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J Vet Res 66, 629-635, 2022
DOI:10.2478/jvetres-2022-0053

Proteomic analysis of tear film in canine diabetic patients with and without retinopathy

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Received: June 2, 2022 *Accepted: October 24, 2022*

Abstract

Introduction: Diabetic retinopathy (DR) is the leading cause of blindness in human and animal patients. Early detection and treatment of the disease are important and can be facilitated by proteomic approaches providing biomarkers. **Material and Methods:** Tear films were collected on Schirmer strips from 32 canine patients (12 diabetic dogs without changes in the retina, 8 diabetic dogs with signs of DR, and 12 control dogs). Two-dimensional electrophoresis was used to separate tear film proteins prior to their identification with matrix-assisted laser desorption/ionisation–tandem time-of-flight mass spectrometry and interrogation of protein function databases to find matches. **Results:** Five significantly differentially expressed proteins were identified; of those, one was downregulated (2′-5′-oligoadenylate synthase 3) and four were upregulated in the tear film of two diabetic groups (Ras-related protein RAB-13; aldo-keto-reductase family 1 member C3; 28S ribosomal protein S31, mitochondrial; and 60S ribosomal protein L5). The differentially expressed proteins identified in the tear film were involved in signalling pathways associated with impaired protein clearance, persistent inflammation and oxidative stress. **Conclusion:** The results of our study confirm that the pathological process in the retina in the course of diabetes mellitus causes changes in the tear film proteome.

Keywords: proteomics, tear film, protein, animal model, diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a common disease in dogs. The most common form of diabetes in dogs resembles type 1 diabetes in humans (19). The canine model seems to be appropriate for research on a wide diversity of issues relevant to human disease, and is so in the case of DM; especially noteworthy is the resemblance of complications, both ocular and systemic, following the natural course of DM in dogs and humans (6).

A common early lesion of diabetic retinopathy (DR) in human patients and in animal models is retinal capillary basement membrane thickening, which occurs in animal models within six months of the induction of diabetes. This hallmark pathology of diabetic microvascular disease is used in the evaluation of drug-mediated modulation of DR in a range of animal models. Moreover, results from published clinical practice have shown that more than 22% of dogs with naturally contracted DM develop ophthalmoscopic signs of retinal haemorrhages or microaneurysms in a relatively short time from the onset of the disease (1–4 years).

The tear film is a thin fluid layer that overspreads the surface of the eye. It contains thousands of molecules including a large variety of proteins with different functions. In-depth analysis of its composition is a source of information useful in the diagnosis, prognosis and treatment of not only ocular disorders but also systemic disease in humans (8, 14, 15, 23). Proteomics has the ability to identify, characterise, and quantify the proteins and peptides in the tear film on a large scale in a single analysis. Some of the proteins and peptides thus identified may serve as new biomarkers for biomedical and veterinary research, corroborating the importance proteomic analysis has assumed as a research tool (5, 9, 25, 26, 28, 29, 31).

Currently, there are few publications in the field of proteomics concerning the canine tear film (4, 9, 10, 27, 29). In our previous study we identified 125 proteins in

the tear film of healthy dogs (30), and in another study we identified 9 differentiating tear proteins in diabetic dogs (28). In the current research, the intention was to deepen knowledge of the tear film protein composition, or proteome, in canine patients with DR, with the aim of identifying proteins that would be specific for the disease. The objective of this study was to analyse the protein content of the tear film in dogs with naturally contracted DM with and without clinical signs of retinopathy, and to compare that content with the content of a control group. An attempt was made to determine whether the pathological process in the retina can result in tear film proteome changes. The research results may be of diagnostic or therapeutic value and contribute to a better understanding of the pathophysiological mechanisms underlying this disease. This investigation may also facilitate the development of a non-invasive method to detect potential protein candidates for DR biomarkers in tear film.

