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Review of Ines Simoes's doctoral dissertation entitled "Effects of Western diet in the development and progression of Non-alcoholic fatty liver disease"

The assessed doctoral dissertation conducted under the supervision of Prof. Mariusz Wieckowski from Nencki Institute of Experimental Biology in Warsaw and Dr. Paulo J. Oliviera from University of Coimbra, Portugal is an original, new approach to the problem of the impact of high-calorie diets on the course and development of non-alcoholic fatty liver (NAFL) disease, with particular emphasis on the role of mitochondria. NAFL disease is the most common liver disease in the world, affecting approximately 25% of the world's population, and is associated with a build-up of extra fat in liver cells that is not caused by alcohol. Ines Simoes's doctoral thesis focuses on the early mechanisms induced by a high calorie diet that can lead to changes in the liver and consequently may cause disease progression to non-alcoholic steatohepatitis (NASH) in the C57BL/6J mouse model of NAFL. In the first chapter, the Doctoral Student investigated redox-related hepatic and mitochondrial changes, characterized the liver proteome, mitochondrial structure and function, production of reactive oxygen species (ROS), and antioxidant defense in the mouse model of early-stage NAFL resulting from chronic 16-week feeding of mice with a high-fat diet, a high-sucrose diet, or a high-fat and high-sucrose diet (representing Western diet, WD). The second chapter describes the study of disease progression from early stage to more progressive disease, through 24 weeks of WD feeding, focusing on successive mitochondrial changes. The third chapter describes the study of the mitochondria-targeted antioxidant AntiOxCIN$_4$ as a new potential therapeutic agent for NAFL disease.

Formal description of the doctoral dissertation

The doctoral thesis submitted for review is written in nice, concise and clear English. Only a few typos, editorial and minor stylistic errors can be found in the entire work. For example:

- The legend of Figure 61 erroneously repeats the legend of Figure 60.
- The beginning of a new paragraph is not marked several times.
- UCP2 abbreviation is not explained (p. 19).

However, the Doctoral Student's great diligence in the preparation of this very extensive doctoral dissertation can be noticed.

The doctoral dissertation, 201 pages in total, contains 68 figures and 8 tables. The layout of the work is typical and includes: table of contents, acknowledgments, funding, author's publications, list of figures and tables, list of abbreviations, summary in English and Polish, general introduction, hypothesis and objectives, materials and methods, results and discussion, summary and final conclusions, and bibliography of 354 publications, mainly from literature published in the last 10 years.

The presented list of the author's publications shows that Ines Simoes is a co-author of 9 publications with a total IF ~42, which at this stage of scientific career is a very good/outstanding scientific achievement. Among these publications, three experimental papers published in *Antioxidants* (2020), *Cells* (2019) and *International Journal of Molecular Sciences* (2021), in which the Doctoral Student is the first (twice) or second (once) author,

present the results described in the assessed doctoral dissertation. In the thesis, the Doctoral Student states her major contribution to the conceptual design, experimental execution, data analysis and interpretation, and manuscript preparation of all the work presented along this thesis dissertation.

Preceding the experimental part, the 34-page General Introduction is a well-written overview of the current state of knowledge on various aspects of NAFL disease, including clinical diagnosis, pathophysiology with a focus on mitochondrial pathophysiology, ROS homeostasis imbalance and oxidative stress, autophagy and NAFL progression. The Doctoral Student also described antioxidant-based NAFL disease therapies, with particular emphasis on the new antioxidant AntiOxCIN$_4$ targeting the mitochondria. When writing the General Introduction, the Author referred to the most important literature on the issues discussed.

Comments and questions to the General Introduction:

- Page 15. " ETC is constituted by four complexes … and several carriers that allow the flux of electrons from Complex I to Complex IV…". Why "several"? There are only two electron carriers, i.e., coenzyme Q and cytochrome *c*.
- Figure 4. Complex II is a transmembrane protein.

In the further part of the doctoral dissertation, the Doctoral Student skillfully presents the hypotheses and 3 main objectives of the research.: "1) study of the hepatic and mitochondrial redox-associated alterations in a NAFL stage; 2) investigation of the specific end-points for mitochondrial dysfunction that represent a point of no return and which drive NAFL progression along time; 3) validation of a next generation of therapeutics for NAFL based on smart antioxidant delivery to mitochondria."

