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Ocular complications associated with diabetes mellitus

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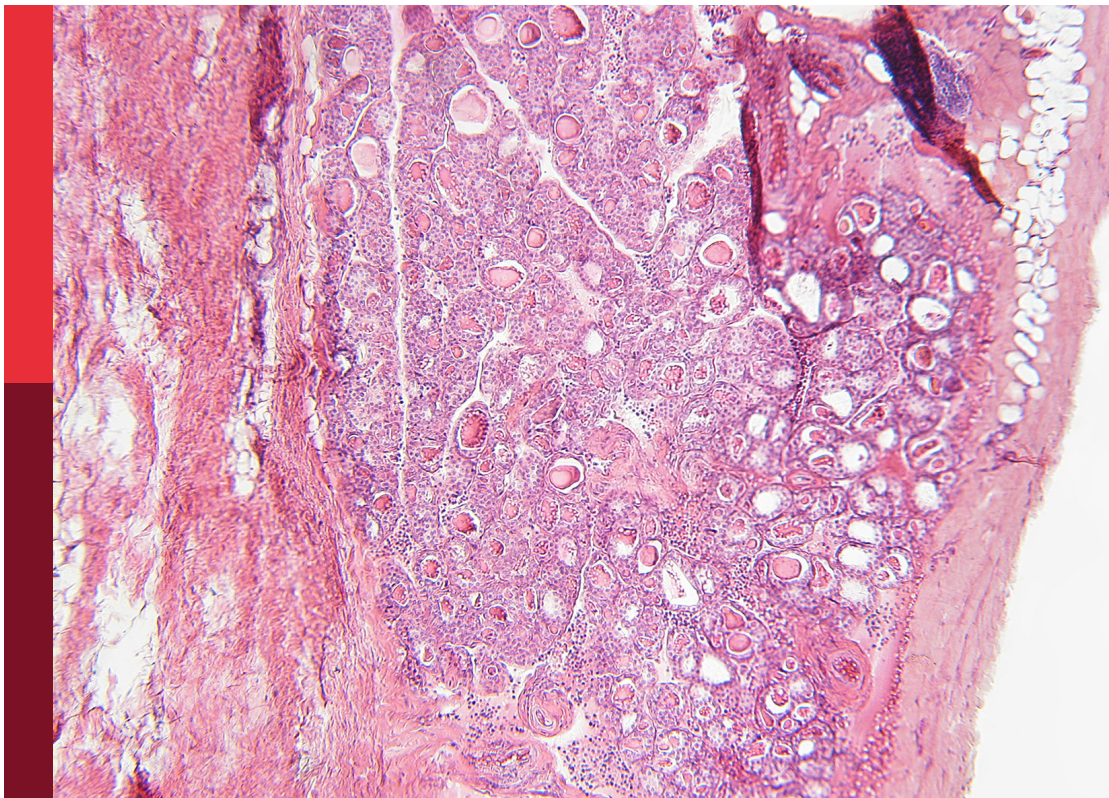

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Ocular complications associated with diabetes mellitus

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Editorial: Ocular complications associated with diabetes mellitus

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Editorial on the Research Topic
Ocular complications associated with diabetes mellitus

Diabetes mellitus (DM) is a major expanding health problem with the fastest growing health challenges in the 21st century. DM is strongly associated with both microvascular and macrovascular complications. The eye is a window to several systemic and neuro-ophthalmic complications and associations of diabetes (1). The retinal vascular fractals reflect long-term microvasculopathy and the pathological and morphological changes of corneal nerve reflect severity of diabetic neuropathy. Preventive measures and early intervention can significantly reduce the morbidity caused by ocular complications associated with diabetes.

This Research Topic highlight the wide spectrum of latest advancements in basic and clinical concepts in the diagnosis, progression, treatment outcomes, and the application of AI techniques in clinical research for diabetes related complications.

Diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of blindness in the working age population in both developed and developing countries. DR and diabetic nephropathy (DKD) are the commonest microvascular complications of DM. Early diagnosis and prompt intervention of sight threatening DR are critical in patients with DM to achieve a good visual outcome. Molecular and imaging biomarkers and artificial intelligence (AI), as well as gene and stem cell research are gathering pace in both early prevention and management of DR. In this Research Topic, Tan and Wong discusses the major trends in DR in 2023 that includes epidemiology especially the global burden of DR, the pathophysiological understanding of DR especially retinal neural dysfunction and, the

Abbreviations: AI, Artificial intelligence; DED, Dry eye disease; DKD, Diabetic nephropathy; DM, Diabetes mellitus; DME, Diabetic macular edema; DR, Diabetic retinopathy; GLP-1R, Glucagon-like peptide-1 receptor; NVG, Neovascular glaucoma; PDR, Proliferative diabetic retinopathy; POAG, Primary open angle glaucoma; SGLT, Sodium-glucose co-transporter; GLUT1, Glucose transporter type 1.

application of new imaging modalities and AI in DR. Xie and Xiao provide insight into the recent advancement and progress made on inflammatory and arteriosclerosis-associated biomarkers; novel therapeutic strategies including anti-VEGF therapy, renin-angiotensin-aldosterone system therapy, nanotechnology on DR and diabetic nephropathy (DKD). Zhang et al. investigate the mechanisms underlying the correlations between DR and DKD in patients with T2DM. The findings highlight the predictive role of Albumin-to-creatinine ratio on DR severity and progression, indicating the link between DR and DKD and the association with dyslipidemia and upregulated circulating level of angiogenic cytokines. Furthermore, Chen et al., Liu et al., Huang et al. describe potential circulating molecular biomarkers and targets for DR including Glucagon-like peptide-1 receptor (GLP-1R), sodium-glucose co-transporter (SGLT) 1, SGLT2, Glucose transporter type 1 (GLUT1) and GLUT2 ferroptosis-related, L-Citrulline, hexanoylcarnitine, chenodeoxycholic acid and eicosapentaenoic acid. By using a non-parametric technique, Wang et al. identified eight predictive risk factors for DR including disease duration, body mass index, fasting blood glucose, glycated hemoglobin homeostatic model assessment-insulin resistance, triglyceride, total cholesterol and vitamin D-T3. These interesting discoveries from bench or bedside will likely become important components of translational research.

Several AI techniques, such as machine learning and deep learning, have been applied in automated screening, diagnosis and prognosis prediction of DR and diabetic macular edema (DME). The integration of AI with imaging technologies such as digital fundus photography and optical coherent tomography will continue to be an important area of DR research, with the potential to further enhance our clinical practice. In this Research Topic, Sheng et al. highlighted the fundamental concepts of AI and its application in DR and further discuss the current challenges and prospects of AI in ophthalmology. A machine learning based and molecular docking methods were also applied to identify the potential ferroptosis-related biomarkers and pharmacological compound in DR by Liu et al. An overview of global publications on machine learning in DR from 2011 to 2021 is also presented by Shao et al. They conclude that diverse and multiple modalities of medical data, new ML techniques and constantly optimized algorithms are the future research areas in DR.

Diabetic ocular surface diseases

While DR is the most well-known complication of DM, ocular surface diseases, including dry eye disease (DED) and diabetic keratopathy are also common in the diabetic population. These diabetic ocular surface diseases may seriously affect the quality of life. Accumulation of advanced glycation end-products impaired neurotrophic innervation and limbal stem cell function, dysregulated growth factor signaling, and inflammation contribute to the pathogenesis of diabetic keratopathy. Lacrimal Functional Unit dysfunction, abnormal tear dynamics, and film dysfunction have been implicated in the pathogenesis of DED. In this Research Topic, Zhou et al. highlight the important roles of the dense innervations in the homeostatic maintenance of cornea and the lacrimal gland. The clinical manifestation, potential treatment options and underlying pathological mechanisms of diabetic keratopathy (diabetic corneal epitheliopathy and corneal neuropathy), diabetic corneal endotheliopathy, diabetic dry eye, diabetic meibomian gland dysfunction have been illustrated in detail. They further emphasize that studies on the neuroepithelial and neuroimmune interactions will likely reveal predominant pathogenic mechanisms and contribute to the development of intervention strategies of diabetic ocular surface complications. Liu et al. further provide an overview of the morphological changes of diabetic corneal neuropathy using *in-vivo* confocal microscopy in both animal and clinical studies. They introduce the pathological changes in maturation stages of corneal dendritic cells (DCs) in DM, emphasizing the relationship between corneal DCs and clinical parameters including age, corneal nerve status and metabolism parameters., The two comprehensive reviews provide valuable insight into the development of diagnostic, preventive, and therapeutic strategies for DM-associated ocular surface complications.

Diabetes associated glaucoma

Glaucoma is a significant cause of blindness worldwide. Primary open angle glaucoma (POAG) is the most common type of glaucoma in patients with DM (2). The commonest type of secondary glaucoma in patients with DM in clinical practice is neovascular glaucoma (NVG), which is characterized by the appearance of neovascular over the iris and the proliferation of fibrovascular tissue in the anterior chamber angle mainly due to DR. In this Research Topic, Tang et al. outline the underlying mechanisms management strategies of NVG in patients with DM in eyes with proliferative DR (PDR). In a mini review article, Cheng et al. describe the correlations between biomechanical dyshomeostasis and glaucoma and other ocular diseases, providing novel diagnostic and treatment strategies targeting mechanobiology of these disorders.

Summary

In conclusion, DM associated ocular complications has progressively and rapidly becoming the most significant cause of morbidity, which are preventable with early detection and timely management. Besides DR, diabetic ocular surface disorder and glaucoma, other DM associated ocular complications including cataract, DM related refractive changes, eye infection, optic neuropathies (diabetic papillopathy, non-arteritic anterior ischemic optic neuropathy) etc. can also be caused by chronic hyperglycemia. Routine eye examinations and intervention at the right point as well as systemic interventions including blood glucose, hypertension and hyperlipidemia control are essential for the reduction of DM related vision loss. The convergence of technologies and the proliferation of biologics as therapeutics promise to provide more novel and effective treatment options as

augmentations or through other delivery methods. This Research Topic provided new insight into the mechanisms, molecular biomarkers, AI, and intervention strategies for DM associated ocular complications.

Author contributions

XZ drafted and revised the manuscript, SS and DT provided comments and made revisions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Metabolomic comparison followed by cross-validation of enzyme-linked immunosorbent assay to reveal potential biomarkers of diabetic retinopathy in Chinese with type 2 diabetes

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Purpose: To identify the biomarkers in the critical period of development in diabetic retinopathy (DR) in Chinese with type 2 diabetes using targeted and untargeted metabolomics, and to explore the feasibility of their clinical application

Methods: This case-control study described the differential metabolites between 83 Chinese type 2 diabetes mellitus (T2DM) samples with disease duration ≥ 10 years and 27 controls matched cases. Targeted metabolomics using high-resolution mass spectrometry with liquid chromatography was performed on plasma samples of subjects. The results were compared to our previous untargeted metabolomics study and ELISA was performed to validate the mutual differential metabolites of targeted and untargeted metabolomics on plasma. Multiple linear regression analyses were performed to adjust for the significance of different metabolites between groups.

Result: Mean age of the subjects was 66.3 years and mean T2DM duration was 16.5 years. By cross-validating with results from previous untargeted metabolomic assays, we found that L-Citrulline (Cit), indoleacetic acid (IAA), 1-methylhistidine (1-MH), phosphatidylcholines (PCs), hexanoylcarnitine, chenodeoxycholic acid (CDCA) and eicosapentaenoic acid (EPA) were the most distinctive metabolites biomarkers to distinguish the severity of DR for two different metabolomic approaches in our study. We mainly found that samples in the DR stage showed lower serum level of Cit and higher serum

level of IAA compared with samples in the T2DM stage, while during the progression of diabetic retinopathy, the serum levels of CDCA and EPA in PDR stage were significantly lower than NPDR stage. Among them, 4 differential key metabolites including Cit, IAA, CDCA and EPA were confirmed with ELISA.

Conclusion: This is the first study to compare the results of targeted and untargeted metabolomics *via* liquid chromatography-mass spectrometry to find the serum biomarkers which could reflect the metabolic variations among different stages of DR in Chinese. The progression of DR in Chinese at different critical stages was related to the serum levels of specific differential metabolites, of which there is a significant correlation between DR progression and increased IAA and decreased Cit, hexanoylcarnitine, CDCA, and EPA. However, larger studies are needed to confirm our results. Based on this study, it could be inferred that the accuracy of targeted metabolomics for metabolite expression in serum is to some extent higher than that of untargeted metabolomics.

KEYWORDS

biomarker, diabetic retinopathy, enzyme-linked immunosorbent assay, metabolomics, non-proliferative diabetic retinopathy, proliferative diabetic retinopathy, type 2 diabetes, targeted metabolomics

1 Introduction

Diabetic retinopathy (DR) as a destructive disease is the most serious microvascular complication of diabetes in eyes (1–3) and the main cause of hypopsia and blindness among 20 to 74 year-old adults in developing and developed countries (4–6). A study showed that China had 114 million diabetics, ranking first in the world (7, 8). In China, the prevalence of DR in the general population was 1.7%, while the prevalence of DR in the diabetic population was 22.4%, with the greatest prevalence in North China (27.7%) (8). Currently, the treatments of DR, including retinal laser photocoagulation, intravitreal injection of anti-vascular endothelial growth factor and vitrectomy are only aimed at controlling the late development of DR, and there is no effective treatment to limit neurovascular dysfunction or promote repair in the early stages of DR (9). In addition, for a long time, the blood glucose level and duration of diabetes have been considered to be the main risk factors for the development of DR (2, 10). However, in clinical practice, these risk factors cannot well explain the huge difference in the rate of individual progression of DR (11, 12) which indicates that there may be other unknown factors that can better screen and predict the occurrence and development of DR.

Although many metabolomic studies of DR have been conducted, the identification of differential metabolites in critical periods of DR development (periods of T2DM and NPDR) has been rarely attempted, especially in Chinese populations. In our previous untargeted metabolomics study of DR in Chinese, we found that in addition to the dysregulation of the classic amino acid metabolic pathway, many small molecules such as long-chain polyunsaturated fatty acids, phosphatidylcholines (PCs) and bile acids were up- or down-regulated to varying degrees during the critical periods of DR (13). The main purpose of untargeted metabolomics is to discover the metabolites in the sample as many as possible and reflect the information of total metabolites to the greatest extent, which helps to discover the unknown key metabolites. Targeted metabolomics uses target compound standards as a reference to detect and analyze specific metabolites in biological samples in a targeted manner, which can more accurately identify the target metabolites (14, 15).

To our best knowledge, there have been no studies using the same detection platform to compare the untargeted and targeted metabolomic outcomes in different stages of DR samples. To fill this gap, this study aimed to perform targeted metabolomics via liquid chromatography-mass spectrometry (LC-MS) in serum of the T2DM Chinese with and without DR. And the results of the targeted metabolomics were compared with those of previous untargeted metabolomics to identify the biomarkers which have a positive or negative impact on the development of DR and are associated with DR prognosis. In addition, we further used

ELISA to revalidate these differential metabolites critical to the course of DR.

2 Methods

2.1 Study participants and study design

We conducted this case-control study, which was registered on May 13th, 2022, and included diabetic patients at Peking University People's Hospital Ophthalmologic Center from June 1st, 2021, to May 1st 2022. A total of 530 samples with type 2 diabetes were screened and a cohort of 110 samples was recruited. This case-control study was approved by the Ethical Committee of Peking University People's Hospital (Approval Number: 2021PHB112-001). This research adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to study enrollment. To match clinical parameters between case and control subjects, the control subjects (n = 27) were healthy individuals, the T2DM group (n = 27) included samples with a diagnosis of type 2 diabetes for at least 10 years with no clinical signs of DR, while DR cases including NPDR group (n = 28) and PDR group (n = 28) were type 2 diabetes samples with clinical signs of DR. In this study, the control group (n = 27), T2DM group (n = 27), NPDR group (n = 28) and PDR group (n = 28) were respectively and randomly divided into 9 control, 9 T2DM, 10 NPDR and 10 PDR samples for targeted metabolomics research and the other samples included control group (n = 18), T2DM group (n = 18), NPDR group (n = 18) and PDR group (n = 18) were conducted for ELISA test (Figure 1).

2.2 Diabetic retinopathy phenotyping

In accordance with Early Treatment Diabetic Retinopathy Study (ETDRS) criteria, DR was graded into three categories: no DR, NPDR or PDR (16, 17). All participants were diagnosed upon dilated fundus examination by two retina specialists. Presence of DR was confirmed and documented with color fundus photography, fluorescein angiography (FA) and optical coherence tomography (OCT), classifying study eyes as NPDR (n = 28) or PDR (n = 28) eyes. Color fundus photography and fluorescein angiography (FA) were obtained with FF 540 Plus (Carl Zeiss Meditech, Jena, Germany) or Optos 200Tx (Optos plc, Dunfermline, Scotland, UK). Optical coherence tomography (OCT) was performed with RTVue XR Avanti (Optovue, Fremont, CA, USA) or Cirrus HD-OCT 5000 (Carl Zeiss Meditec Inc, Dublin, CA, USA). Two or more ophthalmologists evaluated the DR status based on the results of the exams to avoid potential diagnosis bias. If there was discordance between the evaluators, they reviewed the images and agreed on the final interpretation. Participants with following situation would be excluded: (1) presence or history of other eye diseases (retinal degeneration, glaucoma, active ocular inflammation etc.); or history of intraocular surgery (vitreoretinal surgery, intravitreal injection of anti- vascular endothelial growth factor (VEGF) or other drugs, laser therapy) or trauma; (2) cancer, infectious disease, hyperuricemia, inherited metabolic diseases, mental disorder, heart failure, severe hypertension, acute myocardial infarction, stroke or any other severe chronic systemic disease; (3) corneal and lens pathologies that prevent a clear view of the fundus. Only those following none of the exclusion criteria for both cases and controls were potential participants.

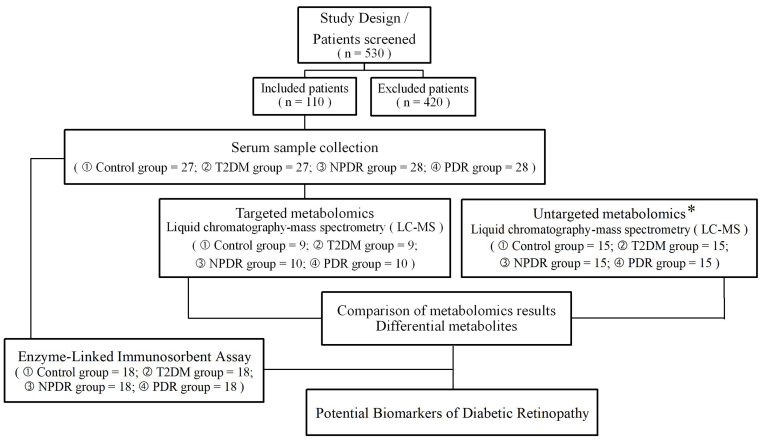

FIGURE 1
An overview of the metabolomics analysis workflow, and the inclusion and exclusion flowchart of the case-control study. * The sample collection and testing in untargeted metabolomics were conducted in our previous study. T2DM, type 2 diabetes mellitus; DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

2.3 Data collection and definitions

All of the participants' medical history and relevant personal history, including age, sex, duration of DM, past medical history, current status of smoking and alcohol consumption, duration of diabetes, treatment history, clinical and laboratory measurements, medication history and disease status were obtained. All participants underwent a physical examination. Blood pressure and body mass index (BMI) measurements were recorded. Blood laboratory tests taken on the closest date (within 3 days) to blood draw including fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), serum creatinine (SCr), hemoglobin A1c (HbA1c), and blood urea nitrogen (BUN) were measured using standard automated assays and were recorded in the electronic case report form.

2.4 Sample preparation for metabolomic study

After at least 8 hours of overnight fasting, 6 mL of venous blood samples were collected under complete aseptic precautions from each study participant with tubes and stored at 4°C. The serum was separated by centrifugation at 3000 rpm for 10 min (4°C) within 30 minutes to separate plasma from whole blood, then the plasma was transferred into a 1.5 mL sterile tube and stored at −80°C ultra- low temperature freeze immediately. Well-trained professional technicians would then carry out further measurements.

2.5 Targeted metabolomics analysis

Targeted quantitative metabolomics analysis was performed on the Biocrates P500 platform using the MxP500 Quant kit (Biocrates Life Science AG, Innsbruck, Austria). Thawed frozen plasma samples (10 µL) were transferred to a 56-well plate, dried under a nitrogen stream and added 5% phenylisothiocyanate (PITC) solution for derivatization. After 1 hour of incubation in the dark, the samples were dried for two hours under nitrogen stream. The filtered extracts (obtained before adding 300 µl of extraction solvent and mixing at 450 rpm for 30 min) were collected by centrifugation at 600 rpm for 10 minutes for subsequent analysis after further dilution.

Metabolites which were extracted on a MetLMS system (Biocrates Life Science AG, Innsbruck, Austria) were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) and flow injection analysis-tandem mass spectrometry (FIA-MS/MS) using multiple reaction monitoring to detect the analytes. Five microlitres of diluted sample extract was used for the LC-MS/MS in positive and negative mode and injected onto a Biocrates® MxP® Quant 500 UHPLC column (Biocrates® Part No.: 22005) at 50 °C using solvent A (water containing 0.2% formic acid) and solvent B (acetonitrile containing 0.2% formic acid). For the FIA-MS/MS, twenty microlitres of the diluted sample extract (diluted in the Biocrates MS Running Solvent) was used for flow injection analysis via tandem-mass spectrometry (FIA-MS/MS) acquisition in positive mode. LC–MS/MS and FIA-MS/MS analysis were performed using a SCIEX Triple QuadTM 6500+ system (Sciex, Darmstadt, Germany) and Acquity H-Class ultra-high performance liquid chromatograph system (Waters).

2.6 Untargeted metabolomics analysis

In our previous untargeted metabolomics study (13), we used Vanquish UHPLC system (ThermoFisher, Germany) with an Orbitrap Q ExactiveTM HF mass spectrometer (Thermo Fisher, Germany) for untargeted metabolomic analysis. The raw data files generated by UHPLC-MS/MS (Ultra High-Performance Liquid Chromatography coupled to Tandem Mass Spectrometry) were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. Normalized data was used to predict molecular formulas based on additive ions, molecular ion peaks, and fragment ions. Peaks were matched with the mzCloud, mzVault and MassList database for accurate qualitative and relative quantitative results. Statistical analysis was performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS version 6.6). As with targeted metabolomics, $P < 0.05$ was considered statistically significant. Fold change (FC) ratios > 1.2 and < 0.833 were used to indicate significantly up- and down-regulated differential metabolites, respectively. Detailed information is presented in the GitHub page (https://github.com/zoe19930939/metabolomic2022.github.io.git). Through untargeted metabolomics, we compared the differential metabolites that met the above conditions with the differential metabolites of targeted metabolomics to determine the key metabolites that appeared in both targeted and untargeted metabolomics.

2.7 The detection method of ELISA

The level of Cit, EPA and IAA for each sample were measured using an ELISA kit (CEA505Ge, CEO122Ge and CEA737Ge, Wuhan CLOUD-CLONE CORP. technology Co., Ltd., China). And the level of CDCA for each sample was measured using an ELISA kit (MET-5008, Cell Biolabs Inc., San Diego, USA). Manufactures instructions were followed for each kit.

2.8 Statistical analysis

Descriptive statistics for demographic and clinical variables of study population were used. Analysis of variance (ANOVA) was used to compare means of normally distributed data with homogeneity of variances. Chi-square test was used for analysis of categoric data (e.g., gender and presence of comorbidities). Wilcoxon rank sum test was performed to compare age, diabetes duration and biochemical parameters. Multiple linear regression was adopted to analyze the differential metabolites between groups and introduce dummy variable to analyze the influence of groups on dependent variables. P-value < 0.05 was considered statistically significant.

The raw data from targeted metabolomics analysis were analyzed in MetILMS version Oxygent-DB110-3005 (Biocrates Life Science AG, Innsbruck, Austria). R statistical software (vision 3.5.2) was used for statistical analysis and visualization of the results. P < 0.05 was considered as statistically significant. Fold change (FC) ratios > 1.2 were considered to indicate up-regulation, and FC ratios < 0.833 were considered to indicate down-regulation. Orthogonal partial least squares- discriminant analysis (OPLS-DA), a volcano map and heat map were used as complementary approaches to identify metabolic features that distinguish different stages of DR samples from controls. Receiver operating characteristic (ROC) curve analysis indicated that the area under the ROC curve (AUC), 95% CI and the AUC ≥0.8 were considered good assessments of the utility of a biomarker.

For detailed information, please refer to: https://github.com/zoe19930939/metabonomic2022.github.io.git.

3 Result

3.1 Baseline characteristics

Of the 123 subjects recruited in the study, clinical data and samples were collected from 110 subjects who gave consent and completed ophthalmologic exams. The mean age of the participants was 66.3 years, the median duration of diabetes mellitus was 16.5 years, and 47.4% of all participants were females. Among a total of 110 participants who underwent ophthalmologic assessment, 27 were T2DM samples with no sign of DR (mean age of 65.75 ± 7.64 years, 39.3% males), 28 were NPDR samples (mean age of 68.72 ± 9.31 years, 69.0% males), 28 were PDR samples (mean age of 63.59 ± 6.97 years, 55.2% males) and 27 were controls (mean age of 67.18 ± 7.77 years, 46.4% males). Samples and controls with no significant differences in clinical characteristics except for blood urea nitrogen and the presence or absence of DR were selected. The demographic characteristics of the study population are shown in Table 1.

TABLE 1 Demographics, comorbidities and serum test results across groups.

Subjects, n	Control 27	T2DM 27	NPDR 28	PDR 28	P-value	P^a	P^b	P^c	P^d	P^e	P^f
Age Mean ± SD	67.18 ± 7.77	65.75 ± 7.64	68.72 ± 9.31	63.59 ± 6.97	0.107	0.913	0.891	0.348	0.516	0.749	0.083
Gender, n (%) Male	13 (46.4%)	11 (39.3%)	20 (69.0%)	16 (55.2%)	0.132						
BMI	23.86 ± 3.11	24.28 ± 3.65	24.88 ± 3.19	24.92 ± 2.92	0.563	0.963	0.646	0.614	0.903	0.883	1.000
Diabetes duration, y	0	10.54 ± 5.19	15.10 ± 7.95	23.83 ± 8.42	<0.001	<0.001	<0.001	<0.001	0.045	<0.001	<0.001
FPG (mm/L)	5.58 ± 0.71	7.40 ± 1.68	7.77 ± 2.09	8.69 ± 3.54	<0.001	0.021	0.003	<0.001	0.928	0.155	0.433
HbA1 (mm/L)	5.11 ± 0.59	6.99 ± 1.06	7.22 ± 1.04	7.46 ± 0.93	<0.001	<0.001	<0.001	<0.001	0.798	0.243	0.764
HDL-c (mm/L)	1.47 ± 0.36	1.28 ± 0.27	1.22 ± 0.26	1.20 ± 0.32	0.005	0.097	0.015	0.007	0.898	0.788	0.995
LDL-c (mm/L)	3.18 ± 0.86	3.00 ± 0.77	2.70 ± 1.17	2.79 ± 0.88	0.227	0.888	0.231	0.403	0.641	0.837	0.986
SCr (mm/L)	73.75 ± 17.50	70.1 ± 14.24	78.00 ± 19.70	95.14 ± 44.46	0.005	0.976	0.937	0.021	0.749	0.006	0.087
TG (mm/L)	1.55 ± 0.59	1.37 ± 0.58	1.83 ± 1.17	1.93 ± 1.49	0.172	0.916	0.743	0.532	0.347	0.192	0.986
BUN (mm/L)	5.04 ± 1.32	5.75 ± 1.46	6.08 ± 1.77	7.83 ± 3.11	<0.001	0.588	0.245	<0.001	0.932	0.002	0.010
HTN%	42.8%	42.9%	58.6.0%	65.5%	0.213						
Treatment					0.226						
OAD	—	14	10	6							
SII	—	5	6	7							
OAD + SII	—	8	12	15							

For age, diabetes duration, FPG, HbA1c, HDL-c, LDL-c, SCr, TG and BUN the mean and standard deviations are presented, and comparisons were made by Wilcoxon rank sum test. Gender, rates of comorbidities and treatment of diabetes were compared by X2 test. T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, Hemoglobin A1c; HDL-c, High density lipoprotein- cholesterol; LDL-c, Low density lipoprotein-cholesterol; SCr, serum creatinine; TG, triglycerides; BUN, blood urea nitrogen; HTN, hypertension; OAD, oral antidiabetic drug; SII, subcutaneous insulin injection. P^a, P-value of control subjects versus T2DM samples. P^b, P-value of control subjects versus NPDR samples. P^c, P-value of control subjects versus PDR samples. P^d, P-value of T2DM samples versus NPDR samples. P^e, P-value of T2DM samples versus PDR samples. P^f, P-value of NPDR samples versus PDR samples.

3.2 Plasma metabolite differences between subjects grouped by different DR status

In the targeted metabolomics datasets, the OPLS-DA model with supervised methods in Figure 2 showed that all four groups were clearly separated, which indicated the significant metabolic differences between each group. The principal component analysis (PCA) model for samples collected from the 4 isolates of sample data is shown in Figure 2. Clustering heatmap showed the relationship between the metabolite content clustering between groups. The identified metabolites in the controls, T2DM, NPDR and PDR groups showed distinguishable clusters in groups, even though the sample clusters overlapped slightly (Figure 3). UHPLC-MS/MS of targeted metabolomics was used to investigated and analyze 541 metabolites in the plasma samples of control subjects and different DR stages, of which 201 biomarkers significantly distinguished. According to the changes of these differential metabolites at different DR stages, 41 of these metabolites were considered as the potential markers to explain the key period variability in DR development. They were classified into 12 subcategories, of which glycerophospholipids had the highest percentage (31.7%) (Figure 4). To identify the metabolites responsible for these separations, variable importance in the projection (VIP), fold changes (FC) and p-value were mainly used. The VIP value is an important parameter for detecting potential biomarker candidates that reflects the correlation of the metabolites with different biological states. In our study, VIP values > 1.0 of OPLS–DAs were used. For evaluating statistical significance, $p < 0.05$ derived from t-test was applied. The relative metabolite levels were converted into FC which is the ratio of each metabolite to the mean of all biological repeat quantitative values between groups. FC > 1.2 and < 0.833 were used respectively to indicate the significantly up-regulated and down-regulated differential metabolites.

3.3 Potential biomarkers for targeted metabolomics in critical periods of DR development

3.3.1 T2DM Versus NPDR

Compared with T2DM and NPDR groups, 65 of the total 201 differential metabolites were detected, of which 57 biomarkers were higher in T2DM group, while the other 8 were lower (Figure 5A). Of the 41 metabolites we identified that distinguish critical period metabolites in DR development, 26 showed in this comparison group. Compared with T2DM group, the serum levels of alpha-aminobutyric acid (AABA), lactic acid, IAA, octadecanecarnitine and fatty acid 20:1 in NPDR group were higher, while the serum levels of Cit, taurocholic acid (TCA), carnitine, hexanoylcarnitine, cholesterol ester (CE) 16:1, 4 PCs (C32:1, C32:2, C36:6 and C42:4) and 12 triglycerides (TC) were lower.

3.3.2 T2DM Versus PDR

The 48 of total 201 differential metabolites were found in T2DM versus PDR groups. Thirty-six of the differential metabolites were higher in T2DM group and the others were lower than PDR group (Figure 5B). Compared with PDR and T2DM groups, we found 28 differential metabolites in the critical periods of DR. The serum levels of beta-aminobutyric acid (BABA), 1-MH and phenylalanine betaine of amino acid, TCA of bile acid, p-cresol sulfate, acylcarnitine (C18:2) and fatty acid 20:1 in PDR group were higher than T2DM group. And the serum levels of CE 16:1, CE 22:5, 3 LPAs (C16:1, C26:0 and C28:1), butyrylcarnitine, 5 PCs (C32:1, C32:2, C34:4, C36:6 and C42:4) and 12 triglycerides in PDR group were lower.

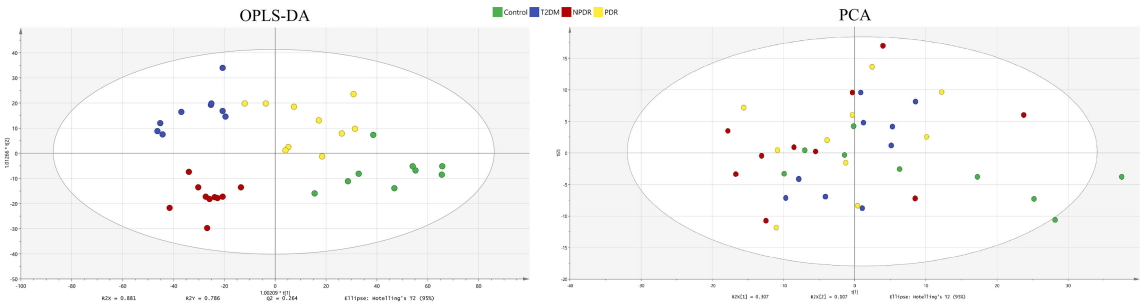

FIGURE 2
OPLS-DA model and PCA model. The four groups were well separated in the OPLS-DA score plot (R2X = 0.881, R2Y = 0.786 and Q2 = 0.264.) The PCA model for samples collected from 4 isolates of sample data. Green dot: control. Blue dot: T2DM sample. Red dot: NPDR sample. Yellow dot: PDR sample. OPLS-DA, orthogonal partial least squares- discriminant analysis; PCA, principal component analysis; T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

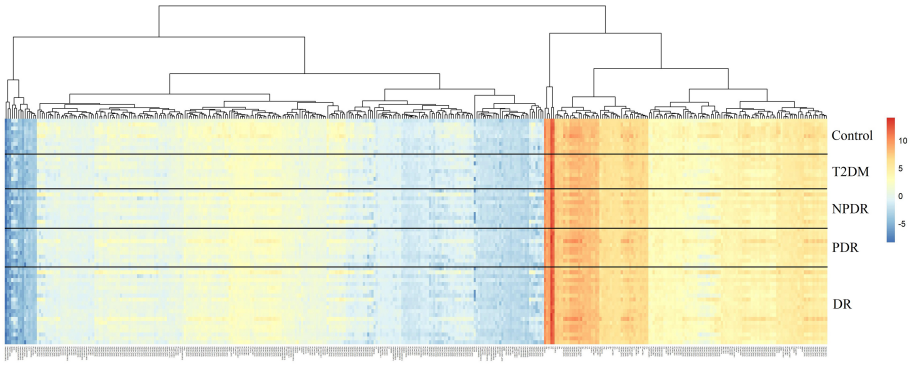

FIGURE 3
Cluster analysis showed that the identified metabolites were clearly grouped into controls, T2DM, NPDR and PDR sample clusters with high repeatability and the resulting data were reliable and logical. The distinctness of each group in the right and center could clearly be seen, and the blending of the groups were shown in the lefts. Significant metabolic features increased (red) or decreased (blue) compared with the others group. T2DM, type 2 diabetes mellitus; DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

3.3.3 T2DM Versus DR (including NPDR and PDR)

In the comparison of T2DM and DR groups, 56 of the 201 differential metabolites were detected, of which the serum levels of 41 biomarkers in DR group were higher in T2DM group, while the serum levels of the other 15 biomarkers were lower in T2DM group (Figure 5C). Furthermore, in the 41 discriminating metabolites we identified contributed to the critical periods of DR development, as 31 of which could be found in this comparison group. Compared with T2DM group, the serum levels of 5 amino acid-related metabolites (BABA, 1-MH, 3-methylhistidine (3-MH), AABA and phenylalanine betaine), lactic acid, IAA, acylcarnitine (C18:2), and fatty acid 20:1 were higher, and the levels of TCA, carnitine, hexanoylcarnitine, butyrylcarnitine, CE 16:1, 5 PCs (C32:1, C32:2, C34:4, C36:6 and C42:4) and 13 triglycerides were lower in DR group.

3.3.4 NPDR Versus PDR

Through targeted metabolomics, a total of 31 of the 201 differential metabolite were found in the comparison of NPDR

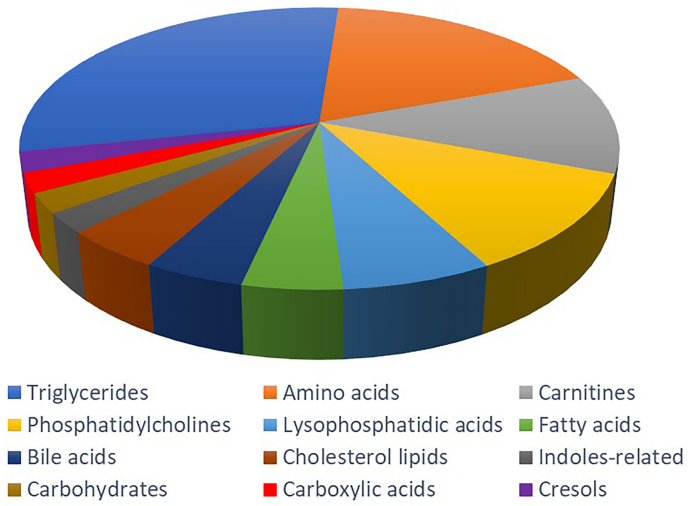

FIGURE 4
Metabolite classification analysis. The pie chart shows the 41 metabolites, including triglycerides (31.70%), amino acid (19.51%), carnitine (12.20%), phosphatidylcholine (12.20%), lysophosphatidic acid (7.32%), fatty acid (4.88%), bile acid (4.88%), cholesterol lipids (4.88%), indoles-related metabolites (2.44%), carbohydrate (2.44%), carboxylic acid (2.44%) and cresol (2.44%).

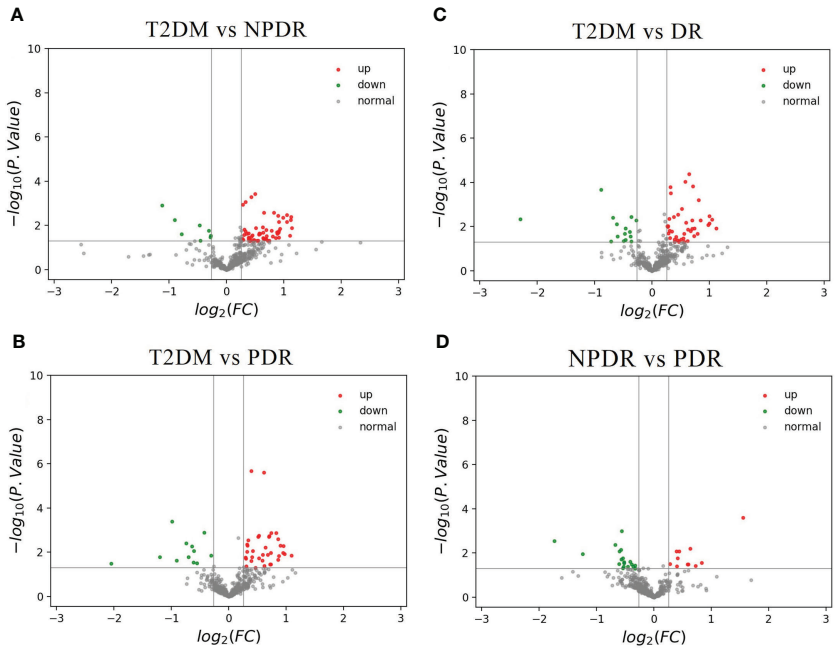

FIGURE 5
(A) The volcano map of the log2 (FC) and −log10 (p-value) showed that 65 differential metabolites were significantly different between T2DM samples (n = 9) and NPDR samples (n = 10). Compared with NPDR samples, 57 metabolic features were significantly increased (red dots) and 8 metabolic features were significantly decreased (green dots) in T2DM samples. **(B)** The volcano map of the log2 (FC) and −log10 (p-value) showed that 48 differential metabolites were significantly different between T2DM samples (n = 9) and PDR samples (n = 10). Compared with PDR samples, 36 metabolic features were significantly increased (red dots) and 12 metabolic features were significantly decreased (green dots) in T2DM samples. **(C)** The volcano map of the log2 (FC) and −log10 (p-value) showed that 56 differential metabolites were significantly different between T2DM samples (n = 9) and DR samples (n = 20). Compared with DR samples, 41 metabolic features were significantly increased (red dots) and 15 metabolic features were significantly decreased (green dots) in T2DM samples. **(D)** The volcano map of the log2 (FC) and −log10 (p-value) showed that 31 differential metabolites were significantly different between NPDR samples (n = 10) and PDR samples (n = 10). Compared with PDR samples, 12 metabolic features were significantly increased (red dots) and 19 metabolic features were significantly decreased (green dots) in NPDR samples. FC, fold change; T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

and PDR, of which serum levels of 12 key metabolites were higher in NPDR group, while 19 were lower (Figure 5D). And 10 of 41 critical metabolites were further detected. Serum levels of 5-aminovaleric acid and p-cresol sulfate in PDR group were higher than that of NPDR group, while serum levels of alanine, CDCA, IAA, butyrylcarnitine, EPA, CE 22:5 and 2 PCs (C34:4 and C36:6) in PDR group were lower than that of NPDR group.

3.4 Intercomparison and validation of the result of targeted and untargeted metabolomics

After searching as many differential metabolites as possible through targeted metabolomics, we compared the results with our previous untargeted metabolomics results and found that a total of 7 biomarkers in the critical period of DR, including Cit, IAA, 1-MH, PCs, hexanoylcarnitine, CDCA and EPA were detected in both targeted and untargeted metabolomic analyses (Table 2). In targeted metabolomics, we found that the serum level of Cit in NPDR group were lower than those in the T2DM group (AUC = 0.794, Figure 6A), whereas our previous untargeted metabolomic analysis showed that the serum Cit level in DR group were higher than in T2DM group. In terms of serum IAA, we found that serum IAA levels in NPDR group and DR group were significantly higher than those in T2DM group (AUC = 0.867, Figure 6B and AUC = 0.767, Figure 6C) through targeted metabolomics. Our previous untargeted metabolomics studies also observed the higher IAA level in DR group than in T2DM group. In addition, we further found that the serum level of IAA was significantly lower in PDR group than in NPDR group (AUC = 0.780, Figure 6D) in the targeted metabolomics, which has not been reported. In our study, we found that serum level of 1-MH in PDR group were significantly higher than those in T2DM group in both targeted metabolomic (AUC = 0.744, Figure 6E) and untargeted metabolomic analyses, and were also significantly higher in DR group compared with T2DM group in targeted

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TABLE 2 Metabolites of DR critical period identified from targeted and untargeted metabolomic profiling (13).

	Targeted Metabolomics				Untargeted Metabolomics			
	T2DM vs. NPDR	T2DM vs. PDR	T2DM vs. DR	NPDR vs. PDR	T2DM vs. NPDR	T2DM vs. PDR	T2DM vs. DR	NPDR vs. PDR
L-Citrulline								
Ratio	1.501	—	—	—	—	—	0.619	—
P-value	0.008						0.036	
Indoleacetic acid								
Ratio	0.536	—	0.653	1.516	—	—	0.681	—
P-value	0.001		0.009	0.007			0.013	
1-Methylhistidine								
Ratio	—	0.683	0.771	—	—	0.486	—	—
P-value		0.031	0.028			0.031		
Hexanoylcarnitine								
Ratio	1.396	—	1.385	—	—	1.594	—	—
P-value	0.044		0.043			0.008		
Chenodeoxycholic acid	—	—	—	9.819	0.344	—	0.316	—
Ratio				0.011	0.011		< 0.001	
P-value								
Eicosapentaenoic acid								
Ratio	—	—	—	1.784	—	1.667	—	1.948
P-value				0.010		< 0.001		0.012
Phosphatidylcholines								
PC C16:0	—	—	—	—	—	0.680	0.753	0.805
Ratio						< 0.001	0.003	0.014
P-value								
PC C32:1								
Ratio	1.427	1.440	1.434	—	—	—	—	—
P-value	0.012	0.002	0.002					
PC C32:2								
Ratio	1.366	1.632	1.494	—	—	—	—	—
P-value	0.027	0.002	< 0.001					
PC C34:4								
Ratio	—	1.862	1.592	1.321	—	—	—	—
P-value		0.005	0.016	0.039				
PC C36:6								
Ratio	1.309	1.773	1.506	1.354	—	—	—	—
P-value	0.041	0.001	0.007	0.009				
PC C42:4								
Ratio	1.260	1.252	1.256	—	—	—	—	—
P-value	0.001	0.005	<0.001					

PC, Phosphatidylcholine; T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; DR, diabetic retinopathy.

metabolomic analysis (AUC = 0.728, Figure 6F). In our previously diabetic retinopathy-untargeted metabolomics, the level of PC C16:0 in serum was significantly positively correlated with the severity of DR. Conversely, in targeted metabolomic analyses, the serum of PCs (including PC C32:1, C32:2, 34:4, C36:6 and C42:2) were inversely proportional to the degree of progression of DR. In terms of serum carnitine levels, both carnitine and hexyl carnitine (AUC = 0.839, Figure 6G) in the NPDR group were lower than those in the T2DM group in our targeted and untargeted metabolomics analysis. These results were the same as the comparison between the DR group and the T2DM group (AUC = 0.767, Figure 6H). Besides, the serum level of butylcarnitine in PDR group was significantly lower than that in NPDR group and T2DM group in targeted metabolomics, while according to our previously untargeted metabolomic analysis, the serum levels of caproylcarnitine and palmitoylcarnitine in PDR group were significantly lower than those in T2DM group, and the serum palmitylcarnitine level was even lower than that in NPDR group. Furthermore, in our previous studies, the serum level of CDCA in NPDR group and DR group were significantly higher than that in T2DM group by untargeted metabolomics, but in targeted metabolomics, we found that the level of serum CDCA in PDR group was lower compared with NPDR group (AUC = 0.740, Figure 6I). In addition, we also found that the level of serum UDCA in PDR group and DR group were significantly

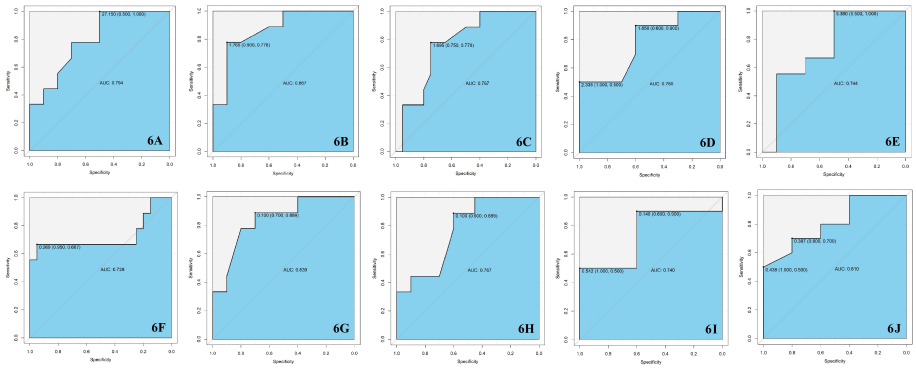

FIGURE 6
(A) The serum level of Cit in T2DM group was higher than NPDR group with the AUC = 0.794. **(B)** The serum level of IAA in T2DM group was lower than NPDR group with the AUC = 0.867. **(C)** The serum level of IAA in T2DM group was lower than DR group with the AUC = 0.767. **(D)** The serum level of IAA in NPDR group was higher than PDR group with the AUC = 0.780. **(E)** The serum level of 1-MH in T2DM group was lower than PDR group with the AUC = 0.744. **(F)** The serum level of 1-MH in T2DM group was lower than DR group with the AUC = 0.728. **(G)** The serum level of hexanoylcarnitine in T2DM group was higher than NPDR group with the AUC = 0.839. **(H)** The serum level of hexanoylcarnitine in T2DM group was higher than DR group with the AUC = 0.767. **(I)** The serum level of CDCA in NPDR group was higher than PDR group with the AUC = 0.740. **(J)** The serum level of EPA in NPDR group was higher than PDR group with the AUC = 0.810. AUC, area under the curve; T2DM, type 2 diabetes mellitus; DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; Cit, L-Citrulline; IAA, Indoleacetic acid; 1-MH, 1-Methylhistidine; CDCA, Chenodeoxycholic acid; EPA, Eicosapentaenoic acid.

lower than T2DM group through untargeted metabolomics, and the serum level of TCA in PDR group, NPDR group and DR group were all significantly lower than T2DM group through targeted metabolomics. However, UDCA and TCA have not been found in both targeted and untargeted metabolomics so far. Regarding targeted and untargeted metabolomics, we found that the serum EPA level in PDR group was significantly lower than that in NPDR group (AUC = 0.810, Figure 6J). In addition, in untargeted metabolomics, serum DHA levels in PDR group were significantly lower than those in NPDR group and T2DM group, respectively.

3.5 Revalidate the differential metabolites by ELISA

As the ELISA kits for the detection of 1-MH, hexanoylcarnitine and PC are unavailable commercially, and metabolomics is considered to be the best method for detecting small molecule metabolites such as carnitine and fatty acids currently, we only re-validated the other 4 differential metabolites, including Cit, IAA, CDCA and EPA.

We performed ELISA test on the serum of 18 T2DM samples, 18 PDR samples, 18 PDR samples and 18 controls (Table 3). We found that the serum Cit levels in controls, T2DM, NPDR, PDR and DR (NPDR and PDR) groups were 286.68 ± 85.17 pg/ml, 500.11 ± 276.85 pg/ml, 180.52 ± 110.30 pg/ml, 169.37 ± 141.23 pg/ml and 174.94 ± 126.83 pg/ml, respectively. Compared with controls, NPDR, PDR and DR groups, the serum level of Cit in T2DM group was significantly higher (P = 0.001, < 0.001, < 0.001 and < 0.001). This result was similar to the targeted metabolomics which indicated the serum level of Cit in T2DM group was higher than NPDR group. The serum levels of IAA were 70.47 ± 23.80 ng/ml, 53.33 ± 16.66 ng/ml, 83.48 ± 20.29 ng/ml, 93.16 ± 37.28 ng/ml and 88.32 ± 30.40 ng/ml in controls, T2DM, NPDR, PDR and DR (NPDR and PDR) groups respectively. Compared with T2DM, we found that the serum levels of IAA in NPDR, PDR and DR group were significantly higher (P = 0.014, < 0.001 and < 0.001), which conformed to the results of our targeted and untargeted metabolomics results. The IAA serum level in NPDR group was higher than T2DM group from targeted metabolomics, and the IAA serum levels in DR group were higher than T2DM group from both targeted and untargeted metabolomics. However, although we found higher serum levels of IAA in NPDR group than in PDR group in targeted metabolomics, this was not detected in the ELISA test. Through ELISA test, the serum levels of CDCA in controls, T2DM, NPDR, PDR and DR (NPDR and PDR) groups were 1651.27 ± 577.20 nmol/L, 2650.36 ± 469.08 nmol/L, 2022.46 ± 710.91 nmol/L, 826.51 ± 667.37 nmol/L and 1426.30 ± 888.79 nmol/L. The serum level of CDCA in T2DM group was significantly higher than those in controls, PDR and DR groups, respectively (P = 0.001, <0.001 and <0.001). Compared with PDR group, the serum level of CDCA was also higher in controls and NPDR group (P = 0.013 and < 0.001) which was consistent with our targeted metabolomics results. The results of ELISA test in EPA showed that the serum EPA levels in controls, T2DM, NPDR, PDR and DR (NPDR and PDR) groups were 312.45 ± 47.91 pg/ml, 263.19 ± 38.20 pg/ml, 256.05 ± 27.69 pg/ml, 196.51 ± 22.55 pg/ml and 226.28 ± 39.03 pg/ml, respectively. Serum EPA levels in the control group were significantly higher than those in the others

TABLE 3 Validate the Differential Metabolites in control, T2DM, NPDR, PDR and DR groups by ELISA.

Subjects, n	L-Citrulline(pg/ml) 18	Indoleacetic Acid(ng/ml) 18	Chenodeoxycholic Acid (nmol/L) 18	Eicosapentaenoic Acid (pg/ml) 18
Control	286.68 ± 85.17	70.47 ± 23.80	1651.27 ± 577.20	312.45 ± 47.91
T2DM	500.11 ± 276.85	53.33 ± 16.66	2650.36 ± 469.08	263.19 ± 38.20
NPDR	180.52 ± 110.30	83.48 ± 20.29	2022.46 ± 710.91	256.05 ± 27.69
PDR	169.37 ± 141.23	93.16 ± 37.28	826.51 ± 667.37	196.51 ± 22.55
DR	174.94 ± 126.83	88.32 ± 30.40	1426.30 ± 888.79	226.28 ± 39.03
P-value	< 0.001	< 0.001	< 0.001	< 0.001
P^a	0.001	0.359	0.001	0.001
P^b	0.284	0.633	0.570	< 0.001
P^c	0.193	0.116	0.013	< 0.001
P^d	0.122	0.185	0.830	< 0.001
P^e	< 0.001	0.014	0.093	0.979
P^f	< 0.001	< 0.001	< 0.001	0.008
P^g	< 0.001	< 0.001	< 0.001	< 0.001
P^h	> 0.999	0.838	< 0.001	< 0.001
P^i	> 0.999	0.975	0.050	0.054
P^j	> 0.999	0.975	0.061	0.054

T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; DR, diabetic retinopathy. P^a, P-value of control subjects versus T2DM samples. P^b, P-value of control subjects versus NPDR samples. P^c, P-value of control subjects versus PDR samples. P^d, P-value of control subjects versus DR samples. P^e, P-value of T2DM samples versus NPDR samples. P^f, P-value of T2DM samples versus PDR samples. P^g, P-value of T2DM samples versus DR samples. P^h, P-value of NPDR samples versus PDR samples. P^i, P-value of NPDR samples versus DR samples. P^j, P-value of PDR samples versus DR samples.

groups (P = 0.001 in control vs. T2DM, P < 0.001 in control vs. NPDR, P < 0.001 in control vs. PDR and P < 0.001 in control vs. DR). And compared with PDR and DR groups, the serum level of EPA was higher in T2DM group (P < 0.001 and 0.008). Of note, the level of EPA in serum was also higher in NPDR group than in PDR group (P < 0.001), which was fully consistent with the results from both targeted and untargeted metabolomics.

3.6 Analysis the differential metabolites between groups by multiple linear regression

After comparison of targeted and untargeted metabolomics results, and cross-validation by Elisa, we mainly indicated that the DR stage showed lower serum level of Cit and higher serum level of IAA compared with the T2DM stage, and the serum levels of CDCA and EPA in PDR stage were significantly lower than NPDR stage. However, since age, diabetes duration, FPG, and HbA1 based on Table 1 may influence the significance of differential metabolites between groups, we performed multiple linear regression analysis. We found that after adjusting age, diabetes duration, FPG and HbA1 of patients in each group, the serum levels of IAA were statistically significant in NPDR vs Control (P = 0.035), T2DM vs NPDR (P < 0.001) and NPDR vs PDR (P = 0.001), the serum CDCA level was statistically significant in NPDR vs PDR (P = 0.028), and the serum Cit level was also of borderline statistical significance in T2DM vs NPDR (P = 0.056). However, the serum levels of EPA showed no statistically significant difference among the groups. (Table 4 and Figure 7).

4 Discussion

Metabolomics as a powerful approach for studying pathophysiological processes can be divided into untargeted metabolomics and targeted metabolomics. Untargeted metabolomics reflects the multivariate dynamic changes of all metabolite levels as much as possible which is helpful to identify unknown disease mechanisms, while targeted metabolomics more accurately detects and analyzes specific metabolites in biological samples. Since the results of metabolomics tests are influenced by the selection of different test methods and sample characteristics (e.g., race, gender, age, dietary structure, environment, and drugs), the results of studies in different regions have a certain degree of difference. At present, there have been many metabolomics studies on diabetic retinopathy, among which the serum (1, 18–21), vitreous and aqueous humor (22–25) of samples are mainly used as samples for metabolomics detection. However, since sampling of the vitreous and the aqueous humor are invasive and their repeatability of detection are difficult, greatly limit their value in studying the metabolomics of DR. In contrast, serum remains the best sample choice for metabolomic testing. To our knowledge, we are the first double comparison study of untargeted metabolomics and

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TABLE 4 The multiple linear regression result of the differential metabolites between groups.

Groups	Indoleacetic acid		Chenodeoxycholic acid		L-Citrulline		Eicosapentaenoic acid	
	Unstandardized Coefficients B (95.0% CI)	P-value	Unstandardized Coefficients B (95.0% CI)	P-value	Unstandardized Coefficients B (95.0% CI)	P-value	Unstandardized Coefficients B (95.0% CI)	P-value
Age	-0.021 (-0.055, 0.014)	0.229	0.006 (-0.020, 0.032)	0.635	0.034 (-0.546, 0.614)	0.905	0.013 (-0.003, 0.030)	0.105
Diabetes duration	0.013 (-0.023, 0.048)	0.472	-0.008 (-0.035, 0.018)	0.535	-0.450 (-1.050, 0.150)	0.136	0.000 (-0.017, 0.016)	0.967
FPG	0.057 (-0.028, 0.143)	0.181	0.042 (-0.022, 0.106)	0.193	1.019 (-0.432, 2.470)	0.162	-0.003 (-0.044, 0.038)	0.881
HbA1	-0.176 (-0.423, 0.070)	0.154	-0.102 (-0.287, 0.082)	0.266	-1.907 (-6.083, 2.269)	0.358	0.019 (-0.098, 0.136)	0.746
T2DM vs. Control	0.187 (-0.604, 0.978)	0.632	-0.415 (-1.007, 0.178)	0.163	-11.207 (-24.613, 2.199)	0.098	0.220 (-0.156, 0.596)	0.241
T2DM vs. NPDR	1.323 (0.663, 1.984)	< 0.001	0.197 (-0.298, 0.692)	0.423	-10.923 (-22.122, 0.276)	0.056	0.087 (-0.227, 0.401)	0.577
T2DM vs. PDR	0.305 (-0.436, 1.046)	0.407	-0.283 (-0.838, 0.272)	0.306	-2.905 (-15.463, 9.654)	0.640	-0.131 (-0.483, 0.221)	0.452
NPDR vs. Control	-1.136 (-2.188, -0.084)	0.035	-0.612 (-1.399, 0.176)	0.123	-0.284 (-18.108, 17.541)	0.974	0.134 (-0.366, 0.633)	0.589
NPDR vs. PDR	-1.019 (-1.583, -0.454)	0.001	-0.480 (-0.903, -0.057)	0.028	8.018 (-1.555, 17.592)	0.097	-0.218 (-0.486, 0.050)	0.108
PDR vs. Control	-0.118 (-1.290, 1.055)	0.839	-0.132 (-1.010, 0.746)	0.761	-8.302 (-28.177, 11.573)	0.400	0.351 (-0.206, 0.909)	0.207

T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; FPG, fasting plasma glucose; HbA1c, Hemoglobin A1c; CI, confidence interval.

targeted metabolomics by LC-MS in Chinese with different severities of DR and using ELISA to further cross-validate the key metabolites.

We found that in both targeted and untargeted metabolomic assays, Cit, IAA, 1-MH, PCs, hexanoylcarnitine, CDCA and EPA were detected and showed significantly different between groups of samples with different degrees of DR. After further analysis, we mainly concluded that samples in the DR stage showed lower serum level of Cit and higher serum level of IAA compared with samples in the T2DM stage, while during the progression of diabetic retinopathy, the serum levels of CDCA and EPA in PDR stage were significantly lower than NPDR stage. Although these biomarkers were regarded as differential metabolites in both targeted and untargeted metabolomics, there were still differences in their expression levels between groups.

Under normal circumstances, L-arginine and Cit can be converted into each other through various pathways in humans. L-arginine is metabolized by nitric oxide synthase (NOS) to produce nitric oxide and Cit. Cit can be recycled back to L-arginine by argininosuccinate synthase and argininosuccinate lyase (26). Due to the dysregulation of nitrogen metabolites-related pathways in DR samples, particularly maladjusted arginine and citrulline, the serum Cit level in diabetic samples is disordered, which leads to the dysfunction of retinal endothelial cell (1). The result of untargeted metabolomics analysis in our study indicated that the serum level of Cit was higher in DR group compared with T2DM group, which was consistent with the results of the serum non-targeted metabolomics of DR by Sumarriva K et al. (1) and also similar to a vitreous untargeted metabolomics of DR in 2018 (27). However, we found that in targeted metabolomics, NPDR group had lower serum Cit level than T2DM group (AUC = 0.794, Figure 6A), which also have been reported in the global amino acid profile of DR status (28). In addition, a targeted metabolomics report in 2021 also showed that serum Cit level was lower in samples with impaired fasting glucose (IFG) compared to normal individuals (18). Therefore, we speculated that the difference in serum Cit levels between targeted and untargeted metabolomics may be due to the choice of different metabolomics methods and the comparison between different DR stages in each study.

Tryptophan is the main precursor of IAA synthesis, which is similar in chemical structure to IAA, and its degradation products include indoxyl sulfate and indoleacetic acid (29) (30). According to KEGG global metabolic network, tryptophan metabolism is one of the most disturbed metabolic pathways. Several studies have already demonstrated dysregulation of serum tryptophan level in DR samples (28) (18, 20, 31). However, the changes of IAA in serum level of DR have been rarely reported. Kong et al. suggested that increasing the levels of tryptophan and IAA and decreasing the level of indole acetaldehyde by drugs may modulate tryptophan metabolism to protect the nervous system of T2DM samples (32). Besides, there was a human trial showed that oral IAA can reduce blood glucose in diabetic samples (33). In 2022, Guo et al. observed that compared with T2DM samples, the serum level of

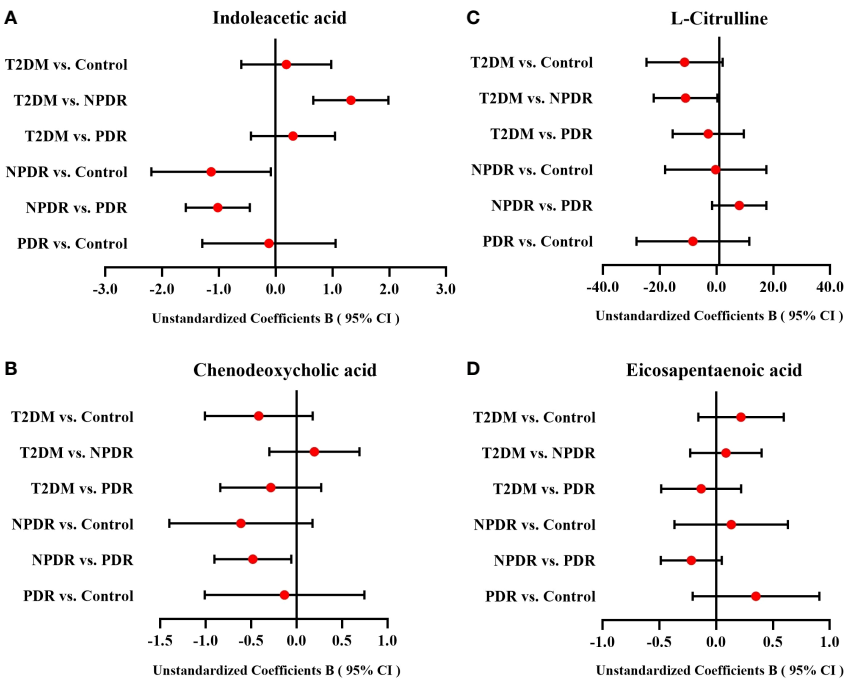

FIGURE 7
The forest plots of IAA, CDCA, Cit and EPA among groups. After multiple linear regression analyses of age, diabetes duration, FPG and HbA1 for differential metabolites between groups, **(A)** the serum level of IAA between T2DM group and NPDR group, NPDR group and control group, and NPDR group and PDR group were statistically significant, **(B)** the serum level of CDCA between NPDR group and PDR group was also statistically significant, and **(C)** the serum Cit level between T2DM group and NPDR group was of borderline statistical significance. **(D)** The serum levels of EPA which showed no statistically significant difference among the groups. CI, confidence interval; T2DM, type 2 diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; Cit, L-Citrulline; IAA, Indoleacetic acid; 1-MH, 1-Methylhistidine; CDCA, Chenodeoxycholic acid; EPA, Eicosapentaenoic acid.

IAA in DR samples was significantly higher (34), which was consistent with our results of targeted and untargeted metabolomics. Through targeted metabolomics, we found that serum IAA levels in both NPDR and DR groups were significantly higher than those in T2DM group (AUC = 0.867, Figure 6B and AUC = 0.767, Figure 6C), and our previous untargeted metabolomics studies also observed higher serum IAA level in DR group than T2DM group. Notably, we further found that the serum level of IAA in PDR group was significantly lower than NPDR group in targeted metabolomics (AUC = 0.780, Figure 6D), which has not been reported before.

Bile acids (BAs) are cholesterol catabolites that are mainly synthesized in the liver (35). In alternative pathways of BA synthesis, CDCA and cholic acid (CA) as two primary BA are formed predominantly in the pericentral hepatocytes over several steps from cholesterol (36) (37). Studies have shown that BAs can be involved in glucose metabolism and energy regulation. Some of the level of serum BAs are also affected by drugs and other biochemical indicators. In 2021 a cross-sectional study comparing serum bile acid levels in T2DM samples and non-T2DM samples, Mantovani et al. concluded that the level of serum CDCA in T2DM samples was not affected by statin, metformin, or incretins, and was significantly different from nondiabetic control individuals and T2DM samples with no drug therapy. In addition, level of serum TCA was lower in T2DM samples treated with incretins, and was significantly correlated with fasting glucose levels, while serum triglycerides were only significantly correlated with UDCA (38). UDCA was considered to have neuroprotective effects in retinal diseases (39) (40), and its inhibitory activity against to VEGF-induced pro-angiogenic and pro-permeabilization of human retinal microvascular endothelial cells was confirmed in the oxygen-induced retinopathy (OIR) mouse models (41). The conclusions of above studies are similar to our findings. In untargeted metabolomics, the serum level of CDCA in NPDR group and DR group was significantly higher than that in T2DM group, but in targeted metabolomics, we found that the level of serum CDCA in PDR group was lower compared with NPDR group (AUC = 0.740, Figure 6I). In addition, through untargeted metabolomics, we also found that the level of serum UDCA in PDR group and DR group was significantly lower than T2DM group, and the serum level of TCA in PDR group, NPDR group and DR group were significantly lower than T2DM group

through targeted metabolomics. These results were similar to the previous studies on the relationship between T2DM and BAs.

Omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) as essential fatty acids in the human diet mainly including EPA and DHA which are expressed at high levels in the retina (42, 43). They have the function of regulating many biological processes, such as regulating vascular endothelial growth factor (VEGF) expression, preventing pericyte loss from retinal vascular inflammation, maintaining retinal capillary structure and integrity, and inhibiting retinal neovascularization (44–46). Numerous studies have found that n-3 LC-PUFAs are reduced in diabetic samples' retina and serum, and researchers believed that increasing the intake of n-3 LC-PUFAs could help reduce the occurrence and development of DR (Saenz 47–50), which has been proved in diabetic animal models (45, 51). Our DR metabolomics study also confirmed the above statement. The serum level of EPA in the PDR group was significantly lower than NPDR group in both targeted (AUC = 0.810, Figure 6J) and untargeted metabolomic analysis. Besides, in the untargeted metabolomics, we also observed that the serum level of DHA in the PDR group was significantly lower compared with the NPDR group and the T2DM group, respectively. We hypothesize that DR could lead to damage of retinal vascular endothelial cells, excessive production of intracellular reactive oxygen species (ROS) and imbalance of VEGF expression, thus affecting the changes of n-3 LC-PUFAs levels in serum. However, a 2018 metabolomic study of NPDR samples found there was no difference in the serum levels of DHA and EPA in the NPDR group compared with health control (52). The other study on the relationship between diabetic retinopathy and lipid metabolism suggested that n-6 PUFAs (including linoleic acid, γ-linolenic acid, eicosadienoic acid, dihomo-γlinolenic acid and arachidonicacid) may be the potential indicators in distinguishing DR from other T2DM samples (53).

