Reviewer's Report – Diploma Thesis Authored by Mônica Jandová

Mônica Jandová's diploma thesis focuses on elucidating the effects of a gluten-free diet on an MPTP-induced mouse model of Parkinson's disease. Since Parkinson's disease (PD) is a growing problem and impacts a substantial number of the aged population, there is the potential for significant health benefits from this study, especially when an inexpensive and simple modification such as a gluten-free diet is suggested. Moreover, it is known that microbiome and diet interventions have an impact on PD, but the effects of a gluten-free diet have not yet been completely investigated and this topic is important and novel.

Generally, the student demonstrated considerable effort in writing the thesis. However, the work contains numerous substantial flaws that should have been identified and addressed before submission. Notably, there appears to be a lack of scientific guidance and language corrections, which could improve the scientific writing skills of the students substantially.

To begin with the positive aspects, the introduction shows that the student put significant effort into gathering various literature sources and spent considerable time writing an extensive and detailed text. The aims of the project are clearly stated and well-structured, with a well-defined project scope. The materials and method sections are arguably the best part of the thesis, featuring graphical photographs of brain dissection and detailed descriptions of all methods used, including the diet formula. The data presented is not overinterpreted, and the discussion section follows all measured parameters, attempting to contextualize them within the framework of published studies.

However, there are numerous weak points in the work. Firstly, there are typographical errors in the title, including two mistakes in the Czech title that went unnoticed on the first representative page. Almost all Czech translations contain typos, suggesting they lacked review by a Czech-speaking person.

The introduction, though thorough, is 35 pages long and exhibits varying levels of English language quality, with numerous typos and unfinished sentences. It lacks a main message, causing the reader to become lost in information that is irrelevant to the overall work. For instance, chapters 2.1 to 2.3 are particularly chaotic and are not linked to the subsequent results or discussion. These chapters seem more connected to the aim of working with patient samples, which was not part of this thesis. This aim should be completely omitted from the thesis, including the methods that were not used in the work (all human sample processing etc). Overall, it reads like a general book about Parkinson's disease, without a deeper connection and understanding of the specific thesis topic. Additionally, some

literature sources are missing, such as references for PD patient numbers in the Czech Republic.

The results section is problematic as all experiments appear to have been conducted only once, using one group of 2 STD and 3 GFD mice. This is an insufficient number of replicates to draw any valid conclusions. Consequently, all conclusions include the sentence that they are not statistically significant due to the limited number of mice, which was constrained by unexpected deaths from the MPTP effect. This limitation means that the thesis does not fulfill its aim to draw any conclusions about the effect of a gluten-free diet (GFD) on Parkinson's disease (PD) development. Not talking about the absence of non-injected mice as an important control. More mice should have been included from the beginning of the study, and the experiment should be repeated at least once more with an appropriate number of mice, ideally five surviving animals per group. Moreover, the effect of MPTP treatment should be shown on brain sections of GFD vs SD mice together with a control that the treatment worked. The use of ANOVA for all statistical comparisons is questioned, as a simple t-test is generally standard for comparing two groups. Additionally, the results section often lacks clarity regarding when chronic and acute models are used.

Figure 1, although showing a visible effect of MPTP treatment, does not clarify whether the image is representative or the best one and how big the tested group was. More than one mouse should be shown with an appropriate scale bar. There are no statistics provided to evaluate the treatment effect, and section slides might not show the same sectioned area, with the arbitrary line not representing anything meaningful. A bright field image for neuromelanin should be added to show the same section area. Quantification of either the number of DOPA neurons or mean fluorescence per specific area should be conducted, or any other quantification that could objectively show differences and make sure the same sectioning area.

In section 5.2, only one mouse per group is shown in the Open Field Test. Conclusions based on this test are suggested, however, importantly the noninjected controls proving the PD model efficacy are missing.

Section 5.3.1 lacks a discussion on why the selected immune parameters were used, their importance for PD development, or their relevance to a GFD. These parameters are first mentioned in the conclusion but should be part of the results section. Otherwise, it appears as a random antibody selection. Please comment on selected antibodies in your oral presentation.

Graphs and axes are not clearly labeled, with the assumption that they represent percentages of parent populations. However, the percentage of live gates or counts (count per 100,000 cells at least) should be added.