- After reading the entire dissertation, it is unclear what processes described can be considered the endpoints of mitochondrial dysfunction that influence NAFL progression. The Author does not mention these processes.

The Materials and Methods section describes the C57BL/6J mouse model of NAFL and the high-calorie diets used in the work. There are also comprehensive and careful descriptions of the numerous research methods used. The wide variety of techniques used is noteworthy, which proves the high versatility of the Doctoral Student in experimental work. The studies included: plasma and histological analyzes, isolation of mitochondria and the cytosolic fraction of the liver, various methods of measuring oxygen consumption rate (OCR), determination of the mitochondrial membrane potential, oxidative phosphorylation (OXPHOS) complexes, ROS production, analysis of oxidative damage, antioxidant activity, immunological protein detection, and finally the proteomic, lipidomic and metabolomic analyses.

Comments and questions to the Material and Methods:

- Throughout the work, I did not notice the information on how many rats were used in particular parts of the work and how many technical repetitions of individual measurements and analyses were (n = ?).

The most extensive part of the thesis, Results and Discussion, which covers 91 pages, is divided into 3 complementary chapters devoted to the three main objectives described in this dissertation. In individual chapters, the Author presents the results of subsequent research tasks in a clear and concise manner. It is worth emphasizing how the reader is guided through the different stages of the experimental work and how the stages are explained. Each point begins with a short introduction and ends with a short summary that organizes the various stages of the research. The results in the individual chapters are presented in a logical

sequence and form a coherent whole. It should be emphasized that the Doctoral Student skillfully and critically analyzed the obtained results within individual chapters, and then discussed them against the results of other researchers. Importantly, each chapter ends with a diagram that summarizes the results obtained.

- General remarks regarding the presentation of the results. It would be more clear for the reader to mark the compared bars with a horizontal line when marking statistical significance. A different scale of blue and red intensity was used in the figures presenting the results of the proteomic analysis. When can a change be assumed to be significant?

In the first Chapter entitled "Fat and sucrose induce the development of fatty liver with the impairment of autophagic flux in mice", the Doctoral Student has shown that a high intake of fat and/or sucrose induces steatosis with an accumulation of monounsaturated fatty acids (FA), likely protecting hepatocytes from saturated FA-induced lipotoxicity, and liver fat accumulation triggers the remodeling of mitochondrial phospholipids with increased levels of cardiolipin. Additionally, the Doctoral Student has performed a very comprehensive characterization of oxidative stress-related parameters in mitochondria and in cytosol of hepatocytes. She has shown that there is no excess of mitochondrial ROS in NAFL, suggesting that other organelles, such as peroxisomes, contribute to hepatic oxidative stress probably due to peroxisomal FA oxidation-induced ROS generation and the impairment of hepatic proteolysis. Moreover, the Doctoral Student has found that fat and sucrose (components of WD) interfere with autophagy in different ways. While fat interferes with the acidification of the lysosomes, sucrose causes the defective formation of autophagolysosomes.

Comments and questions to the first Chapter of Results and Discussion:

- Figure 20. The # symbol above the last bar is described as $P = 0.08$ (?)
- Figure 21B, right panel. Representative images of the in-gel activity assays for Complexes I, II, IV and V are too small. It is difficult to analyze changes (if any?) in individual CI, CIII and CIV supercomplexes. What was the loading control?
- In this and the following chapters, it is unclear whether a high-calorie diet increases mitochondrial biogenesis.

In the second Chapter entitled "Effects of Western diet in the development and progression of Non-alcoholic fatty liver disease", the Doctoral Student has studied the progression of the disease from an early stage into a more progressive stage (up to 24 weeks of feeding), demonstrating for the first time the sequential events of mitochondrial alterations during NAFL development. By week 20 of WD diet, there was an early mitochondrial remodeling with increased levels of OXPHOS subunits and higher mitochondrial respiration, followed by a progressive loss of mitochondrial respiration accompanied by higher susceptibility to mitochondrial permeability transition pore (mPTP) opening. The Doctoral Student has shown that these mitochondrial alterations and subsequent impairment are independent of an excessive mitochondrial ROS generation, which progressively diminished along with disease progression. Instead, increased peroxisomal abundance and peroxisomal FA oxidation-related pathway suggest that peroxisomes may contribute to hepatic ROS generation and oxidative damage, which may accelerate hepatic injury and disease progression.