The results of our targeted metabolomics were basically consistent with those of ELISA in Cit, IAA, CDCA and EPA, while the results of untargeted metabolomics were only partially the same as those of ELISA in IAA and EPA. We believed that this may be due to the difference in metabolomics detection methods and the different thresholds of metabolites that can be detected by different metabolomics. Untargeted metabolomics is the identification of metabolites by comparing the obtained data with the standard product database after quantitative analysis. Targeted metabolomics, on the other hand, is to identify the specific target metabolite more precisely through kits of known metabolites. Therefore, through this comparative study of targeted and untargeted metabolomics, we believe that the accuracy of targeted metabolomics for the expression of the metabolites in serum is higher than that of untargeted metabolomics to a certain extent. To sum up, since the results of targeted and untargeted metabolomics were not completely consistent, in order to have a more comprehensive understanding of the occurrence and development of DR samples, the results of both methods should be evaluated at the same time, and the analysis and judgment should be made based on the sample's current DR stage and the levels of differential metabolites in the sample's serum.

In this study, after comparing targeted and untargeted metabolomics, we found that some of the major differential metabolites seemed not to appear in only one comparison group. Therefore, by performing the cross-validation of differential metabolites of Elisa, we further concluded that the serum level of Cit might be one of the main differential metabolites between T2DM stage and DR stage, and the serum level of CDCA might be a key biomarker which was significant different between NPDR stage and PDR stage. However, IAA and EPA need further discussions to clarify their meanings in different DR stages. In terms of IAA, through the double verification of metabolomics and Elisa, we found that the serum levels of IAA in both DR and NPDR groups were significantly higher than that in the T2DM group. However, since the DR group is composed of the NPDR group and PDR group, and the natural course of DR is mostly from the T2DM stage without DR to the DR stage including NPDR and PDR, we can reasonably infer that IAA may be the main differential metabolite that mainly appears in the progression of T2DM stage to DR stage. In terms of EPA, similarly, through metabolomics and Elisa, we found that compared with the PDR group, the serum IAA levels were significantly higher in both NPDR group and T2DM group, but there was no statistical difference between the NPDR group and T2DM group. As mentioned above, for most of the T2DM patients, the regular process of the DR progression is from the manifestation of non-DR to NPDR, and finally to PDR. Therefore, in contrast, we ultimately indicated that during the progression of DR, the change in the serum level of EPA from NPDR period to PDR period was more markedly different. In summary, we speculated that the serum levels of Cit and IAA might be the main differential metabolites between the periods of T2DM and DR, while the serum levels of CDCA and EPA might be the key biomarkers between the NPDR and PDR stages.

Several advantages can be found in the current study compared with previous studies. First, we used the widely targeted metabolomics approach to detect serum metabolites at different stages of DR samples, compared to our previous high resolution untargeted metabolomic results, and re-validated the differences of these biomarkers in different critical periods of DR. Compared with the traditional studies that only used untargeted metabolomic or targeted metabolomic analyses, our study seems be more comprehensive and accurate in comparing targeted and untargeted metabolomics and obtaining predefined metabolites. Secondly, participants in this study were recruited from the same region and were matched for age and gender to avoid potential confounding factors, making it more comparable between DR groups and controls. Thirdly, different from the grouping method of previous diabetic retinopathy metabolomics

studies, we divided the participants into 4 groups, including the controls, T2DM, NPDR and PDR, and further analyzed the differences between the DR group and T2DM group. To our knowledge, this study is the first to confirm that IAA, 1-MH and CDCA are closely related to the progression of DR in humans. The changes in serum levels of Cit, PC, caproylcarnitine and EPA in our findings were not completely the same to those in previous studies. Thus, more rigorous and well-designed studies are needed to validate our findings. In addition, we further adopted multiple linear regression to analyze the differential metabolites between groups. After adjusting age, diabetes duration, FPG and HbA1, we found that the serum level of IAA, CDCA and Cit were still statistically significant in certain groups which were consistent with our results of metabolomics analysis and Elisa. However, the serum level of EPA was not statistically significant different among the groups. We speculated that this may be due to the small sample size of the study which may affect the reliability of the results to some extent. We are recruiting more participants based on the results of this study, and we plan to proceed with a larger clinical trial to obtain more meaningful and accurate results and further validate our results. In conclusion, our findings may provide some new clues and ideas for research on the prevention and development of DR, have the opportunity to better identify early NPDR samples in T2DM samples, and help distinguish NPDR samples from PDR samples. The results of all these findings will likely contribute to better management of DR samples in the future and hopefully provide a foundation for future research on the screening of new therapeutic targets for DR. In addition, our findings may provide clinicians with a new insight into making better treatment decisions for DR samples.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethical Committee of Peking University People's Hospital (2021PHB112-001). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZW drafted the work, revised it critically for important intellectual content, and provided approval for publication of the content. JT revised and reviewed the work critically for important intellectual content. EJ, CR and SL analyzed and interpreted data for the work. Material preparation and data collection were performed by LZ, YZ and YC. JW, WZ and MZ reviewed, edited and supervised the work. LH and JQ agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Associations between psycho-behavioral risk factors and diabetic retinopathy: NHANES (2005–2018)

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Introduction: Diabetes mellitus (DM) and diabetic retinopathy (DR) increase the global burden. Since their pathogenesis is complex, it is necessary to use the biopsychosocial model to discover the most effective strategies. The study is aimed to investigate the psycho-behavioral factors of DR and confirm the discrepancies from previous studies.

Research design and methods: The study comprised seven cycles of cross-sectional data of the National Health and Nutrition Examination Survey (NHANES) from 2005–2006 to 2017–2018. Samples of DM were selected from this complex multi-stage probability sample and divided into the non-DR and DR groups, where 4,426 samples represented 18,990,825 individuals after weighting. This study comprehensively explored the biological, social, and psychological risk factors of DR, among which the biological factors included blood pressure, blood routine, HbA1c%, blood glucose, the duration of DM, family history, comorbidities, and treatment methods. Social aspects include gender, education, income, insurance, smoking, drinking, sleep habits, and recreational activities. The Patient Health Questionnaire-9 (PHQ-9) was used to assess the psychological state. Taylor series regression was used to examine the connection between factors and DR.

Results: Men accounted for 55.5% of the DR group ($P = 0.0174$). Lymphocyte count, insulin treatment, heart failure, stroke, liver condition, and renal failure showed significant differences in DR ($P < 0.05$). The incidence of depression in DR was 40.5%. Mild to moderate depression [odds ratio was associated with DR [(OR) = 1.37, 95% confidence interval (CI): 1.06—1.79], but there was no statistical difference in severe depression (OR = 1.34, 95% CI: 0.83–2.17). Although ≤6 h of sleep was associated with DR (OR = 1.38, 95% CI: 1.01–1.88), we found no statistical differences in alcohol consumption, recreational activities, or sedentary time between the two groups in our current study ($P > 0.05$).

Conclusions: The biological risk factors of DR are significant. It showed that stroke is associated with DR, and retinal exams have the potential value as a screening tool for the brain. Besides, psycho-behavioral risk factors

of DR should also be paid attention. Our study highlights that mild and moderate depression and ≤6 h of sleep are distinguishably associated with DM complicated with DR. It indicates that psycho-behavioral risk factors confer a vital influence on diabetic health care and DR.

KEYWORDS

diabetic retinopathy, depression, sleep duration, NHANES, recreational activity

Introduction

Diabetic retinopathy (DR) is one of the most prevalent microvascular complications of diabetes mellitus (DM) and a leading cause of blindness globally (1). With the global incidence of DM quadrupling over the past four decades (2), the visual impairment caused by DM has snowballed. According to reports, the global prevalence of DR is 34.6%, with 1 in 10 people suffering from sight-threatening DR (3). Apart from vision loss, DR also signifies a heightened risk of life-threatening systemic vascular complications (4) and causes a significant financial burden (3), making DR a serious public health problem. For instance, the number of DR is predicted to reach 16 million by 2050, and diabetes-related vision loss is expected to cost US $500 million annually (2).

The DR Barometer Report Global Findings 2020 (5) estimated that seven out of ten individuals with diabetic-related ocular complications had experienced days of poor physical and mental health. Increasing studies implied that patients with DR are prone to depression, loss of confidence, and other adverse emotional reactions (6, 7) and behaviors (8). Is there a positive correlation between psycho-behavioral risk factors and DR?

Prior research established that DM is more likely to suffer from depression (9), and the occurrence of DR accompanied by psychopathy, particularly depression, is growing year by year. In Australia, vision-threatening DR and moderate or severe vision impairment were considered independent risk factors for increased depressive symptoms in adults with DM (10). According to several studies, depression is linked to unhealthy behaviors, lack of exercise, and neuroendocrine changes, all of which may accelerate the progression of DM and its complications (11, 12). The progression of chronic diseases is often a process of mutual influence and interaction of biopsychosocial factors. The co-occurrence of a psychiatric condition and unhealthy behaviors are related to worse glycemic control, higher incidences of poor metabolic outcomes, and a higher risk of complications in DM. Tobacco or alcohol consumption, lack of physical activity, sedentary lifestyle (13), poor medication adherence, and self-management all aggravate retinopathy (14). Notably, potential psychological stress could accelerate the progression of DR through common biologic pathways (8). As a result, the American Diabetes Association (ADA) suggests a routine screening for depression in patients with diabetics (15). It also suggests that it is necessary to pay attention to the influence of psycho-behavioral factors on DM and its related visual impairment.

However, due to the relatively small sample size, specialized populations or hospitals, and short follow-up, the research on the exact association between psycho-behavioral factors and DR is still limited, and the results are controversial (16). A systematic review concluded that the incidence and progression of DR had a bidirectional relationship with depression (7), though others disagree (17). Other studies showed that DR had no effect on depression (18–21) and health-related quality of life scores in patients (19, 22). Hence, whether depression is a risk factor for DR and whether behavioral patterns reduce the risk of DR deterioration remain to be fully explored.

Therefore, we updated the incidence of DR and depression among DM in the US population and analyzed the risk factors of DR based on the data published in the NHANES 2005–2018. We aimed to enhance the psycho-behavioral assessment of DR by revealing the association between DR and psychological state and behavioral factors, which would provide further concern in DR screening guidelines to minimize the rate of DR-related blindness and improve the quality of life (3).

Research design and methods

Study population

The National Health and Nutrition Examination Survey is a national cross-sectional survey that represents the non-institutionalized civilian resident US population and is distinguished by its complex sampling strategy. Data are collected from a home interview and standardized physical mobile examination centers (MECs) released in 2-year cycles. The National Center approved the study procedures of the Health Statistics Research Ethics Review Board. Participants were given informed consent before any data was collected and the NHANES protocol details are available in the website of the Centers for Disease Control and Prevention (CDC) (23). We applied seven cycles from 2005 to 2018 to assess the association between clinical, psychological, and behavioral factors and diabetic visual impairment. Respondents aged 18 or older

with DM were selected. Patients with incomplete depression screening questionnaires and pregnant women during the interview were excluded.

Assessment of DM and diabetic visual impairment

Diabetes was defined as having a fasting plasma glucose (FPG) level of more than 126 mg/dL or a glycated hemoglobin (HbA1c) level of at least 6.5% or having a physician-diagnosed diagnosis of DM (13, 24, 25). Diabetic visual impairment was confirmed using a dichotomous, self-reported item, indicating that a doctor had informed the respondent that diabetes had affected their eyes.

Assessment of biological factors

We focused on the diabetic-related clinical variables of DR, encompassing the family history of DM, the duration of DM, the last time of the dilated eye examination, the frequency of self-monitoring blood glucose, the level of blood glucose control, and the therapy of DM. Body mass index (BMI) was computed by dividing kilogram weight by height in meters squared. Urine albumin and creatinine levels were measured with a fluorescent immunoassay and the Jaffe rate reaction method, respectively.

We chose the presence of comorbidities based on previous research: (1) hypertension (2) hypercholesterolemia; (3) heart disease covering congestive heart failure (CHF), coronary heart disease (CHD), angina, and myocardial infarction (MI); (4) stroke; (5) cancer (any); (6) renal failure; and (7) hepatic failure.

Assessment of psychological factor

The psychological status of patients has been assessed using a scale for 14 years. The Patient Health Questionnaire-9 (PHQ-9), as one of the scales for depression state, can assess the psychological state of patients to a certain extent and is regarded as a unified way and method to measure the psychological state of patients by the database (26). PHQ-9 adds the scores of each item and ranges from 0 to 27. In fact, 5, 10, 15, and 20 points represent thresholds demarcating the lower limits of mild, moderate, moderately severe, and severe depression. A score between 0 and 4 is considered normal, and scores higher than 15 signify a possible clinical level of depression (26). Subsequently, the depression categories were collapsed into three groups: no depression, mild or moderate depression, and major depression in this setting.

Assessment of social factors

In addition to gender, education, income, and insurance status, the social factors also include indicators of smoking, drinking, sleeping, and exercise habits of patients with DM. Smoking and drinking were classified according to the questionnaire. The NHANES guidance defines recreational activities as those lasting longer than 10 min per week and that do not include exercise caused by work or traffic. Recreational activities are further classified as vigorous recreational activities or moderate recreational activities. High-intensity activities, such as running and basketball, can produce breathing and an increase in heart rate, but moderate-intensity exercises, such as walking, cycling, or swimming, cause only slight breathing and an increase in the heart rate. In addition, high-intensity exercise corresponded to two times the moderate-intensity exercise score, which was surveyed by uniformly trained professional interviewers. We collected data about the duration of different types of recreational activities, sedentary time, and sleeping habits. Moreover, we paid attention to both physician-diagnosed and self-reported sleeping disorders.

Assessment of covariates

We analyzed the risk factors related to DR as comprehensively as possible from the biological, psychological, and social perspectives in univariate analysis.

Clinical biological indicators, together with traditional social indicators, should be included as confounding background factors in the multivariate analysis from the biopsychosocial model, with a focus on the long-term impact of psychological and social behavior factors on chronic diseases. In other words, clinical biological factors should be used as a baseline and gradually calibrated, allowing for a more accurate comparison of indicators of psychological and social-behavioral factors between the DM and DR groups.

A household interview was undertaken to get information such as age, gender, race, marital status, education, and income. The non-Hispanic Asian subgroup was not available before 2011 due to the survey design; individuals were categorized into five groups (27). Thus, we added the extra group for Non-Hispanic Asians into the fifth group to keep the research consistent.

Statistical analysis

Our statistical analysis was divided into three parts to investigate the connection between risk factors and DR. First, the participants were divided into two groups based on whether or not they had DR. Differences in baseline characteristics between the groups were compared *via* *t*-tests in continuous variables and $\chi 2$ tests in categorical variables. They were

presented as mean ± standard deviation (SD) and frequency or percentage. The complex sample was then analyzed using univariate and multivariate logistic regression models (28). The variance estimation was performed to determine the relationship between factors and DR. Finally, based on the results of the previous step, four multiple regression models with expanding adjustment were used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) for examining relationships. Additionally, multiple imputations were employed to account for missing data (since triglyceride and cholesterol deficiency rates were >25%, these variables were excluded).

Notably, this complex sampling (23) includes stratified, cluster, multistage sampling, and unequal probability proportional to a measure of size (PPS), and this sampling weight needs to be considered. On the one hand, this design makes it possible to merge more cycles and enables more excellent statistical reliability (WTMEC2YR/7). On the other hand, traditional regression will lead to wrong inference conclusions. In particular, the standard error and CI of parameter estimates may be seriously underestimated, and the probability of class I error in hypothesis testing is much higher. Therefore, the existing research (11, 13, 27) and NHANES tutorials recommend SURVEYMEANS, SURVEYREG, and SURVEYLOGISTIC (29) to achieve statistical description and complex sampling logistic regression analysis. Statistical analysis was executed with SAS 9.4 (SAS Institute, Cary, North Carolina).

Result

Characteristics of participants

About 6,783 patients with DM were enrolled in the NHANES from 2005 to 2018 (Figure 1), excluding those under the age of 18 ($n = 98$), who were pregnant ($n = 13$), and with incomplete PHQ-9 data ($n = 889$). Among the 5,783 initially enrolled respondents, 1,357 were removed due to missing data on whether they had DR. Ultimately, 4,426 unweighted samples were included in the analysis, representing 19 million non-institutionalized US population.

We made the following statistics on the missing values in the final 4,426 samples: missing data were found for education ($n = 9$ [0.1%]), income ($n = 473$ [9.8%]), PIR ($n = 431$ [9.0%]), alcohol consumption ($n = 14$ [0.2%]), family history of DM ($n = 112$ [2.4%]), pupils dilated exam ($n = 37$ [0.7%]), treatment of DM ($n = 8$ [0.1%]), and sleeping trouble ($n = 18$ [0.6%]); none of the missing data of the comorbidities were >1%. Moreover, there were missing values in the duration of DM and frequency of self-monitoring blood glucose ($n = 41$ and 28, respectively). Missing data in the categorized variables are grouped separately, and the totals are unweighted.

Characteristics of variables

Eligible DM was divided into two groups according to visual impairment, with a total of 935 (21.8%) patients having impaired eyes. Selected characteristics were comparable in both groups, and all were weighted proportions by SURVEYMEANS and SURVEYREG modules in SAS 9.4 (29, 30).

The four parts of the risk factors are summarized in Table 1, (more details can be seen in Supplementary Tables 1–4). The mean ages for DM without and with DR were 60.6 (SE, 0.8) and 61.0 (SE, 1.3) years, respectively. Participants with DR were predominately to be men (55.5% vs. 48.8%, $P = 0.0174$) and had a lower poverty/income ratio (35.7% vs. 29.4%, $P = 0.0322$). Although there was no significant difference in insurance coverage between them, the number of private insurance purchases was statistically significant ($P = 0.0019$). In contrast, we did not find significant differences in race, marital status, education level, and income on the sociodemographic part of the baseline.

It seems that diabetic relatives, duration of DM, frequency of self-monitoring blood glucose, pupils dilated exams, and insulin therapy indicated significant differences between the two groups. In addition, the DR group had significantly higher systolic blood pressure (133.3 ± 2.1 vs. 130.6 ± 1.0), blood levels of fasting glucose (171.7 ± 6.8 vs. 153.9 ± 3.3), glycosylated hemoglobin (7.8 ± 0.2 vs. 7.3 ± 0.1), the count of RBC (4.6 ± 0.1 vs. 4.6 ± 0.03), and a significantly higher urine albumin/creatinine ratio (312.3 ± 87.0 vs. 118.7 ± 19.3) (all $P < 0.05$). Otherwise, the count of lymphocytes (2.1 ± 0.1 vs. 2.3 ± 0.1) was significantly lower (all $P < 0.05$). Surprisingly, BMI, waist circumference, and diastolic blood pressure were not significantly different.

The DR group had more comorbidities. It had higher percentages of cardiovascular disease (HF, CHD, and MI), stroke, liver condition, and kidney failure than the non-DR group (all $P < 0.05$). In terms of psychological state, 40.5% of the participants with DR met the depression criteria based on their PHQ-9 questionnaire scores (32.8% vs. 26.8 in mild and moderate, 7.7% vs. 4.8 in severe group, $P = 0.0007$.

In the behavioral features, alcohol consumption and moderate recreational activities showed a significant difference between treatment groups ($P = 0.0145$ and 0.0205, respectively).

Association of biological factors with DR

The estimated ORs of univariate logistic regression and the descriptions carried out by the Chi-square tests were consistent. Factors with a P-value < 0.05 in the univariate analysis were selected for the multivariable analysis using the adjusted OR criterion to retain variables according to the univariate analysis (Figures 2, 3 and Supplementary Tables 5–8). It revealed that female sex (adjusted OR = 0.73, 95% CI: 0.55–0.94), having a low lymphocyte count (adjusted OR = 0.94, 95% CI: 0.89–0.99),

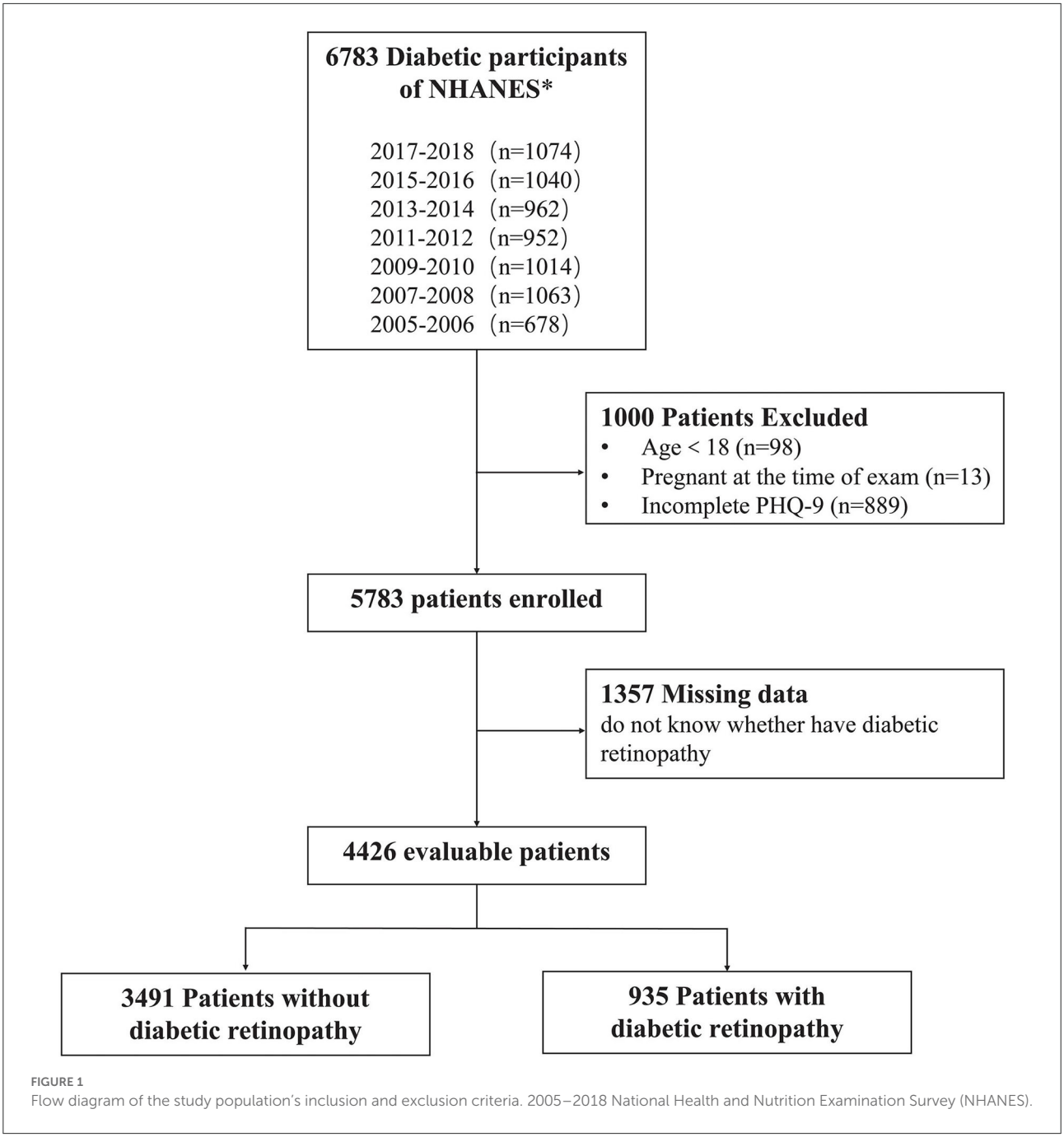

FIGURE 1
Flow diagram of the study population's inclusion and exclusion criteria. 2005–2018 National Health and Nutrition Examination Survey (NHANES).

longer duration of DM (adjusted OR = 1.03, 95% CI: 1.02–1.04), having pupils dilated exam within one month (adjusted OR = 2.11, 95% CI: 1.17–3.79), having received insulin treatment (insulin only, adjusted OR = 2.61, 95% CI: 1.66–4.10; pills and insulin, adjusted OR = 2.48, 95% CI: 1.58–3.88), and having comorbidities (HF, adjusted OR = 1.51, 95% CI: 1.03–2.23; stroke, adjusted OR = 1.47, 95% CI: 1.03–2.08; liver condition, OR = 1.99, 95% CI: 1.40–2.85; and renal failure, OR = 2.36, 95% CI: 1.68–3.33) were significantly associated with DR.

Association of psychological factor with DR

The prevalence of mild and moderate depression of DR was 32.8% (95% CI: 28.6–37.0). Table 2 presents a stratified logistic regression analysis for depression and DR. In both the univariate and multivariate logistic regression models, we found that mild and moderate depression (adjusted OR = 1.38, 95% CI: 1.06–1.78, $P = 0.0160$) were independently associated with DR.

TABLE 1 Baseline characteristics of diabetes in NHANES.

Variables	Total	Diabetes without DR	Diabetes with DR	*P*-value[a]
	Mean or % (95% Cl)	Mean or % (95% Cl)	Mean or % (95% Cl)	
N[b]	4426	3491	935	
Frequency (weighted) [c]	18990825	14857122	4133703	
Sociodemographic variables				
Age (years)	60.7 ± 0.7	60.6 ± 0.8	61.0 ± 1.3	0.5790
Gender (male, %)	50.3 (48.0–52.6)	48.8 (46.2–51.5)	55.5 (50.8–60.3)	0.0174
PIR (%) [d]				0.0322
<1.3 (low)	30.8 (28.2–33.4)	29.4 (26.8–32.1)	35.7 (30.3–41.1)	
1.3–4.9 (medium)	47.1 (44.8–49.5)	48.5 (46.1–50.9)	42.2 (36.6–47.7)	
5 (high)	13.0 (11.2–14.9)	13.4 (11.4–15.4)	11.7 (8.3–15.0)	
Private insurance (%) [e]				0.0019
0	49.4 (46.9–51.9)	47.3 (44.5–50.1)	56.9 (52.1–61.7)	
1	47.5 (45.1–49.9)	49.4 (46.7–52.1)	40.7 (36.0–45.3)	
2	3.1 (2.2–4.0)	3.3 (2.3–4.3)	2.4 (0.8–4.0)	
Clinical variables				
SBP (mmHg)	131.1 ± 0.91	130.6 ± 1.0	133.2 ± 2.1	0.0212
Lymphocyte count ($\times 10^9$/L)	2.3 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	0.0004
Fasting glucose (mg/dL)	157.7 ± 3.0	153.9 ± 3.3	171.7 ± 6.8	<0.0001
HbA1c (%)	7.4 ± 0.1	7.3 ± 0.1	7.8 ± 0.2	<0.0001
ACR (mg/g)	160.9 ± 25.0	118.7 ± 19.3	312.3 ± 87.0	<0.0001
Family history (yes %)	69.7 (67.5–71.9)	68.2 (65.8–70.6)	75.0 (70.5–79.5)	0.0313
Duration of diabetes (years)	11.5 ± 0.46	10.3 ± 0.5	15.7 ± 1.1	<0.0001
Frequency of self–monitoring blood	1.9 ± 0.09	1.8 ± 0.1	2.2 ± 0.2	0.0062
Treatment (%)				<0.0001
Pills only	56.4 (53.9–58.8)	60.5 (57.9–63.2)	41.4 (36.3–46.5)	
Insulin only	13.4 (12.6–15.4)	10.0 (8.4 −11.7)	25.3 (21.2–29.5)	
Pills and insulin	14.0 (12.6–15.4)	11.6 (10.1–13.0)	22.6 (18.8–26.4)	
Neither	16.1 (14.3–17.9)	17.7 (15.7–19.7)	10.5 (6.8–14.3)	
Comorbidities [f]				
HF (yes %)	10.4 (9.1–11.7)	8.6 (7.2–10.0)	17.1 (13.6–20.6)	0.0003
CHD (yes %)	12.0 (10.6–13.4)	10.7 (9.2–12.3)	16.6 (12.8–20.4)	0.0002
Heart attack [e] (yes %)	12.3 (10.7–13.8)	11.3 (9.7–12.9)	15.7 (12.0–19.4)	0.0029
Stroke (yes %)	10.3 (9.0– 11.7)	9.0 (7.7–10.3)	15.2 (11.5–19.0)	<0.0001
Liver condition (yes %)	9.1 (7.7–10.4)	7.8 (6.4–9.3)	13.5 (1.6–10.3)	0.0006
Renal failure (yes %)	9.5 (8.3–10.8)	6.8 (5.7–7.9)	19.4 (15.8–23.0)	<0.0001
Depression (%)				0.0007
None (0 to 4)	66.5 (64.3–68.7)	68.5 (66.0–70.9)	59.5 (55.1–64.0)	
Mild and moderate (5 to 14)	28.1 (25.9–30.2)	26.8 (24.4–29.1)	32.8 (28.6–37.0)	
Severe (≥15)	5.4 (4.5–6.3)	4.8 (3.8–5.8)	7.7 (5.2–10.1)	
Behavioral variables				
Drink (%)				0.0145
Being drinking	11.0 (9.6–12.4)	10.5 (9.1–11.9)	12.6 (9.3–15.9)	
Seldom	46.4 (43.3–49.5)	47.9 (44.6–51.1)	41.0 (35.8–46.3)	
Former	6.7 (5.5–8.0)	6.1 (4.9–7.3)	8.8 (5.7–11.9)	
Never	35.7 (33.0–38.5)	35.2 (32.2–38.2)	37.5 (32.8–42.2)	
Moderate recreational activities [g] (yes %)	34.2 (32.0–36.4)	35.4 (32.9–38.0)	29.8 (25.8–33.8)	0.0205
Sleep hours (%)				0.0054

(Continued)

TABLE 1 (Continued)

Variables	Total	Diabetes without DR	Diabetes with DR	*P*-value[a]
	Mean or % (95% Cl)	Mean or % (95% Cl)	Mean or % (95% Cl)	
<6h	16.2 (14.4–18.0)	15.2 (13.4–16.9)	20.0 (15.7–24.4)	
6 to 8h	65.0 (62.4–67.6)	67.0 (64.5–69.5)	57.8 (52.0–63.6)	
>8h	18.8 (15.9–21.6)	17.8 (15.2–20.4)	22.2 (16.3–28.1)	

Abbreviations: NHANES, National Health and Nutrition Examination Surveys; CI, confidence interval; DR, diabetic retinopathy; GED, General Educational Development; AA, Associate of Arts; PIR, Ratio of family income to poverty level; SBP, systolic blood pressure; HbA1c, glycosylated hemoglobin; ACR, urinary microalbumin/creatinine ratio; it was computed as albumin in milligrams per liter divided by creatinine in grams per liter; HF, Heart failure; CHD, coronary heart disease.
[a]This is a comparison between non–DR and DR adults having diabetes.
[b]The unweighted number of cases.
[c]All cases are weighted to be nationally representative.
[d]PIR was calculated by dividing the family income by the poverty guidelines specific to the survey year. The respondent only reported income as < $20,000 or ≥ $20,000, and the value was not computed.
[e]Number of private insurances covered by Medigap and single service plan.
[f]Doctors or health professionals diagnosed them.
[g]Sports, fitness, and recreational activities exclude the work and transport activities for at least 10 min continuously in a typical week.

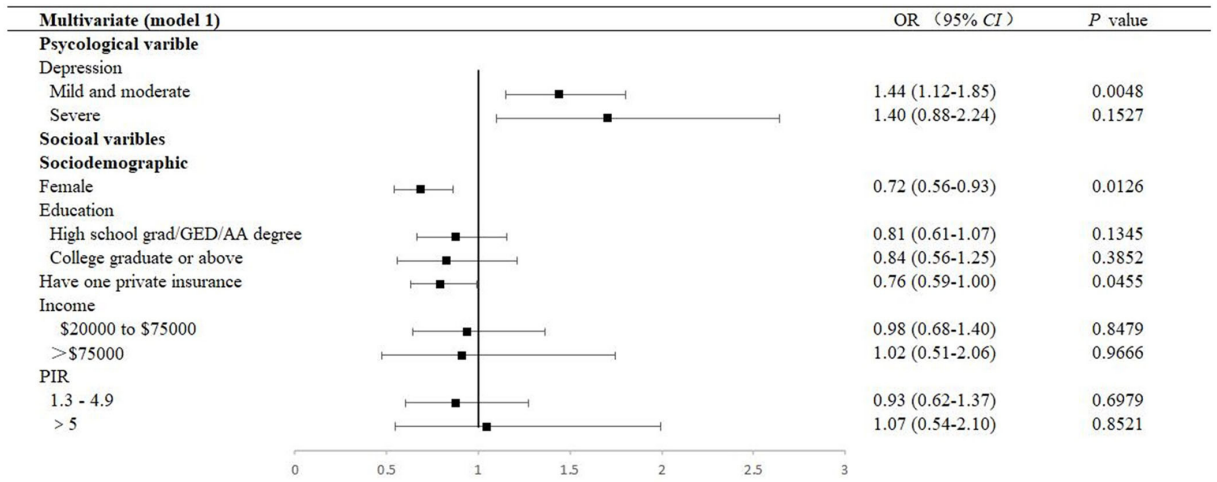

FIGURE 2
Multivariable forest plot of the association between factors and DR (Model 1). Model 1 was performed by adjusting sociodemographic variables. DR, diabetic retinopathy; OR, odds ratios; CI, confidence interval; GED, General Educational Development; AA, Associate of Arts; PIR, Ratio of family income to the poverty level, PIR was calculated by dividing family income by the poverty guidelines specific to the survey year, and the respondent only reported income as < $20,000 or ≥ $20,000, the value was not computed.

However, in adjusted models, severe depression was no longer significant (Figures 2, 3 and Supplementary Figures 1, 2).

Association of behavioral factors with DR

As depicted, 9.3% and 29.8% participated in moderate and high-intensity recreational activities, respectively, among patients with DR. There was an association between behavioral factors, including alcohol consumption and moderate recreational activities (P = 0.0145 and 0.0205, respectively). However, in multivariate analysis, they were not identified as a significant indicator of DR (alcohol consumption, adjusted OR = 1.03, 95% CI: 0.74–1.43, and moderate recreational activities, adjusted OR = 0.98, 95% CI: 0.77–1.26, respectively). At the same time, the mean sleep duration was 7.08 ± 0.23 h a day, with an adjusted OR of 1.38 (95% CI: 1.01–1.88) in those with less than 6 h of sleep and 1.20 (95% CI: 0.88–1.64) in those with more than 8 h of sleep, compared to patients with 6–8 h of sleep (Figure 2, Supplementary Tables 3, 7).

Discussion

The main objective of this study was to investigate the relationship between biological, social, and psychological risk factors and DR. In the analysis of biological factors, our

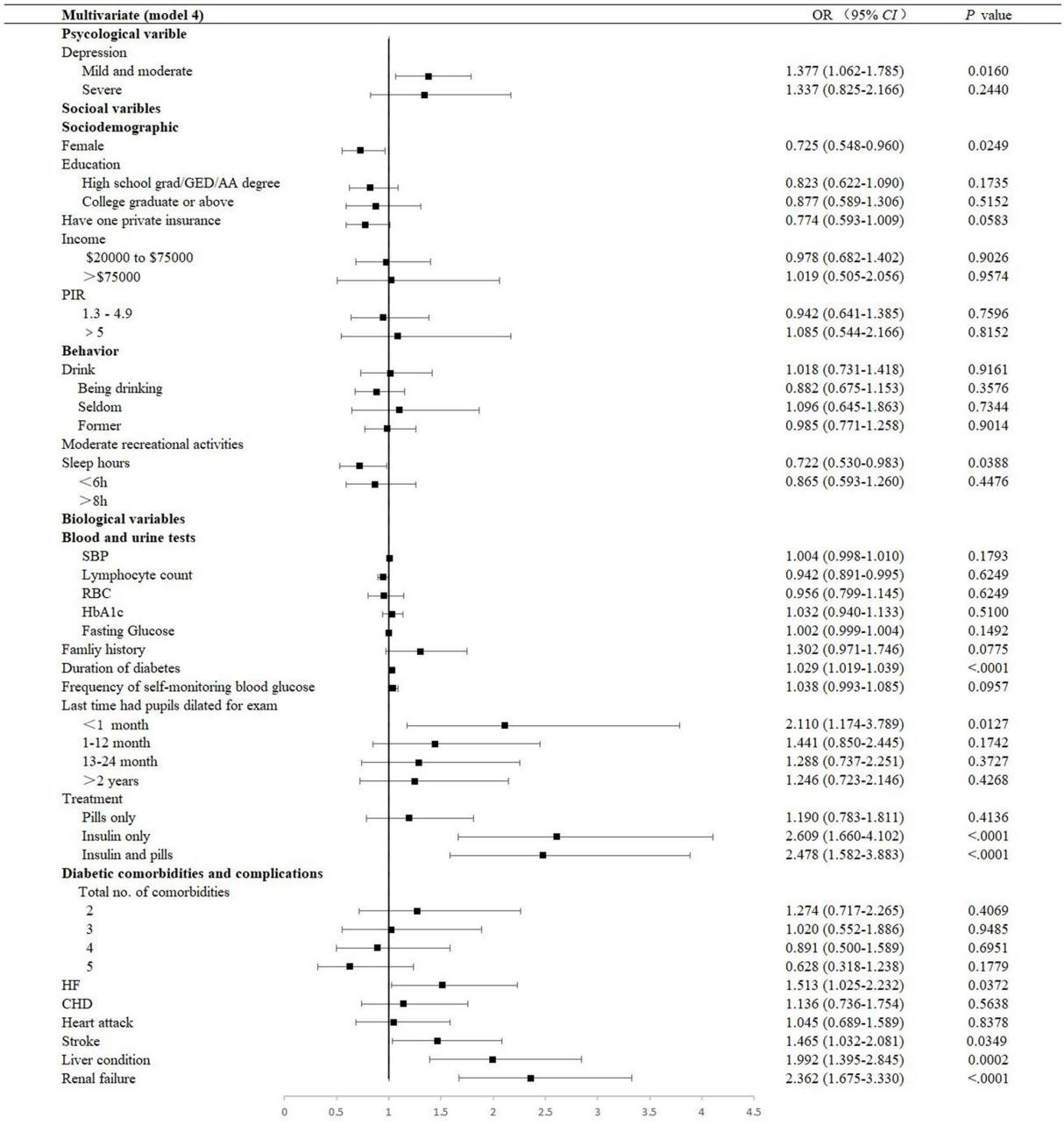

FIGURE 3
Multivariate forest plot of the association between factors and DR (Model 4). Model 4 was performed by adjusting all covariables, including sociodemographic variables, diabetic-related clinical variables, diabetic comorbidities, behavioral variables, and sociodemographic variables. DR, diabetic retinopathy; OR, odds ratios; CI, confidence interval; GED, General Educational Development; AA, Associate of Arts; PIR, Ratio of family income to the poverty level. PIR was calculated by dividing the family income by the poverty guidelines specific to the survey year; the respondent only reported income as < $20,000 or ≥ $20,000, and the value was not computed; SBP, systolic blood pressure; RBC, red blood cell; HbA1c, glycosylated hemoglobin; HF, heart failure; CHD, coronary heart disease.

findings confirmed that stroke was associated with DR, and the strength of the connection was not changed by controlling for confounders. On the contrary, retinal-related exams are expected to become a screening tool for brain lesions. We also found that mild and moderate depressions were independent risk factors for an increased prevalence of retinopathy in adult DM, but severe depression was not in the study of psychological factors. Furthermore, in the study of social behavioral factors, only a short sleep time may affect DR, while other behavioral factors, such as smoking and alcohol

TABLE 2 Associations of depressive symptom severity and DR.

OR (95% CI)	Depressive symptom severity				
	None (0–4)	Mild and moderate (5–14)	*P*	Severe (≥15)	*P*
Proportion (%)[a]	59.5	32.8		7.7	
Model 1[b]	Ref	1.440 (1.149 to 1.804)	0.0017	1.704 (1.100 to 2.640)	0.0173
Model 2[c]	Ref	1.504 (1.184 to 1.910)	0.0009	1.576 (1.006 to 2.470)	0.0473
Model 3[d]	Ref	1.437 (1.118 to 1.846)	0.0048	1.404 (0.881 to 2.238)	0.1527
Model 4[e]	Ref	1.377 (1.062 to 1.785)	0.0160	1.337 (0.825 to 2.166)	0.2377

[a]Prevalence of depressive symptom severity in DR.
[b]Model 1 adjusted for sociodemographic variables (gender, education, income, PIR, and private insurance).
[c]Model 2 adjusted for sociodemographic and diabetic-related clinical variables (systolic blood pressure, lymphocyte count, RBC, HbA1c%, fasting glucose, relatives having diabetes, duration of diabetes, frequency of self-monitoring of blood, the last time had pupils dilated for exams, and treatment).
[d]Model 3 adjusted for sociodemographic, diabetic-related clinical variables, and diabetic comorbidities and complications (the total number of comorbidities, HF, CHD, MI, stroke, liver condition, and renal failure).
[e]Model 4 adjusted for all covariables, including sociodemographic variables, diabetic-related clinical variables, comorbidities and complications, and behavioral variables (drinking, moderate recreational activities, and sleep hours).

consumption, higher recreational activity, and less sedentary activity, had no effect on the progression of retinopathy in patients with diabetics.

It is necessary to use a public database to extensively investigate the risk factors of DR. Early evidence already showed a bidirectional relationship between depression and DM (7, 9). Nonetheless, these investigations have considered diabetes complications as a composite outcome and have given little attention to DR (31). Furthermore, most current studies have a small sample size, and qualitative research is limited to a specific state or ethnicity. To the best of our knowledge, our inquiry is the first to illustrate an overall picture of risk factors for DR using the NHANES database. Out study covers 50 states of the United States (30), has a professional survey organization, which is representative and reliable, and can also update, supplement, and verify the existing conclusions very well.

The indirect impact of depression on clinical risk factors should not be overlooked. A previous study (16) showed that psychological factors can directly affect the occurrence and development of DR by themselves and indirectly affect the process of DR through clinical characteristics. In terms of clinical factors, our findings support previous findings that a longer duration of DM (32–34) and insulin therapy were independently associated with DR (1). These correlations not only highlight that long illness or using insulin may heighten more severe diabetes and a higher risk of complications (35) but also suggest that negative emotion may lead to reduced willingness and worse adherence to treatment (11), poorer glycemic control (21, 36), and then exacerbating DR. Roy et al. (36) and Zou et al. (31) speculated that the hypothalamic-pituitary-adrenal (HPA) axis dysregulation is the cause of depression. It probably affects the pathogenesis of DR *via* hypercortisolemia and insulin resistance. Korczak et al. (37) and Wang et al. (38) added that circulating cytokine and insulin deficiency might explain blood glucose fluctuations, neurodevelopmental abnormalities, and neurocognitive deficits (7). In this study, after adjusting for confounding factors, the lymphocyte count in the DR group was still significantly higher than that in the non-DR group, which also provided evidence for the inflammatory mechanism. However, unlike previous studies (4, 32–34), we did not find a correlation between blood pressure, glycosylated hemoglobin, and DR in the multivariate regression analysis. It is most likely because over half of the respondents did not meet the standard for early morning fasting venous blood collection, which might lead to negative results.

The influence of psychological and behavioral factors on clinical indicators of DR should not be ignored when considering the biological, psychological, and social aspects of chronic diseases. Previous studies found that psychological variables are related to diabetic visual impairment, and the association between DR visual impairment and psychology deserves special clinical attention (10, 16). On this basis, we should fully apply the bio-psycho-social model of chronic disease to investigate the impact of psychological and behavioral factors on DR in the context of clinical factors.

In our study, 21.7% of DM had self-reported DR, which is lower than previous NHANES data (32.8%, 95% CI: 28.6–37.2) (1) but is not significantly different from the globally estimated prevalence of DR in the diabetic population (22.27%, 95% CI: 19.7–25.0) (39). This may be due to the fact that the previous database used retinal images of patients with DM over 40 years old as a reference, and the limitation of age and images improved the estimation of DR prevalence in the diabetic population. Our estimates from 2005 to 2018 indicated that there were 19 million of patients with DM in the United States, 4.1 million of whom were with DR, with the remainder having non-retinopathy, and this represents, on average, 0.1% of the entire population in this country. On the PHQ-9 questionnaire, 1.3 million (32.8%) and 0.3 million (7.7%) participants with DR between 5 and

14 scores and more than 15 scores have different severity of depressive symptoms.

Consistent with the conclusion from the critical meta-analysis of 11 cross-sectional and prospective cohort studies, it was found that depression is significantly associated with DR in patients with type 2 DM (31). Additionally, our results are in line with the results of Krystal Khoo et al., who found that DR is significantly associated with poor psychosocial outcomes after analyzing data from 42 studies (7). A 5-year prospective cohort study found that, for every significant 5-point increase in the severity level of depressive symptoms, the risk of incident DR would increase by approximately 15% (35). However, although our findings, like those of other studies, found that DR was independently linked with mild to moderate depression, we were the first to provide the conclusion that DR did not correlate with severe or major depression, which set us apart from the others. Why is there no correlation between major depression and DR? First, in the process of model adjustment, we noticed that the correlation between major depression status and DR disappeared when adjusting the related comorbidities and behavioral factors. We suspected that this might be due to the reason that DM associated with cardiac insufficiency, liver condition, kidney failure, etc., can also cause or aggravate the severe depressive state. Although the discrepancies between the univariate and multivariate logistic analyses of severe depression may account for collinearity or interaction between other risk factors and major depressive condition, there was a strong association between these comorbidities and DR, and the univariate association between depression and DR disappeared after adjustment for confounders. Second, a cross-sectional study from the Diabetes Management Project in the United Kingdom found that a history of depression or anxiety is the leading cause of DM accompanied by depression, and severe non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) can only explain 19.1% of DM accompanied with depression symptoms (10). When other risk factors, such as the history of depression, were taken into account, major depression was uncorrelated with DR, and we hypothesize that the relationship between DR and severe depression may have been overestimated. Additionally, there may be a correlation between severe depressive state and DR. We have outstanding representative data for the American population, representing 1,027,913 DM with severe depression after weight. However, in terms of the proportion, severe depression accounted for only 5.4% of DM and 7.7% of DR in our study population. It is insufficient to analyze the potential correlation with a small proportion, covering up the possible correlation.

A preliminary study based on NHANES retinal images was presented at the American Stroke Association's International Stroke Congress in 2021. Retinal photographs may indicate an elevated risk of stroke, serving as an early warning signal for stroke prevention and treatment (40). Our study also discovered that DR is associated with stroke, and the association remains after adjusting for confounding factors. Similarly, a population-based cohort study found that retinal microvascular abnormalities provided a window into the brain for over a decade (41). There is a possibility for using retinal images as a screening tool to quickly screen out high-risk DR, compared to time-consuming and expensive magnetic resonance imaging (MRI). Although we examined the association between DR and stroke, more research is needed both on its causality and whether DR with depressive emotion will increase the risk of stroke so that they can have one more tool to screen for depression besides emotional scales.

Behavioral factors frequently coexist with depression to affect DM, and there are substantial differences in recreational activity and sleep between the DR and non-DR groups (13). According to the results, engaging in certain types of physical activities, such as exposure to sunlight and nature and social interactions, is associated with a decreased risk of depression and promotes resistance to stress (42). Studies revealed that moderate-to-vigorous physical activities are associated with DM *via* better glycemic control (43). Sedentary behavior and screen time levels, on the contrary, are associated with risk factors for chronic diseases, such as obesity, high fasting insulin levels, and metabolic syndrome during adolescence (44). A meta-analysis of 22 studies showed that moderate-intensity exercises were beneficial, while sedentary behavior increased DR risk significantly; nevertheless, the evidence was still insufficient (45), and findings are not convincingly consistent (46, 47). Interestingly, our study did not find positive results. We analyzed the association between the frequency of self-reported recreational activity and DR. First, as mentioned in reports (42), not all exercise alleviates depression. For DR with depression, concerns about hypoglycemia and retinal hemorrhage resulted in less high-intensity recreational activity in patients with DM and DR. Second, no difference in the HbA1c level between the DR and non-DR groups may indirectly reflect the same level of exercise between the two groups. Moreover, self-reporting produces a bias in representing actual physical activities, such as exercise types, duration, and intensity of recreational activity levels. All of these may affect the results.

Evidence on associations between sleep duration and DR is still lacking and inconsistent (17, 48). We observed the association between short sleep duration ($\leq$6 h) and DR. It is possible that short and long sleep may have an influence on DR development through the disruption of circadian rhythm or abnormal glucose metabolism (12). Another NHANES study found that sleeping patterns might affect the psychological health (49). Similarly, intermittent hypoxia may increase the levels of vascular endothelial growth factors and other inflammatory cytokines that contribute to the progression of DR (50). However, our study found that sleeping trouble was not significantly associated with DR based on both self-reported and diagnosed data.

We cannot conclude that psychological or behavioral factors enhance the risk of DR or *vice versa*. In addition, interviews could not avoid certain recall biases. Furthermore, since DR diagnosis and severity are inaccurate, objective retinal imaging is required to determine whether depression is related to a specific subtype of DR. Thus, more profound observations and long-term cohort studies are necessary.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical approval for the use of the NHANES survey data from 2005 to 2010, 2011 to 2016 and 2017 to 2018 were obtained from the National Center for Health Statistics (NCHS) Research Ethics Review Board (ERB) through Protocol Number #2005-06, #2011-17 and #2018-01. All participants in this study were provided written informed consent. The information collected by the NCHS was kept with strict confidentiality bound to law. The patients/participants provided their written informed consent to participate in this study.

Author contributions

X-JS was responsible for the statistical analysis and wrote the draft. G-HZ and C-MG assisted to collect the data. Z-YZ and Y-LN assisted to revise this draft. LW and G-RD designed this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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An overview of artificial intelligence in diabetic retinopathy and other ocular diseases

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Artificial intelligence (AI), also known as machine intelligence, is a branch of science that empowers machines using human intelligence. AI refers to the technology of rendering human intelligence through computer programs. From healthcare to the precise prevention, diagnosis, and management of diseases, AI is progressing rapidly in various interdisciplinary fields, including ophthalmology. Ophthalmology is at the forefront of AI in medicine because the diagnosis of ocular diseases heavy reliance on imaging. Recently, deep learning-based AI screening and prediction models have been applied to the most common visual impairment and blindness diseases, including glaucoma, cataract, age-related macular degeneration (ARMD), and diabetic retinopathy (DR). The success of AI in medicine is primarily attributed to the development of deep learning algorithms, which are computational models composed of multiple layers of simulated neurons. These models can learn the representations of data at multiple levels of abstraction. The Inception-v3 algorithm and transfer learning concept have been applied in DR and ARMD to reuse fundus image features learned from natural images (non-medical images) to train an AI system with a fraction of the commonly used training data (<1%). The trained AI system achieved performance comparable to that of human experts in classifying ARMD and diabetic macular edema on optical coherence tomography images. In this study, we highlight the fundamental concepts of AI and its application in these four major ocular diseases and further discuss the current challenges, as well as the prospects in ophthalmology.

KEYWORDS

artificial intelligence, diabetic retinopathy, glaucoma, cataract, age-related macular degeneration

Introduction

Artificial intelligence (AI) is a broad branch of computer science that develops theories, methods, technologies, and application systems to simulate, extend, and expand human intelligence in machines (1). Machine learning (ML) (2) is a subcategory of AI that uses statistical techniques to build intelligent systems. Using either a supervised or unsupervised approach, the intelligent system can learn and improve its performance automatically, such as accuracy, without being explicitly programmed. Deep learning (DL) (3), which uses advanced ML techniques, has achieved great success in computer vision and natural language processing tasks. This success is primarily attributed to its excellent feature extraction and pattern recognition capabilities, which use multiple processing layers (artificial neurons) to learn representations of data with different levels of abstraction (4) such that it associates the input with a diagnostic output. Because of this outstanding success, many investigators have applied DL to medical and healthcare-related tasks, such that DL has become a powerful tool in intelligent screening, diagnosis, and treatment of various diseases recently. DL has been used for COVID-19 detection from chest X-rays (5), thyroid classification from ultrasound imaging (6, 7), and lung nodule detection and staging from computed tomography (CT) images (8, 9).

Currently, AI has achieved radiologist-level diagnosis of medical images by learning from example images, which has significantly improved clinical workflows. The application of AI for medical image analysis plays an important role in maximizing efficiency and enhancing the accuracy of diagnosis and treatment for physicians. AI application also plays a significant role in improving current logistic and economic issues, which could influence the healthcare system by expanding clinical capacity and augments. Furthermore, AI is useful as an important auxiliary tool in the early detection of diseases, particularly in low-resource clinical settings. Based on fundus photographs and optical coherence tomography (OCT), in the field of ophthalmology, DL has been applied to four major eye diseases that cause blindness, including diabetic retinopathy (DR) (10–13), glaucoma (13, 14), age-related macular degeneration (ARMD) (13, 15, 16), and cataracts (17). AI has shown great promise in the auxiliary diagnosis of refractive error (18), retinopathy of prematurity (ROP) (19), retinal detachment (20), choroidal disease (21), and ocular tumors (22). Early detection is particularly crucial to prevent delays in treatment and vision loss.

AI simulates the thinking and diagnostic capabilities of doctors by learning their expertise and medical data to provide efficient and accurate diagnoses and personalized treatment plans in a short period using medical images and other relevant data. The IBM Watson System, a question-answering system, can effectively provide diagnostic and treatment strategies for patients with lung, prostate, and other cancers. This system was successfully developed by learning from empirical evidence-based medical articles, publications, treatment plans, clinical data, and experimental reports.

Personal health data in the future can be dynamically monitored through wearable devices and smart home devices, which will provide a wealth of data for medical diagnosis. Modeling with these personal health data will allow accurate personal health information to predict disease risk in a standardized and accurate manner. Artificial intelligence provides accurate guidance on the management of blood glucose and blood pressure, serves as a medication reminder, monitors health elements, and offers the population with comprehensive, full-cycle health services in a high-quality, intelligent, and daily manner.

The recent development of AI algorithms is providing unprecedented opportunities to address some major challenges in DR and other ocular diseases. For instance, the Inception-v3 algorithm trained with annotated fundus images can achieve diagnosis performance comparable with human experts. Although there exist several related reviews in the community, the technical background, unfortunately, has not been thoroughly investigated. In this study, we highlight the fundamental concepts of AI and its application in four major ocular diseases, and further discuss the current challenges, as well as the prospects in ophthalmology, providing unexplored insight in this area. The ability to introduce the fundamental concept of AI with reference to its clinical applications will increase the awareness of using AI in the community and discover new capabilities in the analysis of ocular diseases.

Method of literature research

In this overview, we retrieved English articles from the commonly used database engines, including Pubmed/MEDLINE, Springerlink, the Cochrane Library, Google Scholar, and EMbase Medline with the keywords "Artificial Intelligence," "Machine Learning," "Deep Learning," combined with keywords, including "diabetic retinopathy," "cataract," "glaucoma," and "age-related macular degeneration." The end date for the retrieval is December 2021. Studies retrieved by each pair of keywords were then combined to build an objective dataset of articles. A comprehensive review by several authors was performed of all references cited in the dataset. Proposals protocols, reviews, letters, opinions, and studies and/or articles that were not peer-reviewed were excluded. Publications relevant to our topic were selected and are found in the references. In this study, we focused on giving an overview of the application of AI in DR and other ocular diseases. We, therefore, attempt to select representative AI techniques for each disease category. We acknowledge that not all the articles under these keywords' combinations were

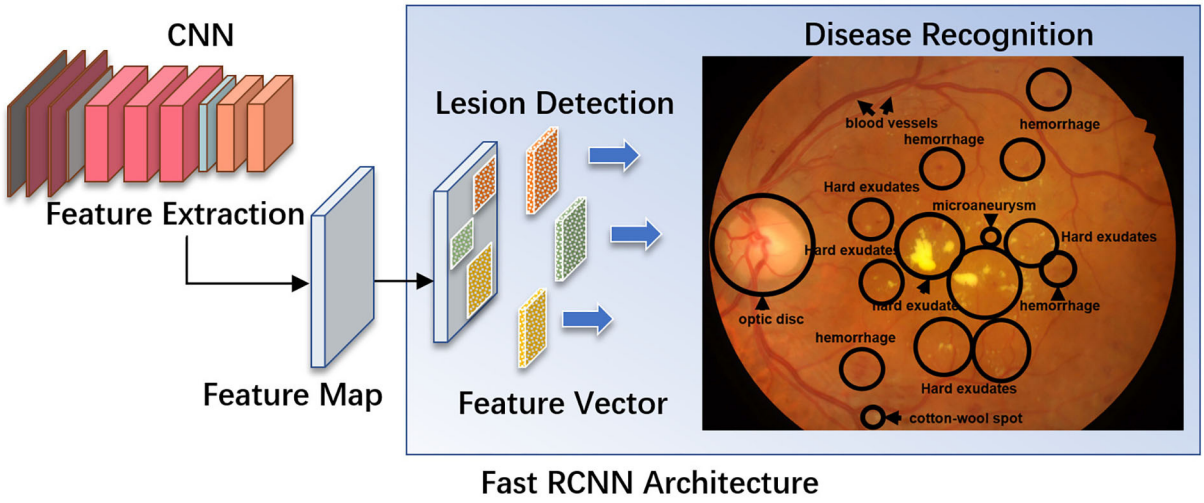

FIGURE 1
A diagram illustrating a fast R-CNN algorithm for automatic lesion detection and disease recognition from fundus images. The input fundus image will be fed into the CNN network to get the corresponding feature map. The derived feature map will be used to estimate region proposals (candidate lesion regions in squared boxes), which will then be classified and predicted as different disease categories. FC, fully connected layers; DM, diabetes mellitus; DR, diabetic retinopathy; R-CNN, region-based convolutional neural network; ROI, region of interest.

included for discussion, providing more of a perspective and opinion review.

AI's impact on human ocular diseases

Diabetic retinopathy

DR is a leading cause of blindness in working populations in both developed and developing countries and is the most serious eye complication of diabetes mellitus (DM). The International Diabetes Federation estimates that by 2040, approximately 600 million people worldwide will have DM, one-third of whom will eventually develop DR. According to a meta-analysis consisting of 35 cohort studies with 22,869 subjects, the global prevalence of DR is 34.6% and vision-threatening DR is 10.2%, accounting for 51% of blindness cases worldwide (23).

Regular DR screening is important for the timely treatment and prevention of vision loss (24). Time and financial constraints are major issues for both ophthalmologists and endocrinologists. The effectiveness of fundus photograph-based screening is significantly impacted by the limited number of registered ophthalmologists, particularly retinal specialists. DR is the most common retinal vascular disease, with typical fundus characteristics, including microaneurysms, hemorrhages, exudates, and neovascularization. For automatic screening of disease, these lesions must first be manually labeled on fundus images, and then a preliminary diagnosis using ML is made (Figure 1). In April 2018, the U.S. Food and Drug Administration (U.S. FDA) approved the first AI-assisted DR detection device, IDx-DR, for primary eye care, to aid physicians in DR screening (25, 26).

Compared to humans, ML can detect DR in a faster and more accurate manner (27). Furthermore, deep neural networks offer significantly higher predictive performance for DR screening using retinal images (28, 29). The AI-based DR screening model is feasible and acceptable for patients in endocrinology outpatient settings (30).

The performance of DL models relies heavily on the availability of sufficient training datasets. However, owing to complicated data acquisition procedures and ethical constraints, it is challenging to acquire sufficient data in the real world. To overcome this limitation, investigators have used migration learning to train a neural network with a small fraction of data and have used features from conventional methods. This method provides comparable performance to human experts in the classification of ARMD and diabetic macular edema (DME) (31). Other researchers have developed a self-supervised training scheme to train neural networks with many unlabeled medical images (32).

Diabetic choroidal vasculopathy (DCV) has been a hot research topic recently. Early detection of DCV could offer warning information regarding the occurrence of DR in patients with DR. However, automatic segmentation of the choroidal layer remains a challenging task because of the low contrast, inhomogeneous intensity, inconsistent texture, and blurred boundaries between the choroid and sclera in the OCT images. Currently used methods continue to emphasize manually or semi-automatically segmenting areas of interest. The researchers

proposed segmenting the Bruch's membrane (BM) in OCT images using a series of morphological operations, while the choroidal layer was segmented using a DL approach (21). Moreover, a segmentation method based on the adaptive appearance and prior shape information was developed to separate the retinal layers (33).

Recently, DL systems for detecting DR have developed rapidly, with remarkable results (12, 13, 26, 34), greatly improving the diagnostic performance of non-proliferative DR (NPDR) and middle- and late-stage PDR (Table 1). Researchers have also extended their research to the grading and prediction of DR based on lesion identification. The International Clinical Classification of Referable DR (rDR) defines DR as moderate, severe non-proliferative DR (NPDR), proliferative DR (PDR), and/or macular edema (ME). Abràmoff et al. showed that AlexNet and VGG achieved 96.8% sensitivity, 87% specificity, and 98% area under the curve (AUC), respectively (10). The team defined mild and beyond classification DR (mtmDR) as ETDRS grade 35 or higher, and/or DME in at least one eye, based on the early treatment diabetic retinopathy study severity scale (ETDRS) and diabetic macular edema (DME). This AI system had a sensitivity of 87.2%, a specificity of 90.7%, and an imageability of 96.1% (34). Gulshan et al. used CNN to classify the referable diabetic retinopathy (rDR) as moderate or worse diabetic retinopathy, referable diabetic macular edema, or both and achieved 97.05% sensitivity, 93.4% specificity, and 99.1% AUC (12). DL system from Google AI Healthcare identified image features to grade fundus lesions derived from 128,175 retinal images (labeled by 54 ophthalmologists) and discovered that these image features could quickly identify DR and identify signs of DR. Ting et al. reported a clinically acceptable diagnostic performance with an AUC of 93.6%, sensitivity of 90.5%, and specificity of 91.6%, in detecting DR using a development dataset acquired from Singapore integrated DR Program and several external datasets from six different countries (13). In another study, investigators from Aalto University trained a DL model based on Inception-v3 and found that DL could accurately separate DR and macular edema (35). Feng Li et al. optimized the Inception-v4 algorithm with a multiple-improvement depth ensemble to detect DR and DMO and achieved an AUC, sensitivity, and specificity of 99.2%, 92.5%, and 96.1% (36), respectively. Reguant et al. visualized the neural network decision process and analyzed image features; discovered that Inception-v3, recognition deep residual learning (ResNet) 50, InceptionresNet50, and Xception achieved 89–95% accuracy with AUC, sensitivity, and specificity of 95–98%, 74–86%, and 93–97%, respectively, for disease classification of DR (37). Ryu et al. proposed a convolutional neural network (CNN) model for diagnosing DR based on optical coherence tomography angiography (OCTA) images, achieving 91–98% accuracy, 86–97% sensitivity, 94–99% specificity, and 91.9–97.6% AUC (38).

Researchers from Shanghai Jiao Tong University proposed a deep neural network-based AI algorithm for detecting early DR and microaneurysms, which significantly improves the accuracy of the automatic detection of early DR and STDR, including proliferative DR and DME (39). A system for the automatic diagnosis of diabetic fundus lesions has been developed to assist in understanding the grading of fundus lesions and the severity of the disease in patients. The investigators have also developed a portable fundus photography device, which consists of a detector lens, smartphone, and fixed holder, allowing users to take fundus photographs anywhere. The fundus photographs obtained can be transmitted to a server for diagnostic analysis, including optic disc and macular localization, vascular segmentation, lesion detection, and lesion grading. The diagnostic results of this system were compared with those of ophthalmologists and achieved an accuracy rate of 85% (16). Furthermore, the researchers proposed an algorithm for optic disc and macular region detection based on a kernel least squares classifier. This algorithm uses several already labeled optic disc and macular region images to complete optic disc boundary localization and establish an accurate mapping from the image to the region. Based on this, the researchers constructed a method to accurately detect the optic disc region and locate the center of the optic disc for color retinal images, which is based on a kernel least-squares classifier to calculate the optic disc area. The method is then based on multimodal information to detect the site of vascular aggregation and obtain the optic disc center with higher accuracy. Particularly, in terms of optic disc localization, this method successfully detected 332 images out of 340 testing images, with a detection success rate of 97.65%. For optic disc boundary detection, the method achieved a success rate of 94.54% among all 112 images in the digital retinal images for vessel extraction (DRIVE) and structured analysis of the retina (STARE) databases; in macular area detection, 330 images were detected on all 340 test images, achieving a detection success rate of 97.06%. In the global finals of intelligent reading of fundus images at the 2018 IEEE International Symposium on Biomedical Imaging (ISBI), the optic disc detection and macular center detection technologies developed independently by researchers won first place worldwide (40). Furthermore, the detection and analysis of blood vessels in fundus images are crucial for the diagnosis of related diseases (41). Researchers have proposed an automatic extraction algorithm for blood vessels in fundus images based on direction-aware detectors, which constructs an orientation-aware detector that can accurately extract blood vessels from fundus images. The detector learns the orientation and distribution characteristics of blood vessels using the energy distribution of the Fourier transform and then extracts the blood vessel morphology using a dual-scale segmentation method, in which a linear operator is used for the large-scale, and a Gabor filter bank is used for the small-scale, making the detector more robust and structure-aware. According to the authoritative

TABLE 1 Typical deep learning systems for NPDR and PDR.

References	Year	Modality	Diseases	Test set	Number of images in test set	CNN	AUC	Sensitivity (%)	Specificity (%)
Abràmoff et al. (10)	2016	CFP	No DR, rDR, vtDR, ME	Messidor-2	1,748	AlexNet/VGG	0.98	96.8	87.0
Gulshan et al. (12)	2016	CFP	No DR, mild DR, moderate DR, severe DR, PDR, rDME, rDR	EyePACS-1	9,963	—	0.991	97.5	93.4
Ting et al. (13)	2017	CFP	DR, possible glaucoma, AMD	SiDRP 14-15	71,896	VGG-19	0.936	90.5	91.6
Abràmoff et al. (34)	2018	OCT	DR, DME	Data from 10 clinical centers in the United States	892 patients	AlexNet/VGG	—	87.2	90.7
Li et al. (36)	2021	CFP	DR, DMO	Messidor-2	8,739	Improved Inception-v4	0.992	92.5	96.1
Reguant et al. (37)	2021	CFP	No DR, mild NPDR, moderate NPDR, severe NPDR, PDR	EyePACS and DIARETDB1	35,122	CNNs	0.95–0.98	74–86	93–97
Ryu et al. (38)	2021	OCTA	DR	OCTA	240	ResNet101	0.919–0.976	86–97	94–99
Dai et al. (39)	2021	CFP	No DR, mild NPDR, moderate NPDR, severe NPDR, PDR, DME	NDSP/ EyePACS	27,948/88,702	DeepDR	0.944/0.943	—	—

AI, Artificial intelligence; AlexNet, Deep Convolutional Neural Networks; AMD, age-related macular degeneration; AUC, area under the curve; CFP, Color fundus photography; CNN, convolutional neural network; DIARETDB1, Standard Diabetic Retinopathy Database Calibration level 1; DMO, diabetic macular edema; EyePACS, Kaggle EyePACS dataset; SiDRP, Singapore Integrated Diabetic Retinopathy Program; ME, macular edema; Messidor, Methods to evaluate segmentation and indexing techniques in the field of retinal ophthalmology dataset; NDSP, Nicheng Diabetes Screening Project cohort; NPDR, Non-proliferative diabetic retinopathy; OCT, optical coherence tomography; OCTA, optical coherence tomography angiography; PDR, Proliferative diabetic retinopathy; rDME, referable diabetic macular edema, rDR, referable diabetic retinopathy; ResNet, recognition deep residual learning; VGG, Very Deep Convolutional Networks; vtDR, vision-threatening diabetic retinopathy.