Figure 15 shows results and gating for an INFγ antibody that did not work, with no positive control used. Figure 16 contains an unfinished sentence. All graphs combine parameters for all organs together, showing “total percentages,” which requires an explanation of the rationale and representation. There is also no validation that the GFD

changed the microbiome or had any effect. Figures 22 and 24 lack an FMO control, so the staining could be not reliable. This issue is particularly evident in Figure 24, where the gating of NK markers NKp46 and NKG2D is unclear and likely incorrect.

Additionally, many abbreviations mentioned in the text, such as SNCA, LB, DA neurons, COMPT inhibitor, HDCAC, and BBB, are not explained upon their first mention. Figure 4 lacks a citation, and there are numerous unfinished sentences throughout the work. The components of the complete media are not described, and there are discrepancies in the thickness of brain slices. Figure 12 should have a visible scale bar added.

In summary, while the student showed an effort to write the thesis, there are numerous areas needing substantial improvement, including correcting typographical errors (including Czech translations), clarifying experimental details, and providing adequate statistical analysis and justifications of the methods and number of mice used.

Since some parts need clarification, please incorporate the following elements into your defense presentation:

- I would like a student to clarify her role in establishing MPTP induced PD model, as it is not clear if it was part of her thesis work, or if she relied on other lab members to establish the model. If so, please provide more information on the different regiments tested and the journey that led to the successful model establishment.

- Please justify the very low number of animals used and explain why experiments were not repeated to meet scientific standards for statistical analysis.

- Provide the brain sections stained for Tyrosine Hydroxylase from all experimental mice (GFD vs. SD mice, along with non-injected control mice) and include statistical evidence that supports the effectiveness of the model in the experimental group.

- Justify the use of ANOVA for comparing two groups instead of a t-test.

- Please in your defense present FACS data as a percentage of the live gate or counts per 100,000 live cells (these can be recalculated from actual counts). Show FACS plots from each mouse (GFD vs. SD) at least for the results with observed trends, but preferentially for all graphs shown. Showing the final gate used for statistical analysis should be sufficient after presenting the general gating strategy.

- Include the open field test results for all animals, similar to Figure 14, and compare the brain sections with those of healthy animals. Non-injected healthy controls are particularly important for comparing values in the open field test, where PD mice should perform significantly worse (as this is one of the main readouts of PD in this model). Please provide an analysis of the motoric function of healthy mice for comparison.

- Provide a visible scale bar and statistics if Figure 12 is shown in the presentation.

Reviewer's Questions:

1. Why do you expect a gluten-free diet (GFD) to affect Parkinson's disease (PD) development? Please discuss GFD-induced changes in the microbiome that could potentially benefit PD. Provide context by comparing it with high-fat diets (HFD) or ketogenic diets.

2. How well does the PD mouse model replicate human disease?

3. Were chronic and acute animal models measured on the same group of animals? Or was the acute measurement simply the initial time point within the chronic model?

4. Why was data from different organs pooled together and presented as total values? Justify the rationale for pooling the different organ data, particularly when it represents a percentage of the parent gate. Clearly state what the y-axis represents in the FACS statistical analysis.

5. What were the gender and age distributions of the experimental mice? Were they littermates?

6. Elaborate on the specific immune cells measured in the experiment and the rationale for selecting these cells for analysis.

7. How does blood-brain barrier integrity look in the PD mouse model? Do you expect immune cell infiltration into the brain? If yes, would you expect differences in the infiltrating immune cells as an effect of different diets?

8. Can you comment on other mouse models of PD, particularly the LRRK2 mutant knock-in mouse model? Compare this model to MPTP-treated mice. How do they differ, and what are the advantages of each model?

9. What is the expected impact of a GFD on the T regulatory (Treg) cell compartment? Do you anticipate differences between organs, and if so, why? Refer to the literature in your presentation and provide the suggested mechanism for the regulation of Tregs by diet.

Despite notable shortcomings, particularly evident in the results section, many aspects of the work can be adequately justified and elucidated during the oral presentation. Then I request a comprehensive preparation of responses to my inquiries, encompassing a thorough clarification of all highlighted issues and incorporating them into the defense presentation (see the part "incorporate into the presentation"). Please also prepare the answers to the reviewer's questions (which don't need to be added to the presentation).

I refrain from proposing a specific mark, leaving it to the committee's evaluation.

Mgr. Jarmila Sekerešová Králová, Ph.D.