Comments and questions to the second Chapter of Results and Discussion:

- Figure 38. The representative Western blot does not reflect the changes shown in the bar graphs for week 22 of the WD diet (decrease in complex II and complex III subunits).

- Figures 40 and 41, representative OCR profiles. The timing of administration of some reagents is incorrectly marked.

In the third Chapter entitled "AntiOxCIN$_4$ - a new mitochondrially targeted antioxidant that prevents the development of Non-alcoholic fatty liver phenotype", the Doctoral Student has tested for the first time the potential therapeutic effect of AntiOxCIN$_4$ supplementation in in a mouse NAFL model of 16-week feeding with WD. She has shown that AntiOxCIN$_4$ supplementation induces a healthier phenotype of hepatocytes in NAFL rats. Namely, a reduction in body weight, an improvement in the plasma markers of liver damage, and a reduction in the accumulation of fat in the liver with a remodeling of the composition of fatty acyl chains were observed. These positive changes can be explained by the stimulation of liver oxidative metabolism (mainly cellular FA oxidation-related pathways), stimulation of the endogenous antioxidant defense system, and the prevention of autophagic flux blockage by the stimulation of lysosomal proteolytic activity. In addition, AntiOxCIN4 appears to improve mitochondrial function by increasing the amount of certain subunits of the OXPHOS complexes and remodeling mitochondrial phospholipids to prevent mPTP from opening.

Comments and questions to the second Chapter of Results and Discussion:

- The title of the Figure 60 legend only refers to part B.
- Page 171. "In addition, AntiOxCIN$_4$ appears to improve mitochondrial function, including higher OXPHOS complexes subunits…" This generalization is incorrect. Increased protein levels were observed mainly/only in the case of complex I subunits Figures 61 and 62).
- Complex II subunits decreased in mitochondria of WD rats (Figure 62) and complex II activity increased under phosphorylating and uncoupling conditions (Figures 59 and 60). How to explain it?
- Page 169. "By stimulating mitochondrial biogenesis, mitochondrial respiration and mitochondrial antioxidant defense enzymes, AntiOxCIN$_4$ demonstrates…". I do not see clear evidence that AntiOxCIN$_4$ stimulates mitochondrial biogenesis in livers of AntiOxCIN$_4$-treated rats (Figure 61B - VDAC1 detection, Figures 61C and 62 – decrease in some OXPHOS subunits, Figure 63 B – no increased level of VDAC1).

Summary

The above comments and the few linguistic and editorial errors do not affect my high assessment of the presented work, its substantive value and the importance of the research carried out. The results of the experiments presented in the dissertation are important for understanding the molecular basis of the etiology and development of non-alcoholic fatty acid liver disease in connection with the Western diet, as well as for its diagnosis and treatment in the future. In a broader sense, the research described in the peer-reviewed dissertation is also important for understanding the importance of mitochondria in the function and development of cell/organism pathology. I believe that the assessed doctoral dissertation meets the conditions set out in Art. 187 of the Act of July 20, 2018, Law on Higher Education and Science (Journal of Laws of 2021, items 478, 619, 1630). Additionally, taking into account the substantive and innovative value of the work, the importance of research, the huge amount of work and the variety of experiences and analyzes, publishing a significant part of the experimental work in good journals, as well as the ability to present and discuss the results, I believe that Ines Simoes's doctoral dissertation deserves to be awarded with the appropriate award.

W. Jarmuszkiewicz

Prof. Wiesława Jarmuszkiewicz

Written Assessment and Review
Doctoral Dissertation

Tittle: Effects of Western diet in the development and progression of non-alcoholic fatty liver disease
Student: Inês Simões

Inês Simões was an early stage researcher of the FOIE GRAS European Training Network, under the supervision of Prof. Mariusz Wieckowski, Nencki Institute of Experimental Disease of the Polish Academy of Science, Warsaw, Poland and Dr. Paulo Oliveira, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal. The results presented in this thesis have already been published in international peer reviewed journals as first author (four) and co-author (three) papers. Inês Simões has contributed to the conceptual design, experimental work, data analysis and interpretation, and writing of the manuscripts. In addition, other manuscripts have been published, or are under review or in preparation.