Material and Methods

Study animals and study design. All the dogs were consecutively enrolled through the four-year period 2017–2021 in the Innovative Centre for Animal Pathology and Therapy in the Faculty of Veterinary Medicine in Lublin, Poland. All samples were obtained during standard veterinary diagnostic procedures as previously described (1). The experiments were conducted in conformance with European Union Directive 2010/63/EU. The dogs' owners were informed about the methods and purpose of the study and gave their written informed consent. The study involved 32 mixed-breed dogs (18 females and 14 males), weighing 5.4–38 kg (median 17.3 kg) and aged 7–16 years (median 10.8 years). The dogs were selected on the basis of anamnesis and clinical status, including a detailed ophthalmological investigation with a slit lamp, optical coherence tomography (OCT) of the retina, biochemical blood serum testing, haematological examination and urinalysis. On the basis of these examinations and analytical tests results, three groups of dogs were selected: Group 1 comprised 12 dogs with no ocular pathology as a control group, showing no other clinical symptoms of the disease and with laboratory parameters within physiological ranges; Group 2 included 12 diabetic dogs without changes in the retina; and Group 3 was 8 diabetic dogs with signs of DR. Exclusion criteria were any ocular pathology, pancreatitis, adrenal gland hyperactivity, purulent inflammation of the uterus, or bacterial infections of the urinary tract.

Diabetes mellitus was diagnosed by finding persistent marked hyperglycaemia (plasma glucose >200 mg/dL; 11 mmol/L) and glucosuria in dogs with clinical signs consistent with the disease (polyuria, polydipsia and weight loss). Diabetic retinopathy was determined by first capturing and then grading standard 7-field fundus images. The images were taken with a Handy NM200D retinal fundus camera (Nidek, Gamagori, Japan). The anterior and posterior segments of the eye were examined by OCT using a 3D OCT 2000 optical tomograph (Topcon, Tokyo, Japan). The results were assessed by two independent ophthalmologists.

Urinalysis and haematological and biochemical analyses of blood were performed. Voided midstream urine samples were collected in sterile tubes in the morning. The urine analysis and urine sediment analysis were performed within 30 min of collection. Blood was collected in tubes with and tubes without ethylenediaminetetraacetic acid from the cephalic vein from animals in the sitting position using a vacuum system. The blood was subjected to a full haematological test (Exigo EOS analyser; Boule, Spånga, Sweden) and centrifuged at 3,000 rpm. The obtained sera were examined in a biochemical analyser (BS-130; Mindray, Shenzhen, China). The chemistry panel included alanine transferase, aspartate aminotransferase, total bilirubin, urea, creatinine, alkaline phosphatase, glucose, albumin, total protein, amylase, lipase and glutamyltransferase. The concentration of the serum cortisol was also determined using an enzyme-amplified chemiluminescent assay (Immulite 1000 analyser; Siemens Healthineers, Erlangen, Germany). For diabetic dogs, glycaemic control was estimated using serum fructosamine concentration. The results of the haematological and biochemical blood analysis are presented in Supplementary Table 1.

Proteomic analysis. For the collection of tear film, Schirmer strips (TearFlo; HUB Pharmaceuticals, North Liberty, IA, USA) were placed without anaesthesia into the lower sacs of both eyes at 1/3 of the distance of the eyelid from the lateral canthus. Each collection was carried out between 7 and 9 a.m. using sterile gloves. After the strips had been in place for 5 min, they were removed and transferred to a 1.5 mL Eppendorf tube without any buffer. The strips were then immediately frozen at −80°C and stored until the extraction step. After thawing, extraction of proteins in urea buffer was performed for 3 h at 4°C with the addition of a protease inhibitor cocktail (catalogue number P8340; Sigma-Aldrich, St Louis, MO, USA). The strips were removed from the solution, and the extracts were centrifuged at 5,000 rpm for 10 min at 4°C. The obtained supernatants were collected and stored at −80°C until they were further analysed.

Concentration of the proteins was determined by measuring absorbance at 280 nm (MaestroNano Spectrophotometer; Maestrogen, Hsinchu City, Taiwan). A tear fluid sample containing 150 µg of proteins was placed in a 1.5 mL test tube and water was added to give a volume of 100 µL. To improve the results of electrophoresis and for quantitative protein precipitation, a ReadyPrep 2-D cleanup kit was applied (Bio-Rad, Hercules, CA, USA). The protein pellets were dissolved in 300 µL of the rehydration buffer (ReadyPrep 2-D Starter Kit Rehydration; Bio-Rad), and the obtained solutions were then applied directly to

immobilised pH gradient strips (17 cm, pH 3–10; Bio-Rad) for 12 h in-gel rehydration.

