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ORIGINAL RESEARCH **PERIOPERATIVE MEDICINE**

Potential role of S100A8/A9 and RNA-binding protein in microvascular complications of type2 diabetes mellitus

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ABSTRACT

Background & objective: Persistent hyperglycemia is the driving force for the progression of diabetic vascular complications and inflammatory response. S100A8 and S100A9 are small calcium-binding proteins involved in various cellular processes, including inflammation and immune responses. Tristetraprolin (TTP), alternatively known as zinc finger protein 36, acts as an RNA-binding molecule that has an important role in regulating the expression of messenger RNAs containing AU-rich elements. We aimed to address the involvement of inflammatory mediators like S100A8/A9 proteins and, RNA-binding proteins, in microvascular complications of type 2 diabetes mellitus (T2DM).

Methodology: The study was conducted from October 2022 to April 2023. We enrolled 200 subjects in this study involved in five equal groups: T2DM, diabetic nephropathy (DN), diabetic retinopathy (DR), diabetic neuropathy (DNR) and 40 normal healthy subjects as control group. CBC analysis was performed directly using the hematology analyzer CBC (Sysmex, Japan) technique. Serum S100 A8/A9 were measured by ELISA, and TTP gene expression was measured by RT-qPCR.

Results: The study's findings revealed a notable increase in neutrophil/lymphocytes ratio (NLR) and S100A8/A9 levels in patients groups compared to the healthy group ($P < 0.05$), while decreased TTP mRNA expression was observed in all patient groups compared to control ($P < 0.05$)

Conclusion: An increase in S1008A/9A levels with down regulation of anti-inflammatory binding protein (TTP) in patients suffering from type 2 diabetes mellitus with diabetic nephropathy, diabetic retinopathy, or diabetic neuropathy, suggests to be the therapeutic targets to regulate inflammatory response in type 2 diabetes mellitus and its complications.

Abbreviations: T2DM - Type 2 Diabetes Mellitus; DN - Diabetic Nephropathy; DR - Diabetic Retinopathy; DNR - Diabetic Neuropathy; NLR- Neutrophil/Lymphocyte Ratio; TTP - Tristetraprolin

Key words: Diabetes Mellitus; Inflammation; S100A8/A9; Inflammation; RNA-binding protein; T2DM; Neutrophil/Lymphocyte Ratio

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1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by insufficient insulin production, ineffective insulin use, or a combination of them. Hyperglycemia is the driving force for the progression of diabetic vascular complications and inflammatory response.[1] Diabetic nephropathy (DN), retinopathy (DR), and neuropathy (DNR) are the microvascular effects of hyperglycemia, in addition to macrovascular complication like cardiovascular disorders (CVD) including stroke, heart disease and chronic kidney disease (CKD).[2]

S100A8/A9, alternatively called calgranulins, form a heterodimer complex upon binding to calcium.[3] The interaction between S100A8 and S100A9 with calcium is vital for their structural stability and activation, enabling them to engage in various cellular processes, particularly those associated with inflammatory and immune responses.[4] Nevertheless, it is essential to acknowledge that the precise functions and roles of calprotectin in various cellular contexts may differ, and ongoing research is continuously exploring this dynamic area of study.[5] Calcium plays a pivotal role in maintaining vascular integrity and modulating endothelial function.[6] Endothelial dysfunction is a contributing factor to microvascular damage, compromised blood flow, and the development of retinal lesions.[7]

The increased expression of S100A8/A9 in micro complications is believed to have a role in their development through diverse mechanisms.[8] These mechanisms enhanced the pro-inflammatory responses, oxidative stress, activation of endothelial cells and fibroblasts, and the remodeling processes in the extracellular matrix. Elevated levels of S100A8/A9 can trigger the activation of various signaling pathways, including the Receptor for Advanced Glycation End products (RAGE) and Toll-like receptor 4 (TLR4). S100A8/A9 serves as a Ca^{++} sensor and is consistently expressed in neutrophils and monocytes. It fulfills vital functions in the reorganization of the metabolism of arachidonic acid.[9] During inflammation, S100A8/A9 is generated in an active state, playing a crucial role in governing the inflammatory reaction. Its function involves enhancing the recruitment of leukocytes and initiating the release of cytokines, thereby influencing the broader inflammatory pathway. S100A8/A9 represents a potential biomarker for diagnosing and monitoring inflammation-associated diseases. Additionally, it holds promise as a predictive indicator for assessing therapeutic responses to such conditions.[10]

