Docosahexaenoic acid prevents peroxisomal and mitochondrial protein loss in a murine hepatic organoid model of severe malnutrition

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Manuscript in preparation
Chapter 4

ABSTRACT

Introduction

Acute and chronic exposure to low amino acid conditions have been shown to lead to a reduction in hepatic peroxisomal and mitochondrial content. There is limited understanding of the underlying mechanisms behind this loss, but data suggests degradation through autophagy. Both organelles play a key role in fatty acid metabolism, which may explain why dysfunction in either one of them might lead to hepatic steatosis.

Methods

Using a previously established murine hepatic organoid model of severe malnutrition, we characterized the effects of prolonged amino-acid restriction on peroxisomal and mitochondrial protein levels and on autophagic flux. To do so, we developed concatemers of $^{13}$C-labelled peptide standards for quantification of over 50 different peroxisomal proteins. To assess the autophagic flux, we transduced hepatic organoids with a GFP-LC3-RFP-LC3ΔG probe. Finally, the effect of PPAR-α activation on peroxisomal loss was determined with various agonists.

Results

Prolonged (96 h) amino-acid restriction led to a more severe loss of peroxisomes than a 48 h restriction, and with a substantial induction of autophagic flux. This was accompanied by accumulation of intracellular triglycerides, loss of mitochondrial and peroxisomal proteins, and loss of peroxisomal functionality. While PPAR-α agonists WY-14643 and linoleic acid (LA) had no effect, docosahexaenoic acid (DHA) supplementation partly prevented peroxisomal and mitochondrial loss under amino-acid restricted conditions and partly inhibited autophagy.

Discussion

The potential of DHA to prevent loss of peroxisomes and mitochondrial functions in low protein diets and severe malnutrition warrants further causal and translational testing in preclinical models and clinical trials, including its use as nutritional supplement.
INTRODUCTION

Recent studies have identified the loss of peroxisomes and mitochondrial dysfunction as key consequences of low protein diets in rodents\textsuperscript{1-3}, leading to metabolic aberrations, similarly as was previously observed in malnourished children\textsuperscript{4}. These results were recapitulated in vitro using hepatic organoids grown in a low amino acid environment\textsuperscript{5}. Although these models have shed some light on the interplay of mitochondria and peroxisomes in malnutrition, little is known about the pathophysiology behind the loss of both organelles. Degradation of organelles in low amino acid concentrations has been previously linked to an increase in autophagic turnover\textsuperscript{6}, which was also observed in malnourished rodents\textsuperscript{1}. Organelles such as peroxisomes and mitochondria are frequently degraded via selective autophagy\textsuperscript{7,8}. Autophagy helps to remove and degrade misfolded proteins or damaged organelles. Moreover, autophagy also helps maintaining adequate cellular levels of amino acids as precursors for biosynthesis or as fuels, when there is a shortage of these nutrients\textsuperscript{9}. Prolonged autophagy and loss of peroxisomes and mitochondria, however, may affect their essential functions in lipid metabolism.

Staple foods high in carbohydrates, lack of access to varied diets, and food insecurity are common in developing countries. These factors, and specifically low protein diets, often lead to severe malnutrition in young children\textsuperscript{10}, characterized by an imbalance in energy and nutrient intake in relation to the body’s requirements. Malnutrition is regarded as one of the most severe deficiencies of macronutrients and it affects metabolic homeostasis, thus hampering healthy development of children. Severe acute malnutrition (SAM) is known as the most life-threatening type of malnutrition\textsuperscript{11}. Children with severe malnutrition commonly develop symptoms of hepatic dysfunction including hypoglycaemia, hypoalbuminemia and hepatic steatosis\textsuperscript{12,13}. Impaired lipid oxidation has also been observed in malnourished children, and is thought to be the main cause of hepatic steatosis\textsuperscript{14,15}. Post-mortem electron microscopy images showed a decreased number of peroxisomes and compromised mitochondria in the liver of malnourished children\textsuperscript{4}. In agreement with the key role of these organelles in lipid metabolism, this is accompanied by the development of hepatic steatosis and increased oxidative stress in malnourished children.

Current management protocols often do not lead to rapid metabolic recovery\textsuperscript{16} and mortality remains high in acutely ill children with severe malnutrition\textsuperscript{17}, which makes the need for new treatment approaches of high importance. Peroxisome proliferator-activated receptor (PPAR)-α is a transcriptional
factor within the family of nuclear receptors known to induce proliferation of peroxisomes and highly expressed in tissues involved in fatty acid metabolism, such as the liver\textsuperscript{18}. Activation of PPAR-\(\alpha\) increases the expression of genes involved in peroxisomal and mitochondrial \(\beta\)-oxidation, thereby upregulating the uptake, activation and oxidation of fatty acids\textsuperscript{19-22}. Therefore, PPAR-\(\alpha\) activation might recover peroxisomal number and mitochondrial health by upregulation of \(\beta\)-oxidation genes, as observed in malnourished rats supplemented with fenofibrate\textsuperscript{1}, and thereby reduce fat accumulation, at least in rodents. Some of the most commonly described PPAR-\(\alpha\) ligands are synthetic compounds, such as fibrates. In addition, naturally occurring compounds, including long-chain polyunsaturated fatty acids (LCPUFA), have also been reported to be PPAR-\(\alpha\) agonists\textsuperscript{23}. Promising results in improvement of cognition of severely malnourished children highlight the latter compounds as interesting dietary supplements\textsuperscript{24}.

Discovery of the organoid technology in 2009\textsuperscript{25} has transformed the field of biomedicine, providing scientists with physiologically relevant in vitro disease models. Organoids are proliferative 3D structures, derived from primary tissue, adult stem cells or pluripotent stem cells. These structures contain different cell types and mimic the functions of the organ of origin\textsuperscript{26}. Organoids are valuable tools for the study of organ development, disease modelling and drug screening\textsuperscript{27,28}. They have also been shown to be a relevant system to study nutrient metabolism\textsuperscript{29,30} and metabolic diseases\textsuperscript{31}. We have previously used organoids to model severe malnutrition using long-term exposure to low amino-acid levels. Although no amino acids were added to the medium, all amino acids were found in low concentrations, probably coming from degradation of the Matrigel or the cytokines and growth factors present in the medium\textsuperscript{5}. The latter study recapitulated liver-specific phenotypes of malnutrition, including peroxisomal loss and mitochondrial dysfunction.

The aim of the present study was to use the previously established murine hepatic malnutrition in vitro model\textsuperscript{5} to gain more insight into the mechanisms of peroxisomal loss under different durations of exposure to low-amino acid conditions and assess the role of autophagy therein. Moreover three different PPAR-\(\alpha\) agonists, including a synthetic ligand (WY-14643) and two long-chain polyunsaturated fatty acids (linoleic acid and docosahexaenoic acid), were tested to determine their ability to prevent peroxisomal loss. We used a GFP/RFP autophagic flux reporter system to quantify the effect of amino acid restriction and PPAR-\(\alpha\) activation on autophagic flux. We conclude that the natural lipid docosahexaenoic acid (DHA) is a promising compound to explore...
as pharmacological treatment for the protection of peroxisomal matrix and mitochondrial proteins in malnutrition.

RESULTS

Liver progenitor organoids generated from mice were differentiated into hepatocyte-like organoids and used for the study of amino-acid restriction, as previously described⁵.

