, to study involved processes (e.g. disrupted organelle homeostasis) and to allow testing of potential interventions in a high-throughput manner, I established the first malnutrition intestinal organoid model. I characterized the impact of amino acid starvation on the structure and function of the organoids. This differs from earlier organoid studies that focused on the effect of starvation for one amino acid on intestinal stem cells[60,61]. The intestinal organoid malnutrition model can be used to link alterations in structure (e.g. crypt atrophy) and function (e.g. barrier dysfunction) to disrupted pathways and metabolic disturbances.

The findings from both preclinical models strongly suggest key roles for dysregulation of the mTOR/autophagy pathway and for mitochondrial dysfunction in malnutrition enteropathy. In the malnutrition mouse model, we found that mTORC1 hyperactivation, decreased autophagy and decreased mitochondrial numbers accompanied intestinal barrier dysfunction and carbohydrate malabsorption (**chapter 5**). Similarly, intestinal barrier dysfunction and morphological changes in starved intestinal organoids coincided with reduced markers of mitochondrial mass and autophagy (**chapter 6**). Importantly, rapamycin administration to malnourished mice and starved organoids could maintain better barrier function, preserve mitochondria and inhibit hyperactivated mTORC1 (only in mice).

In the sections below I will further discuss 1) the mechanisms underlying changes in the mTOR/autophagy pathway and in mitochondrial homeostasis, 2) the interrelations between mTOR, autophagy and mitochondria leading to a potential vicious cycle in malnutrition enteropathy, and 3) the implications of the disruption of the identified processes for intestinal epithelial regeneration in malnutrition enteropathy.

1. The underlying mechanisms of observed changes in mTOR, autophagy and mitochondria

Here I will discuss the mechanisms that may lead to enhanced mTORC1 activation, decreased autophagy and mitochondrial alterations (**chapter 5 and 6**).

Autophagy

Data from the preclinical data suggests a reduction in autophagy in low-protein diet (LPD) fed mice, which was unexpected since amino acid or protein deprivation is widely considered to enhance autophagy[62,63]. The activation of autophagy suppressor mTORC1[64] represents a plausible explanation for the possible reduced autophagy activity in the small intestine of LPD-fed mice. Upon mTORC1 inhibition with rapamycin, however, autophagy did not significantly increase. Yet, we cannot exclude the possibility that autophagy was actually increased, because we did not directly measure autophagic flux, but a marker thereof (LC3-II/LC3-I)[65].

mTORC1

Activation of mTORC1 in LPD-fed mice was also surprising, considering that the exogenous protein supply was low. The fact that mTORC1 activity in starved intestinal organoids was decreased (**Figure 2**) suggests that malnutrition-related changes in systemic factors may be involved in the mTORC1 hyperactivation (**chapter 5 and 6**). Endogenous amino acids, such as from the breakdown of muscle proteins[66], can also stimulate mTORC1. We found that plasma levels of glycine, histidine and serine were increased in LPD-fed mice and these amino acids have the ability to stimulate mTORC1[67]. Alternatively, stimuli other than amino acids can activate mTORC1 including oxidative stress[68]. Damaged or dysfunctional mitochondria can increase reactive oxygen species (ROS) production[69], which could stimulate mTORC1 in malnutrition enteropathy[70]. It is also possible that autophagy itself through the release of degraded intracellular components reactivates mTORC1 to avoid excess autophagy, which has been reported in prolonged amino acid starvation in vitro[71]. Further studies should aim to elucidate the underlying mechanisms of mTORC1 hyperactivation because sustained mTORC1 activity is associated with dysregulated epithelial repair and can promote unwarranted cellular dedifferentiation and epithelial inflammation[72].

Mitochondrial homeostasis

The lower number of mitochondria per cell in the small intestine of malnourished mice could be due to decreased mitochondrial biogenesis or increased turnover (**chapter 5**). Mitochondrial biogenesis was likely decreased in malnourished mice as indicated by reduced proteins levels of peroxisomal proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and mitochondrial transcription factor A (TFAM) (personal communication Cino Ling and Robert Bandsma). Degradation of mitochondria through mitophagy can be induced by nutrient deprivation. However, increased mitophagy seems less likely in malnourished mice since the observed decrease in autophagy activation plausibly also affects the specific sequestration of mitochondria in autophagosomes. The decrease in number could also point to changes in mitochondrial fusion and fission mechanisms[73]. Increased fusion or decreased fission will result in larger but fewer mitochondria, which corresponds with the presence of wider mitochondria in the small intestine of malnourished mice. Increased fusion of mitochondria has been reported as a response to amino acid starvation to protect mitochondria from degradation in autophagosomes[74,75]. Finally, the enlarged mitochondria combined with reduced differentiation of epithelial cells into the secretory lineage (e.g. goblet cells, Paneth cells), which was recently linked to impaired mitochondrial fission[76], could indicate that fission is impaired in malnutrition (**chapter 5**).