Isoelectric focusing was performed in an IEF100 device at 60 kVh with a maximum current of 50 µA per strip (Hoefer, Holliston, MA, USA). The strips with focused proteins were subsequently equalised in 15-min-duration steps in two buffers: the first containing dithiothreitol (2%) and the second containing iodoacetamide (2.5%). Two-dimensional electrophoresis was performed using a 12.5% polyacrylamide gel. The separation took place in a PROTEAN II xi Cell (Bio-Rad) filled with Tris/glycine sodium dodecyl sulphate running buffer. The obtained gels were silver stained and digitalised using an Imagescanner III (GE Healthcare, Boston, MA, USA). The images of the gels were visually and statistically analysed in Delta2D software (DECODON, Greifswald, Germany), which facilitated the quantification of spots and creation of protein expression profiles. This program warps images of gels to each other (correcting positional spot variations and matching images), producing a fused image. This fused image is a proteome map containing every protein spot obtained on every gel during the whole experiment. After this image was produced, spots were detected. The ratio of the mean group values of the relative spot volumes was calculated in a manner where the volume of a given spot in the control group is the denominator of the ratio parameter (Rt > 1.5 means overexpression, Rt < 0.67 means suppression). False-negative and false-positive protein spots were determined manually. Normalised volumes were analysed using a one-way analysis of variance test. The P-value in each test was <0.05.

The spots chosen for further identification were cut out from their gels, put into microtubes, rinsed with water and destained. They were subsequently reduced and alkylated using dithiothreitol and iodoacetamide solutions. The gel fragments were covered with trypsin. After 20 min, 50 mM ammonium bicarbonate solution was added to the gel spots, and the prepared tubes with gel spots were transferred to an autoclave set at 37°C for at least 8 h of digestion. After digestion, peptides were obtained by three-step extraction in an ultrasonic bath with a solution composed of acetonitrile : water : trifluoroacetic acid; v/v/v 50/45/5). The peptide extracts were then concentrated in a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA) and peptide pellets were created. The pellets were dissolved in 10 µL of trifluoroacetic acid and purified with ZipTip C18 pipette tips (Merck, Darmstadt, Germany) according to the standard procedure.

Differentially expressed proteins were identified with an Ultraflex III matrix-assisted laser desorption/ ionisation–tandem time-of-flight mass spectrometry (MALDI TOF/TOF-MS) device (Bruker, Bremen, Germany) as previously described (1). The obtained results were analysed with the BioTools 3.2 bionformatics application (Bruker, Bremen, Germany). The created lists were then compared by Mascot 2.2 (Matrix Science, London, UK) using the UniProtKB/Swiss-Prot database. To further evaluate the five differentiating proteins, the Panther program (http://www.pantherdb.org) was used to assign them to the appropriate biological process.

Results

All the diabetic dogs met the clinical criteria for DM: fasting blood glucose level ≥11 mmol/L (200 mg/dL) and glucosuria. The study groups are described in outline in Table 1.

In this study, we found 489 common tear film protein spots from the diabetic groups and control group. Of these proteins, 11 showed statistically significant different expression levels ($P \leq 0.05$), and so they were excised from the electrophoretic gel. Of these 11 tear film proteins, 5 were positively identified by MALDI-TOF/TOF-MS. Table 2 describes the proteins that were positively identified with their names, genes, and UniProt database accession numbers. Four of the five proteins were downregulated in both diabetic groups, and the fifth protein was upregulated (Table 2, Fig. 1).

Table 1. Clinical characteristics of the study subjects

Variable	Healthy subject n = 12	With diabetic retinopathy n = 8	Diabetic without retinopathy n = 12
Age, Y			
Mean (±SD)	10.8 ± 1.8	10.3 ± 2.8	11.4 ± 2.1
Range	8–13	7–16	8–12
Sex			
Male	7	3	4
Female	5	5	8
Weight, kg			
Mean (±SD)	23.1 ± 7.7	13.9 ± 8.6	14.4 ± 5.3
Range	10–37	5.4–38	6.3–21
Duration of diabetes, y			
Mean (±SD)	0	1.2 ± 0.6	2.4 ± 0.2
Duration	0	0.5–2	1–4
Blood glucose, mmol/L			
Mean (±SD)	5.9 ± 0.2	17.8 ± 6	18.3 ± 2.3
Range	5.6–6.2	8.3–29.7	9.3–22.3
Cataract	0	0	0

Fig. 1 shows representative two-dimensional electrophoresis gel spots of significantly ($P \leq 0.05$) differentially expressed proteins in the control group *versus* the two diabetic groups. Fig. 2 shows a fused image of the condensed electrophoretic gels from the whole experiment. The downregulated protein was 2′-5′-oligoadenylate synthase 3 and the upregulated proteins were Ras-related protein RAB-13; aldo-keto reductase family 1 member C3; 28S ribosomal protein S31, mitochondrial; and 60S ribosomal protein L5. The aldo-keto reductase family 1 member C3 protein was found to be upregulated in both diabetic groups compared to the control, but in the diabetic group with retinopathy the expression of this protein was markedly higher than in the diabetic group without retinopathy.

