The role of peroxisomes in malnutrition and metabolic disease
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Chapter 7

General Discussion
This thesis focuses on the development of new in vitro and in silico translational models to study two diseases affecting fatty acid metabolism. Liver and intestinal organoids are presented as suitable tools to study malnutrition and medium-chain acyl-CoA dehydrogenase deficiency (MCADD). Moreover, I also present two in silico models that helped complement the findings of the in vitro systems as well as to predict metabolic responses. During the thesis, I also focused on the effects of both diseases on peroxisomal and mitochondrial homeostasis and their interplay.

In this chapter, I focus on (i) a general discussion about different aspects related to these new translational models and (ii) the importance of the peroxisomal-mitochondrial interplay in health and disease.

PART 1. ESTABLISHING TRANSLATIONAL IN VITRO AND IN SILICO MODELS FOR THE STUDY OF MALNUTRITION AND MCADD

In this thesis, I established two in vitro organoid models to study malnutrition and medium chain acyl-CoA dehydrogenase deficiency. I have also focused on the development of two in silico models. First, I developed a detailed kinetic model to study fluxes and metabolite concentrations of the peroxisomal β-oxidation. Secondly, I contributed to the development of a deep learning (DL) model to measure and track organoid size. This was done by providing data, defining requirements and validating the outcomes. In this section, I discuss about the establishment of these models for the study of the above-mentioned diseases and how to tailor them to metabolic research. Next, I highlight the limitations of these models and focus on the potential approaches and optimizations needed to overcome these limitations.

Applications and advantages of translational models for metabolic research

In chapter 2, I focused on the development of two in vitro models to study malnutrition. These models were established using primary tissue from murine liver and intestine to set up hepatic and intestinal organoids respectively. To the best of our knowledge, these are the first hepatic and intestinal organoid models of malnutrition described. A variation of the intestinal model was recently described in the literature to understand the role of mitochondrial homeostasis and intestinal barrier function. More recently, human intestinal organoids have also been established for the study of malnutrition in vitro.
However, this study focused on removal of not only amino acids but also proteins and carbohydrates. Moreover, they studied intestinal permeability of the organoids but did not characterize metabolic (mitochondrial or peroxisomal) function.

Organoids are three-dimensional cell structures that recapitulate multiple functions of the organ of origin whilst continuing to proliferate in culture. Since their discovery, tens of different organoid models have been described, including the pancreas, stomach, lungs, and brain. Organoids have been used for a wide range of purposes such as developmental studies, modeling genetic diseases, and drug screening. Here, I demonstrated that hepatic and intestinal organoids make suitable models to study malnutrition in vitro. In order to mimic low protein diets (LPD), used in in vivo models to induce malnutrition, organoids were grown in culture medium without any amino acids. However, CG-MS analysis of the organoid supernatant revealed that all amino acids were present, probably coming from the hydrogel or from the degradation of the growth factors in the medium, yet in much lower levels than in the control cultures. These in vitro systems present a wide range of advantages and new possibilities when compared to in vivo studies. One main point of interest is the possibility to study one specific organ at a time. This allowed us to study the effects of amino-acid restriction on the liver and the intestine in an isolated system without a systemic response. A key example highlighting the importance of organ-specific studies is discussed in chapter 4. Here I reported that, opposite to what was expected from in vivo studies, the autophagic flux was clearly upregulated in hepatic and intestinal organoids upon amino-acid restriction. While these results might seem contradictory, it is not uncommon to see different responses between in vivo and in vitro models. Moreover, this organ-specific approach paves the way for characterizing and studying different organ-specific-pathophysiological processes which can provide great insight into the disease of interest.

Another advantage of these translational in vitro models is the possibility to pre-screen large numbers of drugs prior to in vivo testing. In chapter 2, to illustrate the potential of the models, I tested the effects of fenofibrate and rapamycin in the liver and the intestine, respectively, as a proof of principle. These compounds showed a similar response as to that observed in vivo. Moreover, in chapter 4 I focused on a more extensive drug screening to prevent peroxisomal loss in the hepatic malnutrition model. I this chapter I evaluated the potential of different PPAR-α activators including synthetic and naturally occurring compounds. These studies led us to the findings that docosahexaenoic acid (DHA), an omega-3 fatty acid, could prevent the loss
of multiple peroxisomal and mitochondrial proteins in amino-acid restricted organoids. While these results are promising, they should be interpreted as a first step into the characterization of the biological effect of such compound and this should be further tested and validated in vivo.