Prolonged amino-acid deprivation leads to severe peroxisomal loss in mature hepatic organoids

To investigate the effect of the duration of amino-acid restriction on peroxisomal loss, hepatic organoids were exposed to amino-acid free medium for 48 or 96 h. Clearly, 96 h of amino acid restriction led to a more severe and significant decline in the levels of the peroxisomal marker proteins than 48 h (Figure 1a and 1b). Levels of PMP70, an ATP binding cassette transporter of fatty acids into the peroxisome, and one of the most abundant peroxisomal membrane proteins, were strongly reduced after amino acid starvation. Acyl-CoA oxidase 1 (ACOX1), the first enzyme of the peroxisomal β-oxidation, and catalase, the enzyme metabolising the hydrogen peroxide produced by ACOX1 and multiple other oxidases, were also significantly reduced after 48 or 96 h of amino-acid restriction compared to control cells (Figure 1a and 1b).

To obtain a more comprehensive insight into the different peroxisomal proteins affected by amino acid deprivation, we quantified several peroxisomal proteins by targeted proteomics³⁰. For the proteomic analysis, we designed and used as internal standards a panel of ¹³C-labelled peptides with the QconCAT technology³². These standards target 57 murine peroxisomal proteins as well as their human orthologues. Up to 28 peroxisomal proteins (depending on the experimental condition and amount of organoids), 4 of which have dual localization, were sufficiently abundant to be detected and quantified in the hepatic organoids. These included metabolite transporters, as well as enzymes of the β-oxidation and α-oxidation (Supplementary Table 1). Overall, a time-dependent reduction of these peroxisomal proteins was observed in response to amino-acid deprivation (Figure 1c).

Immunofluorescence analysis using antibodies against PMP70 revealed a reduced number of green punctae in the starved organoids, indicating a reduced number of peroxisomes, but only after 96 hours of amino-acid...
deprivation (Figure 1d and e). In electron micrographs, double-membrane bound electron-dense structures, with peroxisome-like structures inside, appeared in the organoids depleted of amino acids for either 48 or 96 hours (Figure 1f). These structures probably represent autophagosomes or autolysosomes, suggesting autophagy is occurring, most likely also including degradation of peroxisomes. These results are in agreement with previous observations in amino-acid deprived cell cultures\textsuperscript{33}.

We then wondered if amino acid restriction also affected the biogenesis of peroxisomes. Peroxins (PEX) are proteins involved in various aspects of peroxisome biogenesis. Gene expression of these peroxins was assessed by qPCR. PEX3, PEX6 and PEX19 are essential peroxins for the assembly of peroxisomal membranes. Targeting and insertion of membrane proteins involved PEX19 as a cytosolic receptor protein, PEX3 works as a membrane anchoring site for cargo-loaded PEX19 and PEX16 as possible receptor for PEX3\textsuperscript{34}. The mRNA level of \textit{PEX3} was significantly upregulated in amino-acid deprived organoids, while \textit{PEX19} showed a downwards trend and \textit{PEX16} remained unchanged. For the import of proteins into the peroxisome, PEX5 and PEX7 recognize cargo with Peroxisome Targeting Sequence 1 and 2 (PTS-1 and 2), respectively. Interestingly, mRNA level of \textit{PEX7} was significantly downregulated, while \textit{PEX5} was upregulated in amino-acid deprived organoids. The mRNA levels of \textit{PEX1} and \textit{PEX6}, peroxins involved in the protein import, remained unchanged. The mRNA level of \textit{PEX11}, involved in peroxisomal fission\textsuperscript{35}, was increased upon amino acid removal (Figure 1g). These results indicate that amino-acid deprivation in hepatic organoids differently affects expression of mRNAs encoding peroxisomal biogenesis proteins.
Docosahexaenoic acid prevents peroxisomal and mitochondrial protein loss in a murine hepatic organoid model

Figure 1. Longer amino-acid restriction lead to more severe loss of peroxisomal proteins and induces the formation of autophagosome-like structures. (A) Representative immunoblot images. (B) Protein levels relative to β-actin. Quantification of data shown in A and replicates thereof. For ACOX1 quantification, antibody intensities of both bands were summed. Organoids were grown in complete medium (control) or in amino-acid restricted medium for 48h or 96h. Data represent mean ± SEM from 3 biological replicates obtained from independent experiments (*P < 0.05, **P < 0.01, ordinary one-way ANOVA with Tukey’s post-hoc test). (C) Heatmap representing targeted proteomics data from control conditions, 48 h of amino-acid restriction and 96 h of amino-acid restriction. Data represents 4 biological replicates. (D) Representative single Airyscan slice immunofluorescence images. On the left side control organoids and on the right organoids after 96 hours in low amino acid conditions. Peroxisomes stained for PMP70 in green, with DAPI nuclei counter stain (blue) and phalloidin (red). Scale bar, 10 μm. (E) Quantification of number of peroxisomes per μm² per cell based on PMP70 staining in D from 3 biological replicates (*P < 0.05, **P < 0.01, ***P<0.001 ordinary one-way ANOVA). (F) Electron microscopy images upon 48 and 96 hour amino-acid restricted organoids showing double-membrane electron-dense structures. N; nucleus. (G) Relative gene expression of peroxins in organoids in control condition or in amino-acid restricted for 96 hours. Data represent mean ± SEM from 3 biological replicates (*P < 0.05, **P < 0.01, unpaired t-test).
**Amino-acid restriction increases autophagic flux in mature hepatocyte-like organoids**

The previously reported reduction of mitochondrial proteins, together with reduction of peroxisomal proteins and the appearance of structures resembling autophagosomes, suggests altered autophagy in the amino-acid deprived cultures. Increased appearance of autophagosomes might result either from a stabilization of autophagosomes due to an impairment in the last steps of the autophagic process, or from an increase in the formation of autophagosomes, coinciding with an elevated autophagic flux.

To functionally quantify the autophagic flux in the organoids, we established a system based on expression of the GFP-LC3-RFP-LC3ΔG probe, as reported by Kaizuka et al. Upon autophagy activation, ATG4 cleaves the probe into equal amounts of GFP-LC3 and RFP-LC3ΔG. GFP-LC3 is then degraded in a normal way, while RFP-LC3ΔG is stable, and therefore not degraded, and works as an internal standard. Liver progenitor organoids were transduced to express the autophagic flux probe as described on Figure 2a. Two days post-transduction, stable progenitors were harvested and the cells were sorted to get an enriched population of double positives for the expression of both GFP and RFP (Figure 2b,c).