During the initial phases of T2DM the innate immune system becomes activated, and as the condition progresses, the circulating levels of acute-phase inflammatory proteins increase, exacerbating the disease.[11] In obese and/or diabetic patients, prolong inflammation has been observed in various tissues, including fat tissue, liver, vascular endothelial cells, leukocytes, and pancreatic islets.[12,13] Persistent islet inflammation leads to a reduction in beta-cell mass, which is a defining characteristic of T2DM, primarily due to the promotion of beta-cell apoptosis.[14] The Neutrophil/Lymphocyte Ratio (NLR), indicates the equilibrium between pro-inflammatory and anti-inflammatory reactions.[15,16] An increased NLR suggests a predominance of neutrophils over lymphocytes, indicating a shift towards a pro-inflammatory state.[17]

Patients with T2DM, who have increased NLR levels also have elevated HbA1c and poor glycemic control. It can be used as a diabetic patient's follow-up tool for disease monitoring.[18] Furthermore, research has demonstrated a link between increased NLR and a susceptibility to cardiovascular incidents in T2DM.[19]

Tristetraprolin (TTP), alternatively known as zinc finger protein 36, acts as an RNA-binding molecule that has important role in regulating the expression of messenger RNAs containing AU-rich elements. These RNA molecules frequently encode proteins linked to processes involving inflammation, immune responses, and the regulation of metabolism.[20]

In micro complications of diabetes like retinopathy and neuropathy, decreased TTP levels may lead to reduced degradation of specific mRNA molecules involved in inflammatory processes or vascular homeostasis in the retina or kidney.[21] This, in turn, could contribute to increased inflammation and abnormal blood vessel growth, which are characteristic features of diabetic micro complication. Decreased TTP levels may affect mRNA targets involved in neural function, nerve regeneration, or the regulation of pain perception [22] In the context of DR, decreased TTP levels may lead to reduced degradation of specific mRNA molecules involved in inflammatory processes or vascular homeostasis in the retina.[23] This, in turn, could contribute to increased inflammation and abnormal blood vessel growth, which are characteristic features of DR.[24]

In the case of DNR, decreased TTP levels may affect mRNA targets involved in neural function, nerve regeneration, or the regulation of pain perception.[25] Altered expression of TTP and its target genes may impact the inflammatory response within nerves, nerve conduction, or neuronal survival.[26] The decrease in TTP levels in DN may have several implications. TTP is known to regulate the stability of mRNAs encoding pro-inflammatory cytokines and chemokines.[27]

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The study was aimed to identify the effect of S100A8/9 activation and TTP mRNA expression on the prognosis of T2DM in patients with microvascular complications.

2. METHODOLOGY

A total of 160 T2DM patients were enrolled between October 2022 and April 2023 at Murjan Teaching Hospital and Al-Qasim General Hospital and divided into four groups: T2DM group (n = 40), DN group (n = 40), DR group (n = 40) and DNR (n = 40). Additionally, a control group of 40 subjects, without any history of diabetes mellitus, endocrine disorders, metabolic renal diseases, acute illnesses, or infections was included who were visiting the hospital for routine check-ups.

All laboratory examinations were carried out at the Clinical Biochemistry Research Laboratory, situated in the College of Medicine at the University of Al-Qadisiyah. The calculation of body mass index (BMI) involved dividing the weight in kilograms by the square of the height in meters. Relevant basic details such as age, sex, and medical background were recorded. Before enrolling in the study, each participant granted written informed consent, and the research protocols gained endorsement from both the Ethics Committee of the College of Medicine.

A blood sample (5 mL) was collected from each participant, and 1 mL of the blood was promptly transferred to dipotassium-EDTA Vacutainer® tubes. And divided into two parts, each one (0.5 ml). The complete blood count (CBC) neutrophil/lymphocyte ratio was directly analyzed

using the hematology analyzer CBC (Sysmex, Japan), the other part of the blood (0.5 ml) was immediately put in EDTA tubes, and (0.5 ml) of TRIzol® was added. The tubes are then kept at -80 ºC to measure the expression of anti-inflammatory RNA binding protein TTP. RNA was extracted, cDNA synthesized by using the GoTaq®1-Step RT-qPCR (Promega, USA). Specific primers were used (forward: **TGGCAAAGGAGCAGATTAGTAGG**, reverse (**CTGCCACAAGAACTAGAGGATAAGA**). For the housekeeping gene, GAPDH was used. Other 4 ml of blood allowed to coagulate for 30 min at room temperature, followed by a centrifugation process at 1000 xg for (20 min) at 4 °C to separate the serum. The serum is then aliquoted using Eppendorf tubes (0.3 ml) and stored at (-80 °C) for biochemical analysis. Ca^{++} was measured by colorimetric method. S100 A8 and S100 A9 were analyzed by Sandwich ELISA, following the manufacturer's recommendations (Elabscience, China).

