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RESEARCH ARTICLE

Study on the Relationship between TGF-β1, MMP-9 and Diabetic Retinopathy in Diabetic Rat Model

Yan Liu[1*], Guozhi Zhang[2], Xiao Zhang[3], Xiaofei Li[4] and Yiyue Wang[4]

[1]North China University of Science and Technology Affiliated Hospital, Hebei Province, Tangshan, 063000, China
[2]School of Pharmacy, North China University of Science and Technology, Hebei Province, Tangshan, 063000, China
[3]School of Medical, North China University of Science and Technology, Hebei Province, Tangshan, 063000, China
[4]College of Life Science, North China University of Science and Technology, Hebei Province, Tangshan, 063000, China
*Corresponding author: littleangel_keke@163.com

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A B S T R A C T

Diabetic Retinopathy is a common complication of diabetes, stems from intricate molecular interactions. It is characterized by progressive damage to the microvasculature of the retina, and ultimately leads to vision impairment and blindness. A total of 48 female Sprague-Dawley (SD) rats were divided into four groups as follows: the Normal Control (NC) group (n=12), the High-Fat High-Sugar (HFHS) group (n=12), the HFHS + Streptozotocin (STZ) group (n=12), and the HFHS + Metformin (MR) group (n=12). The TGF-β1 and MMP-9 expressions in lens epithelial cells (LECs) were assessed at two-time points post-modeling (week 1 and week 2). We extracted the tissue sections, mounted using coverslips and observed under the microscope to investigate the follicle of the tear duct associated lymphoid tissue (TALT). Statistical analyses, including correlation tests, were performed using GraphPad software. Our results showed that the MMP-9 expression significantly increased from week 1 to week 2 in the HFHS+STZ, HFHS+MR, and HFHS groups ($p<0.001$), indicative of potential kidney complications related to diabetes. Conversely, the control group (NC) maintained stable MMP-9 levels. Regarding TGF-β1, the HFHS+STZ group exhibited a significant increase from week 1 to week 2 ($p<0.001$), while the NC group remained stable ($p<0.001$). Similar trends were observed in the HFHS+MR and HFHS groups, indicating rising TGF-β1 levels over time. Immunostaining of TALT showed brown B-cell aggregates and TALT lymphocytes in the Harderian glands. In conclusion, our findings highlight the complex interplay between TGF-β1 and MMP-9 in diabetic retinopathy.

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INTRODUCTION

Diabetic Retinopathy (DR), is one of the most prevalent and incapacitating complications arising from diabetes mellitus, a chronic metabolic disorder (Ansari *et al*., 2022). DR is characterized by progressive damage to the microvasculature of the retina, and ultimately leads to vision impairment and blindness. As researchers strive to comprehend the intricate mechanisms underlying DR, the interplay between various molecular factors gains significance such as Transforming Growth Factor-beta (TGF-β1) and Matrix Metalloproteinase-9 (MMP-9) (Roy *et al*., 2021; Nebbioso *et al*., 2022).

The lens contains two main types of cells: epithelial and fiber cells. In its early stages, a single layer of lens epithelial cells (LECs) covers the front half of the lens (Griep and Zhang, 2004; Kumar *et al*., 2019). As the eye develops, LECs close to the lens equator change into fiber cells due to varying growth stimulant levels. Lens growth and differentiation continue into adulthood, although at a slower rate than during embryonic stages, especially near the equator (Liu *et al*., 2022). However, certain clinical scenarios such as ocular injuries, surgeries, or systemic conditions like atopic dermatitis and retinitis pigmentosa can trigger proliferation and multilayer formation of anterior LECs. In some cases, these cells undergo epithelial-to-mesenchymal transition (EMT), evolving into myofibroblasts (Fong *et al*., 2003; Simó-Servat *et al*., 2019).

Retinal illness involves complex interactions between vascular and neurodegenerative processes, significantly affecting sensory perception and quality of life (Sivak and Fini 2002; Kaya *et al*., 2021). Key molecules in

understanding the intricate pathogenesis of diabetic retinopathy are transforming growth factor-beta (TGF-β1) and matrix metalloproteinase-9 (MMP-9) (Stitt *et al.* 2016). TGF-β1, a multifunctional cytokine, exerts potent regulatory influence over cell proliferation, differentiation, apoptosis, and extracellular matrix synthesis. Its involvement in wound healing, inflammation, and tissue fibrosis is well-documented, yet its exact implications in DR are not fully elucidated (Xue *et al.*, 2020). Concurrently, MMP-9, an enzyme belonging to the matrix metalloproteinase family, plays a crucial role in extracellular matrix remodeling and degradation. The disruption of this equilibrium has been implicated in various pathological conditions, including ocular diseases (Mondal *et al.*, 2020; Ucgun *et al.*, 2020). In DR, the potential synergy between TGF-β1 and MMP-9 holds promise as a contributing factor to disease progression and analysis.

The diabetic rat model, owing to its similarities to human and most of the other animal's diabetic physiology and retinal architecture, offers a valuable platform for investigating the molecular pathways involved in DR development. This model enables researchers to replicate hyperglycemia-induced retinal changes akin to those observed in diabetic patients (Wang-Fischer and Garyantes, 2018). Olivares *et al.* (2017) found that retinas of diabetic rats show notably higher TGF-β1 level than non-diabetic controls. This correlation between elevated TGF-β1 and increased retinal thickness suggests TGF-β1 's involvement in retinal edema, a hallmark of early DR. TGF-β1 has also been tied to promoting fibrosis in diabetic retinopathy. Heng *et al.* (2013) showed that TGF-β1 - driven fibrosis thickens the basement membrane, compromising the blood-retinal barrier. This compromised barrier facilitates the entry of inflammatory proteins and cells into the retina, leading to retinal damage.

MacIsaac *et al.* (2014) demonstrated that inhibiting TGF-β1 signaling with a specific inhibitor reduced retinal inflammation and angiogenesis in a diabetic rat model. These findings imply that curtailing TGF-β1 signaling could be a promising treatment strategy for mitigating the pathological changes induced by diabetic retinopathy among patients.

