

ARTICLE

Reverse Translational Study of Fenofibrate's Observed Effects in Diabetes-Associated Retinopathy

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Clinical trials suggest that fenofibrate reduces the progression of retinopathies in patients with type 2 diabetes. Furthermore, patients with retinopathies have elevated levels of inflammatory chemokines and dysfunctional retinal angiogenesis. Therefore, we investigated the effects of fenofibrate on the production of inflammatory chemokines and genes associated with angiogenesis. Retinal pigment epithelial cells (RPEs) were cultured with IL-1 β and fenofibrate ranging from 1–50 μ M. ENA-78, IL-8, and RANTES were measured in cell culture by ELISA. ENA-78, ABCA1, and ABCG1 gene expression were tested by RT-PCR. IL-1 β significantly induced the production of ENA-78, IL-8, and RANTES. Fenofibrate at concentrations of 25–50 μ M blunted the IL-1 β induced production of ENA-78 ($p < 0.05$) with no significant effects on RANTES and IL-8. Fenofibrate also reduced the expression of the ENA-78 gene as well as ABCA1 and ABCG1, which are genes involved in angiogenesis. Fenofibrate decreases ENA-78 production and ABCA1/ABCG1 gene expression in RPEs.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Fenofibrate reduces the requirement of surgical interventions and the progression of retinopathies in patients with type 2 diabetes. However, the mechanisms associated with the observed effects remain elusive.

WHAT QUESTION DID THE STUDY ADDRESS?

✓ The study addresses the hypothesis that fenofibrate's anti-inflammatory and anti-angiogenic properties may contribute to the observed benefits in patients with retinopathies.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE?

✓ The results demonstrate that fenofibrate attenuates neutrophilic inflammation via ENA-78 and decreases the expression of genes required for angiogenesis (ABCA1

and ABCG1). ENA-78 is a soluble biomarker that can be easily measured from the serum/plasma of patients with type 2 diabetes. Therefore, this study identifies a biomarker that could be rapidly investigated in fresh or stored plasma/serum from patients with type 2 diabetes.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✓ This study suggests that fenofibrate lowers the production of ENA-78, which is known to be elevated in the plasma/serum with the increasing severity of diabetic retinopathy. Therefore, ENA-78 is identified as a measurable biomarker that can be investigated for future clinical utility. Additionally, we identified ABCA1 and ABCG1 as genes that can be investigated for future clinical utility.

The incidence of diabetes has tripled over the past 30 years and is predicted to continue to rise in the foreseeable future.¹ The majority of this increase is due to the epidemic of type 2 diabetes mellitus. Patients with type 2 diabetes mellitus commonly experience microvascular complications; namely diabetic retinopathy, peripheral neuropathy, and nephropathy. Retinopathy is the most common microvascular complication in diabetics and is the leading cause of blindness in the United States.²

Until very recently, patients with diabetes mellitus had more stringent treatment goals for hypertension than patients

with uncomplicated hypertension (130/80 mmHg vs. 140/90 mmHg, respectively). However, data suggest that achieving these lower blood pressure goals may not lead to fewer new or worsening cases of retinopathy as expected.^{3–5} Similarly, intensive treatment of blood glucose levels in diabetics does not translate to improved retinopathy outcomes.^{6–8} However, two large clinical trials recently showed that fenofibrate, a medication commonly prescribed to modulate lipids, effectively decreases the rate of retinopathies in patients with type 2 diabetes mellitus.^{5,9–11}

The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) and Action to Control Cardiovascular Risk

in Diabetes (ACCORD) eye studies showed remarkable reductions in progression of retinopathy on treatment of patients with diabetes with fenofibrate. A systematic review and meta-analysis of multiple studies (including FIELD and ACCORD) on the effects of the fibrates revealed a nearly 40% risk reduction of diabetic retinopathy.¹² However, the benefits of fenofibrate associated with retinopathies did not correlate with reductions in lipids. Baseline triglyceride levels were equal in patients who required laser therapy to treat retinopathy and in those who did not.¹¹ Therefore, the mechanism of action underlying this preventive effect of fenofibrate on retinopathy risk in patients with type 2 diabetes mellitus remains unclear.