Caloric excess and sedentary lifestyle have led to a global epidemic of obesity and metabolic syndrome. The hepatic consequence, non-alcoholic fatty liver disease (NAFLD), is estimated to affect up to 1/3 of the overall population. The spectrum of liver disease ranges from NAFL with simple steatosis to non-alcoholic steatohepatitis (NASH), and ultimately fibrosis/cirrhosis. Owing to high prevalence and consequent burden of disease progression, NAFLD has rapidly become a leading aetiology underlying hepatocellular carcinoma. The initial stage or NAFL is characterised by intense metabolic remodelling of the liver to compensate fat accumulation, along with mitochondrial function impairment and oxidative stress that later trigger NAFLD progression with inflammation, fibrosis and cirrhosis. Inês Simões has been an active contributor to this field.

General

First, Inês Simões investigated the hepatic proteome, mitochondrial structure and dysfunction, reactive oxygen species (ROS) production and antioxidant defences in a mouse model of early NAFL. She shows that mitochondrial reactive oxygen species (ROS) production are not a major player in NAFL pathogenesis in mice fed high-fat, high-sucrose or high-fat plus high-sucrose for 16 weeks, but rather peroxisomes contribute to hepatic oxidative stress. Fat and sucrose were reported to differentially impair autophagy. Second, Inês Simões described the sequential mitochondrial alterations during NAFL development and progression after high-fat diet up to 24 weeks of feeding. Initial

mitochondrial adaptation was followed by progressive decrease in mitochondrial respiration and higher susceptibility to mitochondrial permeability transition pore opening. Peroxisomes, but not mitochondria, were confirmed to be key contributors to hepatic oxidative damage. Finally, a mitochondria-targeted antioxidant was tested as new therapeutics in NAFLD. AntiOxCIN4 supplementation improved the NAFL phenotype in a diet-fed mouse model, including enhanced hepatic fatty acid oxidation, antioxidant defence and autophagic flux.

These studies represent a large amount of work and entail many and important research suggestions, although some limitations can be recognized and should be further discussed by the candidate. First, several aspects of the work focused on descriptive and associative facts. However, no further functional or mechanistic research was conducted. Cell specific data and gain and loss of function experiments as well as pharmacological approaches should pinpoint molecular targets and signaling pathways of interest. Second, studies were conducted *in vivo*. While the wealth of information is impressive and relevant for humans, indeed, the use of human samples would nicely complement animal studies and confirm the link between proteome signatures, fatty acid oxidation, antioxidant defense systems and autophagic flux, and pathological stages. No doubt, these studies open up new avenues to further research expanding on the above-mentioned findings.

The introduction is very well written, briefly presenting NAFLD epidemiology, risk factors and clinical diagnosis. The pathophysiology section deserves an extensive introduction and focus mainly on lipid accumulation, associated mitochondrial pathophysiology and imbalance of ROS homeostasis and oxidative stress. A short reference to antioxidant-based therapies concludes the introductory part of the thesis. It is missed a more robust discussion about diagnosis and treatment options in NAFLD in what related to lipid accumulation stages of the disease. Pharmacological strategies are not enough expanded to appreciate the effort of developing a new antioxidant molecule. Clinical trial data on the use of antioxidant strategies is also not developed. And finally, surprisingly, the ongoing debate about NAFLD versus MAFLD nomenclature and its implications are not introduced in the thesis.

The hypothesis is that a pro-oxidant state is the trigger of mitochondrial-related alterations that culminate in NAFLD progression. The Objectives are clear and further expanded upon in each chapter in Results and Discussion section. Each objective 1-3 corresponds to a Chapter 1-3. Next, the Materials and Methods section is informative and well balanced. However, it is unclear how many experiments, replicates, animals were included in each experimental setup. The candidate performed a diverse array of experiments, which attests the quality of the doctoral raining provided by the host laboratories.

Specific

Chapter 1 of results is entitled “Fat and sucrose induce the development of fatty liver with the impairment of autophagic flux in mice”. In the first chapter, phenotypic data and liver enzymes show increases in liver weight, body weight and ALT, while histology confirms steatosis but not significant inflammation nor fibrosis. The existence of liver injury in this steatosis model is not further explained. Next lipidomic and proteome analysis revealed that a high fat and/or sucrose diet induces steatosis with accumulation of saturated and unsaturated fatty acids. Mitochondrial ROS and oxidative damage were not evident. In contrast, peroxisomal FAO proteins and cellular ROS were increased. Both fat and sucrose impaired the autophagic response. Together, these changes may be responsible for initial hepatic injury in NAFL.

