GUT REGION-SPECIFIC INTERLEUKIN 1β INDUCTION AND NLRP3 DOWNREGULATION IN THE MYENTERIC NEURONS OF A STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL

PhD thesis

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ABBREVIATIONS

CGRP	calcitonin gene-related peptide
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
IL1β	interleukin 1 beta
IR	immunoreactive
NLRP3	nucleotide-binding oligomerization domain-, Leucin rich repeats-, and pyrin domain-containing protein 3
nNOS	neuronal nitric oxide synthase
STZ	streptozotocin
T1D	type 1 diabetes

1. INTRODUCTION

The enteric nervous system (ENS) represents the intrinsic neuronal innervations of the gastrointestinal tract. The enteric neurons contain sensory, motor, as well as interneurons that are organized into two ganglionated plexuses: myenteric and submucous plexus. Enteric neurons are in dynamic interaction with the gut layers and microbiota and therefore they are involved in the regulation of different gastrointestinal functions including motility, secretion, absorption, and immune modulation. Disruption in the ENS can result in gastrointestinal complications such as altered food transit time, diarrhoea, constipation bloating, hypersensitivity, nausea, or vomiting.

Patients with type 1 diabetes (T1D) often experience gastrointestinal dysmotility symptoms related to myenteric neuropathy. We found that neuronal nitric oxide synthase (nNOS)-immunoreactive (IR) subpopulation of myenteric neurons which have key role in peristalsis are largely impacted in diabetic damage. This may be the result of the upregulated oxidative and pro-inflammatory mechanisms during long-lasting hyperglycaemia in T1D.

Many of these pro-inflammatory cascades ultimately lead to the activation of the interleukin 1β (IL1β) pathway. IL1β, an isoform of interleukin 1, is expressed as a pro-IL1β and activated by caspase-1 enzyme inside an inflammasome complex.

In the gut, activated IL1β represents a pleiotropic cytokine that is crucial for gut homeostasis and may exert divergent effects on distinct neuronal populations within the myenteric plexus. However, excessive IL1β expression due to hyperglycaemia could disrupt gut homeostasis and possibly contribute to diabetes-related regional damage of nNOS-IR neurons. Additionally, IL1β is implicated in the signalling pathway of calcitonin gene-related peptide (CGRP), suggesting its potential effects on the subset of CGRP-expressing myenteric neurons most of which represent intrinsic primary afferent neurons. Therefore, it is plausible that IL1β may have potential effects on the different populations of myenteric neurons under different metabolic, neuroinflammatory, and spatial circumstances.

Among others, the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome is extensively studied due to its involvement in autoimmune and inflammatory diseases including T1D. Furthermore, this inflammasome-forming protein was suggested to contribute to maintaining gastrointestinal tract homeostasis and microbial compositions. However, fewer studies have focused on the role of NLRP3 in myenteric neurons with discrepancies in its involvement in enteric neuropathy depending on the investigated disease. In T1D, Toll-like receptor 4 expression and immunoreactivity were changed in a gut region-specific manner in the myenteric neurons. Since it is an inducer for NLRP3 inflammasome, it is reasonable to examine the perturbation of neuronal NLRP3 inflammasome protein and investigate its possible association with diabetic IL1β expression.

2. AIMS

This dissertation aims to explore the effect of hyperglycaemia and insulin treatment on IL1β and NLRP3 expression in different myenteric neuronal populations and intestinal layers of a T1D rat model. For that, we applied fluorescent immunohistochemistry, immunogold electron microscopy, enzyme-linked immunosorbent assay (ELISA), and RNAscope multiplex fluorescent V2 assay to answer the following questions:

In the intestine

- Is there any difference in the proportions of IL1β-IR, IL1β-nNOS-IR, and IL1β-CGRP-IR myenteric neurons along the duodenum-ileum-colon axis in healthy controls?
- How do hyperglycaemia and insulin treatment influence the proportions of IL1β-IR, IL1β-nNOS-IR, and IL1β-CGRP-IR myenteric neurons in the different intestinal segments?
- Does diabetes-related alteration of IL1β expression contribute to neuronal population-specific diabetic damage?
- What is the effect of diabetes and insulin replacement on IL1β protein level in the tissue homogenates of the myenteric plexus and smooth muscle layers?
- Is there any difference in the density of NLRP3-labelling gold particles within the myenteric ganglia along the duodenum-ileum-colon axis of control rats?
- How do hyperglycaemia and insulin treatment influence the density of NLRP3 gold labels in the myenteric ganglia of the different gut regions?
- Are there any differences in the expression patterns of IL1β and NLRP3 mRNA in the myenteric ganglia, smooth muscle, and mucosa layer of the intestinal wall along the duodenum-ileum-colon axis of control, diabetic, and insulin-treated diabetic rats?

