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**Oponentský posudek diplomové práce Bc. Anny Zelenské**

**A review of the diploma thesis of BSc. Anna Zelenska**

## Effect of low-carbohydrate diet on development of type 1 diabetes and immune parameters in NOD mice

This thesis research focused on the role of low-carbohydrate diet (LCD) in the incidence and onset of autoimmune diabetes in non-obese diabetic (NOD) mice. NOD mice represent a well-established and unique model of type I diabetes mellitus (T1D). It is well known that the diet and microbial colonization are factors modulating the incidence and onset of the disease. The candidate presented interesting results that the administration of the LCD prenatally (to pregnant mothers), substantially accelerates the onset of diabetes in NOD mice. This effect was not observed when the diet was introduced post-weaning. Subsequently, the candidate was trying to find the mechanistic explanation of this phenomenon by flow cytometry (FC) analyses of lymphocytes. Although this analysis seems to suffer from some technical issues (see below), the results indicated that the LCD decreases the frequency of FOXP3+ regulatory cells among CD4+ T cells in the spleen and inguinal lymph nodes.

The thesis is written in good English with an acceptable level of typos. I have received the thesis in two parts – the main thesis and a 21-page long erratum (without page numbers), which complicated my review process.

The **Introduction** is a well-written overview of type I diabetes in humans and in the NOD mouse model with the emphasis on the environmental factors. The candidate did not mention at least three studies that described the effects of high- or low-protein diets on diabetes onset and incidence in NOD mice (see below). Moreover, I was missing a bit deeper discussion on the apparent contradiction between the hygiene hypothesis and hypothesis that particular viral infections trigger diabetes. Both these hypotheses are explained, but not reconciled. The introduction contains three figures, which are not

original, but taken from published articles. The legend is not always sufficient. E.g., it is not clear if Figure 3 illustrates human patients or NOD mice.

The **Aim** of the project is clearly defined.

The **Methods** are generally sufficient in providing experimental details. However, the two additional diets used in Figure 9 are not described in Methods. There is no information on the assignment of individual mice to experimental groups, blinding, and selection of the cohort size, which is now required by many journals.

The **Results** consist of the diabetes incidence curves, examples of gating strategies, summarized FC data, and summarized data on insulitis. The volume of experimental work and the variety of different experiments is adequate for a master thesis in immunology. However, this part contains a considerable number of issues mostly in the terms of data presentation, experimental setup, and "strange" FC results. Below is the summary of the most striking ones:

1. I suspect serious technical issues in some flow cytometry experiments, which substantially harm the credibility of the results.

The percentage of CD62L+ cells out of CD8+ T cells is extremely low. Together with high levels of CD44+ cells (caused by wrong gating - Figure 18; the non-naïve cells should be gated as $CD44^{high}$ especially in BALB/C and NOD mice, where the levels of CD44 on T cells are known to be higher than in C57BL/6 mice), this leads to a ridiculously low frequency of naive cells among $CD8^+$ T cells (0.5-3%) in the secondary lymphoid organs (Figure 19). I would suspect a technical issue with the CD62L staining. The results in Figure 20 also depend on this staining/gating. In the second part of the results, which focuse on the prenatal administration of LDC, the frequencies of naïve T cells are higher, but still much lower than expected (Figure 32).

The FOXP3 staining seems not to work properly, resulting in 1-2% of FOXP3+ cells out of CD4+ T cells in all lymphoid organs (Figure 10). The expected frequency would be around 5-10%. Actually, in the second part of the results (Figure 25), the Treg proportions closer to the expectation, which leads to more convincing data.

There is a suspicious correlation between the CD49b and LAG3 staining in Figure 27 (bottom), suggesting a potential staining/compensation artifact. Instead of the FMO controls, a staining without both of these markers (*fluorescence minus two*?) is shown for an unclear reason.

2. There are multiple issues with data presentation, some of which are listed in the Appendix at the end of this review. Their number is larger than usual for a master thesis.

3. It is unclear, how the cytokines were measured. The standard FC protocols include ex vivo activation using PMA/ionomycin (or a TCR agonist) and an incubation of the cells in the presence of an inhibitor of the secretion pathway for a couple of hours. The thesis only mentions adding Monensin to the FACS buffer, which I doubt is sufficient.

The **Discussion** is well written, since the applicant places their results into the context of published studies. However, it seems that the candidate omitted key previous studies in this area of research. The candidate writes in the **Abstract**: "*A very rare acceleration of the development of T1D in NOD mice by an environmental factor – the LC diet, may shed more light on the pathogenesis of the disease*", in the **Discussion**: "*Our data present a surprising finding - an accelerated development of T1D in NOD mice if exposed to the LC diet prenatally (…). Because LC diet is one of a few, rare manipulations that leads to the disease acceleration*", and in the **Results**: „*there have been very few environmental exposures which had been shown to accelerate T1D onset in NOD mice. This finding was so unusual…*". Based on these statements, I got the impression that these observations are unprecedented and unique. However, there are at least three studies that have shown findings, which seem to be similar, complementary, or at least related to the results obtained by the applicant. None of these relevant studies is mentioned in the thesis.

