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To whom it may concern

Evaluation of the doctoral dissertation

of Ms. Patrycja Bielawiec

"Evaluation of cannabidiol influence on lipid metabolism in the skeletal muscle

in a rat model of obesity induced by a high-fat diet"

This evaluation follows a letter of Professor Irina Kowalska with her invitation to review the doctoral dissertation submitted by **Ms. Patrycja Bielawiec**. I am honored to accept this nomination and present my report here.

General comments

Obscured by the latest political headlines and the pandemic, there hides a public health crisis of interest to Ms Patrycja Bielawiec. Obesity has been developing around the world for over 30 years. For example, in 1985 no state in the United States had an obesity rate greater than 15%. Today, five states exceed 35% [1]. Although less dramatic than the opioid epidemic, obesity is just as fatal and serious. As noted by Ms Patrycja Bielawiec, obesity is associated with type 2 diabetes, hyperlipidemia, cardiovascular diseases, cancer, and other maladies. An international team of experts found that obese people who contracted SARS-CoV-2 were twice as likely as people of healthy constitution to land in the hospital, 74% more likely to be admitted to an intensive care unit, and 48% more likely to die [2]. Many of the proposed solutions to fight obesity emphasize diet and exercise to bring balance between energy intake and expenditure. While these measures

can help some people in a short run, dieters often end up weighing more after completing their program than before. This creates a need for other remedies such as weight loss medications that would lower blood glucose levels and unhealthy lipids along with the weight loss.

The series of studies presented in the dissertation by Ms Patrycja Bielawiec are well positioned to fill that void as she ventures to evaluates the influence of cannabidiol on lipid metabolism in skeletal muscles in a rat model of obesity. The idea of exploring cannabidiol is certainly not accidental. Cannabidiol rose to the mainstream attention in 2018 after its breakthrough approval by the U.S. Food and Drug Administration for children with rare and severe forms of epilepsy. My guess is this event coincided with the time when Ms Patrycja Bielawiec began her studies. The literature regarding the use of cannabidiol to treat obesity and type 2 diabetes remains sparse. I counted one animal study investigating the anti-hyperglycemic properties of cannabidiol in non-obese diabetic mice [3], one case report [4] and one small human study of 62 patients [5]. Of the 397 clinical trials registered at https://clinicaltrials.gov/ to test cannabidiol in various clinical settings, four studies looked at its effect in obesity (Prader-Willi syndrome) and five others on diabetic subjects. This basically means that Ms Patrycja Bielawiec's findings are original and of great importance, and she has the pre-clinical entire field almost exclusively to herself.

The dissertation is 104 pages long and consists of 14 chapters. The structure is appropriate for modern doctoral dissertations that are underpinned by student's own research findings from already published thematically related works as one unit. This thesis is a collection of three multi-author publications (2 original papers and 1 review), in which Ms Patrycja Bielawiec is the first author. They appeared in 2020 and 2021 in peer-reviewed journals featured in the Journal Citation Reports and recognized in the international arena. They have already been evaluated by independent experts and have appeared in print. The total impact factor and the combined scores of the Ministry of Science and Higher Education are 12,262 and 340, respectively. It is worth pointing out that academic achievements of Ms Patrycja Bielawiec are not limited to the works related to her dissertation. At the beginning of the dissertation the list reads 4 other papers of Ms Patrycja Bielawiec that are not included in her thesis, and 8 conference abstracts. Noteworthy is also the fact that her studies were backed by the funds from the European Union and from the National Science Centre of Poland. The actual dissertation is proceeded by an exceptionally long list of abbreviations (3 pages), which makes it much easier to navigate through the work. Following

are sections for introduction (5 pages), goals (1 page), materials and methods (4 pages), overview of the results (7 pages) and conclusions (1 page). The three articles there constitute most of the volume of the doctoral dissertation (53 pages), followed by abstracts in Polish and English (total of 4 pages). The documentation also includes statements by Ms Patrycja Bielawiec and all co-authors with an indication of their contributions to the selected publications and their consents to use them in this dissertation. They clearly indicate the leading role of Ms Patrycja Bielawiec in the development of the concept and execution of experiments, interpretation of the results and preparation of all manuscripts. This is further reflected in the fact that Ms Patrycja Bielawiec is a corresponding author in all three articles. The dissertation ends with a list of 43 references (3 pages), of which 67% are papers published within the last 12 years.