Another in vitro model developed during this thesis is introduced in chapter 6. Together with my colleague Ligia Kiyuna, we developed an iPSC-derived hepatobiliary model to study MCADD. To the best of our knowledge, this is the first patient-specific liver organoid model for the study of MCAD deficiency. Similar to primary organoids, iPSC-derived organoids allow us to understand different pathophysiological processes within a given disease. Moreover, they present a very valuable advantage as they are derived from patient tissue\textsuperscript{19,20}. This offers the opportunity to understand the effect of different mutations on different factors such as development, cell fate, or cell metabolism.

In the case of MCADD, it is not uncommon to see patients who are asymptomatic even if they carry the same mutations as other symptomatic patient\textsuperscript{21,22}. In this first study, we have only used fibroblasts coming from patients with the classical mutation c.985A>G. However, the model offers the opportunity to generate organoids from fibroblasts from patients with different mutations to understand and determine the differences between symptomatic and asymptomatic patients.

I developed two in silico models applied to the study of malnutrition in vitro and in vivo. In chapter 3, together with my colleague Asmaa Haja from the Perico ITN consortium, I contributed to the development of an automated tool to measure and track organoid size and morphology in brightfield images. As my contribution, I provided data to train the algorithm, defined the requirements of the system and validated the results. The need for this tool stemmed from the demand to quantify large sets of images containing, at times, hundreds of organoids. This deep-learning model was developed to speed up the quantification and characterization of the organoid work done in chapter 2, and to be applied for future experiments. In addition to minimizing the workload, another reason to develop this model was to reduce the intrinsic variability associated to organoid measuring and counting. The goal was to identify as many organoids as possible per image and track their individual growth rate in time. During the data analysis phase, distribution profiles were generated, avoiding solely relying on the average of all the data points. This model represents one of the many applications of artificial intelligence to in vitro research.
In chapter 5, I focused on the development of another in silico model, aimed to provide insight into the effects of amino-acid restriction on the peroxisomal β-oxidation of fatty acids. This model relies on kinetic information of the enzymes involved, including rate equations describing the kinetic behavior of the enzymes, kinetic parameters such as $V_{\text{max}}$ and $K_{\text{ms}}$, and ordinary differential equations. Kinetic models have been used to understand multiple metabolic pathways as well as to provide insight into the regulation of the pathway, for instance by understanding what enzyme or enzymes have the most control on the flux. One very interesting application of these models, is the combination with proteomics data from in vitro or in vivo studies to approximate it to physiological conditions\textsuperscript{23,24}. The use of proteomics data also leads to the development of personalized models, very important tools in the case of rare diseases\textsuperscript{25}. Moreover, these models can provide information on the effects of different drugs to the pathway and predict potential accumulations of toxic metabolites. While the model of peroxisomal β-oxidation presented in this thesis is a first approximation which needs further optimization, the long-term goal is to characterize the effects of amino-acid restriction on the peroxisomal β-oxidation of fatty acids and combine it with our previously established mitochondrial counterpart. We believe that this combination of both models might shed some light into the metabolism of fatty acids in the liver not only in malnutrition but also in all diseases where FAO is implicated. Moreover, I have performed metabolic control analysis\textsuperscript{26} under different conditions. The results from these studies pointed towards ACOX1 as the enzyme with the highest control on the pathway, in line with previous studies describing the enzyme as the rate limiting step of the pathway\textsuperscript{27,28}. Moreover, I have used proteomics data from the in vitro hepatic model to understand the effects of amino-acid restriction on the flux control of the different enzymes of the pathway.

**Limitations of new translational models and future perspectives**

Although it is undeniable that the discovery of organoids has transformed the field of biomedical research, it is crucial to be aware of their limitations. Most of the early work on organoids focused on the study of developmental biology, recapitulation of the organ of origin, and disease modelling. Some studies have highlighted some limitations of these in vitro models, such as lack of different cell populations or failure to recapitulate some functions of the organ of origin\textsuperscript{29,30}. 
During the course of the PhD, I have encountered multiple challenges in the establishment and application of metabolic techniques onto organoid cultures. Most of these issues stemmed from either low reproducibility between organoid cultures, low number of cells, or the presence of hydrogels such as Matrigel or BME (Figure 1).

