The central role of mitochondrial health in malnutrition induced enterohepatic dysfunction

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Chapter 7. General discussion and conclusion
The aim of this thesis was to provide insight into the intestinal and hepatic pathophysiology of severe malnutrition, through a combination of clinical studies and pre-clinical studies using a murine model of severe malnutrition. By improving our understanding of the pathophysiology of severe malnutrition, I aimed to identify novel pathways that can be targeted to improve acute and long-term outcome in this population. The WHO estimated that 149.2 million children were stunted and 45.5 million children wasted in 2020. Mortality rates are 20 percent in ill children with severe malnutrition and 11 percent of these children dying post-discharge documented in a recent multinational cohort study. While great reductions have been made in mortality in all children under the age of five, from 93 per 1000 children in 1990 to 38 per 1000 children in 2021, it is unclear why mortality remains so high in severely malnourished children who develop an acute infectious illness, indicating a need for further research to improve their standard of care. A better understanding of the pathophysiological changes occurring in ill children with severe malnutrition is expected to lead to novel interventions that could reduce childhood mortality in this vulnerable population. This thesis identified mitochondrial health as an important factor in reducing the metabolic, intestinal and hepatic dysfunction associated with severe malnutrition. Mitochondrial numbers, but also dynamics and autophagy were found to contribute to the mitochondrial dysfunction seen in our malnourished murine model. Rapamycin, an activator of autophagy via inhibition of mTOR, improved intestinal and hepatic function in chapter 4 and 5 potentially through improved mitochondrial health. Liver-specific ablation of the bile acid receptor fxr in chapter 6 increased mitochondrial and peroxisomal numbers, as well as autophagy, improving hepatic β-oxidation and this was accompanied by reduced steatosis in our mouse model of malnutrition. The research in this thesis shows that promoting mitochondrial health could be a potential target to be incorporated into the treatment of ill children with severe malnutrition.

1. Intestinal dysfunction in severe malnutrition

1.1 The consequence of intestinal dysfunction in ill hospitalized children suffering from severe malnutrition

Diarrheal illness indicating gastrointestinal infections as well as other infections, such as pneumonia in severely malnourished children are highly associated with increased mortality. Children with severe malnutrition have been found to have small intestinal villous blunting, reduced intestinal mucosa and increased intestinal permeability. The loss of intestinal integrity and decreased surface area reduces absorptive function and is thought to lead to increased microbial translocation and thereby increased risk of systemic inflammation and sepsis, although direct evidence is lacking. An underlying hypothesis is that a vicious cycle of malnutrition and recurrent infections causes a shift in microbiota, increases inflammatory cytokines and intraepithelial lymphocytes promoting chronic inflammation and other signs of enteropathy including nutrient malabsorption.
In chapter 2 we systematically reviewed the literature on carbohydrate malabsorption in severely malnourished children. Carbohydrate malabsorption can lead to osmotic diarrhea when the intake of carbohydrates surpasses the absorptive capacity of the intestine. Our systematic review showed an increased occurrence of monosaccharide and disaccharide malabsorption in severely malnourished children compared to healthy controls which was associated with diarrhea. As both F75 and F100 treatment formulas have a high carbohydrate content, with F75 having 65% of their calories derived from carbohydrates, this raised the question whether the high carbohydrate content in F75 could worsen clinical outcomes in in-patient treated malnourished children. To address this, a randomized clinical control trial was carried out comparing the WHO recommended F75 formula to a reduced carbohydrate and lactose free F75 formula (mF75)\textsuperscript{18}. In this study, a reduced carbohydrate and lactose free F75 formula, compared to the original F75 formula, had no effect on the time to clinical stabilization, no effect on mortality or the occurrence of diarrhea\textsuperscript{18}. These findings are potentially explained by the fact that the modified F75 carbohydrate content could still be above the absorptive capacity of the intestine in these children, or that the carbohydrate malabsorption in this group is not severe enough to affect clinical outcomes. This second conclusion would be welcomed since that would mean that the current F75 formula has not endangered children’s recovery from severe malnutrition. To further address the question of carbohydrate induced osmotic diarrhea in children with severe malnutrition, another study should be performed using an even lower carbohydrate content F75 formula.

1.2 mTOR inhibition restores intestinal dysfunction in a murine model of severe malnutrition