Chapter 2 is entitled “Non-alcoholic fatty liver development and further progression is independent of mitochondrial oxidative stress generation”. The results show that Western diet up to 24 weeks induced early signs of disease progression to NASH, associated with decreased mitochondrial respiration, mPTP opening, but without increased mitochondrial ROS, thus confirming peroxisomes as the main source of hepatic oxidative stress

Hints on potential therapeutic targets from the studies in these two chapters deserved further discussion and exploitation in experimental models. Further, NAFL patient samples, if available, could be used in selective mRNA analysis of specific pathways highlighted by in vivo data. Overall, conclusion paragraphs and schematic diagrams summarizing the main findings in each chapter are very useful and carefully prepared. Of note, the terms NAFLD and NAFL represent different stages of the disease process, and yet are often used interchangeably throughout the thesis. For instance, see Figure 31 and respective legend. This should be made clear.

In the 3rd chapter, entitled “Antioxcin4 – a new mitochondrial targeted antioxidant that prevents the development of non-alcoholic fatty liver phenotype”, the mitochondrial antioxidant AntiOxCIN4 was tested in vivo for the first time as a potential preventive therapeutic strategy in NAFL. The results show improved body weight and liver enzymes after AntiOxCIN4 in the prevention of NAFL. Fat accumulation was reduced, FAO, OXPHOS complex subunits, antioxidant defenses and autophagic flux increased. Linking mitochondria as an important source of ROS, several antioxidant molecules have been designed to target mitochondria. The main medicinal chemistry properties of these compounds that target AntiOxCIN4 to mitochondria should be explained and so should be the evidence that it definitively targets mitochondria. Nevertheless, in light of data in previous chapters, it is not clear why targeting mitochondria would be favorable, since peroxisomes may be the main ROS producer as claimed in chapters 1 and 2. An expanded discussion on the use of antioxidants in clinical trials to treat NAFL patients would further elucidate the reader about the potential of this compounds.

Conclusion

Overall, the written document is of high quality and the candidate shows very good understanding of the topic. Attesting the relevance and quality of the work, several articles have been published in international peer reviewed journals. I believe that Inês Simões doctoral dissertation deserves the appropriate award.

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Review of the doctoral dissertation - Ines Simoes

"Effects of Western diet in the development and progression of non-alcoholic fatty liver disease"

Non-alcoholic fatty liver disease (NAFLD) frequently regarded as the hepatic manifestation of the metabolic syndrome with a prevalence of 20 – 40 % of the population, occurs in many countries. NAFLD is detected in about 50% of people with dyslipidemia, about 70% of people with diabetes mellitus type 2 (DM2), and even 90% of obese patients with BMI ≥40 kg/m^2. Moreover, male gender, elderly, and multi-ethnic countries, Hispanic origin are factors which increase the risk of NAFLD. It is estimated that NAFLD is developed in 20-86 cases per 1000 people per year. Notwithstanding, the difference between estimated occurrence of NAFLD and detected cases is extremely high.

In Poland, the prevalence of NAFLD in the general population was 37.2%; 51.4% in participants 65-70 years old (2019 data) and the incidence of advanced fibrosis was 7.79% (14.8% in the NAFLD population) and additionally increased with age ($p < 0.005$). Despite the fact that this disease is mostly diagnosed in patients with high BMI (i.e. >25 kg/m^2) it can also be detected in subjects with normal BMI (i.e. 20-25 kg/m^2) as well as in malnourished sarcopenic patients (BMI <18.5kg/m^2). This fact was written in the introduction of the dissertation by Ines Simoes – PhD student.

This dissertation is an attempt to answer the question how Western diet rich in fat and monosaccharides affects redox processes in liver's mitochondria in early stage of liver

disease. The PhD student is particularly describing processes in first stage of steatosis as effect of Western diet.

From my point of view, the change of lifestyle is the most important aspect. Simple modifications of NAFLD patient's lifestyle, such as weight loss, introduction of healthy eating habits, and regular physical activity are significant in the context of therapy. I cannot agree with extremely abbreviated assessment of the diet as method of therapy. The results of studies show that weight loss is fundamental for reduction of steatosis. In meta-analysis which includes 78 trials (38 trials regarding NASH – non-alcoholic steatohepatitis, 40 trials regarding NAFLD which also include studies with liver biopsy) it was shown that baseline 5% reduction of body mass contributes to the decrease of histological steatosis of the liver without affecting the degree of fibrosis. The loss ≥ 7% baseline of body mass significantly improves NASH (Musso, 2012). In clinical practice, the reduction of 30% daily calories (in regards to total calories needs) is recommended. It means the reduction of meals calories about 750-1000 per day (1200 kcal - minimal daily consumption). It is worth to emphasise that it is the caloric restrictions (fasting, fasting), which the PhD student does not mention in the introduction, are the one of the most effective methods of activating the autophagy process.