The **Introduction** is clear and informative, and critical to understanding of the work. It begins with a section covering the main topic of the dissertation, which is the utilization of energy substrates by skeletal muscles. In simple words, it acquaints the reader with the term 'metabolic flexibility' and the intricate machinery involved in the use of glucose and fatty acids as a source of fuel for energy production. One also learns about the factors associated with maintaining a delicate balance between glucose- and fatty acid β-oxidation and the impact of the latter on the use of glucose in red and white skeletal muscles. This allows to better understand the scenarios in which the physiology turns into a ceramide-driven pathology. In the next section, Ms Patrycja Bielawiec describes the components of the endocannabinoid system and their role in the regulation of lipid metabolism in skeletal muscles. She rightfully uses the term 'expanded endocannabinoid system', or endocannabidiome (eCBome), as it encompasses a much larger network of ligands and targets e.g., GPR55 and GPR119, ion channel receptors TRPV1, nuclear receptor PPAR-γ, and mediators such as N-acyl amino acids [6]. This section is also supported by the review article of Ms Patrycja Bielawiec, which introduces the hero of the dissertation the eCBome – cannabidiol. We learn that the cannabidiol molecule wears many hats as it binds to CB_1, CB_2, GPR55 or TRPV1 receptors. This number of points of action of the drug is, of course, a major source of challenges in cannabidiol research.

The study has three well-defined and ambitious **Goals**: to assess the effect of cannabidiol on (i) plasma glucose and insulin levels, (ii) composition of fatty acids and (iii) ceramides in white and red skeletal muscles of diet-induced obese rats. The achievement of these goals required a detailed

planning and use of labor-intensive methods listed in **Material and Methods** section. These include extraction of sphingolipids and fatty acids from plasma and skeletal muscles, separation of lipid fractions by thin layer chromatography, derivatization of sphingolipids, quantitative analysis by gas-liquid and liquid chromatography, quantification of proteins by Western Blot, ELISA and colorimetric tests, just to name a few. I guess the chromatographic analysis was personally performed by Ms Patrycja Bielawiec, as this technique was not mentioned in anyone's contribution statements. In her analyses, Ms Patrycja Bielawiec used two types of skeletal muscles from rats exposed to a standard chaw or high fat diet for 7 weeks and treated with cannabidiol or its vehicle for the last two weeks on the diet. The tissues were harvested at the end of the study. My only concern here is that the entire work relies solely on a single animal model and the end-point tissue examination. I would expect a bit more extensive selection of experimental models to complement your primary observations. On the other hand, being aware of the continuous efforts to reduce the use of research animals and replace them with other techniques Ms Patrycja Bielawiec's approach was fully justified. She was still successful in the implementation of her research objectives with the current model.

Ms Patrycja Bielawiec generated a massive number of results. Yet, despite the huge volume, she was able to present her findings in a simple and accessible way, a clear indication of an in-depth understanding of the subject. The outcomes are presented in the **Results** chapter in the form of a concise report, which navigates you through 18 pages of results of her two original articles. They are supported by 10 tables showing quantitative changes in polyunsaturated fatty acids in five different red and white gastrocnemius fractions and are supported by 10 figures showing cannabidiol-induced changes in the endocannabidiome, insulin signaling, and sphingolipid signaling pathways. Ms Patrycja Bielawiec made several new observations. Noteworthy, (i) she found an increase in *de novo* ceremide synthesis and accumulation of *n-6* free fatty acids in skeletal muscles of rats on the high fat diet. (ii) The oversupply of fatty acids was associated with the development of oxidative stress and inflammation, reversible or attenable by the chronic cannabidiol treatment. In addition, cannabidiol prevented (iv) the formation of lipid peroxidation products (v) and shifted the n-6/n-3 PUFA ratio towards the anti-inflammatory n-3 species. (vi) The drug restored the level of sphingosine-1-phosphate and (vii) improved muscle insulin signaling and glycogen recovery. The Results section is summarized by the three clear and correct **Concluding** remarks. The final message is that these observations offer a new look into therapeutic