Kerkvliet *et al.* (2003) noted heightened MMP-9 levels in the retinas of those with proliferative diabetic retinopathy, suggesting its potential role in neovascularization. MMP-9 has also been implicated in breaching the blood-retinal barrier. Lei *et al.* (2002) demonstrated that increased MMP-9 activity in diabetic rat retinas correlates with basement membrane breakdown and heightened vascular permeability. This disturbance enables inflammatory cells to infiltrate, worsening retinal damage. In a diabetic retinopathy model, MMP-9 knockout mice displayed diminished retinal vascular leakage and reduced neovascularization. These findings indicate that addressing MMP-9 activity might have treatment implications for preserving retinal integrity in diabetic retinopathy.

The connection between TGF-β1 and MMP-9 in diabetic retinopathy is intricate. As shown by Iturriaga-Goyon *et al.* (2021), TGF signaling stimulates MMP-9 production and activation, contributing to vascular changes and ECM remodeling. Additionally, TGF-β1 -induced fibrosis may prompt other cell types to release MMP-9, intensifying tissue damage.

The main objective of our study was to analyze the relationship between TGF-β1, MMP-9 and Diabetic Retinopathy in diabetic rat model.

MATERIALS AND METHODS

Ethical Approval: This study was approved by the Ethics Committee of Hebei University of Technology with approval number (Lx: Hb20220903).

Animals: The Institution Medical Laboratory Animal Centre (Nantong city, Hebei province, China) provided 48 female Sprague-Dawley (SD) rats. Rats were fed LAD0011 feed from Nantong Teluofei Feed, China. The animals' length, age, and weight were measured and recorded. They were kept in a controlled environment with an indoor temperature of 22.5 ± 2°C and a humidity level of between 40 to 60% under fluorescent illumination for 12 hours a day. The animals had full access to food and water during the experiment.

Reagents: The following main reagents were utilized: rabbit anti-rat TGF-β1 polyclonal antibody and rabbit anti-rat MMP-9 polyclonal antibody sourced from Sigma-Aldrich (MO, USA), streptozotocin from Sigma-Aldrich (MO, USA) for the induction of diabetic retinopathy, as well as the DAB developing kit and SP immunohistochemistry kit obtained from Bio SB (CA, USA) for immunohistochemical staining.

DR Model: A total of 48 rats were initially divided into four groups as follows: the Normal Control (NC) group (n=12), the High-Fat High-Sugar (HFHS) group (n=12), the HFHS + Streptozotocin (STZ) group (n=12), and the HFHS + Metformin (MR) group (n=12).

Rats in the Normal Control (NC) group were fed a standard diet whose total energy was 3.5 kcal/g (Fat: 15%, Protein: 20% and Carbohydrates: 65%). Rats in the HFHS group were fed a diet consisting of 60% basic feed, 20% sucrose, 10% cooked lard, 5% cholesterol, and 5% cholat. The total energy obtained from the HFHS diet was 4.5 kcal/g, with 40% derived from fat, 15% from protein, and 45% from carbohydrates.

Rats in the HFHS+STZ group were also fed the HFHS diet as described above. Additionally, they received a single intraperitoneal injection of 2% streptozotocin (55 mg/kg). Rats in the HFHS+MR group were fed the same HFHS diet as described above. In addition to the diet, they were administered metformin orally at a dose of 150 mg/kg of body weight once daily. The metformin was mixed with their food to ensure consistent intake. Blood samples from the rats' tail veins were collected after a three-day break to measure blood glucose levels (Olivares *et al.* 2017). After the induction, a slit-lamp inspection of the control group's lenses indicated they were transparent. However, the HFHS+STZ, HFHS+MR, and HFHS group's lenses had altered due to flocculent turbidity, and vacuoles.

Tissue collection: At week 1 and week 2, 6 rats from each group were euthanized using intraperitoneal injections of pentobarbital solution (50 mg/kg), respectively. The eyeballs were promptly extracted, and the lenses were isolated. The isolated lenses were immediately preserved in

a neutral formalin solution with a 10% volume fraction, facilitating the preparation of tissue slices for subsequent analysis. The expression of TGF-1 and MMP-9 within LECs was examined using immunohistochemistry.

Isolation of tear duct associated lymphoid tissue (TALT): The rat's head was carefully positioned to expose the tear duct situated near the eyes. Fine scissors and a pair of forceps were used to gently dissect the tear duct-associated tissue from its neighboring structures. The utmost care was taken to minimize any potential contamination from surrounding tissues. After successful isolation, it was transferred to a beaker filled with a neutral formalin solution at a 10% volume fraction. After which, immunostaining was performed by fixing the tissue in 4% paraformaldehyde for 24 hours, followed by dehydration and paraffin embedding. Sections were then cut at a thickness of 5μm and mounted on slides. The sections were deparaffinized, rehydrated, and treated with hydrogen peroxide to block endogenous peroxidase activity. The rat anti-B-cell marker antibody, was applied at a working concentration of 1:200. After overnight incubation, sections were treated with a secondary biotinylated antibody, followed by an avidin-biotin peroxidase complex. The reaction was visualized using 3,3'-diaminobenzidine (DAB) substrate, resulting in a brown staining of B-cell aggregates associated with the lacrimal duct.

In histological examination, the sections were deparaffinized and rehydrated through a series of graded ethanol solutions (99%). Haematoxylin solution was prepared using a 0.5% haematoxylin dye solution in distilled water. The sections were immersed in the haematoxylin solution for 5 minutes to stain cell nuclei. After washing with distilled water, the sections were differentiated in 1% acid alcohol for 20 seconds until the desired shade of blue was achieved. This was followed by a thorough wash with running tap water for several minutes to remove excess stain. Eosin Y solution was prepared as a 1% solution in distilled water. The sections were stained in the eosin solution for 2 minutes to provide contrast to the nuclei staining. The sections were then dehydrated through a series of graded ethanol solutions, followed by clearing with xylene. Finally, the sections were mounted with a coverslip using a mounting medium and observed under the microscope to investigate the follicle of the TALT.

Quantitative Expression of TGF-β1 and MMP-9: After the completion of immunohistochemical staining for TGF-β1 and MMP-9, the prepared tissue sections containing lens samples were carefully positioned beneath a Leica DM1000 microscope equipped with a Leica DFC7000T digital camera (Sigma Aldrich, United States). High-resolution images of the stained sections were captured using a 20x objective lens, providing a resolution of 1380 x 1035 pixels. The images were then saved in uncompressed TIFF format.

