**FINAL REPORT**
**December 1, 2019 – November 30, 2023**

In the present project, we investigated further those regional molecular differences, which are thought to be responsible for the diabetes-related gut region-specific damage of the enteric neurons [1,2] of streptozotocin (STZ)-induced chronic diabetic rat model.

According to the work plan, we used STZ-induced rats to model type 1 diabetes. The rats were divided randomly into three groups: STZ-induced diabetics, insulin-treated diabetics, and sex- and age-matched controls. Hyperglycaemia was induced by a single intraperitoneal injection of STZ (60 mg/body weight kg). The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mmol/L. From this time on, one group of hyperglycaemic rats received a subcutaneous injection of insulin each morning and afternoon. Equivalent volumes of saline were given subcutaneously to the rats in the other hyperglycaemic and the control groups. Non-fasting blood glucose levels were determined, and the animals were weighed weekly in each group. Ten weeks after the onset of hyperglycaemia, animals were anesthetized and sacrificed by cervical dislocation. Blood samples, gut segments from duodenum, ileum and colon as well as the pancreas were collected and stored under appropriate conditions and processed for haematological analysis, histological, immunohistochemical and molecular studies. To ensure sufficient amount of samples for the detailed immunohistochemical and molecular investigations the STZ-induced diabetic rat model was reproduced in all years of the project period.

At the end of the 10-weeks experimental period, the diabetic rats were characterized by a reduced body weight and an increased blood glucose concentration as compared with the age- and sex-matched controls. The insulin-treated diabetic rats did not differ significantly from the control animals in weight or blood glucose concentration. The blood samples of experimental animals were investigated by blood chemistry and haematological tests (routine blood count) in each year.

The quantitative immunohistochemical studies planned within the present project were extremely labour intensive; therefore, these were started in the first year and continued throughout the entire project period as we planned. According to the work plan, in the **first year** of the project, we evaluated the **matrix metalloproteinase 9 (MMP9) and its tissue inhibitor (TIMP1)** expression in myenteric ganglia and their microenvironment from different gut segments and conditions. Using electron microscopic morphometry, double-labelling fluorescent immunohistochemistry, post-embedding immunogold electron microscopy and qPCR, we demonstrated that ten weeks after the onset of hyperglycaemia, the ganglionic basement membrane was significantly thickened in the diabetic ileum, while it remained intact in the duodenum. The immediate insulin treatment prevented the diabetes-related thickening of the basement membrane surrounding the ileal myenteric ganglia. Quantification of particle density showed an increasing tendency for MMP9 and a decreasing tendency for TIMP1 from the proximal to the distal small intestine under control conditions. In the diabetic ileum, the number of MMP9-indicating gold particles decreased in myenteric ganglia, endothelial cells of capillaries and intestinal smooth muscle cells, however, it remained unchanged in all duodenal compartments. The MMP9/TIMP1 ratio was also decreased in ileal ganglia only. However, a marked segment-specific induction was revealed in MMP9 and TIMP1 at the mRNA levels. These findings support that the regional decrease in MMP9 expression in myenteric ganglia and their microenvironment may contribute to extracellular matrix accumulation, resulting in a region-specific thickening of ganglionic basement membrane (published in World Journal of Diabetes 12(5):658-672, 2021).

In the **second year** of the project period, we focused our attention on the investigation of diabetes-related expressional changes of different pro-inflammatory cytokines and their receptors. For that, fluorescent immunohistochemistry on paraffin sections, quantitative

immunogold electron microscopy on ultrathin sections and ELISA on homogenized tissue samples were applied, and revealed a gut region-specific and also intestinal layer-dependent induction of **tumour necrosis factor alpha (TNFα)** in rats with STZ-induced diabetes and after insulin replacement. Increasing density of TNFα-labelling gold particles was observed in myenteric ganglia from proximal to distal segments and TNFα tissue levels were much more elevated in smooth muscle/myenteric plexus homogenates than in mucosa/submucosa/submucous plexus samples in healthy controls. In the diabetics, the number of TNFα gold labels was significantly increased in the duodenum, decreased in the colon and remained unchanged in the ileal ganglia, while insulin did not prevent these diabetes-related TNFα changes. TNFα tissue concentration was also increased in muscle/myenteric plexus homogenates of diabetic duodenum, while decreased in mucosa/submucosa/submucous plexus samples of diabetic ileum and colon (published in Cells 10(9):2410, 2021).