Figure 2b shows the gating strategy used for the enrichment of double positive cells. From the whole population (gate A) we excluded duplets and cellular aggregates (gate B); the enriched population is depicted in gate C (Figure 2b). As expected, amino-acid deprivation activated the autophagy flux in the organoids, as can be inferred from a reduced GFP/RFP ratio (Figure 2d, e). While after 48 hours of amino-acid restriction there was only a mild downward trend of the GFP/RFP ratio (Figure 2d upper panel, e), after 96 hours the GFP/RFP ratio was substantially and significantly reduced, indicating a clear time-dependent activation of autophagy (Figure 2d lower panel, e). Surprisingly, the autophagy inhibitor bafilomycin A1 caused only minor increase of the GFP/RFP ratio in 48 h starved organoids, without any effect after 96 h (Figure 2d, e). Interestingly, while amino-acid restriction-induced autophagy could not be prevented with chloroquine, chemically-induced autophagy (using rapamycin) could be prevented by chloroquine co-treatment (Supplementary Figure 1). This may indicate that prolonged amino-acid-restriction induced autophagy is not sensitive to classical autophagy inhibitors such as Bafilomycin A1 (Figure 2d lower panel, e) and chloroquine (Supplementary Figure 1).
Docosahexaenoic acid prevents peroxisomal and mitochondrial protein loss in a murine hepatic organoid model

**Figure 2.** Amino-acid restriction induces activation of autophagic flux in pMRX-IP-GFP-LC3-RFP-LC3ΔG liver organoids. (A) Scheme of generation of pMRX-IP-GFP-LC3-RFP-LC3ΔG organoids. (B) Gating strategy for organoid sorting. First graph indicates the whole population. Second graph depicts the gating for single cells excluding aggregates. Third graph illustrates the separating based on GFP and RFP expression. (C) Representative brightfield and IF images of pMRX-IP-GFP-LC3-RFP-LC3ΔG liver organoids. Scale bar 100 µm. (D) Representative cytograms depicting the GFP and RFP intensities measured in the cells under different conditions. Wild type organoids (grey), organoids expressing pMRX-IP-GFP-LC3-RFP-LC3ΔG grown in complete amino acid medium (green), in amino acid restricted medium (blue), or in amino-acid restricted medium with bafilomycin A1 (maroon). Analysis was performed after 48 h (upper two graphs) or 96 h (bottom two graphs). (E) Left graph represents GFP/RFP fluorescence intensities ratio in organoids under control condition or in amino-acid restricted medium 48 h (left) or 96 h (right) and treated or untreated with 100nM bafilomycin. Data represent mean ± SEM from at least 3 biological replicates. Right graph shows GFP/RFP fluorescence intensities ratio in organoids under control conditions or in amino-acid restricted medium for 96 hours, and treated or untreated with bafilomycin. Data represent mean ± SEM from at least 3 biological replicates (*P < 0.05, **P < 0.01, one sample t test)
Screening PPAR-α agonist for the prevention of peroxisomal loss in hepatic organoids

Peroxisome proliferator-activated receptor α (PPAR-α) is a nuclear receptor that works as a ligand-regulated transcriptional factor. PPAR-α is activated by a wide range of ligands, of which synthetic fibrates are most widely described\textsuperscript{37}. It can, however, also be activated by natural ligands such as various fatty acids and eicosanoid derivatives\textsuperscript{21}.

Here, we assessed the effect of three PPAR-α agonists on peroxisomal proliferation and fatty acid metabolism in amino-acid restricted organoids. We tested one synthetic compound (WY-14643) and two polyunsaturated fatty acids: linoleic acid (18:2ω6 fatty acid) and docosahexaenoic acid (DHA, C26:ω3). Linoleic acid (LA) did not rescue any of the peroxisomal protein markers PMP70, ACOX1, or catalase (Figure 3a,b), whereas PPAR-α agonist WY-14643 had only a minor rescuing effect on ACOX1. ACOX1, is synthesized as 75 kDa polypeptide, which upon import into the peroxisome is proteolytically cleaved into two smaller peptides, 21 kDa and 55 kDa\textsuperscript{38}. Supplementation of the highest dose (200 μM) of WY-14343 prevented the loss of the uncleaved, 75 kDa protein, while the smaller (53 kDa) was almost significant. (Figure 3c,d). In contrast, the natural compound DHA prevented the loss of catalase and the 55 kDa form of ACOX1 in dose dependent manner, with a complete rescue to control levels at the highest dose. DHA did not affect levels of PMP70 nor of the uncleaved form of ACOX1 (75 kDa) (Figure 3e, f). Since LA and WY-14643 did not show any protection against peroxisomal loss, the effect of DHA might be mediated via a PPAR-α-independent mechanism.
Docosahexaenoic acid prevents peroxisomal and mitochondrial protein loss in a murine hepatic organoid model

Figure 3. Effects of the different PPAR-α agonists on peroxisomal protein levels in amino-acid restriction conditions. Organoids were grown in complete medium (Control) or in restriction medium for 96 hours (Starved), restriction medium with vehicle (DMSO) or (BSA), and in the presence of two different concentrations of LA (50 µM and 150 µM), WY-14643 (100 µM and 200 µM) or DHA (50 µM and 100 µM) (A, C, E) Representative immunoblot images (B,D,F) Peroxisomal protein levels relative to β-actin. Quantification of data shown in A, C, and E, respectively, and replicates thereof. Data represent mean ± SEM from 3 biological replicates (biological replicates are obtained from independent experiments) (*P < 0.05, **P < 0.01, ordinary one-way ANOVA with Tukey’s post-hoc test).

Effect of DHA on mitochondrial and peroxisomal proteome

Since DHA had the largest rescuing effect on peroxisomal marker proteins, we set out to analyse the effect of this compound more comprehensively, using quantitative, targeted proteomics. Next to the above described peroxisomal protein panel, also previously validated panels of proteins involved in mitochondrial metabolism\(^{32}\), and glucose and glycogen metabolism\(^{39}\) were analysed. The results replicated the overall reduction of peroxisomal proteins upon amino-acid deprivation (Fig. 4a), and additionally showed a clear reduction of proteins involved in mitochondrial fatty-acid β-oxidation, TCA cycle and oxidative phosphorylation (Fig. 4b). The highest dose of DHA
supplementation prevented this loss of peroxisomal and mitochondrial proteins (Fig. 4a and b). Enzymes involved in glycolysis, gluconeogenesis and glycogen metabolism showed a milder downregulation upon amino acid removal, some of which were normalised by DHA (Supplementary Figure 4).

**Effects of DHA supplementation on peroxisomal function and health**

Since DHA supplementation recovered peroxisomal and mitochondrial proteins, including enzymes involved in fatty acid oxidation (FAO), we hypothesized that it could also decrease the levels of intracellular triglycerides (TG). In line with our earlier observations amino-acid restriction led to a mildly elevated TG level in the hepatic organoids. Contrarily to what was expected, DHA supplementation did not reduce the TG level. However, given that the organoids were stimulated with a fatty acid, we cannot rule out an increase in \( \beta \)-oxidation. The higher concentration of DHA even led to a further accumulation of intracellular triglycerides when compared to the restriction conditions (Figure 4c). This could be caused by incorporation of DHA into phospholipids, inducing other fatty acids to be used to TG synthesis or even by direct incorporation of DHA into TG.