The captured TIFF images were subsequently transferred and imported into the Image Proplus 7.0 image analysis software. Within the software, the colour threshold was set to detect staining in the brown colour range corresponding to the TGF-β1 and MMP-9 markers. A threshold value of 50-200 grayscale units was applied to measure the staining intensity. An area filter of 20-500 pixels was employed to focus the analysis on stained regions of appropriate size. The Image Proplus software employed thresholding techniques to distinguish positively stained areas (indicative of TGF-β1 expression or MMP-9 expression levels) from the background and other structures. The automated analysis generated quantitative data, expressed in picograms per millilitre (pg/ml), encompassing the mean staining intensity of TGF-β1 and MMP-9.

Data analysis: The statistical analysis used GraphPad Prism Version 9.5.4 (New York, USA). The data obtained were presented as mean ± standard deviation. Independent samples t-test was performed to compare the baseline features of age, length and blood glucose between the study and control groups. For intra-group comparisons at distinct time points, repeated measures analysis of variance was employed, complemented by the Least Significant Difference test to compare the levels of TGF-β1 and MMP-9 at week 1 and 2. Pearson correlation analysis was employed to assess relationships between variables. In all analyses, statistical significance was determined at a threshold of $p<0.05$.

RESULTS

Baseline characteristics for the normal control, HFHS+STZ, HFHS+MR, and HFHS groups: The rats had an average age of 8.3 ± 0.40 weeks and weight of 205.6 ± 2.5g. The consequent data of the age and weight of the animals in the four categories showed no statistical difference ($p<0.05$) (Fig. 1 and 2).

According to Fig. 3 and Table 1, there were no statistically significant differences in blood glucose across all groups ($p>0.05$). HFHS+STZ group had the highest mean blood glucose level, while the Normal Control group had the lowest.

Quantitative expression of TGF: The HFHS+STZ group showed a significant increase in TGF-β1 expression from week 1 (Mean: 0.052 pg/ml) to week 2 (Mean: 0.095 pg/ml) ($p<0.001$). In contrast, the NC group did not show a significant change in TGF-β1 expression from week 1 (Mean: 0.025 pg/ml) to week 2 (Mean: 0.031 pg/ml). The difference between these two time points was not statistically significant ($p<0.001$), suggesting that TGF-β1 expression in the control group remained relatively stable over time. Similar to the HFHS+STZ group, the HFHS+MR group also exhibited a significant increase in TGF-β1 expression from week 1 (Mean: 0.048 pg/ml) to week 2 (Mean: 0.092 pg/ml) ($p<0.001$). The HFHS group also showed a significant increase in TGF-β1 expression from week 1 (Mean: 0.035 pg/ml) to week 2 (Mean: 0.07 pg/ml) ($p<0.001$), suggesting that TGF-β1 expression in the HFHS group significantly increased over time (Fig. 4 and Table 2).

Quantitative expression of MMP-9: In the HFHS+STZ group, the mean expression of MMP-9 at week 1 was 0.084 pg/ml. This indicates a baseline level of MMP-9 expression. However, by week 2, there was a significant increase to 0.167 pg/ml ($p < 0.001$). In contrast, the control group (NC) had low and stable MMP-9 expression levels.

Table 1: Blood glucose levels in different treatment groups.

Glucose Conc. (mg/ml)	Baseline	Normal Control	HFHS	HFHS + STZ	HFHS + MR
M (SD)	13.88 (2.5)	14.17 (1.9)	14.58 (2.9)	14.99 (1.97)	14.21 (1.3)

Note: M (SD) = Mean and Standard Deviation.

Table 2: Expression levels of TGF- β1 in different treatment group.

TGF- β1 (pg/ml)	Baseline	Normal Control	HFHS	HFHS + STZ	HFHS + MR
Week 1, M (SD)	0.006	0.025 (0.002)	0.035 (0.005)	0.052 (0.005)	0.048 (0.007)
Week 2, M (SD)	0.009	0.031 (0.006)	0.07 (0.007)	0.095 (0.01)	0.092 (0.008)

Note: M (SD) = Mean and Standard Deviation.

Table 3: Expression of MMP-9 in different treatment groups.

MMP-9 (pg/ml)	Baseline	Normal Control	HFHS	HFHS + STZ	HFHS + MR
Week 1, M (SD)	0.005	0.01 (0.0001)	0.062 (0.006)	0.084 (0.008)	0.071 (0.005)
Week 2, M (SD)	0.008	0.009 (0.0001)	0.1 (0.009)	0.167 (0.016)	0.14 (0.01)

Note: M (SD) = Mean and Standard Deviation.

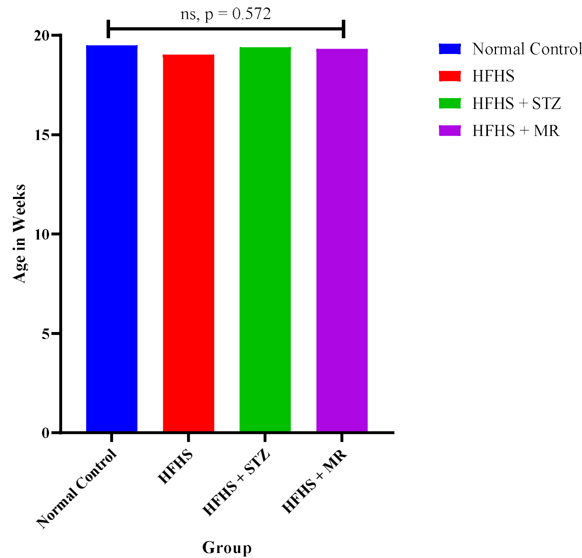

Fig. 1: The differences in age in weeks among various treatment groups.

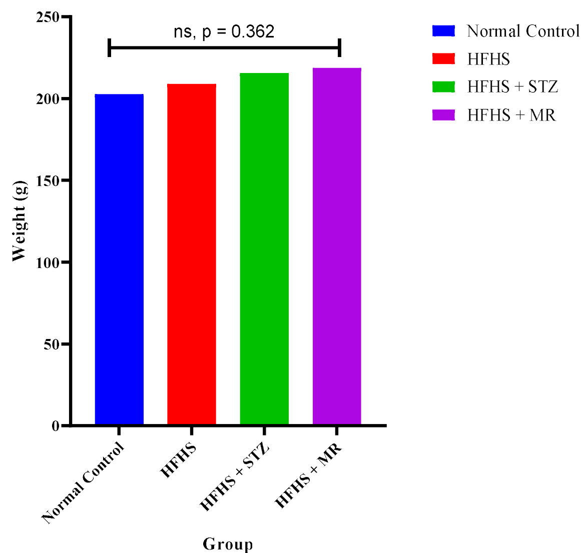

Fig. 2: The differences in weight among various treatment groups.