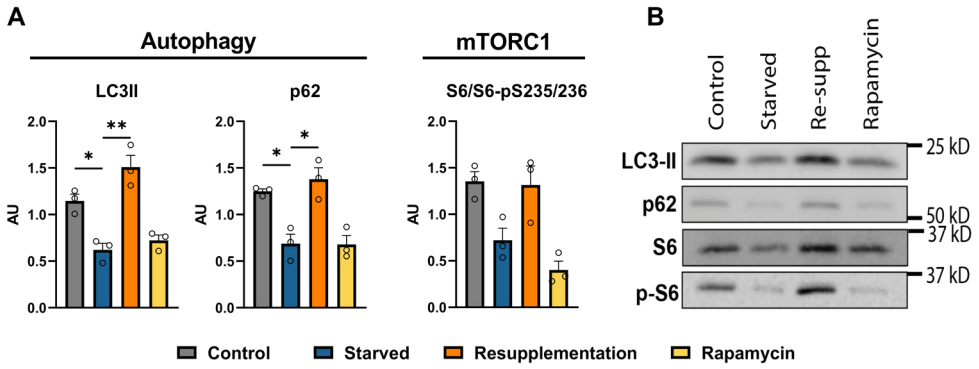

Figure 2. mTOR and autophagy in small intestinal organoids
Protein levels of LC3B-II and p62 (autophagy markers) and RPS6 and RPS6-pS235/236 (mTORC1)(**a**) were measured in intestinal organoids grown in complete culture medium (control), amino-acid-free medium (starved) and amino-acid-free medium supplemented with 2nM rapamycin (rapamycin). Data represents mean ± SEM from 3 biological replicates (*P<0.05, **P<0.01, Two-way ANOVA with Tukey's post-hoc test). (**b**) Representative blots of the detected proteins.

2. Interconnections between mTORC1, autophagy and mitochondria in severe malnutrition

A growing body of evidence highlights the existence of interconnections between mTORC1, autophagy and mitochondria. The disruption in the three parts and the interplay between them could lead to a vicious cycle or self-perpetuating situation in malnutrition enteropathy. I will discuss the existing knowledge of each interconnection separately and finish with the potential vicious cycle in malnutrition enteropathy (**Figure 3**).

Crosstalk between mTORC1 and autophagy

mTORC1 is considered a master regulator of autophagy, because mTORC1 inhibition is required to initiate the autophagy process and mTORC1 has regulatory roles in the subsequent autophagy steps. To illustrate, mTORC1 mediates in the nucleation process through targeting components of the class III PI3K complex I required for the membrane formation[77]. The regulatory roles of mTORC1 in the various autophagy steps are extensively reviewed in[77]. In turn, autophagy can reactivate mTORC1 in conditions of prolonged starvation, which serves as a feedback loop to avoid excess autophagy and restore lysosome homeostasis[71].

Crosstalk between mTORC1 and mitochondria

mTORC1 emerged as an important regulator of mitochondrial function by regulating mitochondrial mass (biogenesis versus autophagic degradation) and mitochondrial turnover (mitochondrial fission and fusion)[78]. mTORC1 promotes transcription of genes involved in mitochondrial biogenesis (e.g. PGC1-α, NRF1) and translation of nucleus-encoded mRNAs encoding mitochondrial proteins[79–81]. In addition, mTORC1 inhibits mitochondrial degradation by suppressing autophagy[82]. Furthermore, it was shown that mTORC1, via inhibition of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), promotes mitochondrial fission through translational regulation of mitochondrial fission process 1 (MTFP1)[81].

