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The role of Peptidylarginine deiminase 4 (PAD4) and Endothelial–Mesenchymal Transition (EndMT) in pancreas-heart axis of Type 1 diabetes mellitus

PhD Dissertation

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Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disorder defined by an absolute deficiency of insulin. This condition arises when the immune system erroneously targets and destroys the pancreatic beta cells responsible for insulin production. It makes up roughly 5–10% of all diabetes individuals. Studies have found that both genetic predisposition and environmental factors contribute to the development of T1DM by inducing stress in the endoplasmic reticulum of beta cells. Current understanding suggests that pancreatic autoantigens are processed by macrophages, dendritic cells, or B-lymphocytes functioning as antigen-presenting cells, and are then presented to naive T-cells in pancreatic lymph nodes, resulting in the generation of autoreactive $CD4^+$ T-cells. However, the exact mechanisms underlying the initiation and progression of beta-cell loss remain unclear.

Peptidylarginine deiminases (PADs) constitute a group of calcium-dependent enzymes that catalyze the post-translational conversion of arginine residues to citrulline, a process called citrullination or deimination. PAD2 and PAD4 have been studied for their involvement in the central nervous system and immune system. PAD2 is distributed across the brain, spinal cord, skeletal muscles, pancreas, and immune cells, while PAD4 is primarily found in granulocytes and monocytes. Both enzymes participate in

regulating gene transcription via histone citrullination and are involved in processes such as NETosis and pyroptosis.

In T1DM, the formation of neutrophil extracellular traps (NETs) has been identified as a significant factor contributing to pancreatic inflammation and β-cell destruction. The process of NETosis is mediated by the enzyme peptidylarginine deiminase 4 (PAD4), which facilitates the citrullination of histone H3 (CITH3). This modification leads to chromatin decondensation and the subsequent release of DNA-protein complexes known as NETs. Notably, pharmacological inhibition of PAD4 using Cl-amidine considerably reduced NETosis and inflammation, emphasizing the pivotal role of PAD4 in T1DM pathogenesis and its promise as a therapeutic target. Mechanistically, calcium influx activates PAD4, leading to histone citrullination and chromatin release, while neutrophil elastase (NE) further degrades nuclear components, thereby intensifying NETosis. Collectively, these findings indicate that NETosis markers—PAD4, CITH3, and elastase—serve not only as biomarkers of inflammation but also as active mediators of autoimmune and inflammatory damage in T1DM.

Streptozotocin (STZ) is an antibiotic that destroys pancreatic islet β-cells. It is commonly used in experiments to create a model of T1DM. The diabetogenic characteristics of STZ, which was first discovered from *Streptomyces achromogenes* in 1960, were not disclosed until 1963. The ability of STZ-injection to modify the disease makes the rat model of diabetes highly comparable to

human conditions. Rats are favored in diabetes research due to their short gestation period of 21–22 days and their sexual maturity reached at postnatal days 60–70. These characteristics make them ideal for studying the effects of various treatments on disease progression and management. Chemically induced models of T1DM in rats often involve the administration of STZ in varying doses and methods. Researchers may use a single high dose of STZ, ranging from 35 to 65 mg/kg, administered either IV or IP. Through GLUT2, STZ, a cytotoxic glucose analog, tends to accumulate in pancreatic beta cells. The methyl-nitrosourea moiety of STZ's DNA alkylating activity determines how hazardous it is. DNA fragmentation results from the methyl group being transferred from STZ to the DNA molecule, which damages the molecule in a specific sequence of events. In addition to direct cytotoxicity, STZ also triggers oxidative stress and inflammatory responses, further exacerbating beta-cell damage. The loss of beta cells initiates a cascade of immune responses, including the activation of resident macrophages and infiltration of immune cells into the islets.