To continue assessing peroxisomal functionality, we studied the ability of peroxisomes to metabolize phytanoyl-CoA in control and starved organoids as we previously reported. Phytic acid is a branched-chain fatty acid that is primarily oxidized by the peroxisomal \( \alpha \)-oxidation, yielding pristanic acid as a product, which subsequently undergoes peroxisomal \( \beta \)-oxidation\(^{40,4}\) (Figure 4d). Mature hepatic organoids were incubated with 25 \( \mu \)M of phytol. Amino-acid restricted organoids had substantially lower levels of phytanic and pristanic acid than control organoids, suggesting that peroxisomal fatty-acid oxidation is affected. Since pristanic acid is an intermediate metabolite in the pathway, however, the effect on the flux of peroxisomal fatty oxidation is unclear. Moreover, DHA supplementation did not affect the levels of phytanic or pristanoic acid at any concentration (Figure 4e).
Figure 4. DHA supplementation prevents the loss of multiple peroxisomal and mitochondrial proteins and prevents autophagy in amino-acid restricted (96 h) organoids. Organoids were grown in complete medium (Control) or in amino-acid restriction medium for 96 h (Starved) with DMSO or DHA (50 µM and 100 µM) (A) Heat map of peroxisomal proteins detected including several metabolic pathways and transporters quantified using targeted proteomics. (B) Heat map of mitochondrial proteins detected including fatty acid oxidation, TCA cycle, ETC and reactive oxygen species detoxification quantified using targeted proteomics. Z-scores were used to normalize the data and protein abbreviations are used according to Uniprot. (In red high Z-score indicating upregulated proteins and in blue low Z-score indicating downregulated proteins). (C) Triglycerides levels in control, starved (96 hours) and starved treated with DSMO (vehicle) and 50 and 100uM of DHA (as per color legend). Data represents 6 biological replicates from independent experiments ± SEM (*P < 0.05, ordinary one-way ANOVA with Tukey’s post hoc test). (D) Scheme of the metabolism of phytol into phytanoyl-CoA and pristanoyl-CoA in the peroxisome. (E) Concentration of phytanic and pristanic acid in the supernatant of organoids. Data represents 3 biological replicates ± SEM (*P < 0.05, **P < 0.01. RM One-way ANOVA with Tukey’s post-hoc test). (F) GFP/RFP fluorescence intensities ratio in Huh7 cells in control conditions and 48 h of amino-acid restriction treated and untreated with DMSO or DHA (50 and 100 µM). Data represent mean ± SEM from 3 biological replicates (*P < 0.05, **P < 0.01 One-way ANOVA with Tukey’s post-hoc test). (G) GFP/RFP fluorescence intensities ratio in organoids expressing pMRX-IP-GFP-LC3-RFP-LC3ΔG. Data represent mean ± SEM from 3 biological replicates (*P < 0.05, **P < 0.01 One-way ANOVA with Tukey’s post-hoc test).
Effects of DHA on peroxisomal biogenesis and autophagy

Given that DHA supplementation increased the overall pattern of peroxisomal and mitochondrial proteins, we investigated the effect of DHA on both peroxisomal biogenesis and autophagic degradation. DHA nor any of the other compounds tested showed any effect on the peroxins involved in peroxisomal biogenesis (Supplementary figure 2). In a first test on Huh7 cells transduced with the pMRX-IP-GFP-LC3-RFP-LC3ΔG construct, 48 hours of amino-acid restriction induced autophagic flux, similarly to what was observed in the hepatic organoids. Autophagic flux decreased to control levels after DHA treatment in a dose-dependent manner (Figure 4f), but was not affected by the other PPAR-α agonists, WY-14643 or LA (Supplementary Figure 3). These results are in agreement with what was previously observed in oxidative stress-induced autophagy and TNF-α-induced autophagy41,42. In the GFP-LC3-RFP-LC3Δ hepatic organoids, the activation of autophagic flux by 96 h of amino-acid deprivation was replicated, but it was not rescued by DHA (Figure 4g). The effect of DHA on autophagy, at least in the Huh7 cell line, goes in line with the observed prevention of peroxisomal and mitochondrial protein loss. The results suggest, however, that in the organoids the rescue of peroxisomal and mitochondrial proteins by DHA might occur via an autophagy-independent mechanism.

DISCUSSION

In this study we have demonstrated that prolonged amino acid restriction leads to peroxisomal loss with increased autophagic degradation and alterations in peroxisomal biogenesis in a murine hepatic organoid model. To assess autophagic flux we have implemented the use of the novel GFP-LC3-RFP-LC3ΔG probe in the hepatic organoids. Using targeted proteomics, we then demonstrated that DHA supplementation prevented the loss of both peroxisomal and mitochondrial proteins in the context of amino acid restriction.

Mechanisms of peroxisomal loss in amino acid restriction and potential therapeutic interventions

Amino-acid deficiency has been previously linked with peroxisomal loss43, however, the mechanisms behind this process in malnutrition still remain unclear. While the main cause of peroxisomal loss in amino-acid starvation has been described to be pexophagy43, these studies focused on the effect of
complete amino-acid starvations. However, little is known about the longer-term effects of exposure to low amino-acid levels in contrast to complete and shorter starvations. Moreover, there is little information available on the effect of amino-acid restriction on peroxisomal biogenesis.

In this study, hepatic organoids were grown in amino acid restricted medium but not in complete starvation conditions. Organoids were still exposed to low levels of amino acids coming from the Matrigel, in which the organoids are cultured, or potentially from the degradation of growth factors present in the medium as specified in previous literature\(^5\). These conditions mimic low protein diets (LPD) like in in vivo conditions better.

Several groups have demonstrated that autophagy is altered in rodents in response to low protein diets at a very young age\(^1-3\). The results reported here are in agreement with those observed in rats on a 1 week low protein diet. However, when rodents were subjected to a prolonged LPD, autophagy was hampered, both in liver and intestine. In the liver of rats fed a low protein diet for 1 week, LC3II was increased while P62 was downregulated, suggesting autophagy to be increased. In contrast, exposure to LPD for 4 weeks led to an increase in p62, which was interpreted as a block in autophagy\(^1\). Similarly, studies using mice on LPD for 2 weeks reported a reduction of autophagy. However, while in both cases the protein levels of LC3-II were downregulated, one of the studies also reported a downregulation of the LC3II/I\(^2\) while the other did not\(^3\). Given the nature of in vivo work, all of these studies assessed autophagy by western blotting and only a snapshot of the pathway was provided at a given time point.

Here we demonstrated that organoids are a dynamic and accessible tool to study autophagic flux in the context of amino acid restriction. To do so, we implemented the use of a dynamic probe (GFP-LC3-RFP-LC3\(\Delta G\))\(^36\), developed for the study of autophagy in live cells. We reported that 96 h amino acid restriction led to an induction of autophagy, as also observed in rats on LPD for a short time and in other in vitro models under amino acid starvation\(^33\).

With respect to peroxisomal biogenesis, rodents fed a low protein diet for 4 weeks did not show any regulation in gene expression of peroxins involved in peroxisomal biogenesis\(^1\). In the present study, we have demonstrated that amino acid restriction has an impact on the expression of genes involved in peroxisomal biogenesis. Since some genes were upregulated and others downregulated we could not draw any clear conclusions. However, further characterization of this differential regulation might help identify the functional impact. Conspicuously, both catalase and ACOX1 are imported
into the peroxisome via PTS1, which then binds to PEX5. Nonetheless, PEX5 is clearly upregulated in the amino-acid deprived conditions, which does not go in line with the decrease in both catalase and ACOX1. This might indicate a compensatory mechanism in order to promote peroxisomal import in conditions with lower number of peroxisomes.

Different therapeutics, including peroxisomal and mitochondrial biogenesis activators as well as autophagy regulators, have been tested in order to prevent peroxisomal and mitochondrial loss and hepatic dysfunction in the context of malnutrition. Activation of PPAR-α activates peroxisomal and mitochondrial biogenesis as well as genes encoding enzymes involved in peroxisomal and mitochondrial fatty acid oxidation\textsuperscript{22,44,45}. In order to prevent peroxisomal and mitochondrial loss in the context of malnutrition, PPAR-α agonist fenofibrate has been previously tested both in vitro and in vivo\textsuperscript{1,5}. Fenofibrate supplementation recovered the levels of peroxisomal protein marker PMP70 and lowered hepatic triglycerides while increasing the levels of ATP in a LPD rat model\textsuperscript{1}. Fibrates have been widely used for the treatment of dyslipidemia and to induce the biogenesis of peroxisomes and mitochondria ameliorating the hepatic phenotype\textsuperscript{46}. Fibrates however cause some side effects. When PPAR-α null mice were administered fenofibrate, the levels of serum triglycerides were decreased, but intrahepatic TGs were significantly increased\textsuperscript{47}. These results suggest that these compounds might not be ideal candidates to treat malnourished children.