Mitochondria to mTORC1 signaling occurs via ROS signaling and regulation of upstream regulators of mTORC1 (e.g. HIF1α and AMPK). Mitochondrial respiration is a major source for ROS generation[83]. The effects of ROS on mTORC1 are tissue-dependent and likely also concentration-dependent. Low levels of ROS induce mTORC1 activity, whereas higher levels inhibit mTORC1 activity[70]. Different types of ROS may also have a disparate impact on mTORC1 activity, but has not been studied. As a preservation mechanism,

inhibition of mTORC1 due to high ROS can, via enhanced mitophagy, lead to a reduction in mitochondrial number and thereby prevent a further increase in ROS formation[84]. Mitochondrial function also regulates HIF1α, AMPK and BNIP3(L), which all impact on mTORC1 activity[85].

Crosstalk between mitochondria and autophagy

Studies have highlighted that mitochondria may be coordination nodes in the autophagy pathway[86]. Various autophagy and apoptotic signaling pathways converge on mitochondria (reviewed in [87]). Mitochondria and autophagy crosstalk can occur in different ways. First of all, mitochondrial oxidative stress (e.g. via ROS formation) can induce autophagy at different steps in the autophagy pathway (e.g. transcriptional upregulation of Atg genes). Secondly, mitochondrial integrity is indispensable for autophagy. It was shown that mitochondrial respiration is required for the initiation of the autophagy process[88]. Moreover, mitochondrial dynamics (fusion and fission events) can determine the response to autophagy, which is illustrated by the finding that starvation-induced elongated mitochondria are spared from autophagic degradation[75] while mitochondrial fragmentation facilitates autophagic clearance of mitochondria[89]. Finally, growing evidence suggests that mitochondria are donors for membranes and lipids that are required for the expansion and maturation of autophagosomes[90]. Selective autophagy of mitochondria or mitophagy is an essential part of mitochondrial quality control[91]. Coordination between the tightly linked processes mitophagy and mitochondrial biogenesis maintains cellular homeostasis through regulation of mitochondrial content and cellular metabolism.

Vicious cycle in malnutrition enteropathy

Based on our findings and the existing knowledge about the interplay between mTOR, autophagy and mitochondria, I postulate the existence of a specific vicious cycle in malnutrition enteropathy. Enhanced activation of mTORC1 decreases autophagy, which likely also decreases mitophagy. The observed elongated mitochondria in malnourished mice can prevent their autophagic degradation[75]. Decreased mitophagy compromises degradation of damaged mitochondria, which can increase ROS production and in turn stimulate mTORC1 activity. The cycle starts over again (**Figure 3**). It needs to be assessed if mitophagy is decreased and if ROS levels are increased in the preclinical models. So far it is unclear which of the three components deteriorates first and if the interconnections indeed create a self-perpetuating situation. Based on knowledge in severe malnutrition,

damaged mitochondria may be the starting point of the vicious cycle. Firstly, severely malnourished children have reduced levels of antioxidants such as glutathione and vitamin E[92–94], which can damage mitochondria. Secondly, it has been suggested that peroxisome decline precedes mitochondrial damage and dysfunction, which has been observed in the livers of malnourished rats[95]. In light of the reduction in both peroxisomal markers and mitochondrial markers in starved intestinal organoids, this may also be the case for the intestine in severe malnutrition (**chapter 6**). Future studies in the preclinical models are required to elucidate underlying mechanisms and understand if and how these interrelations may prevent restoration of homeostasis.