One relevant point to discuss is the high variability observed between organoid cultures. It is well known that organoids are highly heterogenous, displaying variability between cells within the same organoid (intra-organoid heterogeneity), between organoids within the same plate and between organoids from different patients (inter-organoid heterogeneity). While this heterogeneity is crucial to capture differences between patients, and therefore for personalized-medicine purposes, it is also a setback in reproducibility. While inter-organoid heterogeneity is not a problem, inter-organoid variability can minimize reproducibility in many downstream analyses. When working on metabolic profiling of these organoids, it is common to observe disparate absolute values of metabolites within different organoid cultures when they
are derived from the same patient. This heterogeneity between organoids highlights the need for standardized procedures and protocols\textsuperscript{32}. This is particularly important in the field of metabolism which requires precise quantitation.

In order to avoid data misinterpretation, correct analysis and interpretation are crucial. In some cases, it may be considered to normalize the data of each individual organoid line to its own control prior to processing with the other biological replicates. This approach could help identify trends in responses to different treatments or as a result of different insults. A second approach to this issue is the use of distribution profiles of individual organoids instead of averages. In that instance, observational studies of each organoid in the culture could help reduce the risk of losing individual responses based on the averaged trend. One example on how to prevent this, is illustrated in chapter 3. OrganelX is able to track individual organoids in time, allowing us to get individual growth rates of all the organoids in a culture. The data for each individual organoid can be used to obtain an average response but also allows to identify individual responses. While this approach could help optimize the interpretation of data, it does not prevent the above-mention heterogeneity.

Another limitation of the organoid work is the number of cells and biomass obtained for downstream assays. While organoids are composed of thousands of cells, traditional organoid work is done in 24-well plates containing 50 µl domes. Upon collection, the average number of cells obtained from a 50 µl dome containing hepatic organoids can range from $3 \cdot 10^4$ to $5 \cdot 10^5$ cells. In contrast, the average number of HEPG2 cells in a 10 cm$^2$ dish used for metabolic assays ranges between $4 \cdot 10^6$ and $8 \cdot 10^6$. While two domes of 50 µL can provide enough material to perform qPCR or immunofluorescence, this would not be sufficient for most metabolic assays. For some metabolic techniques performed in this thesis, pooling of 3-4 wells was required, which in some cases still did not yield enough material for reliable quantification. Moreover, the high number of organoids needed also increased the costs of the experiment.

One straightforward approach is to increase the number of organoids, and therefore cells, by upscaling the cultures or optimizing the culture procedures\textsuperscript{33}. This would allow us to obtain more reliable readouts that depend on cell number. However, this can also increase costs associated with the amount of media needed, cytokines and consumables. An alternative solution is to increase the sensitivity of the bioanalytical assays performed in metabolomic studies. Several groups are focused on the profiling of different metabolites in small quantities using small numbers of cells with microscale analytical
techniques such as capillary electrophoresis-mass spectrometry (CE-MS). These new techniques could allow for reliable and reproducible metabolic profiles of organoid samples with limited biomass.

Another important and limiting factor of organoid work is the use of hydrogels. Hydrogels, are extracellular matrix (ECM) proteins that work as a scaffold, allowing organoids to grow in three dimensions. These matrices are commonly from tumor origin and present high batch-to-batch variability. Alternatively to animal-derived undefined matrixes, the biomaterials field is currently moving towards alternatives including decellularized extracellular matrix, synthetic hydrogels and gel-forming recombinant proteins. It is important to mention that biological hydrogels contain high concentrations of proteins and their presence might interfere with downstream analysis, particularly in proteomics analysis. During the work for this thesis, I observed how correct degradation and removal of the hydrogel prior to downstream analysis is essential to obtain reliable proteomics results. This could be caused by the amount of hydrogel left after removal, which can also impact total protein concentrations and cause variations in normalization. Therefore, it is important to establish robust and standardized protocols for the complete removal of matrixes and to avoid issues derived from the hydrogels.

Finally, I would also like to bring the attention to a commonly overlooked limitation of organoid work, namely the economic costs. Organoid culture medium often requires expensive growth factors and cytokines, which are used in large quantities. Moreover, the hydrogels needed to grow organoids in three dimensions are also costly and needed in large amounts. Because of these high costs, the organoid technology is not yet accessible to all research groups. Surprisingly, little information has been published on this topic. While the organoid technology slowly becomes a daily practice in more and more labs, it is still far from being accessible to everyone. There is a clear need for companies and academic labs to work together, towards the development and commercialization of robust, well-defined and inexpensive matrixes to standardize biological research.