- Is there any correlation between the changes in IL1β and NLRP3 expression in the myenteric ganglia and their environment in the investigated segments of the experimental groups?

In the pancreas

- How do acute and chronic hyperglycaemia affect the IL1β protein levels in tissue homogenates of the pancreas?
- What is the effect of insulin treatment on the levels of IL1β in the pancreas?
- How does the pancreatic IL1β mRNA expression change in diabetes and after insulin treatment?

In the serum

- What are the effects of acute and chronic hyperglycaemia on IL1β serum levels?
- Is there any effect of insulin treatment on the IL1β concentration in the serum?

3. MATERIALS AND METHODS

3.1. Animal models

For our experiments, we used a streptozotocin (STZ)-induced diabetic rat model to study the effect of acute and chronic hyperglycaemia on the ENS and its environment along the gastrointestinal tract. For the acute (1-week) diabetic rat model, animals were randomly divided into two groups: STZ-induced diabetics (n=8), and age- and sex-matched controls (n=6). For the chronic (10-weeks) diabetic animal model, rats were divided randomly into three groups: STZ-induced diabetics (n=20), insulin-treated STZ-induced diabetics (n=17), and sex- and age-matched controls (n=20). Hyperglycaemia was induced by a single intraperitoneal injection of STZ at 60 mg/kg. Animals with non-fasting blood glucose concentrations higher than 18 mmol/L were considered to be diabetic. From this time on, one group of

hyperglycaemic animals received subcutaneous injection of insulin (Humulin M3) each morning (2 IU) and afternoon (3 IU). Normal saline was administered subcutaneously to diabetic and control groups. Physiological parameters such as weight and blood glucose levels were measured daily for the acute and weekly for the chronic animal experiments.

3.2. Blood sampling and tissue handling

One week or ten weeks after the onset of hyperglycaemia, blood samples were collected, and pancreas and different intestinal segments (duodenum, ileum, and colon) of diabetic, insulin-treated diabetic, and control rats were processed for fluorescent immunohistochemistry, immunogold electron microscopy, ELISA, and RNAscope multiplex fluorescent V2 assay.

3.3. Fluorescent immunohistochemistry

For double-labelling fluorescent immunohistochemistry, myenteric whole-mount preparations derived from different gut segments were immunostained with primary antibodies against IL1β and HuC/HuD, nNOS, or CGRP, as well as NLRP3 and HuC/HuD followed by incubation with appropriate secondary antibodies. Immunostained whole-mounts were observed and photographed with a Zeiss Imager Z.2 fluorescent microscope. An average of fifty myenteric ganglia were taken from each gut segment per experimental group, and the proportions of IL1β-IR, IL1β-nNOS-IR, and IL1β-CGRP-IR myenteric neurons were calculated per ganglion under different experimental conditions.

3.4. Post-embedding immunohistochemistry

Ultrathin sections of the gut segments were mounted on nickel grids, and incubated with primary antibody against NLRP3, followed by 18 nm gold-conjugated secondary antibody. Sections were counterstained and then examined and photographed with a JEOL JEM 1400 transmission electron

microscope. The subcellular localization and quantitative distribution of the gold particles labelling NLRP3 were determined in the myenteric ganglia. Fifty digital photographs of five myenteric ganglia per intestinal segment per condition were conducted at a magnification of 20,000× with the AnalySIS 3.2 program. The intensity of the labelling was expressed as the total number of gold particles per unit area (μm²).

3.5. Measurement of tissue and serum interleukin 1β concentrations

Pancreatic and intestinal muscle/myenteric plexus tissue samples were homogenised and used to determine the total protein content. IL1β levels in the different tissue samples and sera were determined using quantitative ELISA according to the manufacturer's instructions. Optical density was measured at 450 nm. Tissue concentrations of IL1β were expressed as ng/mg protein whereas blood IL1β levels were expressed as ng/ml serum.