1. A study by Schneider et al. (Acta Diabetol. 1996 Sep;33(3):236-40, PMID: 8904932) concludes that a protein-rich diet (55 %) accelerates the diabetes onset by 4 weeks in comparison to a standard diet with 15 % protein content, when administered upon weaning. Accordingly, a study by Chamson-Reig et al. (J Endocrinol. 2009 May;201(2):231-9, PMID: 19228796) showed that low-protein diet delays the onset of diabetes in NOD mice. Although these experimental setups are different from the one used in the candidate's research, there are definitely some similarities, if we consider that proteins constituted a major energy source in the LCD (49 % LDC, 24% control diet) used by the applicant.

2. A study by Elliott et al. 2009 (*International Journal of Diabetes and Metabolism* (2009) 17 (2): 37–40.) concludes that LCD with a high proportion of proteins and maize fiber increases the incidence and accelerates the onset of diabetes. This diet seems to be similar to the diet used in the candidate's research (Figure 5).

Overall, some parts of the thesis are very good, specifically most of the Introduction and Discussion, and the initial finding that the LCD reproducibly accelerated the onset of diabetes and suppresses the Treg abundance in secondary lymphoid organs. However, the thesis has the apparent flaws mentioned above. The three major problems are the low technical quality of the FC data, high number of data presentation errors, and omitting at least three previous studies in the field which came to similar conclusions.

**Comments/questions to be addressed by the candidate during the defense:**

1. Please, comment on the similarities and differences of your experimental setup and results and those of the studies published by Schneider et al. (*Acta Diabeto*l. 1996 Sep;33(3):236-40, PMID: 8904932), Chamson-Reig et al. (*J Endocrinol*. 2009 May;201(2):231-9, PMID: 19228796), and Elliott et al. 2009 (*International Journal of Diabetes and Metabolism* (2009) 17 (2): 37–40). Could high protein content be the major driver of the early diabetic onset induced by the LDC?

2. How does the applicant explain the reduced number of Treg cells in LCD-fed mice in the peripheral lymph nodes and the spleen, but not in the mesenteric lymph nodes and Peyer's patches (Figure 25)? Please, propose a possible mechanism, which is supported by your and/or published data.

3. The FC data in Figure 18 (and further) show extremely low percentage of CD62L+ cells among CD8+ T cells and low percentage of FOXP3+ cells among CD4+ T cells. Could the applicant explain this? Does it fit with data published by others?

4. For the detection of the production of IFNγ or IL-10, were the cells ex vivo activated e.g., with PMA/ionomycine or a TCR agonist? Were these cells incubated with an inhibitor of protein secretion (Monensin, Brefeldin A)?

5. What are „migratory CD8+ T cells subsets“ (Figure 18)?

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**Appendix** – flaws in the data presentation in the **Results**.

1. Most Figures are not referred to in the text.

2. Some plots have Y axis not starting at Y=0, which might give a false impression about the measured differences (Fig. 13 bottom left – interestingly the top left panel uses a segmented axis which has no benefit, Fig. 17 top left and middle, Fig. 19 top left, Fig. 20,

3. Some formulations in the results are strange, such as „This finding was so unusual, that it has been decided to repeat this experiment."

4. There are two identical Figures 19.

5. The results of the third experiment (Figure 23b) is shown in the errata, but the strange corresponding sentence in the Results was not corrected „The ongoing third experiment confirmed these results (data are being processed), which makes this finding highly reliable."

6. Labeling of Y axes is missing in most column graphs.

7. It is not indicated which organs were used for the FC analysis which shows the gating strategies (Figure 10, 15, 18, 27).

8. The gating strategy in Fig. 27 (bottom, mid panels) shows overlaping gates. The logic behind this is unclear.

9. The claim that no diet showed significant differences in Figures 9, 11-14, 16-17, 19-21 is not supported by any results of the statistical analysis. In some figures, the statistical significance is indicated by a number of asterisks (e.g., **) but it is not indicated which range of p-values it represents (Figure 24, 25, 28, 34). Anyway, the exact p value, not just a range, should be indicated according to the publishing standards in the field. The statistical test used for the analysis of data in Figure 35 is not indicated in the legends.

10. The 1084 Keto diet actually shows delayed incidence (Figure 9), although it might not be statistical significance, this should be discussed. The number of mice in the cohorts is not indicated.

11. Low-carbohydrate diet is inconsistently abbreviated as LC (in the text) or Low-carb (in some Figures and Figure legends).