Regarding the in silico models presented in this thesis, some aspects require further attention. These models are presented as tools to predict in vitro/in vivo responses or behaviors. However, in order to build biologically accurate models, we rely on kinetic parameters from the enzymes in the model. While working on chapter 5, I found that there is a lack of standardized and reliable kinetic parameters of the enzymes involved in the peroxisomal β-oxidation. Most of the studies describing kinetics of the enzymes date back to the 80s.
and 90s of the twentieth century and lack information on oxidation of very-long-chain fatty acids or branched-chain fatty acids\textsuperscript{39}. This was due to the insoluble nature of these compounds making it challenging to work with them. Moreover, very little information on kinetic parameters of the transporters is available. In order to perform kinetic studies, transporters are often reconstituted in liposomes to perform transport assays which can be affected by lipid membrane dynamics\textsuperscript{40}. As a solution, I propose to re-visit the early kinetic studies making use of the current technology to fully characterize the mechanism and kinetic parameters of the enzymes of interest, making for new and more detailed kinetic models.

**PART 2. THE IMPORTANCE OF PEROXISOMES AND MITOCHONDRIAL IN FATTY ACID METABOLISM AND THEIR INTERPLAY IN HEALTH AND DISEASE**

The main aim of the thesis was to develop in vitro and in silico models to study two diseases affecting fatty acid metabolism. In both diseases, peroxisomes and mitochondria are involved, highlighting the importance and interplay of these two metabolic organelles. While mitochondria have been extensively studied and characterized, we have an incomplete overview of the many peroxisomal functions that are still being discovered. Recent studies have described peroxisomes to be involved in different processes such as aging\textsuperscript{41}, immunometabolism\textsuperscript{42}, etc.

**Peroxisomes and mitochondria in Severe Malnutrition**

In chapter 2, I focused on the effects of malnutrition on peroxisomes and mitochondria both in the liver and the intestine and the mechanisms regulating the balance between biosynthesis and degradation of both organelles. As a proxy to malnutrition, I exposed the organoids to amino-acid restricted media for different periods of time. The amino-acid restricted conditions in our studies mimic the effects of low protein diets, leading to a reduction in peroxisomal and mitochondrial mass and function. In the case of the liver, mitochondrial loss is described to follow peroxisomal degradation while this time dependence was not observed in the intestinal organoids. However, while the loss of peroxisomes and mitochondria in the hepatic organoids recapitulates the findings of the in vivo models\textsuperscript{14}, the loss of peroxisomes observed in the malnourished intestinal organoids has not yet been investigated in vivo. In chapter 4, I discussed whether the reduction in peroxisomal number in the
hepatic organoids is caused by increased degradation or reduced biosynthesis. While impaired biogenesis could be a potential explanation, little information is available on the topic. Only one in vivo study using rats on an LPD reported no changes in the expression of any peroxins (PEX) involved in peroxisomal biogenesis and maintenance\textsuperscript{14}. During this chapter I showed how amino-acid restricted organoids showed regulation of several PEX genes. This differential regulation indicates, that although it is hard to determine whether biogenesis is up- or downregulated, it is clearly affected by amino acid restriction. In the case of PEX5, linked to pexophagy in nutrient starvation\textsuperscript{43}, we observed a clear upregulation under the amino-acid restricted conditions. Further studies should be done in order to fully understand the effect of amino-acid restriction on peroxisomal biogenesis.