In chapter 5 we studied intestinal integrity and absorption in our newly established low protein murine model of severe malnutrition. The low protein diet resulted in villous blunting, increased intestinal permeability and decreased glucose and lactose absorption, mimicking the changes seen in malnourished children. Other animal models using a low protein or calorie restriction diets also displayed similar phenotypes including villous blunting\textsuperscript{17,19-24}, increased intestinal permeability\textsuperscript{17,19-26}, and loss of intestinal microbiota diversity\textsuperscript{17,27,28}, and loss of goblet cells\textsuperscript{22,23,28}. The increased intestinal permeability is most likely due to a loss of tight junction proteins\textsuperscript{17,19}, which we also observed as well as a reduction in mucin (chapter 5). The loss of villous height and flattening of microvilli could affect the ability of optimal nutrient absorption, further contributing to the cycle of malnutrition. We were able to improve intestinal integrity and lactose absorption through treatment with the mTOR inhibitor rapamycin (chapter 5). Rapamycin treatment also improved intestinal mitochondrial morphology and numbers, which may be underlying the improved intestinal function. It is not clear exactly how mTOR inhibition improved mitochondrial function in chapter 5. Rapamycin has been shown to induce mitochondrial biogenesis through increased PCG-1α and mitochondrial transcriptional factor A (TFAM) after a two-week period of treatment in cardiomyocytes\textsuperscript{29}. In this study autophagy was also increased at two weeks, indicating that beyond biogenesis degradation of damaged mitochondria could also have contributed to the improved mitochondrial function\textsuperscript{29}. In chapter 5, rapamycin did not increase autophagy markers
LC3-II; however, as autophagy is an active flux this finding is inconclusive of the degree of autophagy. Another study by our group that looked at autophagic flux under low protein conditions showed decreased autophagy flux that increased with rapamycin and nicotinamide treatment in the intestine (Ling et al., in preparation). Mitochondrial health is important in intestinal function and mitochondrial dysfunction with subsequent increased ROS production has a central role in different intestinal diseases, such as, gastrointestinal cancers, enteric infections, inflammatory bowel disease, and ischemic intestinal injury\textsuperscript{30,31}. Excess levels of ROS damage intracellular components, such as cytoskeletal proteins, lipid membranes and DNA, the damages impairing intestinal epithelial function by a number of different effects, such as, a loss of tight junction proteins, reduced proliferation and differentiation, and increased cellular apoptosis\textsuperscript{30}. ROS also triggers migration of polymorphonuclear neutrophils and macrophages to the mucosa that causes tissue damage and further promote a state of inflammation\textsuperscript{31}. Both endogenous sources (mitochondria, the ER and peroxisomes) and exogenous sources (microbial signaling and drugs) of ROS can drive oxidative stress\textsuperscript{30,31}. Promoting mitochondrial health by increased mitophagy through SIRT1-PGC1α activation has been shown to restore intestinal barrier function by reducing oxidative stress levels and improving mitochondrial function in intestinal epithelial cells, restoring intestinal tight junction proteins\textsuperscript{32}. Rapamycin has also been shown to improve intestinal barrier function by increasing tight junction proteins and maintaining Paneth cell function through upregulated autophagy\textsuperscript{23,33}. Paneth cells are secretory cells that provide a stem cell niche in intestinal crypts, secrete antimicrobial peptides, and are crucial in intestinal homeostasis\textsuperscript{34}. Studies using intestinal biopsies have shown a reduction in Paneth cells in severely malnourished\textsuperscript{35} and stunted children\textsuperscript{36}, which has also been observed in postmortem biopsies\textsuperscript{37}. A loss of Paneth cells increases inflammation and reduces the intestines’ ability to regenerate\textsuperscript{38}, impairing the intestinal barrier function. But how exactly severe malnutrition depletes Paneth cells is unknown. In studies that look at acute intestinal inflammation in mice, Paneth cell dysfunction has shown to be caused by mitochondrial dysfunction and can be induced by inhibiting oxidative phosphorylation\textsuperscript{38}. While, a study on infectious intestinal inflammation has shown that impaired autophagy drives Paneth cells loss\textsuperscript{39}. It may be that the improved intestinal barrier function and mitochondrial numbers are both due to increased autophagy, or that restored mitochondrial function reduces oxidative stress, thereby restoring barrier function.