potential of cannabidiol for treating metabolic consequences of obesity. I was somewhat disappointed by not finding a separate discussion but rewarded later by reading discussion sections in the two original papers. It looks as if one benefits from this approach, since you get a more comprehensive and thorough insight into the topic. The two original papers combine all results into two logical stories devoted to the role of cannabidiol in the regulation fatty acids and ceramides in skeletal muscles of obese rats. Each paper is supported by a clear and informative scheme making it easier to follow the findings and, what is very important, it ends with a small section discussing clinical implications of her findings. One of the challenges Ms Patrycja Bielawiec faced was an insufficient number of studies to refer to, as her observation are quite novel. Despite these difficulties, however, the discussions are informative and well conducted.

Critical remarks

I only have a few remarks, even though I read this dissertation with a strong motivation to be critical:

- Supplementary materials to article 2 (*Biomolecules*, 2020,10, 1241) and article 3 (*Nutrients*, 2021, 13, 1603) are not included in the dissertation. However, the materials are still accessible online.
- It would be essential to disclose the exact composition and manufacturer of the high fat diet used in your studies so that others could replicate it. From my experience, the yellow paste diet and blue pellet diet available in the United States provide 60 kcal% from fat, yet they contain different quantities of saturated and unsaturated fatty acid species, which result in different experimental outcomes and conclusions.
- The Material and Methods section (page 17) indicates that body weights were monitored throughout an experiment. Yet, I was not able to find any record on how weights changed during the 7-week exposure to diet, or how it was affected by the 14-day cannabidiol treatment. The dissertation only reported the end-point blood glucose, insulin, and HOMA-IR levels (page 45 of the dissertation; article 2, table 1, page 5).
- Cannabidiol is also orally bioavailable [7] and oral formulations are preferred in clinical settings. It would be prudent to look at the pharmacokinetic profile of cannabidiol and discuss the choice of treatment regimen (reasons for choosing intraperitoneal injections, dose, and treatment duration).

- When comparing the treatment (drug vs vehicle) and diet (lean vs high fat) in the same chart, the use of one-way ANOVA may not be the best choice. Your study looks more like a 2x2 factorial design. The standard analysis for such a study would be a two-way ANOVA, which allows to separate tests for diet and cannabidiol.
- The dissertation reports the content of fatty acids and ceramides after treatment with cannabidiol. It would be useful to know if the drug directly affected skeletal muscles or had an indirect effect (e.g., by reducing systemic inflammation). The latter could be addressed by measuring C-reactive protein as a marker of inflammation.
- HOMA-IR is a surrogate index of the whole-body insulin resistance. It assumes that fasting glucose and insulin levels represent a basal steady-state condition. I wonder how long rats were fasted for the HOMA-IR assessment? The index is reliable in humans. In contrast, there is only a modest correlation between surrogates and insulin clamp in rodents, the latter is considered as gold standard approach to evaluate insulin resistance [8]. Have you considered other tests besides HOMA-IR in your studies e.g., glucose tolerance test or insulin tolerance test, in part because there were no differences in HOMA-IR values between your treatment groups in obese rats?

Recommendation

The dissertation written by Ms Patrycja Bielawiec is at a high level. She showed the required practical skills, theoretical knowledge, analytical thinking, and a passion for research. At the same time, she generated an impressive number of novel results, presented them in a logical way that clearly proved her scientific potential. My remarks regarding minor imperfections are not meant to detract from the overall high value of her research but are intended to excel academic performance. So many questions always arise when results are intriguing. Just as the innocent finding of Professor Raphel Mechoulam from 40 years ago regarding the suppression of epileptic episodes by cannabidiol [9] led to the registration of an effective anticonvulsant drug, the observations of Ms Patrycja Bielawiec have a chance to become the basis for the development of a new class of anti-obesity drugs.

In my opinion, the quality of the dissertation complies with requirements for PhD thesis. Therefore, I am pleased to recommend proceeding with the next stages of the doctoral procedure. I also

propose to recognize Ms Patrycja Bielawiec for choosing an interesting and challenging topic, for carefully conducted experiments and original observations.

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