Inflammation and aberrant angiogenesis are hallmarks of the pathogenesis and progression of diabetic retinopathy. Epithelial neutrophil activating peptide 78 (ENA-78), regulated on activation of normal T-cell expressed and secreted (RANTES) and interleukin 8 (IL-8), are chemokines that have repeatedly been shown to be elevated in patients with diabetic retinopathies.^{13–15} Impaired reverse cholesterol transport (RCT) has been strongly linked to aberrant angiogenesis. This becomes a pathological process in the retina with abnormal new blood vessels hemorrhaging into the vitreous cavity and threatening to cause vision loss. Furthermore, dysfunction of the adenosine triphosphate binding cassette (ABC) genes that are involved in RCT have been observed in pathological angiogenesis. This is especially true in retinal tissue in which the ABC genes, *ABCA1* and *ABCG1*, have been linked to macular degeneration^{16–18} and primary open-angle glaucoma.^{19–21} These RCT genes influence cholesterol transport from peripheral sites *in vivo* and modulate inflammatory responses. For these reasons, it is logical to explore the effects of fenofibrate on the production of the aforementioned inflammatory mediators (ENA-78, RANTES, and IL-8) and the expression of the RCT genes (*ABCA1* and *ABCG1*) that influence angiogenesis in diabetic retinopathy.

For our model system, we cultured retinal pigment epithelial cells (RPECs). The RPECs are a specialized layer of epithelial cells that form the outer layer of the blood-retinal barrier. They are closely packed to form tight junctions that are crucial in regulating fluid and solute balance in the eye as well as preventing entry of toxic substances into the retina. We chose to measure the protein production and gene expression of ENA-78, RANTES, and IL-8 (shown in **Table 1**) in our cultured RPECs. We also measured the gene expression of *ABCA1* and *ABCG1* to determine if fenofibrate modulates angiogenesis via its effects on RCT in RPECs. The purpose of this study was to identify potential biomarkers that can be explored in clinical studies in which fenofibrate reduced the risk of diabetic retinopathies.

METHODS

Cell culture

Human RPECs (Lonza; Walkersville, Walkersville, MD) on their third pass were seeded in 2% fetal bovine serum (FBS) in RPEC-plating media at a density of 2.5×10^4 cells/cm² in 48-well plates (surface area 1.1 cm²) for cytotoxicity experiments and 6-well plates (surface area 9.5 cm²) for stimulated cytokine and protein expression experiments. At 24 h post-seeding, the plating media was removed by aspiration and

the cells were thereafter cultured in retinal epithelial growth media (RtEGM) at 37°C and 5% CO₂. This growth media was then changed every 48 h until the RPECs reached 80% confluency. This cell culture procedure was provided by Lonza and is specific to RPECs. Once the cells reached 80% confluency, growth was stopped by adding serum free media for 24 h followed by cell treatment in 2% FBS in RtEGM. The duration of our cell treatments for stimulated cytokine and protein expression experiments was 24 h and was carried out as follows: fenofibrate and dimethylsulfoxide (DMSO) control were added to the appropriate wells and IL-1 β was added to the appropriate wells 2 h later. Addition of the proinflammatory cytokine IL-1 β marked the beginning of the treatment period. The same treatment duration was used for cytotoxicity experiments, the difference being that no IL-1 β was used; therefore, the addition of fenofibrate marked the beginning of the treatment period.

Cytotoxicity

Fenofibrate's toxicity to RPECs at concentrations ranging from 1–100 μ M was determined by trypan blue staining with cell counts obtained using a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA). The solvent used for fenofibrate solutions, as well as a control, was DMSO. Fenofibrate concentrations causing reductions in cell viability to <80% compared with control were excluded from further studies in RPECs. The positive control for the RPEC cytotoxicity study was 10% Triton X-100 (Sigma Aldrich, St. Louis, MO). It was added to the appropriate wells at the same time as fenofibrate and treatment duration was 24 h.