Table 2. Significantly differentially expressed proteins ($P \leq 0.05$) in diabetic dogs identified by matrix-assisted laser desorption/ionisation–tandem time-of-flight mass spectrometry

ID	Protein	Accession number (UniProtKB)	Score	Match	MW (kDa)*	pI*	Modif.	Seq. cov. (%)	Rt** C:D	Rt** C:D ret.	P-value
1	Ras-related protein Rab-13	F1PTE3	79	6	22881	9.36	Carb, Ox (M)	24	0.655	0.455	0.048
2	Aldo-keto reductase family 1 member C3	Q5R7C9	70	9	37224	7.13	Carb	18	0.793	0.015	0.015
3	28S ribosomal protein S31, mitochondrial	B0BN56	111	8	44220	8.46	Carb, Ox (M)	14	0.189	0.502	0.032
4	2′-5′-oligoadenylate synthase 3	Q8VI93	120	11	127907	8.99	Carb, Ox (M)	8	2.709	1.741	0.030
5	60S ribosomal protein L5	P46777	81	20	34568	9.78	Carb, Ox (M)	42	0.074	0.128	0.001

Modif. – Modification; Seq. cov. – Sequence coverage; Carb – carbamidomethylation of cysteine; Ox (M) – oxidation of methionine; Ox (HW) – oxidation of histidine or tryptophan; * – listed molecular weights (MW) and isoelectric point (pI) values correspond to the Mascot search result; ** – Rt (Ratio); C – Control; D – Diabetic; ret – retinopathy

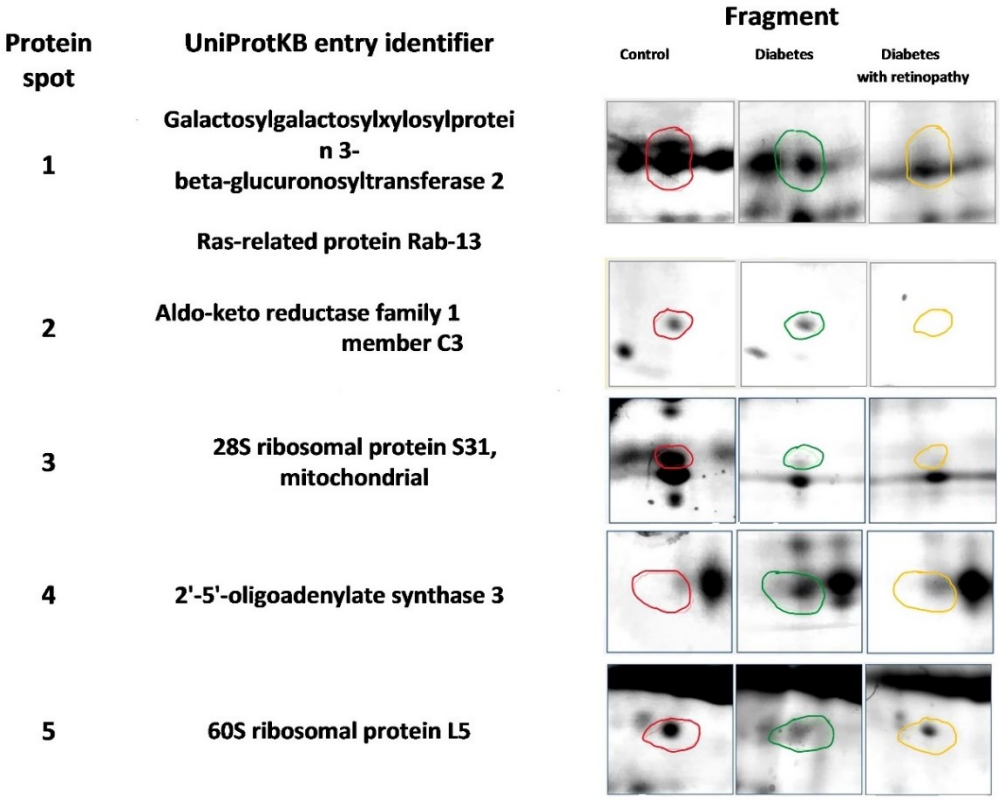

Fig. 1. Statistically significant ($P \leq 0.05$) representatives of two-dimensional electrophoresis gel spots in the diabetic groups of dogs compared to the control group of dogs as revealed by Delta2D software. Protein spots from the healthy group are marked in red, protein spots from the diabetic group without retinopathy are marked in green, and protein spots from the diabetic group with retinopathy are marked in yellow