2. Hepatic and metabolic dysfunction in severe malnutrition

2.1 The central role of mitochondrial health in hepatic dysfunction in a murine model of severe malnutrition

The low protein murine model that we used in this thesis mimicked many of the phenotypic changes seen in children suffering from severe malnutrition. After the two-week low protein diet, mice presented with stunting, wasting, hepatic steatosis, hypoalbuminemia, fasting induced hypoglycemia, and cholestasis. In the pre-clinical chapters of this thesis, reduced mitochondrial health was shown to be an
important feature of malnutrition-induced cellular changes. In *chapter 4* where we focused on the effect of rapamycin on malnutrition induced hepatic dysfunction, we observed improved mitochondrial function as reflected by increased ATP levels and complex I protein levels, as well as reduced mitochondria inclusion bodies on EM and PINK1 protein levels. PINK1 is a marker for dysfunctional mitochondria and functions to mark mitochondria for mitophagic degradation. Rapamycin also improved hepatic function by reducing hepatic steatosis and improving fasting glucose levels (*chapter 4*). In *chapter 6*, we determined the role of FXR in malnutrition induced hepatic steatosis. We treated wild type and liver-specific FXR knock out mice with a FXR ligand and vehicle to determine if FXR activation is protective against low protein induced hepatic steatosis. Surprisingly, FXR ligand did not reduce steatosis in our murine model, possible due to the fact that under low protein conditions FXR activation did not cause a classical FXR genetic response. RNAseq data showed instead that inflammatory and pro-apoptotic pathways were increased by the ligand. That GW4064 did not result in a classical FXR hepatic gene response may be due the fact that this nuclear receptor is a homeostat, which depending on the nutritional state coordinates the body’s response from a fed to fasting state. In a fed state FXR suppresses fatty acid oxidation, VLDL secretion, gluconeogenesis, glycogenolysis, amino acid catabolism, while stimulating lipogenesis, glycogen synthesis and protein synthesis. Yet, in a fasted state FXR has shown to regulate these pathways in the opposite manner. Instead, hepatic *Fxr* ablation lowered hepatic steatosis and signs of inflammation, with increased mitochondrial and peroxisome numbers and improved β-oxidation. I believe that the improved β-oxidation was due to a loss of PPARα suppression, as shown by increased PPARα gene expression and its downstream marker CPT1 (a rate limiting enzyme for β-oxidation) gene expression. The loss of FXR has previously been shown to cause the upregulation of PPARα downstream markers, and FXR activation through bile acids, CA and CDCA, has shown to inhibit PPARα activation in mice. Our group has previously shown that the PPARα ligand fenofibrate reduces malnutrition induced hepatic dysfunction and steatosis through improved mitochondrial and peroxisomal function in a rat model. These chapters showed improved hepatic function under low protein condition through the clearance of dysfunctional mitochondria, as well as, through the increased numbers of mitochondria and peroxisomes. Damaged and dysfunctional mitochondria have been previously reported by our group using a malnutrition rat model and in biopsy samples, from both liver and intestine, of malnourished children in the 60s and 70s. In animal studies and in children suffering from severe malnutrition, oxidative stress has been found. Oxidative stress is an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and antioxidant defense, and the mitochondrial respiratory chain is a major source of ROS. ROS have an unpaired electron and are therefore damaging to cells and oxidative stress can cause mitochondrial dysfunction through mtDNA, protein and lipid damage, as well as, be a byproduct of mitochondrial dysfunction through impaired oxidative phosphorylation. Increased mitophagy has been shown to be protective against overproduction of ROS as it removes dysfunctional mitochondria that produce
excessive amounts of ROS. Severely malnourished children have reduced levels of antioxidants, such as glutathione and vitamin E, as well as increased levels of oxidative stress markers. Antioxidants reduce oxidative stress by neutralizing ROS, glutathione for example reacts with ROS and becomes oxidized into glutathione disulfide. In animal models, increased oxidative stress has been shown by reduced levels of hepatic thiobarbituric acid reactive substances and reduced levels of antioxidants. While these studies indicate mitochondrial dysfunction, in this thesis we take those speculations further by showing that impaired mitochondrial function is central in maladaptation of both enterocytes and hepatocytes to severe malnutrition.

2.2 Upregulation of autophagy clears dysfunctional mitochondria, improving cellular hepatic function
In chapter 4 we found that rapamycin treatment increased autophagic markers LC3-II and autophagosomes on EM images, which likely more effectively degraded dysfunctional mitochondria. To get more insight in the role of autophagy in malnutrition induced hepatic steatosis, we looked more closely at this process using the autophagy inhibitor chloroquine to quantify the autophagic flux in our model. Autophagy is an important process in catabolic states such as fasting, and has shown to be crucial in glycogenolysis, gluconeogenesis and β-oxidation, as well as, elective turnover of specific cargos. Its role in severe malnutrition, however, is not well known, as it is usually studied only after short term starvation and amino acid deprivation. In chapter 6 we observed an impaired autophagic flux in the low protein fed wild type mice, which was also observed by Hu et al. and thought to be caused by low Sirtuin 1 (SIRT1) levels. SIRT1 induce autophagy through direct deacetylation of autophagy related genes and LC3, and by inhibition of mTOR through AMPK activation. In chapter 6 the low protein diet reduced autophagic gene expression in the wild type mice compared to the control diet fed wild type mice; however, we did not look further into the underlying pathway that caused this reduction. FXR has been shown to impair autophagy on both a transcriptional and post transcriptional level, however, FXR gene expression was reduced in the low protein fed wild type mice and downstream FXR targets did not differ compared to the control diet. However, in the FXR hepatic knock out mice we observed an increased autophagic flux compared to wild type mice, indicating that FXR somehow suppresses autophagy in our malnutrition model. This increased autophagic flux could facilitate increased turnover of dysfunctional mitochondria and peroxisomes, but also that the loss of PPARα suppression by FXR allows for increased mitochondrial and peroxisomal biogenesis further improving cellular health. The reduction in hepatic steatosis by the loss of hepatic Fxr may partly be due to increased lipophagy. Hu et al. also reported that increased autophagy and mitochondrial biogenesis by nicotinamide (NAM) treatment, through the SIRT1-PGC-1α pathway, improved mitochondrial function under low protein conditions in mice. As previously stated, the oxidative stress activates mitophagy to reduce ROS formation; however, autophagy is not only a response to mitochondrial dysfunction but healthy mitochondria are also needed for autophagy. Thomas et al. showed that a defects in complex I suppresses autophagy, and in chapter 4 we observed
a reduction complex I protein levels on the low protein diet that may have contributed to an impairment in autophagy. It is also known that a mitochondrial oxidative phosphorylation stimulates autophagy, while a mitochondrial promotion of aerobic glycolysis inhibits autophagy, further showing the intricate relationship between mitochondria and autophagy. Mitophagy is crucial in the maintenance of healthy mitochondria, especially in conditions of high stress. Mitophagy and mitochondrial biogenesis are two interrelated processes and AMPK activation of mitophagy also stimulates mitochondrial biogenesis through PGC-1α stimulation, which is also activated by the mitophagic protein Parkin.