In the case of malnourished children, treatment with dietary supplements is attractive as a simple and safe approach when compared to synthetic compounds such as fibrates. Conveniently, polyunsaturated fatty acids have also been reported to be potent PPAR activators\textsuperscript{48}. These compounds can be found in foods and can be easily supplemented on ready-to-use foods (RTUF) already used in refeeding protocols for malnourished children.

**Docosahexaenoic acid is a potential candidate to prevent peroxisomal loss in in vivo conditions**

Docosahexaenoic acid is an \( \omega-3 \) LCPUFA commonly found in seafood, seaweed and breast milk. It can also be synthesized from \( \alpha \)-linolenic acid (ALA)\textsuperscript{49}. DHA plays an important role in many metabolic processes, and can be found in the bloodstream, lipid stores and cell membranes. Adequate levels of DHA in infants have been linked with optimal development\textsuperscript{50}. Moreover, low levels of DHA were found in breast milk from mothers from malnourished children and
it was suggested that DHA levels in malnourished children highly depend on the DHA intake from breast milk.\textsuperscript{51}

The effects of DHA supplementation have been previously tested in the context of malnutrition. For instance, supplementation in malnourished rats led to a decrease in the production of malondialdehyde (MDA) in blood, a lipid peroxidation marker that reports oxidative stress. It also increased the levels of superoxide dismutase, together suggesting that DHA supplementation may reduce oxidative stress. A clinical study in malnourished Malawian children showed that RUTF supplemented with DHA led to an increase in the Malawi Developmental Assessment Tool (MDAT) score. This could be attributed to its clear cognitive benefit and increase in levels of in 1-palmitoyl-2-eicosapentaenoyl-sn-glycero-3-phosphocholine, an ω3 fatty acid that is important for regulation of cognition.\textsuperscript{53} In line with this, breast-milk LCPUFA levels were linked to neural maturation of breastfed infants.\textsuperscript{54,55} These reports in combination emphasize the potential of DHA as a beneficial supplement for the treatment of malnourished children.

To the best of our knowledge, there are no reports on the effect of DHA on the peroxisomal and mitochondrial content in the liver of malnourished children. Moreover, the fact that WY-14463 and LA showed no effect on peroxisomal recovery suggests that the effects of DHA might not be PPAR-α related. In fibroblasts from patients carrying defects in peroxisomal β-oxidation, DHA supplementation induced peroxisomal elongation via oligomerization of PEX11β and peroxisomal division via DLP1, thus boosting peroxisomal content.\textsuperscript{56} This mechanism might potentially explain the higher levels of peroxisomal proteins in DHA treated organoids in our study.

It needs to be investigated whether DHA prevents peroxisomal loss in vivo during exposure to an LPD. When combining all information, both the effect of LDP and of DHA seem context dependent. The LPD induced autophagy in a short-term experiment. DHA blocked autophagy activation in Huh7 cells, had no effect on autophagy in amino-acid deprived hepatic organoids (this study), and was previously reported to activate autophagy in the presence of amino acids, via mTORC.\textsuperscript{57} One study reported that DHA inhibited oxidative stressed-induced autophagy via the AMPK-dependent signaling pathway, while another showed a decrease in autophagy markers in TNF-α-induced autophagy. This suggests that DHA may act via multiple routes in a context dependent manner. That PPAR-α activators (WY-14463 and LA) had no effect on the Huh7 (Supplementary Figure 3), may be explained by the generally low effect of PPAR-α activators on human cells in contrast to rodent cells.\textsuperscript{58}
In a broader perspective it will be interesting to test co-supplementation with vitamin B3 (nicotinamide or nicotinamide ribose), which was previously found to rescue mitochondrial function in the liver of LPD-treated mice. A combination of nutritional supplements might synergistically improve mitochondrial and peroxisomal function as well as organ function.

**Conclusions**

In conclusion, we found that docosahexaenoic acid prevents the loss of some peroxisomal and mitochondrial proteins in a hepatic organoid model of malnutrition. Our results warrant testing of DHA as a potential dietary supplement in a more physiologically relevant animal model of severe malnutrition.

**METHODS**

**Isolation of biliary ducts and organoid culture**

Hepatic organoids lines were established using mouse biliary ducts isolated from the liver of male C57BL/6J mice between 3 and 5 weeks of age (Jackson Laboratory, Bar Harbor, ME, USA) following the previously reported protocol. Ductal fragments were placed in isolation medium (IM) consisting of Advanced DMEM/F12 (Gibco), supplemented with 10 mM HEPES, 1x GlutaMax, 1% Penicillin-Streptomycin (all Gibco), 1x N2-Supplement (Invitrogen), 1X B27-Supplement without vitamin A (Invitrogen), 10 mM Nicotinamide (Sigma Aldrich), 1.25 mM N-AcetylCysteine (Sigma Aldrich), 10% Rspoon-din-1 condition medium (kindly provided by Calvin J. Kuo), 30% Wnt3a conditioned medium (kindly provided by Hans Clevers), 100 ng/ml Noggin, 50 ng/ml HGF, 100 ng/ml FGF-10, 50 ng/ml EGF (all Peprotech), 10 nM Leu-gastrin (Sigma Aldrich) and 10 µM Y 27632 dihydrochloride (Axon Medchem). Three days after isolation, Wnt3a CM and Noggin were removed from the medium. Y 27632 dihydrochloride was removed 4 days after isolation. Medium without Wnt3a-CM and Noggin was used to keep the organoids in culture and referred to as expansion medium (EM).

Medium was changed every 2-3 days and they were passaged every 7-9 days in a split ratio 1:6 – 1:9 on average.

In order to differentiate the organoids into more mature hepatocyte-like organoids, after passage they were kept in EM medium for 3 days and then
changed into differentiation medium (DM) containing: Advanced DMEM/F12 (Gibco), supplemented with 10 mM HEPES, 1x GlutaMax, 1% Penicillin-Streptomycin, 1x N2-Supplement, 1X B27-Supplement without vitamin A, 10 mM Nicotinamide, 1 mM N-AcetylCysteine, 100 ng/ml FGF-10, 50 ng/ml EGF, 10 nM Leu-gastrin, 50 nM A-83-01 (Axon Medchem) and 10 µM DAPT (Sigma). Between day 13 and 16, the medium was supplemented with 3 µM dexamethasone (Sigma Aldrich). These protocols were based on published literature59.

**Malnutrition in organoids using amino acid free medium**

In order to mimic the effects of a low protein diet, mature hepatic organoids were cultured in a custom-made amino acid-free medium as previously reported5.

Organoids were kept in complete DM until day 12, when they were changed into restriction differentiation medium that contained all the same supplements but was completely depleted of all amino acids. Organoids were kept in starvation for 96 h until the end of day 16 when they were collected for further studies. In the case of the shorter (48 h) amino acid restrictions, organoids were kept in complete DM until day 14, when they were changed into restriction medium and collected at the end of day 16.