3.6. RNAscope multiplex fluorescent V2 assay

To detect IL1β mRNA (for pancreas and intestinal layers) and NLRP3 mRNA (for intestinal layers), the RNAscope multiplex fluorescent V2 assay was applied on fresh-frozen cryosections hybridized with rat IL1β probe or NLRP3 probe according to the manufacturer's instructions. An average of fifteen digital photographs were taken from the pancreas and the different intestinal layers (myenteric ganglia, smooth muscle, and mucosa) of each gut segment and experimental group. The number of punctate dots labelling IL1β or NLRP3 mRNA was quantified per unit area (mm²).

3.7. Statistical analysis

Data were checked for Gaussian distribution by a D'Agostino-Pearson omnibus normality test. Two-tailed T-test with a Mann-Whitney test was used to analyse data from two comparison groups (acute experiment). Data retrieved from multiple comparison groups were analysed by Kruskal–Wallis

test with a Dunn's multiple comparisons test (chronic experiment). All analyses were carried out with GraphPad Prism 6.0. A probability of $p < 0.05$ was set as the level of significance. All data were expressed as mean ± SEM.

4. Results

4.1. Effect of acute and chronic hyperglycaemia on the proportion of interleukin 1β-immunoreactive myenteric neurons

In control rats of the acute and chronic diabetic models, the proportion of IL1β-IR myenteric neurons revealed significant differences between the small and large intestine, in which the colon displayed the highest proportion of that compared to the duodenum and ileum (acute: ~23% *vs* 3-6%, chronic: 50% *vs* 13-20%).

Acute hyperglycaemia significantly induced the proportion of IL1β-IR myenteric neurons in the small intestine (duodenum: 13.83 ± 2.64% vs 3.44 ± 1.02%; ileum: 12.01 ± 2.12% vs 6.76 ± 1.22%) but no significant changes were observed in the colon of acute diabetics compared to controls.

On the other hand, chronic hyperglycaemia significantly induced the proportion of IL1β-IR myenteric neurons in all gut segments, which was completely prevented after insulin treatment. In the colon, a 20% increase resulted in more than 70% of myenteric neurons displaying immunoreactivity to IL1β (71.14 ± 3.29%) in this gut segment, whereas this proportion almost doubled in both the duodenum (34.04 ± 4.06%) and ileum (24.62 ± 3.11%) compared to controls Moreover, both acute and chronic hyperglycaemic animals displayed an increase in the proportion of IL1β-IR myenteric neurons-containing ganglia in all gut segments. Insulin treatment protected against the increase in this proportion only in the small intestine.

4.2. Effect of chronic hyperglycaemia on the proportion of interleukin 1β-immunoreactive nNOS neurons

In controls, the proportion of IL1β-nNOS-IR neurons per total nNOS-IR myenteric neurons was the lowest in the duodenum (12.64 ± 2.03%) and significantly higher in the distal intestinal regions (ileum: 36.36 ± 2.18%; colon: 26.21 ± 2.13%). However, the proportion of IL1β-nNOS-IR neurons per total IL1β-IR myenteric neurons was relatively similar in all gut segments.

In diabetic rats, the proportion of IL1β-nNOS-IR neurons of total nNOS-IR myenteric neurons was significantly induced only in the colon (36.94 ± 2.76% vs 26.21 ± 2.13%), which was not prevented by insulin treatment. Whereas the proportion of IL1β-nNOS-IR neurons per total IL1β-IR neurons was decreased significantly in the small intestine (duodenum: 26.77 ± 2.46% *vs* 40.1 ± 3.65%; ileum: 31.88 ± 2.41% *vs* 43.48 ± 2.37%) and it was prevented by insulin treatment.

4.3. Effect of chronic hyperglycaemia on the proportion of interleukin 1β-immunoreactive CGRP neurons

In control animals, the proportion of IL1β-CGRP-IR neurons per total CGRP-IR myenteric neurons ranged between 40-50% in all gut segments. Whereas the proportion of IL1β-CGRP-IR neurons per total IL1β-IR myenteric neurons ranged between 50-60% which was slightly higher in the small intestine compared to the colon.

In diabetics, the proportion of IL1β-CGRP-IR neurons per total CGRP-IR neurons was significantly increased only in the ileum (64.56 ± 4.17% vs 42.13 ± 3.8%) which was partially prevented by insulin treatment.

4.4. Effect of hyperglycaemia on interleukin 1β protein levels

In the colonic muscle/myenteric plexus homogenates, IL1β protein concentration per total protein content was at low levels in the control samples of the chronic experiment. Chronic diabetic rats showed a 5-fold increase in the IL1β level in the colon compared to controls which was abolished in the insulin-treated group.