Cytokines play a pivotal role in mediating and amplifying the inflammatory response during STZ-induced beta-cell destruction. Proinflammatory cytokines such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) are upregulated and contribute to beta-cell dysfunction by activating nuclear factor-kappa B (NF-κB) signaling pathways, leading to increased expression of inducible nitric oxide synthase (iNOS) and

nitric oxide production, which are toxic to beta cells. Interestingly, several investigations have demonstrated that during beta-cell death, neutrophils might infiltrate the pancreas due to the attraction of cytokines and chemokines. Persistent inflammatory stimuli or unresolved infections can intensify PAD4-driven NETosis. Inchronic inflammatory conditions, prolonged exposure of neutrophils to cytokines and chemokines leads to sustained PAD4 activation and excessive NET generation, which may cause tissue injury and contribute to the development of autoimmune and thrombo-inflammatory disorders. Notably, TNF-α not only stimulates PAD4 but also enhances IL-8 production, serving as a strong chemoattractant and activator for neutrophils. Elastase released during NETosis contributes to extracellular matrix degradation and propagates the inflammatory response. In pathologies such as rheumatoid arthritis and sepsis, elevated concentrations of citrullinated histones and elastase act as biomarkers for NETosis and correlate with disease severity.

DM is also a leading global health burden and a major risk factor for cardiovascular disease (CVD). Despite intensive glucose-lowering strategies, patients with diabetes continue to experience elevated cardiovascular morbidity and mortality, pointing to underlying mechanisms beyond hyperglycemia alone. A growing body of evidence identifies endothelial-to-mesenchymal transition (EndMT) as a crucial pathophysiological process linking diabetes to cardiac dysfunction and vascular complications. EndMT refers

to the transdifferentiation of endothelial cells (ECs) into mesenchymal-like cells. In the diabetic context, hyperglycemia, oxidative stress, and chronic inflammation act synergistically to drive EndMT both locally and systemically. Moreover, cardiac tissues from diabetic patients display increased mesenchymal marker expression, indicating persistent EndMT even under controlled glycemia.

Cytokines and chemokines function as key mediators in both inflammation and fibrosis associated with cardiac pathologies, particularly through their regulatory effects on EndMT. Proinflammatory cytokines, such as IL-1β and TNF-α, can collaborate with TGF-β to intensify EndMT processes and fibrogenic activity. In addition, chemokines such as CXCL-1 and CCL-2 significantly contribute to the recruitment of immune cells into cardiac tissue, perpetuating chronic inflammation and further enhancing EndMT. These molecules also drive the generation of ROS, which activate TGF-β signalling and PAD4-mediated NETosis.

The complex interactions among cytokines, chemokines, ROS, and EndMT indicators, including α-SMA and vimentin, establish a sustained cycle of inflammation and fibrosis within cardiac tissue. TGF-β remains central in this network, integrating signals from oxidative stress and immune mediators to promote EndMT. The resultant mesenchymal cells contribute substantially to extracellular matrix accumulation and tissue rigidity, which are

defining features of cardiac fibrosis. Therapeutic approaches targeting these molecular pathways—particularly those involving TGF-β signalling and ROS production—show promise in mitigating or reversing fibrotic cardiac remodelling.

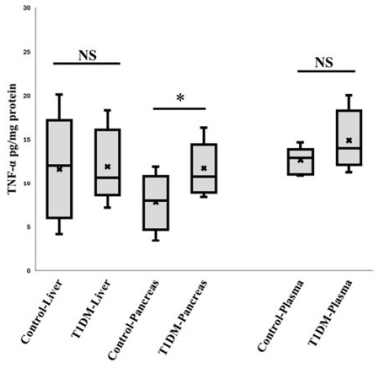
Moreover, citrullination has been identified in heart failure patients, affecting the function of myofilament proteins and potentially linking inflammation to structural changes in the heart. This modification can alter protein function and contribute to the progression of heart failure. Understanding these complex interactions and modifications is essential for developing targeted therapies that address both the inflammatory and reparative processes within cardiac tissues, ultimately improving heart disease outcomes and patient health.