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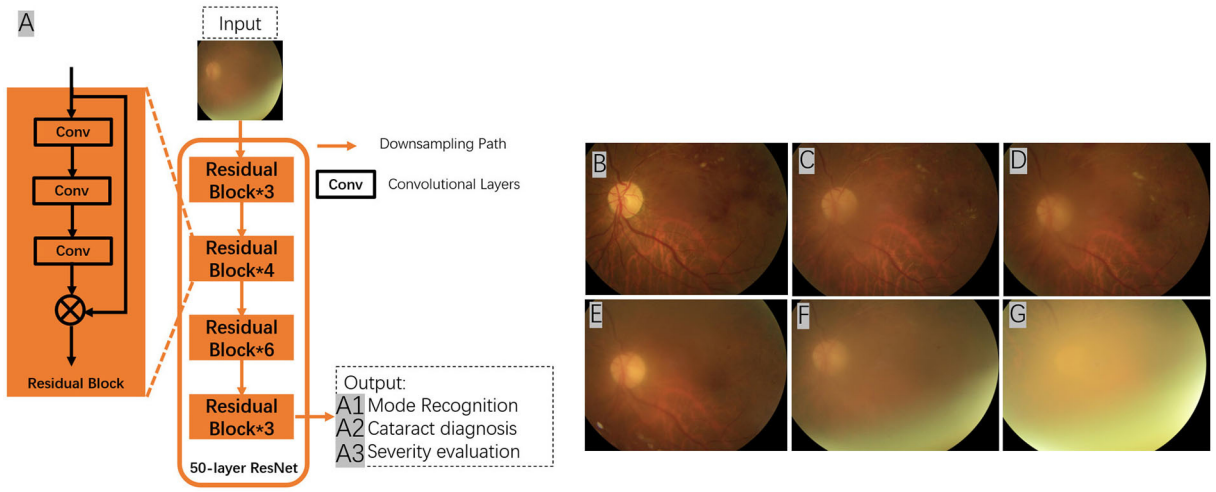

FIGURE 2
Using deep residual network (ResNet) for cataract recognition and grading. The overall architecture of the ResNet consists of 16 residual blocks and each residual block consists of three convolutional layers. The output of the ResNet includes: (A1) mode recognition - identify the capture mode between mydriatic and non-mydriatic images, and between optical section and diffuse slit lamp illimitation; (A2) cataract recognition - the system could classify the images as normal (no cataract), cataractous, or postoperative intraocular lens (IOL); and (A3) severity evaluation - classify the type and severity of the cataract **(A–G)**, and assess the subsequent follow-up or referral arrangements for the patient. Conv, convolutional layers.

standard connectivity, area, and length (CAL) proposed by Gegndez-Arias, the algorithm achieved an accuracy of 80.82% on the international public dataset DRIVE and 68.94% on the STARE dataset. Experimental results show that the proposed method outperforms the existing segmentation methods and has high accuracy and robustness. Furthermore, investigators added a weakly supervised sensitive heat map (WSSH) to the CNN to create a CNN-WSSH model, combining the automatic detection of DR classification with a weakly supervised localization method to address the localization challenge (42).

DL methods enable regular screening in various locations, particularly in rural areas, making the early detection of common chronic diseases easier. To address the lack of medical resources, researchers have evaluated the role of automated AI-based software in DR and STDR, providing an initial tool for mass retinal screening for patients with diabetes using smartphone devices to take fundus photos and validate them against an ophthalmologist's score (43). Furthermore, fundus images acquired by patients using self-filming fundus imaging (SFI) are comparable in image quality to those acquired by trained specialists (44).

Through a prospective study of fundus images taken with smartphones, the researchers concluded that DL models are generalizable in identifying chronic kidney disease and type 2 diabetes, and feasible in predicting disease progression in a longitudinal cohort (45).

We anticipate that AI algorithms will improve their ability to predict the onset and progression of DR more effectively and concisely.

Cataract

A cataract is a metabolic dysfunction disorder with variable pathological factors, such as aging, genetics, local nutritional disorders, immune and metabolic abnormalities, trauma, poisoning, and radiation, resulting in protein denaturation in the lens. Cataracts account for up to 18.4% of visual impairment and 33.4% of blindness worldwide (46). It is critical to screen people with diabetes for age-related cataracts to prevent blindness. Slit lamp examination and iris projection methods are mostly used in the examination of cataracts. However, compared with these two methods, the non-dilated fundus photography method has convenient and effective features.

AI algorithms are important for the automatic detection and grading of cataracts based on slit lamp photographs or color fundus photographs (Figure 2). Wu et al. (17) used a DL system for the diagnosis and referral of cataracts based on slit-lamp photographs. Three steps are performed sequentially in this system: (i) identify the capture mode between mydriatic and non-mydriatic images, and between optical section and diffuse slit lamp imaging; (ii) classify the images as normal (no cataract), cataractous, or postoperative intraocular lens (IOL); and (iii) classify the type and severity of the cataract or posterior capsular opacification and assess the subsequent follow-up or referral arrangements for the patient. The AUC of the CMAAI validation set was more than 99% for both capture mode recognition and cataract detection. For cataract severity evaluation, using mydriatic images with optical sections achieved the best performance (AUC 0.99), whereas using

nonmydriatic images with diffuse illumination was less effective (AUC 0.9328).

A limited number of studies have been conducted on automated cataract assessment systems using color fundus photographs. Dong et al. (47) used a CNN for feature extraction and a SoftMax function for cataract detection and severity grading. Ran et al. (48) used a CNN and random forest for the same task. Pratap and Kokil (49) performed transfer learning, in which a pre-trained CNN was trained on natural images (non-medical images), which were further refined with 400 fundus images. The training and test data used in this study are available from open-source databases, including the high-resolution fundus image database (HRF), STARE, standard DR database calibration level 0 (DIARETDB0), methods to evaluate segmentation and indexing techniques in the field of retinal ophthalmology (MESSIDOR), DRIVE, fundus image registration dataset (FIRE), digital retinal images for optic nerve segmentation database (DRIONS_DB), and Indian diabetic retinopathy image dataset (IDRiD). Li et al. (50) developed a DL system using training data from the clinical database of the Beijing Tongren Eye Center. ResNet-18 and ResNet-50 were used for cataract detection and severity grading (non-cataracts, mild, moderate, and severe cataracts). Therefore, explainable attention maps can be used to illustrate the presence and severity of cataract.

The treatment strategy for cataracts is surgical removal accompanied by intraocular lens (IOL) implantation. AI has also been used to calculate IOL power, which significantly improves the prognosis and visual outcome of cataract surgery.

Glaucoma

Glaucoma is an optic nerve degenerative disease characterized by typical pathological changes in the optic nerve head, retinal nerve fiber layer (RNFL), and visual field. Glaucoma is the second leading cause of irreversible blindness worldwide, and approximately 50% of glaucoma cases remain undiagnosed. Early diagnosis and intervention are essential for preventing blindness. Glaucoma can be classified as open-angle glaucoma or closure-angle glaucoma. Early diagnosis of glaucoma requires a combination of several examination results, including IOP, disc compression/decompression (C/D) ratio, morphology, visual field, and RNFL changes. The C/D ratio is a common index used to evaluate glaucomatous optic nerve damage. The difficulty of the computerized automatic diagnosis system is in segmenting the optic disc and optic cup areas from the fundus image. There is also an association between diabetes and the development of glaucoma, and screening for open-angle and closed-angle glaucoma in the population with diabetes is clinically and scientifically relevant.

The prerequisite for segmentation is localization. Researchers have recently proposed a method for localizing the optic disc based on vessel tracking, using a minimum variance classifier based to predict the region containing the optic disc. The connected partial markers and luminance information are used to identify the fundus vessels, which eventually assist to predict the optic disc (51). Other researchers created a comprehensive dataset of retinal images containing both normal and glaucomatous eyes, which were manually segmented by several ophthalmologists to provide information on other optic nerve head (ONH) regions, including disc rim cuts (52). This dataset is openly accessible and is anticipated to facilitate further research on glaucoma AI diagnosis.

Different imaging characteristics were thoroughly evaluated to determine the most significant characteristics of glaucoma. The researchers trained a multimodal model incorporating multiple deep neural networks and used it for the early detection of glaucoma by training macular volumes on OCT and color fundus photographs and combining demographic and clinical data. The accurate prediction of posttraumatic growth (PTG) through interpretable analysis highlighted the variables that change with the progression of glaucoma, including age and lung function (53). Other investigators have demonstrated the importance of the spatial structure of the thickness map data of the retinal neural fiber layer in the diagnosis of glaucoma using multiple ML models, including two traditional ML algorithms, the support vector machine (SVM) and K-nearest neighbor (KNN), as well as two CNNs, ResNet-18 and Glaucoma Net, to detect glaucoma diagnostic accuracy and support further efforts to optimize the use of these data (54).

Christopher (55) evaluated the ability of DL methods to identify glaucomatous optic neuropathy (GON) using fundus photographs. Two independent ophthalmologists evaluated a large database of fundus photographs of a racially and ethnically diverse group of individuals. The best DL model achieved an AUC of 0.91 in distinguishing GON eyes from healthy eyes, 0.97 for identifying GON eyes with moderate-to-severe functional loss, and 0.89 for GON eyes with mild functional loss. The visualization results indicated that the DL model focused on the anatomical features of the inferior and superior regions of the optic disc. These results suggest that the DL-based assessment of fundus images could be useful in the automation of large-scale glaucoma detection and screening programs. Shibata et al. (56) also developed a deep residual learning algorithm to screen for glaucoma using fundus photography and measured its diagnostic performance compared with that of ophthalmology residents. The DL algorithm achieved a significantly higher diagnostic performance than residents in ophthalmology. Berchuck et al. (57) developed a DL algorithm to improve the estimation of the rate of progression of glaucoma vision loss and the prediction of future patterns. A low-dimensional representation of the standard automatic visual field (SAP) was learned by training a generalized variational self-encoder (VAE) using 29161 visual fields from 3,832 patients. The VAE was trained with 90% of the data sample and randomized at the patient level. Using the remaining 10%, progression rates and predictions were generated and compared to SAP

mean deviation (MD) rates and point-by-point (PW) regression predictions, respectively. Longitudinal rates of change through the VAE latent space detected significantly higher rates of progression than MD at 2 and 4 years after baseline. Deep VAE can be used to assess the incidence and trajectory of glaucoma and has an added benefit as a generative technique that can predict future patterns of visual field damage. Wu et al. (58) evaluated the effect of five glaucoma treatments (medication, laser, non-laser surgery (NLS), laser + medication, and NLS + medication) on a 1-year IOP change, which provides important evidence of clinical outcomes for glaucoma patients. Li et al. (59) developed and evaluated the performance of "iGlaucoma," a smartphone application-based DL system in detecting visual field (VF) changes in glaucoma. In this study, which was divided into two phases, 1,614,808 data points from 10,784 VF (5 542 patients) from seven centers in China were included. The first phase involves training, validating, and testing the diagnostic performance of the DL system. In the second phase, the iGlaucoma cloud-based application was further tested with 33,748 data points from 649 VFs from 437 patients from three glaucoma clinics. In the second stage, the accuracy of iGlaucoma for identifying different patterns in the probability plot region of pattern deviation was 0.99, and the corresponding AUC, sensitivity, and specificity were 0.966 (0.953–0.979), 0.954 (0.930–0.977), and 0.873 (0.838–0.908), respectively.

A longitudinal dataset combining VF and clinical data was used to evaluate the performance of the convolutional long short-term memory (LSTM) neural network. Models trained on VF and clinical data (AUC, 0.89–0.93) performed better than models trained on VF results only (AUC, 0.79–0.82; $P < 0.001$), demonstrating that supplementing VF results with clinical data improves the ability of the model to assess glaucoma progression (60). Furthermore, the investigator validated the traditional artificial neural networks and discovered that they can perform well in detecting spinal field defects in glaucoma cohorts and in detecting visual field defects caused by pituitary disease in a glaucoma population (60). Other researchers have developed hybrid deep learning model (HDLM) algorithms that can quantitatively predict the thickness of the macular ganglion intracellular reticular layer (mGCIPL) from non-red retinal neurofibrillary layer photographs (RNFLPs) with good performance (61). Researchers developed a DL algorithm called image ResNet to discriminate glaucoma and obtained test data with an area under the curve (ROC) of 96.5 (95% confidence interval [CI]: 93.5–99.6), indicating that the DL algorithm outperformed ophthalmology residents in diagnosis (56). The investigators evaluated the external validity of the dynamic structure–function (DSF) model through studies tested in an independent dataset (intraocular pressure treatment study-focal scanning laser fundoscopy [OHTS-CSLO]-assisted study; $N = 178$ eyes) and the Glaucoma Diagnostic Innovations Study or the African Descent and Glaucoma Assessment Study (DIGS/ADAGES) dataset, demonstrating the external validity of the DSF model and its potential to develop it into a useful clinical tool (62). Some investigators have demonstrated the value of ML models in predicting trabeculectomy outcomes in patients with refractory glaucoma using models of random forests, SVMs, artificial neural networks, and multivariate logistic regression to predict the surgical outcome of trabeculectomy (63). A Bayesian deep multi-source learning (BDMSL) model is proposed, which introduces an information-centric multi-source learning framework to integrate multi-source data while employing Bayesian DL to obtain uncertainty information of the model and achieve better performance than other methods (64). The CNN was trained using OCT images and adjusted by the Humphrey field analyzer (HFA) 24–2 to establish a prediction model of the 10-degree central field of VF for glaucoma patients (65). The researchers have also used the DL model that uses fundus photographs to detect superficial anterior chamber depth (ACD) as a screening tool for angle-closure glaucoma (ACG). The cycle generative adversarial network (cycle GAN)—based feature maps show hidden features of superficial ACD that are undetectable by traditional techniques and ophthalmologists and help detect early ACD (66). Some investigators have analyzed multiple features and introduced new cross-sectional ONH features from OCT images to facilitate the current diagnostic evaluation of glaucoma, demonstrating that selected features and cross-sectional ONH cup areas trained using DL have great potential as preliminary screening tools for glaucoma (67). These results will help clinicians make more accurate decisions in the future.

The investigators developed and evaluated the performance of a DL system based on a smartphone app through efficient glaucoma diagnostic workers based on VFs, providing keening to detect visual field changes in glaucoma with smartphones (67). Glaucoma is a disease associated with the loss of retinal ganglion cells (RGCs). The main research efforts are currently being conducted with the help of rodent models, making a tool that reliably quantifies the survival of RGCs. Therefore, some researchers have designed software called RGCode (DL-based quantification of RGCs), which is capable of fully automated RGC quantification in the entire mouse retina (68). Researchers have developed a non-species specificity, which can be extended to the tools of glaucoma AxoNet. It can be calculated from various animal models of glaucoma RGC axons in the optic nerve (ON) organization image, and use the depth study to return to the pixel-level counting axon density estimation and then integrate it into the image area to determine the axon count (68).

Age-related macular degeneration

ARMD is an acquired and complex macular degenerative disease that is the leading cause of blindness in the elderly worldwide. The prevalence increases exponentially every decade

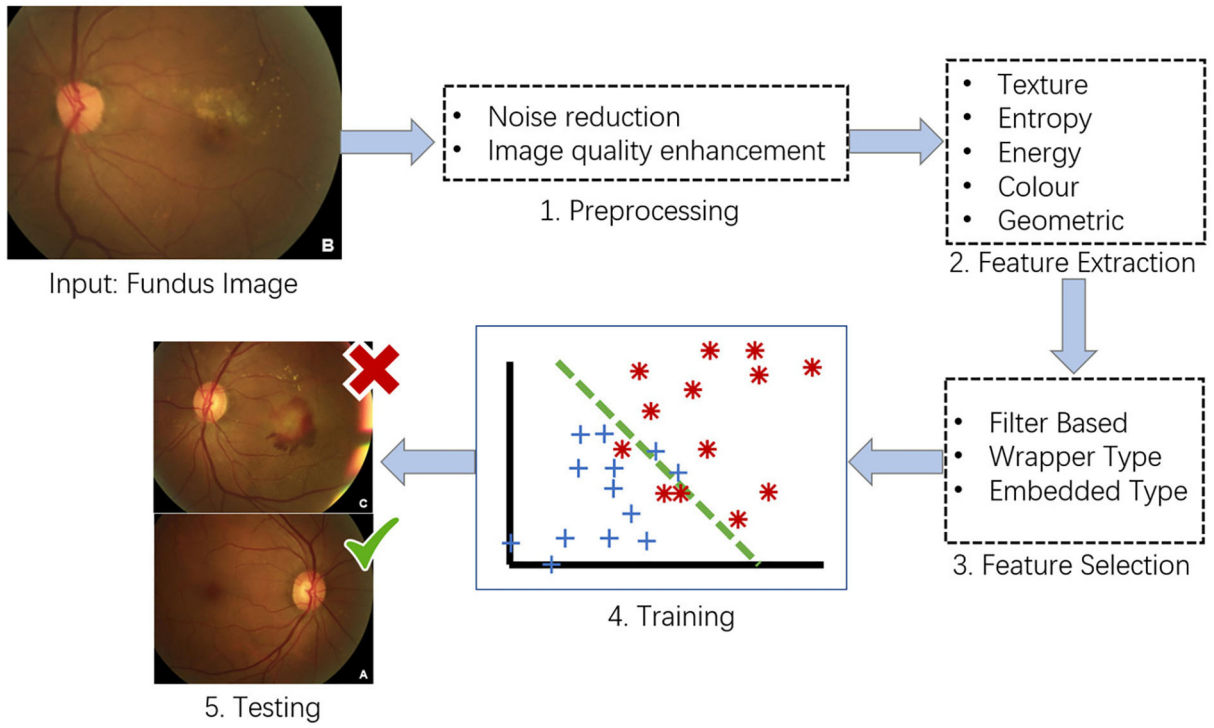

FIGURE 3
Conventional framework for ARMD detection from fundus images. **(1)** Preprocessing – image preprocessing is performed on the input fundus image to reduce noise and enhance image quality. **(2)** Feature Extraction – image features such as texture, entropy and color features will be extracted from the preprocessed images. **(3)** Feature Selection – feature selection will be conducted on the extracted image features to select the best representative features of an image. **(4)** Training – at the training phase, a model such as support vector machine (SVM) will be built that tries to separate the training data into different categories e.g., ARMD and non-ARMD. **(5)** Testing – testing phase will apply the trained model to unseen fundus images and classify them to the known categories e.g., ARMD and non-ARMD.

after the age of 50 (69). Aging, smoking, genetic susceptibility, dysregulated lipid metabolism, oxidative stress, cardiovascular disease, female sex, white race, obesity hyperopia, and other risk factors contribute to ARMD development. The clinical characteristics include the presence of drusen, retinal pigment epithelium (RPE) abnormalities, geographical atrophy, and neovascular derangement. ARMD can be classified into early ARMD [characterized by numerous small (< 63 microns, hard) or intermediate (≥63 microns but <125 microns, soft) drusen]; intermediate ARMD [defined by extensive drusen with small or intermediate size, or any large drusen (≥125 microns)]; and advanced ARMD (characterized by a choroidal neovascular membrane or geographic atrophy). ARMD can be categorized into two subtypes: dry (presence of drusen, RPE abnormalities, or geographical atrophy) or wet (macular neovascularization). The diagnosis of ARMD frequently relies on various examinations, such as fundus photography, fundus fluorescein angiography, indocyanine green angiography, OCT, and OCTA. Early- and mid-stage ARMD can be asymptomatic, leading to easy underdiagnosis, while advanced ARMD progresses faster and has a greater impact on vision, with limited treatment options available (Figure 3). AI can be an essential tool for the early identification of macular lesions that can assist ophthalmologists in the early intervention of the disease.

Recent studies have proposed DL algorithms based on fundus color photography to identify drusen or retinal pigment epithelium (RPE) abnormalities in ARMD. Researchers from Johns Hopkins University achieved an accuracy of 88.1–91.6% for the identification of drusen, which is competitive with manual interpretation (13, 15, 16). AI based on convolutional neural networks (CNNs) has also been used for telemedicine. In this study, an annotated dataset consists of 35,900 ARMD OCT images (acquired from two types of OCT devices including Zeiss Cirrus HD-OCT 4000 and Optovue RTVue-XR Avanti) was used for AI algorithm training and validation groups, respectively, and the CNN architectures named ResNet 50, Inception V3, and VGG 16 were used for image recognition. The detection accuracy of the AI-based system achieved the same image discrimination rate as that of retinal specialists (92.73 vs. 91.9%, $p = 0.99$) and generally higher than that of medical students (69.4 and 68.9%) (70). However, the testing performance of current AI algorithms is still largely dependent on different clinical datasets; therefore, their generalization performance among external clinical datasets is limited. Future

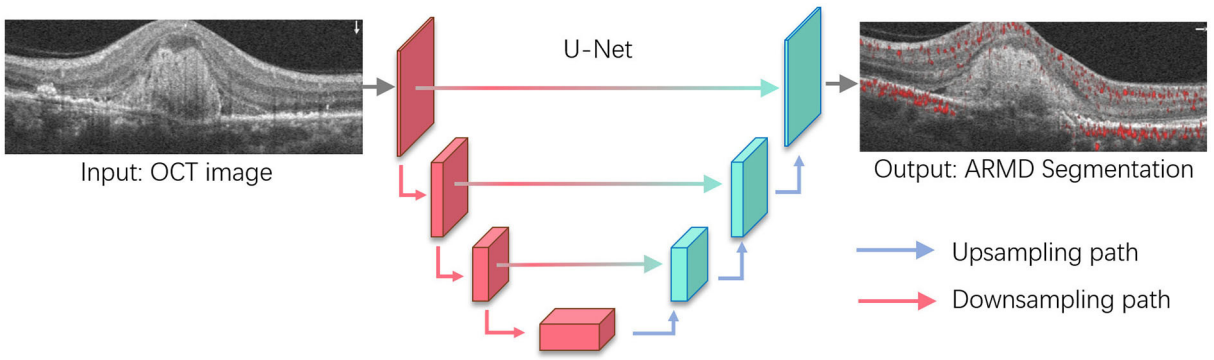

FIGURE 4
Age-related macular degeneration (ARMD) lesions segmentation based on U-Net. U-Net is one of the most widely used segmentation architectures for biomedical images and stemmed from the fully convolutional network. The U-Net model consists of a downsampling path and upsampling path, where downsampling path has convolutional and max-pooling layers to extract high-level abstract information while the upsampling path has convolutional and deconvolutional layers that upsample the feature maps to output the segmentation outcomes. For ARMD segmentation, U-Net will take OCT images as the input and progressively extract semantic features that allow to separate the lesions from the surrounding background and output the lesion segmentation results.

work on the applicability and portability of these algorithms remains challenging.

Owing to the high reliance on OCT images for the diagnosis of the wet form of ARMD (Figure 4), the recognition of ML is no longer limited to color fundus photography. AI research is beginning to focus on large databases of multimodal images and is expected to uncover more adequate information. Several intelligent decision systems based on OCT technology have been developed using ML (41). Meanwhile, the DL technique has achieved higher accuracy in distinguishing a healthy fundus from exudative ARMD (71). Related AI research teams have developed algorithms for the simultaneous recognition of multiple disease types, including macular edema, ARMD, and central serous choroidal retinopathy, which can not only discriminate the presence of retinopathy in the subject but also further indicate the type of retinopathy with satisfactory accuracy (72). This suggests that OCT is a natural fit for AI in the detection of macular diseases. Progression to exudative "wet" age-related macular degeneration (wARMD) is a major cause of visual impairment. For patients with unilateral eye wARMD, Yim et al. (73) introduced an AI system to predict the progression to wARMD of another eye using OCT images and corresponding automatic tissue maps. This system predicts conversion to exARMD within a clinically actionable 6-month time window and demonstrates the potential of using AI to predict disease progression.

Other studies have combined multimodal data to predict ARMD progression. Banerjee et al. (74) proposed a hybrid sequential prediction model called "Deep Sequence" that integrates radionics-based imaging features, demographic, and visual factors, with a recurrent neural network (RNN) model to predict the risk of exudation within a future time frame in non-exudative ARMD eyes. The proposed model provides scores associated with the risk of exudation in the short term (within 3 months) and long term (within 21 months), which allows for addressing challenges related to the variability of OCT scan characteristics and the size of the training cohort. Thakoor et al. (75) proposed a DL approach for multi-class detection of non-ARMD vs. non-neovascular (NNV) ARMD vs. NV ARMD from a combination of OCTA, OCT structure, 2D B-scan flow images, and high-definition (HD) 5-line b-scan cubes. DL also detects ocular biomarkers indicative of ARMD risk. Choroidal neovascularization and geographic atrophy were found to be significant biomarkers for ARMD detection by both CNNs and clinical experts. Detection of ARMD and its biomarkers from OCTA images *via* CNNs has tremendous potential to expedite the screening of patients with early and late-stage ARMD. Yeh et al. (76) proposed a heterogeneous data fusion network (HDF-Net) to predict visual acuity (VA) and to evaluate the prognosis and risk of progression of neovascular age-related macular degeneration (nARMD). The clinical decision-making process was simulated using a mixture of pre-processed information from raw OCT images and digital data, and HDF-Net performed well in predicting individualized treatment outcomes. This new approach is an important step toward personalized treatment strategies for typical nARMD.

Genetic and environmental factors influence the etiology of ARMD. Genome-wide association studies (GWAS) for late-stage ARMD have identified 52 independent genetic variants with genome-wide significance at 34 genomic loci. Yan et al. (77) used the Age-Related Eye Disease Study (AREDS) dataset and a modified CNN with genotype and fundus images to predict whether an eye had progressed to advanced ARMD, showing that the CNN with fundus images plus genotype achieved a mean AUC of 0.85 in

predicting the progression of advanced ARMD, while the CNN with fundus images only achieved a mean AUC of 0.81. Strunz et al. (78) conducted a transcriptome-wide association study (TWAS) that predicted the impact of ARMD-associated genetic variants on gene expression, which addressed the shortcomings of current GWAS analyses that rarely identify functional variants associated with specific genes in the disease process. This study further highlights the fact that the expression of genes associated with ARMD is not restricted to retinal issues but is a systemic pathology.

Other ocular diseases

In addition to the common ocular diseases discussed above, AI has shown promise in the diagnosis of the epidermal membrane (ERM), chronic central serous chorioretinopathy (CSC), bacterial keratitis (BK), pathological myopia, and macular edema (ME). Furthermore, ophthalmic image for AI analysis is not limited to color fundus photography but covers various ophthalmic images, including anterior segment photography, corneal topography, anterior or posterior segment OCT, and ultrasound biomicroscopy (UBM) (79).

A deep neural network-based AI model has been applied for epidermal membrane (ERM) detection based on color fundus photographs (80, 81). A random forest-based regression model was used to infer local retinal sensitivity from the retinal structure and the model was applied to the CSC patients for personalized treatment (81). Yoon et al. (82) used convolutional neural networks and achieved performance of 93.8, 90.0, 99.1, and 98.9% in accuracy, sensitivity, specificity, and AUC for the diagnosis of CSC. Kuo et al. (83) evaluated various DL algorithms, including ResNet50, ResNeXt50, DenseNet121, SE-ResNet50, EfficientNets, and DeepLab framework, and identified that DL algorithms could accurately diagnose BK based on eye anterior segment photographs (84). Besides, the DL algorithm has also been applied to ultra-wide-field fundus (UWF) images for the detection of ME and retinal exudates (85).

Challenges of artificial intelligence in the medical field

Although the application of AI technology in the medical field, particularly in ophthalmology, is becoming more widespread, many problems need to be solved with the application of AI technology in current clinical practice. OCT is an indispensable component of healthcare in ophthalmology and plays a significant role in the diagnosis, grading, and assessment of treatment responses in eye diseases. These challenges can be attributed to the fact that eye diseases have various imaging characteristics, such as size and shape, fuzzy boundaries, low contrast to the surrounding background, and heterogeneity. These challenges have motivated the development of numerous AI-aided systems that can assist clinicians in image interpretation and offer opportunities to enhance clinical analytics and decision-making.

Data quality control

Because the use of AI technology is predicated on a large amount of treatment data, the corresponding labels and data quality directly determine the performance of the model to an extent. Data quality may have the following problems: (i) poor quality of the data itself, such as blurred pictures and artifacts; (ii) poor quality of the data labels, such as incorrect labels; and (iii) insufficient data, where only a small portion of data has been labeled.

Privacy protection

Cloud-based data management and storage platforms are commonly used to facilitate data acquisition across multiple cohorts, such as multiple hospitals. Data security in AI algorithms presents a significant challenge.

Establishment of laws and regulations

The application of AI in ocular diseases remains a big challenge. Erroneous predictions by AI algorithms e.g., due to poor data quality, are unavoidable, which can lead to liability issues for physicians. Therefore, the role of physicians in the perspective of AI diagnosis and treatment process needs to be further refined in future medical regulations. In addition, the compliance of different AI algorithms for the diagnosis of various ocular diseases would also require dedicated regulations. In July 2019, to strengthen the guidance of the registration declaration of AI medical devices and further improve the quality of the review, the State Drug Administration Medical Device Technical Review Center in China organized the development of "DL-assisted decision-making medical device software review points." On January 15, 2020, the State Drug Administration reviewed and approved the first artificial intelligence Class III medical device "Coronary Blood Flow Reserve Fraction Calculation Software" in China. The product is based on coronary CT vessel images and consists of an installation CD and encryption lock. The functional modules include basic image operations, vessel segmentation, and reconstruction based on DL technology, vessel centerline extraction, and blood flow reserve fraction calculation based on DL technology, which pioneered the application of domestic artificial intelligence-aided diagnosis

and treatment software in clinical practice. Internationally, the U.S. FDA approved IDx's Idx-DR DR screening software in April 2018, which detects the severity of glucose retinal symptoms in adult patients with diabetes based on fundus photographs, and provides recommendations on whether a referral for examination is needed. This is the first product approved by the U.S. FDA using a new generation of AI technology for glucose retinal screening software, and the review and approval of its products will help further promote the approval and supervision of AI-aided diagnostic software for diabetic fundus disease in China. Existing silicon-based intelligence, somatotropic technology, Shanggong Medical Information, Deep et al. (39), and many other diabetes AI-aided diagnostic products have been actively involved in registration declarations. The means and efficiency of DR screening and auxiliary diagnosis are expected to become more efficient and accurate in the future.

Lack of clinical context

AI programs are driven by data interpretation, and frequently lack consideration of the underlying clinical context. In particular, AI programs have difficulty holistically processing clinical scenarios, nor can they fully account for the psychological and social aspects of human nature that skilled physicians would normally consider (86). Cabitza et al. discussed the importance of clinical settings and provided an example of an ML prognostic model that, although technically valid, led to the interpretation of clinical data for treating patients with pneumonia. The AI program, which targeted 14 199 patients with pneumonia, showed that those with asthma had a lower risk of dying of pneumonia than those without concurrent asthma. The correctly coded program predicted asthma as a protective feature because asthma patients are frequently admitted to the intensive care unit (ICU) to prevent complications; however, mortality in ICU patients was 50% lower than in patients with pneumonia alone, and patients with asthma and pneumonia had a better prognosis than those with pneumonia alone (86, 87).

Future directions

AI technology has made significant progress not only in treating ophthalmic diseases but also in other systemic diseases with initial results. The direct observation of retinal vessels in the fundus, combined with several physiological and biochemical indicators of the entire body supplemented with AI algorithms for learning and analysis, provides a new method for evaluating risk factors for cardiovascular diseases. In the management of patients with diabetes, it can also be used to predict the risk factors for diabetes-related complications (diabetic nephropathy, cardiovascular disease, diabetic peripheral neuropathy, etc.). Establishing a model to identify complicated eye diseases (DR complicated with glaucoma or cataract) with multiple imaging modalities, such as OCT, OCTA, and fundus photography, is highly desirable. Although there are still some challenges in current clinical practice, the promising developments demonstrated with AI technology in the above applications suggest that it will be of great clinical significance in the future.

Author contributions

XZ and BS: conceptualization. BS, XC, TL, TM, LB, YY, and XZ: writing. XZ, LB, and BS: review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Diabetic retinopathy risk prediction in patients with type 2 diabetes mellitus using a nomogram model

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Background: This study aims to develop a diabetic retinopathy (DR) hazard nomogram for a Chinese population of patients with type 2 diabetes mellitus (T2DM).

Methods: We constructed a nomogram model by including data from 213 patients with T2DM between January 2019 and May 2021 in the Affiliated Hospital of Zunyi Medical University. We used basic statistics and biochemical indicator tests to assess the risk of DR in patients with T2DM. The patient data were used to evaluate the DR risk using R software and a least absolute shrinkage and selection operator (LASSO) predictive model. Using multivariable Cox regression, we examined the risk factors of DR to reduce the LASSO penalty. The validation model, decision curve analysis, and C-index were tested on the calibration plot. The bootstrapping methodology was used to internally validate the accuracy of the nomogram.

Results: The LASSO algorithm identified the following eight predictive variables from the 16 independent variables: disease duration, body mass index (BMI), fasting blood glucose (FPG), glycated hemoglobin (HbA1c), homeostatic model

assessment-insulin resistance (HOMA-IR), triglyceride (TG), total cholesterol (TC), and vitamin D (VitD)-T3. The C-index was 0.848 (95% CI: 0.798–0.898), indicating the accuracy of the model. In the interval validation, high scores (0.816) are possible from an analysis of a DR nomogram's decision curve to predict DR.

Conclusion: We developed a non-parametric technique to predict the risk of DR based on disease duration, BMI, FPG, HbA1c, HOMA-IR, TG, TC, and VitD.

KEYWORDS

nomogram model, type 2 diabetes mellitus, diabetic retinopathy, risk, prediction

Introduction

Currently, diabetes mellitus (DM) is one of the fastest-growing chronic diseases worldwide. An epidemiological study conducted between 2015 and 2017 in 31 cities and provinces of China indicated that the prevalence of DM was 11.0% (1). According to the International Diabetes Federation, there were 463 million diabetic patients between 20 and 79 years in 2019 globally. This number is expected to reach 578 million by 2045 and 700 million by 2045 (2). High blood sugar is a crucial marker of DM that damages the body's microvasculature. For instance, diabetic retinopathy (DR) is a type of microangiopathy caused by diabetes. It is becoming increasingly common, as the number of people with DM is rising. By 2030, the estimated number of non-proliferative DR and progressive DR would be 191 million and 56.3 million, respectively. Additionally, due to a lack of awareness and understanding of DR, it has become the leading cause of vision loss in adults aged 30–60 years, significantly affecting the quality of life and posing a health risk. DR is silent at its inception; hence, its early detection, prevention, and therapy are critical for lowering its effect on life and social resources.

Several factors influence the onset and progression of DR. Hyperglycemia, hypertension, dyslipidemia, obesity, smoking, anemia, a lack of health information, and poor treatment adherence are risk factors of DR that can be altered. Whereas ethnicity, family history or inheritance, diabetes onset age, type of diabetes, and diabetes duration are all constant risk factors (3). Previously, studies have built a predictive model for the risk of DR in patients with type 2 diabetes mellitus (T2DM); however, the independent variables were different in all studies (4–6). Thus, this study collected the independent variables that have not appeared in previous studies and aimed to develop a comprehensive model for predicting the risk of DR and the need for early intervention.

Methods

Patients

We included 213 inpatients and outpatients with T2DM at the Affiliated Hospital of Zunyi Medical University between January 2019 and May 2021. The clinical data were collected in Zunyi, Guizhou, a southwestern region of China; hence, the participants were all Han Chinese. All patients with diabetes satisfied the WHO diagnostic criteria, including a fasting plasma glucose level >126 mg/dl and/or an oral glucose level of 75 mg 2 h later. Blood glucose was >200 mg/dl (7). The exclusion criteria were as follows: 1) People with T1DM and other forms of diabetes, including Cushing syndrome; 2) Patients with diabetes with acute diabetic complications, such as ketoacidosis; 3) Pregnant and lactating women; 4) Patients who were unable to perform a fundus examination, such as those with severe refraction in eyes and myopia/hyperopia with a history of >3 days; 5) Patients with eye diseases, such as glaucoma and severe cataracts, that affect fundus observation; 6) Patients with any other disease that can cause fundus hemorrhage; 7) Patients who consumed drugs that affect lipid metabolism and vitamin D (VitD) in the last 6 months. All individuals with T2DM were screened using CR-PGi (Canon). The CR-PGi captured no-dilatation fundus photographs, which have better sensitivity and specificity for screening DR, and high-quality fundus photographs can screen out the most clinically significant DRs (8). Patients screened for suspected DR were then referred to the ophthalmology department and underwent no-dilatation fundus photography, fluorescein fundus angiography, and optical coherence tomography by the same ophthalmologist to verify the diagnosis of DR. Data on gender, age, disease duration, systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), fasting blood glucose (FPG), glycated hemoglobin (HbA1c), homeostatic model assessment-insulin

resistance (HOMA-IR), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), VitD, and creatinine (Cr) were obtained for all individuals *via* medical records. HOMA-IR was used to assess insulin resistance [IR index = FPG (mmol/L) × FINS (mU/L)/22.5] ≥2.8 (9, 10).

Statistical analysis

Using least absolute shrinkage and selection operator (LASSO) regression, we improved the stability of the model by fitting a generalized linear model and performing variable selection and complexity adjustment (regularization). It screens the statistically significant independent variables and calculates the dominance ratio [odds ratios (OR)], 95% confidence interval (CI), and P-value for each independent variable. Finally, we performed a multifactor logistic regression analysis on the statistically significant independent variables (5). The proposed methodology was then built using heterogeneous logistic regression and properties of the cable regression model. Characteristics also included frequency or P-value with 95% CI. We decided to employ bidirectional statistically significant results to construct a predictive nomogram. A P-value <0.05 was considered statistically significant. To forecast the DR incidence, we constructed a prediction model with appropriate adjustments and graphical representations. The accuracy of the DR nomogram was evaluated using the C-index (11, 12), which was validated through bootstrapping. Preference curve analysis was used to determine the predicted results using a copy based on the projected benefit at scene probabilities (6).

Results

Characteristics of the patients

In total, 213 patients with T2DM, including 59.15% men and 40.85% women were included. The patients' mean age was 55.25 ± 9.34 years (range 29–83 years). Based on the fundus examination, the patients were divided into two groups: non-DR (53.05%) and DR (46.95%). Table 1 illustrates the essential characteristics of patients, including the detailed information of 16 clinical indicators.

Variables to be chosen

We analyzed 16 independent variables using R software. Among these, eight statistically significant independent variables or lambda coefficients, namely, disease duration, BMI, FPG, HbA1c, HOMA-IR, TG, TC, and VitD, were screened using LASSO regression analysis (Figures 1A, B).

Model development for diabetic retinopathy prediction

Furthermore, disease duration, BMI, FPG, HbA1c, HOMA-IR, TG, TC, and VitD were examined using multivariate logistic regression analysis. The results of the Cox regression analysis were presented as forest plots (Figure 2). We obtained the following as the DR risk factors: Disease duration (5 < 10, OR = 4.8636; ≥10, OR = 11.8582), BMI (24 < 28, OR = 1.5497; ≥28, OR = 2.1602), FPG (≥7.0, OR = 16.1295), HbA1c (≥7.0, OR = 1.7667), HOMA-IR (≥2.8, OR = 1.5562), TG (≥1.7, OR = 1.4719), TC (≥5.2, OR = 1.7174), and VitD (15 < 30, OR = 0.4177; ≥30, OR = 0.3997). Using the aforementioned independent predictors, we created a model and presented it using a nomogram (Figure 3). Finally, we created a dynamic web-based calculator (https://dxyjiang.shinyapps.io/DRprediction/) that calculates the total score from each patient's clinical indicators for determining the risk of developing DR (Figure 4).

Accuracy of the cohort diabetic retinopathy exposure nomogram

The C-index for evaluating the occurrence of DR in patients with T2DM was 0.848 (95% CI: 0.798–0.898), demonstrating a high validity (Figure 5). Additionally, the result of the bootstrap verification was 0.816. These results indicate that the model has good prediction accuracy.

Clinic application

The DR nomogram was made up of scales for several variables to calculate the likelihood of a given result. According to the judgment curve, non-adherence use of the nomogram raises the projected chance of DR incidence if the thresholds of the patient and doctor are >2% and 85%, respectively. The overlaps were compiled in this study to ensure an equal net gain (Figure 6).

Discussion

Nomograms are simple, quick, cheap, and noninvasive techniques to monitor patients and make appropriate clinical treatment decisions (13). They are used in various medical professions, such as for predicting tumor diagnostic outcomes and therapeutic effects. Nonetheless, only a few studies have attempted to forecast the risk of DR in patients with T2DM. Hence, this study gathered clinical data and demographic characteristics of patients with T2DM and constructed a new prediction model for determining the probability of acquiring DR.

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TABLE 1 Differences between demographic and clinical characteristics of the non-DR and DR groups.

Demographic characteristics	Non-DR (n = 113)	DR (n = 100)	Total (N = 213)
Age (years)			
<50	4 (44.44)	5 (55.56)	9 (4.23)
50–70	107 (55.15)	87 (44.85)	194 (91.08)
>70	2 (0.20)	8 (0.80)	10 (4.69)
Gender			
Male	68 (53.97)	58 (46.03)	126 (59.15)
Female	45 (51.72)	42 (48.28)	87 (40.85)
Disease duration (years)			
<5	65 (76.47)	20 (23.53)	85 (39.91)
5 < 10	46 (40.00)	69 (60.00)	115 (53.99)
≥10	2 (15.38)	11 (84.62)	13 (6.10)
BMI			
<24	66 (72.53)	25 (27.47)	91 (42.72)
24 < 28	37 (38.14)	60 (61.86)	97 (45.54)
≥28	10 (40.00)	15 (60.00)	25 (11.74)
SBP (mmHg)			
<140	102 (52.04)	94 (47.96)	196 (92.02)
≥140	11 (64.71)	6 (35.29)	17 (7.98)
DBP (mmHg)			
<90	99 (51.83)	92 (48.17)	191 (89.67)
≥90	14 (63.64)	8 (36.36)	22 (10.33)
Clinical characteristics			
FPG (mmol/L)			
<7.0	41 (95.35)	2 (4.65)	43 (20.19)
≥7.0	72 (42.35)	98 (57.65)	170 (79.81)
HbA1c (%)			
<7	34 (87.18)	5 (12.82)	39 (18.32)
≥7	79 (45.40)	95 (54.60)	174 (81.69)
FINs (mU/L)			
<5	11 (78.57)	3 (21.43)	14 (6.57)
5 < 20	98 (50.78)	95 (49.22)	193 (90.61)
≥20	4 (66.67)	2 (33.33)	6 (2.82)
HOMA-IR			
<2.8	33 (80.49)	8 (19.51)	41 (19.25)
≥2.8	80 (46.51)	92 (53.49)	172 (80.75)
TG (mmol/L)			
<1.7	48 (66.67)	24 (33.33)	72 (33.80)
≥1.7	65 (46.10)	76 (53.90)	141 (66.20)
TC (mmol/L)			
<5.2	69 (53.91)	59 (46.09)	128 (60.09)
≥5.2	44 (51.76)	41 (48.24)	85 (39.91)
HDL-C (mmol/L)			
>1.0	45 (50.56)	44 (49.44)	89 (41.78)
≤1.0	68 (54.84)	56 (45.16)	124 (58.22)
LDL-C (mmol/L)			
<2.6	42 (58.33)	30 (41.67)	72 (33.80)
≥2.6	71 (50.35)	70 (49.65)	141 (66.20)
VitD (ng/ml)			
≥30	4 (80.00)	1 (20.00)	5 (2.35)
15 < 30	81 (62.31)	49 (37.69)	130 (61.03)
<15	28 (35.90)	50 (64.10)	78 (36.62)

(Continued)

TABLE 1 Continued

Demographic characteristics	Non-DR (n = 113)	DR (n = 100)	Total (N = 213)
Cr (μmol/L)			
≥90	104 (55.03)	85 (44.97)	189 (88.73)
<90	9 (37.50)	15 (52.50)	24 (11.27)

BMI, body mass index; Cr, creatinine; DBP, diastolic blood pressure; DR, diabetic retinopathy; FPG, fasting blood glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; VitD, vitamin D.

Currently, due to high sensitivity and specificity, fundus photography is routinely employed in clinical settings for DR screening (8, 14). Additionally, screening merely evaluates the outcomes and does not reveal the components that play a part in creating the impact. DR is known to be one of the long-term effects of T2DM. It is widely accepted that diabetes is a potential risk for DR; nonetheless, it is unclear whether other factors lead to the development of DR. What is the significance of the relationship between DR and factors? Interestingly, nomograms can provide answers to all of these questions.

According to studies such as the Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS), overweight, disease duration, hypoglycemia, hypertension, high cholesterol, kidney disease, renal failure [diabetic kidney disease (DKD)], pregnancy, and susceptibility genes are common triggers for DR (15, 16). In this study, gender, age, disease duration, SBP, DBP, BMI, FPG, HbA1c, TG, TC, HOMA-IR, LDL-C, HDL-C, VitD-T3, and Cr, which are clinically available clinical indicators, were correlated with the risk of DR using the LASSO method. Furthermore, multivariate logistic regression analysis identified disease duration, BMI, FPG, HbA1c, HOMA-IR, TG, TC, and VitD as risk factors for DR. Except for disease duration, all other risk factors are modifiable. In short, the value of our model is the identification and management of such modifiable risk factors.

Recently, hyperglycemia and disease duration have been identified as risk factors for the pathogenesis of DR (17, 18). Consistently, our prediction model suggests greater risk levels for high fasting glucose and disease progression. Moreover, DR is a metabolic disorder that is difficult to treat and does not develop in patients with reasonable glycemic control. In contrast, patients with poor glycemic control are more likely to develop DR, implying that there are additional secondary contributing factors. According to previous studies, the probability of developing DR increases by approximately 64% per 10% increase in HbA1c and the two have a positive relationship (19–22). Lower serum levels may decrease the risk of severe blindness by 47% when compared with normal serum

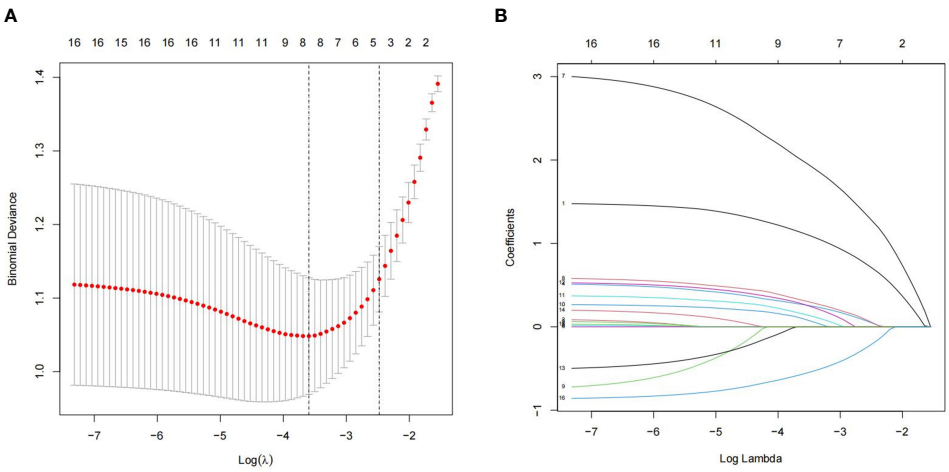

FIGURE 1
Least absolute shrinkage and selection operator (LASSO) logistic regression for assessing the relationship between populations and clinical features. **(A)** The optimum parameter in the LASSO regression model was chosen using a threshold of 16 cross-validations. The partial probability variance curves and the logarithm turn are shown. To get the best estimate, we used the 1-SE technique with minimum standards. **(B)** For each of the 16 characteristics, the LASSO logistic regression coefficients were calculated. The logarithmic lambda was used to determine the profile. The ideal lambda was tested using a cross-validation technique, with the optimal lambda producing eight coefficients.

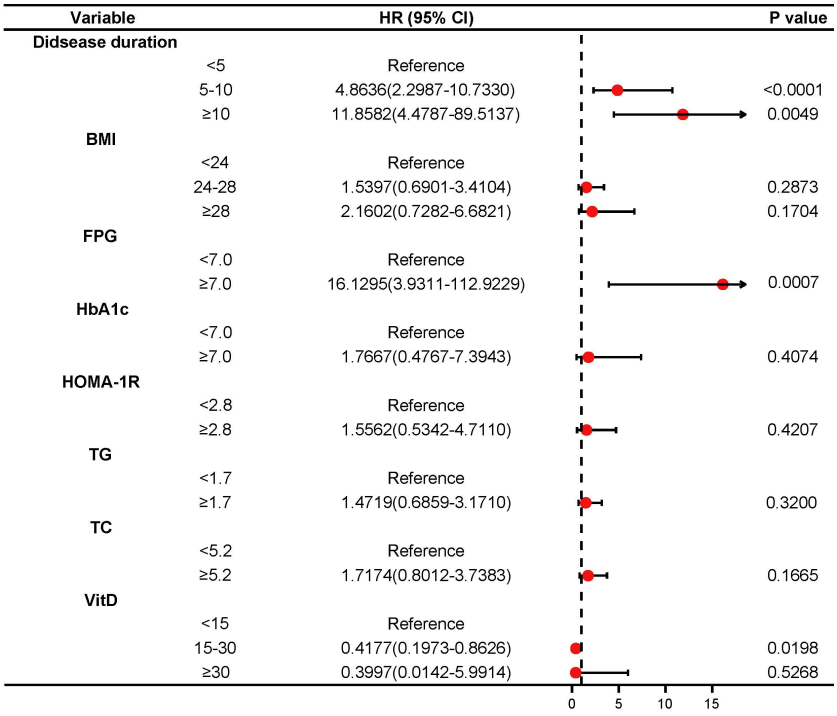

FIGURE 2
The forest plot of the odds ratio (OR) of the Cox regression results.

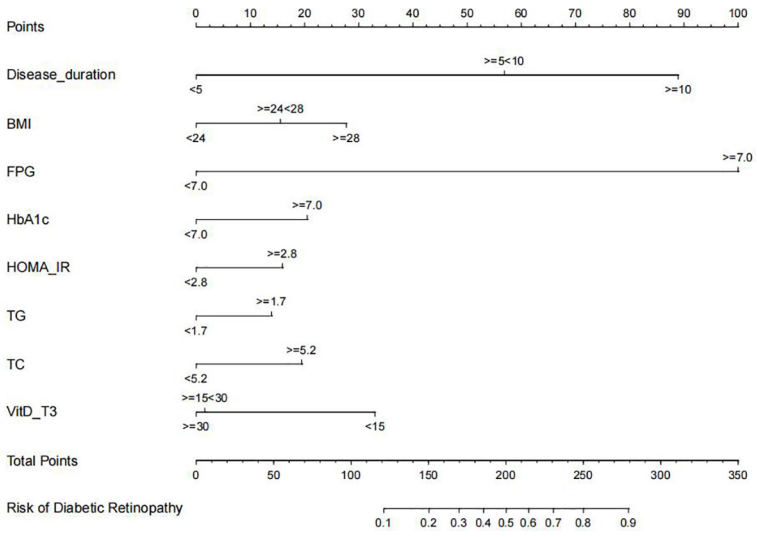

FIGURE 3
Diabetic retinopathy (DR) nomogram. The graph was created using data from the following sources: disease duration, body mass index (BMI), fasting blood glucose (FPG), glycated hemoglobin (Hb1Ac), homeostatic model assessment-insulin resistance (HOMA-IR), triglyceride (TG), total cholesterol (TC), and vitamin D (VitD)-T3.

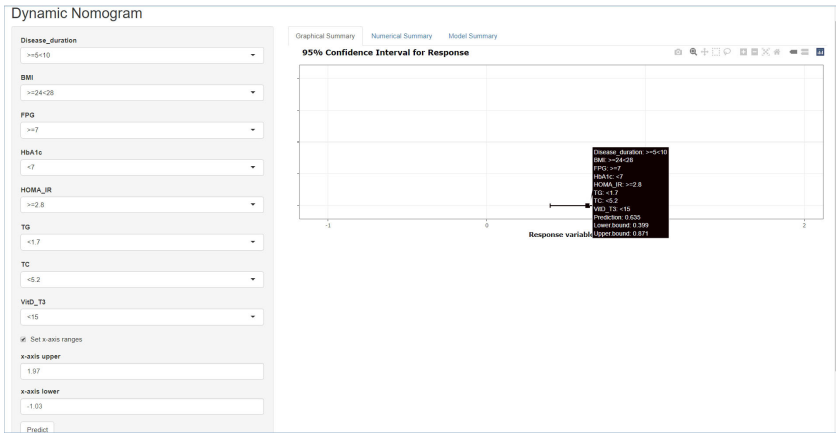

FIGURE 4
A web-based dynamic calculator for predicting the diabetic retinopathy (DR) risk with a 95% confidence interval.

levels after approximately 20 years of follow-up (23). In addition, lipotoxicity, damage to the retinal barrier caused by excessive blood lipids, and exceptionally high TGs are a vital part of DR (24, 25). Thus, controlling dyslipidemia in addition to glycemic management is critical for preventing and treating DR (26). According to the independent variables assessed in our prediction model, HbA1c and lipids could be risk factors for DR. Thus, lowering blood glucose and controlling lipids are among the most critical preventive and therapeutic strategies for DR.

Although nomogram models have been used earlier to predict the risk of DR (6), our model yielded a higher C-index

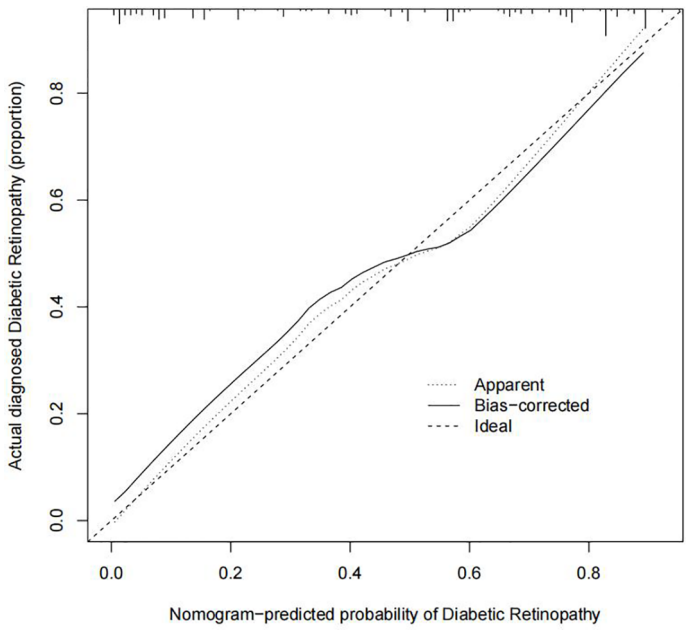

FIGURE 5
Prediction calibration curves of the diabetic retinopathy (DR) nomogram. The x-axis represents the possibility for DR. The x-axis reflects the nomogram-predicted probability. The y-axis reflects the actual predicted probability. A perfect prediction model describes an ideal forecast. The graph depicts the fitness of the nomogram for forecasting outcomes, the dotted line indicating a more reliable prediction.

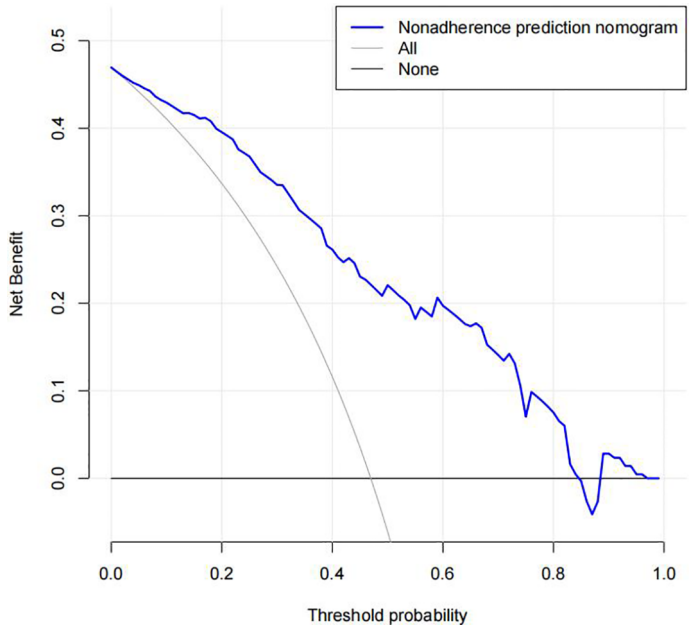

FIGURE 6
Decision curve analysis of the diabetic retinopathy (DR) nomogram. The x-axis represents the threshold probability. The y-axis represents the net benefit. The dotted line represents a DR risk nomogram, and the narrow solid line indicates patients presumed to have DR. The decision curve revealed that if a patient's and a doctor's threshold probabilities are more than 2% and 85%, respectively, using this DR nomogram in the current study to predict DR incidence risk adds more benefit than the intervention-all-patients scheme.

value, indicating a higher accuracy. The indicators included in our model are more comprehensive than those included in the previous models, making it a more accurate predictor of risk. This study included popular Vitamin D (VD) from recent years that other researchers have not used. Low levels of VD are a specific and sensitive sign of proliferative diseases. Additionally, they are adversely associated with the intensity of DR. The AUC recommends VD as a straightforward, sensitive, and specific laboratory test for DR (27). According to a foreign cross-sectional investigation, patients with VD insufficiency were more likely to acquire DR than those with VD sufficiency. Multidisciplinary ordinal regression analysis revealed a link between VD shortage and DR severity (28–30). Consistently, this study identified VD rates as a predictor of DR, and the incidence of DR increases with decreasing VD levels. Relevant mechanisms have been proposed for determining the role of VD in DR, including VD increasing endothelial nitric oxide synthase (eNOS)-dependent NO production, reducing oxidative stress, and enhancing pathophysiological processes, such as vascular endothelial growth factor (VEGF) synthesis and release (31–33). However, according to a prospective observational study, VD deficit is directly linked to all-cause survival and does not predict the development of microvascular complications (34).

Inevitably, there are certain flaws in this research. Firstly, the sample size was small. Secondly, although it was a long-term assessment to evaluate the risk of DR, the study did not account for the bias caused by patients' medications as DR progressed. Thirdly, because lighting affects VitD-T3, we could not collect data from patients during a continuous daytime period. As previously said, the risk or the occurrence of DR is not uniform; hence, we need to incorporate more indicators to derive more preventive methods for DR in patients with T2DM.

Conclusion

This study successfully constructed a nomogram for assessing the risk of DR in patients with T2DM. These findings will guide patients and doctors to develop personalized treatment plans based on these risk factors, eliminate the hazard of DR, and avoid the onset and development of DR.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

QW, NZ, and HT contributed to the conception and design of the study. QW, NZ, XY, QY, LZ, HZ, YZ, XN, and FJ contributed to data collection, analysis, and interpretation. QW and NZ wrote the article. XL and FJ revised the manuscript. The final article was read and approved by all participants before submission. QW and NZ contributed equally to this work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Decreased expression of Glucagon-like peptide-1 receptor and Sodium-glucose co-transporter 2 in patients with proliferative diabetic retinopathy

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Purpose: To investigate the expression of Glucagon-like peptide-1 receptor (GLP-1R), sodium-glucose co-transporter (SGLT) 1, SGLT2, Glucose transporter type 1 (GLUT1) and GLUT2 in patients with diabetic retinopathy (DR).

Methods: We obtained peripheral blood mononuclear cells (PBMCs) and vitreous samples from 26 proliferative DR (PDR) patients, 25 non-proliferative DR (NPDR) patients, 25 non-DR (NDR) patients, and 26 nondiabetic patients with idiopathic epiretinal membranes (ERMs, control). The protein level and mRNA expression level of GLP-1R were quantified by immunoblot and qRT-PCR and the levels of SGLT1, SGLT2, GLUT1, and GLUT2 expression were determined by PCR. Their association with clinical parameters and PBMCs/vitreous cytokine was analyzed. Furthermore, immunofluorescence staining of GLP-1R and SGLT2 was carried out on samples of fibrovascular membranes (FVMs) retrieved from 26 patients with PDR and 26 patients with ERMs.

Results: The transcriptional levels of GLP-1R and SGLT2 in PBMCs were significantly more decreased in PDR patients than in patients without DR and controls, which was simultaneously associated with an increased level of expression of tumor necrosis factor (TNF)-α and interferon (IFN)-γ. The expression levels of GLUT1 and GLUT2 were tightly correlated with their SGLT partners, respectively. Further, Immunofluorescence staining showed no positive staining of GLP-1R and SGLT2 was detected in the FVMs from PDR.

Conclusions: GLP-1R and SGLT2 were significantly decreased in PDR patients which was associated with an increased level of expression of TNF-α and IFN-γ. These findings implicate that defective GLP-1R and SGLT2 signaling may potentially correlate with immune response cytokines in patients with PDR.

KEYWORDS

diabetic retinopathy, glucagon-like peptide-1 receptor, sodium-glucose co-transporter, glucose transporter, cytokines

Introduction

Diabetic retinopathy (DR) is a diabetic microangiopathies commonly occurred as a complication of type 2 diabetes mellitus (T2DM), and the most common cause of sight-threatening blindness worldwide (1). The molecular mechanisms underlying this disease are therefore highly demanded for the development of novel treatment strategies. Intensive studies has been focused on the use of non-insulin anti-hyperglycaemic agents, including agonists of glucagon-like peptide-1 receptor (GLP-1R) and inhibitors of sodium-glucose co-transporter-2 (SGLT-2), in the treatments of T2DM.

Glucagon-like peptide-1 (GLP-1) has becoming a special interest as a treatment target due to its broad regulatory roles in maintaining glucose homeostasis. GLP-1 is postprandially secreted by intestinal enteroendocrine L-cells, and enhances the glucose-induced insulin release from pancreatic beta-cells (2). The G-protein-coupled membrane receptor, GLP-1R, has also been discovered in the pancreatic islets' cells and in various other kinds of tissues as well, including the kidney, heart, blood vessels, central nervous system, and retina (Lin et al., 2018; Shi et al., 2015). On the other hand, several glucose sensors, such as electrogenic glucose transport SGLT 1/SGLT 2 and facilitative glucose transporter (GLUT)1/GLUT 2, have been suggested to associate with the glucose-exposure-induced GLP-1 secretions. It has been proposed that glucose induces GLP-1 release through SGLT1/SGLT 2, and to a lesser extent, GLUT 1/GLUT 2.

At the same time, anti-hyperglycaemic agents are demonstrated to produce protective or neutral influences on eye complications of diabetes. Previous studies have reported the potential beneficial influences of GLP-1 agonists in the treatments of diabetic retina through the functional improvements of blood retina barrier and the inhibition of neuronal apoptosis (3). Study on spontaneously diabetic fatty rats reveals that alleviation of hyperglycaemia by the treatment of SGLT-2 inhibitor is able to limit the development of microvascular complications of diabetes such as diabetic retinopathy (4).

However, underlying mechanisms of the protective effects of anti-hyperglycaemic agents on DR patients remains unclear. Currently, immunity dysregulation is considered as a significant pathogenic mechanism in DR, both locally as well as systematically (5). Metabolic imbalance concerning glucose metabolism potentially results in a dysregulation in the function and dissemination of T-lymphocytes, leading to dysfunctional cell-mediated immune responses, which is considered as a contributor to the pathogenesis of DR (6). In addition, GLP-1Rs have been detected on immune cells, and its anti-inflammatory effects include the inhibition of TNF-α (7). Therefore, it appears feasible to speculate that GLP-1/GLP-1R signaling is related to the functions of T-lymphocytes in DR patients.

In the present study, we quantified the expression levels of GLP-1R, SGLT1, SGLT2, and the respective cognate basolateral transporters (GLUT1 and GLUT2) in peripheral blood mononuclear cells (PBMCs). The pro-inflammatory cytokines associated with T helper cells including TNF-α, and IFN-γ in PBMCs and vitreous fluid were also measured. Subsequently, these results were further confirmed in tissue samples obtained from patients with proliferative DR (PDR).

Materials and methods

Patients

Twenty-six PDR patients, 25 non-proliferative diabetic retinopathy (NPDR) patients, 25 non-DR (NDR) patients, and 26 nondiabetic patients with idiopathic epiretinal membranes (ERMs) who received vitrectomy were recruited from the Zhongshan Ophthalmic Center between Jan and July 2021 (Table 1). Patients with a medical history of intraocular surgery, ocular trauma, ocular inflammatory diseases, trauma, vitreous hemorrhage, uveitis, retinal detachment, systemic or topical steroid treatment, and immunosuppressive drug administration were excluded. All diagnoses were carried out according to the 2002 standards of the American Diabetes Association (8). Exclusion criteria included infectious disease, diabetes-associated nephropathy (including patients with chronic kidney disease in stage 3, proteinuria, and macroalbuminuria, and patients receiving hemodialysis) and patients who received intraocular or intravitreal treatments and photocoagulation within 3 months upon recruitment. Chronic kidney diseases were categorized according to the clinical guidelines of the National Kidney Foundation Disease Outcomes Quality Initiative. DR was diagnosed according to the results of fluorescein fundus angiography (FF450 fundus camera; Carl Zeiss, Germany). Furthermore, the Body mass index (BMI) of patients was also collected. All recruited patients were subcategorized into three groups based on the Diabetic Retinopathy Disease Severity Scale: NDR, NPDR, and PDR (9).

All experimental procedures were carried out following the principles of the Declaration of Helsinki, and authorized by the Human Ethics Committee of Zhongshan Ophthalmic Center of Sun Yat-sen University. Each included patient was fully informed and written informed consents were obtained.

Sample preparation

Whole blood specimens (12 mL, with anticoagulant lithium heparin) were collected from all recruited patients and healthy controls for isolation of PBMCs, protein and mRNA expression tests. Blood sample aliquots were also obtained to conduct

TABLE 1 Clinical and biochemical characteristics of type 2 diabetic patients and healthy control subjects.