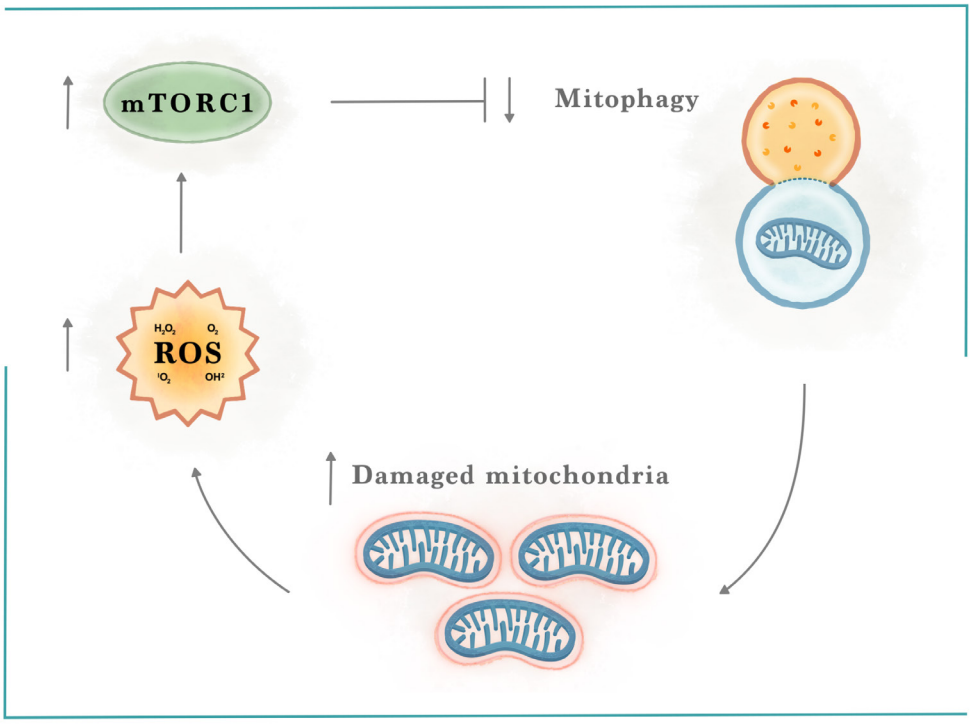

Figure 3. Hypothetical vicious cycle in malnutrition enteropathy.

3. The implications of disruption of mTOR, autophagy and mitochondria for intestinal epithelial regeneration

Intestinal stem cells (ISCs) facilitate rapid and continuous turnover of intestinal epithelial cells (IECs), which is essential for the integrity and functionality of the intestinal epithelium. In the intestinal stem cell niche, different factors such as Paneth cells and growth

factors (e.g. Wnt, Notch signalling) protect stem cells and coordinate their dynamics[96]. Accumulating evidence indicates that cellular metabolism is another key regulator of ISCs activation and differentiation[97,98]. As mTOR, autophagy and mitochondria are all critical regulators of cellular metabolism, disruption in any of these processes may result in decreased intestinal epithelial regeneration and thereby compromise intestinal function. The role of each of these regulators in stem cell maintenance and differentiation will be discussed and placed in the context of the findings from both preclinical malnutrition models.

The role of mTORC1 in proliferation and differentiation of ISCs

The question is what the consequences of mTORC1 hyperactivation are for stem cell proliferation and differentiation in our model. It has been observed that responses of mTORC1 were opposed in ISCs and Paneth cells under calorie restriction. Reduced mTORC1 activity in Paneth cells resulted in activation of mTORC1 in ISCs and fostered an increase in ISC numbers[99,100]. mTORC1 inhibition with rapamycin blocked ISCs expansion, indicating that the mTORC1-S6K1 axis is a prerequisite for ISC expansion[100]. Two other studies showed that mTORC1 enhanced the proliferative activity of stem cells in intestinal crypts in the small intestine and colon[101,102]. In the mouse model, mTORC1 activation in the small intestine of LPD-fed mice coincided with significantly decreased villous height. This indicates reduced stem cell proliferation, which has been reported in the same malnutrition mouse model[103]. Reduced proliferation in spite of mTORC1 activation could be explained by insufficient exogenous protein supply to sustain proliferation. Also, the impact of malnutrition on mTORC1 activity could be disparate in the different intestinal cell types (e.g. low mTORC1 activity in ISCs). Although I did not directly examined this, if increased mTOR1 activity also extended to Paneth cells this could negatively impact ISCs proliferation. In amino-acid deprived organoids, we found that low mTORC1 activity coincided with crypt atrophy, which suggests a reduction in stem cell proliferation, and conversely increased mTORC1 activity upon amino acid re-supplementation aligned with crypt re-growth (**chapter 6**). Interestingly, gene expression of stem cell and proliferation markers were not significantly affected in starved organoids, but these markers were significantly increased in starved organoids that were re-supplemented with amino acids. It needs to be assessed if these observations translate into changes at the protein level. In addition, mTORC1 was not measured for the different cell types. The impact of starvation on mTORC1 activation in the different epithelial cell types such as ISCs and Paneth cells can be further studied in intestinal organoids.

Studies have shown that mTORC1 activity is silenced as IECs differentiate along the crypt-villus axis[104–107]. mTORC1 signalling pathway has an important role in the regulation of goblet and Paneth cell differentiation in mice; (hyper)activation of mTORC1 decreased goblet and Paneth cell numbers, whereas mTORC1 inhibition promoted differentiation into the secretory lineage[99,105,108]. This is in agreement with the observation that the number of goblet cells and Paneth cells were decreased under hyperactivated mTORC1 in low-protein fed mice and increased upon mTORC1 inhibition with rapamycin (**chapter 5**). In intestinal organoids, amino-acid deprivation upregulated the expression of markers of Paneth cells (lysozyme), goblet cells (mucin-2) and enteroendocrine cells (chromogranin A), suggesting increased epithelial cell differentiation. It is important to further investigate if these changes translate to the protein level.