2.3 A block in TCA cycle further indicates mitochondrial dysfunction in ill children with severe malnutrition and in pre-clinical models

In chapter 3 urine metabolomic analysis revealed that children who died from severe malnutrition had increased lactic and succinic acid levels, again indicating mitochondrial dysfunction. Lactic acid is reduced from pyruvate under anaerobic conditions, while in an aerobic setting pyruvate enters the TCA cycle. A mitochondrial dysfunction can impair the utilization of pyruvate and aerobic glycolysis can produce lactic acid in aerobic conditions. The most common laboratory abnormality in children with mitochondrial disease is lactic acidosis, as the dysfunction in the electron transport chain causes a buildup of pyruvate that is converted to lactate. Increased succinic acid could be caused by impairment in TCA complex II (succinate dehydrogenase) that converts succinate to fumarate. Ischemic injury has shown to cause accumulation of succinate due to anaerobic ATP production due to mitochondrial dysfunction, and accumulation of succinate has also shown to drive mitochondrial ROS production.

In line with our findings, Teran-Garcia et al. also reported increased urine levels of both lactic and succinic acid amongst other metabolites, which indicated a potential impairment in mitochondrial function. This has also been supported by a large case-control cohort study by Wen et al. from our group, that compared plasma metabolic and proteomic profiles between children who died from complicated severe malnutrition and children who survived. The children who died had increased metabolites from the β-oxidation and the TCA cycle pathways including pyruvate, fumarate, α-ketoglutarate, succinate, and acetylcarnitine. Bartz et al. reported similar findings of impaired fatty acid oxidation and buildup of acylcarnitines, and that fatty acid metabolism has a crucial role in the response to severe malnutrition. In chapter 4 in our murine model of malnutrition, we found an increase in other TCA metabolites citrate and isocitrate, in hepatic tissue, also indicating a block in the TCA cycle. While in both our human urine metabolic study (chapter 3) and our murine one-carbon metabolomics analysis (chapter 4) we saw buildup of TCA metabolites, the specific metabolites were different between the studies. This may be due to a difference in urine vs hepatic tissue analysis, or a different species response to severe malnutrition. Metabolomics is a snapshot of a metabolic profile and while plasma concentration of a metabolite influences its concentration in urine, other factors also influence the levels of a metabolite in urine, such as glomerular filtration, tubular reabsorption, and the time since last urination. To analyze urine metabolites more accurately, a 24-hour urine collection can
be carried out. Citric acid urine excretion is, for example, decreased under starvation through increased activity of citrate transporters in the kidneys causing lower levels in the urine compared to plasma. Another influencing factor is renal function, which is thought to be impaired in severely malnourished children partially through dehydration and pre-renal dysfunction, as well as, through glomerular filtration and tubular dysfunction. In diabetic induced chronic kidney disease increased levels of TCA metabolites fumarate and malate in urine can be used as a marker of renal dysfunction, as they are reabsorbed by healthy kidneys. These factors need to be kept in mind when interpreting urine metabolic findings, showing that hepatic or plasma metabolites may give a more accurate depiction of metabolic profile. In chapter 5 we observed increased plasma levels of the amino acids glycine, serine and histidine in our murine model. This was in line with human results by Wen et al., reporting increased levels of serine in children who died from severe malnutrition. Pyruvate can be converted to serine and then glycine, which is then used to produce glutathione to reduce ROS. However, it is the conversion of cysteine and glutamate to γ-glutamylcysteine that is the rate limiting step in glutathione production, and not the following step of conversion of γ-glutamylcysteine and glycine. Jahoor et al. observed lower levels of plasma cysteine in edematous malnutrition, as well as decreased glutathione levels and synthesis rates, but not in severe wasting. Glycine levels and synthesis has not shown to be reduced in severely malnourished children compared to their recovered state. The metabolomic analysis in these chapters thus supports the hypothesis of mitochondrial dysfunction in the pathophysiology of severe malnutrition.


To try to answer the above question, I will first discuss the mitochondrial response to a fasted state, followed by a theory of how mitochondrial dysfunction in ill children with severe malnutrition, and then discuss the potential mitochondrial agents that could be given in a low-income setting to try to alleviate the mitochondrial dysfunction.