	Control(N = 26)	NDR(N =25)	NPDR(N = 25)	PDR(N = 26)	p
Sex(m/f)	13/13	12/13	11/14	14/12	0.916
Age(years)	62.8 ± 6.9	64.3 ± 8.7	61.6 ± 8.1	63.7 ± 6.0	0. 623
BMI(kg/m2)	22.5 ± 2.2	23.0 ± 2.5	23.4 ± 2.2	25.1 ± 4.4	0.011
Diabetes Duration(years)	-	8.2 ± 3.4	9.7 ± 3.0	14.0 ± 2.0	<0.001*
FPG(mmol/l)	5.3 ± 0.7	7.8 ± 1.6	9.6 ± 2.1	12.4 ± 1.8	<0.001*
HbAlc(%)	5.1 ± 0.7	7.3 ± 1.4	8.8 ± 1.9	11.4 ± 1.8	<0.001*

DR, diabetic retinopathy; NDR, no apparent retinopathy; NPDR, non-proliferative retinopathy; PDR, proliferative diabetic retinopathy; BMI, Body mass index; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin.
Data are expressed as mean ± SD.
* P ≤ 0.05.

fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) tests.

PBMCs isolation

PBMCs were extracted from heparinized blood samples through Ficoll-Hypaque density gradient centrifugations (Lymphoprep; Nycomed Pharma, Norway). PBMCs (2 × 106 cells/ml) were stimulated with phytohaemagglutinin (PHA) to assess the production of TNF-α and IFN-γ. Isolated PBMCs were stimulated for 48h, and subsequently used for TNF-α and IFN-γ analysis by ELISA.

Vitreous fluid

During pars plana vitrectomy, samples containing undiluted vitreous fluid (0.5 ml) were obtained from 26 PDR patients, 25 NPDR patients, 25 NDR patients, and 26 nondiabetic patients with ERMs. All the samples were preserved at −80°C until they were needed for further analyses.

RNA extraction and quantitative real-time PCR

TRIzol reagent (Carlsbad, USA) was utilized to extract the total RNA of PBMCs and a reverse transcription kit (Toyobo, Japan) was used for reverse transcription to cDNA. qRT-PCR was conducted on a LightCycler CFX96 (BioRad, USA) using QuantiFast SYBR Green PCR Kit (Qiagen, Germany). The primers that were used in this study are described as follows: GLP-1R forward: 5'-GTT TCA TGA TGG CCT GAG GT-3', reverse: 5'-CTG ACT ACT GAA TTG GAA GGG G-3'; SGLT1 forward: 5'-CTC CCT TTC TTA TTC TCC CAG GAT-3', reverse: 5'-GCC CAG GAG ATC AAG GCT ATA GTA-3'; SGLT2 forward: 5'-ATA AAC AGC TGG GCT GTC CC-3', reverse: 5'-CGT AAC CCA TGA GGA TGC AG-3'; GLUT1 forward: 5'-AGG GCT GGA GTG AGG GTA GT-3', reverse: 5'-CAT ACA TCT GTG GGG CAG C-3'; GLUT2 forward: 5'-AAA CAA AGC AAA TGT TCA GTG G-3', reverse: 5'-TGG GTC CCC AAA AGC TTA G-3'; TNF-α forward5′-CCCAGGCAGTCAGATCATCTTC-3′.Reverse:5′-AGCTGCCCCTCAGCTTGA-3′.; IFN-γ forward: 5'-TCAACTT CTTTGGCTTAATTCTCTC-3', reverse: 5'-ATATGGGTC CTGGCAGTAACA-3' and β-actin forward: 5'-GGA CTT CGA GCA AGA GAT GG-3', reverse: 5'-AGC ACT GTG TTG GCG TAC AG-3'. β-actin acted as an internal control. All samples were tested in triplicates. The single peak in the melting curve was used for primer specificity confirmations. The relative mRNA expression levels were estimated according to the ΔΔCt method.

Immunoblotting

Protein samples of PBMCs isolated from T2DM patients as well as healthy controls were prepared in RIPA buffer. Aliquots of 60 μg protein were divided by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and placed on polyvinylidene fluoride (PVDF) membranes *via* semidry electroblotting. The primary antibodies GLP-1R (Abcam, UK) and β-actin (Abcam, UK) were used for immunoblotting: Immunoblot was visualized on radiographic films using the SuperSignal West Pico Substrate Kit (Pierce, USA), and the software Image J (National Institutes of Health, USA) was applied for analysis. β-actin served as internal control.

Cytokine ELISA

The concentration of TNF-α and IFN-γ in the supernatants of collected PBMCs and vitreous fluid were determined by DuoSet ELISA kits (R&D Systems) as instructed by the manufacturer. The lowest detectable concentration of TNF-α was 15.6 pg/ml and 9.4 pg/ml for IFN-γ. These measurements were performed in duplicate.

Immunofluorescence staining of FVMs

The fibrovascular membranes (FVMs) of T2DM patients with PDR (26 cases) were surgically detached through membrane peeling during pars plana vitrectomy. ERM resections were carried out on 26 idiopathic ERM patients as control. As Table 1 shows, significant differences in age and gender were not detected between the groups.

Samples of ERMs were embedded in an ideal cutting compound, fast frozen, and preserved at -80°C within 1h following collection of the fresh samples. The following primary antibodies were used for immunofluorescence staining: anti-GLP-1R polyclonal IgG (Abcam, ab214185, 1:300) and anti-SGLT2 polyclonal IgG (Abcam, ab180799, 1:200). DAPI (Sigma-Aldrich, D9542, 1:1000) was used for visualization of the nuclear morphology. Immunofluorescence staining was examined and images were captured under a fluorescence microscope (DS-Ril-U2; Nikon, Japan)

Statistical analysis

SPSS software (version 22.0, SPSS Inc., USA) was used to carry out statistical analysis. Nonparametric Kruskal-Wallis tests or One-Way Analysis of Variance (ANOVA) was conducted for the group variation analysis between T2DM patients and healthy controls. Mann-Whitney U tests or t-tests were used for the analysis between each group. Spearman's correlation tests were used to establish potential correlations between parameters. The multivariable models of GLP-1R and SGLT2 were utilized to better understand their clinical implications in relation to DR, BMI, diabetes duration, FPG, HbAlc, age and sex. All graphs were generated by GraphPad Prism version 5 and the data were expressed as mean ± SD. $P < 0.05$ was set as the cut-off for statistical significance.

Results

Clinical features

Statistically significant differences ($P = 0.623$) were not found in the average age of T2DM patients (76 patients, of which 37 male and 39 female, average age 63.2 ± 7.6 years old) and normal controls (26 patients, of which 13 male and 13 female, average age 62.8 ± 6.9 years-old), as shown in Table 1. All T2DM patients were divided in either one of the following three groups: NDR (n=25), NPDR (n=25), and PDR (n=26). As a result, the male/female ratios and average ages of each group were as follows: NDR: 12/13, 64.3 ± 8.7 years old; NPDR: 11/14, 61.6 ± 8.1 years old; and PDR: 14/12, 63.7 ± 6.0 years old.In addition, statistically significant differences in gender were also not detected among the groups ($P = 0.916$). However, a significantly higher BMI was found in T2DM patients than healthy controls ($P = 0.011$). A significantly longer course of disease was identified in PDR patients in comparison to NPDR and NDR patients ($P < 0.001$). Statistically significant higher HbA1c and FPG levels were found in PDR patients in comparison to NPDR ($P < 0.001$) and NDR ($P < 0.001$) patients.

mRNA expression levels of GLP-1R, SGLT1, SGLT2, GLUT1, GLUT2, TNF-α and IFN-γ

We investigated the mRNA levels of GLP-1R, SGLT1, SGLT2, GLUT1, GLUT2, TNF-α, and IFN-γ in PBMCs isolated from T2DM patients and healthy controls using qRT-PCR (Figure 1). The findings indicated significantly lower mRNA expression levels of GLP-1R (both $P < 0.001$) and SGLT2 ($P = 0.021$ and $P < 0.001$, respectively) in PBMCs isolated from PDR patients than that of NDR patients and healthy controls. On the contrary, the mRNA expression levels of TNF-α (both $P < 0.001$) and IFN-γ (both $P < 0.001$) were significantly higher in PDR patients in comparison to NDR patients and controls. Meanwhile, we also found a significant intercorrelation between the expression of SGLT1 and GLUT1, and also between SGLT2 and GLUT2 (Figures 2A, B). However, the differences in the expression ratios of SGLT1/GLUT1 ($P = 0.622$) and SGLT2/GLUT2 ($P = 0.087$) were not significant among different groups of patients.

mRNA Levels of GLP-1R and SGLT2 and demographic factors

Figure 3 shows that the detected levels of GLP-1R ($r = -0.605$, $P < 0.001$) and SGLT2 mRNA ($r = -0.281$, $P = 0.014$) both had a negative correlation with the course of disease in T2DM patients. Meanwhile, we also discovered that the mRNA levels of GLP-1R ($r = -0.799$, $P < 0.001$ and $r = -0.788$, $P < 0.001$, respectively) and SGLT2 ($r = -0.512$, $P < 0.001$ and $r = -0.507$, $P < 0.001$, respectively) indicated a negative correlation with the levels of FPG and HbA1c as well.

GLP-1R protein levels in PBMCs

For further verification of the downregulation trend of GLP-1R in DR patients, we tested the protein levels of GLP-1R in the PBMCs isolated from DR patients before receiving any clinical treatments and healthy controls. As shown in Figure 4, we found significantly decreased protein levels of GLP-1R in PDR patients in comparison to both NDR patients ($P < 0.001$) as well as normal controls ($P < 0.001$). Moreover, the protein levels and mRNA expression levels of GLP-1R in every group were significantly positively correlated ($r = 0.604$; $P < 0.001$).

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Multivariate regression analysis of GLP-1R and SGLT2 as dependent variable in the T2D samples

Multiple linear regression analysis revealed that PDR remained independently and negatively associated with GLP-1R protein level and SGLT2 mRNA level after adjustment for age, gender, BMI, diabetes duration, FPG and HbAlc (P=0.003 and P=0.028, respectively). Furthermore, in this model, diabetes duration was found independently and negatively associated with GLP-1R mRNA expression (P=0.028) (Table 2).

Vitreous cytokines

The PBMCs and vitreous concentrations of TNF-α and IFN-γ according to DR status are shown in Figure 5. The detected

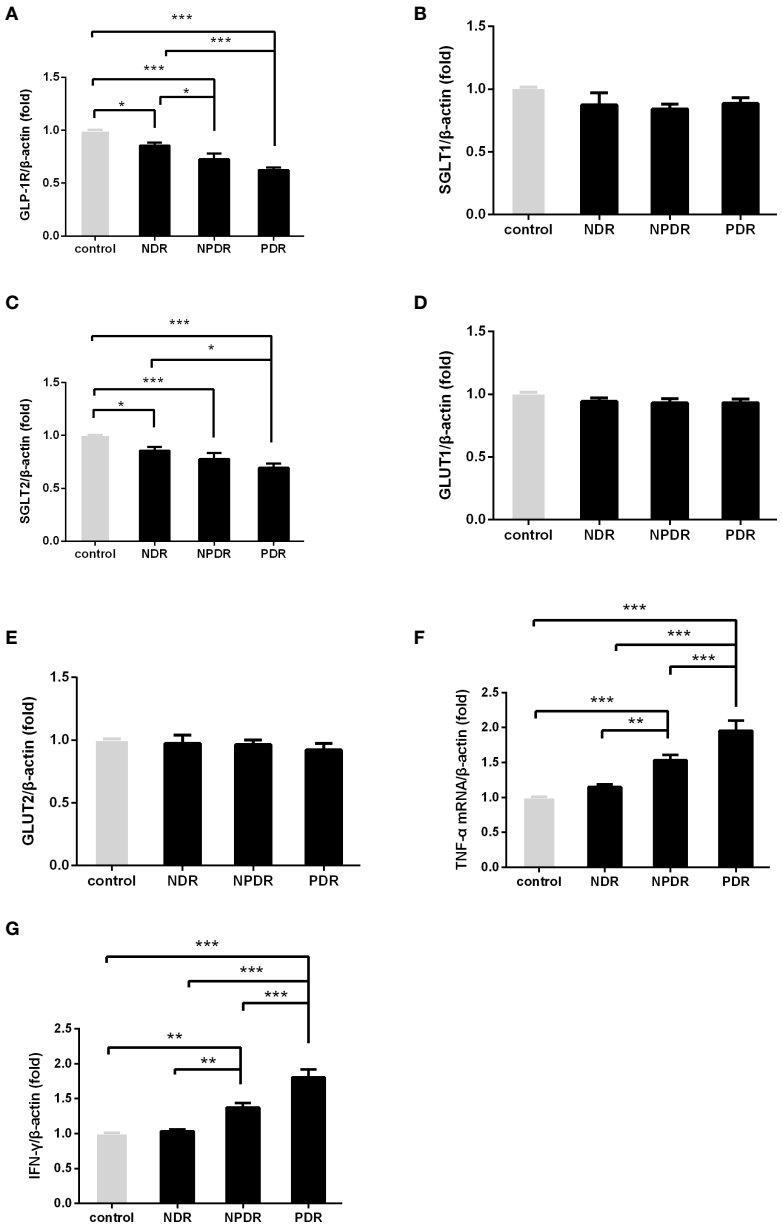

FIGURE 1
The mRNA expression of GLP-1R and SGLT2 was decreased and that of TNF-α and IFN-γ was elevated in DR patients. The mRNA expression of GLP-1R, SGLT1, SGLT2, GULT1, GULT2, TNF-α and IFN-γ in freshly obtained PBMCs was quantified by real-time PCR and normalized to the expression levels of β-actin. (PDR, n=26; NPDR, n=25; NDR, n=25; control, n=26) **(A–G)**. The values represent the fold-change in comparison to the controls. *P < 0.05, **P < 0.01, ***P < 0.001.

levels of TNF-α and IFN-γ in the PBMCs and vitreous fluid of PDR patients (all $P < 0.001$) were statistically significantly enhanced in comparison to those of NDR patients and healthy controls. In addition, statistically significant correlations were found among the concentrations of TNF-α ($r = 0.713$, $P < 0.001$) and IFN-γ ($r = 0.811$, $P < 0.001$) in PBMCs and vitreous fluid (Figure 5C, F).

Correlation between GLP-1R/SGLT2 expression and TNF-α/IFN-γ expression

A negative correlation was found between the mRNA levels of GLP-1R and SGLT2 in PBMCs on the one hand and mRNA levels of TNF-α and IFN-γ on the other (Figure 2C–F). Meanwhile, negative correlations were also discovered between the mRNA levels of GLP-1R and SGLT2 on one hand and TNF-α and IFN-γ on the other in PBMCs and vitreous fluid (Figure 2G–N). These findings show that the expression of GLP-1R and SGLT2 is correlated to TNF-α/IFN-γ Expression.

GLP-1R and SGLT2 Expression in FVMs of PDR patients

In our experiments, positive staining of GLP-1R in the FVMs collected from PDR patients was not identified. Furthermore, positive staining of SGLT2 was also not detected in the membranes collected from PDR patients (Figure 6).

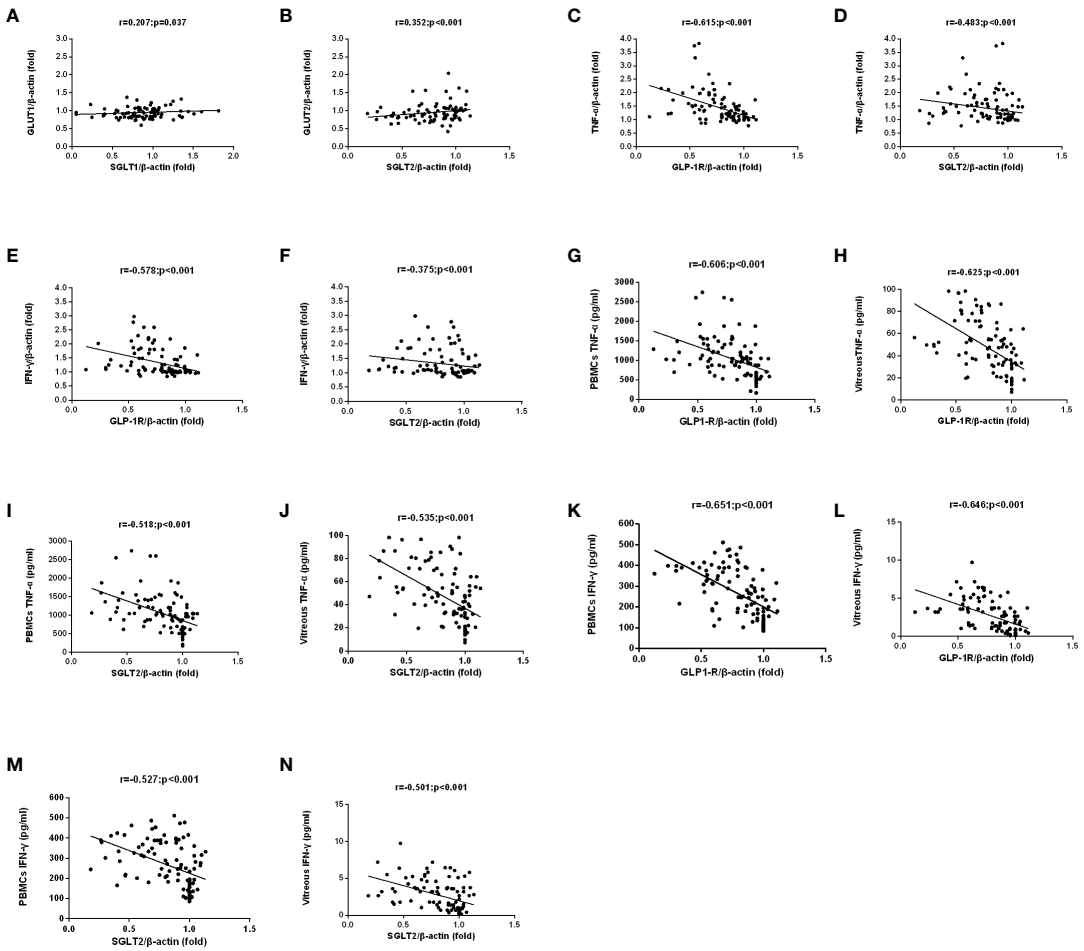

FIGURE 2
The correlation between SGLTs and GLUTs was analyzed in matched samples with the Spearman correlation. GLUT2 and GLUT1 expression were closely correlated to those of the corresponding SGLT partners. **(A, B)**. A negative correlation among TNF-α/IFN-γ mRNA and protein expression and GLP-1R/SGLT2 mRNA expression in PBMCs of all patients were found **(C–N)**.

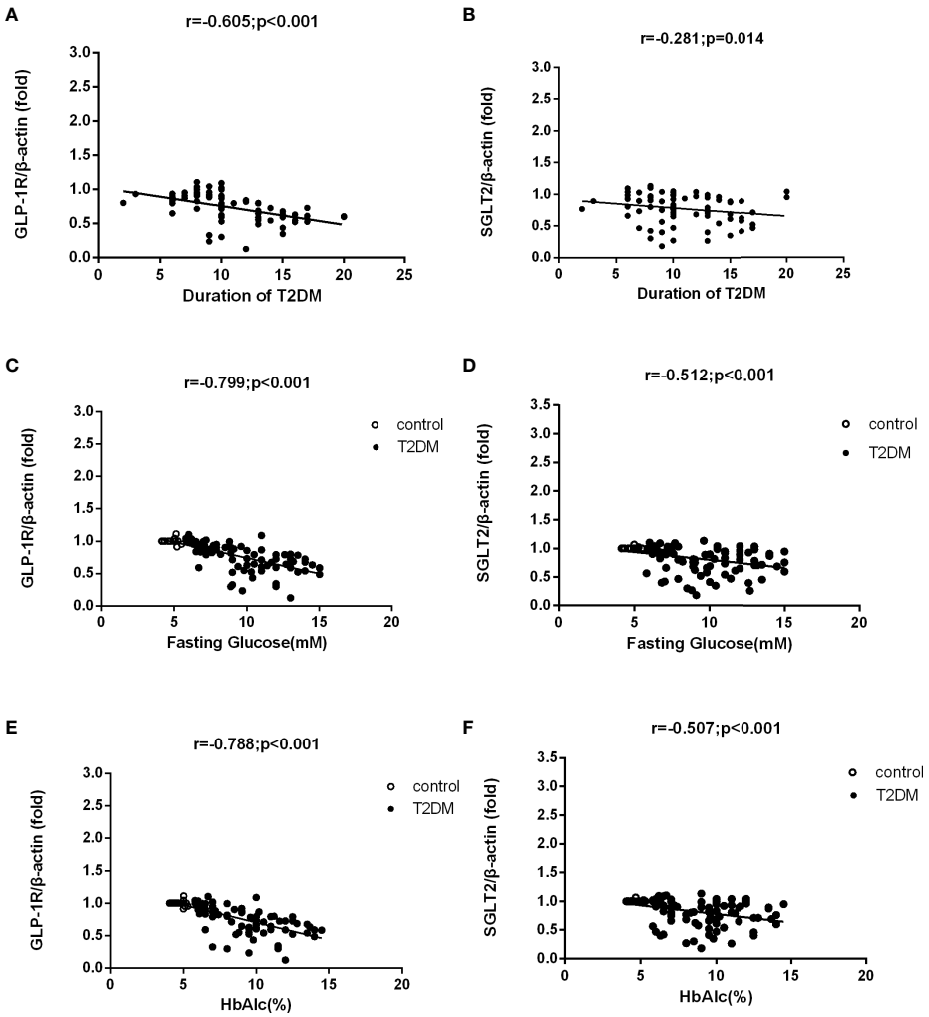

FIGURE 3
Correlation analysis of mRNA levels of GLP-1R and SGLT2 and demographic factors in T2DM patients and control group. mRNA levels of GLP-1R and SGLT2 were both negatively correlated to the course of disease in T2DM patients **(A, B)**. The mRNA levels of GLP-1R and SGLT2 were also negatively correlated to the levels of FPG and HbA1c in T2DM patients and the control group **(C–F)**.

Discussion

In this study, we provided evidence for the changes in GLP-1R expression levels in DR patients. Moreover, we obtained the quantitative expressions of SGLT1/SGLT2 and GLUT1/GLUT2 in DR patients. Our results have indicated that PDR patients were characterized by decreased GLP-1R and SGLT2 expression, which was related to a higher expression of TNF-α and IFN-γ. The expressions of GLP-1R and SGLT2 were subsequently confirmed in the FVM collected from the PDR patients by immunofluorescence. Additionally, we found that the expression ratios of SGLT1/GLUT1 and SGLT2/GLUT2 were similar; meanwhile, both the expression levels of SGLTs showed significant correlations with the expression of respective GLUTs genes. These results are in agreement with the finding that SGLTs interconnect to specific isoforms of GLUTs (10).

In this study, we found that the level of GLP-1R was significantly reduced in PBMCs isolated from PDR patients. We also found that the levels of GLP-1R in eye samples collected from PDR patients in advanced stages was not detectable, which was consisted with previous study (11). As a gut incretin hormone, GLP-1 is produced in intestine by L cells. GLP-1 participates in the regulation of glucose homeostasis through stimulating insulin secretions and suppressing glucagon releasing in response to glucose intakes (12). Meanwhile, GLP-1 also produces functions glycemic independently in different organs (13). The effects produced by GLP-1 in is reflected by the local activations of GLP-1R (14). The expression of GLP-1R in retinal pericytes and ganglion cells was previously demonstrated

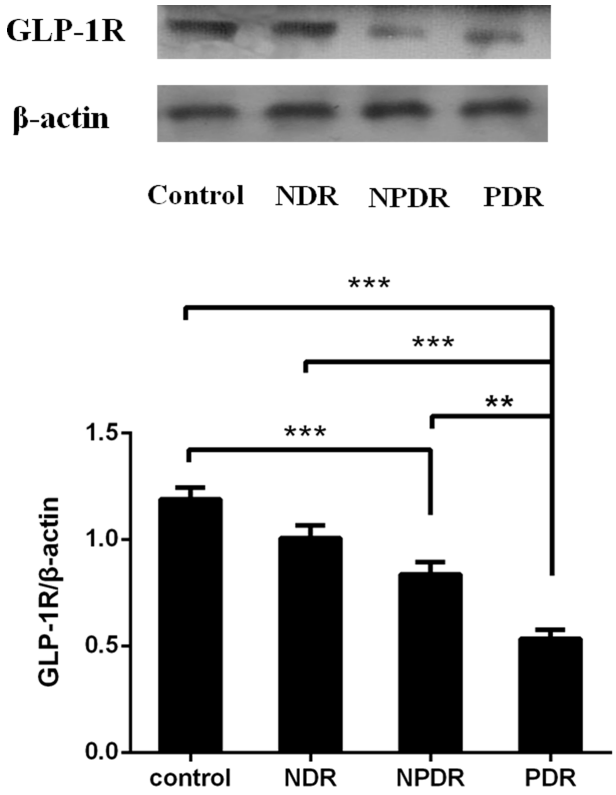

FIGURE 4
The protein expression of GLP-1R was decreased in DR patients (PDR: n=26; NPDR: n=25; NDR: n=25; control: n=26). Western blot analysis (lane 1, healthy control; lane 2, NDR; lane 3, NPDR; and lane 4, PDR) and quantitation of GLP-1R from PBMCs. β-actin was applied as the internal control. **P < 0.01, ***P < 0.001.

(15), while GLP-1/GLP-1R was also reported to produce beneficial effects under the condition of hyperglycemia (16), suggesting that GLP-1/GLP-1R has protective effects on the integrity of retina in the first phases of DR caused by diabetes (3). Consistently, one previous study demonstrated the neuroprotective abilities of GLP-1R agonists in DR of db/db mice (17). Nonetheless, the possible mechanisms responsible for its protective effects are still unknown.

We found a positive correlation between TNF-α and IFN-γ production and expression levels of GLP-1R, which were

TABLE 2 Multivariate Regression Analysis With GLP-1R and SGLT2 as Dependent Variable in T2D samples.

Independent Variables	GLP-1R mRNA		GLP-1R protein		SGLT2 mRNA	
	β (95 %CI)	*P*	β (95 %CI)	*P*	β (95 %CI)	*P*
Groups						
NPDR	-0.046 (-0.147,0.055)	0.369	-0.128 (-0.2821,0.027)	0.106	-0.104 (-0.236,0.027)	0.119
PDR	-0.011 (-0.138,0.160)	0.881	-0.341 (-0.569,-0.113)	0.003*	-0.217 (-0.411,-0.023)	0.028*
Age	0.002 (-0.004,0.007)	0.553	0.000 (-0.008,0.008)	0.978	0.001 (-0.006,0.007)	0.876
Sex	0.011 (-0.067,0.089)	0.774	-0.040 (-0.160,0.079)	0.510	0.008 (-0.093,0.109)	0.877
BMI	0.001 (-0.012,0.013)	0.908	-0.010 (-0.029,0.010)	0.3175	0.003 (-0.014,0.019)	0.727
Diabetes duration	-0.016 (-0.030,0.002)	0.028*	-0.011 (-0.033,0.011)	0.339	-0.007 (-0.025,0.012)	0.476
FPG	-0.017 (-0.074,0.039)	0.551	-0.018 (-0.104,0.069)	0.686	0.026 (-0.048,0.099)	0.493
HbAlc	-0.017 (-0.078,0.044)	0.589	0.008 (-0.086,0.102)	0.868	-0.007 (-0.087,0.073)	0.867

T2D, Type 2 diabetes; NPDR, non-proliferative retinopathy; PDR, proliferative diabetic retinopathy; BMI, Body mass index; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin.
*P ≤ 0.05

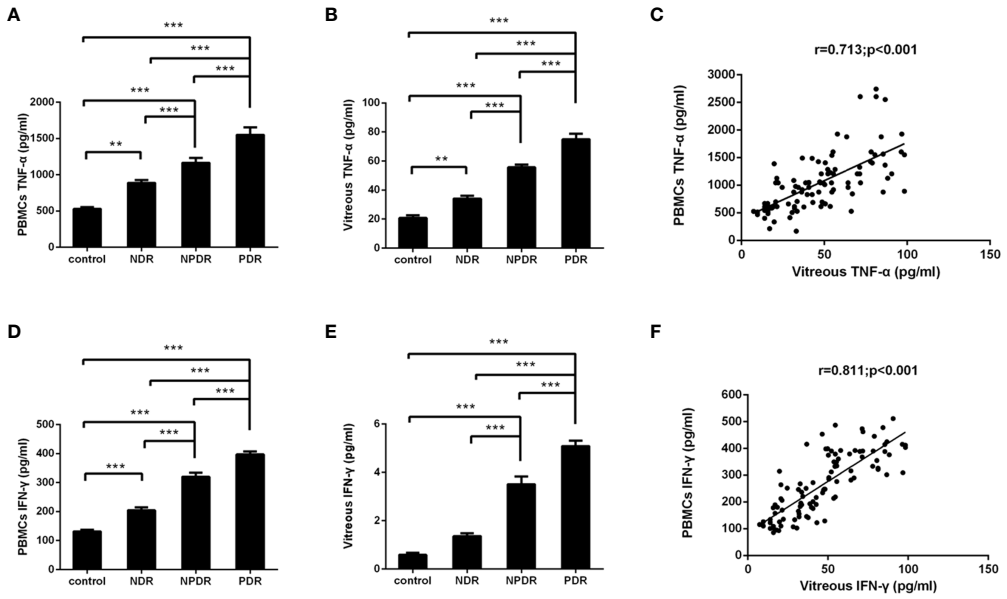

FIGURE 5
Concentrations of TNF-α/IFN-γ in PBMCs supernatants and vitreous fluid of T2DM patients and non-diabetic controls. TNF-α and IFN-γ were measured with ELISA in PBMCs supernatants **(A, D)** and vitreous fluid **(B, E)** of controls (n=26), non-diabetic retinopathy (NDR, n=25), nonproliferative diabetic retinopathy (NPDR, n=25), and proliferative diabetic retinopathy (PDR, n=26). Between group comparisons were conducted with the Kruskal–Wallis test and then the Dunn multiple comparison test. The correlation between concentrations of TNF-α/IFN-γ in the PBMCs supernatants as well as vitreous fluid was analyzed in matched samples with the Spearman correlation **(C, F)**. **P < 0.01, ***P < 0.001.

consistent with the observation that modulation in GLP-1R signaling control host microbial responses and innate immune responses in a mouse model (18).

An accumulating amount of studies have been reporting about the immune dysfunction of T cells in DR (6, 19). A continuous decline in T cell function can be caused by sustained signaling in DR, and it has been well established that TNF-α, IFN-γ, and their distinct receptors are vital components of the innate immune system. Consistent with these outcomes, we also discovered higher expressions of TNF-α and IFN-γ mRNA in PBMCs of DR patients compared to that of non-DR subjects, which simultaneously showed a higher incidence of increased TNF-α and IFN-γ production in correlation to the progression of DR severity.

In our study, we further assessed the role of SGLT1, SGLT2, GLUT1, and GLUT2 in GLP-1 release. GLUTs mainly consist of GLUTs, such as GLUT5, GLUT7, GLUT9, and GLUT11, and SGLTs (10, 20, 21). Of the SGLTs family, subtypes SGLT1 and SGLT2 have been intensively studied. Inhibitors of SGLT2 have already been applied in the clinical treatments of patients with diabetes (22). It is well known that SGLTs and GLUTs produce active and facilitative effects, respectively. The relative roles of SGLTs and GLUTs in GLP-1 secretion induced by glucose have been investigated in seminal studies using pharmacological and genetic interference with SGLTs and GLUTs (23–25), suggesting that they were essential for GLP-1 secretion induced by glucose associated with the cAMP and Ca2+ signaling system (26, 27). Consistent with studies linking decreased SGLT activity with reduced GLP-1, our study found that GLP-1R and SGLT2 were simultaneously decreased in PDR patients (28), these data indicated that release of GLP-1in DR might be a process that requires SGLT2-mediated glucose transport in the signal transduction pathway.

Despite the lack of understanding of the regulation in this process, evidence derived from mouse models and preclinical and clinical research suggested that SGLT2 inhibitors produced effects that reduced tissue inflammation (29–32). A recent study also reported that SGLTs are absent in retinal endothelial cells (33). Meanwhile, no clear evidence has been reported demonstrating SGLT2 expression human retina cells. In the present study, we provided evidence showing the potential function and expression of SGLT2 in DR patients for the first time. Recent studies also demonstrated the downregulation of both SGLT2 and GLUT2 in T2DM patients (34). However, conflicting results also showed upregulated SGLT2 expression in patients with kidney diseases related to diabetes (35). The contrasting results in those clinical studies were potentially caused by multiple reasons, such as different techniques in collecting human tissue and inclusion of different human races, populations, and T2DM patient population. For this

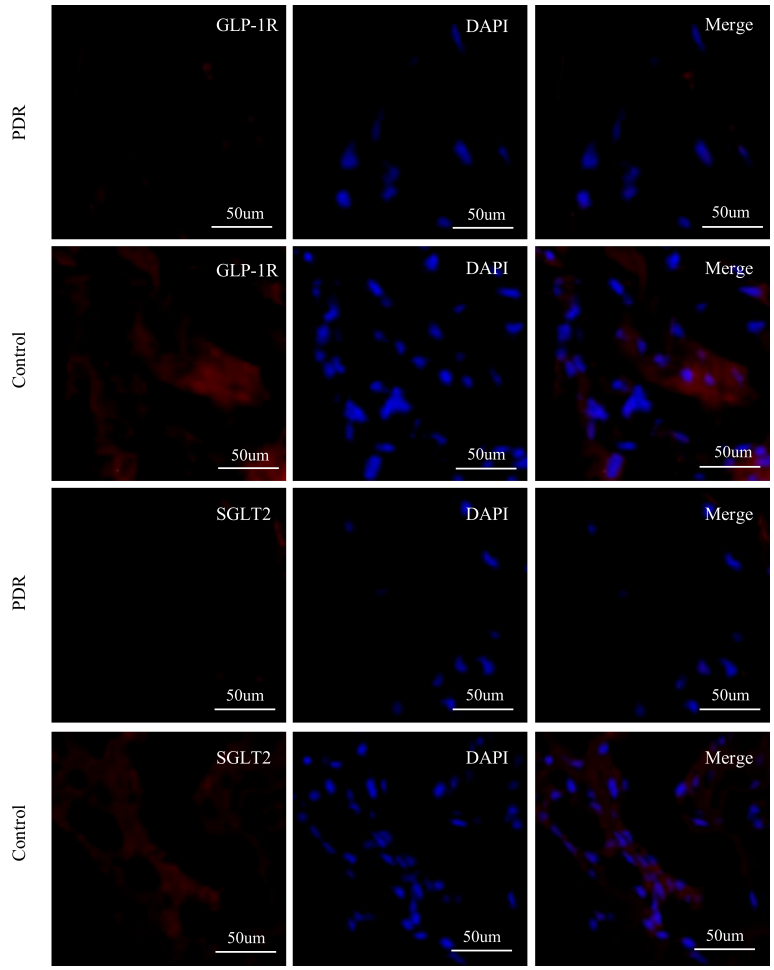

FIGURE 6
Immunofluorescence staining of GLP-1R, SGLT2, and DAPI in fibrovascular membranes of PDR patients. The staining reaction of GLP-1R and SGLT2 (red) was clearly positive on the ERM of a patient of the control group. The DAPI stain (blue) showed numerous nuclei. No GLP-1R-positive reactions and SGLT2-positive are detected on the FVM from PDR patient. Scale bar: 50 μm.

reason, further studies are necessary to explain the regulations of SGLTs in DR.

We also found that the expression of GLUTs was reduced in PBMCs isolated from PDR patients compared to healthy controls. However, this change was not statistically significant and entirely proportionate to the changes in the expressions of SGLTs. As these two types of transporters were anatomically linked, the covariance in the changes of expressions was further proved in our study. In recent studies of the oxidative stress-related inflammation responses caused by hyperglycemia, the downregulated expression of GLUT1 in the retina was found to be correlated to the reduced GLUT1 level on cell membranes due to subcellular redistribution (36, 37). Previous research has also identified the reduction of GLUT1 in retina cells in streptozotocin-induced diabetes in rats (38), and the inhibition of GLUT1 protein translations on the blood-brain barrier (BBB) in diabetes (39).

Finally, we provided evidence indicating negative correlations among disease duration of T2DM, FPG, HbA1c and mRNA levels of GLP-1R and SGLT2 in DR patients, which were consistent with previous studies of animal models in addition to T2DM patients (34, 40). The results above imply that the duration of disease in diabetes and the degree of glycemic maintenance are of critical importance in diabetes treatment and the prevention of related complications.

Our study has some limitations, including its observational design and the proportionately small sample size. As retinal vascular abnormalities are prevalent comorbidities of DR, and GLP-1R analogs have already been used clinically in diabetes and obesity, further pre- and clinical research is necessary to

elucidate the regulative mechanism underlying GLP-1R/SGLT2 signaling in DR.

In conclusion, we investigated the role of glucose sensors, including SGLT1, SGLT2, GLUT1, GLUT2, and GLP-1R in patients with DR in the current study. Our results from PBMCs and FVM demonstrated that GLP-1R and SGLT2 were less expressed in PDR patients than in healthy controls, which was associated with increased TNF-α and IFN-γ production. These outcomes suggest that the restoration of GLP-1R/SGLT2 signaling is potentially involved in regulation of immune checkpoint molecules in DR patients. However, it is still unknown if GLP-1R/SGLT2 signaling and GLP-1R analogs could be used as potential immunomodulatory targets in DR therapy. Further prospective studies are imperative to elucidate the influence of GLP-1R/SGLT2 signaling on the progression of DR.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Human Ethics Committee of Zhongshan Ophthalmic Center of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HC and FW contributed to the conception of the study; XZ, NL,YG and YS performed the experiment; NL, LM contributed significantly to analysis and manuscript preparation; HC performed the data analyses and wrote the manuscript; XZ helped perform the analysis with constructive discussions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Identification of potential ferroptosis-related biomarkers and a pharmacological compound in diabetic retinopathy based on machine learning and molecular docking

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Background: Diabetic retinopathy (DR), a neurovascular disease, is a leading cause of visual loss worldwide and severely affects quality of life. Several studies have shown that ferroptosis plays an important role in the pathogenesis of DR; however, its molecule mechanism remains incompletely elucidated. Hence, this study aimed to investigate the pathogenesis of ferroptosis and explore potential ferroptosis-related gene biomarkers and a pharmacological compound for treating DR.

Methods: Ferroptosis-related differentially expressed genes (DEGs) were identified in the GSE102485 dataset. Functional enrichment analyses were then performed and a protein-protein interaction (PPI) network was constructed to screen candidates of ferroptosis-related hub genes (FRHGs). FRHGs were further screened based on least absolute shrinkage and selection operator (LASSO) regression and random forest algorithms, and were then validated with the GSE60436 dataset and previous studies. A receiver operating characteristic (ROC) curve monofactor analysis was conducted to evaluate the diagnostic performance of the FRHGs, and immune infiltration analysis was performed. Moreover, the pharmacological compound targeting the FRHGs were verified by molecular docking. Finally, the FRHGs were validated using quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Results: The 40 ferroptosis-related DEGs were extracted, and functional enrichment analyses mainly implicated apoptotic signaling, response to oxidative stress, ferroptosis, and lipid and atherosclerosis pathways. By integrating the PPI, LASSO regression, and random forest analyses to screen the FRHGs, and through validation, we identified five FRHGs that performed well in the diagnosis (*CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53*). Immune infiltration analysis revealed that immune microenvironment changes in DR patients may

be related to these five FRHGs. Molecular docking also showed that glutathione strongly bound the CAV1 and TLR4 proteins. Finally, the upregulated expression of FRHGs (*CD44*, *NOX4*, *TLR4*, and *TP53*) was validated by qRT-PCR analysis in human retinal capillary endothelial cells cultured under high-glucose environment.

Conclusions: *CAV1, CD44, NOX4, TLR4*, and *TP53* are potential biomarkers for DR and may be involved in its occurrence and progression by regulating ferroptosis and the immune microenvironment. Further, glutathione exhibits potential therapeutic efficacy on DR by targeting ferroptosis. Our study provides new insights into the ferroptosis-related pathogenesis of DR, as well as its diagnosis and treatment.

KEYWORDS

diabetic retinopathy, ferroptosis, biomarkers, glutathione, *in silico*

1 Introduction

Diabetic retinopathy (DR), a specific neurovascular complication of both type 1 and 2 diabetes, is among the leading causes of visual impairment and blindness in adults worldwide, with an estimated 191 million affected patients by 2030 (1, 2). It is generally acknowledged that the prevalence of DR increases with the duration of diabetes. For type 1 diabetes, approximately 25%, 60%, and 80% of patients will develop DR after 5, 10, and 15 years, respectively. In less than 5 years, the incidence of DR in patients with type 2 diabetes is 40% and 24% for those who do and do not take insulin, respectively. After 19 years, these rates rise to 84% and 53%, respectively (3). The occurrence and progression of DR are so latent that detection is difficult. When visual impairment does occur, the optimal time for diagnosis and therapy has usually passed (4). Thus, it is imperative to further investigate the pathogenesis of DR, distinguish novel biomarkers for diagnosis, and identify pharmacological compounds for targeted treatment.

Ferroptosis is a recently identified type of cell death whose main characteristics are iron-dependent accretion of lipid reactive oxygen species and inhibition of the cystine/glutamate antiporter system Xc^-, leading to decreased cystine uptake and glutathione (GSH) synthesis (5). Ferroptosis may fatally damage cells and result in certain eye diseases, such as glaucoma, retinal ischemia-reperfusion injury, and age-related macular degeneration (6, 7). Recent evidence has revealed the role of ferroptosis in DR. Damage to retinal pigment epithelial (RPE) cells, the resulting destruction to the blood-retina barrier, and increased permeability of human retinal capillary endothelial cells (HRCECs) are key features in the occurrence and progression of DR. It has been reported that ferroptosis serves as a cell death pathway for RPE cells and HRCECs in DR (8, 9). Nevertheless, the DR-related pathologic mechanisms, signaling pathways, and gene biomarkers in ferroptosis have not yet been clarified.

GSH, a bioactive substance involved in cellular metabolism and antioxidant defense, is utilized by glutathione peroxidase 4 (GPX4) to eliminate phospholipid peroxides and protect cells from ferroptosis (10). Studies have shown that enhancement of intracellular GSH activity by natural compounds can alleviate DR by modulating inflammation, oxidative stress, endoplasmic reticulum stress, and autophagy (11). Nevertheless, the pharmacological activity of GSH to target ferroptosis in DR remains unclear.

In this study, we collected RNA-sequencing dataset from the Gene Expression Omnibus (GEO) database and downloaded ferroptosis-related genes from the FerrDb database. We first identified ferroptosis-related differentially expressed genes (DEGs) and performed functional enrichment analyses. Protein-protein interaction (PPI), least absolute shrinkage and selection operator (LASSO) regression, and random forest analyses were further utilized to identify ferroptosis-related hub genes (FRHGs), and another GEO dataset and previous studies were utilized for validation. In addition, we used CIBERSORT to analyze the immune microenvironment in DR. Then, molecular docking between GSH and the FRHG-encoded proteins was performed to validate their prospective application in DR treatment. Finally, the FRHGs were validated using quantitative real-time polymerase chain reaction (qRT-PCR) analysis in the *in vitro* DR model. Our study provides new insights into the potential pathogenesis associated with ferroptosis at the molecular level, novel diagnostic biomarkers, and a pharmacological compound targeting ferroptosis in DR. This is expected to provide valuable information in the future for the accurate diagnosis of DR, as well as drug discovery and development.

2 Materials and methods

2.1 Data collection, preprocessing, and quality control

The two DR datasets used in this study were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/gds). The first transcriptome dataset was the test dataset (GSE102485) and the second microarray dataset was the validation dataset (GSE60436); both are shown in Table 1. Two hundred and fifty-nine ferroptosis-related genes were downloaded from the FerrDb database (http://www.zhounan.org/ferrdb/) (12). The workflow of this study is shown in Figure 1. The GSE102485 dataset also contained non-DR samples, which were excluded. Thus, our study only utilized DR and normal samples for the downstream bioinformatic analyses.

Data processing was performed using R (version 4.1.1) as follows. First, we transformed the Ensembl IDs to gene symbols, and protein-coding genes in the GSE102485 dataset were selected for analyses. Second, we performed ID conversion in the GSE60436 dataset. Third, the average expression value was regarded as the gene expression value when multiple Ensembl IDs/probes corresponded to the same gene symbol.

TABLE 1 Diabetic retinopathy (DR) datasets from the GEO database.

Dataset ID	Platform	DR	Normal	Other retinopathy
GSE102485	GPL18573	22	3	5
GSE60436	GPL6884	6	3	0

The DESeq2 package (13) was utilized to normalize the raw count data of mRNAs for further principal component analysis (PCA). The FactoMineR package (14) for dimensionality reduction was used for PCA to evaluate the data quality.

2.2 Identification of DEGs and ferroptosis-related DEGs

We analyzed gene expression of the GSE102485 dataset *via* the DESeq2 package to identify DEGs. As suggested by the DESeq2 package tutorial, genes with low read counts were not worthy of further analyses. Hence, mRNAs with a mean count less than one and median count equal to zero were excluded in our study. Differential expression analysis then was performed in which the normalization processes of count data were incorporated into the DESeq2 workflow. The false discovery rate was calculated by the Benjamini–Hochberg method and applied to correct the statistical significance of multiple testing (15). The DEGs were screened based on a threshold of $|\log2 \text{fold-change (FC)}| \geq 2$ and false discovery rate < 0.05. Finally, we constructed a volcano plot using the ggplot2 package (16) to visualize the results.

We intersected the 259 ferroptosis-related genes with the DEGs to identify ferroptosis-related DEGs. The online analysis tool Venny2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was utilized to construct a venn diagram to visualize the

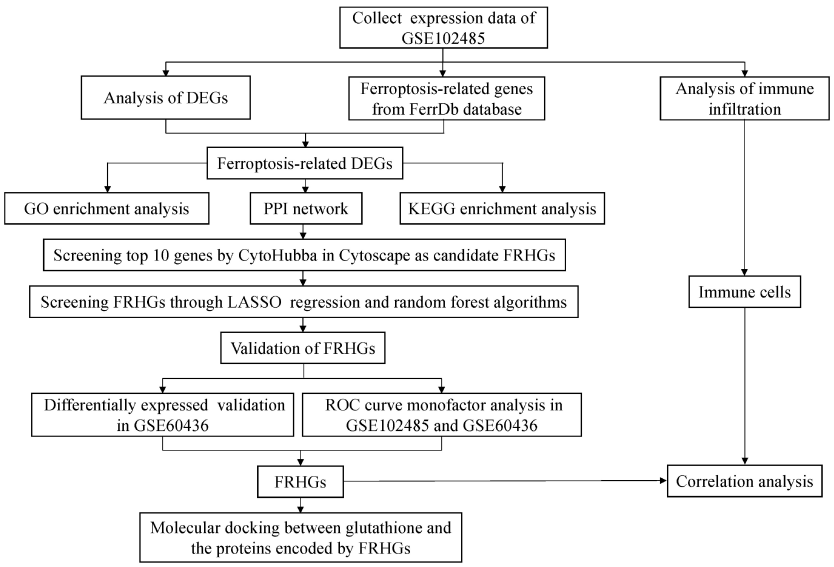


FIGURE 1
Workflow of data analyses utilized in this study. DEGs, differentially expressed genes; GO, Gene Ontology; PPI, protein-protein interaction; KEGG, Kyoto Encyclopedia of Genes and Genomes; FRHGs, ferroptosis-related hub genes; LASSO, least absolute shrinkage and selection operator; ROC, receiver operator characteristic.

results. Then, the pheatmap package was utilized to construct a heat map to visualize expression of the ferroptosis-related DEGs.

2.3 Functional enrichment analyses of ferroptosis-related DEGs

The clusterProfiler (17) and GOplot (18) packages were utilized to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses for the ferroptosis-related DEGs. A bar plot was used to show the top 30 GO terms, including biological process, cellular component, and molecular function, and a chord plot was used to show crosstalk between the ferroptosis-related DEGs and top five GO terms, linking them by ribbons. A circle plot was used to show the top 10 KEGG pathways. A p-value < 0.05 was considered statistically significant.

2.4 PPI network construction and screening of FRHGs

The STRING database (19) was utilized to observe interactions between the ferroptosis-related DEGs. Cytoscape software (version 3.7.2) was used to construct and visualize the PPI network. Candidate FRHGs were then identified using the MCC algorithm of the Cytoscape plug-in CytoHubba. The 10 genes with the highest scores were screened as candidate FRHGs and displayed in the Cytoscape software.

LASSO regression, a machine learning algorithm with dual characteristics of subset selection and ridge regression, is widely utilized to screen the best variables by finding the lambda value when the classification model error is the least (20). The glmnet package (21) was used to perform LASSO regression analysis. Expression of the 10 candidate FRHGs was analyzed using LASSO regression with a binomial model and lambda value equal to the minimum mean cross-validated error to screen most likely FRHGs. Random forest, another machine learning algorithm for training and predicting samples with high accuracy based on constructing a multitude of decision trees, is widely utilized to identify and verify potential predictors (22). Thus, the random forest algorithm was utilized to verify the reliability of the LASSO regression analysis using the randomForest package (23). The out-of-bag error was calculated to evaluate the classification performance of the combined FRHGs identified by LASSO regression. The mean decrease accuracy (MDA) and mean decrease Gini (MDG) were positively correlated with the importance of each variable. Therefore, these FRHGs were sorted by MDA and MDG indexes.

2.5 Dataset validation of FRHGs

First, the normalized expression values of the FRHGs in the GSE60436 dataset were extracted and groups were compared utilizing t-tests; p-values < 0.05 were considered statistically significant. We utilized the ggpubr package to visualize these results. Second, receiver operating characteristic (ROC) curve monofactor analysis was performed on the GSE102485 and GSE60436 datasets to confirm these FRHGs. The ROC curve was visualized using Hiplot software (https://hiplot.com.cn/). Any gene with an area under the ROC curve > 0.9 was considered to have great diagnostic value.

2.6 Immune infiltration analyses

The CIBERSORT package (24) was utilized to analyze immune cell infiltration in DR and normal samples. The normalized gene expression data was transformed into immune cell information by the CIBERSORT deconvolution algorithm. Linear regression analysis was used to analyze the correlation between FRHGs expression and immune cells. A p-value < 0.05 was considered statistically significant. The results were visualized using the ggplot2 package.

2.7 Molecular docking

The 2D chemical structure of GSH was downloaded from PubChem. Various databases were utilized to identify whether the FRHGs were potential targets of GSH, as previously described (25). Molecular docking was utilized to simulate intermolecular binding patterns between GSH and the target proteins. The protein structures of identified GSH targets were obtained from the PDB database (https://www.rcsb.org/). Then, MGLTools (version 1.5.7) in AutoDock (26) was utilized to conduct the docking analysis. After converting the pdbqt format to pdb using OpenBabel, PyMOL was then used to visualize the molecular docking results. The docking parameter setting was assessed according to the binding energy of the ligand.

2.8 External validation of GPX4, SLC7A11 and FRHGs

2.8.1 Cell culture and cell grouping

Human retinal capillary endothelial cells (HRCECs) were cultured in low-glucose DMEM (Solarbio, China) containing 10% foetal bovine serum (Biological Industries, Israel) at 37°C with 5% carbon dioxide. HRCECs cultured in the medium containing 5.5 mmol/L glucose were used as the normal control group (NG group) and cultured in the medium containing 30 mmol/L glucose were used as the high-glucose group (HG group). Mannitol was used as the control to eliminate the influence of osmotic pressure, namely, the MA group. The model cells under HG group were cultured with high-glucose DMEM for 12, 24, and 48 h.

2.8.2 Western blot analysis

Total cellular protein was extracted using Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (Solarbio, China). The proteins were denatured and then separated using 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. Next, these membranes were blocked with 5% non-fat milk at room temperature for 2 h and incubated with primary antibodies against GPX4 (ab125066, Abcam) and SLC7A11 (ab175186, Abcam) at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (BA1054, BOSTER) at room temperature for 1 h. The ECL developer (US EVERBRIGHT, China) was added to the membranes and Imaging System (SYNGENE, Britain) was used to visualize the immunoreactive protein bands. β-actin (AC026, ABclonal) was used as the internal reference.

2.8.3 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total cellular RNA was isolated using RNA Extraction reagent (Servicebio, China) according to manufacturer's instructions. Total RNA was then reverse transcribed to cDNA using SweScript RT I First Strand cDNA Synthesis Kit (Servicebio, China), and RT-PCR was performed using 2 × SYBR Green qPCR Master Mix (None ROX) (Servicebio, China). The primer sequences used in this study were shown in Supplementary Table 1. Relative change in gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method using GAPDH as the internal reference.

2.9 Statistical analysis

All the experimental data, taken from at least three independent experiments, was statistically analyzed using GraphPad Prism software (version 8.0.1). Significance levels were determined by the unpaired Student's t-test between the two groups or the one-way analysis of variance (ANOVA) among multiple groups. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Data collection, preprocessing, and quality control

After expression data collection and preprocessing, all samples were assessed by PCA (Supplementary Figure 1). The results showed that the DR samples were distinctly different from the normal samples, which confirmed the repeatability of the GSE102485 data and suitability for downstream analysis.

3.2 Identification of DEGs and ferroptosis-related DEGs

According to the assigned threshold, 2468 DEGs were detected, of which 1861 were upregulated and 652 were downregulated in DR (Figure 2A). We then intersected 259 ferroptosis-related genes with the DEGs, identifying 40 ferroptosis-related DEGs (Figure 2B), of which 38 were upregulated and 2 were downregulated in DR. The gene symbols of ferroptosis-related DEGs are shown in Figure 2A. A heat map of these ferroptosis-related DEGs revealed variations in relative gene expression among the DR and normal samples (Figure 2C).

3.3 Functional enrichment analyses of ferroptosis-related DEGs

In GO enrichment analysis, ferroptosis-related DEGs were significantly enriched in the intrinsic apoptotic signaling pathway, regulation of apoptotic signaling pathway, intrinsic apoptotic signaling pathway in response to DNA damage, reactive oxygen species metabolic process, and response to oxidative stress under the biological process term; NADPH oxidase complex, secondary lysosome, and lamellipodium membrane under the cellular component term; and heme binding, superoxide-generating NAD(P)H oxidase activity, and iron ion binding under the molecular function term (Figure 3A). The results of crosstalk analyses of genes and GO terms revealed that the functions of ferroptosis-related DEGs in DR might be the result of mutual relationships among multiple gene functions, which are shown in Figure 3B. In KEGG enrichment analysis, the ferroptosis-related DEGs were significantly enriched in ferroptosis, the p53 signaling pathway, and lipid and atherosclerosis pathways (Figure 3C).

3.4 PPI network construction and screening of FRHGs

According to the PPI network results, there were interactions between the identified ferroptosis-related DEGs (Figure 4A). The genes with the top 10 scores were screened as candidate FRHGs, namely, *TXNIP*, *CD44*, *HMOX1*, *NCF2*, *ALOX5*, *TLR4*, *PTGS2*, *TP53*, *NOX4*, and *CAV1* (Figure 4B).

To identify the best FRHGs, LASSO regression was used to analyze the 10 candidate FRHGs. Five genes, *CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53*, were identified (Figures 4C, D). The random forest algorithm was then used to efficiently predict the combined classification performance of these five genes and evaluate the importance of each gene. The out-of-bag error of the random forest model was 0%, and the five genes were ranked

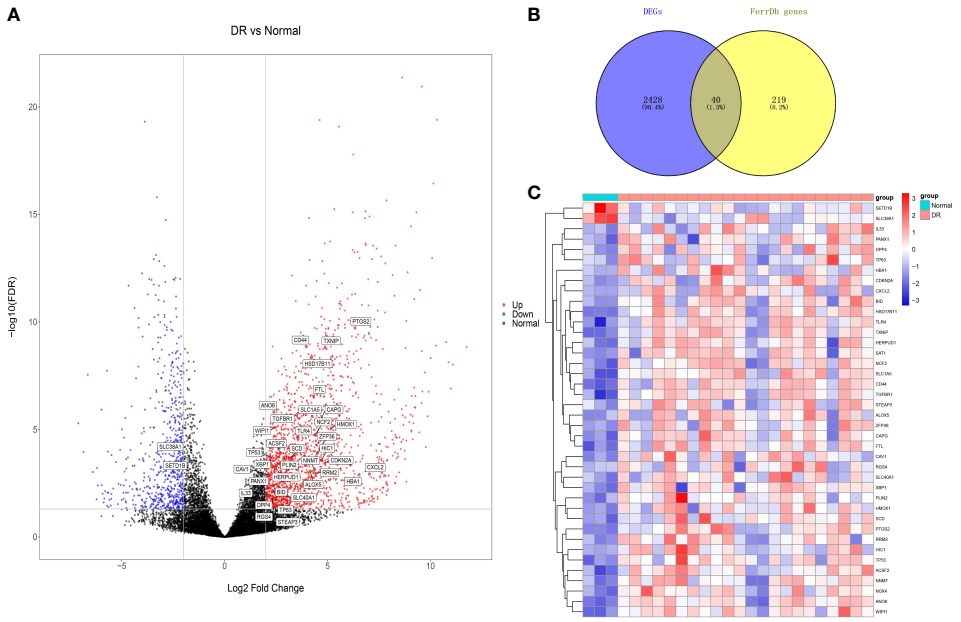


FIGURE 2
Differentially expressed genes (DEGs) analysis. **(A)** Volcano plot of DEGs. **(B)** Venn diagram of ferroptosis-related DEGs, and their gene symbols were shown in **(A)**. **(C)** Heat map of ferroptosis-related DEGs. DR, diabetic retinopathy.

by MDA and MDG indexes (Figures 4E, F). The results showed high reliability and important metrics for the five FRHGs.

3.5 Dataset validation of FRHGs

We utilized the GSE60436 dataset to validate the five FRHGs. The results revealed that the expression of these genes was higher in the DR samples than in the normal samples (all $p < 0.05$) (Figure 5A), which is consistent with the results of the GSE102485 dataset. Subsequently, ROC curve monofactor analysis was performed. The results revealed that the diagnostic accuracies of *CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53* for DR were 96.97%, 100.00%, 96.97%, 96.97%, and 98.48% in the GSE102485 dataset, respectively (Figure 5B), and 100.00%, 94.44%, 100.00%, 100.00%, and 100.00% in the GSE60436 dataset, respectively (Figure 5C). These results showed that the five FRHGs have great diagnostic potential for DR.

3.6 Immune infiltration analyses

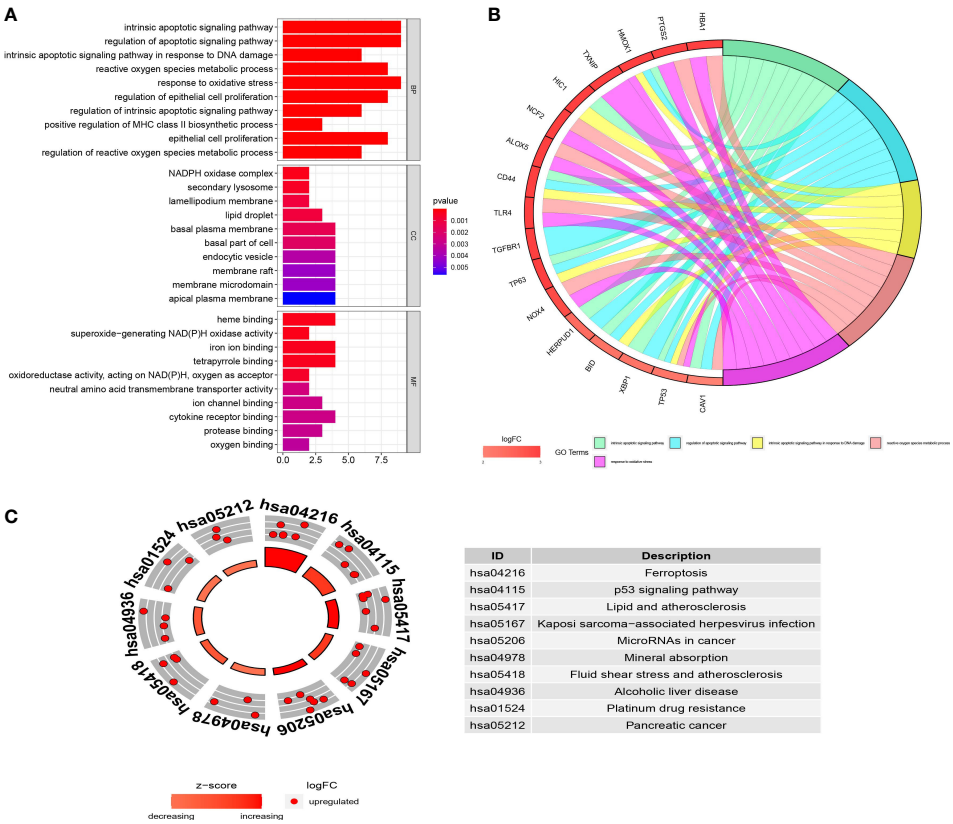
We also performed CIBERSORT immune cell infiltration analyses. The histograms in Figure 6A show the proportions of 22 infiltrating immune cells in each sample. There were some differences in immune infiltration between the DR and normal samples. Specifically, the DR samples had a lower memory B cell ratio, lower T follicular helper cell ratio, and higher neutrophil ratio than in the normal samples (Figure 6B). Regarding the correlation between FRHG expression and immune cell infiltration, the expression of *CD44*, *NOX4*, *TLR4*, and *TP53* showed a significantly negative correlation with the proportion of memory B cells and T follicular helper cells, and the expression of *CAV1* showed a significantly negative correlation with the proportion of memory B cells (Figure 6C).

3.7 Molecular docking

By searching various databases, CAV1, NOX4, and TLR4 were confirmed as potential targets of GSH. Because the protein structure of NOX4 was not found in the PDB database, we only investigated the possibility of direct binding of GSH to CAV1 and TLR4 by molecular docking. The results showed that GSH possessed high binding affinity for CAV1 (PDB ID: 5IJP) (27) and TLR4 (PDB ID: 5JIC) (28). The results demonstrated that GSH forms hydrogen bonds with amino acid residues Gln-16, Asp-23, Tyr-19, Ile-20, and Lys-68 of CAV1; the free binding energy was −3.74 kcal/mol. For TLR4, GSH forms hydrogen bonds with Arg-234, Lys-235, and Asp-239; the free binding energy was −5.65 kcal/mol. The details of the binding affinities are shown in Figures 7A, B.

3.8 External validation of GPX4, SLC7A11 and FRHGs

HRCECs were cultured in the medium containing 30 mmol/L glucose to simulate the DR model *in vitro*. Firstly, we analyzed



ID	Description
hsa04216	Ferroptosis
hsa04115	p53 signaling pathway
hsa05417	Lipid and atherosclerosis
hsa05167	Kaposi sarcoma–associated herpesvirus infection
hsa05206	MicroRNAs in cancer
hsa04978	Mineral absorption
hsa05418	Fluid shear stress and atherosclerosis
hsa04936	Alcoholic liver disease
hsa01524	Platinum drug resistance
hsa05212	Pancreatic cancer

FIGURE 3
The functional enrichment analyses of the ferroptosis-related differentially expressed genes (DEGs). **(A)** GO analysis of the ferroptosis-related DEGs. **(B)** Chord plot shows the distribution of ferroptosis-related DEGs in different GO terms. Symbols of ferroptosis-related DEGs are shown on the left side of the plot with their logFC values indicated by color scale. **(C)** KEGG analysis of the ferroptosis-related DEGs. The inner ring is a bar plot where height displays the significance of the term, and the outer ring displays scatter plots which indicate the expression levels (logFC) for the genes in each term. BP, biological process; CC, cellular component; MF, molecular function; logFC, log2 fold-change.

the differential expression of GPX4 and SCL7A11, the markers of ferroptosis, among NG group, MA group and HG group using western blot. The results showed that the protein levels of GPX4 and SLC7A11 were significantly downregulated in HRCECs under the high-glucose environment for 48 h (Figure 8A). The FRHGs were then validated using qRT-PCR analysis. The results showed that the expression of *CD44*, *NOX4*, *TLR4*, and *TP53* was significantly upregulated in HRCECs under the high-glucose environment for 48 h compared with the low-glucose environment, which was consistent with that of bioinformatics analysis (Figure 8B).

4 Discussion

DR, characterized by ischemic microvascular disease of the retina and retinal neurodegeneration, is a highly specific complication of diabetes with complex multifactorial pathophysiology, which finally leads to visual impairment or blindness (29). Emerging evidence from a series of *in vivo* and *in vitro* studies revealed that ferroptosis, a new type of iron-dependent programmed cell death linking metabolism, disease, immune cells, and targeted therapy, is closely associated with the pathophysiological states of various ocular diseases, such as corneal alkali burn, glaucoma, age-related macular degeneration, and retinitis pigmentosa (6, 30–32). Furthermore, targeting ferroptosis is a promising treatment for ocular diseases (6, 31). Some studies indicated that the inhibition of ferroptosis can alleviate cell death more effectively than the inhibition of apoptosis and necrosis in age-related macular degeneration (9). Most recently, a growing number of studies indicate that ferroptosis may be closely involved in the development and progression of DR (6). Ferroptosis is involved in oxidative stress-induced RPE cell and HRCEC death under high-glucose conditions. An *in vitro* study showed that glia maturation factor beta (GMFB), upregulated in the vitreous at a very early stage of diabetes, can induce ferroptosis in RPE cells by impairing lysosomal acidification and ultimately

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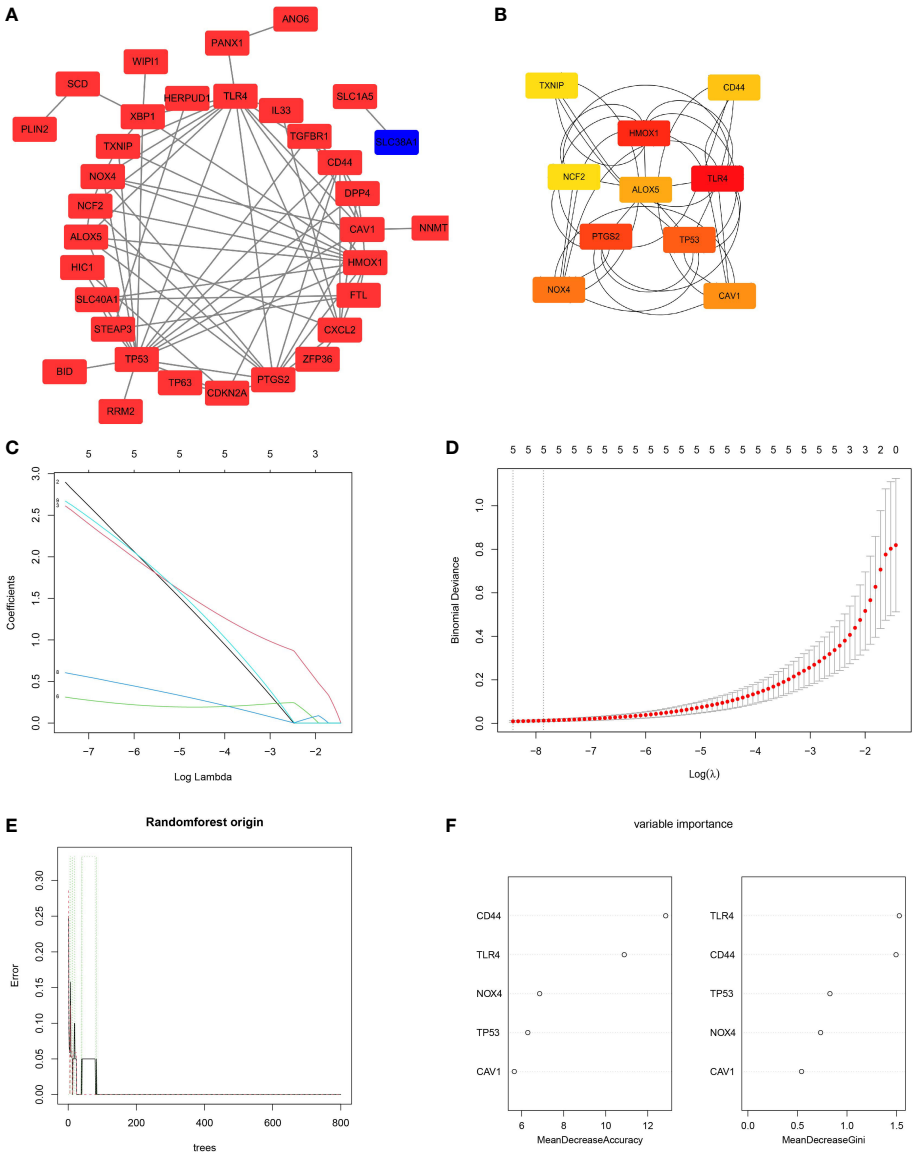

FIGURE 4
Protein-protein interaction (PPI) analysis and screening of ferroptosis-related hub genes (FRHGs). **(A)** The PPI network of ferroptosis-related DEGs. Red rectangles represent upregulated genes, while the blue rectangle represents a downregulated gene. **(B)** Network of the candidate FRHGs. A redder color represents a higher score in Cytoscape based on the MCC algorithm. **(C, D)** Least absolute shrinkage and selection operator (LASSO) regression algorithm to screen five FRHGs. **(E, F)** Construction and evaluation of random forest model based on the five FRHGs screened by LASSO regression. **(E)** Trend of the model errors based on the number of decision trees. **(F)** The importance of all variables in the random forest model.

damaging retinal function (33). Another study showed that high-glucose triggers ferroptosis in HRCECs by upregulating tripartite motif containing 46 (TRIM46) and inducing ubiquitination and accelerated clearance of GPX4 (8). However, existing reports on the underlying molecular mechanisms of ferroptosis in the field of DR are still preliminary and limited in scope. Thus, we analyzed transcriptome datasets based on bioinformatics to investigate the potential pathogenesis of iron metabolism in the occurrence and progression of DR. Clarifying the interrelationship between ferroptosis and DR may identify novel biomarkers for its diagnosis and pharmacological compounds for its targeted treatment, which could provide new ideas for treatment regimens with ferroptosis as the therapeutic target.