**Fluorescence microscopy**

Hepatic organoids were fixed with 1% formaldehyde in 0.1M PBS pH 7.4 for 15 min. Organoids were blocked and permeabilized in 0.1 M PBS with 20mM glycine, + 3% (w/v) BSA (9048-46-8; Fisher Scientific) and 0.1% (w/v) saponin (47036-50G-F; Sigma-Aldrich) pH 7.4 for 60 min at RT. Organoids were incubated with antibodies against PMP70 (Sigma: P0497) 1:400 in the same buffer. Subsequently organoids were washed in PBS three times and incubated with donkey anti-rabbit IgG (H+L) Alexa Fluor 488 (A21206; Thermo Fisher Scientific, Invitrogen) at 1:800 dilution. For actin labeling, phalloidin Alexa Fluor 546 was used (A22283; Thermo Fisher Scientific) at 1:200 dilution in parallel with the labeling with secondary antibodies. Organoids were imaged in PBS containing DAPI. All steps were performed in a glass bottom 96 wells plate. Airyscan images were captured with a confocal microscope (LSM800; Carl Zeiss) equipped with a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT), Zen 2009 software (Carl Zeiss), and a 40 × 1.20 NA objective (Carl Zeiss). Peroxisome numbers per cell were quantified using FIJI. For each cell the grey values are adapted to visualize and quantify the separate peroxisomes because of the difference in staining intensities between the edge and the
middle part of the organoids. For each cell the number of peroxisomes per square micron is calculated from three biological replicates.

**Electron microscopy**

Hepatic organoids were incubated in DMEM on ice to dissolve the Matrigel. Organoids were washed in 0.1M sodium cacodylate (103256; Millipore) pH 7.2 and fixed for 75 min with 2% glutaraldehyde (G7661; Sigma-Aldrich) in sodium cacodylate. Organoids were post fixed with 1% osmium tetroxide (19134; Electron Microscopy Sciences) and 1% ferrocyanide in sodium cacodylate for 60 min. Samples were *en bloc* stained O/N with 0.5% uranyl acetate, dehydrated in series of ethanol and embedded in epon. 80 nm thin sections were collected on carbon evaporated formvar copper grids and analyzed with a CM12 transmission electron microscope (Philips) running at 100 kV.

**Therapeutic interventions with different PPAR-α agonists**

Mature hepatic organoids were cultured in complete DM until day 12. The organoids were then placed in amino acid free DM supplemented with different PPAR-alpha agonists at different concentrations. WY-14643 (Axon Medchem) was tested at two different concentrations 100 and 200 µM based on literature reports\(^60-62\). Docosahexaenoic acid (DHA) (Sigma Aldrich) was tested at two different concentrations 50 and 100 µM\(^63,64\). Linoleic Acid (Sigma Aldrich) was tested at two different concentrations 50 and 150 µM\(^65,66\). The vehicles DMSO and fatty acid-free BSA were tested in the same volume as that of the higher concentration of the compounds used.

**Transfection of Hek293T cells for production of γ-retroviral particles.**

Hek293T packaging cells were seeded in a 10 cm standard tissue culture dish to reach a confluency of 80%. Transfection of cells was performed using 7.0 µg of Retroviral transfer plasmid pMRX-IP/GFP-LC3-RFP-LC3ΔG (kindly provided by Noboru Mizushima, Department of Biochemistry and Molecular Biology, University of Tokyo, Tokyo, Japan; Addgene plasmid #84572; (http://n2t.net/addgene:84572; RRID:Addgene_84572)\(^36\), 7.5 µg pGag/Pol, 2.5 µg pVSV-G packaging vectors and 1 µg pAdvantage expression enhancer. The plasmid mix was added to PEI (Polysciences Inc. Cat N: 23966-2) transfection reagent prepared in Opti-MEMTM Reduced Serum Medium (Gibco, Cat N: 31985062) at 1:5 ratio (plasmid DNA Mix:PEI). The final mix was added to Hek293T cells dropwise. Media from viral particles producing-cells (10mL) was collected.
after 24 h and 48 h and filtered through a 0.45 μm SFCA filter (Corning, Cat N: 431220). Finally, 0.5 μL of polybrene (4mg/mL stock concentration) (SIGMA, Cat N: H9268-5G) per mL of media was added to enhance retroviral transduction. For transduction of target cells, 1 mL or 2 mL (depending on experiment design) of retroviral supernatant was used. Then, target cells were incubated for at least 24 hours at 37°C and 5% CO2.

**Establishment of LC3B-GFP-RFP-LC3ΔG liver organoids**

Organoids were collected in Advanced DMEM/F12 and kept on ice for 10 minutes to disrupt the Matrigel. Organoids were then centrifuged at 290G for 5 minutes and reconstituted in 1mL of pre-warmed Trypsin for 5 minutes. Then, mechanical disruption of the organoids was used to dissociate them into single cells. 10 mL of advanced DMEM/F12 were added and the organoids were centrifuged again to remove the trypsin. Cells were counted and approximately 4*10^5 cells were placed in 500 μL of expansion medium supplemented with 10 μM Y 27632 dihydrochloride. Equal volume of viral medium, produced as described in the previous section, was added and placed into a 24 well plate well (pre-coated with Matrigel). The cells were kept in the incubator at 37°C for 48 h. Cells were then trypsinized and collected. After removal of the trypsin, the cells were reconstituted in advanced DMEM/F12 and Matrigel (1:3) and plated in domes. Expansion medium was supplemented with 10 μM Y 27632 dihydrochloride. After 24 h, the medium was changed to selection medium (EM containing Puromycin 1:500)

**FACS sorting of stable mouse liver organoids expressing the autophagic flux probe**

In order to isolate single clonal progenitors of liver organoids that properly express the autophagic flux probe GFP-LC3-RFP-LC3ΔG (double positive cells) and exclude GFP-LC3ΔG cells caused by homologous recombination during the transfection and transduction procedures reported by Kaizuka et al., 2016^36 we sorted the polyclonal stable liver organoids and performed an enrichment of GFP-RFP double positive cells. In brief, polyclonal liver organoids were harvested and individualized as previously mentioned. Cells were kept on ice in FACS buffer containing 1X HBSS (Gibco), 2% FCS (Gibco), 2% BSA, 1% Penicillin-Streptomycin (P/S). Cell sorting was performed using the MoFlo Astrios EQ Cell Sorter (Beckman Coulter, Life sciences) with a 100 μm nozzle and the 488 nm and 561 nm lasers. Sort rate was set up to 25.000 events/sec and approximately 1.000.000 double positive events were recorded and
harvested in a FACS tube containing 2 mL of expansion medium supplemented with 10\(\mu\)M Y 27632 dihydrochloride and 2\(\mu\)g/mL puromycin selection treatment followed by a centrifugation step at 290G for 5 minutes; then the pellet was reconstituted in 1 mL of pre-warmed expansion medium supplemented with puromycin (2 \(\mu\)g/mL). Cells were counted and viability was determined by trypan blue staining (higher than 90%). Approximately 5000 sorted cells were reconstituted in advanced DMEM/F12 and Matrigel (1:3 ratio) and plated in domes in 24 well plates. The organoids were maintained in 500 \(\mu\)L expansion medium supplemented with 10 \(\mu\)M Y 27632 dihydrochloride and 2 \(\mu\)g/mL puromycin selection treatment and kept in the incubator at 37\(^\circ\)C and 5% CO\(_2\). Enough liver progenitors were seeded and expanded for either performing experiments or storage in liquid nitrogen. Two days after sorting the formation of new liver organoids was confirmed as well as the expression of GFP and RFP were determined by fluorescence microscopy.