The role of autophagy in proliferation and differentiation of ISCs

We found signs of decreased autophagy in malnourished mice, but how does this affect stem cell proliferation and differentiation? Emerging evidence indicates that autophagy is a key player in stem cell activation, differentiation, self-renewal and quiescence in different types of stem cells[109]. In intestinal crypts, autophagy is constitutively active at a steady state[110,111]. The importance of intrinsic autophagy in the maintenance of ISCs derives from various animal models. In mice lacking the autophagy gene Atg5 in IECs, the number of Lgr5+ ISCs was substantially decreased, ROS was higher in the crypts and damage-induced ISC-dependent intestinal regeneration was impaired[111]. In mice lacking autophagy gene Atg7 in IECs, ROS levels were also higher in the crypts and regeneration following irradiation was compromised. Although these mice displayed enhanced loss of Lgr5+ ISCs through apoptosis, the number of these stem cells was not reduced as ISC death was correlated with amplification of the Lgr5+ ISC pool[112]. In line, in Drosophila it was shown that autophagy was required to sustain ISC proliferation and preserve the ISC pool[113]. These studies validate that intrinsic autophagy is important in ISC maintenance and intestinal regeneration. In both preclinical models, reduced autophagy activity can concern the ISCs, increase ROS levels and compromise ISC proliferation. Reduced ISCs proliferation was found in this animal model[103], but the role of autophagy in this process has not been studied. To study this, the malnutrition mouse model could be combined with intestinal organoids. Findings from the intestinal organoids suggest that insufficient basal autophagy may be involved in disturbed ISCs proliferation in severe malnutrition. We found that reduced autophagy in starved organoids aligned with crypt atrophy, while control and re-supplemented intestinal organoids had crypt growth and higher autophagy

levels (**Figure 2**)(**chapter 6**). Future studies should use intestinal organoids to further investigate if insufficient intrinsic autophagy in intestinal crypts plays a role in reduced ISCs proliferation and thereby leading to villus atrophy. Measurements of autophagy or autophagic flux, cell death and ROS production can be measured specifically in Paneth cells and ISCs, and can be validated in the malnutrition mouse model.

Although changes in structure and function of differentiated epithelial cell types have been reported in relation to deficient autophagy in the intestine[114], very little has been described about the role of autophagy in the differentiation process of ISCs. It was shown in the guts of Drosophila lacking Atg16 that pre-enteroendocrine cells failed to mature into fully differentiated EE cells with proper secretory function[115].

The role of mitochondrial homeostasis on ISC proliferation and differentiation

What are the consequences of decreased mitochondrial number and dysmorphic mitochondria for stem-cell proliferation and differentiation in the malnutrition models? A growing body of evidence indicates that mitochondrial metabolism and mitochondrial morphology control ISCs activation and actively determine lineage commitment. In Lgr5+ ISCs mitochondrial OXPHOS activity is high and that this metabolic phenotype is required for proper ISC function[76,116–118]. Mechanistically, it was shown that oxidative phosphorylation can activate p38 MAPK via mitochondrial ROS signalling. p38 MAPK in turn acts as a switch between proliferation and differentiation[117,119]. Furthermore, mitochondrial signalling has been shown to affect Wnt signalling[120], which is one of the key pathways regulating the ISC niche[121]. To illustrate, intestinal-specific depletion of mitochondrial transcription factor A (*Tfam*) lowered mitochondrial respiration and subsequently resulted in decreased Wnt signalling[122]. Additionally, it was shown that mitochondrial ATP maintains endoplasmic reticulum homeostasis thereby sustaining Wnt signalling[123]. Yet, the metabolic transitions that drive differentiation of ISCs remain to be elucidated. Nevertheless, researchers revealed that different IECs have distinct metabolic properties with high rates of mitochondrial respiration in ISCs and enterocytes, while Paneth cells are more glycolytic[124]. In addition, the role of metabolism for proper differentiation was further supported by the finding that differentiation was skewed towards the secretory lineage when the intestinal crypt had a more glycolytic phenotype[125]. Furthermore, IEC-specific loss of Hsp60, the major chaperone of the mitochondrial matrix, decreased mitochondrial respiration, and simultaneously abrogated intestinal stemness and proliferation[126]. Moreover, selective