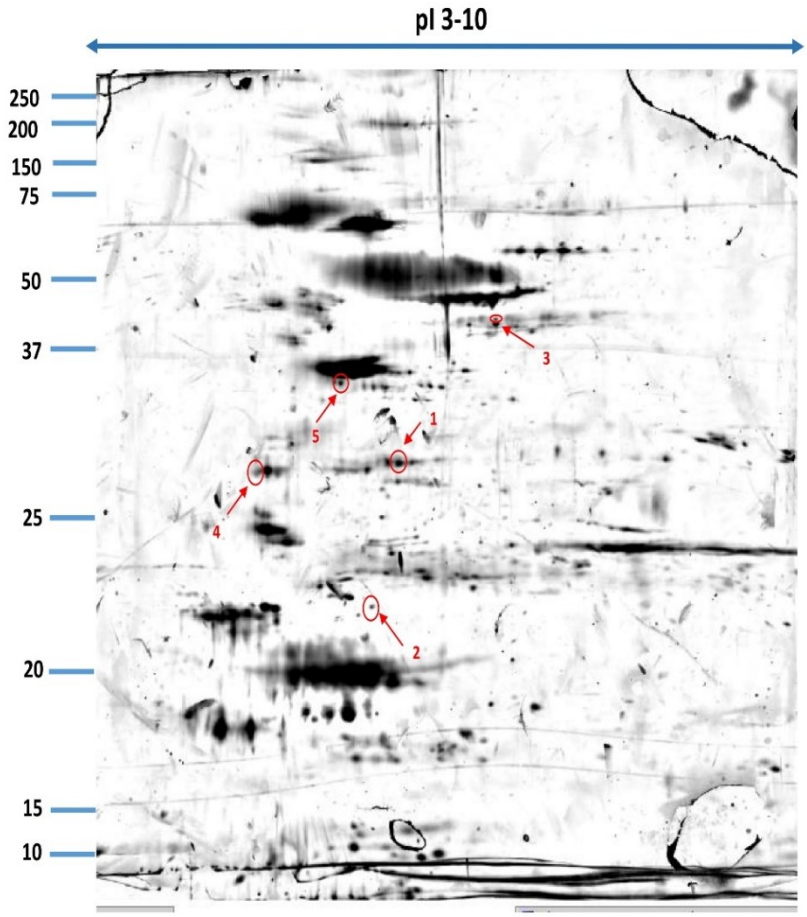

Fig. 2. Proteome map containing every protein spot present on every electrophoretic gel obtained in the experiment. The map is a fused image showing the condensed spot patterns from the experiment. Differentially expressed proteins are marked in red. pI – isoelectric point

Discussion

Diabetes mellitus can have a serious retinal disease called diabetic retinopathy as a sequela, which can lead to blindness in animal and human populations. Early diagnosis and clinical management are critical in slowing the progression of DR, hence the urgent need for biomarkers to better determine the clinical course. In veterinary medicine, the presence of DR is determined by clinical evaluation using retinal screening. Currently, there is no diagnostic test to identify early signs of DR. A non-invasive diagnostic tool to detect early-stage DR would be invaluable in terms of patient treatment and prevention of further retinal and microvasculature damage before a clinical manifestation (12). The tear film is an easily accessible source of proteins, and was previously investigated in human patients and animal models in many ways, also using proteomic approaches. Nevertheless, to the best of our knowledge there is no study regarding the proteomic analysis of canine tear fluid in diabetic retinopathy.

We identified five tear film proteins that were differentially expressed between diabetic groups (with and without DR) and healthy subjects. Four of them were upregulated in both diabetic groups: Ras-related protein Rab-13; aldo-keto reductase family 1 member C3 (AKR1C3); 28S ribosomal protein S31, mitochondrial; and 60S ribosomal protein L5. One protein, 2′-5′-oligoadenylate synthase 3, was downregulated in diabetic groups. Gene ontology analysis using the Panther classification system (pantherbd.org) found that the molecular functions of these differentiating proteins are catalytic activity, regulation and binding. The identified proteins are involved in biological regulation, cellular process, localisation, metabolic process and response to stimulus.