Using post-embedding immunohistochemistry and ELISA, we also evaluated the distribution of **TNF receptor 1 and 2 (TNFR1, TNFR2)** in the myenteric ganglia and intestinal tissue homogenates of different segments and experimental groups. Our findings showed diabetes-related region-dependent changes in TNFR expression and suggested that TNFR2 is more affected than TNFR1 in myenteric ganglia in the duodenum of type 1 diabetic rats. Briefly, a distinct region-dependent TNFRs expression was detected in controls. The density of TNFR1-labeling gold particles was lowest, while TNFR2 density was highest in duodenal ganglia and a decreased TNFRs expression from proximal to distal segments was observed in homogenates. In diabetics, the TNFR2 density was only significantly altered in the duodenum with decrease in the ganglia, while no significant changes in TNFR1 density was observed. In diabetic tissue homogenates, both TNFRs levels significantly decreased in the duodenum, which markedly influenced the TNFR2/TNFR1 proportion in both the ganglia and their muscular environment. Insulin treatment had controversial effects on TNFR expression (published in World Journal of Diabetes 14(1):48-61, 2023).

Our quantitative evaluation on **serotonergic myenteric neurons** in diabetes was also finished in this year. In this study, we revealed that the 5-HT-immunoreactive myenteric neurons represent a small proportion (~2.5%) of the total neuronal number in the investigated gut segments of controls. However, we demonstrated that the proportion of 5-HT- immunoreactive myenteric neurons was enhanced in type 1 diabetes in a region-specific manner, as well as, immediate insulin treatment prevented it and restored the serotonergic neuronal proportion to the control level in each investigated gut segment (published in Applied Sciences 11(13):5949, 2021.)

In the **third year** of the project, we investigated the diabetes-related expressional changes of **toll-like receptors (TLRs)** which play a key role in sensing microbial stimuli. For that, double-labelling fluorescent immunohistochemistry, post-embedding immunogold labelling and ELISA were applied, and revealed a gut region-specific and also intestinal layer-dependent induction of TLR4 in rats with STZ-induced diabetes and after insulin replacement. In controls, the number and proportion of the TLR4-immunoreactive myenteric neurons showed an increasing tendency to aboral direction. These values were significantly higher in diabetics compared to controls in the duodenum and ileum, but were significantly lower in the colon. In diabetics, the distribution of TLR4-labelling gold particles between the perikaryon and neuropil of myenteric neurons varied in a different way by intestinal segment. TLR4 tissue concentration changed only in the diabetic duodenum, and it decreased in muscle/myenteric plexus-containing homogenates, while it increased in mucosa/submucosa/submucous plexus-containing samples relative to controls. Insulin had beneficial effects on TLR4 expression (published in Biomedicines 11(1):129, 2023).

In the **last year** of the project period, we completed all of our ongoing research started in former years. Studying of the diabetes-related regional expression of **interleukin 1 beta (IL1ß)** in myenteric neurons has started in the second year, and continued with investigating different neuronal subpopulations along the duodenum-ileum-colon axis by double-labelling fluorescent

immunohistochemistry. The proportion of IL1β-immunoreactive myenteric neurons was significantly higher in the colon than in the small intestine of controls. In diabetics, this proportion significantly increased in all gut segments, which was prevented by insulin treatment. The proportion of IL1β-nNOS-immunoreactive neurons only increased in the diabetic colon, while the proportion of IL1β-CGRP-immunoreactive neurons only increased in the diabetic ileum. Elevated IL1β levels were also confirmed in tissue homogenates. IL1β mRNA induction was detected in the myenteric ganglia, smooth muscle and intestinal mucosa of diabetics. These findings support that diabetes-related IL1β induction is specific for the different myenteric neuronal subpopulations, which may contribute to diabetic motility disturbances (published in International Journal of Molecular Sciences 24(6):5804, 2023).