We identified 40 ferroptosis-related DEGs between the DR and normal samples. GO and KEGG enrichment analyses revealed that these ferroptosis-related DEGs were mainly enriched in the apoptotic signaling pathway, reactive oxygen

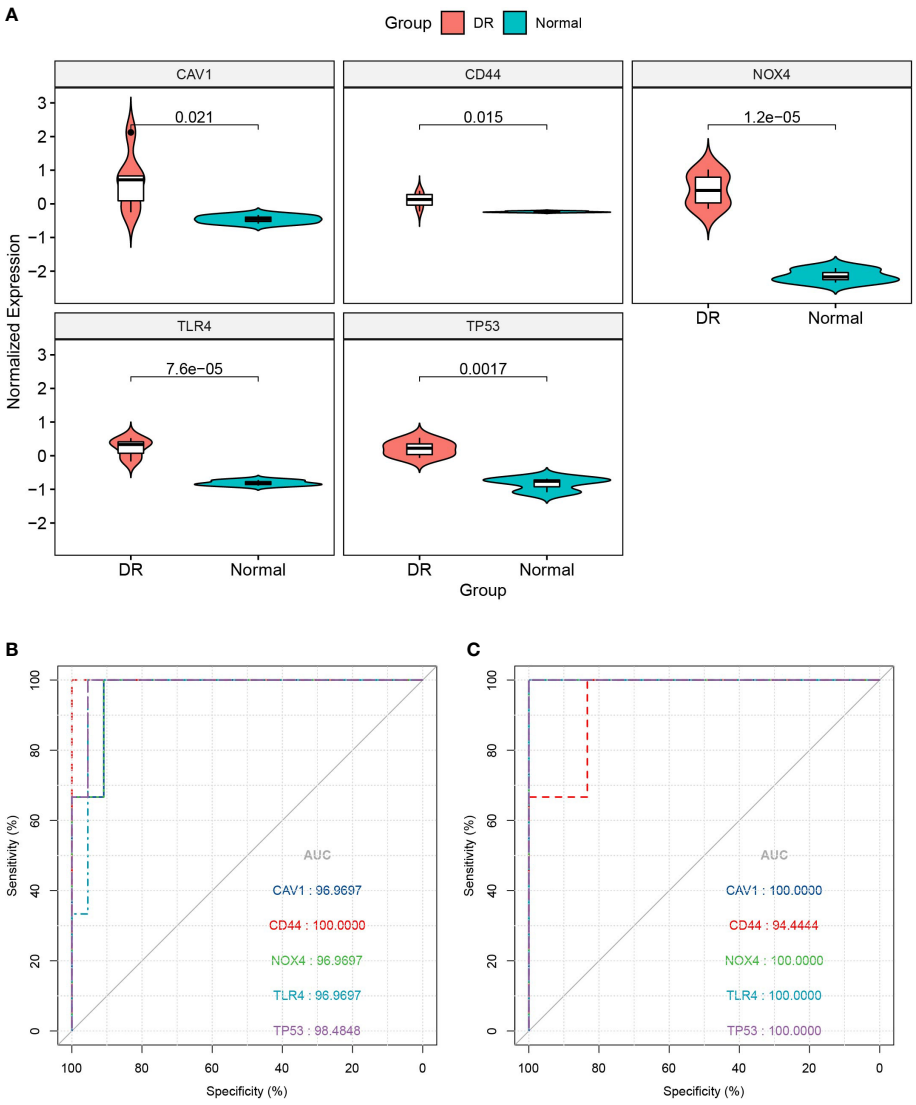

FIGURE 5
Database validation of ferroptosis-related hub genes (FRHGs). **(A)** Validation of FRHGs in the GSE60436 dataset. **(B)** ROC curve of FRHGs in the GSE102485 dataset. **(C)** ROC curve of FRHGs in the GSE60436 dataset. DR, diabetic retinopathy.

species metabolic process, response to oxidative stress, ferroptosis, p53 signaling pathway, and lipid and atherosclerosis terms, which have been reported to be associated with DR pathogenesis (8, 34–37). Interestingly, our results highlighted the involvement of these ferroptosis-related DEGs in the intrinsic apoptotic signaling pathway, regulation of apoptotic signaling pathway, and intrinsic apoptotic signaling pathway in response to DNA damage terms, consistent with previous findings that DR is affected by crosstalk between apoptosis and ferroptosis mechanisms (38, 39). The results of enrichment analyses confirmed the validity of the ferroptosis-related DEGs identified in our study. Theoretically, it is not difficult to determine that ferroptosis contributes greatly to DR. Moreover, we further analyzed the expression of GPX4 and SCL7A11, ferroptosis-related markers, was downregulated in HRCECs under the high-glucose environment, suggesting that ferroptosis is one of the pathologic mechanisms involved in DR. However, the ferroptosis-related genes and their associated terms and pathways found in our study have not been fully elucidated, especially p53 signaling pathway. It is reported that this pathway is involved in regulating metabolism, immune response, neurodegeneration and tissue ischemia/reperfusion injuries by promoting or inhibiting ferroptosis (40). But the specifically ferroptosis-related mechanism of p53 signaling pathway in DR has not been reported yet, and in-depth experimental investigation and discussion are required. We

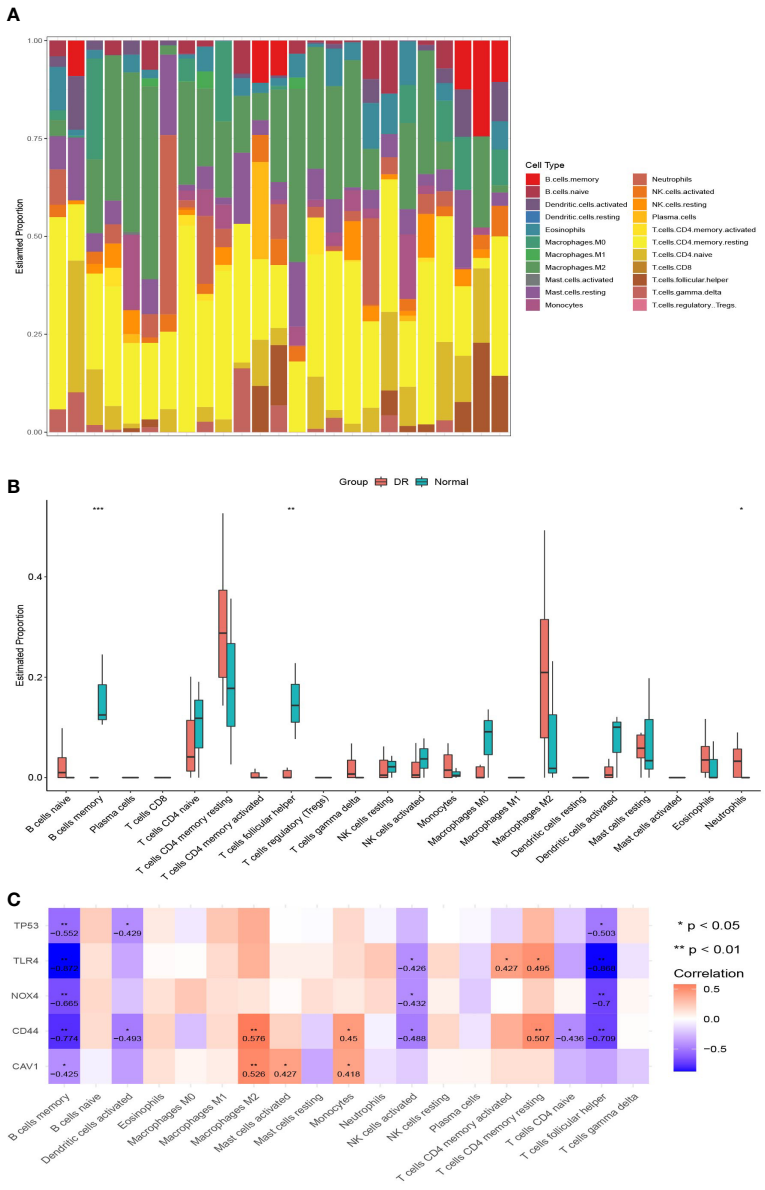

FIGURE 6
Immune infiltration analyses. **(A)** The histograms of 22 immune cell proportions in DR samples and normal samples. **(B)** The box plot of differences in immune infiltration in the two groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(C)** The correlation between ferroptosis-related hub gene expression and different immune cells; the numbers in the cell represent the correlation coefficient. DR, diabetic retinopathy.

speculate that the occurrence and progression of DR are the result of crosstalk among multiple genes and pathways.

Through integrated bioinformatics analyses, we identified and validated five FRHGs (*CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53*) with great diagnostic potential for DR. Interestingly, previous studies have also indicated that these genes were upregulated in DR (41–45), although our qRT-PCR results showed that the expression of CAV1 was inconsistent with the results of bioinformatics analysis and previous studies. We speculated that because the differences in cell culture condition and vitality might provide different results. *CAV1* encodes a transmembrane protein that is the main component of caveolae in plasma membranes and is associated with multiple cellular functions including signal transduction, cholesterol homeostasis, and endocytosis (41). Increased CAV1 expression in the retinas of patients who are diabetic can enhance Toll-like receptor signaling and proinflammatory cytokine release, leading to a breakdown of the blood-retinal barrier (41, 46). CD44 is a receptor for extracellular matrix proteins and polysaccharides, as well as a significant regulator of neovascularization (47).

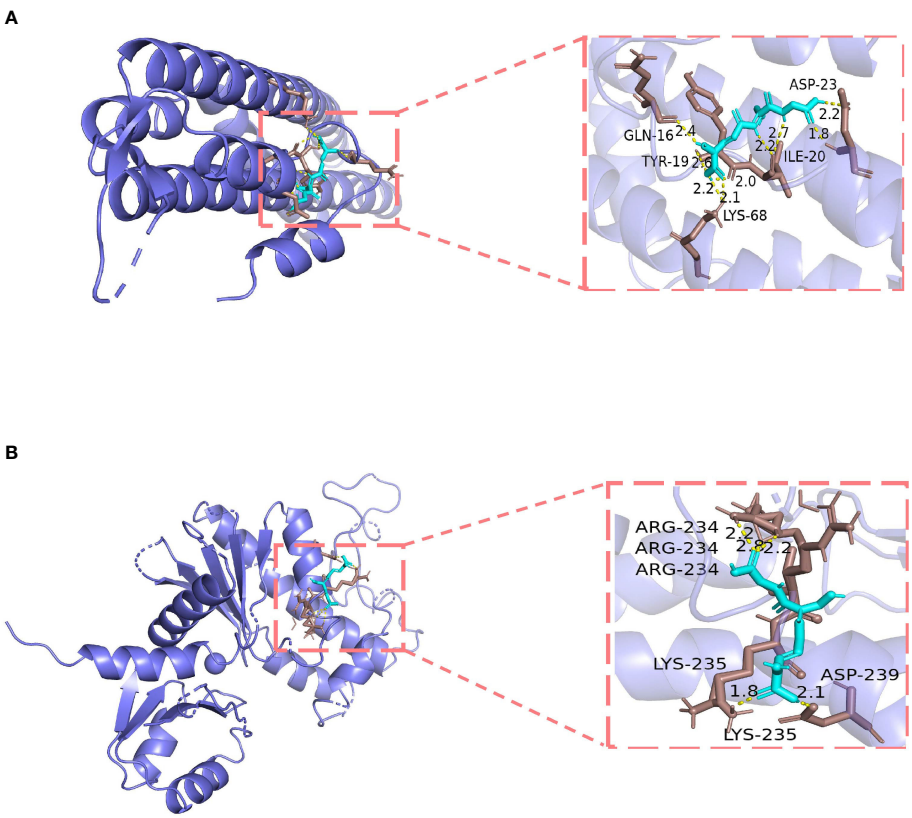

FIGURE 7
Molecular docking models of glutathione (GSH) binding to its targets. **(A)** GSH binds to CAV1. **(B)** GSH binds to TLR4.

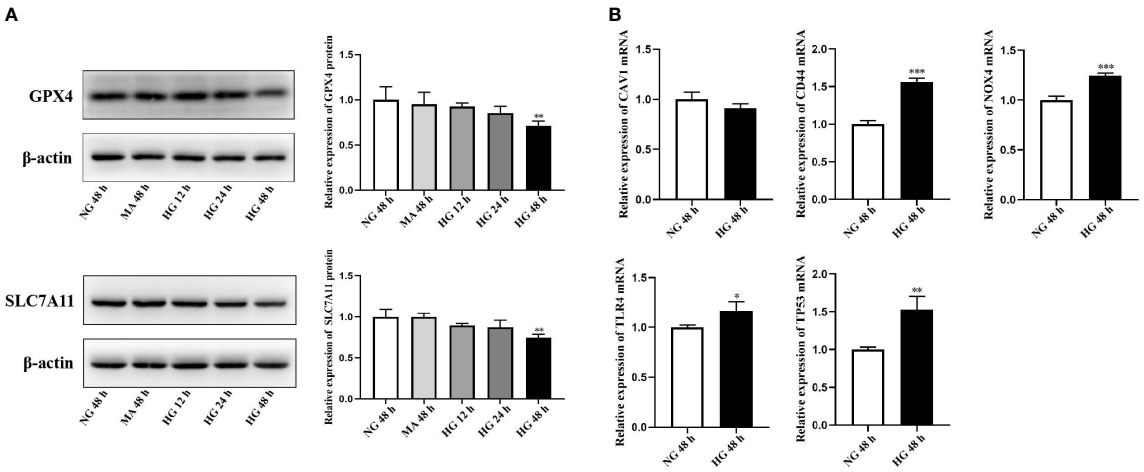

FIGURE 8
External validation of GPX4, SLC7A11 and ferroptosis-related hub genes (FRHGs). **(A)** The protein levels of GPX4 and SLC7A11 were evaluated in cell samples by western blot. **(B)** The mRNA levels of CAV1, CD44, NOX4, TLR4, and TP53 were evaluated in cell samples by qRT-PCR. NG, normal control group; MA, Mannitol; HG, high-glucose group. *$p < 0.05$ vs NG 48 h, **$p < 0.01$ vs NG 48 h, ***$p < 0.001$ vs NG 48 h.

Zhang et al. have shown that the interaction of CD44 with phosphorylated moesin leads to less pericyte coverage and disruption of vessel integrity, which may contribute to neovascularization in DR (48). NOX4 is a member of the NADPH oxidase family of enzymes, which catalyze the reduction of molecular oxygen to various reactive oxygen species (49). Previous studies have implicated the activation of NOX4 in DR blood-retinal barrier breakdown, retinal neovascularization, and inflammation (43, 50). TLR4 is a Toll-like receptor that plays an important role in the initiation of inflammatory and immune responses (39). Recent evidence has shown that TLR4 ligand- TLR4 binding initiates downstream signaling cascades, such as PI3K, p38/MAPK, and NF-κB, resulting in the development of inflammation, neovascularization, oxidative stress, and neurodegeneration, all of which are involved in DR pathogenesis (44, 51). TP53, activated in response to diverse stressors to regulate the expression of target genes inducing cell cycle arrest, apoptosis, senescence, and DNA repair, has recently been recognized as a metabolic regulator (52). Hyperglycemia increases the transcription and expression of TP53, whose codon 72 polymorphism is significantly associated with diabetic complications, including diabetic retinopathy, in patients with type 1 or type 2 diabetes (45, 52, 53). Based on machine learning algorithms, we established a novel reliable model, and these five FRHGs may represent a molecular signature for the diagnosis of patients with DR. Studies have revealed that these ferroptosis-related genes can regulate ferroptosis in liver fibrosis, heart failure, Alzheimer's disease, and tumors (54–58). However, the exact molecular mechanisms involved in influencing DR *via* ferroptosis remain unclear. Thus, further exploration of their ferroptosis-related functions could provide novel research directions.

Chronic inflammation and leukocyte stasis play central roles in the pathogenesis of DR. An imbalance in iron homeostasis can also affect the function, differentiation, and death of immune cells (59). Thus, we utilized CIBERSORT to analyze the immune microenvironment to investigate the molecular immune mechanisms associated with ferroptosis in DR. The results showed significantly decreased proportions of memory B cells and T follicular helper cells and increased proportion of neutrophils in DR samples, which were consistent with previous studies (60, 61). Unsurprisingly, the high expression of *CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53* was linked to lower proportions of memory B cells and T follicular helper cells in DR. According to the above findings, we hypothesize that the five FRHGs are involved in the chronic inflammation and immune processes of DR occurrence and progression by affecting the immune microenvironment. The cooperative interactions of ferroptosis, immune responses, and inflammation in DR might be multilinked and complicated, and remain to be elucidated in future studies.

We investigated the possible use of GSH in treating DR by targeting FRHGs, as GSH depletion triggers ferroptosis (62). As expected, GSH was predicted to act on multiple targets (CAV1, NOX4, and TLR4) to produce synergistic pharmacological activities, and the binding affinities of GSH to CAV1 and TLR4 were predicted to be strong. A previous study showed that ferroptosis susceptibility was enhanced by increased ERK pathway activation due to CAV1 overexpression in human rhabdomyosarcoma cells, and antioxidant molecules such as GSH could alleviate ferroptosis (63). Another study revealed that ferrostatin-1, which could enhance intracellular GSH activity, was able to inhibit the ferroptosis-induced upregulation of TLR4 and the NLRP3 inflammasome to protect rat pulmonary artery endothelial cells (64). Taken together, we believe that GSH might be a promising therapeutic treatment for DR by targeting ferroptosis and undoubtedly deserves in-depth investigation in the future.

This ferroptosis-related gene signature and targeted molecule have not been previously reported in DR. However, our study still has some limitations. First, all results were based on publicly available data and existing research data; more biological experiments or clinical observations are needed to verify these findings. Second, the small sample size in the present study must also be considered. Moreover, the biological functions of these genes and the pharmacological activity of GSH need to be further validated in the *in vitro* and *in vivo* DR model, which will be the focus of our future study.

5 Conclusions

Based on bioinformatics technology, dataset cross-validation, and support from previous studies, we identified five FRHGs (*CAV1*, *CD44*, *NOX4*, *TLR4*, and *TP53*) associated with the pathogenesis and progression of DR. These genes are potential novel biomarkers for the diagnosis of DR and its targeted therapy. CAV1, NOX4, and TLR4 were predicted to be targets of GSH associated with ferroptosis in DR, which might contribute to the development of new DR therapies. Overall, our study provides new insights into the pathogenesis associated with ferroptosis, as well a theoretical basis for exploring new diagnostic indicators and therapeutic strategies in DR.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: GSE102485 and GSE60436 datasets downloaded from GEO database.

Author contributions

JL: designed the study, collected and analyzed data and wrote the initial manuscript. XL: verified the analysis of data. YC, KL,

and HZ: prepared figures and tables. ZY: guided design the study, revised and edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.988506/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Principal component analysis. DR, diabetic retinopathy.

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Novel biomarkers and therapeutic approaches for diabetic retinopathy and nephropathy: Recent progress and future perspectives

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The global burden due to microvascular complications in patients with diabetes mellitus persists and even increases alarmingly, the intervention and management are now encountering many difficulties and challenges. This paper reviews the recent advancement and progress in novel biomarkers, artificial intelligence technology, therapeutic agents and approaches of diabetic retinopathy and nephropathy, providing more insights into the management of microvascular complications.

KEYWORDS

microvascular complications, diabetic retinopathy, diabetic nephropathy, biomarkers, therapy, artificial intelligence

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder affecting more than 400 million people worldwide and is still on the rise. The long-standing hyperglycemia and genetic predisposition contribute to higher risks for macrovascular and microvascular complications among individuals with diabetes, which substantially places large financial and societal burden. Diabetic microvascular complications are associated with long term impairment and dysfunctions of various organs and systems including retina and kidney, which potentially result in blindness and end-stage kidney disorder, contributing significantly to the morbidity and mortality. These complications often already present in newly diagnosed diabetes and most patients may lost the opportunity to be diagnosed in the early stages to achieve clinically significant improvement. Therefore, early detection and novel treatment strategies are mandatory for alleviating progression and improving outcomes of microvascular complications. Nowadays, with the widely application of omics-technique, multiple novel biomarkers emerge as predictive and

therapeutic targets for diabetic complications and increasing potential agents are in clinical trials or undergoing preclinical investigations. Furthermore, artificial intelligence (AI) is also developed and has been applicated in precision medicine, which facilitates the improvement of diagnosis and prognosis of microvascular complications. In this review, we highlighted the recent advances and new frontiers in the diagnosis and management of microvascular complications, especially focused on diabetic retinopathy (DR) and nephropathy (DN), providing perspectives on the clinical applications and implementation of novel biomarkers, diagnostic techniques and therapeutic agents.

Diabetic retinopathy

Novel biomarkers

DR remains the leading cause of visual impairment and blindness in working-age populations globally. Known risk factors including the duration of diabetes and poor glycemic control, however, cannot fully explain and predict the occurrence and progression of DR. And the present DR screening approaches all have different limitations such as poor accuracy, difficulty of obtaining high-quality images, invasive operation and high cost, which largely influence the early detection rate (1). Increasing evidence shows that pathological changes like inflammation and neuronal dysfunction may have occurred before retinal vasculature changes. Therefore, the development of a cost-effective biomarker that facilitates early risk assessment and accurate diagnosis is therefore urgently needed. The potential biomarkers of DR studied in recent years are listed in Table 1.

Inflammatory biomarkers

Due to the indispensable role of inflammation in the pathogenesis of DR, close attention has been paid to inflammatory biomarkers for DR. A variety of clinical studies have provided evidence for the inflammatory biomarkers of DR. Multiple proinflammatory cytokines and adhesion molecules have been found increased in serum and ocular samples from both vitreous and aqueous humor of patients with DR, including interleukin family (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and intercellular adhesion molecule-1 (ICAM-1), etc (2–5). And the level of IL-2, IL-5, IL-4, IL-6, IL-8, TNF-α, MCP-1 and macrophage inflammatory protein (MIP)-1α were significantly higher in early-onset proliferative diabetic retinopathy (PDR) patients compared to non-PDR (NPDR) and late-onset PDR, which facilitate evaluating the severity and predicting prognosis of DR in clinical practice (2, 6, 7). Besides, long pentraxin 3 (PTX3) has also been considered as a novel biomarker in DR. PTX3 is produced by endothelial cells in response to inflammation. Studies have shown elevated levels of PTX3 in the serum or aqueous humor of patients with DR compared with non-retinopathy or non-diabetic controls (9). Recently, King et al. recognized retinol-binding protein 3 (RBP3) as a potential biomarker for DR severity and progression. The level of RBP3 in aqueous humor was found to be reduced from mild NPDR, moderate and severe NPDR to PDR patients gradually (10). Elevated RBP3 level in the vitreous was correlated with lower levels of TNF-α, TNF-β and VEGF were associated with a lower risk of PDR (11).

Recent preclinical experiments also have gained progress in identification of potential biomarkers further used in DR detection and diagnosis. The main characteristics of inflammatory response in DR includes infiltration of immune cells such as macrophages, neutrophils, B and T cells, activation of microglia and enrichment of cytokines and chemokines (50, 51). Under normal condition, there is generally no immune cells within the vitreous body (52). However, in DR, which the blood-retinal barrier is disrupted, multiple immune cells (leukocytes and T, B cells) will enter the vitreous and trigger inflammatory reactions (53). Therefore, some immune cells and their related genes have been reported to be associated with DR, which may act as inflammatory biomarkers. Bioinformatics analysis identified 8 CD8+T lymphocytes-related genes linked to the occurrence and progress of diabetic macular edema (DME) in human macular samples and verified the expressions in DR mouse model (54). Similarly, hub genes of T-helper 17 (Th17) cells gained good diagnostic values in distinguishing PDR patients from normal subjects with the predictive role of the DR progression (NPDR and DME) (55). Furthermore, evidence have shown that inflammatory mediators initially rise in the retina and then enter the vitreous (12), implying the early predictive role of biomarkers in retina. Several monocyte/macrophage markers (e.g.,F4/80mRNA, CCL2) and glial cells markers (e.g.,NF-κB, IL-17) in retina are found correlated with the progression of DR in rat models (8), which need further confirmation in clinical practice.

Angiogenesis biomarkers

Angiogenesis is an critical pathological factor in the occurrence and progression of DR. Vascular endothelial growth factor (VEGF) is the main angiogenic regulator which serves as a promising predictive biomarker and target for the treatment of DR. Accumulating evidence has suggested the circulating or even tear's level of VEGF, significantly increased in PDR compared with NPDR, which predict DR severity (13). Clinical studies indicate that serum VEGF-A, VEGF-C, VEGF-D and placental growth factor (PlGF), and vitreous and aqueous VEGF-A and PlGF are positively correlated with the severity of DR and are strong predictors for DR occurrence in diabetic individuals (12, 14). And anti-VEGF injection in PDR patients significantly reduced the levels of VEGF-A in aqueous, which

TABLE 1 Biomarkers for DR and DN.

Diabetic retinopathy

Role	Profile	Biomarkers	Location	References
Inflammation	Cytokines	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, MCP-1, TNF-α, IFN-γ	Serum, vitreous, aqueous	(2–7)
	Adhesion molecules	ICAM-1,VCAM-1	Serum	(2, 4)
	Immune cell markers	F4/80 mRNA, NF-κB, MIP-1α	Serum	(6, 8)
	Proteins	PTX3	Serum, aqueous	(9)
		RBP3	Vitreous, aqueous	(10, 11)
Angiogenesis	Growth factors	VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGF, FGF23	Serum, vitreous, aqueous	(12–14)
	Angiopoietin	ANGPTL3	Serum	(15)
Extracellular vesicles	Proteins	TNFAIP8, RANTES, CCR5	Plasma	(16–19)
Arteriosclerosis-associated parameters	Lipids	TG, LDL-C, sdLDL-C, apo	Serum	(14, 20, 21)
	Atherogenic index	(TC-(HDL-C))/HDL-C	Serum	(14)
	Atherogenic plasma index	(LDL-C/HDL-C)	Serum	(14)
Multi-omics	Metabolites	12-hydroxyeicosatetraenoic acid, 2-piperidone	Serum	(22)
	Proteins	lipophilin A, lactotransferrin, lysozyme C, lipocalin 1, mammaglobin B	Tears	(23)
	microRNAs	miR-9-3p, miR-431–5p, miR-200b-3p, miR-365-3p, miR-199a-3p, miR-146a-5p, miR-21, miR-34a, miR-145, miR-92a, miR-375	Plasma, serum, vitreous, aqueous	(24–29)

Diabetic nephropathy

Category	Profile	Biomarkers	Location	References
Kidney injury	Proteins	NGAL	Urinary	(30–32)
		Cystatin C	Serum	(33)
		KIM-1	Urinary, plasma	(34)
		L-FABP	Urinary	(34)
		B2M	Plasma	(35)
	Angiopoietin	ANGPT1, ANGPT2	Urinary	(36, 37)
	Growth factors	VEGF, FGF, PDGF	Serum	(38, 39)
Inflammation	Chemokines	CCL19	Tubular sample	(40)
		CCL5	Urinary	(41)
		CCL15	Serum	(42)
	Cytokines	TNF-α, IL-1, IL-6, IFN-γ, MCP1	Urinary, serum	(34)
	Proteins	ICAM-1,VCAM-1, PAl-1, CRP, TNFR1, TNFR2	Serum	(34, 43)
Anti-inflammation	Proteins	Klotho	Serum	(44)
	Carotenoid	Lutein	Serum	(45)
Multi-omics	Proteins	CKD-273	Urinary	(46, 47)
	Metabolites	2-hydroxyisobutyrate,leucine, valine, pseudouridine, threonine, citrate	Urinary	(48)
	MicroRNA	miR192, miR-21, miR-29a-3p, miR-126-3p,miR-192-5p,miR-214-3p, miR-342-3p	Urinary, serum	(49)

suggests the potential role of aqueous VEGF in evaluating the efficacy of PDR therapies (12). Besides, circulating angiopoietin-like 3 (ANGPTL3) is also considered as a promising biomarker which independently and strongly associated with DR onset and development (15). Moreover, recent studies revealed more candidate angiogenic factors like ITGA7, FGF23, THBS1, COL1A1, MAPK13, and AIF1 in early stage of DR (56), which may be novel intervention targets. Exploring the mechanisms of angiogenesis also could facilitate early recognition of DR.

Extracellular vesicles

In recent years, extracellular vesicles (EVs) have gained increasingly attention as potential sources of new biomarkers for multiple diseases. According to their size and biogenesis, EVs

can be classified as exosomes, microvesicles, or apoptotic bodies. It was found that hyperglycemia leads to the increase of EVs from donor cell. The molecular profiles and origins of EVs are distinct between DR and normal, presenting proinflammatory and proangiogenic properties. 90 proteins in the proteomic profiles of plasma EVs significantly changed between DR and non-DR subjects, among them, tumor necrosis factor-α-induced protein 8 (TNFAIP8) was increased in DR patients (16). *In vitro* experiment confirmed the angiogenic role of TNFAIP8 in retinal microvascular endothelial cell, indicating that TNFAIP8 in plasma EVs may act as a new biomarker for DR (16). Aleksandra et al. have described that the plasma level of microvesicles containing RANTES and CCR5 receptors, which both act as inflammatory and proangiogenic factors, were significantly higher in NPDR than diabetic patients without DR, and were positively associated with the progression of DR (17). Furthermore, Ogata et al. have reported gradually elevated levels of platelet- and monocyte-derived EVs in the early and advanced stage of DR (18, 19). These platelet-derived EVs induce oxidative stress and monocyte-derived EVs exaggerate inflammatory responses, leading to retinal vascular damages during the development of DR. Therefore, circulating EVs as carriers of proteins and RNAs, are not only the messengers for cell-cell communications, but serve as important biomarkers for prediction of disease occurrence and progression.

Arteriosclerosis-associated biomarkers

Plasma lipid parameters not only predict cardiovascular outcomes but also act as markers for microvascular complications. Serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are found significantly elevated in DR patients, acting as risk factors for the presence of DR. Besides, the atherogenic plasma index calculated as (LDL-C/HDL-C) and atherogenic index calculated as (TC-(HDL-C))/HDL-C also possess great values in predicting the onset and severity of DR compared with the traditional lipid indexes (14). Apolipoprotein (apo) profiles in non-DR, NPDR and PDR subjects revealed a panel of apos as independent risk factors for the occurrence and severity of DR and two apos as protective factors, which could be further used as biomarkers for predicting DR (20). Additionally, some lipid parameter has also been suggested to guide treatment of DR. For example, small dense low-density lipoprotein cholesterol (sdLDL-C) was identified as a sensitive biomarker for evaluating the need of laser treatment for DR patients (21).

Multi-omics related biomarkers

Development of high-throughput sequencing techniques allows the detection and quantification of the overall and dynamic changes in genome, transcriptome, proteome and metabolome, which produce a large amount of date in a short time and provide insight in identification of novel biomarkers (57). A recent metabolomics study observed a serum metabolites panel associated with DR occurrence and development. Serum 12-hydroxyeicosatetraenoic acid and 2-piperidone exhibited better diagnostic value than hemoglobin A1c (HbA1c) in differentiating DR from non-DR diabetes, which can be used for detection of early-stage DR (22). Besides, proteomics analysis from tears of DR patients uncovered multiple proteins changed correlating with the occurrence and severity of DR, providing evidence and targets for tear-based protein biomarkers for early diagnosis of DR (23). Furthermore, non-coding RNAs, especially microRNAs, are also the most well-studied biomarkers of DR (8). MicroRNAs have been shown to regulate multiple pathological processes during DR, including cell proliferation, apoptosis, inflammation and microcirculation impairments and exerted differential expressions in blood and vitreous samples from DR patients (24, 58). Therefore, microRNAs may act as promising biomarkers for early diagnosis and progression of DR.

Artificial intelligence

Diagnosis and monitoring the progression of DR heavily reliance on imaging, artificial intelligence (AI) has been a pioneer in the early detection and screening of DR. Based on the rich data generated by imaging techniques, using machine learning and deep learning, AI is now being applied in facilitating the early recognition, prognosis and treatment selection. Pivotal studies have demonstrated the clinical benefits of AI system for detection and screening of DR. A prospective multicenter study evaluated the efficacy of the AI DR detection system (EyeArt) in detecting more-than-mild DR and vision-threatening DR (59). The findings found high sensitivity and specificity of the AI system in detecting more-than-mild DR (95.5% and 85%, respectively) and even vision-threatening DR (95.1% and 89%), without physician assistance. Dai et al. (60) developed DeepDR system based on deep learning method, which can automatically detect the whole course of DR from mild to PDR, providing real-time feedback to the quality of fundus images as well as the recognition and segmentation of fundus diseases. Furthermore, AI techniques have also been used in DR prognosis and treatment efficacy judgement. A prospective study evaluated the potential of AI using optical coherence tomography data including segmentation of intraretinal cystoid fluid (IRC), retinal layer segmentation and subretinal fluid, to predict the prognosis of patients with DME. The findings showed that IRC possessed the greatest predictive value for best-corrected visual acuity (BCVA) at baseline, while IRC and total retinal thickness had greater prognostic value for BCVA after 4 and 12 weeks. The application of AI transforms descriptive data into information and factors that can be used for prediction. The establishment of predictive models for the prognosis will encourage patients to pursue aggressive treatment and make optimal treatment options (61). Early detection is critical for DR management and reducing the

blindness rate of DR. However, current DR screening faces the dilemma of insufficient ophthalmologists for standardized image reading and lack of awareness for DR screening. AI with the characteristics of high accuracy, easy to copy and promote, is promising to make up for staff shortages and facilitate early screening, intelligent fundus reading and intelligent diagnosis of DR in clinical practice. Until now, both the EyeArt and DeepDR systems have been approved for clinical practice. Similarly, other AI systems like IDx-DR (62), RetmarkerDR (63), Singapore SERI-NUS (64) and so on, also obtain good results in DR screening in different populations.

Novel therapeutic approaches

Treatment for DR generally includes systemic control, laser photocoagulation, and pharmacotherapies targeting mediators involved in pathogenesis of DR. The recent progress of therapeutic research in DR continues the previous academic concerns including anti-VEGF therapy, anti-inflammatory treatment, traditional Chinese medicine, and precision drug delivery, etc.

Anti-VEGF therapy

Approved in 2013 by US FDA, Anti-VEGF drugs are currently the first line therapy of DME with vision loss. Several studies, such as RESTORE (65), VISTA and VIVID (66) study have shown that the emergence of anti-VEGF therapy led to significant clinical improvements compared to laser therapy alone. Besides, a recent randomized clinical trial observed that combination of intravitreal bevacizumab injections and laser photocoagulation was more effective in preventing neovascularization and ameliorating visual field than pan-retinal photocoagulation (PRP) or bevacizumab alone in PDR patients (67). The combined protocol reduced the adverse effects of full PRP and need fewer injections and visits, suggesting that for some high-risk patients, anti-VEGF combined with PRP therapy may be more helpful in delaying the progression and improving the adherence of injections in DR. Furthermore, PANORAMA study found that anti-VEGF therapy also improve NPDR without DME (68). Compared with sham, intravitreal aflibercept significantly improved retinopathy severity scale and reduced vision-threatening adverse effects in moderately severe to severe NPDR. Though anti-VEGF therapy have gained benefit in clinical trials, frequent injections, close monitoring and heterogeneous response of patients are current barriers to achieve optimal outcomes in real world. Recently, long-acting and slow-release anti-VEGF agents have shown benefits in clinical trials. KESTREL and KITE study demonstrated that brolucizumab, a new agent targeting VEGF-A and facilitating high and sustained molar concentration, improved BCVA and reduced central subfield thickness (CSFT), subretinal and/or intraretinal fluid in DME patients during 52 weeks therapy (69). Additionally, Abicipar pegol, a VEGF-A inhibitor with longer half-life and higher affinity than ranibizumab, has also shown functional and anatomical improvements with fewer injections compared with ranibizumab administered every 4 weeks over a period of 28 weeks in DME patients (70). Alternatively, the port delivery system for anti-VEGF drugs also allows long-term, continuous delivery of ranibizumab into the vitreous, which maintains optimal vision and anatomic outcomes with reduced number of injections and gains high treatment satisfaction from patients (71, 72). This technology is now applied in patients with neovascular age-related macular degeneration in clinical trial stage and is expected to be further used in the treatment of DME. New agents beyond the VEGF pathway are also developed to optimize the efficacy and compliance of DR treatment. Faricimab is a novel antibody inhibiting angiopoietin2 (ANGPT2) and VEGF-A with high affinity and specificity. Two phase III trials indicated that faricimab administration achieved vision gains and improved anatomical outcomes with fewer visits and increased dosing compared with aflibercept in DME treatment (73). Furthermore, gene therapy is of great interest in the treatment of DR. RGX-314 uses the NAV AAV8 vector to deliver anti-VEGF monoclonal antibody fragment and is promising to maintain continuous expression of anti-VEGF-A protein in retina with a single administration (74). And an ongoing phase II clinical trial (ALTITUDE) is now conducted to evaluate the safety, tolerance and efficacy of RGX-314 in patients with moderate to severe NPDR and mild PDR. Further research is still needed to determine the safety, efficacy, durability and targeted population of gene therapy prior to implementing into clinical practice. Anti-VEGF agents targeting DR are summarized in Table 2.

Anti-inflammatory therapy

Inflammation plays a critical role in the pathogenesis of DR, and anti-inflammatory agents have shown functional and anatomical improvement in DR and DME. Clinical studies demonstrated that the visual outcomes of intravitreal dexamethasone injection were comparable with the anti-VEGF group after 1 year (78). DR patients who did not respond to anti-VEGF drugs, switching to dexamethasone sustained-release therapy may still reduce macular edema and improve vision (79). *In vitro* experiments implied that dexamethasone reduced the level of cytokines, including IL-1β, IL-6, and TNF-α in retinal ganglion cell and Müller cells, which alleviated hyperglycemia induced inflammation and improved cell survival rate (80). In addition to glucocorticoids, key inflammatory factors can be used as new targets for DR treatment. CD40 is a critical driver of DR which induces proinflammatory cytokines release in myeloid cells, and treatment blocking CD40 signaling decreased the level of

TABLE 2 Anti-VEGF agents for treatment of DR.

Agents	Mechanism	Study type	Status	Regimen	Outcomes
Ranibizumab (65)	Anti-VEGF	RCT Phase III	Completed	Intravitreal 0.5 mg	Superior visual acuity and central macular thickness improvement in DME compared with laser
Bevacizumab (75)	Anti-VEGF	RCT	Completed	Intravitreal 1.25mg	Significant central macular thickness reduction and visual acuity improvement in DME compared to laser
Aflibercept (66, 68)	Anti-VEGF	RCT Phase II & III	Completed	Intravitreal 2mg	Superior in improving visual acuity vs.laser in DME and PDR; Significantly improving retinopathy severity scale and reducing adverse effects in NPDR
Brolucizumab (69)	Anti-VEGF	RCT Phase III	Completed	Intravitreal 3 mg/6 mg	6 mg showed superior improvements in central subfield thickness and lower adverse effects vs aflibercept
Abicipar pegol (70)	Anti-VEGF	RCT Phase II	Completed	Intravitreal 2 mg	Functional and anatomical improvements with fewer injections compared with ranibizumab in DME
Faricimab (73)	VEGF and ANGPT2 inhibitor	RCT Phase III	Completed	Intravitreal 6 mg	Superior vision gains and anatomical outcomes improvements with fewer visits compared with aflibercept in DME
Conbercept (76)	Anti-VEGF	Retrospective	Completed	Intravitreal	Significant visual acuity improvement compared to baseline in DME Non-inferior to ranibizumab
Pegaptanib (77)	Anti-VEGF	RCT Phase II & III	Completed	Intravitreal 0.3 mg	Superior visual acuity improvement vs. sham in DME
RGX-314	Anti-VEGF (gene therapy)	RCT Phase II	Ongoing	Intravitreal $2.5x10^{11}$ ~$5x10^{11}$genomic copies per eye	Phase II trial ongoing

inflammatory molecules in retina of diabetic mice, providing evidence for the potential of CD40 in DR treatment (81). Moreover, nuclear factor of activated T cells (NFAT) and RBP3 are also important biomarkers involved in retinal inflammation. Inhibition of NFAT prevented retinal vascular leakage and inflammation in DR mice model; while intravitreal injection of recombinant human RBP3 reversed high glucose induced retinal vascular dysfunction and inflammatory cytokine elevations in STZ rats. The role of these inflammatory biomarkers in the progression of DR needs further research, and they are expected to become novel targets in pharmacotherapeutics.

Renin-angiotensin-aldosterone system therapy

RAAS activation potentially has a role in the development of end-organ damage, and has been considered as a risk factor for DR. Activation of the angiotensin receptor 1 (AT1-R) induces the progression of DR by stimulating multiple pathways involved in the pathogenesis including advanced glycosylation end-products (AGE) accumulation, inflammation, oxidative stress, and several crucial mediators of angiogenesis such as VEGF (82). Clinical and animal experiments showed that the RAAS is activated in diabetic retinopathy and its inhibitors may exert protective role against retinal damage (83, 84). Several clinical trials indicated that ACE inhibitors and/or ARBs treatments reduced the incidence of DR in hypertensive diabetic people (85–87). However, there also exists studies that showed no benefit in DR incidence or DR progression from the ACE inhibitor and ARB therapy (85, 88), which indicates that mechanism other than RAAS also responsible for the progression of DR, and further research working on the efficacy of RAAS inhibitor to prevent the progression of DR in diabetic people are still needed.

Nanotechnology

Recently, nanotechnology has been widely applied in the medical field, broaden the horizon of new drug discovery, drug delivery and precision treatment. The diameter of nanomolecules ranges from 1 to 100 nanometers, enabling the drugs pass through the blood-retinal barrier. Nanoparticle-based delivery of triamcinolone acetonide are safe and long-lasting and significantly improved anatomic outcome and functional activity of retina in DR rat model (89). In addition, multiple micro RNAs (miRNA) and DNAs can also be delivered by nanomaterials, which are expected to delay DR progress and promote retinal regeneration.

Diabetic nephropathy

Novel biomarkers

Early recognition of DN is key to preventing renal function reduction, however, biomarkers currently used in clinical practice such as albumin, creatinine and eGFR do not sufficiently predict and assess the progression of DN (90). Therefore, novel

biomarkers to predict the risk of functional decline have been urgently sought. In recent years, multiple efforts have been made to identify new reliable and sensitive biomarkers for the early diagnosis and monitoring of DN (Table 1).

Kidney injury biomarkers

Several biomarkers indicating kidney injury are being developed (34). Neutrophil gelatinase-associated lipocalin (NGAL) is a well-studied tubular damage marker. DN patients with normo-albuminuria have present increased levels of urinary NGAL, which implies that tubular damage may occur in very early stages (30–32). Besides, kidney injury molecule 1 (KIM-1) and β-2-Microglobulin (B2M) have also been extensively studied in DN. Longitudinal studies indicated that high level of KIM-1 had a strong correlation with higher risk of eGFR decline and increased risk of DKD (43, 91). Evidence also showed that diabetic individuals with elevated B2M levels had a higher risk for DN, which may serve as promising predictors of DN progression in diabetic patients (35). ANGPT are vascular growth factors that promote angiogenesis and vascular repair and play a crucial role in the glomerular capillaries (36). Elevated level of urinary ANGPT2 was found in T2D patients with renal damage and was associated with albuminuria (36). Inversely, ANGPT1 has exerted a protective effect against renal function decline and reduced level of ANGPT1 was detected in early diabetic kidney disease (37). Other growth factors such as VEGF and fibroblast growth factor (FGF) have also been previously evaluated as important biomarkers and targets in DN diagnosis and progression (38, 39). Furthermore, recent studies illustrated that serum cystatin C, another marker of tubular damage, shows better predicting value of eGFR decline in diabetes patients than creatinine (33).

Inflammatory/anti-inflammatory biomarkers

Markers involved in inflammation are also highly reported (34). Chemokines such as CCL19,CCL5 and CCL15 have been identified as critical genes of DN. Bioinformatics analysis and *in vitro* experiments revealed that CCL19 was significantly upregulated in tubular samples of DN patients (40). Besides, urinary level of CCL5 was correlated with renal function reducing and the extent of renal interstitial fibrosis (41). While serum CCL15 is found negatively correlated with eGFR and independently associated with high DN risk in T2D patients (42). In addition, prospective studies reported that diabetic patients with higher plasma levels of TNFR1, TNFR2 and MCP1 had increased risk of progression of DN (43). On the other hand, some anti-inflammatory markers have also been studied in DN. Klotho protein mainly expressed in the kidney and can suppress the inflammatory response. A recent meta-analysis found that the soluble Klotho was significantly lower in DN than that in control, and this decrease can be detected in the early stages of DN (44). Lutein is an oxygenated carotenoid with antioxidation and anti-inflammatory effects. Serum lutein level is negatively associated with the risk of DN and possesses a good diagnostic value of DN with an AUC of 0.779 (45).

Multi-omics related biomarkers

The urinary proteomic CKD273 score was used to quantify the risk for the new onset of albuminuria. CKD-273 was validated in T2D cohorts and shown to predict the progression of albuminuria in DN (46, 47). Besides, metabolomics of urine samples from 2670 T1D individuals revealed five urinary metabolites closely associated with kidney disease progressing, and the level of 2-hydroxyisobutyrate reflected the progression of DN in individuals with normo-albuminuria (48). Urinary or serum miRNAs also emerge as new biomarkers for DN depending on the microarray and RNA-sequencing techniques (49).

Novel therapeutic approaches

The current therapy for DN including glycemic control, blood pressure and cholesterol management, focus on the systematic control of DR. As the emergence of abundant targets involved in DN, the direction of DN treatment investigation has been progressed to focus on molecular mechanism and target the critical molecules or signaling pathways in the progression of DN.

Anti-inflammatory agents

Pentoxifylline (PTF) is a methylxanthine derivate and plays an anti-inflammatory role in kidney disorder progression. An early meta-analysis summarized that PTF may reduce proteinuria in patients with DN (92). The PREDIAN Trial and a recent randomized clinical trial both confirmed that the addition of PTF to renin-angiotensin system antagonists resulted in a more significant reduction of urine albumin excretion and slowed the progression of renal function decline (93, 94). Besides, some chemokine antagonists also exert beneficial effects in clinical studies. CCX140-B, a selective MCP-1 inhibitor, significantly reduced albuminuria by 18% in DN patients (95). A phase II study in T2D patients with albuminuria demonstrated that emapticap pegol (NOX-E36), another antagonist of MCP-1, was safe and well tolerated during administration, and significantly improved urinary albumin/creatinine ratio (ACR) compared to the placebo group (96). Klotho could inhibit the expression of MCP-1 and ICAM-1, resulting in lower accumulation of macrophages, thus exerting anti-inflammatory function and reduced tubulointerstitial injury (97).

Anti-fibrotic agents

Some agents have been found to target renal cell function for anti-fibrotic effect in DN. Oxymatrine prevented renal extracellular matrix deposition by inhibiting the epithelial-to-mesenchymal transition (EMT) process and ultimately

attenuated tubulointerstitial fibrosis (98). The extract of P. fallax has been demonstrated to downregulate the expression of ECM proteins, such as FN, Col IV, MMP-9, and MMP-2, therefore protecting glomerular mesangial cell from high glucose induced fibrosis (99).

Renin-angiotensin-aldosterone system therapy

The RAAS plays an important role in renal disease. In DN, the RAAS has been linked with changes in intraglomerular hemodynamics as well as structural alterations in both the glomerulus and tubulointerstitium (100). Growing evidence has revealed that RAAS inhibitors blocked the development of kidney diseases, manifesting as improved proteinuria and well-maintained renal function (101). It has been shown that ACEI or ARB which block the RAAS delayed the development of DN and reduced the incidence of end-stage renal disease in DN patients with large albuminuria (102).

Novel anti-diabetic drugs

Recently, the emergence of novel anti-diabetic drugs not only improve the management of hyperglycemia, but also obtain heart and kidney benefits. SGLT2 inhibitors are oral hypoglycemic drugs and exhibit renoprotective effects. Studies demonstrated that SGLT2 inhibitors therapy decreased albuminuria, prevented GFR decline, and reduced need for renal replacement therapy or death from kidney causes in diabetes patients (103, 104). Besides, data suggested DPP-4 inhibitors and GLP-1 agonists also have renoprotective effect in diabetic patients through antioxidant, anti-inflammatory and antifibrotic mechanisms, which prevent DN occurrence and development (105, 106).

Endothelin receptor blocker

Endothelin (ET) is a kind of vasoconstrictor which has vasoactive, inflammatory, and profibrogenic characters and is significantly correlated with kidney disorders. Animal experiments suggested that ET receptor antagonists reduced proteinuria and exerted nephroprotective effect in experimental models of DN (107). Phase III clinical trials also showed that ET receptor antagonists decreased albuminuria in patients with DN (108). However, the safety and side effects (congestive heart failure) of ET receptor blockers require further investigation.

Vitamin D supplementation

Some clinical trials and observational studies have supported the benefits of vitamin D supplementation in DN treatment. A randomized control study revealed that paricalcitol decreased urinary albumin excretion rate by 18% in T1D patients compared to placebo (109). Similarly, an observational study also showed that oral cholecalciferol therapy for 4 months significantly reduced the albuminuria in T2D patients (110). A meta-analysis including 20 randomized clinical trials with total 1464 DN patients suggested that vitamin D supplement (calcitriol, alfacalcidol and vitamin D3) improved 24-hour urine protein and urine albumin excretion rate, as well as inflammatory indexes, such as hs-CRP, TNF-α and IL-6 (111). But there was no significant difference in serum creatine, eGFR or HbA1c. Therefore, more trials are needed to confirm the therapeutic value of vitamin D supplementation in DN for reaching a consensus and recommendation.

Nanotechnology

Recent research assembled synthetic high-density lipoprotein (sHDL) with a liver X receptor (LXR) agonist, which aim to deliver LXR agonists to kidney and promote the removal of excessive lipids from mesangial cells, thus attenuating inflammation and recovering renal function, and the efficacy of sHDL nanoparticle has been confirmed in DN animal experiments (112).

Link between DR and DN

DR and DN are both major microvascular complications of diabetes. DR is the leading cause of blindness in adults aged 20-74 years, with almost all type 1 diabetes and 60% of type 2 diabetes occurring after 20 years. DN is one of the major causes of end stage renal disease (ESRD), contributing to significant morbidity and cardiovascular mortality (113). The connection between DR and DN can be addressed in many aspects. Firstly, these two complications share common pathogenetic mechanisms (114). The main biological mechanisms of microvascular complications include advanced glycation end products (AGEs) accumulation, polyol pathway activation, oxidative stress, inflammatory responses (e.g., activation of NF-kB, adipokines, chemokines, adhesion molecules and proinflammatory cytokines), hemodynamic alterations (e.g., RAAS activation) and growth factors overexpression (e.g., VEGF is one of the most important factors in the progression of DR and DN). Secondly, the similar underlying pathogenetic mechanisms contribute to the overlap of biomarkers for predicting the progress of DR and DN. As we discussed previously, DR and DN share many common biomarkers, such as inflammatory biomarkers, angiogenetic biomarkers, and lipid profiles. Therefore, combination of these biomarkers could facilitate detecting early disease affecting both systems, and therapeutic strategies targeting their common markers may also gain retinal and renal benefits. Moreover, DR and DN have a predictive effect on each other. Accumulating evidence suggested that the presence of DR increased the incidence of DN in diabetic patients and DR has been considered as the predictor of DN, especially PDR as a more sensitive predictor (115, 116). Yang et al. (117) found that haptoglobin, a urine proteome specific for PDR could serve as

an indicator complementing to urine albumin to predict renal dysfunction in patients with T2D. Likewise, DN can also predict DR to some extent. A prospective ten-year follow-up study demonstrated that eGFR and ratio of urine albumin to creatinine (ACR) served as sensitive biomarkers to predict the incidence of DR (118). And serum creatinine and decreased eGFR has been shown to be associated with the progression and severity of DR in diabetic patients (119, 120). Although the onset of retinopathy symptoms is very hidden, with the development of fundus examination and AI, accurate and early recognition of DR gains clinical feasibility. However, the early stage of DN lacks specific clinical symptoms and requires kidney biopsy to confirm the diagnosis, which is the greatest challenge for clinical promotion. Microalbuminuria, the most used indicator, however, its accuracy remains controversial. Therefore, considering the close link between DR and DN, it is promising to replace invasive and unpredictable detection of other microvascular complications with noninvasive, simple, and low-cost ophthalmoscopy. And vice versa, it also useful to screen patients with kidney disorders for associated retinal diseases. Comprehensive clinical evaluation in patients with CKD or ESRD should include external ophthalmoscopy and direct ophthalmoscopy. These recommendations may facilitate early recognition of both retinal and renal damages in diabetic patients and provide evidence for multi-factorial approach and potential multi-target therapeutic strategies.

Perspectives

In conclusion, sensitive and cost-effective biomarkers facilitating early identification guarantees optimal treatment of DM related microvascular complications. Multi-omics techniques provide huge data for candidate biomarker discovery, however, most of them remains further confirmation in clinical practice. The application of AI technology may also be expected to achieve effective diagnosis, efficacy determination, and prognosis of microvascular complications. Furthermore, in view of the close association between DR and DN, biomarkers and therapeutic strategies targeting their common pathophysiology mechanism such as VEGF and inflammation are key research directions in the future. Novel agents targeting multifactor which will complement current therapies in effects, such as SGLT-2 inhibitor and DPP-4 inhibitor, etc., are expected to benefit more patients. Finally, nanotechnology have shown incomparable benefits in non-invasive and precise drugs delivery. The application of nanomaterial-based drug delivery systems in microvascular complications therapy is of great potential and interests.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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WF SS-OCTA for detecting diabetic retinopathy and evaluating the effect of photocoagulation on posterior vitreous detachment

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Purpose: This study aimed to assess the clinical usefulness of widefield swept source optical coherence tomography angiography (WF SS-OCTA) for detecting microvasculature lesions in diabetic retinopathy (DR) by comparing it with ultra-widefield fluorescein angiography (UWFFA) and to investigate the effect of panretinal photocoagulation (PRP) on posterior vitreous detachment (PVD) status.

Methods: Patients with severe non-proliferative DR (NPDR) or proliferative DR (PDR) who were initially treated with PRP were enrolled. They underwent WF SS-OCTA with a 12×12-mm scan pattern of five visual fixations at baseline and at least a 3-month follow-up after PRP treatment. Patients with no contraindications underwent imaging with UWFFA within a week. Images were evaluated using two methods for the areas of the visible field of view (FOV), non-perfusion area (NPA), presence of neovascularization of the disc (NVD), neovascularization elsewhere (NVE), and PVD status.

Results: In total, 44 eyes of 28 patients with DR that were initially treated with PRP were analyzed. The FOV of the UWFFA was significantly wider than that of the WF SS-OCTA. The quantitative measurement of the NPAs was consistent between the two methods. NPAs more than 5DA outside the panoramic OCTA imaging area were detected in 1 eye with NPDR (8.3%) and in 10 eyes with PDR (47.8%). WF SS-OCTA had high detection rates for NVDs and NVEs, with a low rate of false positives. After PRP treatment, no eyes indicated progression in the PVD stages around the macula, optical disc, or NVEs at the short follow-up.

Conclusion: WF SS-OCTA is clinically useful for evaluating NPAs and neovascularization in DR. PRP treatment does not induce PVD development in the short term.

KEYWORDS

widefield swept source optical coherence tomography angiography, ultra-widefield fluorescein angiography, nonperfusion area, neovascularization, diabetic retinopathy, posterior vitreous detachment

Introduction

According to reports from the International Diabetes Federation, 537 million adults had diabetes in 2021, and this number is predicted to increase to 784 million by 2045 (1). Diabetic retinopathy (DR) is a severe microvascular complication of diabetes characterized by microaneurysms, intraretinal microvascular abnormalities, venous beading, non-perfusion area (NPA), and neovascularization (NV). The diagnosis and treatment of DR are mainly based on fundus photography (including ETDRS 7-standard field 35 mm stereoscopic color 30° fundus photographs and ultra-widefield fundus [UWF] photography), fluorescein angiography (FA), and optical coherence tomography (OCT) (2–4).

FA is currently the gold standard for the clinical evaluation of retinal vascular features in DR. However, as an invasive examination, it has some contraindications, such as renal insufficiency, cardiovascular diseases, and possible risks ranging from nausea and vomiting to anaphylaxis to even death. According to the American Academy of Ophthalmology Preferred Practice Patterns, FA is not a routine examination for patients with diabetes and is not indicated for monitoring the therapeutic effect or progression of DR (5).

OCTA is a noninvasive imaging technique for evaluating vasculature circulation in the choroid and any layer of the retina (6). It can also provide objective information on the vitreoretinal interface (VRI) simultaneously, such as posterior vitreous detachment (PVD), which is important for the growth of NVs. Due to the limited field of view (FOV), conventional OCTA (3 × 3 mm or 6 × 6 mm) only assesses the macular vascular network qualitatively and quantitatively and is meaningful in the evaluation of macular pathologies (6, 7).

Meanwhile, the commercial widefield swept source OCTA (WF SS-OCTA) system (VG200, SVision Imaging, Ltd., Luoyang, China) can capture a 12 × 12-mm angiography image in a single scan. Additionally, with Flexible Montage TM technology, the FOV further expands to 80° × 60° (23.5 × 17.5 mm).

WF SS-OCTA can obtain high-resolution images of the VRI from the macula to the mid-peripheral retina, which is wider than conventional OCT/OCTA. According to previous studies, PVD stage may be related to the pathophysiology of diabetic macula edema (DME) and NVs (8, 9). Thus, prophylactic induction of PVD may benefit patients with DR.

In this study, we explored whether WF SS-OCTA could substitute UWFFA in clinical practice to evaluate the DR lesions and observed the short-term effect of panretinal photocoagulation (PRP) treatment on PVD progression.

Materials and methods

Study participants

This observational study was conducted at Tianjin Medical University Eye Hospital between September 2020 and February 2022 and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients.

The inclusion criteria were as follows: age ≥20 years, confirmed diagnosis of type 1 or type 2 diabetes mellitus, severe non-proliferative DR (NPDR) or proliferative DR (PDR) that required PRP treatment, no previous history of intravitreal injection. Meanwhile, the exclusion criteria were as follows: history of fundus laser treatment, presence of other retinal diseases, history of glaucoma, media opacities such as cataract or severe vitreous hemorrhage, and underwent cataract surgery during the study.

Study protocol

The enrolled patients underwent comprehensive ophthalmological examinations, including measurement of visual acuity and intraocular pressure, slit-lamp biomicroscopy, UWFFA (except for patients with contraindications), WF SS-OCTA at baseline, and a 3-month follow-up after PRP.

UWFFA images were obtained after standard intravenous injection of 5 ml of 10% sodium fluorescein using the Optos Optomap Panoramic 200Tx imaging system (Optos, PLC, Dunfermline, Scotland), which theoretically covered nearly the entire retina (up to 200°). The configurations of retinal vessels and hyperfluorescent areas were evaluated in the early phase to reduce the effect of dye leakage.

Five OCTA en-face images of 12×12-mm regions (center, temporal superior, temporal inferior, nasal superior, and nasal inferior) were acquired using the WF SS-OCTA system (VG200, SVision Imaging, Ltd., Luoyang, China). This instrument was equipped with a swept-source laser with a central wavelength of 1050 nm and operated at a scanning rate of 200,000 A-scans per second. The axial resolution in the tissue and lateral resolution at the retinal surface were 5 μm and 20 μm, respectively. Each image was obtained using a raster scan protocol of 1024 B-scan positions per volume, two repeated B-scans per B-scan position, and 1024 A-scans per B-scan. The system was equipped with an artificial intelligence-assisted tracking system to eliminate eye motion artifacts and retain the original blood vessel signals using the SS-PAR algorithm. In the case of segmentation errors, the segmentation of the different layers was manually corrected. The

WF SS-OCTA en face montage was generated automatically (up to 80°×60°), and only high-quality images were included in our study (signal strength≥6).

Data on age, sex, and stage of DR were obtained from medical records.

Treatment protocol

A 577-nm pattern scan laser photocoagulator was used for PRP. Parameters (power × duration) were determined by visual observation; the dose was considered adequate if the spot turned gray-white immediately after the laser. In total, 1200–1600 spots (300–500-μm diameter for each spot) were delivered in three sessions. Intravitreal anti-vascular endothelial growth factor (anti-VEGF) therapy was added if DME was present.

Image processing and analysis

NPAs were defined as the complete absence of retinal capillaries with dark or gray areas on both UWFFA and OCTA. NVs were characterized by abnormal vessels that grew towards the vitreous cavity. The retinal vasculature slab and VRI slab of OCTA were used to detect NVs combined with B-scan images for further confirmation (10). Neovascularization of the disc (NVD) was defined as a lesion located in the disc or within 1-disc diameter from the margin, whereas neovascularization elsewhere (NVE) was located outside this area (11).

PVD stage and progression were classified according to the study by Itakura and Kishi (12, 13) as follows: stage 0 (no PVD), stage 1 (paramacular PVD), stage 2 (perifoveal PVD), stage 3 (vitreofoveal separation and peripapillary PVD), and stage 4 (complete PVD).

The disc area (DA), FOV, NPAs, and horizontal and vertical lengths in WF SS-OCTA and UWFFA images were measured manually and separately using the ImageJ software (Version 1.53c). The values of FOV/DA and each NPA/DA in the two different widefield devices were calculated and compared. The PVD status around the macula, optical disc, and NVEs at baseline and at the 3-month follow-up after PRP treatment was recorded (Figures 1, 2).

Statistical analysis

Statistical analyses were performed using Statistical Packages for Social Sciences V.21.0 (SPSS V.21.0) and MedCalc (Version 19.4). Continuous variables are expressed as mean values ± standard deviation or median and interquartile range. Data were analyzed using the Kolmogorov–Smirnov test or Shapiro–Wilk test to evaluate the normality of the sample distribution. The agreement of NPAs measured by WF SS-OCTA and UWFFA was assessed using Bland–Altman analysis. The sensitivity and specificity of WF SS-OCTA were calculated based on UWFFA images as the reference standard for evaluating NVD and NVE. Agreement of measurements between readers was assessed using interclass correlation coefficient (ICC).

Results

Patient characteristics

In total, 44 eyes of 28 patients with DR were analyzed. Only seven eyes of five patients were subjected to WF SS-OCTA imaging due to contraindications for UWFFA; the DA in two eyes of two patients could not be measured on UWFFA due to leakage of the NVD. Five eyes of three patients were excluded because of media opacities or poor fixation ability. The patient characteristics are described in Table 1.

Quantitative measurement of the FOV

The images of the UWFFA and WF SS-OCTA en face montage are presented in Figure 3. The mean extension ratios of the horizontal and vertical field to the corresponding diameter of the optic disc were 11.13 ± 0.97 (range, 8.47–12.47), 9.40 ± 0.86 (range, 7.16–10.64) in WF SS-OCTA, and 18.79 ± 2.64 (range, 12.87–23.05) and 10.88 ± 1.63 (range, 7.91–14.70) in UWFFA. The ratios of the horizontal and vertical dimensions between WF SS-OCTA and UWFFA were significantly different ($P<0.05$). The mean FOV to DA in the two methods were 118.28 ± 19.70 and 209.95 ± 37.62, respectively, and a significant difference in FOV/DA values was observed ($P<0.001$).

Quantitative measurement of NPAs

Within the area of the panoramic WF SS-OCTA image

The ratios of NPA/DA in the retinal layer measured using WF SS-OCTA and UWFFA were normally distributed

TABLE 1 Clinical characteristics.

Parameter	Values
Eyes/patients	44/28
Age, Mean ± SD, y	51.43 ± 10.93
Sex	
Male	19
Female	9
DR severity	
NPDR	14
PDR	30

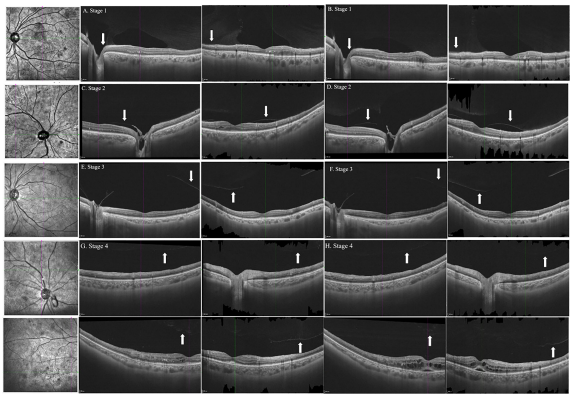

FIGURE 1
B-scan images of horizontal and vertical cross-sections of representative eyes with the stages 1, 2, 3, and 4 PVD in patients with DR at baseline **(A, C, E, G)** and follow-up **(B, D, F, H)**. Arrows indicated the posterior vitreous. PVD, posterior vitreous detachment; DR, diabetic retinopathy.