Stimulated cytokine production

The concentration-dependent effects of fenofibrate on IL-1 β stimulated production of the ENA-78, RANTES, and IL-8 were determined. Cell treatment groups included control (DMSO only), IL-1 β (2 ng/mL), fenofibrate (1–50 μ M), and fenofibrate plus IL-1 β . Fenofibrate and IL-1 β (both from Sigma Aldrich) were diluted to the desired concentration using DMSO and RNase/DNase-free molecular grade water, respectively. Each co-treatment experiment was performed six times. IL-1 β was chosen because it is a potent proinflammatory cytokine and is a prototypical inflammatory mediator implicated in diabetic retinopathy pathology. IL-1 β is also involved in other ocular inflammatory states, including dry eye disease.²²

Protein quantification

Twenty-four h after the experimental conditions were applied, the cell media was collected and the concentrations of the chemokine/cytokine of interest in this study were assessed using standard enzyme-linked immunosorbent assay (ELISA; R+D Systems, Minneapolis, MN). The calculated concentrations were standardized to milligrams of total protein using bicinchoninic acid assays (Pierce, Rockford, IL). All samples were measured in duplicate. The standard curves of best fit for each cytokine was generated using Gen 5 Microplate Data Collection and Analysis Software (Biotek, Winooski, VT) by plotting the baseline corrected mean absorbance (\pm SD) of the supplied analyte standard against their known concentrations.

Table 1 List of chemokines that were assessed in this RPEC model

Chemokine	Abbreviation/gene	Function
Epithelial-derived neutrophil activating peptide 78	ENA-78/CXCL5	Attracts and activates neutrophils
Interleukin 8	IL-8/CXCL8	Attracts and activates neutrophils
Regulated upon activation, normal T-cell expressed and secreted	RANTES/CCL5	Attracts T-cells, basophils, eosinophils, and activates neutrophils

RPEC, retinal pigment epithelial cell.

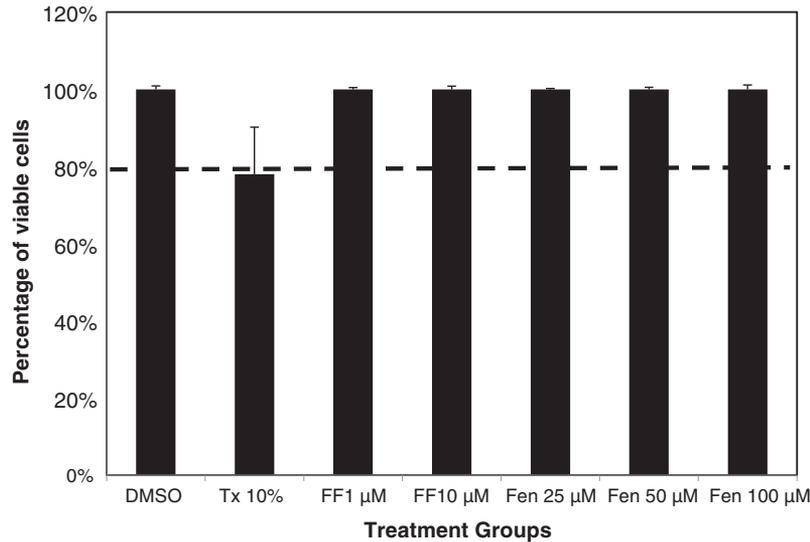


Figure 1 Effect of fenofibrate (FF) on retinal pigment epithelial cell (RPEC) viability. RPECs were treated with FF at concentrations ranging from 1–100 μ M. Dimethylsulfoxide (DMSO) was used as the negative control; Triton-X surfactant was the lysis control.