At week 1, the mean was 0.01 pg/ml, and it remained stable at 0.009 pg/ml by week 2. Importantly, there was no statistically significant difference between these two time points (p<0.001). The HFHS+MR group showed a mean MMP - 9 expression of 0.071 pg/ml at week 1,

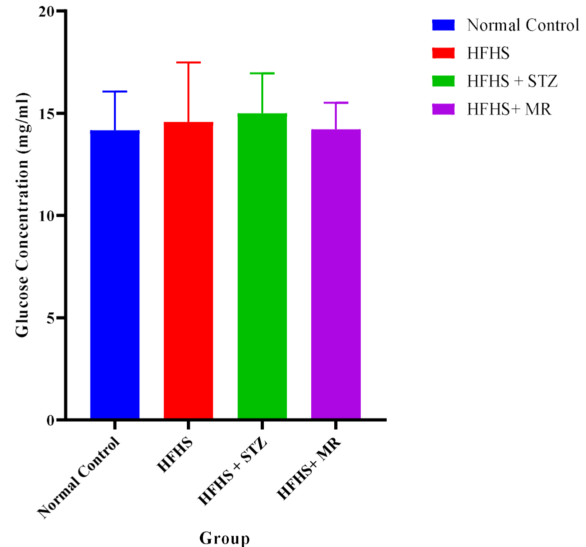

Fig. 3: The differences in blood glucose concentration among various treatment groups.

indicating a baseline level. However, by week 2, there was a significant increase to 0.14 pg/ml (p < 0.001). In the HFHS group, the mean MMP-9 expression at week 1 was 0.062 pg/ml, indicating a baseline level. By week 2, there was a significant increase to 0.1 pg/ml (p<0.001) (Fig. 5 and Table 3).

The correlation analysis of MM-9 and TGF- β1 as expressed in DR Rats: The study found a substantial correlation between the expression levels of these two important components, with a positive correlation coefficient of r=0.862 in the LECs of DR rats (Fig. 6). According to this finding, there is a corresponding increase in the expression of MMP-9 within LECs when TGF-1 expression increases in the HFHS + STZ group.

The examination of our data revealed a statistically significant correlation between the expression of TGF-β1 and MMP-9 within Lens Epithelial Cells (LECs) of DR rats in the HFHS + STZ group.

Isolation of tear duct associated lymphoid tissue (TALT): According to Fig. 7, the position of the TALT tissue was identified using a light microscope before being isolated from the bone. In section A, we revealed the ductal structure consisting of dilated ends to the bone and on the eye side. Evan's blue dye in the ductal structure confirmed

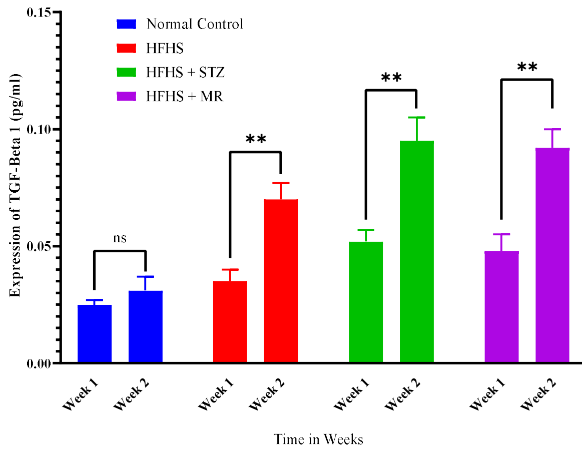

Fig. 4: A comparison of TGF- β1 quantitative expression across various treatment groups.

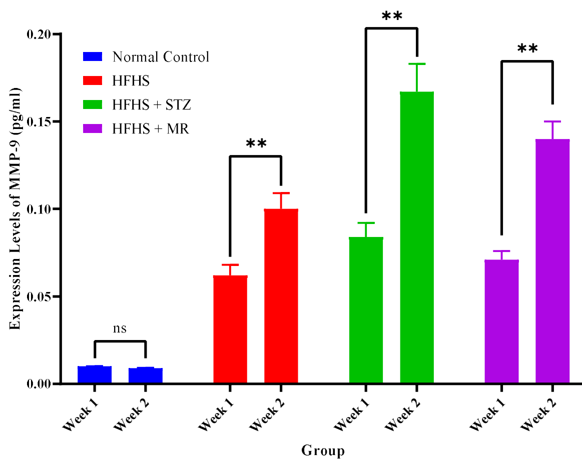

Fig 5: The differences in expression levels of MMP-9 in various treatment groups at week 1 and 2.

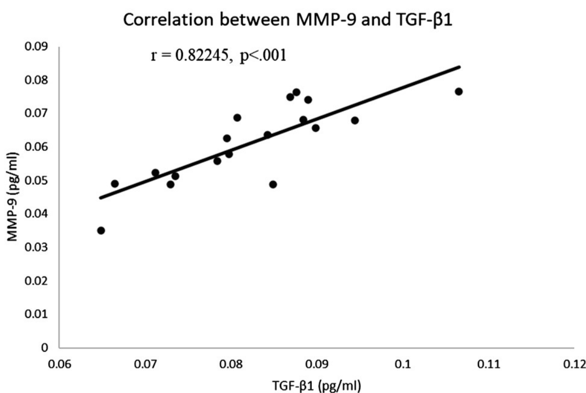

Fig. 6: Pearson's correlation between the expression levels of TGF-β1 and MMP-9 in the HFHS + STZ group.

the presence of a nasolacrimal duct. The nasolacrimal duct connects the nasal cavity to the lacrimal canaliculi. In section B, the immunostaining showed brown B-cell aggregates associated with the lacrimal duct; in sections C and D, the follicle of the TALT was covered with a squamous epithelium of double layers. In sections E and F, H & E staining revealed aggregation of TALT lymphocytes in the Harderian glands.