3.1 Mitochondrial response to a nutrient deprived state

Mitochondrial health is crucial for metabolic flexibility, the ability to adapt to metabolic changes, such as transitioning from a fed to fasted state. During nutrient deprivation the energy sensor AMP-activated protein kinase (AMPK) is activated and causes phosphorylation and inactivation of Drp1 which in turn promotes mitochondrial elongation (Figure 1). Mitochondrial elongation allows mitochondria to avoid mitophagic degradation and can lead to increased ATP production and mitochondrial DNA exchange, as well as breaking-off dysfunctional components of the mitochondria for degradation. Mitochondria also undergo changes in acetylation dependent on the NAD⁺ dependent deacetylase sirtuin-3 (SIRT3), which under fasting conditions promotes fatty acid oxidation and utilization of lipids as the primary source of acetyl-CoA. This process also stimulates ketogenesis, as well accelerates amino acid
catabolism for utilization in the TCA cycle, and protects against ROS\(^87\). It is disputed if nutrient deprivation stimulates mitochondrial biogenesis\(^85\), as some studies show an improvement in mitochondria is due to dynamic changes, increased turnover through mitophagy, and reduced ROS formation\(^85,88\). The endoplasmic reticulum (ER) also plays an important role in metabolic flexibility\(^89\), and through mitochondrial associate membranes (MAMs) the mitochondria and ER can coordinate their responses to changes in nutrient availability\(^90\). ER-mitochondrial interactions increase during low nutrient availability and reduces with increased glucose intake, leading to increased mitochondrial fission and reduced respiration\(^91\). High fat diet studies demonstrated high levels of mitochondrial fission with reduced levels of the fusion protein Mfn2, which is crucial for the docking of mitochondria to the ER to allow for ER-mitochondrial communication\(^85\). Metabolic flexibility of cells has mostly been studied in short-term fasting studies and increased nutrient intake, it is unknown exactly how a state of chronic low nutrient intake effects mitochondrial function.

3.3 A theory hypothesis that explains how chronic malnutrition impairs autophagic flux through blocked fission, hindering the clearance of dysfunctional mitochondrial

The results of this thesis support the hypothesis that changes in mitochondrial dynamics and autophagy play a role in the observed mitochondrial dysfunction. One can speculate that there is a build-up of dysfunctional mitochondria due to the chronicity of malnutrition in these children. Low nutrient availability causes mitochondria to elongate, which initially improves ATP production as the elongation increases oxidative phosphorylation\(^92\). The elongation is initiated by phosphorylation of fission protein Drp1 at S637, making mitochondrial fusion protein Mitofusin 1 (Mfn1), Mfn2, and Optic atrophy 1 (Opa1) unopposed\(^86\) causing outer and inner membrane fusion\(^92\). This elongation protects mitochondria from autophagy that is upregulated in a state of nutrient scarcity, allowing other cellular components to be prioritized for recycling while maintaining energy homestasis\(^86,93\), as well as, allowing mitochondrial membrane to be used for autophagosome biogenesis\(^94\). Mitochondria need to continuously go through

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**Figure 1. Schematic illustration of mitochondrial response to nutrient deprivation.** In step 1 low levels of nutrients activates AMPK which inhibits Drp1 allowing for Mfn2 fusion (step 2.). This causes mitochondrial elongation (step 3) that allows for increased ATP production and mtDNA exchanged, as well as, budding off of dysfunctional parts of mitochondria (step 4) destined for mitophagy (step 5).
quality control and fission is needed to split off dysfunctional parts of mitochondria for degradation, so that the healthy part of the mitochondria can survive\(^a\). Studies show that fission is a prerequisite for mitophagy and inhibition of this results in the accumulation of dysfunctional mitochondria\(^b\). In this thesis I show that a low protein diet causes a loss in mitochondria and an accumulation of dysfunctional, elongated mitochondria, which express PINK1 and contain increased mitochondrial inclusion bodies. PINK1 only accumulate in mitochondria that are severely depolarized and recruits Parkin for autophagic elimination\(^c\). It is unclear exactly how these elongated mitochondria become dysfunctional in our model, but I speculate that the low protein condition hinders the necessarily quality control of mitochondria through suppression of Drp1 fission due to low SIRT1 (as observed by Hu et al.\(^d\)). Since PINK1 and Parkin are able cause the ubiquitination and proteasome degradation of Mfn1 and Mfn2, it is unlikely that fusion proteins hinder fission\(^e\). The lower mitochondrial numbers in the low protein fed mice is most likely due to a loss of mitochondrial biogenesis as shown by Hu et al.\(^f\), autophagic flux has shown to be impaired in the low protein group (chapter 6)\(^g\). In a highly stressed cellular state mitochondrial fission through Drp1 has shown to induce cellular apoptosis, which may be why blocking fission is beneficial in this low protein setting\(^h\) which with time becomes unbenevolent to the cell. In acute starvation fission is inhibited though increased cAMP levels and subsequent activation of protein kinase A that causes the phosphorylation of Drp1\(^i\). A previous study in low protein fed rats showed decreased hepatic cAMP levels\(^j\). SIRT1 activation by NAM-treatment in cells have shown to increase Drp1 fission through AMPK activation and subsequent cAMP/PKA pathway induction causing an increase in mitophagy\(^k\). However, while NAM treatment in our low protein model restored