(P=0.413). Figure 4 presents Bland–Altman plots for NPAs. The horizontal lines represent the mean ratio and 95% confidence interval limits. According to the Bland–Altman method, the mean ratio of the NPAs measured by WF SS-OCTA to UWFFA was 1.00. Here, 132 of 141 paired values (93.62%) were situated within the 95% level of agreement (LoA, 0.84–1.20). The interobserver ICC was 0.890 (95% CI: 0.850–0.920).

Outside the area of the panoramic WF SS-OCTA image

Outside the area of the panoramic OCTA images, the NPA/DA values detected by UWFFA ranged from 0 to 46.67. Of the 35 eyes measured in our study, NPA (>5 DA) was observed in 1 eye (8.3%) in patients with NPDR and in 11 eyes (47.8%) in patients with PDR (Table 2). The interobserver ICC was 0.994 (95% CI: 0.988–0.997).

Detection of NVs

Altogether, 25 eyes with PDR were analyzed in this study. NVDs were detected in nine eyes using UWFFA. The sensitivity and specificity of WF SS-OCTA for NVD detection were 100% and 96.67%, respectively. The ability to accurately identify NVEs was also assessed. 96 NVEs were detected by WF SS-OCTA, of which 94 NVEs were confirmed by UWFFA, two of which may be false positives (FP, 2.2%) because of no evident leakage in the late frames of UWFFA (Table 3). Additionally, 21 NVE lesions were identified using UWFFA in the area of WF SS-OCTA (18.3%), whereas no eyes only existed NVs in this area.

PVD status

Altogether, 20 eyes of 14 patients completed the follow-up, and the median time was 106.0 ± 30.0 days (range: 80–187 days). Owing to DME, three eyes of two patients received three

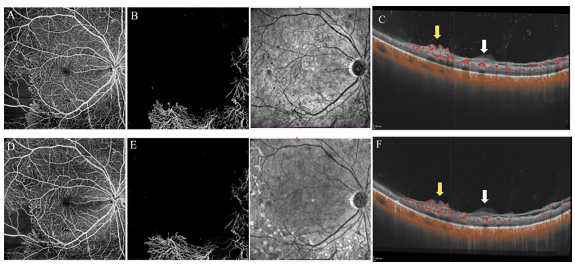

FIGURE 2
12×12mm SS-OCTA slabs of the retina **(A, D)**, VRI **(B, E)**, and B-scan images **(C, F)** showed features of PDR eyes at baseline (top) and 3-month follow-up (bottom). The area of NVs was reduced from 14.77mm^2 **(B)** to 13.11mm^2 **(E)** after PRP treatment (measured by Angiotool, Version 0.6a). B-scan images revealed no PVD around the location of NVs at baseline and after PRP treatment. White arrows indicated the thickened posterior vitreous (PVC). Yellow arrows indicated the NVs. VRI, Vitreoretinal Interface; PVD, posterior vitreous detachment; NVs, Neovascularization; PRP, panretinal photocoagulation.

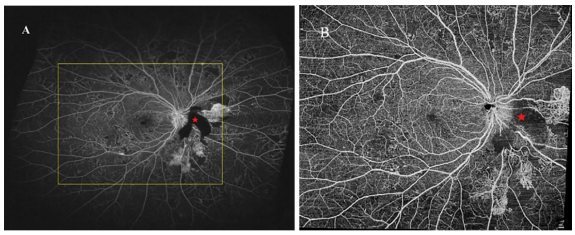

FIGURE 3
UWFFA **(A)** and WF SS-OCTA **(B)** posterior pole montage of a representative eye. UWFFA performed by Optos Optomap Panoramic 200Tx imaging system with the yellow square indicated the same FOV of WF SS-OCTA obtained by VG200, SVision Imaging system. The area noted with red pentagram was caused by funds hemorrhage.

monthly injections of anti-VEGF agents, except for PRP treatment during the investigation. At baseline, 19 eyes had vitreoretinal relationships at stages 1 (50.0%), 2 (35.0%), and 3 (10.0%). Stage 4 vitreoretinal relationship (complete PVD) was observed in only one eye (5.0%) (Table 4). B-scan images of all the eyes revealed no PVD at the NV locations. However, after PRP treatment, PVD around the macula and optical disc did not develop in any of the eyes (20/20, 100%; Figure 1). No evident progress in PVD around the NVs was observed (15/15, 100%; Figure 2).

Discussion

This study compared the clinical utility of WF SS-OCTA and UWFFA for detecting DR lesions. The distribution of diabetic microangiopathy is widely known to be non-uniform within the retina (14, 15), and identifying lesions in the perifoveal and periphery is crucial for evaluating the risk of DR (16). In this study, we calculated the FOV captured by WF SS-OCTA and UWFFA. Although WF SS-OCTA had already expanded the imaging area substantially compared with conventional models of 3 mm × 3 mm and 6 mm × 6 mm, WF SS-OCTA still captured smaller areas of the fundus than UWFFA ($P<0.001$), and the ratios in both horizontal and vertical dimensions were significantly different ($P<0.05$).

Our results accord with those of previous studies demonstrating that OCTA had high sensitivity and specificity in detecting NPAs and NVs, which are crucial for evaluating DR progression and courses of treatment (10, 17–20). As is well established, NPAs represent retinal or macular ischemia, which is associated with DME and NVs. A previous study has reported

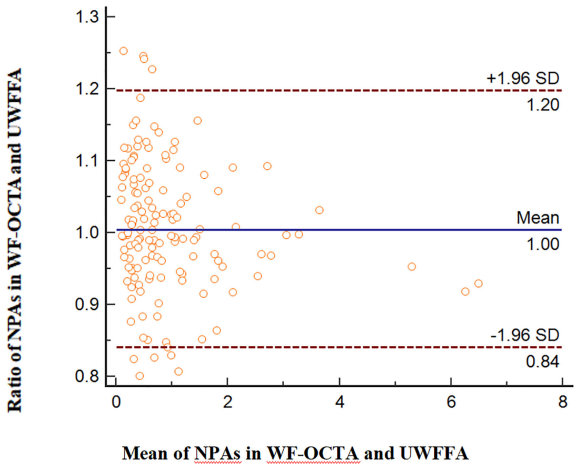

FIGURE 4
Comparison of NPAs in WF SS-OCTA and UWFFA *via* the Bland–Altman approach, displaying bias and the 95% LoA.

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TABLE 2 NPAs measured by UWFFA out of the area of the panoramic OCTA image.

Features (NPAs/DA)	Number (%)
NPDR	
≤5	11 (91.7%)
5-10	1 (8.3%)
≥10	0 (0%)
PDR	
≤5	12 (52.2%)
5-10	3 (13.0%)
≥10	8 (34.8%)

NPAs, Nonperfusion areas; DA, Disc area; NPDR, Nonperfusion proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

that peripheral NPAs were significantly higher in the eyes with PDR than in the eyes with NPDR, whereas NPAs in the posterior pole were not significantly different (21). In this study, we conducted a quantitative assessment of NPAs within and outside WF SS-OCTA images using two checking devices. The Bland–Altman consistency analysis revealed that the points out of 95% LoA were 9 out of 141 (6.38%); therefore, we considered that the consistency for NPAs between WF SS-OCTA and UWFFA was good. However, in our study, out of 35 eyes detected by UWFFA and WF SS-OCTA, NPAs (>5 DA) outside the panoramic OCTA image area were detected in one eye with NPDR (8.3%) and in 11 eyes with PDR (47.8%). Despite the limited number of patients in our study, WF SS-OCTA still had some limitations in scanning peripheral NPAs compared with UWFFA, especially in PDR. The implications of far-peripheral lesions on DR severity and progression over time remain unknown. Some recent studies have reported that the far peripheral retina beyond the FOV of the current WF SS-OCTA instruments did not significantly contribute to the diagnosis and management of these patients (22–24). A possible clinical use for identifying peripheral NPAs is to target PRP in these regions, although no evidence can support that this approach could decrease the anti-VEGF injection burden or improve vision outcomes (25). Hence, the utility of quantifying NPAs in clinical practice requires further investigation.

Compared to NPAs, the meaning of determining and monitoring NVs is well known. In this observational study, WF SS-OCTA has a sensitivity and specificity for detecting NVDs of 100% and 96.67%, respectively, and a high detection rate for NVEs (100%) with a low rate of false positives (2.2%).

TABLE 4 PVD stages at baseline.

Stages	Number (%)
Stage 0	0
Stage 1	10 (50.0%)
Stage 2	7 (35.0%)
Stage 3	2 (10.0%)
Stage 4	1 (5.0%)

PVD, posterior vitreous detachment.

WF SS-OCTA might be clinically adequate for identifying NVs in PDR cases as most NVs were observed within the mid-periphery of the retina, which were covered by WF SS-OCTA images (19).

In recent years, the absence of PVD and presence of an intact VRI have become generally considered essential for the growth of NVs in DR (26, 27). Therefore, identifying treatments to liquefy the vitreous gel and weaken vitreoretinal adhesions may be helpful for patients with diabetes. Prophylactic induction of PVD before the onset of NVs may be effective for preventing PDR progression. However, few studies have reported the frequency of PVD following intravitreal injection. Geck et al. observed a 25% PVD rate after injections within a mean follow-up of 11.1 weeks (28). Özsaygili et al. have reported that PVD occurred in approximately 18 % of the DME cohort during three aflibercept injections (29). However, Veloso et al. have reported PVD in only 7 of 125 eyes after a follow-up of 21.1 months (30).

Moreover, according to previous clinical studies using biomicroscopy, the incidence of PVD was higher in patients with DR who received PRP treatment than in those who did not (the mean follow-up times were 4 years and 4.5 years, respectively) (31, 32). Thus, PRP may induce PVD and provide therapeutic benefits. Moreover, the areas of PVD could serve as strategic locations for pars plana vitrectomy to delaminate fibrovascular membranes (FVM) (33). Therefore, prior to surgery for PDR, according to clinical experience, some vitreoretinal surgeons intend to perform PRP treatment, which might induce PVD progression to avoid exerting undue traction on the retina, complications of iatrogenic retinal break, and inadvertent transection of active FVM. However, our results differed from previous conclusions. To the best of our knowledge, no studies have used OCT/OCTA to evaluate the effects of PRP on inducing PVD in patients with DR. In the present study, we observed no significant change in PVD status

TABLE 3 Detection rates of NVEs in proliferative diabetic retinopathy.

	NVE lesions	Confirmed(%)	Not confirmed(%, FP)
UWFFA	94	Reference	Reference
WF SS-OCTA	96	94(100%)	2(2.2%)

UWFFA, ultra-widefield fluorescein angiography; WF SS-OCTA, widefield swept source optical coherence tomography angiography; NVE, neovascularization elsewhere; FP, False Positive

around the macula, optical disc, and NVs after PRP treatment in the short term. The therapeutic benefit of PRP in regressing retinal neovascularization might not be achieved by mediating the occurrence of PVD. Our conclusions being different from those of Sebag et al. may be attributed to the follow-up time and patient heterogeneity.

The limitations of this study include the small sample size, short follow-up period, and heterogeneity in the PRP parameters. We also measured NPAs manually, which is impractical for clinical use. With the development of technology and deep learning, a wider imaging mode and more accurate automated measurement in OCTA may be available in the future. Additionally, eyelash artifacts prevented clear imaging of the inferior far retinal periphery, which might have overestimated the extent of the non-perfusion areas (34). To avoid eyelash artifacts, tape fixation and the examiner's assistance with cotton swabs were used. However, this might decrease the efficiency of the examination and patient comfort during the examination. Finally, the effect of PRP on PVD is complicated by anti-VEGF injections in eyes with DME because the injections may have a role in inducing PVD. To definitively answer the question of whether, how, and when PRP affects PVD progression, a large, long-term, prospective study utilizing WF SS-OCT/OCTA acquisition protocols is needed.

Conclusion

Overall, WF SS-OCTA was consistent with UWFFA in evaluating NPAs and NVs in DR. However, it still had limitations in terms of clinical value because of the area. PRP treatment could not change the PVD stages around the macula, optical disc, and NVs in patients with DR in the short term.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Tianjin Medical University Eye Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YG and LH were responsible for drafting the manuscript, as well as the acquisition, analysis and interpretation of data. LW collected and interpreted the data. YS and XL contributed to the conception and design of the current study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Overview of global publications on machine learning in diabetic retinopathy from 2011 to 2021: Bibliometric analysis

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Purpose: To comprehensively analyze and discuss the publications on machine learning (ML) in diabetic retinopathy (DR) following a bibliometric approach.

Methods: The global publications on ML in DR from 2011 to 2021 were retrieved from the Web of Science Core Collection (WoSCC) database. We analyzed the publication and citation trend over time and identified highly-cited articles, prolific countries, institutions, journals and the most relevant research domains. VOSviewer and Wordcloud are used to visualize the mainstream research topics and evolution of subtopics in the form of co-occurrence maps of keywords.

Results: By analyzing a total of 1147 relevant publications, this study found a rapid increase in the number of annual publications, with an average growth rate of 42.68%. India and China were the most productive countries. *IEEE Access* was the most productive journal in this field. In addition, some notable common points were found in the highly-cited articles. The keywords analysis showed that "diabetic retinopathy", "classification", and "fundus images" were the most frequent keywords for the entire period, as automatic diagnosis of DR was always the mainstream topic in the relevant field. The evolution of keywords highlighted some breakthroughs, including "deep learning" and "optical coherence tomography", indicating the advance in technologies and changes in the research attention.

Conclusions: As new research topics have emerged and evolved, studies are becoming increasingly diverse and extensive. Multiple modalities of medical data, new ML techniques and constantly optimized algorithms are the future trends in this multidisciplinary field.

KEYWORDS

machine learning, diabetic retinopathy, global publication trend, topic analysis, bibliometric analysis

Introduction

Diabetic retinopathy (DR), as one of the characterized microvascular complications of diabetes mellitus, has already become the leading cause of vision loss in the worldwide working-age population (1). Most patients with early-stage DR appear normal without any visual disruptions, however, the potential pathological changes, such as microvascular damage and neurodegeneration, are progressing (2, 3). Severe DR can cause visual impairment and finally lead to irreversible blindness, seriously affecting the quality of life. To prevent or manage DR, screening, early detection and intervention are crucial (4). In clinical practice, fundus examinations are recommended during the process of screening, diagnosis and follow-up of DR. The mainstream examinations include digital retinal photography, optical coherence tomography (invasive technologies such as fundus fluorescein photography are less common), etc. (1, 5, 6). Ophthalmologists can diagnose DR based on the typical lesions (e.g., exudates, microaneurysms) that appeared in the digital images (7). In addition to forming the basis of clinical diagnosis, the massive medical data from examinations has significant value for academic research.

With the development of artificial intelligence (AI) technologies, machine learning (ML), as an advanced field of AI, has gradually intertwined with various aspects of modern medicine (8). Machine learning focuses on enabling computers to automatically learn from the data of different modalities without being explicitly programmed (9). ML is a general name including many technological terms, such as deep learning (DL), supervised learning or neural networks. The implementation of ML in medicine is usually related to disease detection, survival prediction and risk evaluation, and so on (10–12). When compared to other medical specialties, ophthalmology features a wide application of imaging techniques with abundant data resources and an urgent need for computer-aided diagnosis due to the shortage of ophthalmologists (13). This leads to the emergence and rapid development of ML in ophthalmology. DR is one of the widely researched diseases in this field because of its increasing prevalence and the high risk of blindness in severe cases. Automatic DR grading/identification, automatic DR lesion detection and other related achievements have been reported in various conferences or journal articles (14, 15). Moreover, a number of review articles discuss the overall development of ML techniques in DR (4, 16). Thus far, however, no bibliometric analysis has been conducted on this topic.

The bibliometric analysis uses mathematical and statistical methodologies to obtain quantifiable and objective data from intangible features of the literature (17, 18). It has been applied in numerous topics and disciplines. To our knowledge, this is the bibliometric study focused on the literature related to ML in DR. To search for as much relevant literature as possible, we prepared a keyword list based on related books and articles. However, the search based on these keywords leads to the retrieval of documents with diversified purposes, study design and topics, or some irrelevant records. Thereby we generated the inclusion criteria and manually screened all the retrieved documents to confirm that included articles focused on ML in DR. Moreover, as the topics of included documents are relatively diverse and impractical to summarize one by one, we utilized VOSviewer and Wordcloud to visualize these topics in the form of co-occurrence maps of keywords. In addition, we also interpret our results based on the overall progress of ML techniques and the status of DR during 2011-2021 to make our analysis reasonable.

This paper has three objectives: first, to summarize the publication trend and identify the outstanding achievements; second, to reveal the contributions of countries/institutions/journals and visualize the collaboration networks; third, to uncover the mainstream topic and study the evolution of subtopics in this area.

Methods

Search strategy

All of the reference data used in this study were collected from the Web of Science Core Collection (WoSCC), which incorporates articles in over 20,000 high-quality peer-reviewed scholarly journals published worldwide in addition to a large number of proceedings papers (a single set of 28 criteria was made to evaluate journals). To search for the relevant data, a set of DR-related keywords and a set of ML-related keywords were prepared based on relevant literature and books (1, 19). Specific keywords were shown in Table 1. We searched for documents containing at least one DR-related keyword and one ML-related keyword in the "topic" of records (including title, abstract, author and keywords), for example, the documents that include both "diabetic retinopathy" and "machine learning". As many state-of-the-art achievements in computer science involving machine learning technologies would publish in conference proceedings besides journal articles, the scope of document types included journal articles, proceedings papers and reviews. The timespan was from 2011 to 2021. The last search was conducted on September 24, 2021. A total of 2960 retrieved documents from the WoSCC were prepared for the following screening (Figure 1).

Screening strategy

As we searched based on a relatively big set of keywords, some irrelevant documents may also be retrieved. Three authors (A.S., K.J. and L.L.) made the inclusion criteria by reviewing the first 500 documents (primary screening). The practical inclusion

TABLE 1 List of the search keywords.

Related to	Sample search keywords in the publication topic
Diabetic retinopathy	"diabetic retinopathy", "diabetic macular edema", "exudates", "intraretinal microvascular abnormality", "microaneurysm", "neovascularization".
Machine learning	"machine learning", "deep learning", "supervised learning", "unsupervised learning", "adversarial learning", "classification", "neural networks", "predictive model", "random forest", "decision trees", "pattern mining", "support vector machine", "multitask learning", "probabilistic graphical model", "association rules".

criteria: 1. Involves ML technologies. 2. Involves DR, including: (1) Studies focused on DR; (2) Studies focused on the characterized clinical features of DR, for example, microaneurysms and exudates detection (20); (3) Studies focused on multiple diseases and DR was included (21); (4) Studies focused on a topic that is beneficial to the various clinical scenarios of DR, for example, blood-vessel segmentation in the fundus images is beneficial for the following diagnosis of DR (22). After carefully reviewing the 2960 retrieved documents (secondary screening by A.S. and K.J.), 1147 documents were included for the bibliometric analysis (Figure 1).

Bibliometric analysis

Bibliometric analysis was conducted on 1147 documents for obtaining insights into the current trends and topics on ML for DR. In this study, we conducted a trend analysis of publications and citations, publication pattern and collaboration analysis, research domains and targeted sources analysis, as well as the analysis of the keywords.

The analytic tool of the WoSCC database, and Microsoft Excel were used for data mining and representation. The summarized data included publication count and citation count of years/countries/institutions/journals/research domains. Self-citations were included. The Hirsch-index (H-index) was used originally to reflect the academic impact of a researcher, which describes that a researcher has published h number of articles, and each of the h articles has at least h times of citations (23, 24). Currently, the H-index is commonly used for assessing the academic influence of countries/institutions/journals in the bibliometric analysis (25). The growth rate of publications was calculated as follows:

$$Growth\ \ rate = \left(\sqrt[t2-t1]{p_2 \div p_1} - 1 \right) \times 100$$

where t_1: First year; t_2: Last year; p_1: Publication count of the first year; p_2: Publication count of the last year.

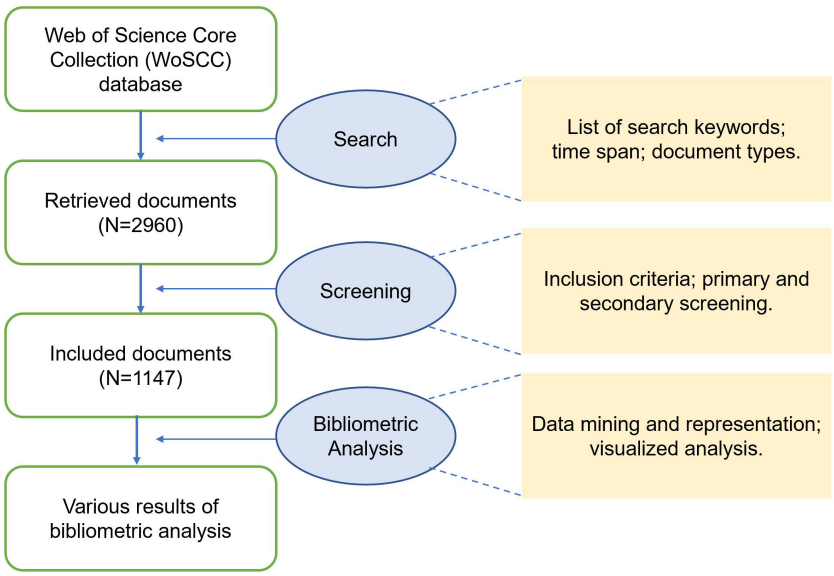

FIGURE 1
Flowchart of search, screening and bibliometric analysis.

Visualized analysis

VOSviewer and Wordcloud were also applied to visualize the collaboration of countries/institutions, co-occurrence of keywords and evolutions of hotspots in the target field. VOSviewer is an analytical tool for constructing and displaying bibliometric maps in an easy-to-interpret manner. To use VOSviewer, we first exported the entire record and cited references of included documents in plain text form, and then the data were imported into VOSviwer (version 1.6.17) (26). By adjusting the options of types and other parameters (type of analysis: co-occurrence; unit of analysis: author keywords; counting method: full counting; the size variation of items, labels and lines between two items were also adjusted for the best presentation), we generated the primary bibliometric maps. Based on the observation of these maps, we generated a text file of the thesaurus to avoid the appearance of synonyms (e.g., "automated detection" & "automatic detection") in the keyword map. At last, certain meaningless keywords (e.g., "level") were also deleted to generate the final diagram. To use Wordcloud, we loaded the Wordcloud Python package. The data of title, abstract and keywords were exported and stored as 5 text documents corresponding to 5 periods of time. The thesaurus file was also applied so that synonyms will be regarded as the same word/phrase. After deleting the meaningless characters in the files (e.g., "TI", the abbreviation before each title), we generated 5 diagrams, representing the 5 studied periods.

Results

Trend analysis of publications and citations

Figure 2 plots the annual trends of publications and citations on machine learning in diabetic retinopathy. We included 1147 articles for the analysis in this study (658 journal articles, 449 proceedings and 40 reviews). From 2017, the annual publication number exceeded 100, and the last 5 years (2017-2021) contributed 78.12% (896/1147) of all articles. The average growth rate from 2011 to 2020 was 42.68%. Polynomial regression analysis was conducted to model the publication and citation trends (2021 was excluded because of incomplete indexing). The estimated models of y1 = 2.6591x2 - 3.2742x + 15.433 and y2 = 102.63x2 - 670.7x + 911.78 indicate changes in the quantities of publications and citations with time, respectively. Both the results of the growth rate and polynomial regression model demonstrate the significant and rapid increase in publications and citations, indicating that machine learning in diabetic retinopathy keeps gaining researchers' attention and the field is generally at the growth phase. The detailed publication number of different article types and study designs(retrospective/prospective) during 2011-2021 were shown in Table 2.

Moreover, we listed the top 10 articles ranked by annual citation count in Table 3. Of these 10 articles, all were journals, and 6 articles were published in the last 5 years. The earliest article was by Wang et al. in 2015 (33), introducing a new retinal blood-vessel segmentation method that was beneficial to the screening of DR. The most impactful article was by Gulshan et al. in 2016 (14). They developed a deep learning algorithm to identify referable/non-referable DR and DME, which was a milestone in this field. The algorithm achieved fairly high performance with the area under the receiver operating curve above 0.99 in 2 publicly available datasets (EyePACS and Messidor-2). It is noteworthy that the medical device called IDx-DR mentioned in the pragmatic trial by Abramoff et al. is the first device authorized for marketing by the FDA to automatically detect DR based on fundus images without the need for the interpretation of an additional specialist. The year 2016 and 2017 witnessed 7 of the top 10 articles in this field. The

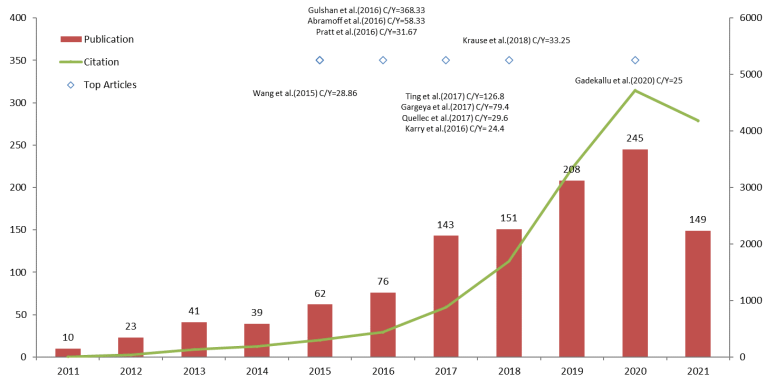

FIGURE 2
Trend analysis of publications and citations.

TABLE 2 The yearly publication count.

Year	Article type			Count	Study Design	
	Journals	Proceedings	Reviews		Retrospective	Prospective
2011	3	7	0	10	0	0
2012	12	10	1	23	2	0
2013	19	20	2	41	3	0
2014	22	17	0	39	3	0
2015	26	36	0	62	3	0
2016	26	47	3	76	7	1
2017	59	84	0	143	7	3
2018	73	75	3	151	18	3
2019	111	85	12	208	20	8
2020	181	51	13	245	38	16
2021	126	17	6	149	23	11
Total	658	449	40	1147	124	42

TABLE 3 Top articles ranked by annual citations and total citations.

References	Title	Year	C/Y	Source Title
Top 10 articles ranked by annual citations (C/Y)				
Gulshan et al. (14)	Development and Validation of a Deep Learning Algorithm for Detection of Diabetic Retinopathy in Retinal Fundus Photographs	2016	368.3	JAMA
Ting et al. (27)	Development and Validation of a Deep Learning System for Diabetic Retinopathy and Related Eye Diseases Using Retinal Images From Multiethnic Populations With Diabetes	2017	126.8	JAMA
Gargeya et al. (28)	Automated Identification of Diabetic Retinopathy Using Deep Learning	2017	79.4	Ophthalmology
Abramoff et al. (29)	Improved Automated Detection of Diabetic Retinopathy on a Publicly Available Dataset Through Integration of Deep Learning	2016	58.3	IOVS
Krause et al. (30)	Grader Variability and the Importance of Reference Standards for Evaluating Machine Learning Models for Diabetic Retinopathy	2018	33.3	Ophthalmology
Pratt et al. (31)	Convolutional Neural Networks for Diabetic Retinopathy	2016	31.7	Procedia Computer Science
Quellec et al. (32)	Deep image mining for diabetic retinopathy screening	2017	29.6	Medical Image Analysis
Wang et al. (33)	Hierarchical retinal blood vessel segmentation based on feature and ensemble learning	2015	28.9	Neurocomputing
Gadekallu et al. (34)	Early Detection of Diabetic Retinopathy Using PCA-Firefly Based Deep Learning Model	2020	25	Electronics (Switzerland)
Karri et al. (35)	Transfer learning based classification of optical coherence tomography images with diabetic macular edema and dry age-related macular degeneration	2017	24.4	Biomedical Optics Express
References	Title	Year	TC	Source Title
Top 10 articles ranked by total citations (TC)				

(Continued)

TABLE 3 Continued

References	Title	Year	C/Y	Source Title
Gulshan et al. (14)	Development and Validation of a Deep Learning Algorithm for Detection of Diabetic Retinopathy in Retinal Fundus Photographs	2016	2210	JAMA
Ting et al. (27)	Development and Validation of a Deep Learning System for Diabetic Retinopathy and Related Eye Diseases Using Retinal Images From Multiethnic Populations With Diabetes	2017	634	JAMA
Gargeya et al. (28)	Automated Identification of Diabetic Retinopathy Using Deep Learning	2017	397	Ophthalmology
Abramoff et al. (29)	Improved Automated Detection of Diabetic Retinopathy on a Publicly Available Dataset Through Integration of Deep Learning	2016	350	IOVS
Mookiah et al. (36)	Computer-aided diagnosis of diabetic retinopathy: A review	2013	217	Computers in Biology and Medicine
Wang et al. (33)	Hierarchical retinal blood vessel segmentation based on feature and ensemble learning	2015	202	Neurocomputing
Pratt et al. (31)	Convolutional Neural Networks for Diabetic Retinopathy	2016	190	Procedia Computer Science
Antal et al. (37)	An Ensemble-Based System for Microaneurysm Detection and Diabetic Retinopathy Grading	2012	171	IEEE Transactions on Biomedical Engineering
Srinivasan et al. (38)	Fully automated detection of diabetic macular edema and dry age-related macular degeneration from optical coherence tomography images	2014	168	Biomedical Optics Express
Zhang et al. (39)	Exudate detection in color retinal images for mass screening of diabetic retinopathy	2014	151	Medical Image Analysis

top 10 articles ranked by total citation count were also listed in Table 3. Three journals and one review published before 2015 were newly on the list.

Publication pattern and collaboration analysis

Overall, 58 countries contributed to the publications on this topic. The top 10 countries ranked by total publication output accounted for 92.50% (1061/1147) of all included studies and were listed in Table 4. India published the most documents (350/1147), accounting for 30.51% of all included studies. China was the second leading country (222/1147, 19.35%), followed by the USA (161/1147, 14.04%). It is worth noting that the USA ranked 1st in terms of citation count while it ranked 3rd in the publication count, and the citation ranks of Singapore, Malaysia, and Australia were also higher than their publication ranks. Institutions with at least 15 documents were also listed in Table 4 ranked by total publications. National University of Singapore is the most prolific institution (26/1147, 2.27%), followed by National University of Sciences Technology, Pakistan (25/1147, 2.18%) and Singapore National Eye Center (24/1147, 2.09%). However, the publication of reviews cannot directly indicate the active research of a certain institution. Therefore, we also calculate the number of publications except for the reviews. National University of Sciences Technology was the most active institution in research (23/1147, 2.01%), followed by Sun Yat-Sen University (22/1147, 1.92%). There were 3 institutions from Singapore with the highest overall H-index (11, 14, 14). Three Chinese institutions, 2 Indian institutions, 2 American institutions and 2 Pakistan institutions were listed in Table 4. Figure 3 demonstrates the collaboration networks of countries (documents ≥5, 36 countries were included) and institutions (documents ≥5, 65 institutions met the criteria, 16 institutions had no connections to other institutions and were excluded, hence 49 institutions were included).

Research domains and targeted sources

Table 5 shows the 10 most common research domains that the included documents belong to. Computer Science (537/1147, 46.82%), Engineering (527/1147, 45.95%) and Radiology Nuclear Medicine Medical Imaging (128/1147, 11.16%) were the 3 main research domains.

Journals with H-index ≥5 and publications ≥10 were listed in Table 6, ranked by publication count. We also referred to the Journal Citation Reports (JCR)(2020) to demonstrate the academic impact of these journals. *IEEE Access* was the journal with the most articles published (36, 3.14%) while *IEEE Transactions on Medical Imaging* was the most impactful among all included journals at the time of analysis. The top 12 journals, which only accounted for 5.31% of 226 journals that have published articles in this field, published 29.23% of all

TABLE 4 Top countries and institutions ranked by publication count.

Country	Documents	%	Citations (Rank)	H-index	Article type		
					Journals	Proceedings	Reviews
India	350	30.57	4183(2)	25	185	159	6
China	222	19.39	2732(3)	26	162	55	5
USA	161	14.06	6393(1)	31	101	55	5
Pakistan	66	5.76	936(7)	17	43	19	4
England	58	5.07	1610(5)	17	40	13	5
Singapore	49	4.28	1909(4)	20	34	9	6
Canada	42	3.67	435(9)	12	22	19	1
Malaysia	40	3.49	1102(6)	18	25	13	2
Australia	37	3.23	663(8)	16	23	12	2
Saudi Arabia	36	3.14	358(10)	12	31	4	1
Institution (Country)	Documents	%	Citations	H-index	Article type		
					Journals	Proceedings	Reviews
National University of Singapore (Singapore)	26	2.27	1075	14	14	7	5
National University of Sciences Technology Pakistan (Pakistan)	25	2.18	599	12	13	10	2
Singapore National Eye Center (Singapore)	24	2.09	1082	14	17	4	3
Sun Yat-Sen University (China)	22	1.92	899	8	18	4	0
Indian Institute of Technology System (IIT System) (India)	20	1.74	218	6	11	9	0
Northeastern University China (China)	20	1.74	209	7	12	8	0
COMSATS University Islamabad (CUI) (Pakistan)	19	1.66	224	9	13	2	4
National Institute of Technology (Nit System) (India)	18	1.57	76	4	9	9	0
Chinese Academy of Sciences (China)	17	1.48	175	5	13	4	0
Ngee Ann Polytech (Singapore)	16	1.4	777	11	14	0	2
Mansoura University (USA)	15	1.31	118	4	9	6	0
University of Louisville (USA)	15	1.31	148	6	8	7	0

journal articles and reviews (204/689). Seven journals ranked "Q1" in JCR, two journals ranked "Q2" and one journal ranked "Q3". As for the conference documents, only the *International Conference on Medical Imaging Computer-Aided Diagnosis* published more than 5 documents (7/1147, 0.61%).

Keywords analysis

To obtain a deeper understanding of research topics and how they are interconnected, we visualized the hotspots of included studies by conducting a keyword co-occurrence analysis using VOSviewer (Figure 4). For the total of 2088 automatically identified keywords, 84 keywords occurred at least 10 times, which were shown in Figure 4. This map of keywords illustrates the hotspots related to machine learning in diabetic retinopathy. All included keywords were divided into 3 clusters, indicated by red, green and blue colors, representing the ML techniques (e.g., "deep learning", "convolutional neural networks", etc.), applications of ML techniques (e.g., "classification", "segmentation", etc.) and the DR-related diseases, clinical features and medical data (e.g., "microaneurysms", "fundus images", etc.), respectively. From Figure 4, we can identify the hot topics represented by strongly linked keywords and the weakly-explored subareas between 2 relatively isolated keywords.

To understand when these hotspots emerged and how they evolved, we divided the documents into 5 groups by publication time:1.2014-2015; 2.2016-2017; 3.2018-2019; 4.2020-2021;

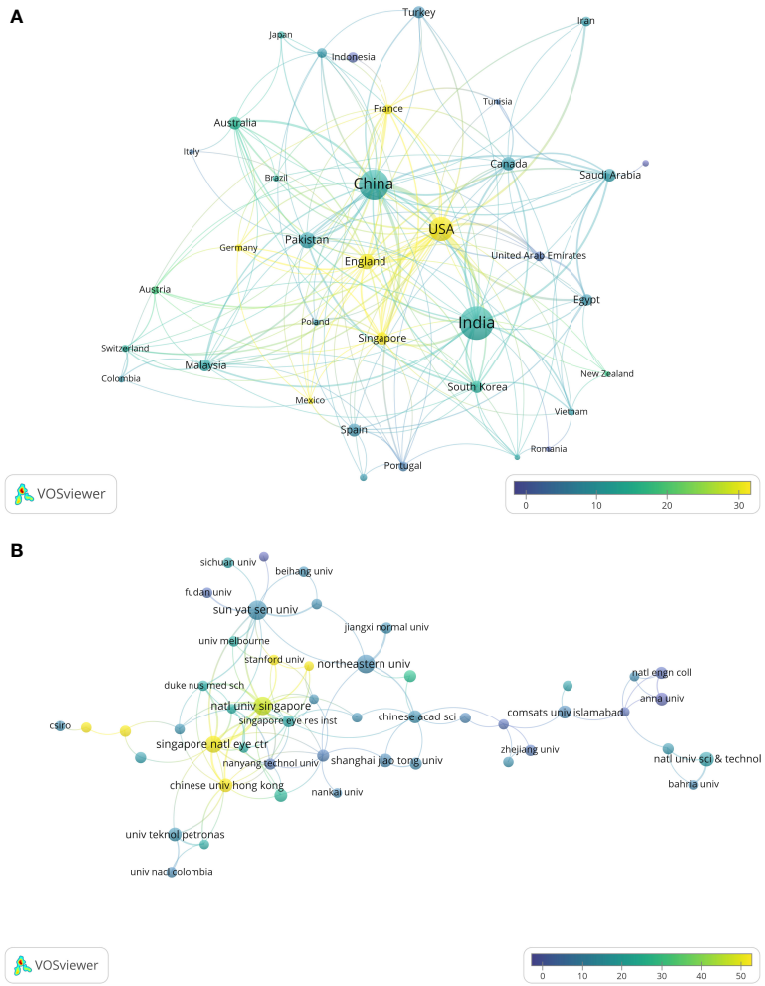

FIGURE 3
Collaboration maps between countries and institutions.**(A)** Highly contributed countries. **(B)** Highly contributed institutions. Circle size represents the publication count; circle color represents average citations; links represent the collaboration.

5.2011-2013 (the only 3-year group, considering that in the first 3 years, the academic output was relatively small when compared with other periods). Five corresponding maps of keywords were conducted by Wordcloud (Figure 5). Each map includes the top 30 keywords ranked by the frequency of occurrence. The size of the font represents the frequency (the more frequently-occurred, the bigger scale). "Diabetic retinopathy" was the most dominant keyword for the entire period. Other frequent keywords included "classification", "fundus image", "deep learning", indicating that most studies in this field focused on applying the classification ability of ML techniques into DR based on the medical images. Figure 5A displays the top keywords identified during 2011-2013, where the dominant keywords besides "diabetic retinopathy" were "microaneurysm", "exudate", and "blood vessel" (ranked 1st to 15th, red color), whereas "neural network", "diagnosis" and "database" were less dominant (ranked 16th to 30th, green color). In 2014-2015, "detection", "segmentation", and "support vector machine" were more dominant, while "blindness", "image processing", and "vessel segmentation" were less dominant (Figure 5B). In 2016-2017, "detection", "neural network" and "diabetic macular edema" were more dominant; "deep learning", "convolutional neural network", and "support vector machine" were less dominant (Figure 5C). In 2018-2019, "deep learning", "optical coherence tomography" and "dataset" were more dominant; "exudate", "microaneurysm" and "grading" were less dominant (Figure 5D). In 2020-2021, "deep learning", "convolutional neural network" and "grading" were more dominant; "lesion", "blood vessel" and "exudate" were less dominant (Figure 5E). The detailed frequency of keywords was listed in Supplementary Table 1.

TABLE 5 The most related research domains ranked by publication count.

Research Domain (WoS categories)	Count[a]	%
Computer Science	537	46.82
Engineering	527	45.95
Radiology Nuclear Medicine Medical Imaging	128	11.16
Telecommunications	116	10.11
Medical Informatics	97	8.46
Imaging Science Photographic Technology	93	8.11
Ophthalmology	85	7.41
Mathematical Computational Biology	71	6.19
Optics	70	6.10
Science Technology Other Topics	47	4.10
[a] Some documents belong to multiple research areas.		

TABLE 6 Most productive journals ranked by publication count.

Source Title	Count	Citations	H-index	%	Journal Citation Reports 2020 Impact	Rank
IEEE ACCESS	36	399	11	3.14	3.367	Q2
LECTURE NOTES IN COMPUTER SCIENCE	32	196	7	2.79		
COMPUTER METHODS AND PROGRAMS IN BIOMEDICINE	19	652	13	1.66	5.428	Q1
COMPUTERS IN BIOLOGY AND MEDICINE	18	750	14	1.57	4.589	Q1
TRANSLATIONAL VISION SCIENCE TECHNOLOGY	16	110	6	1.40		
JOURNAL OF MEDICAL SYSTEMS	15	411	9	1.31	4.460	Q1
BIOMEDICAL OPTICS EXPRESS	14	599	9	1.22	3.372	Q2
PLOS ONE	13	356	9	1.13	3.240	Q1
IET IMAGE PROCESSING	11	99	5	0.96	2.373	Q3
COMPUTERIZED MEDICAL IMAGING AND GRAPHICS	10	373	9	0.87	4.790	Q1
ARTIFICIAL INTELLIGENCE IN MEDICINE	10	191	8	0.87	5.326	Q1
IEEE TRANSACTIONS ON MEDICAL IMAGING	10	403	8	0.87	10.048	Q1

Discussion

Trend analysis of publications and citations

From 2011 to 2020, the number of publications grew from 10 to 245 and the overall growth rate reached 42.68%, indicating significant growth in research interests in this field. In addition, the rapid expansion of the annual citations reflected the increasing impact of related publications. On the one hand, this growing trend is due to the breakthroughs in AI technology and its wide application in medicine: in 2012, a well-trained deep convolutional neural network won the ImageNet challenge (40); in 2014, the generative adversarial network was invented (41). As a subarea of ML, DL was gradually applied to various domains of medicine, including radiology, pathology, dermatology, ophthalmology, and so on (42). On the other hand, multiple public ophthalmic datasets were set up around 2010, which accelerated the development of relevant research. For example, Kaggle EyePACS (2015) consists of over 80000 annotated fundus images with DR staging; the Messidor dataset (2008) consists of 1200 fundus images accompanied with medical diagnosis. These public large-scale datasets have created a great opportunity for academic groups worldwide to test and benchmark their models/

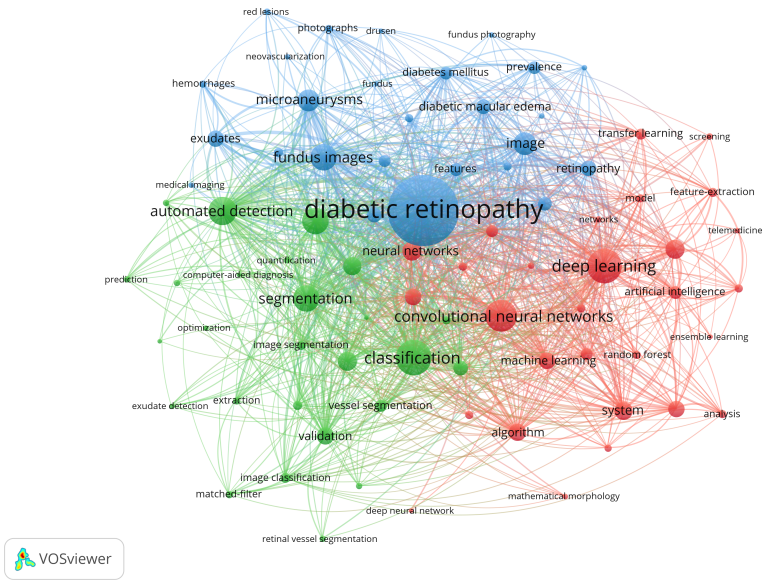

FIGURE 4
The co-occurrence map of keywords; reveals 3 clusters (in 3 colors): ML techniques; applications of ML techniques; relevant diseases, clinical features and medical data. Circle size represents the frequency of occurrence; links represent the co-occurrence.

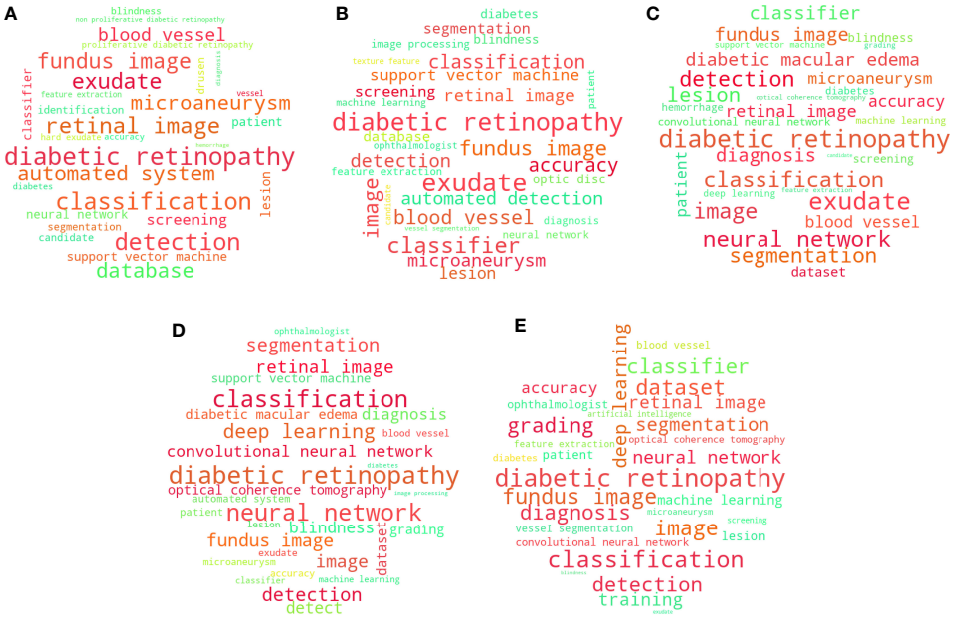

FIGURE 5
Cloud maps of keywords in 5 periods (the top 30 most frequently-occurred keywords in each map, red keywords are more dominant, green keywords are less dominant): **(A)** during periods 2011-2013; **(B)** during periods 2014-2015; **(C)** during periods 2016-2017; **(D)** during periods 2018-2019; **(E)** during periods 2020-2021.

systems/algorithms. Furthermore, the establishment of recognized DR grading standards (e.g., ICDRSS scale) (7) also promoted the comparison of diagnostic ability between different models or between man and machine. In general, there is still a distance between the current ML in DR and the clinical practice as most studies are in silico and aim to optimize algorithms and propose new techniques based on recognized public datasets or local private datasets. The prospective studies in this field mainly focus on the real-world viability test, clinical validation of algorithms/software and human-machine comparison (43–45). However, as machine learning becomes mature in this area, the number and proportion of real-world-oriented studies are increasing.

Table 3 shows that the most impactful articles were published after 2015. After Gulshan et al. published the most impactful in 2016 and received widespread attention from ophthalmic researchers, many DL-based studies have sprung up, which is also consistent with the publication trend and the development of technologies and databases. Some common points of impactful articles were found out: 1. Published by influential journals (e.g. JAMA - IF:56.27; Ophthalmology, IOVS – the top journals of ophthalmology); 2. New techniques (e.g. deep learning in 2016, 2017); 3. Excellent results (e.g., great performance of algorithms with an area under the receiver operating curve > 0.99); 4. Involved in multiple tasks (e.g. automatic grading of DR severity or detection of multiple diseases including DR). These articles led the developing trend in this field and many articles were based on these achievements.

Publication pattern and collaboration analysis

Researchers all over the world have contributed to the field of ML in DR. The publication pattern reveals that India and China have been the most productive countries. The two densely populated developing countries contributed to nearly half of the relevant publications, which is uncommon in other bibliometric studies on the topic of AI technologies in medicine (17, 25), as developed countries such as the USA or England are usually the main force. In addition, there are 5 developing countries in the top 10 countries ranked by publication count, all with considerable academic output. However, in terms of the H-index and citations of different countries, developed countries performed relatively better compared to developing countries. This can be explained by differences in social medical resources and technologies between countries. With the global epidemic of diabetes, the prevalence of DR is also rising predominantly, especially in densely populated countries like India and China (46, 47). There is a clear but unmet need to comprehensively screen DR in the diabetic population in the rural area of these developing countries due to the disproportionally low ophthalmic population (13). Developing countries are urgently calling for a cost-effective way to manage DR. Therefore, the automatic system based on ML is widely explored by academic groups from developing countries. As for developed countries, institutions and researchers benefit from technological breakthroughs and the mature ophthalmic system. Researchers are more likely to publish impactful articles. The National University of Singapore is the most productive institution and most publications also belong to Singapore National Eye Center. The two institutions tend to publish articles that push forward the clinical application of ML techniques in DR, including the clinical validation of DL systems based on the Singapore National Diabetic Retinopathy Screening Program or other multiethnic DR screening data and reviews that discussed the current status of AI techniques in the real-word DR screening (48, 49). By analyzing the top institutions (documents ≥ 20), we found that most studies from National University of Sciences Technology Pakistan, Indian Institute of Technology System and Northeastern University China are ML technique-oriented. Most studies from two Singaporean institutions are medicine-oriented. Researchers from Sun Yat-Sen University published both technique-based studies and clinical validation studies as they collaborated a lot with hospitals and computer science laboratories. From the perspective of citations, those medicine-oriented and pragmatic studies are more popular than technique-oriented studies.

The collaboration analysis also revealed that productive countries/institutions have more options for international collaborations. In addition, the nodes in the middle of Figure 3A tend to appear yellow, indicating that countries/institutions with more external collaborations have a greater chance of publishing impactful articles (i.e., high average citations).

Research domains and targeted sources

As included documents are mainly related to computer techniques and imaging systems, journals that specialized in these domains were productive in this field. On the one hand, the advancement of computer science and engineering accelerated the pace of applying AI technologies in medicine. On the other hand, imaging techniques are commonly used in ophthalmology and produce lots of valuable data on DR patients, which is useful for developing ML algorithms. The impact factor (mostly around five) and the JCR rank of the twelve "core journals" indicate the overall impact and quality of relevant publications. Only *Computers in Biology and Medicine* and *Biomedical Optics Express* have published impactful articles, as shown in Table 3. Impactful journals such as *JAMA* and *Ophthalmology* are not shown due to the publication count.

Keywords analysis

The frequently occurred keywords in the literature always indicate the research hotspots. The co-occurrence of several keywords represents the widely discussed topic containing several basic components. By dividing the relevant literature by time, the emergence and evolution of keywords can be visualized on the word clouds. Keywords analysis reveals the mainstream topics in the field, the research focuses on different periods and the subareas that are currently popular or remained to be explored.

Overall, the application of machine learning techniques in diabetic retinopathy is extensive and diverse, while most documents aim to diagnose DR automatically. "Diabetic retinopathy" is the most dominant keyword for the whole period, along with other frequent keywords such as "classification", "segmentation" and "fundus images". Thus, fundus images are the most commonly used data for research. Classification and segmentation are the tasks for ML or the processing steps for the data. Some keywords relating to DR lesions (e.g., "microaneurysms", "exudates") are also dominant in Figure 5, as many documents focus on detecting characterized lesions of DR to mimic the diagnostic process of ophthalmologists. A tiny microaneurysm can be the key to distinguishing between diseases and normality, thereby the automatic detection of these lesions makes the diagnosis of ML algorithms reasonable (20). Some keywords of ML techniques were prominent in the keywords co-occurrence analysis (e.g., "deep learning", "support vector machine", "convolutional neural network"), representing the popular tool applied in DR. By linking up the keywords, the mainstream concepts are immediately visible, for example, the diagnosis of "diabetic retinopathy" based on the "automated detection" of "exudates" in "retinal images" by "deep learning".

However, both techniques and clinical focuses change over time. From 2011 to 2021, the evolution of topics mainly focused on computer methods, clinical tasks and data modalities. First, "deep learning" and "convolutional neural network" appeared in 2016-2017 for the first time and subsequently became larger in the word cloud, indicating that deep learning and related techniques gained increased research attention, which was consistent with the publication time of the paper by Gulshan et al. and the overall development of DL techniques. The traditional technique "support vector machine" became less popular in this field due to the remarkable performance of DL in feature extraction and representation. Second, the keywords of DR features (e.g., exudate, microaneurysm) became less frequent, indicating that simple lesion-detection algorithms were gradually dismissed. Many comprehensive DR grading systems and multi-disease diagnosis systems have sprung up recently as the keyword "grading" gradually become frequent (21). Third, due to the limited information offered by digital fundus images, the data from new imaging techniques such as optical coherence tomography, gradually emerged in this field (Figures 5C–E). Other imaging techniques like fundus fluorescein angiography were also considered but not shown in the word cloud, which needs to be further studied (50). Moreover, the ML algorithms usually focused on the simple data modality while doctors would refer to different types of examination data and complaints of patients. As the keyword "dataset" and "database" has become much more dominant from 2011 to 2021, the integration of multi-modal data from different sources might be the future direction for automated diagnosis. In addition, we found that although the keyword "patient" was less prominent from 2011 to 2021, the frequency rank kept rising. From a clinical point of view, patients are always the main components of all relevant studies. With the ML techniques in DR getting matured, more researchers designed studies that better reflect the real-world effectiveness of AI systems. These studies not only included the existing datasets but also test their algorithm/software in broader patient groups. To utilize AI as tools in real clinical settings, the algorithms in this field are constantly optimized in both the techniques (from "support vector machine" to "deep learning") and the capacity of dealing with more complex conditions which mimic the clinical settings (e.g., grading DR based on multi-modal data).

This study is the first bibliometric analysis of ML in DR and aims to provide a holistic view of the relevant research. The results discussed in this study are objective, quantifiable and macroscopical, which would be suitable for any researchers interested in this field to get familiar with the basic knowledge structure (e.g., the mainstream topic, the outstanding achievements, the emerging trends, global publication pattern, and relevant research domains, etc.) and can help them find potential collaborators and develop relevant studies. Moreover, the change in publication trends and keywords from 2011 to 2021 indicated the potential directions of further studies in this field, including the incorporation of optimized ML techniques, multi-modal data, real-world-oriented study design, etc.

Limitations

This study has some limitations. First, we only used reference data from a single database (WoSCC) and the results of the bibliometric analysis may not be as robust as studies that collect data from multiple databases due to some unpredictable bias when we search for documents in WoSCC. However, WoSCC is a well-indexed database that represents one of the largest multidisciplinary collections of indexed published literature. Moreover, the list of keywords may not be comprehensive enough to retrieve all related documents even if we referred to the relevant literature and books. Second, although some meaningless keywords were deleted in the figure conducted by

VOSviewer and Wordcloud, not all keywords are informative enough in the figure, such as "system". These general keywords occur frequently but do not refer to any deeper subfields, therefore, these keywords cannot be analyzed. Finally, like other bibliometric analyses, this study didn't focus on the content of every single article; the uniqueness and novelty of most articles were ignored and only top articles were analyzed. Third, the emerging novel topics discussed in this study may have stagnations to practice as the co-occurrence maps of keywords are based on frequency. A breakthrough was reflected on these maps only when it gradually became recognized in the research community and also it takes time for researchers to cite these articles (to be listed as top articles in the bibliometric analysis).

Conclusions

In this study, we provided a comprehensive overview of all retrieved articles on ML in DR following a bibliometric approach for the first time. It's a growing research area and has been studied by researchers from multiple countries and institutes. As new topics have emerged and evolved since 2011, studies in this field are becoming more diverse and extensive. Real-world-oriented studies with multi-modal data and optimized ML techniques are the further directions as clinical application is the ultimate goal in this field. Further studies can focus on larger research fields (e.g., AI techniques in ophthalmology) and the integration of data from multiple databases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

Design of the work: AS, KJ, WZ and JY. Collection and screening of the data for the work: AS, KJ and LL. Analysis of the data: YL. Drafting the work: AS. Revising of the manuscript: KJ, LL, WZ and JY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1032144/full#supplementary-material

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Mechanistic investigations of diabetic ocular surface diseases

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With the global prevalence of diabetes mellitus over recent decades, more patients suffered from various diabetic complications, including diabetic ocular surface diseases that may seriously affect the quality of life and even vision sight. The major diabetic ocular surface diseases include diabetic keratopathy and dry eye. Diabetic keratopathy is characterized with the delayed corneal epithelial wound healing, reduced corneal nerve density, decreased corneal sensation and feeling of burning or dryness. Diabetic dry eye is manifested as the reduction of tear secretion accompanied with the ocular discomfort. The early clinical symptoms include dry eye and corneal nerve degeneration, suggesting the early diagnosis should be focused on the examination of confocal microscopy and dry eye symptoms. The pathogenesis of diabetic keratopathy involves the accumulation of advanced glycation end-products, impaired neurotrophic innervations and limbal stem cell function, and dysregulated growth factor signaling, and inflammation alterations. Diabetic dry eye may be associated with the abnormal mitochondrial metabolism of lacrimal gland caused by the overactivation of sympathetic nervous system. Considering the important roles of the dense innervations in the homeostatic maintenance of cornea and lacrimal gland, further studies on the neuroepithelial and neuroimmune interactions will reveal the predominant pathogenic mechanisms and develop the targeting intervention strategies of diabetic ocular surface complications.

KEYWORDS

diabetic keratopathy, dry eye, neuropathy, epitheliopathy, lacrimal gland, pathogenesis

Introduction

Diabetes mellitus (DM) is an endemic disease that occurs all over the world, imposing extensive health burden on society (1, 2). Diabetics with prolonged periods of hyperglycemia suffer from numerous complications affecting almost every organ system, including the ocular tissues (3, 4). DM-related ocular complications are the leading cause of blindness, especially in developed countries. Although diabetic

retinopathy is the most common and well-known ophthalmic complication, DM also has profound clinically relevant effects on the ocular surface (5, 6).

The corneal tissue composes five stratified layers: the epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelium (7, 8). Corneal epithelium is the cornea's outermost layer, whose integrity is essential to maintaining healthy vision. Corneal stroma, which is populated by keratocytes, represents almost 90% of the thickness of the cornea. Corneal endothelium, a single cell layer between the corneal stroma and anterior chamber, exhibits barrier and 'pump' functions to maintain corneal dehydration. In addtition, to maintain a healthy ocular surface, the lacrimal gland and meibomian glands produce tears and lipids to prevent excessive evaporation of the tear film. Dysfunctions of these glands will cause dry eye disease (9, 10).

Although the structure of the ocular surface is relatively uncomplicated, problems with either component may have serious consequences. For DM-related ocular complications, various primary pathological manifestations occur, such as decreased corneal sensitivity, delayed epithelialization after corneal abrasions, basement membrane abnormality, corneal neuropathy, and endothelial decompensation (11, 12). Generally, these changes are referred to as diabetic keratopathy or diabetic neurotrophic keratopathy. Another common diabetic complication in the ocular surface is dry eye, with the involvement of lacrimal functional unit dysfunction (LFUD) (13). These complications drastically influence on the quality of life of patients and are frequently underdiagnosed and underestimated.

Current therapies for DK mainly include topical lubricants, antibiotic ointments, patching, bandage soft contact lenses, and corneal transplantation (14). Nevertheless, these treatments are usually incurable for serious DK, even if in combination. For the treatment of dry eye, identifying effective therapeutics remains an urgent challenge. Thus, research on novel drug targets is vital to the prevention and treatment for diabetic complications on the ocular surface.

Herein, we review recent advances in the pathogenesis of diabetic keratopathy and dry eye. We also evaluated the progress in diagnosis and treatment. These novel findings will shed new light on potential intervention strategies for diabetic ocular surface complications.

Diabetic keratopathy

Diabetic keratopathy is the most common clinical disease in which diabetes affects the ocular surface. It is a potential vision threatening disease, mainly including epitheliopathy, neuropathy and endotheliopathy.

Diabetic corneal epitheliopathy

The corneal epithelium consists of 5-7 layers of non-keratinized stratified squamous epithelium, which plays a key role in maintaining corneal transparency and stability. Because the cornea has no blood vessels, and the level of tear glucose level is far less than that of aqueous humor and serum in diabetic patients (15, 16), it is believed that the glucose in corneal epithelial cells is mainly transported from aqueous humor (17). The level of glycosylation in the corneas of diabetic patients increased significantly (18), and the accumulation of glycogen granules was observed in diabetic corneal epithelial cells (19). In diabetic patients, corneal epithelial cells are exposed to persistent high levels of glucose, resulting in various clinical epithelial abnormalities.

Several studies have found that corneal epithelium in diabetic patients tends to have increased fragility, lower cell density, thinner thickness and reduced barrier function (20–22). An electron-microscopic examination of corneal epithelium showed an increased epithelial fragility in specimens of diabetic patients (23). Saini and Khandalavla measured the corneal epithelial fragility of healthy people and diabetic patients using an esthesiometer (20). The results revealed that the average corneal epithelial fragility of diabetic patients was significantly higher than that of healthy people, and that the epithelial fragility of diabetic retinopathy patients increased more significantly. Increased corneal epithelial fragility was also found in Goto Kakizaki rats with type 2 DM (24). A few studies reported that there was no statistical significance in the reduction of corneal basal epithelial cell density in diabetic patients (25, 26). However, more clinical studies have demonstrated that the density of corneal basal epithelial cells was significantly reduced in type 1 and type 2 diabetic patients (21, 27–29), which may be related to the reduction of corneal innervation, impaired of basement membrane and higher turnover rate (21). In the diabetic patients, the mean corneal epithelium thickness was thinner (22, 30) which is associated with the stage of the disease. Similarly, Cai et al. verified the characteristics of the thinning of corneal epithelium and the decreasing density of basal epithelial cells in the rodent model of type 1 diabetes induced by streptozotocin (31). The changes of corneal epithelial density and thickness reflect the imbalance between cell proliferation, differentiation, migration and death. The corneal epithelium has a strong barrier function, making it the first line of defense for the eyeball to resist the external environment. It has long been found that the barrier function of diabetic corneal epithelium is weakened (32–34) which is related to the increase of glycosylated hemoglobin level (34), and correspondingly, diabetic corneas are more prone to infection than healthy people (35–39). *In vitro* studies have proven that high glucose exposure leads to the impairment of the human

corneal epithelial cell barrier function, but this change was not caused by the reduced expression of tight junction protein (40).

Clinically, epitheliopathy is characterized by superficial punctate keratitis, recurrent epithelial erosion, persistent epithelial defect and delayed and often incomplete wound healing. In our previous review, according to Semeraro's classification criteria (41), we summarized the manifestations of mild, moderate and severe diabetic corneal epithelial lesions found in our hospital (4). Corneal abrasions in diabetic patients can cause more serious damage, in some cases leading to basement membrane detachment, and in other cases leading to recurrent corneal erosion (42). Epithelial wound healing is critical for restoring corneal barrier function after injury. Corneal epithelial damage in diabetic patients often takes longer to heal, even does not heal, which is also the main reason why diabetic corneal erosion is difficult to treat (14).

The surgical treatment on diabetic patients will more often lead to subsequent epithelial lesions, such as long-term erosion of epithelial cells and poor healing of epithelial cell defects. It has been confirmed that patients with diabetes who have undergone corneal refractive surgery are at greater risk of developing various epithelial diseases (43–45). Therefore, some ophthalmologists suggest that refractive surgery for diabetic patients should be carefully considered, especially for patients with poor blood glucose control (44–47). A recent study showed that DM is an important risk factor of corneal epithelial defect after vitreoretinal surgery (48). Frequently, diabetic patients with epithelial keratitis after cataract surgery have the characteristics of rapid development, severe epithelial damage, and slow corneal epithelial repair (49). Patients with diabetes are at a greater risk of epithelial debridement due to impaired epithelial wound healing (50).

Diabetic corneal neuropathy

Corneal nerves, a branch of the ophthalmic division of the trigeminal nerve, enter the peripheral cornea in a radial fashion parallel and then penetrate Bowman's layer to form the corneal sub-basal nerve plexus, which terminate in free nerve endings in the corneal epithelium and comprises the outermost layer of the cornea and protects cornea from microbial invasion (51, 52). Diabetic peripheral neuropathy (DPN) is the most common complication of diabetes, affecting up to 50% of diabetic patients (53). Recent study reported that the density of corneal nerve fiber and branch, and the corneal nerve fiber length are significantly decreased in diabetic patients (12). Moreover, the loss of 6% or more of corneal nerve fibers per year has been found in 17% of diabetic patients (54, 55). Approximately 39% of diabetic patients experience painful DPN when left untreated (56).

In type 1 and type 2 diabetic patients and animal models, the length, branch and density of corneal nerve fibers in the sub-basal nerve plexus near the corneal epithelium have been found to be reduced, which relates to the severity of diabetic polyneuropathy (24, 31, 57–63). Detailed examination by *in vivo* confocal microscopy has revealed increased corneal nerve tortuosity and thickness in diabetic patients (60, 64–68). Moreover, reduced corneal sensitivity is observed in diabetic patients and animals, and the degree is correlated with the severity of diabetes (60, 63, 67, 69–71). Pritchard et al. reported that corneal sensation threshold was significantly higher for patients with neuropathy compared to those without neuropathy and controls (72). Recent studies have identified corneal sensitivity as a potential marker of diabetic neuropathy (73). In addition, the regeneration of corneal sub-basal nerves is significantly slower in diabetic animals during corneal epithelial wound healing (24, 74). Importantly, the reduction of sub-basal nerve plexus density and corneal sensitivity, which precedes other clinical and electrophysiology tests, could be used as markers for DPN assessment (75, 76). In addition, patients with diabetes often have burning, dryness or painful feeling in the eye (77).

Pathologic mechanisms

The pathogenesis is difficult to investigate through human epidemiological studies due to too many confounding factors. Therefore, researchers often use animal diabetes models and *in vitro* cell models to study pathogenesis (78, 79). The changes in growth factors, immune cells and signal pathways in diabetic keratopathy have been elaborated in previous reviews (4, 14, 17, 78, 80). Here, we mainly discuss the following aspects.

Chronic inflammation

As a significant characteristic of DM, low-grade chronic inflammation is regarded as an important mechanism for the development of DM and its complications, including diabetic nephropathy, diabetic retinopathy, and diabetic cardiomyopathy (81, 82). These chronic inflammatory scenarios was triggered and sustained by immune cells and structural cells of specific organs/tissues, which activated innate immunity mainly through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (82, 83). Therefore, chronic inflammation theoretically also contributes to the development of DK. Several compelling evidence we found consolidated the pathogenic involvement of chronic inflammation in the development of DK (Figure 1).