Statistical analysis

The date was expressed as means ± standard deviation (SD). SPSS version 23, a statistical software package typically used in the social sciences, was used for the statistical study. The Chi-square test was used to analyze differences between groups, and quantitative measurements were reported as numbers and percentages. The Andersen-Darling test was used to determine the data's normality. The Student's t-test was used to determine whether there were significant differences between the control and experimental groups. To compare significant differences among several groups, a one-way analysis of variance (ANOVA) was performed, followed by a post hoc analysis using Tukey's test. A level of $P < 0.05$ was considered statistically significant for all analyses.

3. RESULTS

The demographic variables of individuals with T2DM, DN, DR, DNR and healthy group were involved in Table 1. No significant changes were observed in the mean age

Table 1: The demographic characteristics of patients and control subject

Parameters	T2DM (n = 40)	DN (n = 40)	DR (n = 40)	DNR (n = 40)	Control (n = 40)	P-value
Age (y)	56 ± 1.3 (44-70)	55.67 ± 10.90 (45-67)	55.65 ± 9.28 (37-70)	54.75 ± 11.596 (36-71)	52.9 ± 1.2 39-65	0.402 NS
BMI (kg/m^2)	30.27 ± 3.12 (27.3 ± 34,51)	31.38 ± 5.33 (22-33.62)	31.34 ± 6.31 (22.89-41.02)	30.57 ± 5.575 (22.2 ± 42.43)	25.82 ± 2.51 (19.11-30.04)	0.251 NS
Gender male	19 (47.5)	14 (35.1)	18 (45)	15 (37.5)	19 (47.5)	0.23
female	21 (52.5)	26 (65)	22 (55)	25 (62.5)	21 (52.5)	NS
Family history of renal disease	10 (25)**	11 (27.5)**	14 (35.1)**	14 (35.1)**	0	< 0.01

NS: Not significant; Data presented as mean ± SD (Range) or n (%)

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Table 2: WBC, PCV, neutrophils counts, lymphocytes count and NLR

Parameters	T2DM (n = 40)	DN (n = 40)	DR (n = 40)	DNR (n = 40)	Control (n = 40)	P-value
WBC (x109/L)	15.54 ± 3.46* (10.32-23.1)	15.54 ± 3.46* (11.6-22.6)	12.2 ± 3.66* (5.9-20)	19.47 ± 7.133* (9.7-39.98)	5.4 ± 0.67 (4.6-6.3)	< 0.05
Neutrophils (x109/L)	13.26 ± 2.4* (5.32-17.34)	11.8 ± 2.3* (6.9-14.7)	6.07 ± 2.76* (2.68 ± 12.4)	14.6 ± 6.65 (9.9-35.9)	3.71 ± 0.96 (2.5-6.3)	< 0.0 5
Lymphocyte (x109/L)	2.51 ± 0.4 (0.6-1.8)	3.95 ± 0.87* (2.9-5.32)	2.21 ± 0.99 (2.9-4.2)	3.55 ± 1.22 (1.45-5.65)	2.18 ± 0.47 (1.34-3.12)	< 0.05
NLR	3.04 ± 0.43* (2.2-3.6)	3.02 ± 0.39* (2.56-3.96)	2.7 ± 0.83*` (1.4-5.3)	4.27 ± 1.82* (2.76-11.8)	1.7 ± 0.3 (1.32-2.98)	< 0.05

**P < 0.05; ** P < 0.01 NS: significant; Data presented as Mean ± SD and Range.*

of all patient groups compared to the control (P = 0.451). Also, non-significant changes in BMI were found between patient groups and control (P = 0.321) (Table 1).