HSP60 deletion in Lgr5+ ISCs resulted in mitochondrial dysfunction with concomitant reduced Lgr5+ expression and differentiation into dysfunctional Paneth cells[127]. This is in line with the observation that reduction in mitochondrial marker proteins Hsp60 and Tom20 in starved organoids coincided with crypt atrophy. Besides metabolism, mitochondrial quality control has been shown indispensable for intestinal stemness and differentiation[76,126,128,129]. In Drosophila it was shown that mitochondrial fusion is required for IEC differentiation[129]. In mice and organoids mitochondrial fission has been shown indispensable for differentiation of stem cells into the secretory lineage (e.g. Paneth and goblet cells)[76], but not for differentiation into the absorptive enterocyte lineage. Further work should focus on investigating the impact of severe malnutrition on mitochondrial function and dynamics both in malnourished mice as well as in intestinal organoids. Intestinal organoids are suitable to study if 1) mitochondrial dysfunction occurs in severe malnutrition, and 2) how this affects ISC proliferation and differentiation.

In conclusion, autophagy, mitochondria and mTORC1 have important roles in regulation of ISC proliferation and differentiation into the different IECs. Data from both preclinical models suggests that dysregulation of these processes may be involved in the impaired regeneration of the intestinal epithelium (e.g. villous atrophy) in severely malnourished children. Both preclinical models are equipped to further study these processes in malnutrition enteropathy. Furthermore, these malfunctioning processes and the risk factors for inpatient mortality from part I can serve as potential therapeutic targets. The implications of these findings for (new) therapeutic interventions and potential policy changes will be discussed in the next section.

III - Directions for future therapies and suggestions for potential policy changes

The findings from the clinical studies (Part I) indicate that intestinal dysfunction has a key role in mortality in severely malnourished children. Low faecal SCFAs and systemic inflammation were directly associated with death. Impaired intestinal barrier function and compromised epithelial integrity play an important part in systemic inflammation. In the preclinical models, we discovered that the key pathways mTORC1 and autophagy, and mitochondrial homeostasis were affected in malnutrition enteropathy (Part II). These findings provide opportunities to explore novel interventions to improve malnutrition enteropathy and thereby improve survival. In the following sections, the therapeutic potential will be discussed of 1) increasing intestinal SCFA levels and 2) reducing systemic inflammation through improving intestinal barrier function and structure.

1. Interventions to restore intestinal SCFA levels

Microbiota dysbiosis may have a central role in the risk of death during admission. Lower microbial metabolites such as SCFAs can lead to intestinal inflammation and potentially favour translocation of pathogenic and commensal species across an impaired intestinal barrier (**chapter 2-4**).

Based on our findings, the potential positive impact of increasing SCFAs via direct supplementation or indirectly through activation of SCFAs-producing bacteria on reducing mortality deserves further exploration (**chapter 2**). SCFAs have various beneficial properties such as supporting epithelial cell proliferation, modulating the intestinal barrier and taking part in immune regulation[31]. Furthermore, SCFAs can affect mTORC1, autophagy and mitochondria. Reports on the impact of SCFAs on mTORC1 in intestinal epithelial cells have been contradictory as mTORC1 activity was reduced in mice treated with a mixture of SCFAs[130] while butyrate activated mTORC1 in vitro[131]. Studies indicate that SCFAs can stimulate autophagy[31,132]. Mitochondrial function can be improved by SCFAs through various mechanisms, which include reducing ROS levels, stimulating mitochondrial biogenesis and influencing mitochondrial dynamics (reviewed in [133]). This illustrates that SCFAs supplementation may have the ability to restore mTORC1, autophagy and mitochondrial homeostasis in severe malnutrition.

The use of SCFAs supplementation has not been tested in severely malnourished children. Animal studies have reported beneficial effects of oral SCFAs supplementation on the intestine. However, translation of these effects to the human situation is difficult in particular as delivering sufficient amounts of SCFAs to the large intestine is challenging[134]. Clinical studies have reported clinical and histological improvement in IBD patient when SCFAs were delivered via enemas[31]. Yet, in severely malnourished children, often with diarrhoea, another method is preferred. Therefore, intestinal SCFAs level restoration by microbiota modulation through the use of pre-, pro- or synbiotics may be more feasible.