Objectives:

I. Investigate whether **PAD4-mediated histone citrullination and NETosis** represent a central, unifying mechanism in the pathogenesis of type 1 diabetes mellitus (T1DM).
II. Assess how increased **PAD4 activity in the pancreas** contributes to systemic inflammation.
III. Explore whether the resulting **proinflammatory and oxidative milieu** facilitates **endothelial–mesenchymal transition (EndMT)** in the diabetic heart.
IV. Integrate pancreatic and cardiac endpoints to establish PAD4 as a **common upstream driver of multi-organ diabetic complications**.
V. Provide a mechanistic rationale for the development of **PAD4-targeted therapeutic strategies**.

Results

- **The expression of TNF- α in Plasma, Liver and Pancreas.**



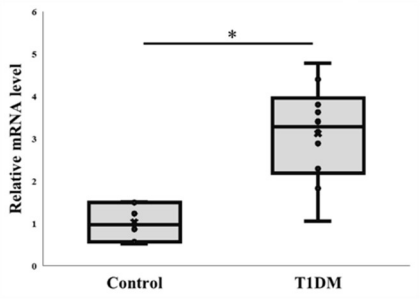
The expression of TNF- α in the plasma, liver, and pancreas. According to the comparison, over the first six weeks, STZ may only damage DNA in the pancreas through GLUT2. Neither the liver nor the other organs were damaged. (n = 10), * $p < 0.05$.

- **Concentration of various inflammatory and anti-inflammatory cytokines in the pancreas.**

Summary of the analysis of various proinflammatory/anti-inflammatory cytokines in the pancreas of STZ-treated diabetic rats and control. * $p \leq 0.05$, n = 10 (unit: pg/mg protein).

Group	CXCL-1	IFN-γ	IL-6	IL-18	IL-33	IL-10
Control	33.02 ±3.34	21.62 ± 2.44	40.81 ± 5.63	77.67 ± 4.83	708.38 ± 76.19	451.95 ± 152.31 *
STZ	46.85 ± 3.32 *	29.46 ± 2.00 *	55.44 ± 3.29 *	130.75 ± 17.46 *	1027.26 ± 113.47 *	153.71 ± 18.73
Cytokine	inflammatory	inflammatory	inflammatory	inflammatory	inflammatory	anti-inflammatory

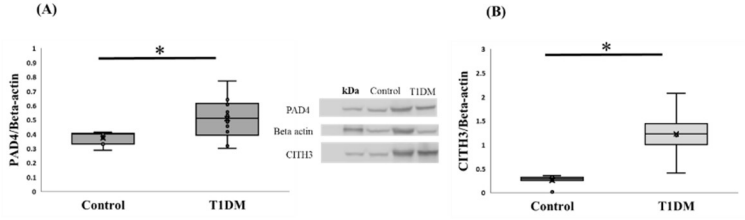
- **PAD4 mRNA levels in Pancreas.**



Change of PAD4 mRNA in pancreas between control and T1DM group. ($P < 0.05$). Results are presented as mean ± S.E.M. (Control, n=7; T1DM, n=10)

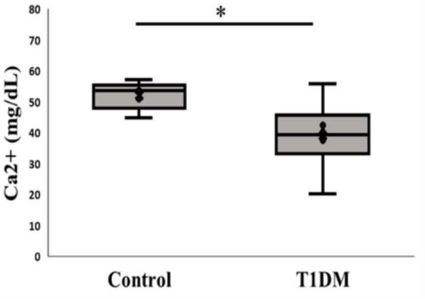
- **PAD4 and CITH3 protein expression in Pancreas**