Gene expression

The effects of fenofibrate on IL-1 β stimulated gene expression were also tested. Treatment groups included control (DMSO), IL-1 β (2 ng/mL), and fenofibrate (10–25 μ M). Each experiment was repeated six times. Gene expression studies followed those experiments in which fenofibrate modulated the IL-1 β induced protein concentrations. Ribonucleic acid was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA) 24 h after cell treatments. Complimentary DNA (cDNA) was synthesized by high capacity cDNA reverse transcriptase using 500 ng of total RNA (7500 Fast Real-Time Polymerase Chain Reaction [RT-PCR]; Applied Biosystems, Foster City, CA). RNA and cDNA quality and quantity were measured using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE). Quantitative RT-PCR was performed using a probe for the protein of interest as well as our selected reference gene (18S).

Statistical analysis

Cytokine concentrations were compared by one-way analysis of variance with Tukey's correction for multiple comparisons, where appropriate. Values of $p < 0.05$ were considered significant. Gene expression was reported using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

RPECs were viable across all concentrations of fenofibrate used in the cytotoxicity experiments (**Figure 1**). Concentrations of fenofibrate 1–50 μ M were used in the experimental

treatment groups as they represent expected plasma concentrations across the clinical spectrum (i.e., the range of fenofibrate's peak plasma concentration C_{max} in plasma is between 26 and 30 μ M).^{23–25}

IL-1 β induced the production of ENA-78, RANTES, and IL-8 in RPECs, as expected. ENA-78 protein production was increased by eightfold (from 43.6 ± 48.2 pg/mg to 350.8 ± 69.4 pg/mg). Co-treatment with fenofibrate significantly attenuated the stimulated increases in ENA-78 production (**Figure 2**). Fenofibrate also decreased the gene expression of ENA-78 (CXCL5) relative to control and IL-1 β treatment (**Figure 3**). RANTES production was increased by over sixfold (from 6.5 ± 0.3 pg/mg to 41.0 ± 24.7 pg/mg). Co-treatment with fenofibrate attenuated the stimulated increases in RANTES production at 25 μ M and 50 μ M concentrations, but this effect failed to reach statistical significance (**Figure 4**). IL-1 β increased production of IL-8 by over three orders of magnitude (from 6.5 ± 5.3 pg/mg to 6579 ± 3046 pg/mg), but fenofibrate did not significantly decrease its production (**Figure 5**).

The RCT genes involved in angiogenesis, *ABCA1* and *ABCG1*, were downregulated compared with control with the addition of fenofibrate and further downregulated in a seemingly dose-dependent fashion with fenofibrate and IL-1 β co-treatment (**Figure 6**).

DISCUSSION

In patients with type 2 diabetes mellitus, fenofibrate doses of 160–200 mg/d have shown positive effects on retinopathies.

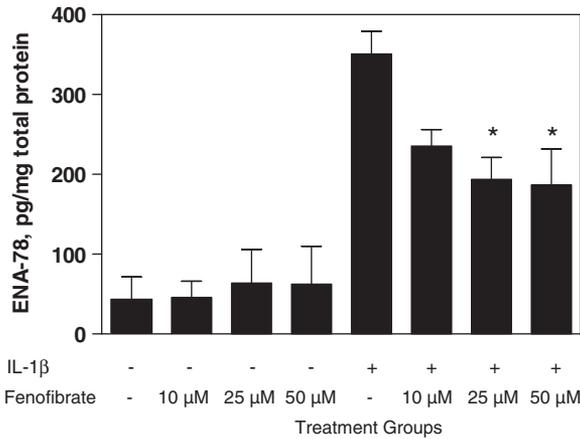


Figure 2 Effect of fenofibrate on interleukin 8-beta (IL-8β) stimulated epithelial neutrophil activating peptide 78 (ENA-78) protein production. IL-1β significantly induces production of ENA-78 (p value < 0.0001). Fenofibrate 25–50 μM significantly decreased the production of ENA-78 (p value < 0.05 denoted by *) compared with the IL-1β control. Data are presented as mean ± SEM.