1. Nutrient \(\rightarrow\)
2. Upregulated fusion
3. Elongation

AMPK
Drp1
\(\beta\)-oxidation
ATP

6. Enterohepatic dysfunctional

5. Dysfunctional mitochondria
4. Impaired fission

cAMP
PINK1
ROS

Figure 2. Schematic overview of hypothesis behind malnutrition induced mitochondrial dysfunction. In step 1 low levels of nutrients activates AMPK which inhibits Drp1 allowing for fusion (step 2.). This causes mitochondrial elongation (step 3) that allows for increased ATP production and mtDNA exchanged and avoidance of autophagic degradation. Increased cAMP levels impair Drp1 fission (step 4), blocking mitochondrial quality control and the budding off and degradation of dysfunctional mitochondria. Buildup of dysfunctional mitochondria (step 5) leads to enterohepatic dysfunction (step 6).
mitochondrial numbers and function, it did not upregulate autophagy\textsuperscript{53}. Potentially NAM upregulation of fission allowed for earlier mitochondrial quality control, budding off and proteasomal degradation of the smaller dysfunctional mitochondrial component. To understand the role of fission in malnutrition induced hepatic dysfunction, we first need to know the mitochondrial dynamical changes occur in low protein conditions. It would be important to compare mitochondria function, numbers, morphology and expression of fusion-fission proteins at weaning, one week into the diet and after two weeks of the diet. Thereafter, a genetic hepatic overexpression of Drp1 model on a low protein diet could be used to test the beneficial effects of upregulating fission in our model.

3.4 Mitochondrial targeted treatment therapies in ill malnourished children

In chapter 4 and 5 we used Rapamycin as an intervention in treating malnutrition induced enterohepatic dysfunction. In both chapters we observed that Rapamycin’s beneficial effects were through improved mitochondrial health. While these studies provided insight into potential areas of intervention, Rapamycin might not be a suitable treatment in critically ill malnourished children. Rapamycin triggers diverse metabolic effects and is an immunosuppressant, which could be dangerous in this patient group that already often suffers from recurrent infections and are already immunosuppressed\textsuperscript{98}. However, a pilot study by Jones et al.\textsuperscript{99} used an immunosuppressant to reduce the intestinal inflammation seen in environmental enteric dysfunction in severely malnourished children. This treatment reduced inflammatory markers and did not have adverse effects in severely malnourished children\textsuperscript{99}. Other mTOR inhibitors have similar broad effects on a diverse set of metabolic functions. In chapter 6 we explored the role of FXR in malnutrition. In our malnutrition model, however, FXR activation did not reduce hepatic steatosis, and this may be due to the fact that FXR is a homeostat that, depending on the nutritional state of body, has different functions, coordinating the body’s response from a fed to fasting state\textsuperscript{40}. FXR activation is also known to reduce inflammation in NAFLD, yet our transcriptome analysis showed an upregulation of inflammatory pathways in low protein fed wild type mice receiving the FXR ligand. We instead observed that hepatic Fxr ablation was beneficial in our murine model, suggesting that FXR antagonism may have therapeutic value in malnutrition. FXR antagonists have been less extensively researched than FXR agonists, but have been shown to be beneficial in the treatment of type 2 diabetes mellitus through the suppression of gluconeogenesis\textsuperscript{100} or improved insulin sensitivity\textsuperscript{101}. Different FXR antagonists have been reported including natural ligands such as, α- and β-Muricholic acids (MCA) and guggulsterone, or synthetic ligands such as Ivermectin (a drug used for parasite infection)\textsuperscript{102}. The suppression of gluconeogenesis would increase the risk of hypoglycemia in severely malnourished children, making it less ideal as a treatment option in this population. As we believe that the beneficial effects of hepatic Fxr ablation is due to a loss of PPARα suppression, a more logic therapy would be PPARα stimulation. Fenofibrate is a PPARα agonist that has been used in a rat model of severe malnutrition\textsuperscript{43}, which improved peroxisome numbers and mitochondrial morphology while restoring hepatic ATP content and increased fatty acid oxidation and
reducing hepatic steatosis. Activation of the PPARα target PGC-1α by nicotinamide under low protein condition, has also been shown to increase mitochondrial amount and function through upregulated biogenesis and mitophagy, leading to an improved metabolic function.