(A) PAD4 protein expression in Pancreas. PAD4 protein level between Control and T1DM against internal control (Beta-actin), elucidating PAD4 expression in T1DM was greater than in Control within pancreas (*$P < 0.05$). Results arepresented as mean ± S.E.M. (Control, n=7; T1DM, n=10) (B) CITH3 levels in Pancreas. Change of the expression of citrullinated histone-3 (CITH3) between Control and T1DM group. The result indicated successful induction of T1DM according to citrullinated level (*$P < 0.05$). Results arepresented as mean ± S.E.M. (Control, n=8; T1DM, n=6)



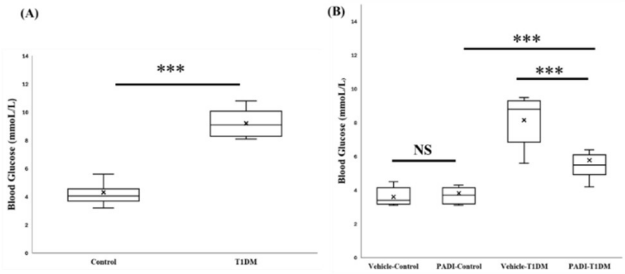
- **Ca^{2+} content in Pancreas**

Change of the expression of Calcium ion between Control and T1DM group, Ca^{2+} is a cofactor for PAD4 and our measurement presented profound impact in pancreas between Control and T1DM group (*P < 0.05). Results are presented as mean ± S.E.M. (Control, n=5; T1DM, n=6)



- **Blood Glucose level before termination**

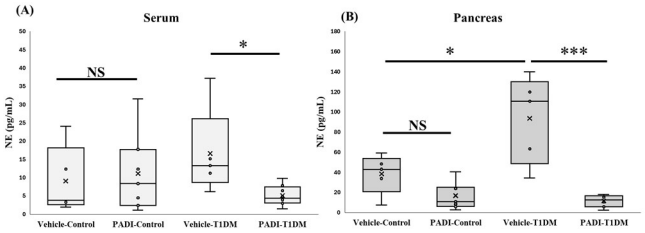
(A) At 6th week, by confirming blood glucose levels, T1DM was successfully induced. (B) In experiment B, we used a PADs inhibitor (PADI) to further examine PAD4 expression. After ten days of dosing, we also checked the blood glucose level. ***P < 0.001 (All groups, n=10)



- **NETosis level in Serum and Pancreas**

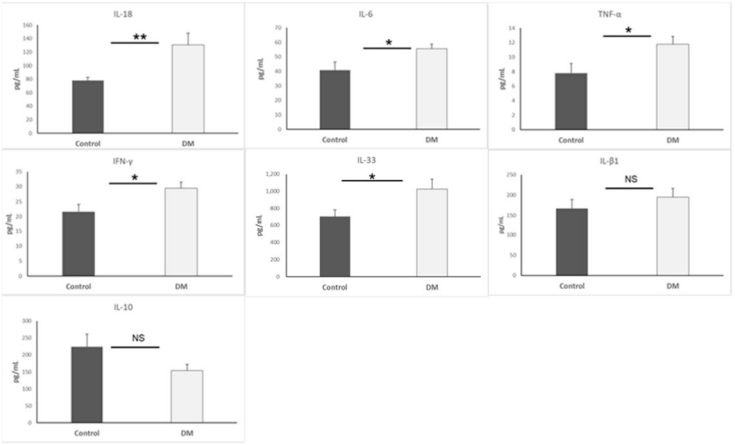
A component of extracellular trap formation is neutrophil elastase (NE). We employed Cl-amidine, one of the PADs inhibitors (PADI). *P < 0.05, ***P < 0.001, (A) Vehicle-Control, n=5; PADI-Control,

n=7, Vehicle-TIDM, n=5, PADI-T1DM, n=8 (B) Vehicle-Control, n=5; PADI-Control, n=7, Vehicle-TIDM, n=5, PADI-T1DM, n=7