DISCUSSION

Our study revealed a strong positive correlation between TGF-β1 and MMP-9 expression in the LECs of DR rats. In the HFHS+STZ group, MMP-9 expression started at 0.084 pg/ml in week 1, denoting a baseline level, but surged significantly to 0.167 pg/ml by week 2 ($p<0.001$), signifying a substantial increase possibly associated with diabetes-related kidney complications. Conversely, the control group (NC) maintained consistently low MMP-9 levels at both week 1 (0.01 pg/ml) and week 2 (0.009 pg/ml), with no significant difference between the two time points ($p<0.001$), indicating stability without evident complications. In the HFHS+MR group, week 1 showed MMP-9 expression at 0.071 pg/ml, and it escalated significantly to 0.14 pg/ml by week 2 ($p<0.001$), implying a rise potentially linked to dietary factors and metformin treatment. Similarly, the HFHS group exhibited an increase in MMP-9 expression, starting at 0.062 pg/ml in week 1 and rising significantly to 0.1 pg/ml by week 2 ($p<0.001$), suggesting an increase attributable to the high-fat high-sugar diet.

Regarding TGF-β1, The HFHS+STZ group displayed a significant TGF-β1 expression increase from week 1 (Mean: 0.052 pg/ml) to week 2 (Mean: 0.095 pg/ml) ($p<0.001$), while the NC group remained stable (week 1 Mean: 0.025 pg/ml; week 2 Mean: 0.031 pg/ml) with no significant difference ($p<0.001$). Similarly, the HFHS+MR group showed a notable TGF-β1 increase from week 1 (Mean: 0.048 pg/ml) to week 2 (Mean: 0.092 pg/ml) ($p<0.001$), mirroring the HFHS group (week 1 Mean: 0.035 pg/ml; week 2 Mean: 0.07 pg/ml), indicating a significant TGF-β1 rise over time.

The observed positive correlation between TGF-β1 and MMP-9 expressions within the LECs of rats with DR holds valuable insights for potential therapeutic interventions. For instance, modulating TGF-β1 levels or its downstream signaling, lead to changes in MMP-9 expression, subsequently affecting extracellular matrix remodeling and angiogenesis. These findings were consistent with Winkler *et al.* (2020), who suggested that the correlation implies that controlling one of these factors helps regulate the other. Therefore, the development of therapeutic strategies aimed at controlling both TGF-β1 and MMP-9 are required to mitigate the detrimental effects of DR.

Findings were consistent with Li *et al.* (2018), who postulated that TGF-β1's involvement in DR lies in its ability to promote fibrosis and alters the extracellular matrix. Fibrosis, driven by TGF-β1-induced myofibroblast activation, contributes to thickening the basement membrane of retinal blood vessels (Li *et al.*, 2018). Moreover, they found that these thickening compromises the blood-retinal barrier, allowing inflammatory proteins and cells to infiltrate the retina and exacerbate damage. Additionally, Li *et al.* (2018) suggested that TGF-β1-induced fibrosis encourages other cell types to secrete MMP-9, intensifying the degradation of ECM components, including the basement membrane. In contrast, Abudukeyoumu *et al.* (2020) postulated that MMP-9 has an extensive role in ECM remodeling and angiogenesis. In DR, elevated MMP-9 expression is associated with increased breakdown of the basement membrane and

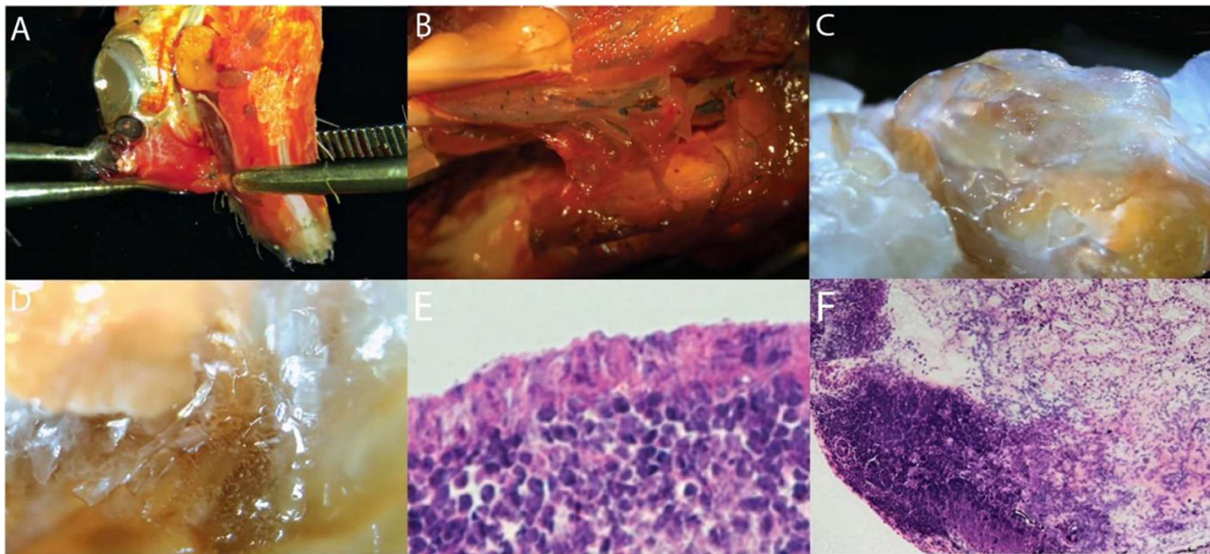

Fig. 7: Isolation of TALT from nasolacrimal duct in the HFHS + STZ group.

elevated vascular permeability. This enhanced vascular permeability allows inflammatory cells to enter the retina, further contributing to retinal damage.

We suggest that the positive correlation suggests that TGF-β1's influence on MMP-9 contributes to these processes. TGF-β1's signaling pathways activate pathways that increase MMP-9 expression. Consequently, targeting TGF-β1 signaling indirectly impacts MMP-9 expression, potentially mitigating the disruption of the blood-retinal barrier, inflammation, and angiogenesis observed in DR.

This study established that MMP-9 expression increased from week 1 to week 2 within the HFHS+STZ, HFHS+MR, and HFHS groups, while remaining consistently low in the control group. The observed increase in MMP-9 expression within the HFHS+STZ, HFHS+MR, and HFHS groups suggests a temporal dimension to their involvement in DR. These findings were consistent with Kowluru *et al*. (2012), who showed that the increase over time reflects a heightened response to ongoing pathological processes within the retina. Furthermore, they suggested that MMP-9, known for its involvement in ECM remodeling and angiogenesis, responds to the evolving microenvironment in DR, where vascular changes and tissue remodeling are prominent.