**Generation of stable HuH7 cells expressing the autophagic flux probe**

Wild-type HuH7 cells were seeded in control medium containing DMEM supplemented with 1% P/S and 10% FCS in a 6 well plate a day before transduction and kept in the incubator at 37\(^\circ\)C and 5% CO\(_2\). Cells were transduced with 2 mL of retroviral supernatant and incubated for 48 h. Next day and 48h after transduction cells were checked for positive GFP and RFP expression by fluorescence microscopy. After 48 h post-transduction, cells were harvested and expanded for two consecutive passages. The transduced-HuH7 cells were maintained in DMEM-GlutaMAX (Gibco, NL) media supplemented with 10% FCS, 1% P/S and 2 \(\mu\)g/mL puromycin. The cells generated in this step were considered polyclonal stable HuH7 cells expressing the autophagic flux probe GFP-LC3-RFP-LC3\(\Delta\)G, in short cells were called Poly-HuH7-3\(\Delta\)G. In order to produce single monoclonal HuH7 cells that properly express the autophagic flux probe GFP-LC3-RFP-LC3\(\Delta\)G (double-positive cells) and exclude homologous recombination during the transfection and transduction procedures reported by Kaizuka et al., 2016\(^36\) we sorted the polyclonal cells. In short, polyclonal HuH7 cells were harvested and kept on ice in FACS buffer containing 1X HBSS, 2% FCS, 2% BSA, 1% P/S. Cell sorting was performed using the MoFlo Astrios EQ Cell Sorter (Beckman Coulter, Life sciences) with a 100 \(\mu\)m nozzle equipped with the 488 nm and 561 nm lasers. Sort rate was set up to 25,000 events/sec and individual sort mode was used. Single double-positive (GFP/RFP) cells were dripped in 96-well plates previously filled with 200 \(\mu\)L of standard cultured media supplemented with 2 \(\mu\)g/mL puromycin. A total of 3 96-well
plates were filled with single double-positive cells to increase the chance of colonies growing. Sorted cells in 96-well plates were followed for the formation of single colonies with homogeneous shape for not longer than 12 days; wells with more than one colony were discarded. Seven colonies were followed and expanded under selection media with puromycin (2 μg/mL) and storage for further characterization.

**Measuring of autophagic flux by FACS**

Stable monoclonal-HuH7-3ΔG cells or enriched-LivOrg-3ΔG were grown in either control conditions (Ad. DMEM containing 1% P/S and 10% FCS) or in amino-acid restricted medium (a custom-made amino acid-free medium containing 1% P/S and 10% FCS). Organoids were treated with 150 nM of rapamycin to chemically induce autophagy. Bafilomycin A1 was used at 100 nM and Chloroquine at 50 μM. Cells were harvested using Accutase (Sigma, Cat N A9664-500ML) and individualized according with the methodology previously described. Cells were kept on ice in FACS buffer containing 1X HBSS, 2% FCS, 2% BSA. Cells were analyzed with the BD LSR-II cytometer (BD, Biosciences) equipped with a 488 nm laser and 561 nm laser. Samples were acquired using DIVA 8.0 software and at least 10,000 events for each sample were acquired. Data was saved as FCS 3.0 or 3.1 files and compensation was not required in any case. Data was processed with Kaluza software (Beckman Coulter). For calculating the autophagic activity, the GFP and RFP mean fluorescence intensity (MFI) (Geometric mean) was used to calculate the GFP/RFP ratio which reversely correlates with autophagic activity.

**Immunoblotting**

The protocol used for Western blotting was based on a previously published paper\(^67\). Ice cold Ad.DMEM/F12 (with or without amino acids) was used to collect organoids, and these were kept on ice for 10 minutes. Organoids were then centrifuged at 290G for 5 minutes and washed with PBS once. After a second centrifugation, they were reconstituted in 200 μL of radio immunoprecipitation assay buffer (1% IGEPAL CA-630, 0.1% SDS, and 0.5% sodium deoxycholate in PBS) supplemented with Complete Protease Inhibitor Cocktail (Cat. No. 1186145001; Sigma-Aldrich), Phosphatase Inhibitor Cocktail 2 (Cat. No. P5726; Sigma Aldrich) and Cocktail 3 (Cat. No. P0044; Sigma Aldrich). Organoids were then sonicated using a 30% amplitude four times for 10 seconds using Sonics Vibra cell VCX130 (Sonics & Materials inc.). Protein concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientific) and all the
samples were adjusted to the lowest concentration of all the samples. The lysates were further processed in the same way as previously described. Signals were normalized to a loading control (β-actin) as indicated. The list of antibodies used can be found in Supplementary Table 2.

RNA isolation, reverse transcription and real time qPCR

Total RNA was extracted from the organoids using the commercially available RNase easy Kit (Qiagen) as described by the manufacturer. The purity and quantity of the RNA was assessed using NanoDrop (NanoDrop Technologies). For the reverse transcription M-MLV Reverse Transcriptase (200 U/µl) (Invitrogen) was used as established by the manufacturer. qPCR was performed in 384 well format in duplicates (10 ng per well) using FastStart Universal SYBR Green Master (Rox) (Sigma Aldrich). qPCR was performed using QuantStudio 7 Flex (Thermo Fischer Scientific). All primer (Integrated DNA technologies Inc.) sequences are listed in Supplementary Table 3.

Fat isolation and quantification of triglycerides

Organoids were collected in ice-cold Ad.DMEM/F12 (with or without amino acids) and left in ice for 10 minutes to disrupt the Matrigel. The organoids were pelleted and washed with PBS. They were then reconstituted in ice-cold 1X TBS. Fat was isolated using chloroform: methanol in a 2:1 ratio and levels of intracellular triglycerides were quantitatively determined using the DiaSys Triglyceride FS kit. All the results were normalized to protein content.

Quantitative targeted proteomics

Quantitative targeted proteomics were performed following the published protocol by Wolters et al. Organoids were collected in ice-cold Ad. DMEM/ F12 (with or without amino acids) and incubated in ice for 10 minutes to disrupt Matrigel. The organoids were then centrifuged at 290G and the pellets were washed with cold PBS to ensure Matrigel removal. Pellets were then reconstituted in lysis buffer (0.1% v/v NP40, 0.4 M NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) supplemented with Complete Protease Inhibitor Cocktail (Cat. No. 1186145001; Sigma-Aldrich), Phosphatase Inhibitor Cocktail 2 (Cat. No. P5726; Sigma Aldrich) and Cocktail 3 (Cat. No. P0044; Sigma Aldrich). Organoids were then sonicated using a 30% amplitude for four times for 10 seconds using Sonics Vibra cell VCX130 (Sonics & Materials inc.). Protein concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientific) and all the samples were adjusted to the lowest concentration of all the samples.
Isotopically-labelled concatemer derived standard peptides were selected for the targets of interest (QconCAT technology, PolyQuant GmbH). In-gel digestion, LC-MS and analysis were performed as previously described by Wolters et al. The list of peptides used for targeted proteomics of peroxisomal proteins can be found in Supplementary Table 1.