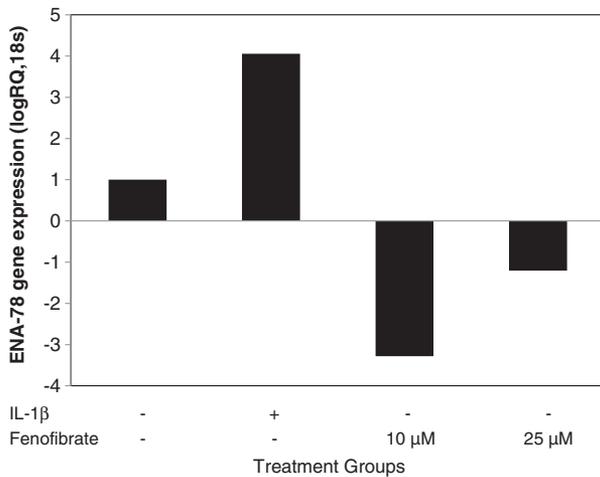


Figure 3 Effect of fenofibrate on interleukin 8-beta (IL-8β) stimulated epithelial neutrophil activating peptide 78 (ENA-78; CXCL5) mRNA production. IL-1β induces the expression of ENA-78 (CXCL5) mRNA. Fenofibrate 10–25 μM decreased basal expression of ENA-78 (CXCL5) mRNA. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and analysis of variance with post-hoc Tukey's analysis.

These effects do not seem to correlate with standard lipid parameters and, therefore, may be related to fenofibrate's anti-inflammatory and pleiotropic properties.^{23,26,27} We desired to understand the mechanisms by which the peroxisome proliferator-activated receptor alpha (PPARα) agonists caused clinically significant reductions in retinopathy progression by testing their effects on inflammatory pathways in RPEs.

Our group and others have shown that fenofibrate modulates inflammatory cytokines in various cell models but data in RPEs were lacking. We explored the effects of fenofibrate on ENA-78, RANTES, and IL-8, as well as the RCT genes, *ABCA1* and *ABCG1*, in an RPEC model of retinopathy.

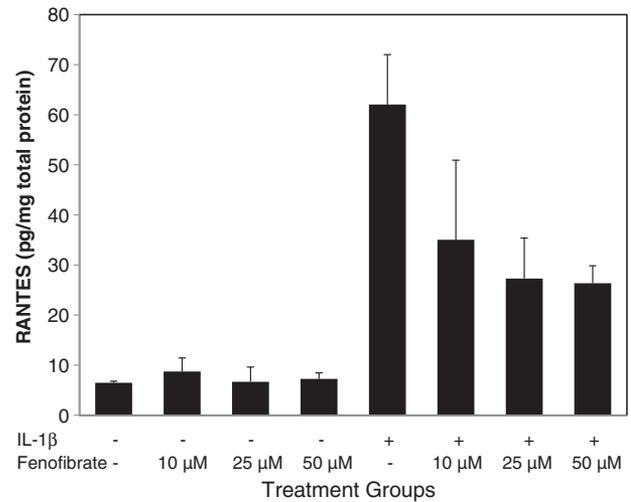


Figure 4 Effect of fenofibrate on interleukin 8-beta (IL-8β) stimulated regulated on activation of normal T-cell expressed and secreted (RANTES) protein production. IL-1β significantly induces production of RANTES (p value < 0.001). Fenofibrate 10–50 μM concentrations co-treatment decreased RANTES production but failed to reach statistical significance. Data are presented as mean ± SEM.