Next, I will discuss three mitochondrial health promoting therapies that could be considered for use in a low-income setting: fibrates, α-lipoic acid and MitoQ/ Idebenone. Fibrates are a group of drugs that target PPARα and are known to lower plasma triglyceride and LDL levels, while increasing HDL. They have an essential role in glucose homeostasis, insulin sensitivity, lipid uptake and fatty acid oxidation. Fibrates stimulate mitochondrial biogenesis through the PPAR-PCG-1α axis and thereby improve β-oxidation, as well as induce autophagy and reduce lipogenesis. Bezafibrate is a fibrate that has been extensively studied in pre-clinical and clinical trials, with a well-known safety profile and low cost. Chapter 6 showed that the loss of hepatic FXR reduced hepatic steatosis through improved beta oxidation due to increased PPARα expression. Therefore, a fibrate is expected to be beneficial in reducing the malnutrition induced hepatic steatosis seen in our murine model of severe malnutrition. This is further supported by a previous study by our group in a malnutrition rat model, where fenofibrate alleviated malnutrition induced hepatic dysfunction through restoring mitochondrial and peroxisomal health. Two common side effects that may be harmful specifically in this patient population is decreased appetite and increased diarrhea.

A second potential drug for treatment of ill children with severe malnutrition that could be used in a low-income setting is α-lipoic acid, another mitochondrial health promoting compound. It is essential for pyruvate dehydrogenase and ketoglutarate dehydrogenase and is a potent antioxidant that has been shown to decrease oxidative stress and been used as a treatment in mitochondrial diseases. α-lipoic acid acts as a free radical scavenger, promotes endogenous antioxidant (GSH, vitamin E and C), and has been shown to be beneficial in diabetes, high blood pressure, obesity, neuropathy and multiple sclerosis. It, however has a low bioavailability, but this can be improved by providing it in a liquid form, which would be easier to combine in the F75 formula. Other studies have shown increased oxidative stress in both animal and human studies of severe malnutrition. In chapter 6 we observed an increase in the unfolded protein response in the wild type mice on the low protein diet, potentially indicating increased oxidative stress in our model. A pilot study by Becker et al. compared α-lipoic acid and glutathione supplemented treatment to standard WHO treatment in children with nutritional edema, and reported an increase in survival in the children receiving the antioxidants. One recorded adverse event of α-lipoic acid that may be dangerous for our patient population is hypoglycemia.

Finally, MitoQ or CoenzymeQ (CoQ) or CoQ₁₀ is a third treatment option to promote mitochondrial health in a low-income setting. MitoQ acts as an electron carrier in the mitochondria respiratory chain improving electron flow, and also has antioxidant effects by reducing ROS and inhibiting HIFα induction. MitoQ accumulates in the mitochondria and also improves lipid peroxidation.
Idebenone, an analogue of MitoQ/CoQ₁₀, also promotes mitochondrial health and has been tested in multiple clinical trials for Friederich’s ataxia (FRDA), Duchenne muscular dystrophy (DMD), Alzheimer’s disease, and has been approved by the European Medicine Agency for the treatment of Leber’s hereditary optic neuropathy (LHON). In chapter 4 we observed that an increase in mitochondrial respiratory complex I and hepatic ATP levels was associated with reduced hepatic dysfunction. This may indicate that improving the mitochondrial respiration is a potential area of intervention. The adverse effects reported on MitoQ have been small with light gastrointestinal discomfort being the most common.

4. Fundamental science has an important part in global health research

4.1 The need for more pre-clinical studies in understanding malnutrition induced mitochondrial dysfunction

To be able to select a suitable mitochondrial intervention, more pre-clinical studies are needed to better understand malnutrition induced mitochondrial dysfunction and its progression. We do not know if the impaired β-oxidation is the origin of mitochondrial dysfunction or if mitochondrial dysfunction causes impairment in β-oxidation. In patients with inborn fatty acid oxidation defects an impairment in β-oxidation causes an accumulation of long chain carnitines and fatty acids subsequently causing mitochondrial dysfunction through increased oxidative stress and respiratory chain inhibition. However, we speculate that it is an impairment in mitochondrial function that causes the impairment in β-oxidation. Murine models of impaired fatty acid oxidation, such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency or medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, would unlikely survive the low protein diet as even acute fasting is usually lethal in these mice. We therefore need to look more specifically at mitochondrial function in our murine model by isolating mitochondria and performing respirometry to look at both electron transfer chain and β-oxidation. Mitochondrial oxidative capacity has been measured in our low protein murine malnutrition model by Hu et al., which showed a reduced capacity with decreased expression of genes involved in β-oxidation. The actual β-oxidation capacity, however, was not measured in that study. We are currently carrying out a study that looks at PPARα activation in our murine model of severe malnutrition. With this study we will test whether a PPARα ligand, by stimulating β-oxidation, is able to improve mitochondrial function under low protein conditions and reduce malnutrition induced hepatic dysfunction. We also need to understand how the mitochondria in severely malnourished children respond to refeeding. Pre-clinical studies are needed to understand other aspects of the pathophysiological changes associated with malnutrition. It is too costly and unethical to carry out treatment studies in such a vulnerable patient population without having a solid foundation of pre-clinical studies. This insight can then be translated in to human clinical trials to test if the promotion of mitochondrial health can aid severely malnourished children in their recovery. Our lab is part of a
collaboration called the CHAIN network that aims to identify biological mechanisms and socio-economic factors that determine a child’s risk of death from malnutrition\^{118}. This network has clinical sites in Pakistan, Bangladesh, Kenya, Uganda, Malawi, and Burkina Faso that allows for the quick implementation and clinical trials that can determine the effect of promoting mitochondrial health in children being treated for complicated severe malnutrition. This type of translational research is ideal and greatly reduces the time from bench to bedside. An example of this is the carbohydrate malabsorption review published in this thesis that was the foundation for the randomized control trial that compared a low carbohydrate and lactose free modified F75 to the original F75.