Neutrophil / lymphocyte counts and NLR

The hematological characteristics between individuals with T2DM, DN, DNR, and the healthy group are summarized in Table 2. The neutrophils/ lymphocytes ratio (NLR) was calculated in the present study as a marker of inflammatory responses in patients. A significant increase was indicated in WBC and neutrophil counts in all patient groups compared to the control (P < 0.05). A significant increase was observed in lymphocytes counts in patients with DN (P < 0.05), while non-significant increase was observed in lymphocyte counts in other patient groups (T2DM, DR, and DNR) compared to control (P > 0.05, Table 2). Results indicate that NLR is significantly higher in all patient groups than control (P < 0.05).

Serum S100A8/9 levels

The activation of calcium-binding protein S100A8/A9 is involved in T2DM. The present results showed that levels of S100A8 were increased significantly in patients with DN and DNR compared to the control group (P < 0.05, Figure 1). High levels were observed in DN group. Non-significant increase was observed in S100A8 in patients with T2DM in DR and DR groups.

In line with S100A8, S100A9 was increased significantly in DR and DNR patient groups compared to the control (P < 0.05, Figure 2). Non-significant increase was observed in T2DM compared to control (P > 0.05, Figure 2).

Serum calcium levels

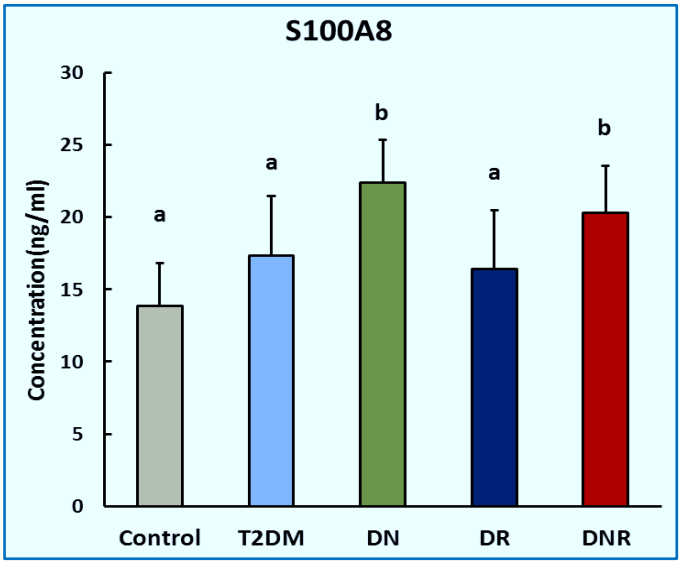

Figure 1: Serum S100A8 levels in patients with T2DM, DN, DR, DNR and control groups. Data are expressed as means ± SD. The same letters indicated non-significant changes, while different letters indicated a significant difference between groups (P < 0.05

Serum calcium levels were measured in this study. The present results showed that levels of Ca+2 significantly decreased in all patients compared to the control group(P > 0.05, figure 3).no significant decrease was observed in Ca+2 between patient groups.

Anti-inflammatory RNA-binding protein (Tristetraprolin) gene expression

It is well known that the activity of Tristetraprolin (TPP) is regulated during the inflammatory response. TPP gene expression was analyzed by RT-PCR(qPCR).

The results demonstrated a significant decrease in TTP mRNA expression in T2DM, DR and DNR groups compared to the control (P < 0.01, Figure 4). Non-significant decrease in TPP mRNA expression was

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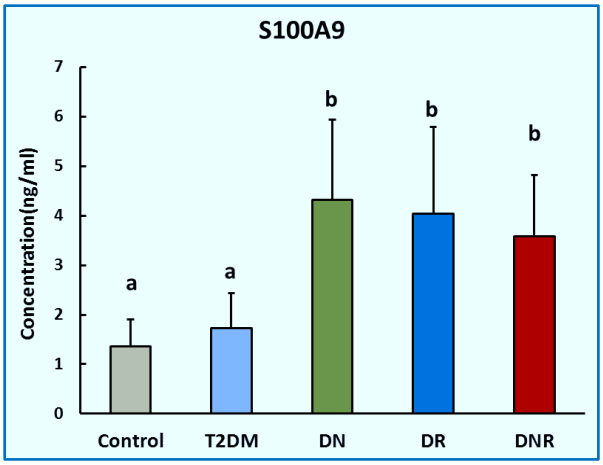

Figure 2: Serum S100A9 levels in patients with T2DM, DN, DR, DNR and control groups. Data are expressed as mean ± SD. The same letters indicated no significant changes, while different letters indicated a significant difference between groups (P < 0.05)

observed in patients with DN compared to control (P > 0.05, Figure 4)

Anti-inflammatory RNA-binding protein (Tristetraprolin) gene expression

It is well known that the activity of Tristetraprolin (TPP) is regulated during the inflammatory response. TPP gene expression was analyzed by RT-PCR (qPCR).