NOD-like receptor protein 3 (NLRP3) inflammasome, a fully characterized inflammasome, contains NLRP3, adaptor protein ASC, and pro-caspase-1(pro-CASP1), and can be activated by various stimuli, including pathogenic molecules, sterile insults, and metabolic products (84, 85). NLRP3 inflammasome-mediated inflammation plays key roles in the development and progression of DM and its complications, such as diabetic nephropathy (83),

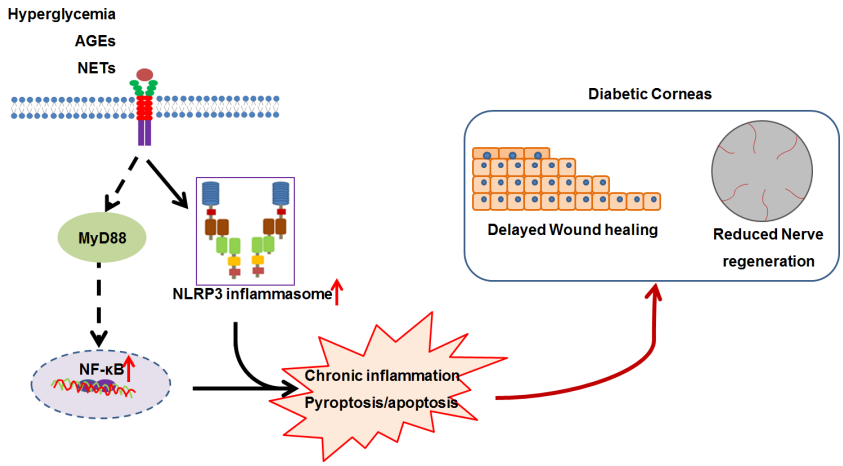

FIGURE 1
The working model for the low-grade chronic inflammation contributing to diabetic keratopathy. In diabetic mellitus, numerous diabetes-associated danger molecules (such as hyperglycemia, AGEs and NETs), persistently activate NF-κB signaling and NLRP3 inflammasome, resulting in chronic inflammation and pyroptosis, which ultimately postpones corneal epithelial wound healing and impairs re-generation.

diabetic retinopathy (86, 87), and diabetic cardiomyopathy (88). Using genetic and pharmacological approach, we revealed that persistent activation of NLRP3 inflammasome resulted in delayed diabetic corneal wound healing and impaired re-innervation (89, 90). This was supported by the findings of hyper-activation of NLRP3 inflammasome responsible for delayed diabetic skin wound healing and diabetic foot ulcer closure (91–93). Furthermore, we mechanistically revealed that the accumulated advanced glycation end-products (AGEs) promoted hyperactivation of NLRP3 inflammasome through ROS production, ultimately resulting in impaired corneal wound healing and nerve regeneration (89). The findings of AGEs accumulation on the basement membrane of corneal epithelium and Descemet's membrane in diabetic patients (94–96) were therefore mirrored the possibility of AGEs involving in the DK progression via NLRP3 inflammasome signaling. Generally, the assembly and activation of NLRP3 inflammasome results in the CASP1-dependent secretion of interleukin (IL)-1β and IL-18, as well as gasdermin D (GSDMD)-mediated pyroptosis (97). Yan et al. found that the imbalance of IL-1β and IL-1RA (IL-1 receptor antagonist) in DM corneas inhibited epithelial proliferation and promoted apoptosis, further delaying corneal epithelial healing and re-innervation (98). Inhibition of IL-1β signaling using recombinant IL-1RA and IL-1β neutralizing antibody significantly reversed the postponed diabetic corneal epithelial closure and restored re-innervation (90, 99). In addition to the elevated matured form of IL-1β, the activated form of GSDMD in diabetic corneas after abrasion was also significantly increased (89), which suggested that the GSDMD-executed pyroptosis could be also probably responsible for the excessive inflammation and the impaired corneal would healing and nerve regeneration. Therefore, NLRP3 inflammasome-mediated chronic inflammation is one of important contributors to the pathogenesis of DK, and targeting NLRP3 inflammasome could a promising for DK treatment. Moreover, we also found that blocking TLR4 signaling via TAK-242 expedited diabetic corneal re-epithelialization and nerve regeneration. In addition to receptor of AGEs (RAGE), AGEs also elicit inflammatory response through TLR4 and myeloid differentiation 2 (MD2) (100). AGEs/TLR4 mediated inflammatory response could be another factor attributed to the postponed diabetic corneal wound healing and impaired nerve regeneration.

Under normal conditions, the cornea is endowed with a heterogeneous resident population of antigen-presenting cells, including dendritic cells and macrophages (101–103). Several lines of evidence revealed that specific deletion of dendritic cells or macrophages results in a delayed corneal wound healing in healthy or DM corneas (74, 104–106). Although accumulative evidence indicates the essential role of macrophages and dendritic cells in the pathogenesis and development of DM and its complications (107–110), whether chronic inflammation triggered by macrophages and dendritic cells contributes to DK pathogenesis and progression remains elusive. Fewer neutrophils are usually distributed in normal corneas, but more are recruited after tissue injury or infection. During diabetic corneal wound closure, the number of neutrophils was significantly heightened (111), suggesting a pathogenic role for postponed corneal wound healing and impaired nerve regeneration.

As a component of innate immune system, neutrophils carry out numerous functions, including wound repair (112). During normal wound healing, neutrophils undergo apoptosis after accomplishing their functions, and are subsequently engulfed

by macrophages to resolve inflammation (112). However, the DM triggered the neutrophils to NETosis (99, 113). During NETosis, the neutrophils die through releasing web-like chromatin structures loaded with cytotoxic proteins, which is termed as neutrophil extracellular traps (NET) (114). A series of evidence has revealed that NETosis primed by DM resulted in the delayed wound healing and sterile inflammation (99, 113). During diabetic corneal wound healing, NETs production was pronouncedly elevated, and blockade of NETs formation using DNase I or Cl-amidine not only improved inflammation resolution, but also promoted corneal epithelial wound healing and mechanical sensation restoration (115). Besides its crucial role in innate host defense, NETs also fuel inflammatory and autoimmune response, including NLRP3 inflammasome (92, 93, 116, 117). In this regard, NETs would be an essential driver for chronic inflammation during DK pathogenesis and progression.

Neurotrophic function

The relationship between corneal nerves and epithelium has been found interdependency and mutual support. The corneal nerves maintain the integrity of corneal epithelium by releasing neurotrophic factors (118). Our laboratory has been committed to studying the role and mechanism of neurotrophic functions in diabetic keratopathy. We found that the levels of many neuropeptides, neurotrophic factors and axon guidance molecules in diabetic corneas were lower than in normal corneas, suggesting that the imbalance of neurotrophic function may be among the critical mechanisms of diabetic keratopathy (4).

Neuropeptides released from the sensory nerve terminals, such as substance P (SP), vasoactive-intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), and insulin–like growth factor -1 (IGF-1), play important roles in maintenance and nutrition of the corneal epithelium by promoting migration and proliferation (111, 119–127). Substance P (SP) is an 11-amino acid (ARG-PRO-LYS-PRO-GLN-GLN-PHE-PHE-GLY-LEU-MET) neuropeptide expressed in the corneal nerves, cornealepithelium and stromal keratocytes in the cornea (128–131). However, there has been no report on the expression of SP in the resident immune cell population of cornea. We found that SP content in cornea of type 1 diabetic mice decreased significantly. Exogenous SP supplementation markedly promoted epithelial wound healing and cornealsensation recovery by augmenting mitochondrial function, which was blocked by the antagonist of its NK-1R receptor, indicating that SP-NK-1R signaling played a notable role in regulating diabetic epithelial repair (Figure 2) (126). Many studies have illustraed that SP-NK-1R pathway can activate multiple signal pathways that promote epithelial growth, migration and adhesion (122, 132–135). Moreover, administration of eye drops containing SP and IGF-1 ameliorated the barrier function by promoting corneal wound healing in rats and rabbits with neurophic keratopathy (121, 136, 137). FGLM, a SP-derivedpeptide (PHE-GLY-LEU-MET), combined with IGF-1, promoted the corneal epithelialwound healing and has been used for the neurotrophic keratopathy treatment in clinical successfully (138, 139). In addition, a tetrapeptide (SSSR) derived from the IGF-1 combination with FGLM also has the synergy in corneal epithelial wound healing (140, 141).

Neurotrophin (NT) is a kind of protein molecule produced by tissues and astrocytes dominated by nerves and necessary for the growth and survival of neurons. Nerves can nourish corneal epithelium, and neurotrophic factors derived from corneal epithelium can also nourish nerves by promoting the growth and survival of nerves. Hyperglycemia attenuates the expression of nerve growth factor (NGF) and glial cell-derived nerve growth factor (GDNF) in the corneal epithelium, while exogenous NGF and GDNF increased the sub-basal nerve fiber density and corneal sensitivity (142). In diabetic mellitus, the content of CNTF and netrin-1 is lessened in diabetic mouse corneas, and we have demonstrated exogenous CNTF improves the corneal epithelial wound healing and nerve regeneration markedly (143). Gao et al. pointed out that dendritic cells are also the main source of CNTF. The reduction of CNTF levels caused by the decrease in dendritic cells during diabetic corneal wound healing is the potential mechanism of diabetic corneal neuropathy (106).

Mesencephalic astrocyte-derived neurotrophic factor (MANF), first discovered as secreted proteins with trophic activity, was expressed in the neuronal and non-neuronal systems especially in high metabolic tissues (144–146). MANF also plays an important role in diabetes. Notably, mice with the konckout of MANF developed diabetes due to increasing apoptotic cell death and reduced proliferation of pancreatic β cells, while recombinant MANF could promote proliferation and prevent cell death (146, 147). In addition, MANF has anti-inflammatory abilities in human pancreatic β cells that protect cells from cell death by repressing the NF-κB signaling pathway (148). MANF has been newly identified in corneas and is reduced in both unwounded and wounded corneal epithelium of diabetic mice. Moreover, recombinant MANF significantly promoted the wound healing of epithelium and nerve regeneration by inhibiting hyperglycemia-induced ER stress and ER-stress related apoptosis (149). Hence, MANF might be a potential therapeutic target for treating diabetic keratopathy.

Besides neuropeptides and neurotrophic factors, there is also a class of factors that play a key role in the repair of nerve innervation, namely axon guidance molecules. These molecules mainly include the Slits family, Netrins family, Ephrins family, Semaphorins family, etc (150). We found that hyperglycemia downregulates netrin-1 expression in corneal epithelium, and the subconjunctival injection of netrin-1 promotes corneal epithelial wound healing and nerve regeneration in diabetic mice. Netrin-1 facilitates the proliferation and migration of corneal epithelial cells under high-glucose conditions.

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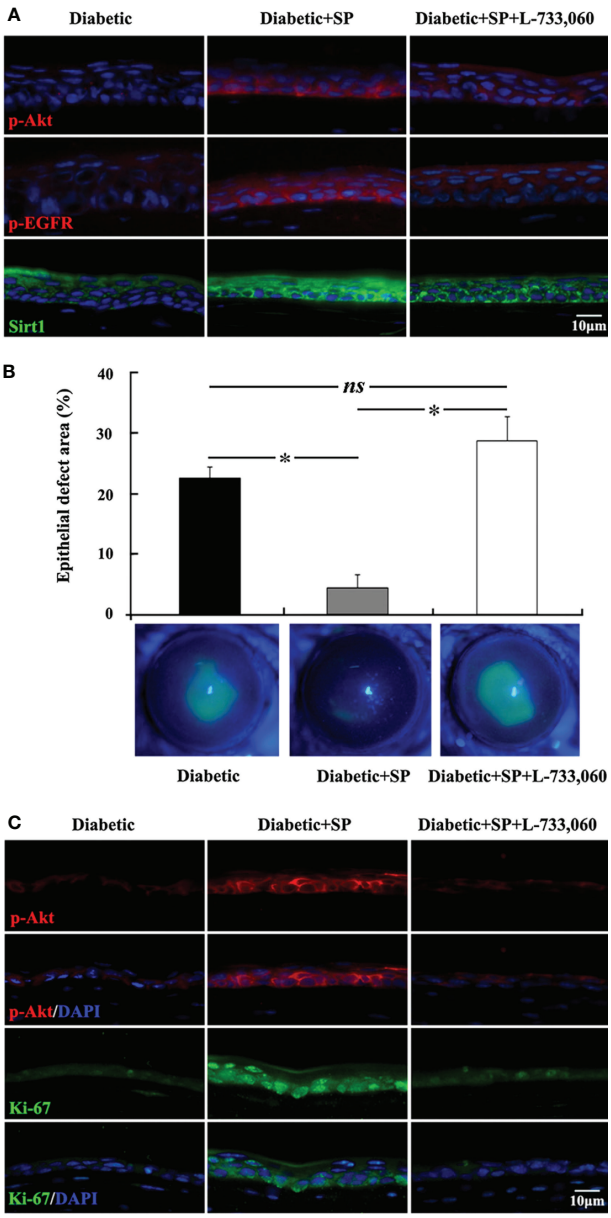

FIGURE 2
SP-NK-1R signaling regulates diabetic corneal epithelial wound healing. **(A)** In the unwounded corneal epithelium, the elevation of p-Akt, p-EGFR, and Sirt1 level by SP application was attenuated in NK-1 receptor antagonist L-733,060-injected SP-treated diabetic mice. **(B)** L-733,060 injection reversed the promotion of SP on diabetic corneal epithelial wound healing. **(C)** L-733,060 treatment reversed the promotion of SP on p-Akt activation and proliferation in the regenerated corneal epithelium. ns, no significance; *p< 0.05. (ref 126).

Furthermore, we revealed that netrin-1 inhibited neutrophil infltration, enhanced M2 macrophage transition, and attenuated the expression of pro-infammatory factors in diabetic mouse corneal epithelium *via* adenosine 2B receptor (151). Bettahi et al. revealed that diabetes inhibited the upregulation of Sema3c induced by corneal epithelial injury, but had no effect on Sema3a (152). The diabetic corneal epithelium and nerve regeneration can be promoted by exogenous supplementation with Sema3c (153). The above-mentioned studies suggest that the reduction of axon guidance factors, such as netrin and Sema3c, is partly responsible for diabetic keratopathy.

Some growth factors and metabolites also have neuroprotective effects. The expression of vascular endothelial growth factor (VEGF)-B is decreased in the regenerated diabetic corneal epithelium, and exogenous VEGF-B promotes the

regeneration of diabetic corneal nerve fibers by reactivating the PI-3K/Akt-Gsk3ß-mTOR signaling (154). Moreover, VEGF-B also elevates the corneal content of pigment epithelial-derived factor (PEDF). He et al. found PEDF plus docosahexaenoic acid (DHA) could accelerate corneal nerve regeneration in diabetic mice (155). Nicotinamide adenine dinucleotide (NAD) is involved in glycolysis, gluconeogenesis, tricarboxylic acid cycle, and other cellular metabolic reactions, and has essential biological functions (156). Our group demonstrated that NAD^+ biosynthesis plays an important role in maintaining corneal homeostasis and innervation (157). In diabetic corneas, NAD^+ content was decreased, and elevated the levels of NAD+ and its precursors NMN and nicotinamide riboside (NR) markedly promoted epithelial and nerve repair by activating SIRT1 and pEGFR, pAKT, and pERK1/2 signaling (158). Another study found that nicotinamide mononucleotide is helpful in improving cell viability and tight junctions in high glucose treated human corneal epithelial cells through the SIRT1/Nrf2/HO-1 pathway (159).

Neural ion channels changes

Corneal neurons express a range of membrane channels, including chemical or polymodal nociceptors, mechanonociceptors, and thermal or cold receptors (160, 161). Among corneal afferent neurons, approximately 45% expressed TRPV1, 28% expressed Piezo2, and 8% expressed TRPM8, with 6% of TRPV1 neurons co-expressing TRPM8 (162). The transient receptor potential (TRP) family is thought to transduce environmental and endogenous stimuli to electrophysiological signals. TRPV1 is a well-characterised channel expressed by a subset of peripheral sensory neurons, and canonically mediates inflammatory and neuropathic pain (163). TRPV1 sensitization can be induced by capsaicin. Nowadays, capsaicin 8% patch has been used to alleviate pain in patients with peripheral neuropathic pain, which induced fewer systemic side effects (164–169). Corneal TRPV1 is involved in the maintenance of the corneal structure, re-epithelialization, and inflammation in corneal injury (170). In addition, blinking behavior in guinea pigs related to ocular discomfort is reversed by treatment with the TRPV1 blocker, capsazepine (171). Therefore, corneal TRPV1 may be important for healing corneal tissue, and alleviating the pain in inflammatory disorders of the ocular surface. The depletion of TRPV1+ sensory nerves delayed corneal wound healing by enhancing the recruitment of neutrophils and γδ T cells, increasing the number and TNF-α expression of CCR2+ macrophages and decreasing the number of CCR2− macrophages and IL-10 expression (172). In diabetic conditions, the TRPV1 expression in trigeminal ganglia is increased and the integrity of TRPV1 neurons is important for avoiding alveolar bone resorption and inflammation (173).

Recently, cold receptors have come under greater scrutiny. TRPM8, which is activated by temperatures lower than 25-28°C and menthol, is widely expressed in corneal afferent fibers (174–176). We found that in Trpm8-deficient mice, corneal wound healing is accelerated, while squamous metaplasia occurred in the central corneal opacity after multiple injuries (unpublished data). TRPV1-dependent neuronal sensitization facilitates the release of SP from TRPM8+ cold-sensing neurons to signal nociception in response to cold (177, 178). Overexpression of TRPV1 in TRPM8+ sensory neurons leads to cold allodynia in both corneal and non-corneal tissues without affecting their thermal sensitivity (177). Type 1 diabetic mice exhibit heightened sensitivity to both heat and cold. In diabetic hyperalgesic mice, the thermal hyperalgesia induced by an increase in TRPV1 function is further aggravated by decreased TRPM8 function (179). Abdulhakeem S. Alamri et al. found that the density of corneal nerve fibers in mice fed a high-fat and high-cholesterol diet and those with hyperglycemia had a similar reduction. The reduction of nerve fibers expressing TRPM8 receptors in the corneas of the two models was more significant than that of TrpV1 positive nerve fibers (180).

Diabetic autonomic neuropathy

Several influencing factors have been implicated in the pathogenesis of diabetic neuropathy. The hyperglycemic activation of the polyol pathway and protein kinase C may reduce the neuronal blood flow causing direct neuronal damage (181–183). In addition, the increased oxidative stress induced excess nitric oxide production may result in the formation of peroxynitrite and damage to neurons (184, 185). Moreover, the reduction of neurotrophic growth factors, the deficiency of essential fatty acids, and the accumulation of advanced glycosylation end products may also cause less endoneurial blood flow and nerve hypoxia which altered nerve function (183, 186–188). Diabetic neuropathy has been classified as diabetic peripheral and autonomic neuropathies based on pathophysiological characteristics (127). However, few studies have focused on the changes of autonomic nervous system in diabetes keratopathy and its regulatory mechanism.

Diabetic autonomic neuropathy (DAN) is a serious and common complication which has negative impact on the survival and quality of life in patients' with diabetes (189). DAN may affect many organ systems throughout the body, such as gastrointestinal, genitourinary, and cardiovascular (190). The autonomic nervous system is divided into the sympathetic and the parasympathetic nervous systems. In mammalian corneas, the density of the sympathetic innervations which are from the superior cervical ganglion, vary among species (191). The sympathetic innervations compose about 10–15% of corneal innervations in rabbit, mouse, rat and cat, whereas in primates, they are rarely reported (52, 104, 192). The activation of the

sympathetic nervous system has been found in type 1 and type 2 diabetic mice (193–196). In cornea, the activation of sympathetic nervous system may inhibit the wound healing of corneal epithelium and induce the expression of proinflammatory genes in the CD64+CCR2+ macrophages through the β-2 adrenergic receptor (ADRB2) (104). Moreover, we found that the abnormal activation of sympathetic nerve in diabetic mice resulted in the partial depletion of multiple neurotrophins in corneal epithelial cells and dysfunction of limbal stem cells through ADRB2, which further delayed the corneal sensory nerve regeneration and epithelial wound healing (Unpublished data).

The parasympathetic innervations, which are from the ciliary ganglion, exist in different species and vary among in rats, cats, and mice (52, 104, 197). Conversely, the activation of parasympathetic nerves promotes the wound healing of corneal epithelium and enhances the expression of the anti-inflammatory genes in CD64+CCR2- macrophages through α-7 nicotinic acetylcholine receptor (α7nAChR) (104). VIP is secreted predominantly by parasympathetic nervous system. The distinct local macrophages have been found to be activated by VIP, which further modulated inflammation and epithelial renewal. Recently, we found VIP and its receptor are decreased in diabetic corneas in the process of wound healing compared with normal, while exogenous VIP attenuates the wound healing of DM corneas by regulating the wounding inflammatory response and nerve regeneration through Sonic Hedgehog signaling pathway (111).

miRNAs and long noncoding RNAs

Generally, miRNA has been proven to be a key regulator of gene expression and can target a variety of molecules that affect cell physiology and disease development. Numerous reports have shown that miRNA relates to the pathology of the diabetic corneal epithelium and nerve damage, making miRNA becoming a promising therapeutic approach for the treatment of diabetic keratopathy.

As the source of corneal nerve fibers, changes in the trigeminal ganglion (TG) caused by diabetes may contribute to corneal neuropathy. Through RNA sequencing, our group found that 68 miRNAs and 114 mRNAs in the TG tissues of diabetic mice diverged from those in normal TG tissues. We predicted that the interaction of miR-350-5p and Mup20, miR-592-5p and Angptl7, and miR-351-5p and Elovl6 may be related to diabetic corneal neuropathy (198). Jianzhang Hu et al. found that inhibiting the expression of miR-181a and miR-34c in TG of diabetic mice promoted the growth of trigeminal sensory neural cells and the regeneration of corneal nerve fibers by regulating autophagic activation (199, 200). Our study revealed that the expression of miR-182 was downregulated in the TG tissue of diabetic mice, which was a key molecule downstream of the endogenous protective gene Sirt1 in TG. And NOX4 was a key target gene for miR-182 to regulate diabetic corneal epithelial and nerve repair (201). Targeting NOX4 and Sirt1 could effectively mitigate the severity of diabetic keratopathy (201, 202).

We also screened differentially expressed miRNAs in the regenerated corneal epithelium of normal and type 1 diabetic mice, and found that miR-223-5p was significantly upregulated, which may be involved in regulating the delay of diabetic corneal wound healing. In the next validation experiment, we confirmed that inhibition of miR-223-5p accelerated the regeneration of diabetic corneal epithelium and nerves, which mediates inflammation response and epithelial cell proliferation through its target gene Hpgds (203). In 2016, our group also found that miR-204-5p, which can directly regulate sirt1, has increased expression in diabetic corneas, and inhibition of miR-204-5p promotes corneal epithelial regeneration by accelerating cell cycle (204).

Compared with normal diabetic mice, diabetic miR-146a KO mice had significantly delayed epithelial wound healing of cornea and skin, and increased neutrophil infiltration. The potential mechanism was that miR-146a KO induced an imbalance in the IL-1β, TNF-α, IRAK1, TRAF6 and NF-κB signaling pathways. Interestingly, there was no difference in corneal wound healing between miR-146a KO and normal mice with normal blood glucose (205). Subsequently, another group's research in cultured human limbal epithelial cells showed that overexpression of miR-146a reduced the expression of proinflammatory TRAF6, IRAK1 and downstream target NF-κB; and inhibited the expression of cytokine IL-1α, IL-1β, IL-6 and IL-8 and chemokines CXCL1, CXCL2, and CXCL5, which were significantly upregulated in diabetic corneal limbal epithelial cells (206). These studies indicate that miR-146a plays an important role in the regulation of corneal epithelial homeostasis and regeneration under diabetic conditions.

lncRNAs are a class of noncoding RNA molecules with a length of more than 200 nucleotides, which have been reported to play a regulatory role in diabetic complications, retinopathy, pterygium and other eye diseases. Xiaxue Chen and Jianzhang Hu analyzed the differentially expressed lncRNAs (DELs) in the regenerated corneal epithelium of type 1 diabetic and normal corneas. In the diabetic group, 111 upregulated DELs and 117 downregulated DELs were detected. The authors conducted in-depth research on lncRNAs Rik, which is significantly downregulated in diabetes, and found that Rik can be combined with miR-181a-5p as a ceRNA, thus promoting the healing of diabetic corneal epithelial wounds (207).

Limbal stem cell dysfunction

The corneal epithelium is self-renewed and regenerated by limbal stem cells (LSCs) that reside in the basal epithelial layer of the limbus, which plays a key role in corneal epithelial wound healing (208–211). A study based on the alteration of LSCs in patients with diabetes found that the expression of markers of

LSCs such as ΔNp63α, ATP-binding cassette sub-family G member 2 (ABCG2), N-cadherin, K15, K17, K19, and β1 integrin was decreased significantly in the diabetic limbus (212). *In vitro* cultured LSCs from healthy and diabetic patients were subjected to immunofluorescence staining with LSC markers, and it was also found that the expression of LSCs markers ΔNp63α, PAX6, ABCG2, K15 and K17 in diabetic patients was reduced markedly, especially K15 and K17 (213). Similarly, type 1 and type 2 diabetic mice also showed a significant reduction of LSCs markers in corneal limbus (143, 214). Thus, the loss or dysfunction of the resident LSCs could be responsible for clinically observed delayed corneal epithelial wound healing in diabetic corneas. Therefore, improving the function of diabetic LSCs through genes or growth factors is expected to be an effective means to promote diabetic corneal epithelial wound healing.

We found that the expression of neurotrophic factor CNTF was significantly reduced in corneal epithelium of STZ-induced type 1 diabetic mice. Studies in cultured mouse corneal epithelial stem/progenitor cells found that CNTF increases the efficiency of clone formation, promotes cell proliferation, and upregulates the expression level of corneal epithelial stem/progenitor cell-related transcription factors by activating Stat3 signal (143). It can also upregulate MMPs by activating Akt signal to promote the migration of corneal epithelial stem/progenitor cells (215). CNTF supplementation by subconjunctival injection can promote the corneal epithelial would healing both in normal and diabetic mice, and is accompanied by the enhancement of corneal epithelial stem/progenitor cell proliferation activity (Figure 3). In contrast, the application of CNTF neutralizing antibody significantly impairs the normal repair function of corneal epithelium. Hiroki Ueno et al. reported that insulin-like growth factor-I (IGF-I) is capable of protecting against corneal stem/progenitor cells and nerve damage in diabetes (214). Taken together, growth factors, such as CNTF and IGF-1, have potential effects in ameliorating limbal stem cell deficiency and treating diabetic keratopathy by enhancing LSCs functions.

Some compounds also have the effect of enhancing the stemness of limbal stem cells, such as ascorbic acid (216), ROCK inhibitor Y-27632 (217), and pluripotin (218). Recently, we found that the proinflammatory cytokines IL-1β and TNF-α were overexpressed during diabetic corneal epithelial wound healing (219). Proinflammatory cytokines can suppress the LSCs markers expression and the colony-forming capacity of corneal epithelial stem cells, as well as destroy the normal ability for corneal epithelial wound healing in a mouse model (220). Proinflammatory cytokines regulate corneal epithelial wound healing through p16Ink4a-STAT3 signaling, and knockdown of p16Ink4a partially restores diabetic corneal epithelial repair defects (221). Yuka Okada et al. confirmed that the sensory nerve TRPV4 is essential for maintaining the stemness of LSCs and is one of the main mechanisms for maintaining corneal epithelial homeostasis (221). Thus, controlling inflammation and maintaining sensory nerve function are beneficial to diabetic corneal epithelial wound healing.

Diabetic corneal endotheliopathy

Clinical manifestation

Corneal endothelial cells (CECs) can be characterized according to the percentage of hexagonal cells (HEX) and the coefficient of variation (CV) (222–225). The previous researches are inconsistent regarding the effect of DM on CEC pleomorphism and polymegathism. Many studies report that the CECs of diabetic patients have a decreased HEX and an increased CV compared to healthy controls (226–230), whereas other studies show no differences (224, 225, 231, 232). Most studies support the hypothesis that DM is associated with worsening CEC pleomorphism and polymegathism. Especially, studies comparing patients with type-1 and type-2 DM (T1DM and T2DM, respectively) found that individuals with T1DM had more remarkable changes in CEC morphology (230, 233, 234).

The rate of cell density loss stabilizes to approximately 0.5% per year (235). Endothelial cell density (ECD) is an indirect marker of endothelial health and function (223–225, 235–239). The rate of CEC loss and the subsequent decrease in ECD speed up in patients with DM (225, 230, 234, 237, 238, 240–244). It should be noted that patients with T1DM (compared to T2DM) and those with a longer disease duration sustain a more severe decline in ECD.

It is widely known that an increase in central corneal thickness (CCT) could serve as one of the earliest signs of CEC dysfunction (245). Many researchers found that T1DM subjects have a higher CCT (238, 245–247). In fact, there have also been reports of a difference in CCT between T1DM and T2DM while few studies have found CCT and DM are unrelated.

Pathologic mechanisms

The pathogenesis of corneal endotheliopathy in diabetes is still less studied. The reported mechanisms mainly include mitophagy impairment, endoplasmic reticulum (ER) stress and pyroptosis.

Mitophagy is a highly selective form of autophagy that eliminates dysfunctional or excess mitochondria under stressful conditions, such as hypoxia (248). In our recent study, we demonstrated that hyperglycemia causes abnormal endothelial cell morphology and impaired mitophagy, leading to the accumulation of damaged mitochondria. *In vivo* data also confirmed that increased mitophagy had a protective effect on the CE of diabetic mice. Our results suggest that regulating mitophagy may be a promising strategy for the treatment of diabetic corneal endothelial dysfunction (249).

The ER stress response is a vital regulatory mechanism that maintains intracellular homeostasis (250, 251). The overactivation of the ER stress response and mitochondrial dysfunction are

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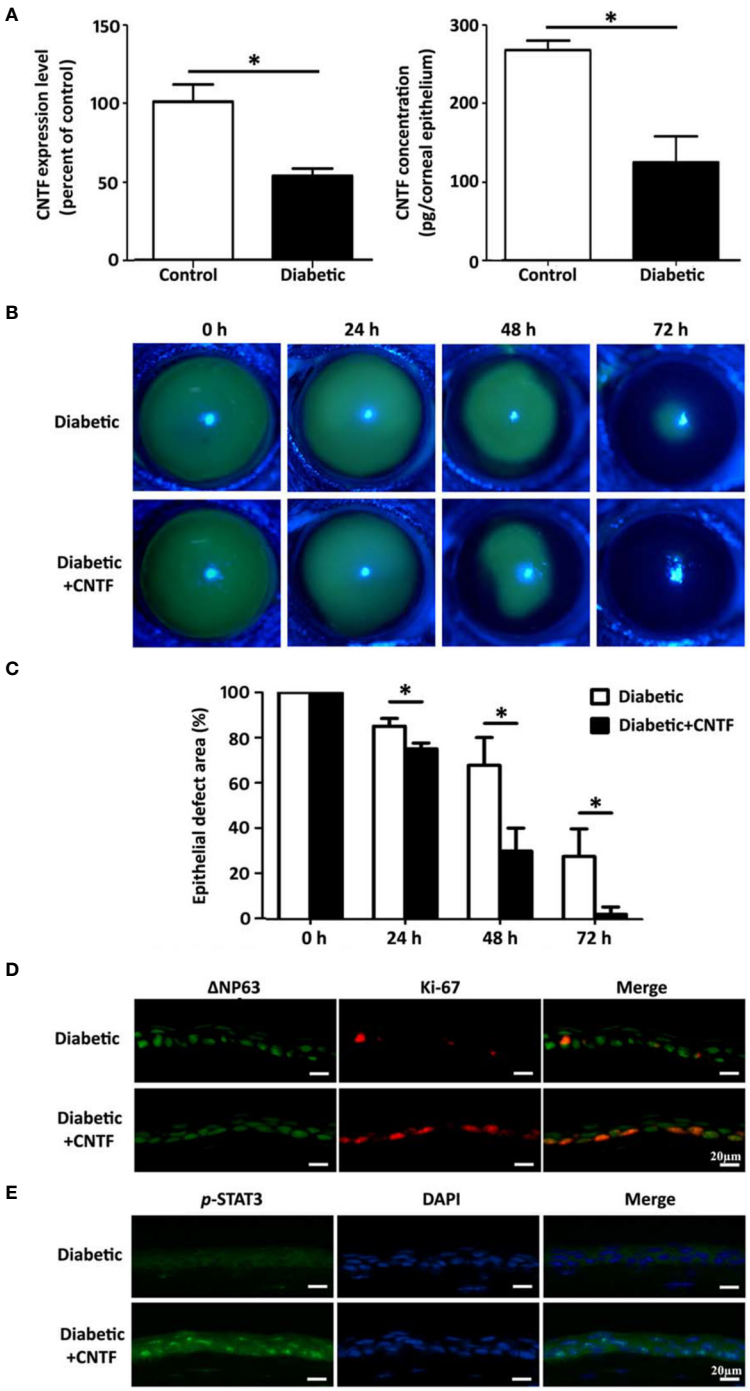

FIGURE 3
CNTF promotes corneal epithelial wound healing in diabetic mice. **(A)** CNTF is decreased in diabetic corneas both in mRNA and in protein level. **(B, C)** Subconjunctival injection of 50 ng CNTF significantly promotes the corneal epithelial wound healign in diabetic mice. **(D)** The expression of ΔNp63 and Ki-67 in the regenerating corneal epithelium is upregulated after CNTF treatment. **(E)** CNTF activated Stat3 signaling in diabetic wounded corneas. "*p< 0.05. (ref 215).

prominent etiological factors in the development of diabetes (252). We observed ER stress response activation in diabetic mice and diabetic human corneal endothelial cells, which induced CEC-specific morphological changes. Persistent ER stress response activation can cause CEC loss and corneal endothelial dysfunction (Figure 4). Consequently, in DM, the inhibition of ER stress could mitigate endothelial cell loss and corneal edema *via* the mitochondrial pathway (253).

Pyroptosis is a recently discovered form of programmed cell death that is related to inflammation (254–256). Zhang et al. unraveled the novel role of long non-coding (lnc) RNA KCNQ1OT1 in pyroptosis, whereby KCNQ1OT1-repressed micro-RNA (miR)-214 expression upregulated the expression of the inflammatory molecule Caspase-1 and promoted pyroptosis *in vitro* and *in vivo*. Additionally, KCNQ1OT1 acts as a competing endogenous (ce)RNA that competitively binds miR-214 to regulate Caspase-1 activity, thus promoting diabetic corneal endothelium dysfunction. Further study of the role of KCNQ1OT1 will be critical for understanding the pathogenesis of diabetic corneal endothelium dysfunction and will help identify new biomarkers or potential therapeutic targets to treat this debilitating condition (257).

Diabetic related dry eye

Diabetic lacrimal gland disorder

Characteristics of diabetic lacrimal gland

Patients with DM may have a higher prevalence of dry eye than the healthy population (258). It has been reported that dry eye disease affects about one-fifth of patients with T2DM and reduces the patients' quality of life (13, 259, 260). Dry eye may be caused by impaired tear production or excessive tear evaporation and is associated with photophobia, red eyes, vision impairment, local pain, and pruritus. The tear film is the interface between the ocular surface and the environment, and it contains a tightly controlled complement of water, proteins and lipids. LG secretion of proteins and fluid into the tear film is essential for maintaining the health of the ocular surface.

DM impairs tear secretion and induces LG changes. Early studies have identified the involvement of insulin in disorder of LG, such as impaired secretion and a reduction in protein secretion (261, 262). Subsequent studies validated that lipid accumulation in the LG acinar increased with age in a non-obese diabetic (NOD) mouse model. This change is along with lymphocytic infiltration and destruction of the acini. In addition, LG cholesteryl esters obviously increased in these mice (263). Similarly, the polyol pathway was triggered by hyperglycemia in type-2 diabetes, and the accumulation of sorbitol within cells led to cellular edema and dysfunction, which finally resulted in LG dysfunction and decreased tear secretion (264). Recently, He et al. reported that hyperlipidemia affects LG function, including the inhibition of tear secretion, rising lipid accumulation, inflammation, and oxidative stress levels (265). Nakata et al. demonstrated that diabetes suppresses hemodialysis-induced increases in tear fluid secretion, which suggests that the autonomic control of the LG function may be compromised by neuropathy in patients with DM (266). Most recently, our results suggested that streptozotocin-induced type-1 diabetic mice exhibited the early onset of reduced tear secretion and LG weight compared to the symptoms of diabetic keratopathy (267).

Pathogenesis of diabetic lacrimal gland

Hyperglycemia, oxidative stress, nerve alterations may play an important role in the development of LG impairment (268) in DM. The detailed mechanisms have become clearer.

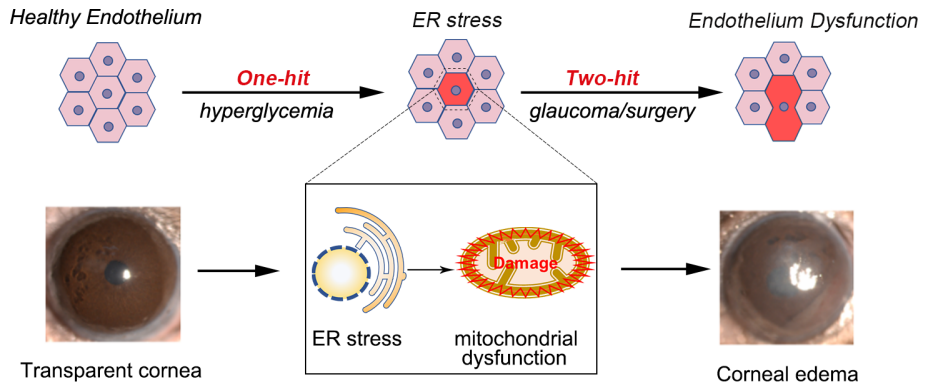

FIGURE 4
Proposed mechanism of endothelium dysfunction in the diabetic cornea. Endothelial cells from diabetic mice exhibit high levels of ER stress and ER stress appears before morphological changes of the endothelium. Activation of ER stress can promote corneal endothelial dysfunction by triggering mitochondrial dysfunction. Acute injuries, such as glaucoma, can lead to corneal edema and endothelial cell loss.

Mitochondria is the major source of intracellular reactive oxygen species and the target of oxidative damage (269–271). Previous studies had confirmed the existence of oxidative stress and mitochondrial dysfunction in the LG of dry eye mice (272, 273). In the type-1 diabetic model, oxygen consumption rate and basal extracellular acidification rate detection results suggested that the early onset of diabetic dry eye may be due to the susceptibility to a mitochondrial bioenergetic deficit in diabetic LG (Figure 5), while the application of mitochondria-targeted antioxidant SKQ1 may ameliorate diabetic dry eye and keratopathy.

It is recognized that inflammation plays a prominent role in the development and propagation of dry eye. Hyperglycemia initiates an inflammatory cascade that generates the innate, adaptive immune responses of the lacrimal functional unit (LFU). The downstream immune-inflammatory regulators have been identified as matrix metalloproteinase-9 (MMP-9), immature antigen-presenting cells (APCs), $CD4^+$ helper T cells (TH) subtype 1, and TH17 cell subsets, interferon-γ (IFN-γ) chemokines, chemokine receptors, cell adhesion molecules (CAMs), and interleukin-17 (IL-17) (274).

The neural response that regulates LG fluid secretion is an integral part of the LFU, which consists of the sensory afferent nerves of the cornea and conjunctiva, the efferent parasympathetic and sympathetic nerves that innervate the LG, the LG secretory cells, and the LG excretory ducts (275). Both anatomically and functionally, the parasympathetic system predominates, with overwhelming evidence indicating that the loss of parasympathetic innervation blocks LG functioning (276–283). Research has demonstrated that different densities of sympathetic innervation in glandular areas and the sympathetic denervation of the rabbit LG by ablating the superior cervical ganglion did not alter the LG acinar morphology and induced the denervation supersensitivity of protein secretion (284, 285). In addition, researchers have reported that the electrostimulation of the superior cervical ganglion increased tear secretion (286). However, the involvement of sympathetic stimulation in LG in DM remains poorly understood. Recently, we illustrated that the sympathetic pathway is activated in the pathogenesis of diabetic dry eye and may provide a potential strategy to counteract diabetic dry eye by interfering with sympathetic activity (Unpublished data).

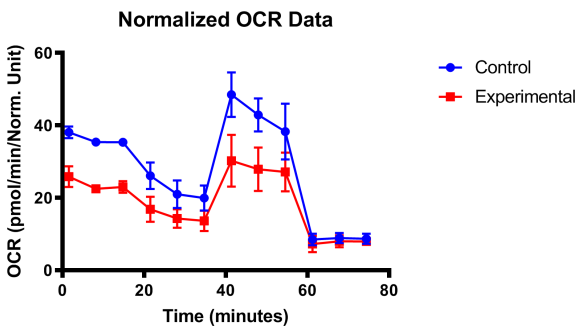

FIGURE 5
Mitochondrial dysfunction in diabetic lacrimal gland. Lacrimal gland cells from the diabetic mice after 16 weeks were evaluated with the seahorse XFp analyzer (ref. 267).

Diabetic meibomian gland dysfunction

Clinical manifestation

Meibomian gland dysfunction (MGD) is an important cause of dry eye, and diabetes may be a risk factor. Studies reveal a high incidence of MGD in patients with diabetes (287–289), especially long-lasting diabetes (290, 291).Yu et al. (287) observed 132 eyes to assess the changes of Meibomian Glands (MGs) in type 2 DM. As the diabetes progressed, they found more MGs dropouts and absence of MGs in the DM group, and MG bubbles density were decreased with shape alterations, such as atrophy, fibrosis, expansion. The opening of glandular duct appeared to be atrophic and cornified. Additionally, lipid layer thickness (LLT), lid margin abnormalities, and tear breakup time (BUT) were significantly changed in diabetic patients; interestingly, the results of LLT were varied in different investigations (289, 290), which deserves further research. More importantly, some studies suggested that diabetes was associated with asymptomatic MGD, and it may be an early sign of ocular discomfort in T2D (290, 292). These findings suggest a lack of association between signs and symptoms. Therefore, it is alert to notice the signs of MGD in the absence of symptoms and perhaps the necessary treatment should be taken to prevent the progression of complications.

While MGD in type 2 DM has been widely investigated in the literature, studies on type 1 DM were very limited. Previous studies reported that BUT were lower in the Type 1 DM group and significantly associated with the duration of DM (293, 294). Semer et al. (295) evaluated the changes of MGs with Type 1 DM and found that in diabetic children, a higher secretion score and total eyelid score appeared. The thinning and shortening of MGs and presence of ghost areas were more common. In Type 1 DM animal model established by streptozotocin (STZ), more signs were founded, such as acini dropout, condensed lipid deposition at the orifice of the MG, disorganized acini and ducts, lipid metabolism disorder compared to those of non-diabetic controls (296, 297). Previous studies have documented peroxisome proliferator activator receptor-γ (PPARγ) plays a dominant role in regulating meibocyte differentiation and lipid synthesis (298, 299). Recent study has confirmed the reduced PPARγ in diabetic MGs, and upregulation of PPARγ could improve the production of lipid (300). Taken together, these indicated that pathological process of MGD could be observed in diabetic model induced by STZ, so, it may be used as vital tool for studying the physiopathology of MGD resulting from hyperglycemia. Generally, the pathogenesis of Type 1 DM differs from that of Type 2 DM, and distinctions in

the presentation and progression of MGs between DM types has been seldom reported. Hence, future comparative investigations are necessary.

Pathogenesis of diabetic meibomian gland dysfunction

Unlike other sebaceous glands, the lipid secretion of MG is controlled by various neurotransmitter-neuromodulator mechanisms, and disparate neuropeptides/neurotransmitters play a role in the functioning of MG cells (301–303). The continuous proliferation and differentiation of MG cells are the basis for maintaining the secretion of lipids. Neuropathy, one of the most common complications of DM, may lead to MG dysfunction by disrupting the function of MG cells. Peripheral neuropathy may also alter meibum delivery to the ocular surface. Clinical studies have revealed that peripheral neuropathy causes a decline in nerve impulses emanating from the brain and corneal hypoesthesia, which leads to reduced blink rates (69, 70, 304). During blink movement, the muscle could produce a compression force to the tarsal plate and facilitate the delivery of the lipid from the MGs. Therefore, it is speculated that neuropathy leads to a decline in the blinking rate and meibum delivery forces, and ultimately leading to greater MGD prevalence in diabetes patients.

In diabetic patients, laser scanning confocal microscopy (LSCM) displayed the infiltration of inflammatory cells in the interstitial of gland bubble (305). In STZ-induced diabetic mouse model, more CD45 positive cells, such as macrophage and neutrophils, accumulated in MGs (297). Similarly, Yuli at al. found more inflammatory cells and overexpressed inflammatory factors in MG of diabetic rat. Genomic analysis techniques revealed that inflammation-related genes were upregulated in type 2 diabetic mice (306). In addition, more studies have demonstrated that the lipid homeostasis is related to the inflammation (307, 308). Many lipid species could regulate inflammatory responses. In turn, inflammation can alter the lipid metabolism. As a systemic metabolic disease, DM is closely associated with the lipid metabolism, and it has been recognized that diabetes induces the disruption of lipid homeostasis in MGs (297, 309). It was suggested that phospholipids (PLs) may play a key role in the inflammatory reaction. A higher level of PLs was observed in the meibum with DM, and the overexpression of PLs could release more inflammatory mediators, such as free fatty acid (FFA). FFA was considered to be toxic hydrolysate generated by microbial lipases from normal lipids, which would conversely induce inflammation and hyperkeratinization, thus damaging the ocular surface and MGs (310).

Potential treatment options

Diabetic ocular surface diseases is treated by local symptomatic treatment (such as the use of steroids to treat epithelial defect) on the premise of systemic control of blood glucose (such as insulin injection). However, the existing primary treatment methods cannot fully meet the treatment needs of diabetic ocular surface diseases, so it is necessary to find alternative treatment targets.

Stem cells therapy have been proposed as an emerging treatment option for diabetic keratopathy. Mesenchymal stem cells (MSCs) are a good choice for stem cell therapy due to their pluripotency and regenerative potential (311–314). MSCs exist in various tissues, including bone marrow, peripheral blood, adipose tissue, placenta, nervous tissue and so on. MSCs are known to play an important role in regulating tissue repair and immune inflammation through direct or indirect mechanisms. Our study based on bone marrow mesenchymal stem cells (BM-MSCs) on diabetic corneal wound healing found that the local transplantation of BM-MSCs significantly promoted the repair of corneal epithelium in type 1 diabetic mice. In mechanism, BM-MSCs alleviate diabetic corneal impairment by promoting the activation of corneal epithelial stem/progenitor cells and accelerating the polarization of macrophages to anti-inflammatory M2 phenotypes by secreting tumor necrosis factor-a–stimulated gene/protein-6 (TSG-6) (315).

Based on its ability to self-renew and promote regeneration, hemopoeitic stem cell (HSC) is another potential adult stem cell for disease therapy. Maha et al. assessed the possible effect of HSC therapy on STZ-induced diabetic keratopathy in albino rat and found that a tail vein injection of HSC ameliorated the changes of cornea and conjunctival epithelium caused by diabetic keratopathy (316).

Many studies in stem cell therapy have been conducted to restore corneal functioning, including autologous/allogeneic limbal stem cell transplantation (317), embryonic stem cells (ES)/induced pluripotent stem cells (iPS)-induced corneal cells (318, 319) and various adult stem cell (320, 321) treatments. Some have entered clinical trials; however, stem cell therapy in the field of diabetic keratopathy is still in its early stages. Although MSC and HSC transplantations have certain application prospects at the animal level, they are still far from clinical application, and further exploration is needed in the future.

Considering that cornea, lacrimal gland and meibomian gland are densely innervated, and neuropathy is one of the most common, complex and serious complications of diabetes patients, treatment based on neural regulation has also been emphasized. Exogenous supplementation of sensory neuropeptide SP, CGRP and parasympathetic neuropeptide VIP has been proven to effectively promote the regeneration of corneal epithelium and nerves in the experimental stage. As mentioned above, the therapeutic effects of various neurotrophic factors and axon guidance molecules on diabetic ocular surface diseases have also been successively verified in diabetic animal models. It is worth mentioning that Cenegermin (Oxervate^{TM}), an ophthalmic eye drops mainly composed of recombinant human NGF, was recently approved by the FDA for the treatment of neurotrophic keratopathy (322). In addition, our

latest study found that sympathetic overactivation caused by diabetes also participated in the pathogenesis of diabetes keratopathy and diabetes related dry eye (Unpublished data). Sympathetic nerve-targeting regulation may also be a potential therapeutic target for diabetic ocular surface disease.

In addition, recent research has also revealed many other new methods to treat corneal epithelial defects, including the application of natural Chinese medicine (such as lycium barbarum polysaccharide) (323), various cell derived exosomes (324), and biological materials (such as hydrogel) (325). The mechanism revealed by these studies has something in common with the pathogenesis of DK, and maybe also used for developing new DK treatment methods, which may eventually open up a new way for developing new treatment methods to improve corneal wound healing.

Conclusion

With increasing clinical evidences of ocular surface damage in diabetic patients, ophthalmologists have gradually recognized the harm of diabetic ocular surface complications, and more basic ophthalmic research has focused on the disclosure of the pathogenesis and potential therapeutic targets of diabetic ocular surface complications.

The defined pathogenesis of diabetes keratopathy includes the accumulation of advanced glycation end products, the imbalance of growth factors and signaling pathways, the occurrence of persistent inflammation, the decline of neurotrophic function, the dysfunction of stem cells, the impairment of mitochondrial function, excessive oxidative stress, etc. Therefore, controlling inflammation and excessive oxidative stress, improving the function of stem cells and mitochondria, and targeting relevant growth factors, neurotrophic factors and signal pathways will be the direction of developing new targets for DK treatment, and guiding the clinical treatment of DK.

Diabetic dry eye was found to be closely associated with the abnormal mitochondrial function of lacrimal gland and the abnormal lipid metabolism of meibomian gland. For the treatment of dry eyes in diabetes, attention should be paid to improving tear secretion and meibomian gland lipid metabolism. Animal experiments have confirmed that promoting mitochondrial function has a good therapeutic effect on diabetic dry eyes, providing a basis for future clinical applications.

Clinical prospective studies have discerned that the early clinical symptoms of diabetic ocular surface complication are dry eye and corneal nerve degeneration, suggesting that early diagnosis should first examine corneal nerves changes using confocal microscopy and examine dry eye related clinical indicators. Further study on the interaction between neuro-epithelium and neuro-immunity will help to reveal the key pathogenic mechanism and formulate targeted intervention strategies for ocular surface complications of diabetes.

Author contributions

LX and QZ contributed to the manuscript design, discussion and revision. LY, QW, YL and CW contributed to the manuscript preparation and writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Classification of diabetic retinopathy: Past, present and future

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Diabetic retinopathy (DR) is a leading cause of visual impairment and blindness worldwide. Since DR was first recognized as an important complication of diabetes, there have been many attempts to accurately classify the severity and stages of disease. These historical classification systems evolved as understanding of disease pathophysiology improved, methods of imaging and assessing DR changed, and effective treatments were developed. Current DR classification systems are effective, and have been the basis of major research trials and clinical management guidelines for decades. However, with further new developments such as recognition of diabetic retinal neurodegeneration, new imaging platforms such as optical coherence tomography and ultra wide-field retinal imaging, artificial intelligence and new treatments, our current classification systems have significant limitations that need to be addressed. In this paper, we provide a historical review of different classification systems for DR, and discuss the limitations of our current classification systems in the context of new developments. We also review the implications of new developments in the field, to see how they might feature in a future, updated classification.

KEYWORDS

diabetic retinopathy, classification, severity staging system, pathophysiology, imaging technology, artificial intelligence, deep learning, quantitative assessment

Introduction

Diabetes mellitus (DM) is one of the fastest growing chronic diseases in terms of global prevalence (1). According to recent data published by the International Diabetes Federation, approximately 537 million adults had diabetes in 2021, while estimates suggest that this figure will increase to 783 million by 2045 (2). Diabetic retinopathy (DR) is an important microvascular complication, and occurs in about 30% of individuals with diabetes (3, 4). DR is therefore a leading cause of preventable vision impairment and blindness among adults, particularly in higher-income countries (5). With the overall incidence of diabetes rapidly increasing, the number of adults worldwide with DR, vision-threatening DR, and diabetic macular edema (DME) are projected to increase to approximately 161 million, 45 million, and 29 million, respectively by 2045 (6).

Since the first description of retinal changes in diabetes, the emphasis has predominantly been on vascular abnormalities in DR. This is not surprising, as the early ophthalmoscopically-visible lesions in DR, such as intraretinal hemorrhages, venous abnormalities, lipid exudates and other changes, primarily reflect retinal capillary abnormalities, which has been confirmed on histopathological studies (7, 8). Eventually, these vascular abnormalities and retinal ischemia result in diabetic macular edema (DME) and retinal vasoproliferative complications, which can lead to vision loss and blindness. Over decades, various DR staging and classification systems have sought to accurately describe the progression of DR, quantify severity of the disease, and stratify risk of progression. Early classifications from the mid-20th century, such as the ophthalmoscopic classification (9) and Hammersmith grading system (10) have been abandoned as our understanding of the disease has improved. More recently, the Early Treatment of Diabetic Retinopathy Study (ETDRS) classification (11) has been considered the "gold standard" for many years, because it was developed and validated on natural history data that demonstrated its ability to prognosticate risk of progression to proliferative disease and vision loss (12). The ETDRS classification is still used for research and clinical trials today, but its widespread clinical application is limited by its complexity. In everyday clinical practice, the International Clinical Diabetic Retinopathy (ICDR) Severity Scale (13), which in essence is a simplified ETDRS system, is currently the most commonly used classification system worldwide. Previous classification systems had to be updated or replaced as our understanding of the disease improved. In the years since the adoption of the ETDRS and ICDR staging systems, there have been major developments, including better understanding of the pathophysiology of DR, recognition of retinal neural dysfunction and neurodegeneration, improvements in imaging technology, and the development of disease modifying treatments, such as anti-vascular endothelial growth factor (anti-VEGF) therapy. Considering these massive strides that have been made in the field, we feel that it is timely to review the progress made, and determine if it is time for an update to our existing classification systems for DR.

Therefore, we aim to provide a historical review of different classification systems for DR, as well as to discuss the limitations of current classification systems in the context of new developments. We also review the implications of technological developments and new treatments for DR, to see how they might feature in an updated classification.

Past: A historical review of classification systems for diabetic retinopathy

Early classifications of DR

Diabetic retinal lesions such as hemorrhages and exudates were first observed by Eduard Jaeger using the direct ophthalmoscope in 1856 (14). However, there was limited evidence of a causal relationship between diabetes mellitus and retinopathy at the time, and many prominent ophthalmologists, such as Albrecht von Graefe, questioned the link (15). In the years that followed, more evidence linking diabetes and retinal complications began to emerge, including reports by Louis Desmarres in 1858 (16) and Henry Noyes in 1869 (17). In 1872, Edward Nettleship published a histopathological study demonstrating "cystoid degeneration of the macula" in diabetes (18). In 1876, German ophthalmologist Wilhelm Manz described fibrovascular proliferations along the blood vessels in a patient with proliferative diabetic retinopathy, which he termed "retinitis proliferans" at the time (19). Julius Hirschberg proposed the first classification of DR in 1890, which he sub-divided into 3 types: retinitis centralis punctata (which affected mainly the posterior pole), retinitis hemorrhagica, and other retinal manifestations (20). "Diabetic retinitis" was a frequently used term at the time, because it was presumed that exudation was related to inflammation. In 1934, Wagener, Dry and Wilder proposed an expanded classification which included 5 stages and incorporated lesions such as hemorrhages, punctate exudates, cotton-wool exudates and venous changes, with proliferative retinopathy being the most severe stage of disease (21). Subsequently in the 1940s, Arthur James Ballantyne described capillary wall alterations and microaneurysms in DR, and included them in a classification of DR (22).

As DR was studied in greater depth, more classification systems for DR were proposed over the next decades. In the early 1950s, Scott suggested a six-stage clinical classification of DR (23). In stages I to III, various lesions that we now understand as pre-proliferative disease were described, including capillary

microaneurysms, intraretinal hemorrhages, exudates and venous changes. At the time, it was not recognized that vitreous hemorrhage was a direct consequence of neovascularization, and so vitreous hemorrhage was classified as a separate stage IV, which was thought to subsequently progress to proliferative disease. Stage V was "retinitis proliferans", which was subdivided into V(a), retinitis proliferans, and V(b), the "vascular type" of retinitis proliferans, while stage VI was retinal detachment and "gross degenerative changes", representing end-stage diabetic retinal disease. One of the major drawbacks of this classification system was the fact that the pre-proliferative stages of disease were still divided primarily by specific lesion type – for example, the presence of exudates necessitated classification as stage III, whereas we now know that the development of hard exudates or macular edema can progress independently of overall retinopathy status.

Grading of individual lesions

In 1966, Lee et al. proposed an updated DR classification system, which started to resemble more modern classification systems. Recognizing that different specific lesion types (such as venous changes, microaneurysms and hemorrhages, exudates) do not necessarily progress together, but can vary in terms of severity, they proposed grading each of these lesions types on individual severity scales (9). Based on detailed examination with binocular indirect ophthalmoscopy, and detailed fundus drawings from 400 patients with DR, they proposed individual 5-point severity gradings for each of four lesion types: 1. angiopathy (with separate sub-gradings for A. venous dilatation, B. microaneurysms & hemorrhages, and C. neovascularization), 2. exudates, 3. proliferative retinopathy, and 4. vitreous hemorrhage. After individual lesion classification, they then looked at the eye as a whole to determine which type of retinopathy predominated. Astutely, they also included separate classification for additional "Other Changes", including macular changes, rubeosis iridis and secondary glaucoma, retinal detachment, and optic nerve changes. Naturally, one of the major drawbacks to this classification system was that it relied on ophthalmoscopy and detailed fundus drawings, which were time-consuming and prone to inter-observer variability.

Photographic classification: Hammersmith grading system

As reproducibility and consistency were clearly important for a universal DR staging system, the introduction of fundus photography into the classification systems represented a major breakthrough. Fundus drawings from indirect ophthalmoscopy were an important method for recording the appearance of the retina and the progress of visible lesions for many years. However, as assessments of DR and individual lesion severity relied on more objective assessment of lesion size, extent, and number, this approach became increasingly impractical. Fundus photographs were more objective, and could even be used to evaluate progression of DR severity in the same patient at different time points. In 1967, the Hammersmith grading system was the first to describe the severity of DR by using the fundus photographs (10). Five components of retinopathy such as microaneurysms and hemorrhages, exudates, new vessels, venous irregularities and retinitis proliferans, were recorded through four standard photographs. The Hammersmith grading system was widely used to document the changes in eyes associated with treatment (24). For example, in a study examining the effect of laser photocoagulation on proliferative DR in 90 eyes of 72 patients, severity grading by color fundus photography (CFP) was performed prior to laser treatment, and following laser treatment at yearly intervals (25). This allowed by objective evaluation of the effect of treatment, and analysis by the number of quadrants affected, It was also significant that in this study they acknowledged and included some patients with neovascularization outside the photographic fields of the Hammersmith grading system, which did highlight a drawback of the system at the time. Other examples of the Hammersmith photographic grading system in use included a large study involving 6792 diabetic patients in South India (26). This large cohort underwent clinical examination and fundus photography, graded according to the Hammersmith grading system. This allowed for estimation of the prevalence rates of DR in a large South Indian cohort.

Airlie house classification

In 1968, over 50 experts from around the world met in the Airlie House, Virginia, USA to analyze current understanding of DR natural history, and to develop a standardized classification for DR (27). This was a major milestone in the classification and staging of DR. Some key elements in the natural history of DR that were identified and described include: "capillary occlusion is an essential early change prior to the formation of arteriovenous shunts in DR, which was contrary to previous popular cognition; newly formed blood vessels undergo a cycle of proliferation and degeneration; and vision will be seriously threatened when fibrous tissue or vitreous attached to the neovascularization shrinks" (28). The Airlie House classification that was produced, on which all our current modern DR classification systems are based, emphasizes a fundamental dichotomy of retinopathy between non-proliferative DR (NPDR) and proliferative DR (PDR). NPDR included various signs such as microaneurysms, hard and/or soft exudates, venous caliber abnormalities, venous sheathing, perivenous exudate, arteriolar abnormalities, intraretinal microvascular abnormalities

(IRMAs), and arteriovenous nicking. PDR included retinal or disc neovascularization, fibrous proliferation, retinal detachment, preretinal and vitreous hemorrhage. This classification system relied on standardized 7-field stereoscopic CFP images, which were compared against a set of 18 standard color photographs.

Modified airlie house classifications – the ETDRS severity scale

Modern DR classification systems that are in use today are largely based on the original Airlie House classification, and are frequently referred to as "modified Airlie House classifications". Minor modifications were made to the Airlie House classification, for application in the Diabetic Retinopathy Study (DRS) (29) and Early Treatment of Diabetic Retinopathy Study (ETDRS) (30) in 1981 and 1991, respectively. Modifications that were made for the DRS classification include: assessment of location, extent, and severity of retinal thickening of macular edema; assessment of five features including hard exudates, soft exudates, arteriovenous nicking, retinal elevation, and vitreous hemorrhage as an additional step for the grading; separating previously combined characteristics into venous abnormalities and arterial abnormalities and grading them individually; addition of some characteristics such as microaneurysms, drusen, hard exudate rings, papillary swelling, and subretinal hemorrhage (30). In the ETDRS classification, fundus lesions and characteristics, such as hemorrhages/microaneurysms (H/Mas), venous beading and loops, hard exudates, IRMAs and neovascularization, were graded individually from standard 7-field 30°C fundus photographs, and based on these individual lesion gradings, an overall retinopathy severity level was determined at the eye level, with 14 levels ranging from level 10 (DR absent) to level 85 (advanced PDR, with posterior fundus obscured, or center of macula detached), excluding level 90 (for ungradable images) (12).

Another key contribution of the ETDRS clinical trials, was that they defined "clinically significant macular edema" (CSME). CSME was observed by using stereoscopic fundus photographs on the basis of the presence of retinal thickening and hard exudate (31), and was defined as: (a) Thickening of the retina at or within 500 μm of the center of the macula; or (b) hard exudates at or within 500 μm of the center of the macula, when associated with adjacent retinal thickening; or (c) a zone or zones of retinal thickening 1 disc area or larger, any part of which was within 1 disc diameter of the center of the macula (32). CSME was a crucial definition that influenced clinical management at the time, as the ETDRS trial established the therapeutic benefit of focal/grid laser photocoagulation for DME meeting the criteria for CSME (32).

Since its introduction in the early 1990s, the ETDRS severity scale has been the gold standard DR classification for both clinical and research clinical trial use. This is because the ETDRS study rigorously validated the severity scale, and demonstrated its prognostic value in predicting risk of progression to PDR, at 1-, 3- and 5-years, in a longitudinal cohort of 3,711 untreated eyes (12). This severity scale has been used in countless clinical and epidemiologic studies of DR over the past few decades, and has been an instrumental factor in improving our understanding and management of DR. One major drawback of this classification though, lies in its complexity. Because it requires detailed grading, it is frequently employed in research studies that have dedicated reading centers for standardized grading, but it is impractical for daily clinical use by ophthalmologists.

The wisconsin epidemiologic study of diabetic retinopathy (WESDR)

One alternative classification system that attempted to overcome the issue of complexity with the ETDRS was proposed by the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) study group. The WESDR was a population-based longitudinal cohort study that was started in 11 counties in southern Wisconsin from 1979 to 1980 (33–35). This cohort study included 1210 young patients with diabetes (age < 30 years) and 1780 older persons with diabetes (age ≥ 30 years) between 1980 and 1982. Over the next few decades, this large cohort of diabetic patients were systemically assessed for DR, and associated risk factors (36–45). DR was evaluated in a standardized manner by masked grading of standard 7-field stereoscopic CFPs throughout the study (46), and this was proposed as a simpler, less cumbersome alternative to the ETDRS severity scale.

Using the ETDRS severity scale, a grader would have to individually evaluate 21 lesions in each of the photographic fields for each eye, and use a computer program based on these gradings to assign the eye one of 14 possible severity levels (excluding ungradable images). In contrast, with the WESDR system, a grader examined all 7 photographic fields as a whole, and assigned the eye a severity level based on the greatest level of retinopathy severity present in any field. There were also fewer retinopathy severity levels in the WESDR system, ranging from level 1 to 7. To validate this simplified classification scale, they graded 4,604 eyes with both the WESDR and ETDRS scales, and demonstrated acceptable agreement. The exact agreement between the two scales was 78.3%, and the WESDR showed interobserver agreement of 78.5%, and intraobserver agreement ranging from 84% to 90% (47).

The international clinical diabetic retinopathy (ICDR) severity scale

The ETDRS severity scale has been further simplified into the International Clinical Diabetic Retinopathy (ICDR) severity scale, for widespread daily clinical use. The ICDR severity scale essentially distills the 14 severity levels of the ETDRS severity scale, into 5 levels of retinopathy severity. Because of its convenience and ease of adoption, the ICDR severity scale is by far the most common classification system in clinical use around the world.

The ICDR severity scale was developed from the ETDRS and WESDR data and classification systems, through an international consensus workshop in 2002. An initial planning meeting including representatives from five countries was held in conjunction with the Annual Meeting of the American Academy of Ophthalmology (AAO) in 2001. Thereafter, in 2002, 14 individuals from 11 countries attended the International Congress of Ophthalmology in Sydney and developed the ICDR through discussion and consensus *via* a modified Delphi system (13). This classification system was deliberately intended to be convenient and easy to use in everyday clinical practice by general ophthalmologists and primary care physicians. The ICDR severity scale, along with the corresponding ETDRS severity scale levels, are shown in Table 1. Various international clinical guidelines for DR management, such as the International Council of Ophthalmology (ICO) guidelines, use the ICDR severity scale for recommendations of management and follow-up surveillance intervals for DR (48).

Present: Limitations of current DR classification systems

Current DR classification systems are reproducible, well-validated, and are robust in prediction of important outcomes of clinical interest. However, major developments over the past few decades since their introduction have resulted in some important limitations.

First, the current DR classifications rely only on 7 standard field photographs to grade the severity of DR. However, these standard photographic fields only cover about 30% of the total retinal surface area (49). Peripheral retinal lesions may have important prognostic significance and may improve prediction of future clinical outcomes. Second, DME is now the most common cause of visual impairment from DR (50), and the presence of DME influences clinical management and treatment. however, our DR classification system prognosticates the risk of progression to PDR, and does not effectively predict the incidence of DME, nor does it adequately account for different levels of DME severity. Third, our classification systems do not take into account measures of visual function, such as best-corrected visual acuity, or other aspects of visual function, such as contrast sensitivity, visual quality, visual fields, low luminance acuity, and metamorphopsia. Inclusion of such outcome measures may be important as new therapies are developed. Beyond measures of visual function alone, patient-reported outcome measures and quality of life may also need to be taken into account.

Fourth, our DR classifications focus only on the vascular aspect of disease, and do not include evaluation of the neural retina or diabetic retinal neurodegeneration. There is evidence now that early neural degeneration may precede or accompany vascular lesions, and these changes may have impact on visual function (51, 52). Fifth, evaluation of systemic health is absent in current classifications, although it is clear that systemic factors such as diabetes duration, glycemic control, co-morbid hypertension and dyslipidemia, and even pregnancy, can influence DR progression and outcomes (3, 53, 54).

Sixth, current classification systems do not record the regression or resolution of retinal neovascularization. If the PDR scale was revised to describe the key levels, it could help to improve the characterization of the natural history of eyes

TABLE 1 ICDR and corresponding ETDRS severity scale levels.

ICDR severity levels	ETDRS severity levels	
No apparent retinopathy	Level 10	No retinopathy
Mild NDPR	Level 20	Very mild NPDR
Moderate NPDR	Level 35	Mild NPDR
	Level 43	Moderate NPDR
	Level 47	Moderately severe NPDR
Sever NPDR	Level 53	Severe NPDR
PDR	Levels 60, 61	Mild PDR
	Level 65	Moderate PDR
	Levels 71, 75	High-risk PDR
	Levels 81, 85	Advanced PDR

ICDR, International Clinical Diabetic Retinopathy; ETDRS, Early Treatment of Diabetic Retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

with PDR and be an outcome measure for treatment. Seventh, the current classification system fails to address reductions of DR severity that are seen after intravitreal anti-VEGF treatment. It is currently unclear how improvements in DR severity level with such treatments modify the undertlying disease process, and affect future clinical outcomes. Finally, current DR severity scales and individual lesion gradings are not quantitative. Quantitative staging systems may facilitate research, and provide better prognostication (55). With these limitations in mind, it is clear that improvements and updates are needed to our existing DR classification systems.