It is well known that oxidative stress is involved in the pathogenesis of DR. An overabundance of reactive oxygen species may damage the tissue in and around retinal vessels, precipitating the development of DR. It has been described that some metabolic disorders are related to hyperglycaemia-induced oxidative damage in the retina: activation of the protein kinase C pathway, polyol pathway flux, activation of the hexosamine pathway and intracellular formation of advanced glycation end-products (13). Persistent oxidative stress along with impairment of autophagy are strongly associated with inflammation. Inflammation is also a well and extensively described process underlying the pathogenesis of diabetic retinopathy (13). Transient inflammation is contributory to host defence in a process called para-inflammation, whereas persistent inflammation leads to

the detrimental tissue changes encountered in DR and other age-related diseases (28). In our study, we noted overexpression of some inflammatory agents, namely Ras-related protein Rab-13; AKR1C3; 28S ribosomal protein S31, mitochondrial; and 60S ribosomal protein L5 in the tear fluid samples obtained from dogs with diabetic retinopathy.

Among the proteins that we found in different abundances between the diabetic group with retinopathy and the group without, AKR1C3 was significantly upregulated in the retinopathy group. AKR1C3 is an enzyme that has been detected in various tissue types, including tumorous and normal tissues, also located in the central nervous system. AKR1C3 is involved in the metabolism of sugars, steroids, prostaglandins and other metabolites (21) and activates PAH dihydrodiols to yield their corresponding reactive and redox-active o-quinones, which can then generate reactive oxygen species that cause oxidative DNA damage. To the authors' knowledge, there is no data on the role of AKR1C3 in the pathogenesis of diabetic ocular complications. One study demonstrated increased expression of 3α-HSDs (isoforms of AKR1C3) in glaucomatous human optic nerve head astrocytes (2). Our findings of increased expression of AKR1C3 in the tear film of canine patients with changes in the retina offer a new insight into possible roles for neurosteroids in the pathophysiology of diabetic retinopathy. Nevertheless, additional studies are required involving larger patient groups.

Diabetes increases oxidative stress in the retina and causes disorders in its mitochondria, accelerating capillary cell apoptosis. Experimental model studies have demonstrated that an increase in cytosolic reactive oxygen species precedes mitochondrial damage, resultant capillary cell apoptosis and the histopathology characteristic of diabetic retinopathy (16). Ras proteins are known to be key molecules initiating the activation of the intracellular protein kinase cascade. Oncogenic Ras proteins constitutively activate the mitogen-activated protein kinase signalling cascade, thereby sending an uninterrupted growth signal. Ras proteins must be translocated to the plasma membrane to function (18). In our study we observed upregulation of the Ras-related protein Rab-13 in both diabetic groups; however, in the retinopathy group, the expression of this protein was more upregulated. These findings suggest that Ras-related proteins may be involved in the development of diabetic retinopathy, and therefore serve as both a potential biomarker and a target for novel therapeutics.

The sole significantly downregulated protein in the tear film obtained from diabetic patients was 2′-5′-oligoadenylate synthase 3. This protein belongs to the oligoadenylate synthase-like (OASL) protein family and is mainly linked to viral infection and various autoimmune diseases, *i.e.* multiple sclerosis or lupus erythematosus (6). It was reported to play a pivotal role in the development of both type 1 and type 2 diabetes in humans (6, 20, 24, 30). Although the natural course of canine diabetes and its aetiopathogenesis are not exactly the same as in humans, there are numerous reports suggesting its similarity to type 1 diabetes, with the autoimmune response playing a significant role. Therefore, we suggest that impaired OASL expression can contribute to the development of diabetic retinopathy, especially given that it showed significantly different expression not only between the control and diabetic groups, but also between the diabetic groups with and without retinopathy.

The results of our study confirm that the pathological process in the retina in the course of diabetes mellitus is reflected by changes in the tear film proteome. Comprehension of the molecular variations leading to the ocular complications caused by hyperglycaemia may contribute to improving the quality of life of diabetic patients through the identification of novel diagnostic biomarkers as well as therapeutic targets.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This work was supported by the Polish National Science Centre (NCN). The first author is in receipt of by the Preludium grant number UMO-2016/23/N/NZ5/02576. The research data supporting this publication can be accessed by contacting the corresponding author.

Animal Rights Statement: All samples were obtained during standard veterinary diagnostic procedures; thus, according to Polish law, the approval by Local Commission for Ethics in Animal Experiments was not required since there was no medical or invasive diagnostic treatment, or procedure causing psychological or social discomfort for the participants.

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