In contrast, several groups have focused on the effects of LPD on autophagic degradation in rodents. It is important to emphasize that different experimental conditions lead to different results, and in some cases comparison between models might be misleading. Low protein diets have been shown to induce peroxisomal and mitochondrial loss as well as dysfunctional mitochondria both in children and laboratory animals\textsuperscript{13,14,44}. Most of the in vivo work on malnutrition has reported that long exposure to LPD leads to an impairment in autophagic turnover\textsuperscript{12,13}. However, the results found in this thesis, using in vitro models, opposed those found in in vivo studies. In chapter 4, I reported that amino-acid restriction leads to a clear upregulation of autophagic degradation, as measured with an autophagic probe. An interesting aspect of this model is the use of amino-acid restriction instead of a complete amino-acid starvation. While it has been previously described that complete amino-acid starvations induce autophagy in vitro\textsuperscript{45,46}, there is no information of the effect of long periods of time of amino-acid restriction. If we try to compare our results with the in vivo models, they are more in line with what was observed in the liver of rats fed a LPD for 1 week\textsuperscript{14}. However, in that same study, the authors reported a block in autophagy after 4 weeks of LPD. These results, together with those of the mouse models which used 2 weeks of LDP, might indicate that autophagy is differentially regulated according to the time of exposure to LPD. On that note, in chapter 4, I also reported a different regulation of autophagy in organoids deprived of amino acids for 48 h and 96 h. These results also point towards a differential regulation based on time. However, in the case of the amino-acid restricted hepatic organoid model, amino-acid restrictions of longer than 4 days were hard to recapitulate using the organoids due to technical limitations. On this note, it is important to emphasize the challenges of translating time length from organoid work to animal models.
During the work on this thesis, I also illustrated the potential of 3D in vitro models to test different therapeutic approaches to prevent peroxisomal and mitochondrial loss in the context of amino-acid restriction. In chapter 2, I briefly focused on the therapeutic effects of fenofibrate and rapamycin in the liver and the intestine, respectively. Fenofibrate prevented the loss of peroxisomal markers in 48h amino-acid restricted hepatic organoids, but failed to prevent loss of peroxisomal function. In chapter 4 I tested the effects of different PPAR-α agonists on preventing peroxisomal and mitochondrial loss during longer amino-acid restrictions (96h). When comparing the effects of docosahexaenoic acid (DHA) supplementation with other PPAR-α agonists we observed disparate results. DHA supplementation prevented the loss of multiple peroxisomal and mitochondrial proteins, whilst the rest of the compounds showed little to no effect. Interestingly, the results were different from those of fenofibrate in chapter 2. However, it is not possible to compare these results directly as the amino-acid restriction time was different between experiments in the two chapters. Given the differences between DHA and the other agonists, we hypothesized a PPAR-α-independent effect of DHA leading to the prevention of peroxisomal and mitochondrial loss. While in this chapter I only focused on a small panel of compounds, this platform is suitable for larger screenings of other compounds to either stimulate peroxisomal biogenesis or to prevent their degradation. The use of targeted proteomics could be applied to larger screenings of compounds, assessing their effect on prevention of peroxisomal loss in the context of amino-acid restriction. Optimization of the number of organoids needed for the protocol would greatly benefit the upscaling process and minimize associated costs.

**Potential roles of peroxisomes in MCADD**

Finally, in chapter 6, I focused on the study of medium chain acyl-CoA dehydrogenase deficiency. This inherited disease affects the metabolism of medium chain acyl-CoA leading to accumulation of medium-chain fatty acids and fatty-acid esters under different circumstances. One interesting point of discussion raised in chapter 6 is the heterogeneity in symptomatology observed amongst MCADD patients. While the main aim of this chapter was to develop an in vitro model to study and characterize the disease, we also explored a potential compensatory role of peroxisomes. Here, we hypothesized that different compensatory mechanisms involving peroxisomes could explain the differences in adaptation to conditions such as fasting or illness. In this chapter we observed no regulation of the peroxisomal β-oxidation of fatty acids in MCADD organoids. However, we identified a clear regulation of several genes
coding for enzymes involved the metabolism of CoA, both in the peroxisome and mitochondria. These results point towards a peroxisomal contribution to MCADD and emphasize the need to further characterize the interplay of both organelles in the disease. For this study, and to set up the model, we purposefully selected fibroblast from symptomatic patients. For that reason, we believe that differentiation of fibroblast from a larger number of patients, including symptomatic and asymptomatic, will provide broader view of the adaption mechanisms of peroxisomes in the disease.

PART 3. CONCLUDING REMARKS AND FUTURE DIRECTIONS

In conclusion, this work illustrates the potential of new translational models to understand and characterize disorders affecting mitochondrial and peroxisomal fatty-acid metabolism. I present two in vitro and two in silico models for the study of malnutrition and MCADD and introduce them as tools for the study of the pathophysiology of the diseases and discovery of therapeutic approaches.

As an proof-of-principle, the intestinal model has already been used to test the effect of amino-acid restriction on mitochondrial homeostasis and intestinal permeability. Moreover, as shown in chapter 2 and 4, the organoid models can be used to test different therapeutic and pharmacological interventions. Future studies, including higher number of compounds should be performed to find the best approach to minimize peroxisomal and mitochondrial defects in malnutrition.

In the case of MCADD, we aim to perform further studies including RNAseq and untargeted proteomics to understand the differences between MCADD and control organoids. Furthermore, we intend to establish organoids from asymptomatic patients in order to compare them to the symptomatic ones. We believe that this approach will help us uncover and understand potential adaptation mechanisms in MCADD.
REFERENCES