5. We should never not be talking about prevention

5.1 The complex challenge of malnutrition prevention

As childhood malnutrition is a complex challenge that is caused by a combination of environmental, social and medical risk factors, its prevention also requires a combination approach\^{78}. To better understand the combination approach to prevention the UNICEF Framework can be used, which was described in the introduction section (Figure 1). The main underlying causes of malnutrition are inequality and poverty, with income inequality most prominently increasing the risk of malnutrition but all forms of marginalization and societal exclusions also contributing\^{119}. This falls under enabling determinants, which are built up of good governance, sufficient resources and positive social and cultural norms. These work together to promote good maternal and child health on a fundamental level. Income inequality is a measure of how unequally income is distributed in a population, and it has been growing in most countries since the 1990s\^{120}. In 2021 the top 10% had 52% of the world’s income, while the bottom 50% had 8.5\%\^{121}. The world social report 2020 highlights the need to reduce inequality by promoting access to opportunity, pursue more progressive taxation and strengthen social protection systems, and tackle prejudices and discrimination to ensure equal participation\^{120}. These big social undertakings are important for promoting health as income inequality is strongly correlated with under the age of 5 child mortality and stunting\^{122}. This thesis does not focus on the strategies used to reduce income inequality, but instead focuses on the next level of the UNICEF Framework, which are the underlying determinants. It is important to keep in mind that most reduction in childhood malnutrition is due to a combination of a country’s financial growth, as well as promoting public sector programs that stimulate good nutritional practice and social factors such as access to healthcare and improved sanitation\^{78}.

5.2 Malnutrition prevention programs that have shown to be effective in reducing childhood malnutrition

Underlying determinants of the UNICEF Framework are made up of food, feeding practices and adequate services. To ensure good maternal and child nutrition adequate health, education, sanitation
and social protective programs are required\textsuperscript{123}. This is necessary to inform mothers of appropriate feeding and dietary practices, as well as, hygiene and food preparation. This includes interventions for newborns such as delayed cord clamping, breast feeding within the first hour of life, and kangaroo mother care for low birth infants\textsuperscript{124}. For infants and young children, it includes breastfeeding support, nutritional counselling for complementary feeding, high dose vitamin A supplementation, zinc treatment with diarrheal disease, and micronutrient and iron supplementation\textsuperscript{124}. Among the underlying determinant subsection food, proven interventions include age appropriate and nutrient rich food, clean drinking water, and exclusive breastfeeding until 6 months, as well as, iron and folic acid supplementation to adolescent girls to be continued until and during pregnancy\textsuperscript{124}. Protein and calcium supplementation have also shown to be important in reducing malnutrition in mothers\textsuperscript{124}. These are interventions that have shown to reduce childhood malnutrition and need to be prioritized to accelerate the reduction in child malnutrition\textsuperscript{125}. While promotion of household wealth, health care, and parental education have been shown to be important in most countries when it came to reducing stunting, other factors differed greatly between countries\textsuperscript{125}. These differences highlight how very different policies and priorities can all reduce childhood malnutrition, such as hygiene interventions, focus on maternal nutrition, or investments in reproductive health, but also that there isn’t a one fit all model to eradicate it\textsuperscript{125}. It is thus important that countries select their own specific strategies to address child malnutrition, tailored towards their geography and unique social and societal complexities.

6. Concluding remarks

Severe malnutrition in children is a global burden that requires a multifactorial approach to prevention as well as treatment to reduce the childhood mortality. While we have an incomplete understanding of the pathophysiological changes in ill children with severe malnutrition, this thesis has shown the importance of mitochondrial health in reducing the associated metabolic, intestinal and hepatic dysfunction. Mitochondrial numbers, but also dynamics and autophagy were found to contribute to the mitochondrial dysfunction seen in our malnourished murine model. The metabolomics study also supported the hypothesis of impaired mitochondrial function by increased urine lactic and succinic acid levels. Rapamycin, an activator of autophagy via inhibition of mTOR, improved intestinal and hepatic function, potentially through improved mitochondrial health. Liver-specific ablation of the bile acid receptor \textit{fxr} increased mitochondrial and peroxisomal numbers, as well as autophagy, improving hepatic \(\beta\)-oxidation and this was accompanied by reduced steatosis in our mouse model of malnutrition. Therefore, a new area for potential intervention is alleviating enterohepatic dysfunction in malnutrition is promoting mitochondrial health. Hopefully, this thesis will be a step towards an improvement in treatment of these vulnerable children.
REFERENCES


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