The results demonstrated a significant decrease in TTP mRNA expression in T2DM, DR and DNR groups compared to the control (P < 0.01, Figure 4). Non-significant decrease in TPP mRNA expression was observed in patients with DN compared to control (P > 0.05, Figure 4)

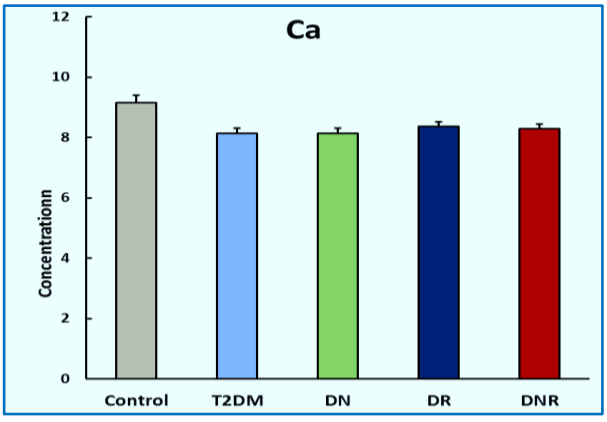

Figure 3: Serum C+2 levls in patients with T2DM, DN, DR, DNR and control groups. Data are expressed as means ± SD. The same letters indicated no significant changes, while different letters indicated a significant difference between groups (P < 0.05).

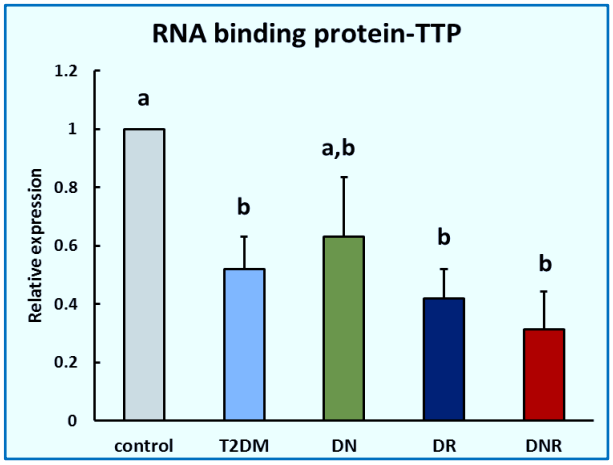

Figure 4: TPP expression by patient groups and control. RNA was extracted from blood; qPCR was used to determine RNA expression in different groups and normalized expression against endogenous controls. Gene expression is expressed as a fold change in ex

4. DISCUSSION

Studies have examined the link between inflammatory biomarkers, the onset and long-term effects of T2DM. DN, DR and DNR are all brought on by inflammation, whereas the toxic consequences of diabetes-related lipotoxicity bring on macrovascular conditions.[28] Non-significant changes were found in all patient groups compared to control. Age and gender may be linked to common risk factors, such as obesity, family history, physical inactivity, or unhealthy dietary habits, which all have a uniform impact across different age and gender groups.[29] In some cases, researchers may not find significant differences in diabetes-related complications or outcomes between different age and gender groups. This could indicate that factors influencing complications, such as glucose control, blood pressure, and lipid management, have a consistent effect across the population studied.[30]

According to this study, family history shows a significant increase in diabetic people in contrast to healthy people (P < 0.01). Family history is a significant risk factor for DM because many diabetic diseases have a genetic component. In some cases of diabetes, mutations in specific genes can lead to retina, nerve damage and contribute to kidney disease, obesity, hypertension.[31]

The results of our study show that levels of S100A8/A9 were increased significantly in DN and DNR, compared to healthy subject (P < 0.05). Many studies examined the association between S100A8/A9 levels and microvascular complications in T2DM. The results indicated that higher circulating levels of S100A8/A9