**Branched chain fatty acids measurements**

Organoids were incubated with 25 µM phytol (Sigma Aldrich) for 48 h prior to quenching with ice-cold methanol. Methanol was then evaporated under a constant stream of N₂. The remaining pellet was reconstituted in PBS and using a 30% amplitude for four times for 10 seconds using Sonics Vibra cell VCX130 (Sonics & Materials inc.). Samples were further processed as previously described.

**Statistical Analysis**

Results are expressed as mean ± standard error of the mean (SEM). Biological replicates are considered as independent experiments using independent organoid lines. Analyses were performed using GraphPad Prism Software Version 9.02 (Graphpad Software). Statistical significance between comparisons is provided in figure’s legends. no indication means no significant changes (ns).

**FUNDING**

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 812968, and by the Netherlands Organisation for Health Research and Development under grant number 435005013.

**ACKNOWLEDGEMENTS**

We would like to thank Niels Kloosterhuis and Marieke Smit for their technical support with animal work. We would like to thank Nicolette Huijkman for the lentivirus production and establishment of stable cell lines. We would also like to thank Tjaša Košir for her advice on the choice of PEX transcript and interpretation.
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Docosahexaenoic acid prevents peroxisomal and mitochondrial protein loss in a murine hepatic organoid model


SUPPLEMENTARY FILES

Supplementary Figure 1. Effect of chloroquine on autophagy flux in hepatic organoids.

**GFP/RFP fluorescence** ratio in organoids in control conditions (Control) and 96 h of amino acid starvation treated and untreated with 50 µM Chloroquine. Organoids were also treated with 150nM Rapamycin with and without Chloroquine. Data represent mean ± SEM from 2 biological replicates.
Supplementary Figure 2. Effect of PPAR-α agonists on peroxisomal biogenesis.

(A) Relative gene expression of different peroxisomal biogenesis markers. Organoids were grown in complete culture medium (Control) or in starvation medium for 96 h (Starved), starvation medium with vehicle (DMSO) and (BSA) and in the presence of two different concentrations of WY-14643 (100 µM and 200 µM), DHA (50 µM and 100 µM) and LA (50 µM and 150 µM). Data represent mean ± SEM from 3 biological replicates (biological replicates are obtained from independent experiments).
Supplementary Figure 3. Effect of PPAR-α agonists on autophagic flux

(A) GFP/RFP fluorescence intensities ratio in Huh7 cells in control conditions and 48 h of amino acid starvation treated and untreated with DMSO (vehicle) or WY-14643 (100 µM or 200 µM). Data represent mean ± SEM from 3 biological replicates (*P < 0.05, **P < 0.01, ****P<0.001 RM One-way ANOVA)

(B) GFP/RFP fluorescence ratio in Huh7 cells in control conditions and 48 h of amino acid starvation treated and untreated with BSA (vehicle) or Linoleic acid (50 µM or 150 µM). Data represent mean ± SEM from 3 biological replicates (*P < 0.05, **P < 0.01 RM One-way ANOVA)
Supplementary figure 4. Effect of DHA on glycolysis, gluconeogenesis and pentose phosphate pathway.

Organoids were grown in complete culture medium (Control) or in starvation medium for 96 hours with vehicle (DMSO) and or DHA (50 µM and 100 µM). (A) Heatmap of enzymes involved in glycolysis, gluconeogenesis and PPP (Z-score normalized per protein). (B) Heatmap of enzymes involved in glycogen metabolism (z-score normalized per protein).
**Supplementary Table 1. List of all proteins included in the peroxisomal targeted proteomics and the peptide sequence.**

M denotes the peptide is mouse and HM for both mouse and human.

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EHHAHDM_M    VVGVPVALDLITSGR
CROT_HM      HLLGLLLIAYK
CROT_HM      SGGGNFVLSTSLVGYLR
AGXT_M       LLLGPGPSNLAPR
AGXT_M       QGIQYVFQTR
HAO1_HM      AIFVTVDTPYLGNR
HAO1_HM      QLDGVPATIDVLPEIVEAVEGK
ECH1_M       AVVVSAGAK
ECH12_M      ASQDFENALNQVK
ECH12_HM     WDAWNLGSLPK
CYP4F3_M     ALSDEDIR
CYP4F3_M     IFHPAFIKPVVLAPLVAPK
GPD1_HM      ITVQEVDTVEICGALK
GPD1_HM      LISEVIGER
CAT_HM       FYTEDGNWDLVGNNTPIFFIR
CAT_M        GAGAFGFVTDHIR
ACBD5_M      GSLNEQIALVLIR
ACBD5_HM     QATEGPCK
PEX11B_HM    FCITVSHLNR
PEX11B_HM    NACDLFlPLDK
### Supplementary Table 2. List of primary antibodies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACOX1</td>
<td>Abcam</td>
<td>Ab184032</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma-Aldrich</td>
<td>A5441</td>
</tr>
<tr>
<td>PMP70</td>
<td>Sigma-Aldrich</td>
<td>P0497</td>
</tr>
<tr>
<td>Catalase</td>
<td>Santa Cruz</td>
<td>SC-271803</td>
</tr>
</tbody>
</table>

### Supplementary Table 3. List of primer sequences used in RT-qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward and reverse primer sequence (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPIA</td>
<td>Fwd: TTCCTCCTTCACAGAATTATTCCA&lt;br&gt;Rev: CCGCCAGTGCCATTAGG</td>
</tr>
<tr>
<td>PEX1</td>
<td>Fwd: AAGGAAGAGCGTATTAAGCTGGA&lt;br&gt;Rev: TCGATTTCCGCACTCTGCTTCT</td>
</tr>
<tr>
<td>PEX5</td>
<td>Fwd: GCTGAGGAGTATCTGGAGCAGT&lt;br&gt;Rev: CCTTGACACAAAGGCTAGG</td>
</tr>
<tr>
<td>PEX7</td>
<td>Fwd: CCGAGTTCTCTCCGATCTTG&lt;br&gt;Rev: ACGTCAAACAGCCGTCATTTC</td>
</tr>
<tr>
<td>PEX11α</td>
<td>Fwd: GACGCCCTTCATCGAGTCG&lt;br&gt;Rev: CGGCTCTTTGTAGCTACTTGA</td>
</tr>
<tr>
<td>PEX13</td>
<td>Fwd: AACAACACTTACAAGAGTGC&lt;br&gt;Rev: CCGTAGGGCTCATATCAGGAGAG</td>
</tr>
<tr>
<td>PEX16</td>
<td>Fwd: AGGTCGCTTCTCCGATTAC&lt;br&gt;Rev: TGAGAGCAATGAGAGCCAAC</td>
</tr>
<tr>
<td>PEX3</td>
<td>Fwd: GCTCGCCGCACAGTACCATTTT&lt;br&gt;Rev: CGAGTTGAGCTGGCATTAAG</td>
</tr>
<tr>
<td>PEX6</td>
<td>Fwd: GGCAAGTTGAGATCCTGGAAAG&lt;br&gt;Rev: GGTGGTATCCAAGCCAAAGGCA</td>
</tr>
<tr>
<td>PEX19</td>
<td>Fwd: CTACTCAGAGGGTCGCTTTG&lt;br&gt;Rev: CCGACAGATTGAGAGCATTCCAG</td>
</tr>
</tbody>
</table>

Fwd, forward primer; Rev, reverse primer.