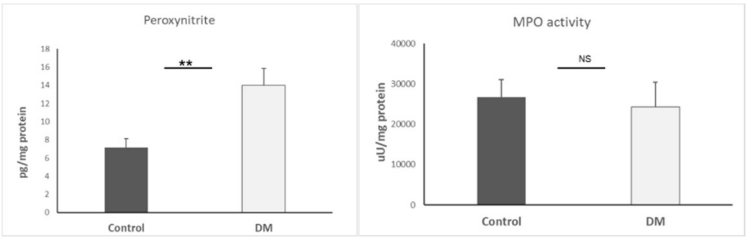
- **The Expression of Various Cytokines in the Heart**

In this study, cardiac cytokines were measured to map the inflammatory status in the heart. We obtained significant results (IL-6, IL-33, IFN-γ, and TNF-α: $p < 0.05$, IL-18:$p < 0.01$) for primary confirmation. The inflamed status of the heart was determined due to a high level of inflammatory cytokines in the STZ-DM group. Results are presented as mean + SEM, n = 5–9/group.



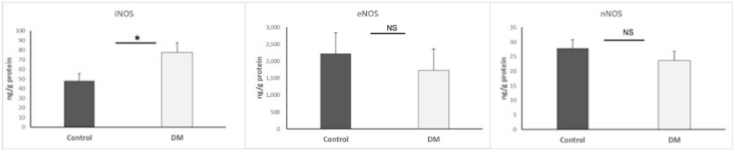
- **Basic ROS Examination in the Heart**

ROS is one of the main root causes of an EndMT microenvironment. We utilized Peroxynitrite (ONOO−) and MPO to estimate the ROS condition. The data only presented increased peroxynitrite ($p < 0.01$)) in STZ, suggesting a high ROS surrounding in the cardiac area. Results are presented as mean + SEM; n = 4–9/group.



- **Nitric Oxide Synthases (NOS) Determination in the Heart**

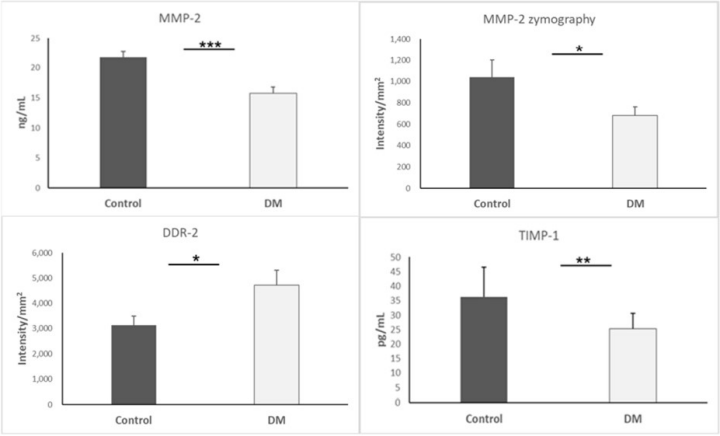
Here, we detected inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal ROS (nROS) in cardiac tissue. Our data showed that iNOS was only significantly higher in the STZ-DM group ($p < 0.05$). As a result, immune cells were likely to accumulate in the cardiovascular system. Results are presented as mean + SEM; n = 5–6/group.



- **The Assessment of EndMT by DDR-2, MMP-2, and TIMP-1 in the Heart**

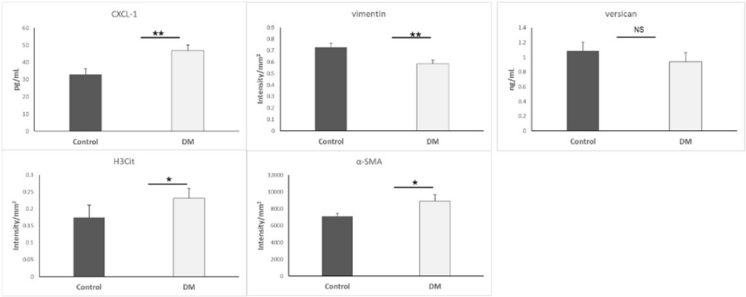
For the purpose of assessing EndMT, we observed DDR-2, MMP-2 activity, and TIMP-1. The underlying mechanism is that TIMP-1