Analysis of **blood and urine samples** collected and analysed from the whole project period revealed hyperglycemia-related metabolic and morphological changes of experimental rats. In chronic diabetic rats, decreases in albumin, total protein, and antioxidant glutation concentration were measured, while glutamic-pyruvic transaminase, alkaline phosphatase, red blood cell (RBC) count, hematocrit, and hemoglobin levels were increased. Moreover, an increased level of the phenotypic variants was detected in the RBC population of the diabetic animals. In conclusion, we verified the sensitivity of RBCs to long-lasting hyperglycemia, and to insulin deficiency, which were both accompanied with an increased level of RBC-derived parameters and the presence of eccentrocytes, hemolyzed RBCs, and codocytes. Moreover, our results showed that the response of the RBC glutation system to oxidative stress depends on the duration of hyperglycemia, and that the short-term activation of this defense system is exhausted in a long-lasting oxidative environment. Insulin therapy was effective in the case of most parameters (published in Applied Sciences 13(17):9920, 2023).

**Pancreatic measurements** were also started in the first year and continued in the entire project period. Regarding the exocrine function, pancreatic ductal $HCO_3^-$ and fluid secretion were investigated by microfluorometric techniques and swelling method. Experiments regarding endocrine pancreatic function involved the measurements of α and β cells' exocytosis; cell capacitance was measured by whole cell configuration of the patch clamp technique. Using single- and double-labelling fluorescent immunohistochemistry, the quantification of the insulin- and glucagon-producing islet cells in different experimental conditions was also finished. The manuscript including these results is under preparation now and we are planning to send it for publication soon.

Moreover, in the last year we summarized our knowledge in a review about the key elements determining the intestinal region-specific environment of enteric neurons in type 1 diabetes (published in World Journal of Gastroenterology 29(18):2704-2716, 2023). Our commitment for the last year of the project was 1 Hungarian and 2 international congresses, as well as 2 research articles, which were completed. We participated and presented our IL1ß results at 65$^{th}$ Congress of Hungarian Society of Gastroenterology (Siófok); results of nuclear factor kappa B p65 expression in diabetes were demonstrated at 1$^{st}$ International Conference on Antioxidants (Barcelona) and we also participated at 31$^{st}$ United European Gastroenterology Week in Copenhagen.

By the end of the project 4 graduates and 1 PhD (Diána Mezei) thesis were performed and 2 PhD (Afnan AL Doghmi, Bence Pál Barta) thesis are in progress. During the project period, I successfully completed my habilitation in 2021.

In summary, in the frame and support of this research project, 9 original and review articles, 2 book chapters and 8 Hungarian or international congress presentations were published. At the end of the final report, the published cooperation works are also listed.

**References**

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**PUBLICATIONS**

Research articles related to this project

1. **Bódi N**, Mezei D, Chakraborty P, Szalai Z, Barta BP, Balázs J, Rázga Z, Hermesz E, Bagyánszki M (2021) Diabetes-related intestinal region-specific thickening of ganglionic basement membrane and regionally decreased matrix metalloproteinase 9 expression in myenteric ganglia. World Journal of Diabetes 12(5):658-672.
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2. **Bódi N**, Chandrakumar L, al Doghmi A, Mezei D, Szalai Z, Barta BP, Balázs J, Bagyánszki M (2021) Intestinal Region-Specific and Layer-Dependent Induction of TNFα in Rats with Streptozotocin-Induced Diabetes and after Insulin Replacement. Cells 10(9):2410.
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3. Mezei D, **Bódi N**, Szalai Z, Márton Zs, Balázs J, Bagyánszki M (2021) Immediate Insulin Treatment Prevents Diabetes-Induced Gut Region-Specific Increase in the Number of Myenteric Serotonergic Neurons. Applied Sciences 11(13):5949.
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6. **Bódi N**, Egyed-Kolumbán A, Onhausz B, Barta BP, Doghmi AA, Balázs J, Szalai Z, Bagyánszki M (2023) Intestinal Region-Dependent Alterations of Toll-Like Receptor 4 Expression in Myenteric Neurons of Type 1 Diabetic Rats. Biomedicines 11(1):129.
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9. Szalai Z, Berkó AM, **Bódi N**, Hermesz E, Ferencz Á, Bagyánszki M (2023) Oxidative-Stress-Related Alterations in Metabolic Panel, Red Blood Cell Indices, and Erythrocyte Morphology in a Type 1 Diabetic Rat Model. Applied Sciences 13(17):9920.
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Book chapters

1. **Bódi N**, Bagyánszki M (2020) Diabetic enteric neuropathy: imbalance between oxidative and antioxidative mechanisms. In Book (Preedy V ed.): Diabetes: Oxidative Stress and Dietary Antioxidants, 2nd Edition, Academic Press, Chapter 3, pages 25-33; Paperback ISBN: 9780128157763; eBook ISBN: 9780128157770; https://doi.org/10.1016/B978-0-12-815776-3.00003-6.