The results of recently published umbrella meta-analysis in JAMA has shown that caloric restrictions (every types) contribute to loss of body mass and fat mass as well as alteration of level of blood glucose, lipids, low-density lipoprotein, cholesterol, triglycerides, insulin levels, and insulin resistance index (HOMA-IR) (JAMA Network Open. 2021;4(12):e2139558). Despite the fact that in this meta-analysis steatosis was not assessed directly, the results clearly indicate that this way is the most interesting in treatment of NAFLD. Additionally, caloric restrictions act as a mild stress factor, increasing endurance and the body's ability to survive in conditions of strong stress (Southam & Ehrlich, 1943). The theory of mitohormesis by Schultz, which assumes that reduction of glucose consumption, causes the temporary increase of oxidative stress and the following increase of stress

protection. Therefore, it is perfect hypothesis model of effective elimination of free radicals from cells. Fasting is also a natural activator of sirtuin - NAD + dependent dacetylases.

The recommended physical activity (including strength training) for patients with NAFLD is an effective method of activating the mitochondriogenesis process.

Originality of the research problem

This doctoral dissertation solves original scientific problem, i.e. it assessed the disruption of redox processes in mice liver which were feeding proinflammatory diets (in different combination WD, high fat diet, high sucrose diet or high fat and high sucrose diet). The PhD student obtained the results which indicate that the progression of fatty liver caused by Western diet can be the results of peroxisomes dysfunction. This result is breakthrough because it seems that mitochondrial respiratory chain and ß oxidation is the main source of reactive oxygen species (ROS). Intensified ß oxidation was associated with development of fatty liver. Meanwhile, the PhD student has shown that peroxisomes are the source of ROS in Western diet. Peroxisomes are commonly known as organelles which play a secondary role in the context of oxidation polyunsaturated fatty acids (PUFAs). Interestingly, this PhD student as a first person worldwide has described the sequences of mitochondrial dysfunction during both development and progression of NAFLD. It is important to recognize the process of NAFLD and NASH development at the beginning. Autophagy is disordered during progression and it is showed by PhD student. The introduction modificatory factors at the stage of fatty liver development can be a significant step in treatment of NAFLD patients.

The importance of the research problem

The alteration of lifestyle (eating habits, physical activity) and the introduction of caloric restrictions is one of the methods of effective solution of problems of patients with NAFL and NAFLD and even NASH. Unfortunately, the data show that maintenance and continuation of dietary recommendations is difficult for most of the patients. Prospective

studies regarding 261 participants have shown that only 50% of patients were able to maintain reduction of body mass (around 7%) per 12 months. This result means that around half of patients can expect the effective pharmacological treatment without changing lifestyle (which is often impossible due to psychological barrier). Thus, the research conducted by PhD student is significant due to cognitive and practical aspects.

Interestingly, hydroxycinnamic acid was described in 2011 as one of the component of Chinese berries which efficiently inhibited OA-induced TG accumulation in HepG2 cells and has potential preventive effect on NAFLD in its early stage (Y Liu et al. J Agric Food Chem . 2011 Nov 23;59(22):12254-63). New product (completely synthetic) gives a hope for repeatability of the effect and its availability. As PhD student showed ANTIOx CIN4 which is directed to mitochondria can inhibit the progression of steatosis. It is an important result for pre-clinical studies. I hope that this product will be studied in clinical studies and it will present itself as effective therapeutic product. I would only like to add that a lot of products do not meet the expectations, for instance medications contributing to caloric restrictions (sirtuin activators STACs). Biguanide, Rapamycin, Thiazolidinedione and natural components (which are described by PhD student in the introduction of dissertation, for instance resveratrol) belong to tested substitutes. However, most of them were not a breakthrough in treatment of obesity. I hope that Anti OxCIN 4 will occur effective in supporting treatment of steatosis in early stage.

The originality and validity of research methods

Doctoral dissertation structure

This doctoral dissertation has a typical structure. The first part is an introduction which was written based on well-prepared literature review. The chapter is well written, carefully with enough amount of citations. The cited papers are modern and extremely well fitted. Moreover, there are numerous figures which increase the quality and allow to better understanding.