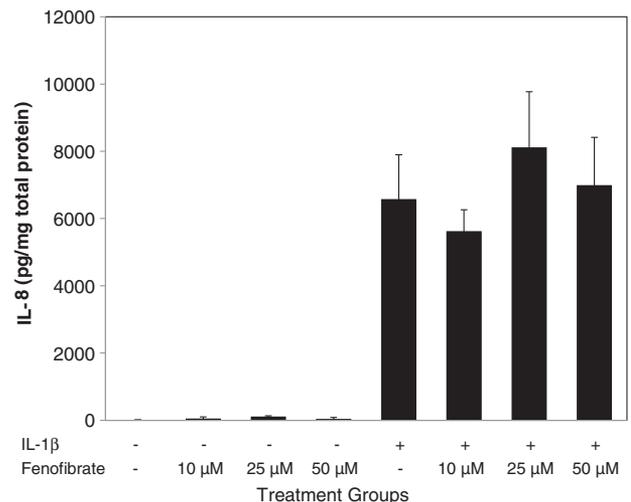


Figure 5 Effect of fenofibrate on interleukin 8-beta (IL-8β) stimulated IL-8 protein production. IL-1β significantly induces production of IL-8 (p < 0.05). Fenofibrate at increasing concentrations does not significantly lower the production of IL-8. Data are presented as mean ± SEM.

These chemokines have all been implicated in other studies as proinflammatory and immunomodulatory in the eye.^{22,28,29}

These types of studies could help tailor therapies to patients who stand to benefit the most from additional pharmacotherapy. Furthermore, these translational studies could lead to the identification of new targets for therapies that treat retinopathy (with possible benefits in the other microvascular dysfunctions).

Fenofibrate exerted no effect on the production of IL-8. The IL-8 was highly induced by IL-1β but fenofibrate did not attenuate this effect. Fenofibrate has displayed disparate

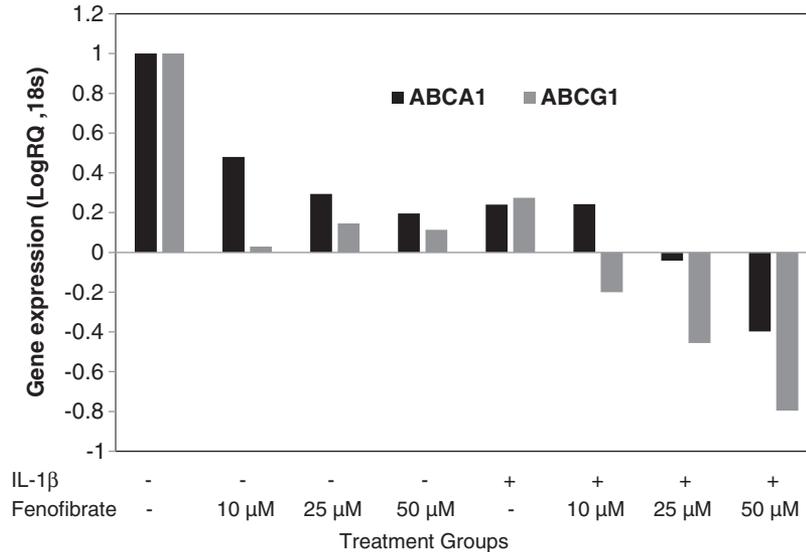


Figure 6 Effect of interleukin 8-beta (IL-8 β) and fenofibrate on the expression of reverse cholesterol transport genes, adenosine triphosphate binding cassette (ABC)A1 and ABCG1. Fenofibrate decreased the expression of ABCA1 and ABCG1 in basal retinal pigment epithelial cells (RPECs). IL-1 β and fenofibrate co-treatment demonstrated additive effects on ABCA1 and ABCG1 mRNA expression. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method and analysis of variance with post-hoc Tukey's analysis.

effects on IL-8 production in various cell models^{30,31} and these data do not suggest an effect on this biomarker in RPECs. The IL-8 modulation by PPAR α agonists seems to be largely tissue and culture model system-dependent. Fenofibrate treatment was associated with reductions in the IL-1 β stimulated production of RANTES but the observed effects failed to reach statistical significance. Finally, IL-1 β stimulated production of ENA-78 protein was significantly reduced by fenofibrate in concentrations of 25 μ M and 50 μ M in this model system, as reported previously in cell lines from other organ systems.^{32,33} This seems to indicate a more prominent role for fenofibrate's anti-inflammatory effects related to stopping the activation of neutrophils. Therefore, ENA-78 seems to be an attractive biomarker for exploring the positive effects of fenofibrate on diabetic retinopathy progression.