The increase in MMP-9 aligns with specific stages of DR progression known for intensified pathological processes such as inflammation. These findings were consistent with Abu El-Asrar *et al*. (2018), who proposed that the rise in MMP-9 expression corresponds to increased inflammatory responses within the diabetic retina. Similarly, some study suggested that MMP-9, with its capability to degrade extracellular matrix components and modulate immune cell infiltration, contributes to the amplification of inflammation in DR. Furthermore, the structural changes due to inflammation, vascular alterations, and tissue damage lead to the structural disarray observed in the diabetic retina.

Khomiak and Kaczmarek (2018) showed that MMP-9 is involved in modulating immune cell infiltration which is consist with the findings of the present study. These findings were replicated by Liu *et al*. (2022), who proposed that during inflammation, immune cells such as macrophages and neutrophils, infiltrate the diabetic retina, contributing to tissue damage. The findings of Hannocks *et al*. (2019) showed that MMP-9's capability to degrade ECM creates paths and facilitates the migration of immune cells into retinal tissues which propagates the inflammatory response within the diabetic retina, the influx of immune cells contributes to the amplification of inflammation observed in DR.

We suggest that the heightened expression of MMP-9 within the HFHS+STZ, HFHS+MR, and HFHS groups offer insights into its potential role in driving the vascular changes characteristic of proliferative features of DR, which includes neovascularization and abnormal permeability of existing vessels. These findings were consistent with Ucgun *et al*. (2020), who suggested that MMP-9's capability to influence angiogenesis, is significant in proliferative DR. Similarly, Zhou *et al*. (2021) showed that neovascularization contributes to the progression of DR. Furthermore, MMP-9's involvement in the degradation of extracellular matrix components provides a conducive environment for growth of new vessels. The increased MMP-9 expression contributes to the disruption of the ECM architecture, enabling the sprouting and growth of new blood vessels in the retina (Zhou *et al*., 2021).

Moreover, Ucgun *et al*. (2020) suggested that MMP-9's influence on ECM remodeling affects the integrity of existing blood vessels. In proliferative DR, abnormal vascular permeability contributes to retinal edema and hemorrhages. MMP-9's ability to modify the ECM composition disrupts the structural integrity of vessel walls, leading to increased permeability. This could facilitate the leakage of fluids and proteins, contributing to retinal edema and further compromising retinal function (Ucgun *et al.,* 2020).

Similarly, our study was consistent with Jayashree *et al*. (2018), who postulated that the increase in MMP-9 expression contributes to exacerbating tissue damage, a significant consequence of advanced DR. The role of MMP-9 in degrading the basement membrane leads to the

disruption of the blood-retinal barrier and facilitates the infiltration of inflammatory cells, ultimately worsening tissue damage and retinal dysfunction (Jayashree *et al*., 2018). The basement membrane is a critical component of the blood-retinal barrier, maintaining the separation between the bloodstream and the retina (Gurler *et al*., 2023). It serves as a protective barrier, preventing the uncontrolled passage of substances from the blood vessels into the retinal tissues (Gurler *et al*., 2023). In DR, particularly in its advanced stages, the integrity of the blood-retinal barrier becomes compromised, leading to increased vascular permeability and retinal edema.

Our findings are similar to Mondal *et al*. (2020) who postulated that MMP-9 is an enzyme with the ability to break down various ECM components, including structural proteins like collagen and the basement membrane. The degradation weakens the structural integrity of the basement membrane, creating gaps or breaches in the barrier that normally prevents the entry of inflammatory cells and other blood-borne substances into the retina (Mondal *et al*., 2020). This infiltration of inflammatory cells is a key contributor to the worsening of tissue damage in advanced DR. Inflammatory cells release cytokines and reactive oxygen species, which lead to further damage of retinal cells and exacerbating the inflammatory response. This cascade of events leads to increased retinal dysfunction, vision impairment, and other complications associated with DR.

Our findings showed significantly higher TGF-β1 expression levels in the HFHS+STZ, HFHS+MR, and HFHS groups compared to the control group. These findings were consistent with previous studies (Vallée and Lecarpentier, 2019; Peng *et al*., 2022), who observed that the observed increase in TGF-β1 expression correlates with its induction of fibrotic processes within the retina. Siani *et al*. (2023), postulated that fibrosis is characterized by the excessive deposition of extracellular matrix components, and is a significant factor in the structural alterations of the diabetic retina. Similarly, Resnikoff *et al*. (2022) showed that the role of TGF-β1 in fibrosis is derived from its ability to stimulate the transformation of fibroblasts into myofibroblasts. In DR, sustained high levels of TGF-β1 trigger the activation of myofibroblasts within the retinal tissue. These myofibroblasts then produce excessive amounts of ECM components, leading to fibrosis (Resnikoff *et al*., 2022).

Zhang *et al*. (2018) observed that TGF-β1 plays a pivotal role in promoting angiogenesis, the formation of new blood vessels. In DR, the retina experiences inadequate oxygen supply due to vascular changes. Similarly, some study postulated that TGF-β1 can stimulate the expression of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). Increased VEGF levels induce the growth of new blood vessels in an attempt to restore oxygen delivery. However, this neovascularization is often abnormal, leading to fragile and leaky vessels that contribute to retinal edema, hemorrhages, and further retinal damage. Bonfiglio *et al*. (2020) showed that TGF-β1 is involved in complex inflammatory responses. However, it has anti-inflammatory effects in certain contexts, it can also promote inflammation under pathological conditions. TGF-β1 stimulates immune cell activation and migration, particularly macrophages and neutrophils. While in certain scenarios, this might be a protective response, in DR, the infiltration of activated immune cells into the retinal tissue can exacerbate inflammation. These immune cells release pro-inflammatory cytokines and reactive oxygen species, contributing to tissue damage and dysfunction. The above viewpoint is consistent with some of the results of our experiment.

In DR, TGF-β1 can disrupt the balance of cytokines, which are signaling molecules that play a crucial role in immune responses. Similarly, Beaulieu Leclerc *et al*. (2018) showed that in DR, TGF-β1 might lead to an imbalance favouring pro-inflammatory cytokines. This shift in cytokine profile amplifies the inflammatory response within the diabetic retina, contributing to the sustained inflammatory environment. TGF-β1's effects on endothelial cells lining blood vessels can also contribute to inflammation. In DR, TGF-β1 can promote endothelial dysfunction, which includes increased expression of adhesion molecules and altered vascular permeability (Beaulieu Leclerc *et al*., 2018). This can facilitate the entry of immune cells and pro-inflammatory molecules into the retina, further increasing the inflammatory cascade.