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were linked to elevated likelihood of microvascular complications, including nephropathy and retinopathy.[32] Also it was suggest that an increase in S100A8/A9 levels in individuals with T2DM, may contribute to chronic inflammation, insulin resistance, and the development of microvascular complications.[33] In DR, the retina may have chronic low-grade inflammation. The presence of inflammatory mediators like S100A8/A9 could result from the activation of immune cells and the release of these proteins as part of the immune response to retinal damage.[34] S100A8/A9 is a protein that binds to calcium and functions as a damage-associated molecular pattern. It can activate either Toll-like receptor (TLR)-4 or the RAGE. While the activation of these receptors is involved in the advancement of renal fibrosis, the specific role of S100A8/A9 in this process remains unclear.[35] Mechanically, high S100A8/A9 expression in tubular epithelial cells during diabetes activates some type of TLR signal pathway which promotes the epithelial to mesenchymal transition - EMT process and finally leads to diabetic nephropathy.[36]

The absence of S100A8/A9 conferred protection to tubular epithelial cells against apoptosis and key epithelial-mesenchymal transition stages induced by unilateral ureteral obstruction (UUO). In vitro investigations unveiled a fresh role for S100A8/A9 as a mediator of epithelial cell damage, characterized by disruptions in cell polarity, cell cycle arrest, and subsequent cell demise.[36] The inhibition of S100A8/A9 presents a potential therapeutic avenue for impeding renal fibrosis in individuals grappling with chronic kidney disease.[35]

Our observations reveal that S100A8/A9 predominantly resides within the cytoplasmic fraction of neutrophils and is not a constituent of the granule content.[37] Moreover, scientists have isolated S100A8/A9 from the cytoplasmic portion of neutrophils and ascertained that it could trigger neutrophil activation, leading to inflammation in organs such as nerves, kidneys, or the retina.[38]

Calcium has a crucial effect in vascular integrity and regulating endothelial function. Endothelial dysfunction contributes to microvascular damage, impaired blood flow, and the formation of retinal lesions.[39] Ca^{++} levels were significantly decreased in all patients compared to the control group ($P < 0.05$) Calcium homeostasis is vital for renal function, and disturbances in calcium levels may contribute to diabetic nephropathy.[40] Decreased calcium levels have been linked to renal dysfunction, including glomerular injury, inflammation, and fibrosis.[41] The relationship between calcium levels and DN is complex and not fully understood. Calcium plays a role in nerve conduction and neurotransmitter release, and disturbances in calcium homeostasis may contribute to nerve damage and dysfunction.[42]

It well known that the activity of tristetraprolin (TPP) is regulated during the inflammatory response; therefore, to indicate whether the TTP contributes to the regulation of inflammatory events in T2DM and its microvascular complication,[43] TPP gene expression was assessed by RT-PCR(qPCR). The results demonstrated a significant down regulation in the expression of TTP in T2DM, DR and DNR groups compared to the control ($P \leq 0.01$). TTP is a protein involved in post-transcriptional regulation of gene expression, particularly in the degradation of mRNA molecules containing AU-rich elements (AREs). These mRNA molecules often encode proteins related to inflammation, immune responses, and metabolic regulation.[44] In the context of DR, decreased TTP levels may lead to reduced degradation of specific mRNA molecules involved in inflammatory processes or vascular homeostasis in the retina. This, in turn, could contribute to increased inflammation and abnormal blood vessel growth, which are characteristic features of diabetic retinopathy.[45]

In the case of DNR, decreased TTP levels may affect mRNA targets involved in neural function, nerve regeneration, or the regulation of pain perception. Altered expression of TTP and its target genes may impact the inflammatory response within nerves, nerve conduction, or neuronal survival.[46] Among patients with diabetic kidney disease (DKD), a significant reduction in TTP expression was observed within glomerulus podocytes. This decrease correlated with podocyte injury, and elevated levels of interleukin (IL)-17.[47] The decrease in TTP levels in DN may have several implications. TTP is known to regulate the stability of mRNAs encoding pro-inflammatory cytokines and chemokines. With decreased TTP, there might be an accumulation of these mRNA molecules, leading to increased expression of pro-inflammatory factors within the kidney.

5. CONCLUSION

The dysregulation of inflammatory mediators like S1008A/9A and RNA-binding protein TTP could contribute to the development and progression of chronic inflammation in microvascular complications of Type 2 diabetes mellitus that can promote tissue damage. This suggests to consider S100A8/A9 and TPP as vital targets to regulate inflammatory response in Type 2 diabetes mellitus and its complications.

6. Data availability

The numerical data generated during this research is available with the authors.

7. Acknowledgement

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8. Conflict of interest

The authors declare no conflict of interest.

10. Authors' contribution

All authors tool equal part in the conduct of this study and preparation of the manuscript.

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