Probiotics are defined as viable microorganisms that exert health benefits on the host when consumed or administered in adequate amounts[135]. Prebiotics are non-viable components that are able to alter the composition and/or activity of the intestinal microbiota leading to a positive effect on the host. Synergistic combinations of probiotics and prebiotics are referred to as synbiotics[135].

Two studies have demonstrated that probiotics and synbiotics tended to reduce mortality and improve clinical outcome in severely malnourished children. In Malawi,

there was a trend towards reduced mortality following hospital discharge when children received 4 probiotics and 4 prebiotics mixed with RUTF in comparison with standard RUTF only[136]. In Uganda, diarrhoea after hospitalization was reduced in the intervention group that received 2 probiotics daily for eight to twelve weeks[137]. Meta-analysis revealed that probiotics reduced mortality[138]. Alou et al. identified 12 bacterial species that are commensal in the gut microbiota of healthy infants with desirable properties for a probiotic mixture (e.g. SCFA production) to be used in severely malnourished children[139,140]. A similar approach could be used to determine the missing repertoire in diseased children compared to survivors. Million et al. proposed the healthy mature, predominantly anaerobic gut microbiota (HMAGM) as a protective factor against diarrhoea, and intestinal and systemic inflammation[141], exactly the risk factors that we identified in **chapter 2**. The ongoing HOPE-SAM trial might give more information about potential microbiota differences between deceased children and survivors[142]. This study aims to evaluate long- and short-term clinical outcomes in children with complicated severe malnutrition, and to investigate the role of enteropathy, gut microbiota and cellular immune function in the pathogenesis of severe malnutrition. Comparing gut microbiota of severely malnourished children that died to that of survivors and well-nourished controls, could lead to identification of differences in SCFA-producing species and guide microbiota-directed therapy.

Although implementation of SCFAs supplementation and microbiota-directed therapy has potential, it requires critical benchmarking of safety (e.g. small risk of invasive infections with probiotic use[143]) and efficacy in severely malnourished children in comparison to the current standard of care. In children with moderate acute malnutrition, the use of microbiota-directed complementary food (MDCF-2) was reported to be safe and to positively impact growth and gut microbiota repair[144]. Moreover, knowledge of the functional activities of other active microbial metabolites (e.g. tryptophan, secondary bile acids) on intestinal barrier function is gradually expanding (reviewed in [41]). New discoveries relevant for severe malnutrition could be tested in our preclinical models and inspire potential future treatments of severely malnourished children.

2. Interventions to reduce systemic inflammation via improved barrier function and intestinal structure

Systemic inflammation may be the result of bacterial translocation across an impaired intestinal barrier in severely malnourished children[5,39,40]. Interventions that are successful in repairing small intestinal mucosal damage could reduce systemic inflammation via

improved barrier function and improved intestinal regeneration. Our findings are in agreement with emerging evidence that persistent intestinal damage constitutes a threat to survival of hospitalized severely malnourished children[5,145]. To our knowledge, the TAME study is the first to test four interventions (budesonide, colostrum, N-acetylglucosamine and teduglutide) that specifically aim for mucosal recovery in severely malnourished children[146]. There is a different rationale behind each of these interventions. The corticosteroid budesonide is an anti-inflammatory drug. This and other anti-inflammatory drugs have been shown to improve the intestinal barrier in IBD[147,148]. Dampening aberrant immune activation in malnutrition enteropathy[40] may improve intestinal barrier function. Colostrum is the first liquid secreted from the mother's breast, which is rich in protein and growth factors. It has been demonstrated to strengthen the intestinal barrier in adults[149]. Along the same lines, N-acetylglucosamine, a natural amino-sugar present on the cell surface, could restore barrier function in CD[150]. Reduced metabolism of glycosaminoglycans has been reported in the intestinal mucosa of children with oedematous malnutrition[151], suggesting that supplementation of N-acetylglucosamine may be beneficial. Teduglutide, a glucagon-like peptide 2 (GLP-2) analog, stimulates villous growth through enhancing stem-cell proliferation and reducing apoptosis[152]. In patients with intestinal failure, this has been shown to improve nutrient absorption[153].