2. **Bódi N**, Bagyánszki M (2020) Az oxidatív és antioxidatív egyensúly felborulásának szerepe az entericus neuropátia kialakulásában 1. típusú diabétesz során. In: Poór P, Mézes M, Blázovics A (ed.) Oxidatív stressz és antioxidáns védekezés a növényvilágtól a klinikumig, Magyar Szabadgyök-Kutató Társaság, ISBN: 9786156203007, pp. 195-201.

*Hungarian and international congresses*

1. Szalai Z, Mezei D, Barta B P, Balázs J, Bagyánszki M, **Bódi N** (2020) A szerotoninerg myentericus neuronok mennyiségének bélszakasz-specifikus és inzulin-függő változásai I. típusú diabéteszes patkányokban. Magyar Gasztroenterológiai Társaság 62. On-line Nagygyűlése, Siófok, Magyarország, Central European Journal of Gastroenterology and Hepatology / Gasztroenterológiai & Hepatológiai Szemle Vol 6, Suppl 2, pp. 82-83.

2. **N Bódi**, D Mezei, P Chakraborty, Z Szalai, BP Barta, J Balázs, Zs Rázga, E Hermesz, M Bagyánszki (2021) Correlation between the intestinal region-specific thickening of ganglionic basement membrane and regionally decreased matrix metalloproteinase 9 expression in myenteric ganglia in type 1 diabetes. 45th FEBS Congress, Ljubljana, Slovenia (online, July 3-8, 2021)

3. BP Barta, D Mezei, Z Szalai, J Balázs, M Bagyánszki, **N Bódi** (2021) Gut region-specific expression of tumor necrosis factor alpha and its receptor in the myenteric neurons of streptozotocin-induced diabetic rats. 11th International Symposium on Cell/Tissue Injury and Cytoprotection/ Organoprotection (ISCTICO), Pécs, Hungary (October 27-30, 2021)

4. M Bagyánszki, BP Barta, B Onhausz, A AL Doghmi, J Balázs, Z Szalai, **N Bódi** (2022) Intestinal Region-dependent Alterations of Toll-like Receptor 4 Expression in Myenteric Neurons of Type 1 Diabetic Rats. 3rd International Conference on Cell and Experimental Biology, Boston, USA (online, 18-20 April, 2022)

5. **N Bódi**, BP Barta, A AL Doghmi, B Onhausz, L Chandrakumar, D Mezei, Z Szalai, J Balázs, M Bagyánszki (2022) Intestinal region- and layer-dependent TNFα induction and TNF receptor expression may contribute to myenteric neuroprotection of duodenum in type 1 diabetic rats. 46th FEBS Congress, Lisbon, Portugal (9-14 July, 2022)

6. A AL Doghmi, BP Barta, A Egyed-Kolumbán, B Onhausz, Sz Kiss, Z Szalai, J Balázs, M Bagyánszki, **N Bódi** (2022) Intestinal segment-specific alterations of Interleukin-1ß expression in the myenteric neurons of streptozotocin-induced diabetic rats. 30th UEGW Congress, Vienna, Austria (8-11 October, 2022)

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myenteric ganglia and its microenvironment of streptozotocin induced diabetic rats. 1st International Conference on Antioxidants, Barcelona, Spain (10-12 May, 2023)

8. Bagyánszki M, AL Doghmi A, Barta B, Egyed-Kolumbán A, Onhausz B, Kiss Sz, Balázs J, Szalai Z, **Bódi N** (2023) Interleukin 1β expresszió bélszakasz-specifikus indukciója különböző myentericus neuronpopulációkban 1-es típusú diabéteszes patkánymodellben. Central European Journal of Gastroenterology and Hepatology / Gasztroenterológiai és Hepatológiai Szemle 9:Suppl. 1 pp. 63-64., Magyar Gasztroenterológiai Társaság 65. Nagygyűlése, Siófok, Magyarország (2023. június 1-4.)

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