The role of neutrophils in the pathogenesis and progression of diabetic retinopathy has been previously described.³⁴⁻³⁸ Modulating the infiltration and activation of neutrophils and subsequent inflammation may be one mechanism by which fenofibrate is able to reduce retinopathies in patients with type 2 diabetes mellitus. ENA-78 is an attractive biomarker for exploration in patients at increased risk of diabetic retinopathy. Meleth *et al.*³⁹ previously reported that ENA-78 serum levels tended to be higher as the severity of diabetic retinopathy increased in patients. Meleth *et al.*³⁹ enrolled 101 subjects with diabetes at the National Eye Institute into a clinical study and the subjects received extensive phenotyping of the eyes. The subjects who were classified as having severe retinopathy had a 55% increase in ENA-78 found in their serum when compared with the subjects with less severe retinopathies (mean and 95% confidence interval = 2,629 pg/mL [2,158-3,102 pg/mL] vs. 1,689 pg/mL [1,377-2,074 pg/mL]). Furthermore, ENA-78 has been shown to be elevated in the serum of patients with type 2 diabetes when

compared with healthy controls.⁴⁰ Therefore, ENA-78 seems to be an attractive clinical biomarker for retinopathy stratification and may identify patients who are candidates for fenofibrate therapy. Moreover, clinical trials that have examined the effects of fenofibrate on the progression and risk of diabetic retinopathy have the potential to serve in the translation of the findings that we report. Specifically, this biomarker could be evaluated in stored plasma/serum samples from recent large fenofibrate clinical trials to explore its usefulness as a biomarker of retinopathy risk, progression, and response to fenofibrate.

Fenofibrate decreased the expression of RCT genes that are implicated in angiogenesis in this experimental model of diabetic retinopathy. RCT and cholesterol efflux are essential for angiogenesis.^{41,42} Panigrahy *et al.*⁴³ previously published a manuscript that established direct and indirect effects of fenofibrate on angiogenesis, which supports our findings. Specifically, Panigrahy *et al.*⁴³ demonstrated that fenofibrate inhibited fibroblast growth factor-2 induced corneal angiogenesis in an animal model by >50%. Our report complements the current literature by suggesting that fenofibrate modulates ABCA1 and ABCG1 expression in the retinal pigment epithelium and, thus, influences angiogenesis. Therefore, we suggest that ABCA1 and ABCG1 are appealing targets for additional studies and may serve as biomarkers of diabetic retinopathy risk or progression.

Limitations of our study include the fact that the RPECs represent only one of the potential sources of pathogenesis of diabetic retinopathy and sites of potential action for fenofibrate. However, this model system was able to express several soluble biomarkers that are elevated in the plasma/serum of patients with diabetic retinopathy. Furthermore, this model system provided data that could be used for generating clinical hypotheses that can be explored inexpensively in stored clinical samples that were obtained from

patients afflicted with diabetic retinopathies and treated with fenofibrate.

In conclusion, we report that fenofibrate decreases the production of the neutrophilic chemokine ENA-78, which is known to be elevated in the plasma/serum of patients with diabetic retinopathies. Furthermore, fenofibrate suppressed the expression of *ABCA1* and *ABCG1* that may contribute to aberrant angiogenesis. Therefore, our data complements the current literature that suggests fenofibrate's beneficial effects on retinopathies are due to anti-inflammatory and anti-angiogenic properties in the retina. Finally, we suggest that translational studies be conducted to confirm the usefulness of plasma/serum levels of ENA-78 and expression of pro-angiogenesis genes (*ABCA1* and *ABCG1*) as biomarkers for the risk, progression, and treatment response in diabetic retinopathies.

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Conflict of Interest. The authors declared no conflict of interest.

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