In the pancreas, the tissue level of IL1β was significantly increased (> 6-fold) in the diabetics of acute hyperglycaemic animals, however, chronic diabetic rats showed no significant changes in IL1β levels relative to controls.

On the other hand, serum IL1β levels from the acute and chronic diabetics were slightly increased compared to their control counterparts with no significant alterations.

4.5. Effect of chronic hyperglycaemia on the expression of interleukin 1β mRNA

In controls, the number of dots labelling IL1β mRNA varied in a gut segment-specific and layer-dependent manner. The number of IL1β mRNA dots was the lowest in the intestinal smooth muscle layer in all gut segments of control rats, while it was highest in the myenteric ganglia of the duodenum and colon. In the ileum, the mucosa contained the highest IL1β mRNA value (4558 ± 486.7 dots/mm^2) which was double that of the duodenum and colon.

In diabetic rats, the number of IL1β mRNA labelling dots was significantly increased in the myenteric ganglia of all the investigated segments with the greatest increase (tripled) detected in the duodenum. In the smooth muscle, the level of IL1β mRNA dots increased significantly in the duodenum and colon. Furthermore, the density of IL1β mRNA dots was significantly increased in the mucosal layers of the small intestine with a robust (5-fold increase) change in the duodenum compared to controls.

Insulin treatment completely prevented the diabetic alterations in the ileum and partially in the duodenum.

In the pancreas, IL1β mRNA expression was robustly increased in diabetic rats compared to controls (23061 ± 708.5 dots/mm² *vs* 3236 ± 267.6 dots/mm²), which was partially prevented by insulin replacement (7918 ± 593.7 dots/mm²).

4.6. Subcellular localization and quantitative evaluation of NLRP3 density within the myenteric ganglia

Myenteric neurons were IR to NLRP3 inflammasome protein with strong localization within neuronal varicosities. Subcellular localization of NLRP3-labelling gold particles appeared in the cytosol of the perikarya, in and near the mitochondria, and in the nucleus. Additionally, NLRP3 gold labels were abundant in the neuropil regions of the myenteric ganglia. Quantitative analysis of NLRP3 density in the myenteric ganglia revealed similar distribution (2.8-3.5 labels/µm²) along the duodenum-ileum-colon axis in control rats.

In diabetics, duodenal NLRP3 gold labels became half of the control level and decreased by 1.5-fold in the colon compared to controls. Insulin treatment caused a robust increase in NLRP3 density in the myenteric ganglia of the duodenum (4.28 ± 0.22 labels/µm²) and colon (5.82 ± 0.36 labels/µm²) compared to controls and diabetics.

4.7. Effect of chronic hyperglycaemia on the expression of NLRP3 mRNA

In controls, the number of NLRP3 mRNA-labelling dots varied in a gut segment-specific and layer-dependent manner. The number of NLRP3 mRNA labelling dots was the lowest in the intestinal smooth muscle layer in all gut segments with relatively similar distribution among the gut segments (800-930 dots/mm²). In the myenteric ganglia, no significant differences were

observed in the number of NLRP3 mRNA dots along the duodenum-ileum-colon axis of controls. In the mucosa, the number of punctate dots labelling NLRP3 mRNA was the highest in the duodenum (3572 ± 148.9 dots/mm²) and the lowest in the colon (1648 ± 206.1 dots/mm²).

In diabetic rats, significant decrease in the number of NLRP3 mRNA dots was observed in the myenteric ganglia of the duodenum (1810 ± 221.5 *vs* 3437 ± 473.6 dots/mm², $p < 0.05$) and in all the intestinal wall layers of the ileum compared to controls (myenteric ganglia: 1449 ± 427.2 dots/mm² *vs* 2394 ± 320.2 dots/mm², $p < 0.05$; smooth muscle: 569.5 ± 68.65 dots/mm² *vs* 877.8 ± 93.75 dots/mm², $p < 0.05$; mucosa: 2375 ± 266.5 dots/mm² *vs* 2873 ± 189.2 dots/mm², $p < 0.05$). Insulin treatment did not protect against the hyperglycaemia-induced decrease in the number of NLRP3 mRNA dots observed in the distinct layers of the small intestine. Moreover, the only observed increase in NLRP3 mRNA expression was detected in the colonic mucosa of insulin-treated diabetic rats compared to controls and diabetics.