is capable of inhibiting MMP-2, and DDR-2 can promote MMP-2-mediated proliferation. Interestingly, our data indicated that MMP-2 ($p < 0.001$) and TIMP-1 ($p < 0.01$) were decreased simultaneously in the hearts of the STZ-DM group. Nevertheless, DDR-2 was increased in the hearts of the STZ-DM group ($p < 0.05$). Results are presented as mean + SEM; n = 5–8/group.



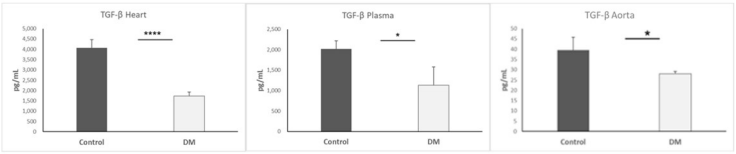
- **Biomarkers of Mesenchymal Cells and Neutrophils in the Heart**

Continuously, we measured citrullinated histone to confirm whether CXCL1 attracted neutrophils. Additionally, we measured some typical biomarkers (vimentin, versican, and α-SMA) to sense mesenchymal cell. Our data indicated that vimentin was lower ($p < 0.01$) but α-SMA was higher ($p < 0.05$) in the STZ-DM group. Additionally, the STZ-DM group presented high CXCL1 ($p < 0.01$) and H3Cit ($p < 0.05$) levels, proving that neutrophils were able to infiltrate the heart during EndMT. Results are presented as mean + SEM; n = 6–15/group.



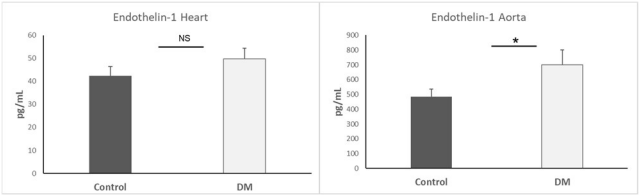
- **TGF-β Level of Heart, Aorta, and Plasma**

TGF-β is an important factor for EndMT and an upstream indicator to trigger EndMT. We continued to detect TGF-β expression since we obtained lower vimentin in the STZ-DM group. The TGF-β level in the heart of the STZ-DM group was exceedingly lower, as proven by statistical significance ($p < 0.0001$), as well as in the aorta ($p < 0.05$) and plasma ($p < 0.05$). Results are presented as mean + SEM; n = 4–8/group.



- **Endothelin-1 Expression in Heart and Aorta**

Endothelin-1 has been linked to the pathophysiology of other biological diseases, especially irregular EndMT. We also measured endothelin-1 expression in the heart and aorta. Our data only indicated that endothelin-1 expression in the aorta of the STZ-DM group was higher than the control group ($p < 0.05$). Results are presented as mean + SEM; n = 5–7/group.



List of publications linked to the thesis

MTMT identification: 10094398

- **Kang, H. L.,** Szász, A., Valkusz, Z., Várkonyi, T., Pósa, A., & Kupai, K. (2025). **Targeting PAD4: A Promising Strategy to Combat β-Cell Loss in Type 1 Diabetes.** *International Journal of Molecular Sciences*, *26*(13), 6113. **IF:4.9, D/Q rank: Q1**
- **Kang, H. L.,** Várkonyi, Á., Csonka, Á., Szász, A., Várkonyi, T., Pósa, A., & Kupai, K. (2025). **Endothelial–Mesenchymal Transition and Possible Role of Cytokines in Streptozotocin-Induced Diabetic Heart.** *Biomedicines*, *13*(5), 1148. **IF:3.9, D/Q rank: Q1**

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