In both preclinical malnutrition models, the use of rapamycin had significant beneficial effects on intestinal barrier function (**chapter 5 and 6**). This suggests that mTORC1 modulation with rapamycin or its analogues (rapalogues) might serve as a promising therapeutic strategy to improve the barrier function in severely malnourished children. Along similar lines, and based on knowledge from other intestinal diseases, mitochondria are therapeutic prospects for improving intestinal barrier function in severe malnutrition.

mTORC1 as therapeutic target

Rapamycin and its analogues have been used to prevent organ transplant rejection and to treat autoimmune diseases and cancers[154]. Over the years, pharmacological modulation of mTOR has gained interest in various diseases including Alzheimer's disease, anti-aging and IBD. Limited studies report that the use of rapamycin was safe and effective in mucosal healing in children and adults with severe refractory IBD[155,156]. In addition, in a rare inflammatory disease with enteropathy (IPEX) the use of rapamycin was also safe and effective in improving the intestinal architecture[157]. The safety, tolerability and efficacy of rapamycin and other rapalogues largely depend on the disease, the dose,

the duration of the treatment and the patient population. The use of mTORC1 inhibitors such as rapamycin could be particularly valuable in early stages of in-hospital treatment of severely malnourished children. Improved mucosal healing could swiftly restore the intestinal barrier and reduce the mortality that is due to bacterial translocation (e.g. sepsis). In addition, nutritional recovery can be supported when rapamycin improves nutrient absorption as observed in the malnutrition mouse model. Furthermore, improved barrier function and mucosal healing may reduce the risk of relapse, as has been suggested in IBD patients[158]. The immunosuppressive effects of rapamycin and rapalogues can present a challenge in this patient population with a high burden of infections[154]. Yet, it also has been shown that in lower doses, as used in the setting of anti-aging, rapamycin can improve immunity in elderly and cancer patients[159,160]. Furthermore, studies reported that rapamycin reduced the risk of CMV infection in organ transplant patients[161], improved antipathogen immunity in mice[162] and protected aged mice against pneumonia[163]. This implicates that treatment with lower doses of rapamycin or rapalogues may be feasible provided that at that dose rapamycin exerts its beneficial intestinal effects. Importantly, natural products such as curcumin and indoles also inhibit mTORC1[164]. Many of these products have been shown to improve barrier function. Some of these natural products might be more readily available in low-resource settings and, if proven safe, they could be added to the rehabilitation diets. Comprehensive clinical studies are required to confirm the safety and efficacy of mTOR inhibitors in treating malnutrition enteropathy.

Mitochondria as therapeutic target

Perturbed mitochondrial health is a novel feature of malnutrition enteropathy and can potentially be targeted to lessen intestinal disease. Antioxidants have been proposed as independent or supportive treatment option in IBD, because of their beneficial properties and promising research results. Promising antioxidants in IBD include synthetic (e.g. N-acetylcysteine), natural (e.g. curcumin, resveratrol) and micronutrient antioxidants, such as vitamin C (reviewed in [165]). Data from a pilot study by Becker et al. suggest that restoring the antioxidative capacity by supplying cysteine equivalents in the form of glutathione or N-acetylcysteine aids in the biochemical and clinical recovery of children with severe edematous malnutrition[166]. No data is available for the potential positive impact of antioxidants on the intestine. Antioxidants that are not specifically targeted to mitochondria may not reach significant amounts in sites with high mitochondrial ROS production[167]. Antioxidant treatment could interfere with the physiological functions

of ROS production, such as signaling and anti-pathogen defense. Another option to reduce excessive ROS production and restrain the immune system is through improved clearance of dysfunctional or redundant mitochondria through induction of autophagy or more specifically mitophagy[168]. Furthermore, stimulating mitochondrial biogenesis, for example with SCFAs, can improve mitochondrial mass and capacity[133]. Future studies will have to demonstrate whether mitochondrial dysfunction indeed occurs in the intestine of severely malnourished children and what the underlying mechanisms are in order to decide which mitochondria-directed treatment has the most potential.

The therapeutic targets that were discussed above have potential in the treatment of malnutrition enteropathy. To translate these findings to patients, it will be necessary to investigate if mTOR, autophagy and mitochondria are also involved in malnutrition enteropathy in children. If this is the case, the readily availability of mTOR-related therapies will speed up the design of clinical trials.

Final remarks

The translational approach used in this thesis provides important insights into intestinal dysfunction in severely malnourished children. The clinical studies underscored that intestinal dysfunction likely persists during inpatient treatment. Mechanistic studies into the intestinal dysfunction in a mouse model and intestinal organoids revealed that dysregulation of mTOR, autophagy and mitochondria may be involved in malnutrition enteropathy. Connecting the models to acquire mechanistic insights into malnutrition enteropathy in a more efficient and targeted way will improve the discovery-to-intervention pipeline and could lead to novel treatments to reduce mortality.

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