We propose that the isolation of TALT improves the understanding of potential interactions between ocular immune responses and the pathogenesis of DR. Similarly, Oya *et al*. (2021) postulated that the ductal structure of TALT serves as a conduit connecting the nasal cavity to the lacrimal canaliculi, suggesting a potential route for immune cells and molecules to travel between the ocular and nasal environments. Immunostaining highlighted the presence of brown B-cell aggregates associated with the lacrimal duct. This observation suggests the presence of immune cell clusters, particularly B-cells, which play a crucial role in adaptive immune responses (Tanaka and Baba 2020). These findings were consistent with Tanaka and Baba (2020), who observed that such immune cell aggregates near the lacrimal duct and influence the local immune environment and modulate responses to DR.

Zimmermann *et al*. (2019) also postulated that B-cells consists of B-cell receptors (BCRs) that enable B-cells to recognize specific antigens. In the presence of an antigen that corresponds to the BCR, B-cells become activated (Tanaka and Baba 2020). In DR, antigens associated with retinal damage, inflammation, or abnormalities trigger B-cell activation in the vicinity of the lacrimal duct (Kang and Yang, 2020). Once activated, B-cells differentiate into plasma cells, which are specialized cells responsible for producing antibodies (Zimmermann *et al*., 2019). These antibodies are specific to the antigens that triggered B-cell activation. In DR, the B-cell aggregates near the lacrimal duct might be producing antibodies that recognize components related to retinal damage or inflammation. Our findings were similar to Obasanmi *et al*. (2020), who showed that antibodies produced by B-cells could potentially influence the progression of DR by interacting with components related to inflammation, angiogenesis, or tissue damage within the retina (Uccelli *et al*., 2019). Depending on the specific antigens recognized by the B-cell aggregates, their presence could either exacerbate or attenuate the pathological processes characteristic of DR.

Limitations: Our study primarily focused on animal models of DR, which may not perfectly replicate the human disease. Additionally, the study mainly examined the expression of TGF-β1 and MMP-9, and while their correlations were established, other factors contributing to diabetic retinopathy's complex pathogenesis were not comprehensively explored. The sample size of the HFHS+STZ, HFHS+MR, and HFHS groups might impact the generalizability of the findings. Furthermore, the isolated Tear Duct Associated Lymphoid Tissue (TALT) was studied mainly in an anatomical context, and its functional role in the context of DR remained speculative. Lastly, the study did not investigate potential therapeutic interventions based on the identified correlations. Overall, while the study provides valuable insights, these limitations should be considered in interpreting and extrapolating the findings.

Conclusion: Our study analysed the intricate relationship between TGF-β1, MMP-9, and DR. The positive correlation observed between TGF-β1 and MMP-9 expression in the LECs of DR rats suggests their interconnected roles in the disease's pathogenesis. The elevation of MMP-9 and TGF- β1 expression over time in the HFHS+STZ, HFHS+MR, and HFHS groups highlights their potential contributions to inflammation, vascular alterations, and tissue damage as DR progresses. Additionally, the identification of TALT underscores the complex ocular immune responses. Despite limitations, this study enhances our understanding of key molecular mechanisms involved in DR, presenting avenues for future research and therapeutic interventions.

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REFERENCES

Abu El-Asrar AM, Ahmad A and Bittoun E, *et al.*, 2018. Differential expression and localization of human tissue inhibitors of metalloproteinases in proliferative diabetic retinopathy. Acta Ophthal 96:27-37.

Abudukeyoumu A, Li MQ and Xie F, 2020. Transforming growth factor-β1 in intrauterine adhesion. American J Reprod Immunol 84:13262.

Ansari P, Tabasumma N and Snigdha NN, *et al.*, 2022. Diabetic retinopathy: an overview on mechanisms, pathophysiology and pharmacotherapy. Diabetology 3:159-75.

Beaulieu Leclerc V, Roy O and Santerre K, *et al.*, 2018. TGF-β1 promotes cell barrier function upon maturation of corneal endothelial cells. Sci Reports 8:4438.

Bonfiglio V, Platania CBM, Lazzara F, *et al.*, 2020. TGF-β serum levels in diabetic retinopathy patients and the role of anti-VEGF therapy. Inter J Mol Sci 21:9558.

Fong DS, Aiello L and Gardner TW, *et al.*, 2003. Diabetic retinopathy. Diabetes care 26: 99-102.

Griep AE and Zhang P, 2004. Lens cell proliferation: the cell cycle. Dev Ocular Lens 191-213.

Gurler G, Belder N and Beker MC, *et al.*, 2023. Reduced folate carrier 1 is present in retinal microvessels and crucial for the inner blood retinal barrier integrity. Fluids Barr CNS 20:1-19.

Hannocks MJ, Zhang X and Gerwien H, *et al.*, 2019. The gelatinases, MMP-2 and MMP-9, as fine tuners of neuroinflammatory processes. Matrix Biol 75:102-13.

Heng LZ, Comyn O, Peto T, *et al.*, 2013. Diabetic retinopathy: pathogenesis, clinical grading, management and future. Diabetic Medicine 30:640-50

Iturriaga-Goyon E, Buentello-Volante B, Magaña-Guerrero FS, *et al.*, 2021. Future perspectives of therapeutic, diagnostic and prognostic aptamers in eye pathological angiogenesis. Cells 10:1455.

Jayashree K, Yasir M, Senthilkumar GP, *et al.*, 2018. Circulating matrix modulators (MMP-9 and TIMP-1) and their association with severity of diabetic retinopathy. Diabetes & Metabolic Syndrome: Clin Res Rev 12:869-73.

Kang Q and Yang C, 2020. Oxidative stress and diabetic retinopathy: Molecular mechanisms, pathogenetic role and therapeutic implications. Redox Biol 37:101799.

Kaya SG, Inanc-Surer S, Cakan-Akdogan G, *et al.*, 2021. Roles of matrix metalloproteinases in the cornea: A special focus on macular corneal dystrophy. Med Drug Discov 11:100095.