5. DISCUSSION

In support of our earlier findings that nitrergic neuronal susceptibility to hyperglycaemia is strictly regional, our current work provides evidence of gut region-specific and layer-dependent induction of IL1β. Moreover, we demonstrate that this induction of IL1β is not mediated by the NLRP3 inflammasome since it is downregulated in hyperglycaemia in a gut region-specific manner.

IL1β, produced by various cell types, plays a role beyond inflammation, influencing glucose metabolism, intestinal barrier homeostasis, and neuronal stimulation. Furthermore, oxidative and inflammatory pathways activating IL1β were found to be altered in T1D. Therefore, our aim was to study the involvement of IL1β in diabetes-induced region-specific myenteric neuropathy and to elucidate its possible impact on diabetic damage of

different myenteric neuronal populations using fluorescent immunohistochemistry, immunogold electron microscopy, protein measurement, as well as RNAscope using acute and chronic STZ-induced T1D rat models. It was crucial to firstly get deeper insight into the expressional patterns of IL1β in the myenteric neurons and their environment in healthy conditions. Control animals showed higher baseline proportion in IL1β-IR myenteric neurons in the colon compared to the small intestine, possibly influenced by the rich anaerobic microbiota and the higher oxidative environment in the colon. Further analysis revealed segment-specific variations in IL1β immunoreactivity of myenteric populations of nNOS and CGRP neurons, suggesting a neuronal population-specific IL1β expression in myenteric ganglia. On the mRNA level, IL1β transcription showed distinct spatial patterns across the intestinal wall layers, concentrating in the myenteric ganglia of both the duodenum and colon, whereas dominating in the mucosal layer of the ileum.

In hyperglycaemic states, a time-dependent and region-specific induction of IL1β was observed in the myenteric neurons of the investigated gut segments. Acute hyperglycaemia promptly induced the immunoreactivity of IL1β in the myenteric neurons of the small intestine, while colonic myenteric neurons only developed that after prolonged hyperglycaemia. This may be explained by the presence of defence mechanisms in the colon to combat high baseline level of microbial load and pro-oxidative environment rendering them more tolerant to the short-term increase in oxidative stress associated with acute hyperglycaemia which could be ineffective under prolonged hyperglycaemia. Moreover, high glucose concentrations activate IL1β enhancers in various cells which ultimately trigger pro-inflammatory cytokines including IL1β. Cytokines like TNFα and IL1β are neurotrophic for myenteric neurons and thus can promote neurite growth which may serve

as a compensatory mechanism to rescue enteric neurons and mediate neuroplasticity.

The overall increase in IL1β-IR myenteric neurons varied among neuronal populations and along the gastrointestinal tract. In diabetics, an increase in IL1β-nNOS-IR neurons was observed only in the colonic segment potentiating its association with nitrergic disruption seen in diabetic colon. Additionally, the proportion of IL1β-containing CGRP neurons increased specifically in the ileal ganglia, possibly linked to microbial rearrangement and Klebsiella invasion in this particular gut segment in diabetes. Given these complex interactions, further studies are needed to determine the role of induced IL1β in nitrergic neuropathy and explore the implications of CGRP modulation in diabetic enteric neuropathy. IL1β mRNA induction was also evident in the myenteric ganglia and to a lower extent in the intestinal smooth muscle. Robust IL1β mRNA induction in the mucosa of the small intestine, particularly the duodenum, may be influenced by alterations in mucosa-associated gut microbiota, potentially contributing to increased intestinal permeability under chronic hyperglycaemia.

Chronic hyperglycaemia caused a myenteric neuronal IL1β induction on both the mRNA and protein levels. For IL1β activation, an inflammasome complex is required to be assembled to enzymatically mediate the activation of pro-IL1β into an active IL1β. Therefore, we aimed to study NLRP3 role in the diabetes-induced region-specific myenteric neuropathy and its correlation with the activation of IL1β in our experiments.