I have a few questions and doubts regarding this part of paper (i.e. introduction):

1. The autophagy process is stimulated physiologically by caloric restrictions. There is a literature regarding both human study and with animal models (mice). I am very unsatisfied with the lack of this solution as one of the physiological aspects for Western diet.

2. The is a lack of small part about intestinal microbiota, which is known as one of the main factors leading to fatty liver development. There are papers which showed that introduction of high-fat diet is a factor which in negative way modulates microbiota. It is caused by alter of metabolites productions, endotoxemia which lead to activation of steatosis. Interestingly, metabolomic assessment of microbiota patients with NAFLD and NASH show that changing of the composition of bacteria (for instance, reduction of the level of *Faecalibacterium prausnitzii*, *Coprococcus comes*, and increase of *Klebsiella pneumoniae* or *Veillonella atypica*) lead to increase of oxidative damage. It is a pity that there is a lack of small part in such a well doctoral dissertation.

3. It would be useful to add a few sentences about distinctiveness diagnostics of NAFL between species (human versus mice).

Hypothesis and Objectives part

There are presented the main hypothesis and three additional good constructed and proved (in further part of this dissertation) hypotheses.

Main hypothesis – “Pro oxidant state is the trigger of mitochondrial – related alterations that culminate in NAFLD progession.”

PhD student proves the hypotheses through testing:

1.Hepatic and mitochondrial alterations in NAFL

2. Specific and points for mitochondrial dysfunction

3. How ANTIOx CIN4 works in animal model

Material and Methods part

There are carefully written procedures. The methods are well described in 33 pages by PhD student. It should be emphasized that there is a wide range of modern methods.

1. I have only question regarding the part of animal breeding – very young subjects were selected to this study while NAFL is detected frequently in middle-age and elderly (age and gender is the predictor of steatosis). Why older subjects were not selected?
2. How was the dietary intake controlled in every combination? Were the leftovers which were not eaten during day weighted?
3. How many animals were placed in one cage? It is important because males in hight crowding can dominate other individuals which then leads to the restriction of food consumption.
4. Does the different content of exogenous fatty acids in the feed pose a problem for further results? Standard diet contains 1.8 % LA while high fat diet only 0,75%? Similarly ALA – 0,23% standards vs 0,13 high fat. The deficiency of essential fatty acids are one of the factor which causes fatty liver in human. Similarly the content of proteins especially in standard diet ENVIGO - 14,3 % vs 20,7 for high fat and 19 % for standard.

The rest of methods represent the enormity of well-planned and hard work done by the PhD student to prove her research hypothesis.

Results and discussion

The results are described with high scientific precision. The PhD student step by step conducted research steps. She used a wide range of research methods.

In Chapter 1, PhD student proved that:

1. HFHS diet (but also other e.g. HF) increases body weight,
2. Only HFHS and HS increased liver weight and ALT activity

How can such a small influence HS diet on liver mass and lack dysfunction of liver enzymes secretion be explained, especially when every diets indicate simple steatosis? Next

important observation is the reduction of glycogen content on HS diet. What was the sugar added to feed and jellies, sucrose or fructose? It should be emphasized that high-sugar diet should increase the glycogen synthesis and lipogenesis *de novo*, while in this doctoral dissertation the content of glycogen is reduced on HS diet similarly as in combination HF and HFFS (Fig. 14). How can it be explained? The difference between synthesis of saturated, monounsaturated, and PUFA in different diets is extremely interesting.

I have a question about alpha linolenic acid (C 18:3 n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosapentaenoic acid (DPA, 22:5n-3) – there is a lack of these acids in analysis (Fig 17, 19 a and b). Additionally, docosapentaenoic acid (DHA, 22:6n3), which is formed as a result of denaturation and elongation of C 18: 3 n3 - which is not in the chromatogram?

The result which shows that high caloric diet (not depending on its ingredients) has an influence on the content some of mitochondrial membrane phospholipids (cardiolipin and sphingomyelin)- Fig 18. It is a significant result for clinical practice. Notably, high sucrose diet alters PC/PE ratio thus *de facto* mitochondrial fluity and integrity (Fig 18). The results from Chapter 1 were analysed and checked with the results obtained by other researchers. It should be emphasized that results presented by the PhD student as a first showed that the hepatocyte damage is not caused by mitochondrial – related oxidative stress but probably due to peroxisomal FAO.