Kerkvliet EH, Jansen ID, Schoenmaker TA, *et al.*, 2003. Low molecular weight inhibitors of matrix metalloproteinases can enhance the expression of matrix metalloproteinase-2 (gelatinase A) without inhibiting its activation. Cancer: Interdiscip Intern J ACS 97:1582-88.

Khomiak D and Kaczmarek L, 2018. Matrix metalloproteinase 9 and epileptogenesis-the crucial role of the enzyme and strategies to prevent the disease development. Postepy Biochem 64:222-30.

Kowluru RA, Zhong Q and Santos JM, 2012. Matrix metalloproteinases in diabetic retinopathy: potential role of MMP-9. Expert Opinion Invest Drugs 21:797-805.

Kumar B, Chandler HL, Plageman T, *et al.*, 2019. Lens stretching modulates lens epithelial cell proliferation via YAP regulation. Invest Ophthal Visual Sci 60:3920-29.

Lei TC, Vieira WD and Hearing VJ, 2002. In vitro migration of melanoblasts requires matrix metalloproteinase-2: implications to vitiligo therapy by photochemotherapy. Pigment Cell Res 15:426-32.

Li Q, Pang L, Yang W, *et al.*, 2018. Long non-coding RNA of myocardial infarction associated transcript (LncRNA-MIAT) promotes diabetic retinopathy by upregulating transforming growth factor-β1 (TGF-β1) signaling. Medical science monitor: Inter J Exp Clinical Res 24:9497.

Liu Y, Bai Q, Yong W, *et al.*, 2022. EMMPRIN promotes the expression of MMP-9 and exacerbates neurological dysfunction in a mouse model of intracerebral hemorrhage. Neurochem Res 47:2383-95.

Liu Z, Huang S, Zheng Y, *et al.*, 2022. The lens epithelium as a major determinant in the development, maintenance, and regeneration of the crystalline lens. Prog Retinal Eye Res 101112.

MacIsaac RJ, Ekinci EI and Jerums G, 2014. Markers of and risk factors for the development and progression of diabetic kidney disease. American J Kidney Dis 63: 39-62.

Mondal S, Adhikari N, Banerjee S, *et al.*, 2020. Matrix metalloproteinase-9 (MMP-9) and its inhibitors in cancer: A minireview. European J Med Chem 194:112260.

Nebbioso M, Lambiase A, Armentano M, *et al.*, 2022. Diabetic retinopathy, oxidative stress and sirtuins: an in depth look in enzymatic patterns and new therapeutic horizons. Survey Ophthal 67:168-83.

Obasanmi G, Lois N, Armstrong D, *et al.*, 2020. Circulating leukocyte alterations and the development/progression of diabetic retinopathy in type 1 diabetic patients-a pilot study. Current Eye Res 45:1144-54.

Olivares AM, Althoff K, Chen GF, *et al.*, 2017. Animal models of diabetic retinopathy. Curr Diab Rep 17:1-17.

Oya Y, Kimura S, Nakamura Y, *et al.*, 2021. Characterization of M cells in tear duct-associated lymphoid tissue of mice: A potential role in immunosurveillance on the ocular surface. Front Immunol 12:779709.

Peng Y, Liao K, Tan F, *et al.*, 2022. Suppression of EZH2 inhibits TGF-β1-induced EMT in human retinal pigment epithelial cells. Exp Eye Res 222:109158.

Resnikoff HA, Miller CG and Schwarzbauer JE, 2022. Implications of fibrotic extracellular matrix in diabetic retinopathy. Exp Biol Med 247:1093-1102.

Roy D, Modi A, Khokhar M, *et al*., 2021. Microrna 21 emerging role in diabetic complications: a critical update. Curr Diab Rev 17:122-35.

Siani A, Infante-Teixeira L, d'Arcy R, *et al*., 2023. Polysulfide nanoparticles inhibit fibroblast-to-myofibroblast transition via extracellular ROS scavenging and have potential anti-fibrotic properties. Biomat Adv 153:213537.

Simó-Servat O, Hernández C and Simó R, 2019. Diabetic retinopathy in the context of patients with diabetes. Ophthal Res 62:211-7.

Sivak JM and Fini ME, 2002. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. Prog Retinal Eye Res 21:1-14.

Stitt AW, Curtis TM, Chen M, *et al*., 2016. The progress in understanding and treatment of diabetic retinopathy. Prog Retinal Eye Res 51:156-86.

Tanaka S and Baba Y, 2020. B Cell Receptor Signaling. In: Wang, JY. (eds) B Cells in Immunity and Tolerance. Advances in Experimental Medicine and Biology, vol 1254. Springer, Singapore. https://doi.org/10.1007/978-981-15-3532-1_2

Uccelli A, Wolff T, Valente P, *et al*., 2019. Vascular endothelial growth factor biology for regenerative angiogenesis. Swiss Med Weekly 149:20011

Ucgun NI, Zeki-Fikret C and Yildirim Z, 2020. Inflammation and diabetic retinopathy. Mol Vision 26:718.

Vallée A and Lecarpentier Y, 2019. TGF-β in fibrosis by acting as a conductor for contractile properties of myofibroblasts. Cell Biosci 9:1-15.

Wang-Fischer Y and Garyantes T, 2018. Improving the reliability and utility of streptozotocin-induced rat diabetic model. J Diab Res 2018:1-14

Winkler J, Abisoye-Ogunniyan A, Metcalf KJ, *et al*., 2020. Concepts of extracellular matrix remodeling in tumour progression and metastasis. Nat Commun 11:5120.

Xue VW, Chung JYF, Córdoba CAG, *et al*., 2020. Transforming growth factor-β: a multifunctional regulator of cancer immunity. Cancers 12:3099.

Zhang D, Qin H, Leng Y, *et al*., 2018. LncRNA MEG3 overexpression inhibits the development of diabetic retinopathy by regulating TGF-β1 and VEGF. Exp Therap Med 16:2337-42.

Zhou L, Li FF and Wang SM, 2021. Circ-ITCH restrains the expression of MMP-2, MMP-9 and TNF-α in diabetic retinopathy by inhibiting miR-22. Exp Mol Pathol 118:104594.

Zimmermann M, Rose N, Lindner JM, *et al*., 2019. Antigen extraction and B cell activation enable identification of rare membrane antigen specific human B cells. Front Immunol 10:829.