In controls, the number of NLRP3-labelling gold particles was evenly distributed within the myenteric ganglia along the duodenum-ileum-colon axis. This was significantly decreased in the duodenal and colonic myenteric ganglia of diabetics compared to controls. The inhibition of NLRP3 may be due to the hyperglycaemia-induced immunoreactivity of adrenergic or cholinergic neurons which were found to antagonise this inflammasome

protein. Quantitative analysis of NLRP3 transcripts of control rats showed regional differences in the mucosal layer with higher NLRP3 transcription detected in the small intestine compared to the colon, consistent with our previous observation regarding IL1β mRNA levels. However, hyperglycaemia decreased NLRP3 mRNA levels specifically in the different compartments of the small intestine which was not prevented by immediate insulin treatment. Similarly, the expression of other inflammasome proteins like NLRP1, NLRP3, and NLRP6 was also decreased in the small intestine of a diabetic obesity-induced animal model. Moreover, another study demonstrated the inhibition of NLRP3 inflammasome by inducing haem oxyegenase1 which has been robustly induced in the myenteric neurons of the ileum in T1D rat model. The intestinal wall layers including the myenteric neurons may downregulate the NLRP3 inflammasome protein as a compensatory mechanism to limit further propagation in intestinal inflammation. A downregulated NLRP1 and NLRP3 in the circulatory system was associated with poor prognosis in T1D patients. Therefore, the downregulation of NLRP3 inflammasome besides others, may play a role in the disrupted intestinal barrier seen in diabetes and myenteric neuropathy.

In conclusion, IL1β is a crucial cytokine expressed by cells in the intestinal tract and display a spatial and segment-specific expressional patterns in healthy rats. Hyperglycaemia causes intestinal region-specific and layer-dependent induction in this cytokine. Specific myenteric neurons react to hyperglycaemia and become a higher source for IL1β production. This highlight the role of IL1β in myenteric subpopulation-specific neuropathy that can give rise to diabetic dysmotility. Moreover, since NLRP3 is regionally downregulated in chronic hyperglycaemia, it is assumable that hyperglycaemia-related IL1β induction is not mediated through the NLRP3 inflammasome protein rendering it dispensable for IL1β activation in the gut in T1D.

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Publications related to the thesis

1. AL Doghmi A, Barta BP, Egyed-Kolumbán A, Onhausz B, Kiss S, Balázs J, Szalai Z, Bagyánszki M, Bódi N. Gut Region-Specific Interleukin 1β Induction in Different Myenteric Neuronal Subpopulations of Type 1 Diabetic Rats. Int J Mol Sci. 2023 Mar 18;24(6):5804. doi: 10.3390/ijms24065804. **IF: 5.6**

2. Bódi N, Chandrakumar L, AL Doghmi A, Mezei D, Szalai Z, Barta BP, Balázs J, Bagyánszki M. Intestinal Region-Specific and Layer-Dependent Induction of TNFα in Rats with Streptozotocin-Induced Diabetes and after Insulin Replacement. Cells. 2021 Sep 13;10(9):2410. doi: 10.3390/cells10092410. **IF: 7.43**

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2. Bódi N, Egyed-Kolumbán A, Onhausz B, Barta BP, AL Doghmi A, Balázs J, Szalai Z, Bagyánszki M. Intestinal Region-Dependent Alterations of Toll-Like Receptor 4 Expression in Myenteric Neurons of Type 1 Diabetic Rats. Biomedicines. 2023 Jan 4;11(1):129. doi: 10.3390/biomedicines11010129. **IF: 4.7**

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CONFERENCES

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Supervisor's/Coauthor's declaration

I, undersigned Nikolett Bódi, the coauthor and supervisor of Afnan Mohammad Rashed AL Doghmi's PhD work hereby certify that I am familiar with the PhD thesis of the applicant Afnan Mohammad Rashed AL Doghmi entitled 'Gut region-specific interleukin 1β induction and NLRP3 downregulation in the myenteric neurons of a streptozotocin-induced diabetic rat model'.

The applicant's contribution was prominent in obtaining the followings: establishment of the diabetic rat model, quantitative fluorescent and post-embedding immunohistochemistry, fluorescent and transmission electron microscopy, enzyme-linked immunosorbent assay, RNAscope multiplex fluorescent V2 assay, and statistical analyses.

I did not and will not use these results in getting academic research degree. There is no other PhD student who can use these results in a doctoral process.

Nikolett Bódi, PhD

supervisor

30 April, 2024

Coauthor's declaration

We, undersigned and the coauthors of Afnan Mohammad Rashed AL Doghmi's papers hereby certify that we are familiar with the PhD thesis of the applicant Afnan Mohammad Rashed AL Doghmi entitled 'Gut region-specific interleukin 1β induction and NLRP3 downregulation in the myenteric neurons of a streptozotocin-induced diabetic rat model'.

We did not and will not use these results in getting academic research degree.