The PhD student very skilfully defended and discussed her results showing the participation of ROS from sources other than the mitochondrial FAO related enzymes in the steatosis process in the early stage of steatosis. The aspects about HF diet and worsen of autophagic flux due to a reduction of lysosomal enzymes activities and defective autophagosomal formation was also interestingly discussed. The PhD student emphasized that HS diet impairs autophagic flux too but by autophagolysosomal formation. This results are similar to the results obtained by other authors early. The observation that HF and HS diet induce simple steatosis with accumulation both saturated and monounsaturated FA is also a significant results for clinical aspects. What according to PhD student may protect

hepatocytes from lipotoxicity (induced by SFA), initiation of cellular apoptosis and autophagy impairment.

In Chapter 2, PhD student collected results which describe development and progression NAFL as process independent from mitochondrial oxidative stress. In this part the results come from the observation animals which were fed with Western or standard diet per 16-24 weeks. These results are dangerous because they show how fast WD lead to serious alteration of liver structure and function. I have doubts about the result Fig 32 – ALT – there is no statistically significant difference between SD and WD for 20, 22, and 24 weeks?

There is a great discussion of this part of doctoral dissertation. In my opinion hypothesis is completely defended – mice which were fed WD longer than 16 weeks developed NAFL with indicating NASH. The PhD student described these results with remodelling of structure and function of mitochondria. On the other hand, she proved that mitochondrial origin ROS do not contribute to first hit for NAFLD progression. As the PhD student proved in Chapter 1 higher peroxisomal abundance with higher peroxisomal - FAO activity overcome fat accumulation at the expense of hepatic oxidative stress.

The results described in Chapter 3 give a hope for possible role Anti Ox CIN 4 in limitation of NAFLD progression. Results are encouraging, because they show that supplement given by 18 weeks significantly reduced body mass, liver mass, and many other parameters, such as hepatic damage, hepatic lipid accumulation. Anti Ox CIN 4 works as mitochondrially target antioxidant. The PhD student concludes that Anti Ox CIN 4 can be used not only in the prevention of NAFLD but also in its treatment.

I’m looking forward to it as a scientist.

Finally, a very personal question. At hepatology congresses every year the question arises how to lose weight quickly and how to use for this purpose a ketogenic diet (consisting of 80% fats, mainly PUFA and MUFA) and about 20-30 g of carbohydrates per day. In the

physiological state of ketosis induced by this diet, patients lose weight. Based on the results, can you recommend this diet for rapid bodyweight reduction in individuals with NAFLD?

Ability to critically analyses data

I rate it as outstanding the ability to critically analyze data. Ms Inez Simoes critically describes obtained results using them to confirm the hypothesis. The work is written with the highest scientific care. It is important to emphasize the excellent selection of literature and the ability to cite it, the use of many research methods, the scientific maturity of the doctoral student in formulating research hypotheses and proving them.

Chapters comprehensively written, supported by a large number of figures, tables and diagrams summarizing the research. I evaluate the construction of the work as correct.

Rozprawa doktorska ”Effects of Western diet in the development and progression of non-alcoholic fatty liver disease” spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018r. Prawo o szkolnictwie Wyższym i Nauce Dz U z 2021r. poz.478,619, 1630).
Praca ta stanowi oryginalne rozwiązanie problemu naukowego, doktorantka - Ines Simoes wykazała się ponadprzeciętną znajomością tematyki związanej z wpływem diety zachodniej na powstawanie i progresję stłuszczenia wątroby, a także umiejętnością samodzielnego prowadzenia pracy naukowej.
Wnoszę o wyróżnienie pracy doktorskiej.

The doctoral dissertation “Effects of Western diet in the development and progression of non-alcoholic fatty liver disease “ submitted for evaluation meets the requirements of Art. 187 of the Ustawy z dnia 20 lipca 2018r. Prawo o szkolnictwie Wyższym i Nauce Dz U z 2021r. poz.478,619, 1630 on academic degrees and academic title as well as degrees and title in the field of art.

This work is an original solution to a scientific problem, the PhD student – Ines Simoes has demonstrated above-average knowledge of the subject of the Western diet for the formation and progression of fatty liver, as well as the ability to independently conduct scientific work.

I am applying for a doctoral dissertation award.

Yours faithfully