Future: New developments that will influence a new DR classification system

Pathophysiologic mechanisms

As we understand more about the pathophysiologic mechanisms that drive DR progression and its complications, this knowledge is likely to influence new classification systems. Current understanding is that hyperglycemia and other metabolic factors, such as hypertension and dyslipidemia, instigate a cascade of physiological and biochemical changes leading to retinal microvascular abnormalities, retinal ischemia, and resultant complications (Figure 1) (5). Upregulation of VEGF has been proven to be closely implicated in the pathogenesis of DR and its vascular complications such as neovascularization and DME (56). Subsequently, VEGF-independent pathways, such as erythropoietin, growth hormone and insulin-like growth factor, and angiopoietin, have been identified, through proteomic and other analyses (57, 58). Erythropoietin and its receptors are synthesized by retinal pigment epithelial cells and are important stimuli for mobilizing endothelial progenitor cells to impaired retinal sites (59, 60). Upregulation of erythropoietin expression in the ischemic retina may promote neovascularization and contribute to the progression of PDR (61). In one study, though the correlation between erythropoietin and VEGF levels were not strong, erythropoietin was more closely correlated with the presence of PDR than VEGF (57). Thus, erythropoietin inhibition has been proposed as a potential

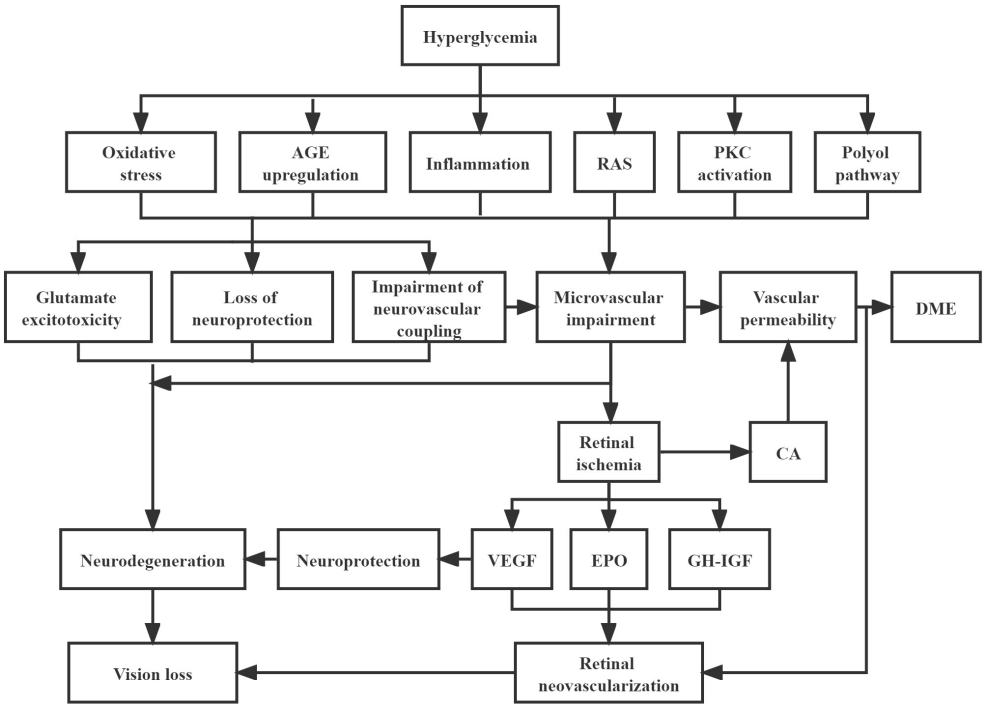

FIGURE 1
Pathophysiology of diabetic retinopathy Hyperglycaemia cascade of events leading to neurodegeneration and microvascular impairment, which are the two key main pathways to result in the development of diabetic retinopathy. Neurodegeneration can be activated by glutamate excitotoxicity, loss of neuroprotection, and impairment of neurovascular coupling. Meanwhile, impairment of neurovascular coupling can lead to microvascular impairment, which can trigger the formation of DME and retinal neovascularization. AGE, advanced glycation end-products; RAS, renin-angiotensin system; PKC, protein kinase C; DME, diabetic macular edema; CA, carbonic anhydrase; VEGF, vascular endothelial growth factor; EPO, erythropoietin; GH-IGF, growth hormone-insulin growth factor.

therapy option for DR, but the potential adverse effects on photoreceptor survival need to be balanced (57). Growth hormone and insulin-like growth factor may play a crucial role in pathological neovascularization in PDR and influence its progression (62). Growth hormone directly stimulates the proliferation of human retinal microvascular endothelial cells (63). Insulin-like growth factor and its binding protein are expressed in blood vessels, neurons, and glial cells throughout the retina and are altered in response to hyperglycemia and hypoxia (64). The angiopoietin pathway has already been trialed for therapeutic benefit. Faricimab, a novel bispecific antibody, provides dual inhibition of both VEGF-A, and angiopoietin-2 (Ang-2) to treat vascular eye diseases, including diabetic eye disease (65). It is thought that inhibition of Ang-2 works synergistically with VEGF inhibition, and helps to promote increased vascular stability (66). Recent phase III clinical trials seem to suggest that this approach may provide greater durability of treatment effect (67). Proteomic analyses have also shown raised levels of extracellular carbonic anhydrase in DR (68), which is thought to increase retinal vascular permeability, with equal potency to VEGF (58). Whether this pathway can be targeted for treatment with carbonic anhydrase inhibitors is an area for further study (69).

Furthermore, the traditional view that DR is purely a microvascular disease process is incomplete. The accumulating evidence indicates that there is a process of diabetic retinal neurodegeneration that accompanies or even precedes vascular damage. Evidence for loss of retinal neural elements can be seen as thinning of the retinal nerve fiber layer (RNFL) and ganglion cell layer on optical coherence tomography (OCT) imaging (70, 71). Functional abnormalities can also be demonstrated by electroretinography (ERG), including pattern ERG and multifocal ERG (72, 73). Some studies have also shown that these structural and functional neural abnormalities may develop early in DR, even before the onset of microvascular changes or retinopathy (74–76). In particular, the multifocal ERG has shown promise for detecting early abnormal alterations of retinal function in diabetic patients without apparent DR, and changes in multifocal ERG implicit time especially could be used as a potential clinical biomarker for providing early diagnosis of diabetic retinal disease and effective prognostication (75–77). It is postulated that chronic hyperglycemia induces retinal neurodegeneration, microvascular damage, and impairment of the neurovascular unit (61). The two key pathogenic factors involved in retinal neurodegeneration are the accumulation of extracellular glutamate, and imbalanced production of the retinal neuroprotective factors (78). The former is thought to result in neuron death (79), while the latter may impair the neuroprotective effect, which is related to the down regulation of neuroprotective factors such as pigment epithelium-derived factor, interstitial retinol-binding protein, somatostatin and several neurotrophins (78). Interestingly, there is some evidence that VEGF may be a survival factor for retinal neurons facing ischemic injury (80, 81). Anti-VEGF treatment certainly helps to reduce vascular leakage and retinal edema and improve visual acuity, but it has been postulated that there may be some deleterious effects on the retina from chronic long-term VEGF inhibition (82, 83). These potential limitations of anti-VEGF therapy need to be examined in further studies, and it would be interesting to see if multifocal ERG or other functional assessment modalities can be informative.

Finally, other pathogenic pathways are also likely to be important in DR, such as inflammation (84–86), increased oxidative stress (87–89), upregulation of receptors for advanced glycosylation end products (90–93), renin-angiotensin system (RAS) activation (88, 89, 94, 95) and dysfunctional endothelial progenitor cells (60). Much of the interaction between the neural and vascular abnormalities in the pathophysiology of DR remains to be clarified. However, as we better understand the relationship and link between these aspects, especially in the early stages of disease, such information will definitely influence our classification of DR, and may additionally promote the development of new potential treatment methods targeting these pathways (78, 96).

Improved imaging technology and novel biomarkers

Major advancements have been made in retinal imaging technology over the past few decades. Up until the 1990s, the traditional retinal imaging modalities were standard color fundus photography (CFP), and fluorescein angiography, which were considered the gold standard for diagnosis, grading and visualization of retinal vasculature. Current DR fundus imaging patterns are summarized in Table 2. However, the development of better imaging techniques, such as OCT, ultra-widefield (UWF) imaging and optical coherence tomography angiography (OCTA), have allowed for new ways to visualize the anatomy of the retina and its vasculature, which will undoubtedly improve the ability to assess, prognosticate and monitor DR. Table 3 summarizes the features of these new retinal imaging modalities in DR.

Optical coherence tomography

OCT is a non-contact and non-invasive imaging method that has become standard of care for diagnosis and monitoring of many retinal diseases (97). With the application of OCT for accurate retinal thickness measurements and imaging of retinal microstructure, new information about disease characteristics that were previously unrecognized is now available.

With OCT, a variety of potential biomarkers and structural abnormalities has been described in DR and DME. OCT can detect the significant reductions in the thickness of RNFL and

TABLE 2 Summary of current fundus imaging modalities in diabetic retinopathy.

Imaging modality	Advantages	Limitations	Clinical findings in DR
Fundus photography	Noncontact Wide application Gold standard for diagnosis and grading	Two-dimensional image Limited field of view Qualitative assessment	Microaneurysms Intraretinal haemorrhages Cotton-wool spot Venous beading Intraretinal microvascular abnormalities Neovascularization of optic disc (NVD) or elsewhere (NVE)
Fluorescein angiography (FA)	Gold standard for retinal vasculature Rapid assessment of retinal vascular changes High sensitivity when detecting low flow vascular lesions Differentiation of intraretinal microvascular anomalies (IRMAs) and neovascularization elsewhere (NVE) Differentiation of focal leak and diffuse capillary bed leak in DME Able to capture peripheral lesions Less liable to show artifacts than OCTA and easier to interpret	Invasive Two-dimensional image Time-consuming Potential adverse reactions to the dyes Leakage of dye can obscure details of vascular structures	Microaneurysms Retinal capillary non-perfusion Vascular telangiectasia Capillary drop outs Enlargement or irregularity of the foveal avascular zone The presence of neovascularization

ganglion cell-inner plexiform layer in DR, but also in diabetic patients without DR compared with healthy controls (98). This retinal thinning is thought to represent diabetic retinal neurodegeneration or neural dysfunction (51). Other quantitative changes that have been described include reduced retinal thickness, retinal volume, and decreased optical reflectivity (99). Qualitative abnormalities are also detectable, such as the presence of intraretinal hyper-reflective foci (HRF)

TABLE 3 Summary of new multiple fundus imaging modalities in diabetic retinopathy.

Imaging modality	Advantages	Limitations	Clinical findings in DR
Optical coherence tomography (OCT)	Noncontact Widely used Cross-sectional and three-dimensional images Objective and quantitative assessment of DME Gold standard for diagnosis of DME and monitoring of treatment response	Fixation requirement Absence of visualizing vascular changes	Retinal thickness Subfoveal choroidal thickness Photoreceptor outer segment Hard exudates Hyperreflective retinal foci (HRF) Hyperreflective choroidal foci (HCF) Intraretinal cystoid spaces Disorganization of retinal inner layers (DRIL) Bridging retinal processes Subfoveal neurosensory detachment Integrity of ELM and EZ Taut posterior hyaloid membrane
Ultra-wide Field Retinal Imaging	Fast acquisition Noncontact High-resolution No pupillary dilatation Wide field of view Improvement of the detection of DR lesions Precise grading of DR	High cost and limited availability Image artifacts Peripheral distortion and magnification Superior and inferior periphery is not well visualized Difficulty to precisely measure the retinal surface area of lesions	Microaneurysms Intraretinal haemorrhages Cotton-wool spot Venous beading Intraretinal microvascular abnormalities Predominantly peripheral lesions Neovascularization of optic disc (NVD) or elsewhere (NVE) Preretinal haemorrhage Vitreous haemorrhage
Optical coherence tomography angiography (OCTA)	Quick and noncontact Cross-sectional and three-dimensional image Visualization and quantification of retinal vascular plexuses Visualization of vascular details Quantification of non-perfusion and vessel density Identification and monitoring of damage	High-resolution images need for good fixation Production of projection artifacts Limited peripheral view Complicate learning curve to capture and interpret images Not widely used	Microaneurysms Venous beading Decreased vascular density Capillary non-perfusion Enlargement of foveal avascular zone Increased vessel diameter index Decreased fractal dimension Increased vessel tortuosity Intraretinal microvascular anomalies (IRMAs) and neovascularization elsewhere (NVE)

that are thought to represent microglial activation and migration. More HRF have been shown to be present in diabetic patients with DR compared to those without DR, and has been associated with the progression of DR (100). Some OCT biomarkers have been associated with visual acuity outcomes in DME and DR, such as disruption of the external limiting membrane (ELM) (101) and ellipsoid zone (EZ) (102), and disorganization of retinal inner layers (DRIL). DRIL has also been shown to be associated with increased severity of DR (103). In addition, other potential biomarkers in characterizing DR include hyperreflective choroidal foci (HCF), intraretinal cystoid spaces, hard exudates, and subfoveal neurosensory detachment, which are shown in Figure 2. However, prospective validation is needed before many of these potential biomarkers can be useful tools in clinical practice.

Based on OCT changes and biomarkers in DR and DME, some groups have proposed OCT-based classification systems for DME or diabetic maculopathy. One such classification describes different types of DME including the sponge-like retinal swelling type, cystoid macular edema type, and serous retinal detachment type (104, 105). Another, more comprehensive, classification takes into account multiple different OCT biomarkers, to classify diabetic maculopathy into four different stages: early diabetic maculopathy (DM), advanced DM, severe DM, and atrophic maculopathy (106).

Ultra-widefield retinal imaging

UWF imaging is defined as retinal imaging providing at least 110°field of view, with visualization including the anterior edge of the vortex vein ampullae (107), though many current commercials systems can capture up to 200° in a single retinal image. Figure 3 shows an UWF retinal image in comparison with the area covered by 7 standard-field CFP images. Although the ETDRS classification has been the gold standard for DR classification and detection for many years, it is important to remember that a single 45° CFP image only covers about 15% of retinal surface area, and the 7 standard fields in total cover about 30% (108). In contrast, UWF images can cover about 82% of total retinal surface area (Figure 4). Recent studies examining UWF imaging in DR have shown that more than 50% of DR graded lesions are located outside the area covered by the 7 standard ETDRS fields, and they also demonstrate that peripheral DR lesions may have powerful

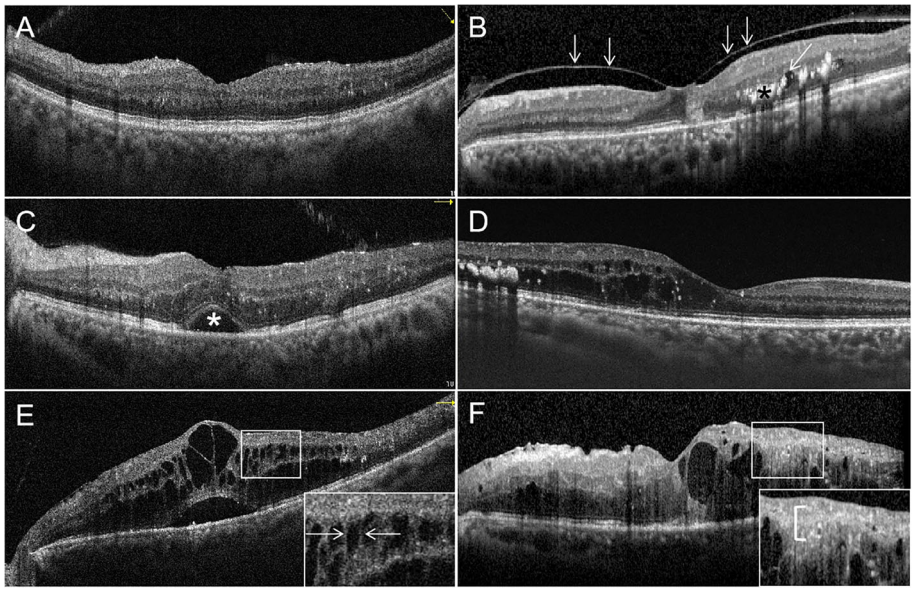

FIGURE 2
Optical coherence tomography (OCT) signs of diabetic macular edema (DME). **(A)** All retinal layers are intact and visible. The retinal profile is not altered. But there is diffuse macular thickening. **(B)** Vitreomacular traction with a thick posterior hyaloid membrane (white arrowheads), small cystoid spaces (oblique white arrowheads) and hard exudates (black asterisk) in the outer plexiform layer and the outer nuclear layer. **(C)** Multiple hyperreflective retinal foci (HRF) are seen. Subretinal fluid causing a neurosensory detachment of the fovea (white asterisk). **(D)** Cystic cavities, hard exudates, and HRF located in the outer retina, but the external limiting membrane (ELM) and ellipsoid zone (EZ) are intact. **(E)** The magnified image (white square) shows the bridging retinal processes (white arrowheads) between the cystic cavities. **(F)** Multiple cystoid spaces and HRF in the inner and outer layers with disorganization of the inner retinal layers (DRIL; white bracket in the magnified image). The ELM and EZ are disrupted under the fovea.

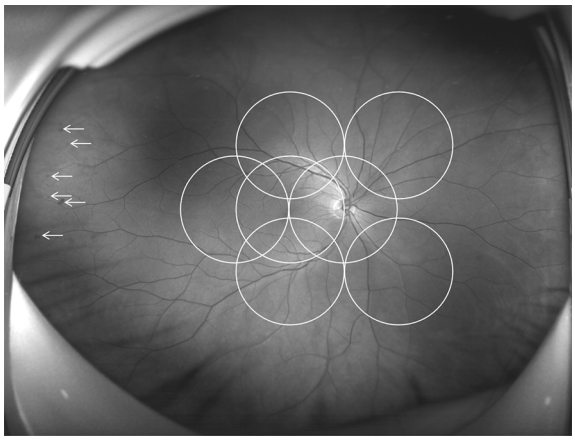

FIGURE 3
Comparison of an ultra-wide field (UWF) retinal image and the Early Treatment Diabetic Retinopathy Study (ETDRS) 7 standard photographic fields. UWF retinal image is superimposed by the ETDRS 7 standard fields in white circles. The white arrowheads showing diabetic retinopathy lesions predominantly peripheral to the ETDRS fields.

prognostic significance (49). One study showed that eyes with predominantly peripheral lesions (PPLs) had a 3.2-fold increased risk of DR progression and a 4.7-fold increased risk of PDR progression compared to eyes without PPLs (109). Meanwhile, UWF imaging has shown that the PPLs outside the ETDRS fields account for 40% in the eyes with DR and that PPLs may lead to a more severe level ETDRS grading in about 10% eyes (Figures 5A, B) (110). In one study, about 50% of neovascularization (new vessels elsewhere) was predominantly peripheral when examined with UWF images (111). Clearly, the peripheral retina as visualized by UWF imaging can provide valuable information about the classification and progression of DR, and visual prognosis, but how to this should be incorporated into a new DR classification is currently unclear.

UWF imaging can also be applied to fluorescein angiography. UWF fluorescein angiography (UWFA), together with color or pseudocolor UWF imaging, has been applied to detect peripheral neovascularization and ischemic areas, and to guide the diagnosis and treatment of DR (Figures 5C, D). In one study on UWFA, parameters such as the areas of non-perfusion, neovascularization and panretinal photocoagulation scars displayed by UWFA images increased by 3.9 times, 1.9 times and 3.8 times, respectively compared with 7 standard field ETDRS images.

Meanwhile, Ehlers et al. demonstrated the relationship between the quantitative angiographic parameters of microaneurysm count, panretinal leakage, and ischemic area on UWFA, and the clinical severity of DR (112). Such parameters derived from UWF photos and UWFA may be used as biomarkers to assess the objective information that may be related to need for therapeutic intervention or therapeutic response.

Optical coherence tomography angiography

OCTA is a novel, non-contact and non-invasive technique capable of capturing high-resolution images of the retinal and choroidal vessels (113, 114). OCTA displays vascular flow information by creating three-dimensional depth-resolved images of the retinal and choroidal vascular system, so as to identify areas with or without flow, which is an important aspect of DR assessment. Although OCTA cannot reveal vascular leakage, it still has many advantages over fluorescein angiography (FA) (115). Most importantly, OCTA is non-invasive, and can provide detailed information about the retinal microvasculature in DR, without the need for intravenous contrast dye (Figure 6) (116, 117). Meanwhile, the acquisition of OCTA image and data is more convenient and rapid than FA. Furthermore, OCTA provides depth-resolved images, and can allow separate visualization of the superficial, middle and deep retinal capillary plexuses, which may provide additional pathological information over traditional dye-based angiography (115).

Vascular changes associated with diabetes can be detected by OCTA even before the appearance of clinically-visible DR (118). Some of the parameters provided by OCTA include vessel density, vessel tortuosity and fractal dimension, of the superficial capillary plexus, deep capillary plexus, and the middle capillary plexus (119). OCTA can also identify foveal avascular zone parameters such as size, circularity and perimeter (Figure 7). Many such parameters have been correlated with severity of DR (120). Although FA has a higher sensitivity than OCTA in detecting microaneurysms, some studies have proven

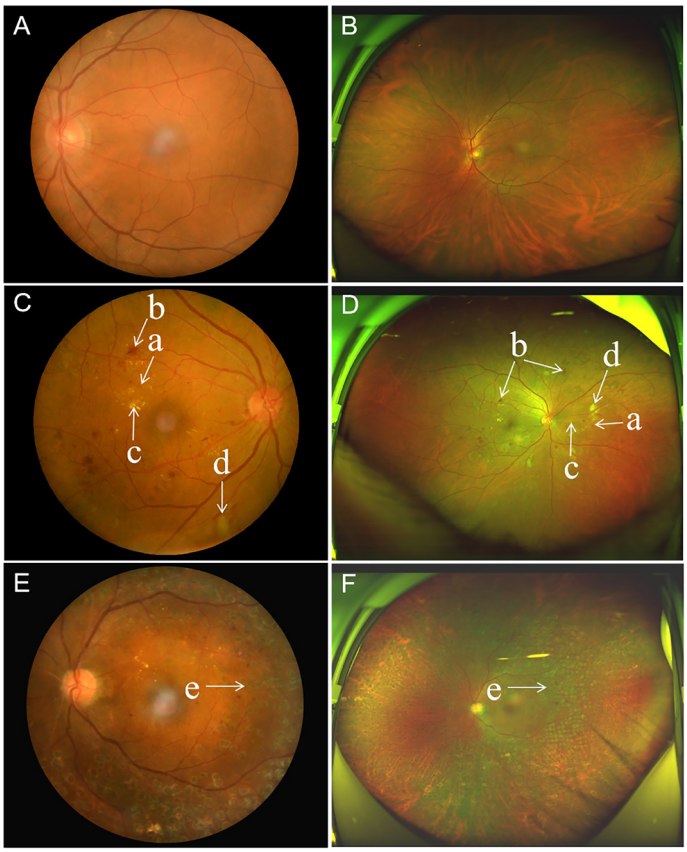

FIGURE 4
Comparison of paired standard 45° fundus photographs and ultra-widefield photographs in three diabetic patients. **(A, B)**, Standard 45° fundus photograph and ultra-widefield photograph from the left eye of the same patient, with no diabetic retinopathy. **(C, D)**, Standard 45° fundus photograph showing microaneurysms, hard exudate, cotton wool spots and dot-blot retinal hemorrhages from diabetic retinopathy in the posterior pole, and accompanying ultra-widefield photograph from the same eye showing more retinal lesions in the periphery. **(E, F)**, Standard 45° fundus photograph showing an eye with diabetic retinopathy that has undergone panretinal laser photocoagulation, and the accompanying ultra-widefield photograph from the same eye showing the peripheral extent of the laser photocoagulation scars. **(a)** microaneurysms, **(b)** hemorrhage, **(c)** hard exudate, **(d)** cotton wool spots, and **(e)** photocoagulation scars.

that OCTA can detect microaneurysms that are not detectable by FA (121, 122). Meanwhile, OCTA can also detect intraretinal microvascular anomalies (IRMAs), neovascularization of the disc (NVD), and neovascularization elsewhere (NVE) in intraretinal and extraretinal neovascularization with excellent reliability (Figure 5E, F) (121, 123). Not only does OCTA provide better detection of IRMAs and neovascularizations compared to FA and CFP, but it also allows for better morphologic characterization of IRMA and NV, because of the absence of late dye leakage. Meanwhile, both widefield OCTA and UWFA have been compared and applied in patients with DR. One study suggested that widefield OCTA had a higher detection rate of capillary non-perfusion areas than ultrawide field fluorescein angiography (124). One research group has proposed a new staging system for DR based on wide-field swept-source OCTA. This classification uses various retinal vascular and structural features to define various disease stages including no DR, subclinical DR, non-proliferative DR, pre-proliferative DR, PDR, and tractional retinal detachment (125). Naturally, such classification systems will need to be validated and refined over time, and new technological advances in OCTA technology will also influence these modifications.

Artificial intelligence and deep learning

Artificial intelligence (AI) was originally proposed in 1956, as a field of study looking to develop computer methods to simulate human intelligence and perform complex cognitive tasks. Deep learning (DL) is a subset of AI, which is designed to mimic neural networks in the human brain, enabling systems to cluster and learn from unstructured data, using this make classification decisions and predictions with incredible accuracy. Today, DL has been widely used in various medical and clinical

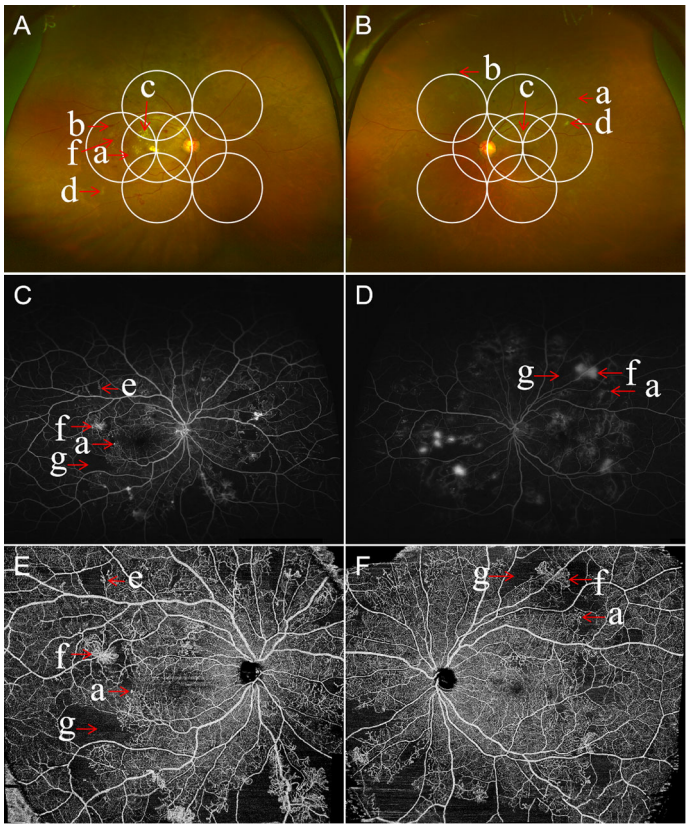

FIGURE 5
Multimodal images of proliferative diabetic retinopathy in both eyes of the same patient. Ultra-wide field (UWF) retinal images with the ETDRS 7-feld, 30-degree fundus images in circles outlined in white. The UWF fundus imaging of right eye **(A)** and left eye **(B)** showing retinal hemorrhages, microaneurysms, hard exudates, cotton wool spots, abnormal vascular loop, intraretinal microvascular abnormalities (IRMA), and retinal neovascularization. The ultra-widefield fluorescein angiography of right eye **(C)** and left eye **(D)** illustrating the corresponding hyperfluorescent dots of microaneurysms, areas of capillary non-perfusion, and multiple small areas of neovascularization identified by the hyperfluorescent leakage of dye. Corresponding wide field swept-source optical coherence tomography angiography (WF SS-OCTA) of right eye **(E)** and left eye **(F)** exhibiting area of non-perfusion, abnormal vascular loop, IRMA, and retinal neovascularization. **(a)** microaneurysms, **(b)** hemorrhage, **(c)** hard exudates, **(d)** cotton wool spots, **(e)** IRMA, **(f)** retinal neovascularization, and **(g)** areas of retinal ischemia.

settings. Particularly within ophthalmology, AI using DL has been adopted by a variety of groups to develop algorithms for automated DR diagnosis and screening. Supplementary Table 1 provides a summary of the AI systems in detection of DR using fundus photographs, UWF fundus images, and OCTA images.

The first DL algorithms for automated DR detection were developed by Gulshan et al. in 2016 (126), and Ting et al. in 2017 (127). These algorithms used standard CFP images as input. Both groups demonstrated that the algorithms had high diagnostic accuracy, with areas under the receiving operating characteristic curves of more than 0.9 on independent datasets. Since then, numerous DL algorithms have been developed for this purpose, and there are multiple that have already received regulatory approval, and are in clinical use. For example, IDx-DR (IDx LLC, Coralville, IA, USA) and EyeArt (Eyenuk, Inc., Woodland Hills, CA. USA) have received USA Food and Drug Administration approval (128, 129), while SELENA+ (EyRIS, Singapore) has received European CE Mark approval. In terms of DL for other imaging modalities, Cheung et al. recently developed an effective DL algorithm for DR detection on UWF images, using a dataset of 9,392 images from 4 different countries (130). As for OCTA, Ryu et al. evaluated the role of DL in diagnosing DR in OCTA images (131). Their DL model could achieve an overall accuracy, sensitivity, and specificity of 91-98%, 86-97%, 94-99%. Automated analysis of different imaging modalities with AI and DL is now possible, and validation and implementation of these algorithms is likely to greatly improve and optimize the efficiency and of DR screening and diagnosis.

AI and DL could feature in a new DR classification system in a few ways. First, if AI-based automated grading is equivalent or better than human graders in terms of accuracy and reproducibility, then a new DR classification system could accept AI-based grading for use in research and clinical practice. Second, AI could be used to optimize or improve

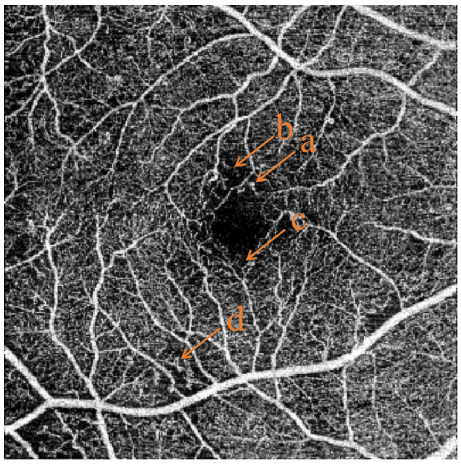

FIGURE 6
Common features of OCTA in non-proliferative diabetic retinopathy. **(A)** microaneurysms, **(B)** capillary non-perfusion area, **(C)** slightly enlarged foveal avascular zone, **(D)** abnormal vascular loops.

prognostication of patient outcomes, over and above existing risk stratification methods. This may be through image analysis, or through the addition or inclusion of multimodal clinical data as well. Third, if classification systems become more quantitative, AI could be used to automate the lesion quantification and counting processes. Nevertheless, significant barriers still remain in this area. Developing and validating robust AI algorithms requires good longitudinal datasets. As new imaging modalities are developed or included, we would need new large datasets of these images, linked to outcomes of interest, in order to develop these AI models. Explainability and clinician acceptance of AI models in clinical practice is also an area that can be improved.

Quantitative assessment of diabetic retinopathy

Current DR classification systems are semi-quantitative and categorical. For example, in the ETDRS, DR lesions such as H/Mas or IRMAs are graded individually based on their severity, which is based on comparison against reference standard photographs. The more lesions such as H/Mas that an eye has, the greater the severity, but the severity is divided into a few severity categories, and is not a continuous quantitative scale. The classification systems were designed this way, because it was not practical at the time to individually count lesions for classification. However, it is possible that objective quantification of lesions and other biomarkers, such as OCTA vessel density or UWFA ischemic areas may provide more accurate disease evaluation and better prediction of treatment response.

For example, Sadda et al. demonstrated that quantitative assessment of DR lesions on UWF images identified new risk factors for DR progression, such as hemorrhage surface area or distance of hemorrhages from the optic nerve head (132). Sears et al. compared subjective and quantitative methods of determining PPLs and the distribution of DR lesion in UWF images, and found that objective quantitative assessment of DR was more accurate. On UWFA (133), Sun et al. analyzed quantitative parameters related to leakage, ischemia and microaneurysm counts, and found that they were strongly associated with DR severity (134), as well as PDR and DME (135). On OCTA, Alam et al. characterized quantitative OCTA features of NPDR and observed that quantitative OCTA metrics such as blood vessel density could be effective for quantification and staging of NPDR (136).

With AI, automated quantification of relevant parameters and metrics from retinal photographs and other imaging modalities is now possible. Quantitative assessment and staging may provide more accurate prognostication for DR outcomes, but this will need to be validated and evaluated in future studies. Also, there are multiple different imaging techniques that can be analyzed quantitatively in DR, and standardization of quantitative method is likely to be important going forward.

Response to new treatments

Our DR classifications at present are all based on grading the presence and severity of visible retinal lesions and photographic appearance. Up until the last decade, the mainstay of treatment for

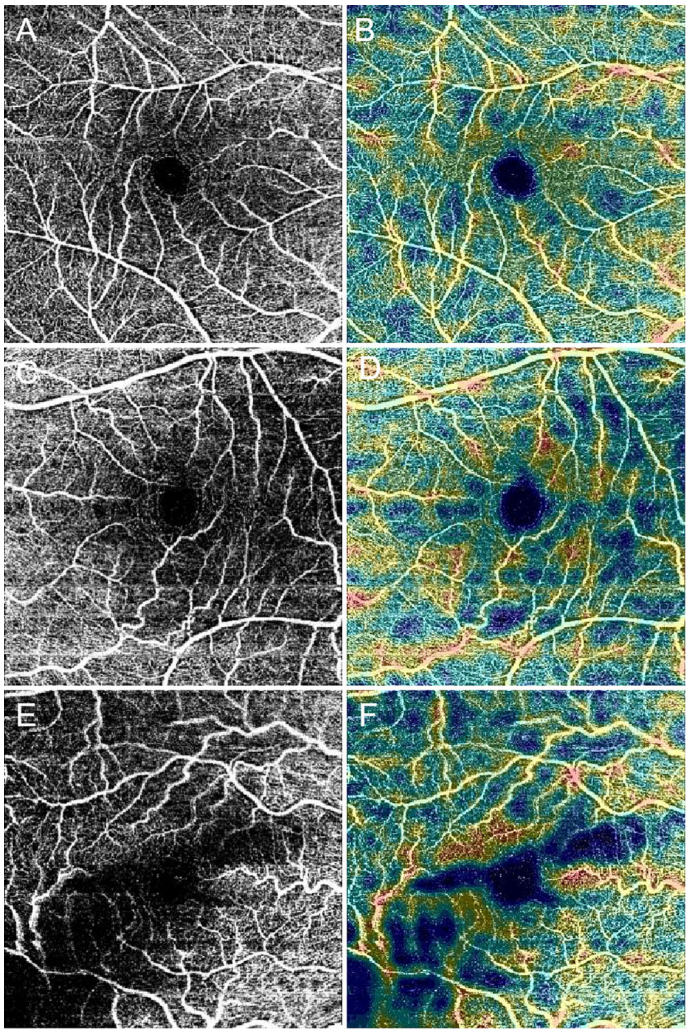

FIGURE 7
Optical coherence tomography angiography (OCTA) images present the foveal avascular zone, macular capillary nonperfusion and vessel density in diabetic patients. Black and white scans **(A**, **C**, and **E)** represent OCTA angiograms. Color map scans **(B**, **D**, and **F)** represent color-coded vessel density in the corresponding OCTA angiograms. With worsening diabetic retinopathy severity level, the foveal avascular zone diameters increase, and the non-perfusion area and the vessel density decrease in these images. **(A, B)**, No diabetic retinopathy. **(C, D)**, nonproliferative diabetic retinopathy (NPDR). **(E, F)**, Proliferative diabetic retinopathy (PDR).

DR was PRP, to reduce the risk of progression to PDR, and therefore to reduce the risk of severe visual loss. After successful PRP, characteristic DR lesions such as H/Mas and neovascularization tend to regress, and our existing DR classification systems cannot be formally applied to prognosticate such eyes that have undergone disease-modifying treatment. There was no strong need to develop a formal classification for such post-PRP eyes, as the effect of PRP in reducing retinal ischemia was persevering and long-lasting. However, this is no longer true with new treatments that we have for DR and DME now. Treatments such as intravitreal anti-VEGF and corticosteroid injections, are known to modify the appearance of the fundus in patients with DR (137–140). Many patients show "improvement" in DR severity scales if these DR lesions regress. However, none of these therapies effectively address the underlying problem of retinal ischemia (124). Thus, the disease tends to recur or progresses rapidly after stopping treatment. Current classification systems may not be applied to accurately prognosticate these post-treatment eyes, and so this is a major need to be addressed in a new classification.

Current classification systems are also based primarily on progression to PDR, which used to be the major cause of visual loss in DR. However, DME is now the leading cause of visual impairment in DR, and there are effective treatments for DME (141). Furthermore, up to 40 to 50% of eyes with DME do not respond fully to anti-VEGF treatment (142), and it has been suggested that different DME phenotypes determined by OCT

appear to have different prognosis and responsiveness to treatment (143). Therefore, an effective updated classification system should also include risk stratification and severity gradings for DME.

Conclusion

Diabetic retinopathy is a complex, multifactorial disease, and our understanding of this disease is constantly evolving. Over the years, our DR classification systems have gone through various iterations, and have had to be modified and updated to keep up with our understanding of the disease, and with technological advancements. Though our current ETDRS and ICDR severity scales have provided the foundation for major research trials and modern clinical management of DR, it is time for an update. The significant advances that have been made over the past few decades in disease pathophysiology, imaging technology, artificial intelligence and treatment, must inform a new classification system. New DR classification systems should be based on available evidence and robustly validated, and will hopefully translate to better outcomes and managements for the millions of patients with DR worldwide.

Author contributions

All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1079217/full#supplementary-material

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Diabetic retinopathy: Looking forward to 2030

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Diabetic retinopathy (DR) is the major ocular complication of diabetes mellitus, and is a problem with significant global health impact. Major advances in diagnostics, technology and treatment have already revolutionized how we manage DR in the early part of the 21st century. For example, the accessibility of imaging with optical coherence tomography, and the development of anti-vascular endothelial growth factor (VEGF) treatment are just some of the landmark developments that have shaped the DR landscape over the last few decades. Yet, there are still more exciting advances being made. Looking forward to 2030, many of these ongoing developments are likely to further transform the field. First, epidemiologic projections show that the global burden of DR is not only increasing, but also shifting from high-income countries towards middle- and low-income areas. Second, better understanding of disease pathophysiology is placing greater emphasis on retinal neural dysfunction and non-vascular aspects of diabetic retinal disease. Third, a wealth of information is becoming available from newer imaging modalities such as widefield imaging systems and optical coherence tomography angiography. Fourth, artificial intelligence for screening, diagnosis and prognostication of DR will become increasingly accessible and important. Fifth, new pharmacologic agents targeting other non-VEGF-driven pathways, and novel therapeutic strategies such as gene therapy are being developed for DR. Finally, the classification system for diabetic retinal disease will need to be continually updated to keep pace with new developments. In this article, we discuss these major trends in DR that we expect to see in 2030 and beyond.

KEYWORDS

diabetic retinopathy, future trends and predictions, epidemiology, pathophysiology, imaging modalities, artificial intelligence, new treatments, classification and staging system

1 Introduction

Diabetic retinopathy (DR) is the major ocular complication of diabetes mellitus, and occurs in about 30 to 40% of diabetic individuals (1, 2). Globally, more than 100 million individuals are living with DR, and DR is a leading cause of blindness and visual impairment, especially among the working-age adult population (1, 3). Fortunately, much of the visual loss from DR is preventable, and the rates of vision loss from diabetes and DR have steadily declined over the past few decades (4, 5). Such improvements in visual outcomes for DR are multifactorial, and are due in large part to a combination of better systemic risk factor control, coupled with advances in ocular disease assessment, screening, imaging and treatment in recent years. For example, the universal adoption of DR classification systems such as the Early Treatment of Diabetic Retinopathy Study (ETDRS) and International Classification of Diabetic Retinopathy (ICDR) severity scales that effectively prognosticate the risk of disease progression, coupled with large-scale DR screening programs around the world, have allowed for appropriate surveillance and early intervention to prevent the onset of vision-threatening complications (5–7). Panretinal laser photocoagulation (PRP) helps to prevent severe vision loss due to proliferative DR (PDR), and the introduction of pattern scan laser (PASCAL) has made the procedure quicker, easier to perform, and more comfortable for patients (8–10). The widespread availability and use of non-invasive imaging such as optical coherence tomography (OCT), together with the introduction of intravitreal anti-vascular endothelial growth factor (anti-VEGF) treatments have revolutionized the assessment and treatment of diabetic macular edema (DME), and dramatically improved visual outcomes for this complication of DR (11–13). Surgical outcomes for tractional retinal detachments and diabetic vitrectomies have also improved over the years, with the availability of more advanced instrumentation and surgical adjuncts such as pre-operative anti-VEGF injections (14–16).

Despite the tremendous progress that the field of DR has already seen, there are yet more exciting advances being made. Looking forward over the next decade, many of these ongoing developments are likely to further transform the clinical and research landscapes. In this article, we review some of the recent progress that has been made, and suggest how these developments may continue to shape the field in 2030 and beyond.

2 Shifts in epidemiology and disease burden

The global prevalence and disease burden of DR is expected to increase significantly over the next few decades, from about 103 million individuals in 2020, to 130 million in 2030, and 161 million in 2045 (17). Such projections are due to a variety of factors, including the increasing prevalence of diabetes around the world, lifestyle changes, and increasing lifespans and aging global populations (17). This sharp increase in DR disease burden by more than 25% in just 10 years, is likely to further strain healthcare systems and resources that are already stretched. The economic costs associated with DR and its complications are substantial. Direct healthcare costs related to DR in the USA were estimated at $493 million per year in 2004 (18). More recent data is lacking, but it is notable that these estimates were arrived at prior to the introduction of anti-VEGF treatment for DME. Subsequent studies have found that economic costs are significantly higher for patients with DME than without, and much of this is due to the need for costly anti-VEGF treatment (19, 20). Global prevalence of DME is also projected by increase by about 25%, to about 24 million individuals by 2030 (17). The resultant increase in healthcare costs are expected to be staggering.

Perhaps just as important as the overall increase in disease burden, is the projected pattern of increase. Based on epidemiologic projections to 2030, the rates of increase in DR prevalence for traditionally high-income regions such as North America and Europe appear to be relatively low, ranging from 10.8 to 18.0%. In contrast, the rates of increase in middle- and low-income regions such as the Western Pacific (WP), South and Central America, Asia, Africa, the Middle East and North Africa (MENA) are much higher, ranging from 20.6% to as high as 47.2%. In absolute terms, the largest increases by far are projected to occur in MENA, and WP, where the numbers of individuals with DR are expected to rise by more than 6 million in each region respectively (17). This geographic shift in disease burden towards Asia, Africa and WP means that global health strategies to combat DR will need to pivot to follow the shifting disease demographic. Healthcare resources for DR screening, diagnosis, follow-up, and treatment are urgently needed in these areas. Large-scale systematic, rather than opportunistic, DR screening programs that target all patients with diabetes in these regions will allow for early detection and intervention, will be cost-effective, and will reduce rates of vision loss, but they require significant investment in infrastructure and time to set up (21–24).

3 Non-vascular aspects of diabetic retinal disease

The clinically-visible retinal lesions associated with DR, such as microaneurysms, hemorrhages and hard exudates, are primarily the result of retinal microvascular damage. Consequently, the focus on DR pathophysiology, diagnosis and assessment has traditionally always centered around the vascular

aspect of the disease. However, with the availability of better structural retinal imaging modalities and functional assessments, evidence has accumulated over the years of significant retinal neural dysfunction as well, which occurs together with, or in some cases precedes, the development of vascular abnormalities. These structural and functional changes have collectively been termed "diabetic retinal neurodegeneration" (DRN) (25–28).

OCT studies have shown that patients with diabetes demonstrate significant thinning of the inner retinal layers, including the retinal nerve fiber layer (RNFL), and ganglion cell layer (GCL) (26, 29–31). Retinal thinning is progressive over time, and can precede the development of clinically-visible DR lesions (26, 30). Histological studies on enucleated eyes also corroborate these findings, showing reductions in retinal ganglion cell density in eyes with DR (32). Functional assessments in diabetes reveal reductions in contrast sensitivity, visual field defects, electrophysiologic deficits, and impaired pupillary responses (33–38).

Despite the clear evidence of DRN occurring in diabetic retinal disease, there remain many important unanswered questions in this area. What is the prognostic significance of DRN in terms of ocular or systemic outcomes in diabetes? What is the functional impact of DRN on quality of life? How and when should DRN be assessed and quantified? Current OCT studies on DRN measure different retinal layers (e.g. RNFL, GCL), and in different, non-standardized locations. Functional assessments such as electrophysiology, visual field perimetry and pupillometry are often time-consuming and resource-intensive. Recently, a portable, handheld chromatic pupillometer was shown to able to provide rapid, clinic-based assessment of retinal neural function in diabetes (38). Such findings, however, need to be replicated and validated in larger cohorts. There is also much ongoing work to determine the prognostic impact of DRN, and to incorporate such assessments of DRN into routine DR classification and staging systems (28, 39, 40). These efforts are likely to change the way we routinely assess and manage DR in the next few decades.

4 New imaging modalities and biomarkers

New imaging modalities such as ultra-widefield (UWF) retinal imaging and OCT angiography (OCTA) have been available for research and commercial clinical use for a number of years now. UWF retinal imaging provides a field of view of about 110° to 220°, and allows for visualization up to at least the anterior edge of the ampullae of the vortex veins (41). These platforms can be used for UWF color or pseudocolor photography (UWFCP), as well as UWF fluorescein angiography (UWFFA). UWF imaging platforms are non-contact and often do not require pupillary mydriasis, but their most important advantage, is that they provide for assessment of the retinal peripheries, and overall a much larger retinal surface area than standard color fundus photography (CFP). With standard CFP, the typical 7 standard ETDRS fields cover only about 30% of total retinal surface area (39, 42). In contrast, UWF imaging systems allow for assessment of approximately 80% of retinal surface area, which is a major advantage (42).

Assessment of the retinal peripheries with UWF images in DR has significant prognostic and management implications. For one, inclusion of the peripheries in UWFCP images results in a greater DR severity level in 10 to 19% of eyes (43–46). Furthermore, studies from a longitudinal cohort showed that various peripheral DR lesions, such as predominantly peripheral lesions (PPLs), and number, surface area, and distance of hemorrhages/microaneurysms and cotton-wool spots from the optic nerve head, were independently associated with greater risk of progression to PDR (47, 48). However, the prospective longitudinal Diabetic Retinopathy Clinical Research Network (DRCR.net) Protocol AA study recently concluded that PPLs in UWFCP images were not correlated with DR worsening, whereas PPLs and non-perfusion on UWFFA were (49, 50). Unfortunately, UWFFA has some major drawbacks that limit its universal use in all DR patients, including the need for invasive dye administration, time needed for acquisition, and the need for tertiary specialist interpretation. At present, the ideal modality for peripheral assessment and the best way to do so in DR remain unclear. Nevertheless, it is clear that as we better define the role of the retinal peripheries, UWF imaging platforms are sure to play an important role in DR assessment and management over the next decade.

OCTA is another imaging platform that will be increasingly important in DR assessment and prognostication. OCTA is a non-invasive, non-contact system that can provide angiographic information without the need for invasive dye administration like fluorescein. Other advantages of OCTA over dye-based fluorescein angiography are better visualization of the capillary microvasculature, and depth-resolved segmentation of the superficial, middle and deep capillaries plexuses, which are differentially affected in diabetes and DR (51–53). OCTA can provide quantitative metrics relating to the retinal microvasculature, and many of these, such as lower vessel density, lower fractal dimension, greater tortuosity, and greater foveal avascular zone area, have been associated in cross-sectional studies with greater DR severity (51–55). The impact of such cross-sectional associations in clinical practice is limited, but the major impact from OCTA will be realized when such OCTA metrics are eventually linked to clinical outcomes of interest on longitudinal studies. At present, longitudinal prospective OCTA studies are limited, but hopefully this need will be addressed in the next few years (56–59). Other barriers to widespread adoption and clinical impact of OCTA include scan quality and gradability, as well as the use of multiple different commercial OCTA machines, with proprietary algorithms and

quantitative metrics that are not standardized or interchangeable between devices. As these barriers are addressed, it is likely that OCTA will become a powerful, non-invasive prognostic tool for clinical assessment in DR.

5 Artificial intelligence

Artificial intelligence (AI) and deep learning (DL) algorithms will play an increasingly important role over the next decade in the areas of medical diagnostics, screening, prognostication, and assisting with management or treatment decisions. Ophthalmology has been a leader in developing AI algorithms for clinical use, and automated diagnosis or detection of DR from CFP images was one of the first use cases developed, from as early as 2016 (60–62). Initial studies already demonstrated that AI algorithms developed on large datasets could reach very high levels of diagnostic performance for detection of referable DR and vision-threatening DR (61, 62). About 5 years later, there are now multiple AI-based systems for DR screening that have been approved for clinical use. IDx-DR (IDx LLC, Coralville, IA, USA) and EyeArt (Eyenuk, Inc., Woodlands Hills, CA, USA) have both received approval by the USA Food and Drug Administration (FDA), and are already in clinical use (63, 64). SELENA+ (EyRIS Pte Ltd, Singapore) has received European CE Mark Approval, and is planned to be deployed as part of the national DR screening program in Singapore soon. An economic modelling study suggested that incorporation of such an AI algorithm as an assistive tool in a large scale DR screening program will be associated with significant cost savings (65). It is likely that by 2030, we will see AI algorithms routinely deployed in many large-scale DR screening programs around the world, either as fully autonomous systems, or in hybrid systems where the algorithms function as assistive tools (65). However, there are still some challenges that need to be overcome for widespread acceptance of large-scale AI screening systems. Retinal images frequently contain signs of other ocular or systemic diseases besides DR, and the medicolegal aspects of this are still uncertain. IDx-DR, for example, only detects DR, and the FDA approval for its use clearly states that the algorithm does not diagnose any other ocular disease. Other AI-based systems take a different approach to this; SELENA+ detects DR, as well as 2 other major eye diseases – age-related macular degeneration and glaucoma (62). Poor image quality can also adversely affect the accuracy of such algorithms, but most commercial AI systems now have in-built automated image quality assessments (62, 63).

Beyond just diagnosis and screening of DR, there are other potential use cases for AI algorithms that are also being developed. AI-based detection of DME from CFP images is promising, and could help to improve and reduce false positive referral rates from DR screening programs (66). Some imaging modalities such as OCT and OCTA have in-built software and segmentation algorithms that provide quantitative parameters, such as central subfield thickness (CST) in OCT, or capillary vessel density in OCTA. However, the capability of these automated software algorithms to provide detailed quantitative information is limited to a few parameters, and is dependent on the accuracy and resolution of automated segmentation. Using AI to improve retinal layer segmentation and to provide precise quantification of fluid volumes in different fluid compartments could have major impact in terms of prognostication, and guiding treatment decisions for DME (67–71). Similarly, there has been a shift in emphasis towards quantitative assessment in modalities that are typically assessed qualitatively or categorically, such as number, size and location of retinal vascular lesions on CFP or UWFCP images, or areas of retinal non-perfusion on UWFFA images (48, 50, 72–74). Manual grading and assessment of these quantitative parameters would be impractical, and AI algorithms for automated quantification will go a long way to making such quantitative parameters accessible, and clinically useful. Finally, the use of AI to process multimodal clinical and imaging data in DR, to provide more accurate prognostication of long-term outcomes, such as visual outcomes, risk of developing incident DME, and anti-VEGF treatment burden in DME, is an exciting area to look forward to (75).

6 New treatment strategies

Intravitreal anti-VEGF therapy is the established first line treatment for center-involved DME, and has also been shown to be a valid treatment option for PDR (12, 76, 77). Observations from the registration trials for anti-VEGF therapy in DME showed that anti-VEGF therapy can also result in significant improvements in DR severity for patients with non-proliferative DR, and this has been confirmed in more recent prospective clinical trials as well (78–81). As a result, intravitreal aflibercept is now FDA-approved for treatment of non-proliferative DR, as well as PDR and DME. However, at this point, it seems unlikely that anti-VEGF therapy will be used on a large scale for routine treatment of non-proliferative DR. The DRCR.net Protocol W trial showed that anti-VEGF therapy for non-proliferative DR could prevent the onset of PDR and DME, but that final visual outcomes were no different from a strategy of initial observation, with treatment for PDR or DME initiated as-needed (81). Furthermore, while anti-VEGF therapy results in regression of vascular lesions and apparent “improvement” in DR severity, reports show that the underlying retinal ischemia is unchanged, and that lesions and retinopathy often recur rapidly after cessation (82, 83). Finally, the cost-effectiveness of treating non-proliferative DR with regular anti-VEGF therapy has not been well-examined, but it is difficult to imagine widespread use outside of high-resource clinical settings.

Instead, new treatments that are more likely to have significant impact on the DR landscape over the next decade are those targeting new pathophysiologic pathways, and those

that improve the durability of treatment effect. For example, faricimab is a bi-specific monoclonal antibody that provides dual inhibition of both the VEGF and the angiopoietin (Ang) and tyrosine kinase with immunoglobulin-like and epidermal growth factor homology domains (Tie) pathways (84, 85). Inhibiting Ang-2 on top of VEGF-A is thought to provide a synergistic effect, with better vascular stability and reduction in vascular leakage (84). The recent phase 3 YOSEMITE and RHINE clinical trials demonstrated that intravitreal faricimab for DME provided substantial visual gains comparable to aflibercept, but with superior anatomic outcomes. More importantly, faricimab had a durable treatment effect, with more than 70% and 50% of eyes reaching dosing intervals of every 12 to 16 weeks, and 16 weeks respectively at 1 year (85). Other promising treatment strategies to provide increased durability and reduced treatment burden include high-dose aflibercept (8 mg), sustained delivery of ranibizumab through a refillable port delivery system (PDS), and gene therapy with agents such as RGX-314 and ADVM-022 for long-term VEGF suppression (86–89). By providing more durable treatment effect, these approaches aim to address real unmet needs in DME treatment, where high treatment burden, problems with compliance to therapy, and under-treatment limit real world visual outcomes (90–93). These treatment approaches will play a major role in DME management in the near future.

7 An updated classification system for diabetic retinal disease

As a consequence of these many exciting advances in the field of DR over the past few decades, our DR classification and severity staging systems need to be updated to keep pace with the latest developments (39, 40, 94). The ETDRS and ICDR severity scales that are in routine use have made tremendous impact to research trials and clinical management, but they are now 2 to 3 decades old, and have significant limitations (7, 95). Some of the key areas that need to be addressed in an updated classification system are: (1) Inclusion of relevant prognostic information from the retinal peripheries that can now be reliably imaged with UWF systems, (2) Recognition and assessment of non-vascular aspects of diabetic retinal disease, such as retinal neural dysfunction or DRN, (3) Incorporating information and biomarkers from available imaging modalities such as OCT and OCTA, (4) Greater emphasis on, and clinically-relevant severity classification for DME, which is now the most common cause of visual impairment from DR, and which drives management decisions, and (5) Accurate prognostication of eyes that have undergone intravitreal anti-VEGF or other treatments.

There are major international efforts ongoing to update the DR classification system, such as the Diabetic Retinal Disease Staging System Update Effort, a project which is part of the Mary Tyler Moore Vision Initiative, which brings together leading scientists and experts on DR, with the overall aim of preventing vision loss from diabetes (94). There are still many gaps and unmet needs in the literature that need to be addressed, to inform a robust, evidence-based updated classification system. Nevertheless, it is likely that we will see a new and improved DR classification and staging system soon, that will have major impact on how we practice and manage DR in 2030. Such a classification system will no doubt need to be validated, regularly reviewed, and further updated to keep pace with new developments in the field. Furthermore, various widely-used international DR management guidelines, such as those by the International Council of Ophthalmology (ICO), will also need to be updated in accordance with new classification systems (76).

8 Conclusion

Clearly, many important strides have been made in the field of DR over the past few years, which will shape and transform the clinical and research landscapes in the years to come. Here, we have attempted to anticipate and predict some of these trends that are likely to be influential over the next decade. While many of these new imaging, assessment and treatment modalities have the potential to significantly improve clinical outcomes in DR, it is important that these advances are translated equally to both high- and low-resource settings around the world. As we have discussed above, epidemiologic projections suggest a continued shift towards increased disease burden in low-resource settings, and advances in DR management must be accessible to these patient populations, if we hope to see continued reductions in the rates of visual loss and blindness from DR in 2030 and beyond.

Author contributions

T-ET and TYW both contributed to conception of the study, and drafting and revising of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

TYW is a coinventor, with patents pending, for a deep learning system for diabetic retinopathy, glaucoma, and age-related macular degeneration (SG Non-Provisional Application number 10201706186V), and a computer-implemented method for training an image classifier using weakly annotated training data (SG Provisional Patent Application number 10201901083Y), and is cofounder and shareholder of EyRIS Pte Ltd, Singapore.

The remaining author declares that the research was conducted in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prophylactic intravitreal injection of aflibercept for preventing postvitrectomy hemorrhage in proliferative diabetic retinopathy: A randomized controlled trial

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Introduction: The aim of this study was to assess the effects of preoperative intravitreal aflibercept (IVA) injection on the incidence of postoperative vitreous hemorrhage (VH) after vitrectomy for proliferative diabetic retinopathy (PDR).

Methods: This study involved a prospective, randomized clinical trial. One hundred twenty-eight eyes of 128 patients of PDR who underwent pars plana vitrectomy (PPV) were enrolled. Sixty-four eyes were assigned randomly to either the IVA group (IVA injection 1 to 5 days before PPV) or the control group (no IVA injection). The primary outcome was the incidence of VH at 1 month after PPV. Secondary outcome measures were best-corrected visual acuity (BCVA) changes from baseline to at 1 week, 1 month, 2 months, and 3 months after surgery.

Results: The VH incidences in the IVA group and the control group were 14.8 and 39.3% at week 1, 8.6 and 31.7% at month 1, 11.7 and 30.5% at month 2, and 8.6 and 30.5% at month 3, respectively. Intergroup differences showed a significantly decreased VH rate in the IVA group compared with that in the control group at week 1, month 1, and month 3 ($p = 0.021$, 0.006, and 0.047, respectively). Compared to the baseline, neither the mean BCVA nor the BCVA change in the Logarithm of the Minimum Angle of Resolution (logMAR) scale did differ significantly between the two groups at each visit point. There are a greater number of eyes with BCVA improvement of more than 2 logMAR in the IVA group than in the control group at week 1 (8 vs. 2, $p = 0.048$).

Conclusions: This study found that the adjunctive use of preoperative IVA reduces early and late postoperative VH in vitrectomy for PDR.

KEYWORDS

aflibercept, vitreous hemorrhage, vitrectomy, diabetic retinopathy, post-vitrectomy hemorrhage

Introduction

Patients with diabetic retinopathy (DR) are becoming more and more predominant in many countries with the increasing prevalence of diabetes worldwide. Diabetic retinopathy is the leading cause of vision loss in patients with diabetes. The standard and effective surgical treatment for vitreous hemorrhage (VH) and tractional retinal detachment (TRD) for proliferative diabetic retinopathy (PDR) is pars plana vitrectomy (PPV). Although the anatomical success rate of vitrectomy for PDR is good, it can have a few postoperative complications sometimes. Postvitrectomy VH is one of the most common complications after vitrectomy in PDR and has been reported with success rates ranging between 17 and 75% (1–6). Patients may have delayed visual rehabilitation because of postvitrectomy VH due to obscuration of the fundus, and postvitrectomy VH may hinder the monitoring of the disease course and/or create the need for additional application of laser treatment.

The level of vascular endothelial growth factor (VEGF) is elevated in the retina of patients with diabetes (7, 8). A previous study reported that a higher VEGF level in the vitreous humor during the primary vitrectomy could be a risk factor for early postoperative VH and neovascular glaucoma (NVG) in patients with PDR (9). Recently, many studies reported that the preoperative vitreous injection of an anti-VEGF agent can reduce VH after PPV for patients with PDR. Most studies reported that providing intravitreal bevacizumab (IVB) injection before PPV can increase the feasibility of PPV and reduce active retinal neovascularization, intraoperative bleeding, surgical time, and postoperative VH (10–21). These findings suggest that a high VEGF level at primary vitrectomy contributes to the development of postoperative VH for PDR.

Aflibercept is a fully humanized recombinant fusion protein that has a molecular weight of 115 kDa and is made by fusing the fragment crystallizable (Fc) region of human immunoglobulin G (IgG) to the second domain of vascular endothelial growth factor receptor 1 (VEGFR-1) and the third domain of vascular endothelial growth factor receptor 2 (VEGFR-2). It can bind to not only all subtypes of VEGF but also placental growth factor (PlGF). The affinity of aflibercept for vascular endothelial growth factor-A165 (VEGF-A165) is 94 times greater than that of ranibizumab and approximately 120 times greater than that of bevacizumab. The intravitreal half-life of aflibercept is greater than those of ranibizumab and bevacizumab (4.7 vs 2.9 days and 4.3 days) (22). It has been proven to be effective in inducing retinal neovascularization regression in patients with PDR, but a well-structured prospective study about the adjunctive use of intravitreal injection of aflibercept (IVA) to reduce postoperative VH in PPV for PDR is still lacking. In this study, we aimed to assess the effect of preoperative IVA on the incidence of postoperative VH after PPV for PDR.

Methods

This was a prospective, randomized clinical trial (NCT 05478967). The study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Peking University People's Hospital. Written informed consent was signed by all participants before enrollment.

The present study enrolled patients with PDR at the Department of Ophthalmology, Xiamen Eye Center of Xiamen University, between August 2019 and March 2021. The inclusion criteria were as follows: (1) patients with type 2 diabetes mellitus (T2DM), (2) patients aged between 35 and 65 years, and (3) patients with PDR who underwent primary pars plana vitrectomy (PPV) for VH. The following cases were excluded from analysis: (1) eyes with retinal tear, (2) eyes with iris or anterior angle neovascularization, (3) eyes with intraoperative use of silicone oil, (4) eyes with choroidal or retinal disease other than PDR or any inflammatory condition, (5) eyes that underwent any previous vitrectomy or scleral buckle surgery, (6) eyes that received intraocular triamcinolone acetonide (TA) injection within 90 days before screening, (7) eyes that received intraocular anti-VEGF treatment within 60 days before screening or contralateral eyes received intraocular anti-VEGF treatment during follow-up, (8) patients who had taken aspirin orally within 7 days before screening, (9) patients who had coagulation mechanism disorder or had taken any other medicine for anticoagulant treatment, (10) patients who had cerebrovascular accident and/or myocardial infarction occurring within 180 days before screening, (11) patients with uncontrolled blood pressure (sitting position > 160/100 mmHg), (12) patients with liver or kidney dysfunction or any severe systemic disease, and (13) patients who accepted any anti-VEGF therapy for the study eye during the follow-up. If both eyes of the same patients were eligible, the eye with worse vision was included in the study.

The enrolled eyes were randomly assigned, according to the Central Randomization System, with a ratio of 1:1 to the IVA group and the control group. Patients in the IVA group received an IVA (0.5 mg/0.05 ml) injection before surgery (1 to 5 days before surgery); patients in the control group did not receive IVA injection before vitrectomy. The preoperative IVA injection was given following a standard protocol. All patients underwent 25-gauge transconjunctival sutureless vitrectomy using the 25-gauge trocar and cannula system under local anesthesia. Procedures, such as fibrovascular membrane dissection, endodiathermy, or endolaser photocoagulation, were performed with 25-gauge instruments, as required. Pan-retinal photocoagulation (PRP) was complicated as much as possible during the surgery. Intraoperative bleeding was controlled either by endodiathermy or by increasing the irrigation pressure.

Patients were examined 1 week, 1 month, 2 months, and 3 months after surgery if there were no postoperative events. If

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TABLE 1 Patient demographics and preoperative clinical findings of the two groups.

		Total	Aflibercept group	Control group	*P* value
Eyes (no.)		128	64	64	
Gender (no.)	Male	82	44	38	0.269
	Female	46	20	26	
Age (years)		54.3 ± 10.9	52.9 ± 10.5	55.3 ± 10.9	0.420
DM duration (years)		8.7 ± 5.1	8.4 ± 5.2	9.0 ± 5.0	0.503
HbA1c		6.7 ± 2.2	6.9 ± 2.8	6.5 ± 1.1	0.190
HTN	Yes	52	27	25	0.896
	No	69	35	34	
Renal dysfunction	Yes	7	5	2	0.438
	No	105	51	54	
Pre-op BCVA (LogMAR)		1.53 ± 0.81	1.49 ± 0.81	1.57 ± 0.80	0.590
IOP (mmHg)		14.8 ± 3.0	14.6 ± 2.9	14.9 ± 3.0	0.375
Lens status (no.)	Phakic	125	63	62	1.000
	Pseudophakic	3	1	2	
Previous PRP	No	48	29	19	0.110
	Partial	12	3	9	
	Complete	14	8	6	
	Cannot grade	54	24	30	
Pre-op VH grade	Mild (visible optic disc and large vessels)	30	15	15	0.268
	Moderate (only optic disc visible)	51	29	22	
	Severe (no view of the fundus)	37	16	21	
Pre-op tractional retinal detachment	No	57	29	28	0.637
	Yes	15	9	6	
	Cannot grade	56	26	30	
Location of proliferation	No	26	11	15	0.639
	Within vascular arcade	62	30	32	
	Involve equator	37	21	16	
	Beyond equator	3	2	1	
Size of proliferation	<1 PD	52	22	30	0.279
	1∼5 PD	58	33	25	
	>5 PD	16	7	9	

postoperative complications, including VH, occurred, patients were instructed to visit the clinic, regardless of the visit schedule. At each visit, any events involving the study eye between the visit schedules were recorded accordingly. At each postoperative visit, slit-lamp biomicroscopy, indirect ophthalmoscopy, and fundus photography were performed.

The primary outcome was the incidence of VH at 1 month after PPV. Secondary outcome measures were best-corrected visual acuity (BCVA) changes from baseline at 1 week, 1 month, 2 months, and 3 months after surgery. Preoperative, intraoperative, and postoperative data were collected for each patient. Preoperative data included age, sex, duration, and status of diabetes mellitus [hemoglobin A1c (HbA1c)]; the presence of other systemic diseases such as hypertension and renal function (serum creatinine); and ophthalmic parameters including best-corrected visual acuity (BCVA), intraocular pressure (IOP), lens status, previous PRP, and indication for surgery. Intraoperative data included phacoemulsification and intraocular lens (IOL) procedures, sulfur hexafluoride (SF6) or air tamponade, and the presence of fibrovascular proliferation and tractional retinal

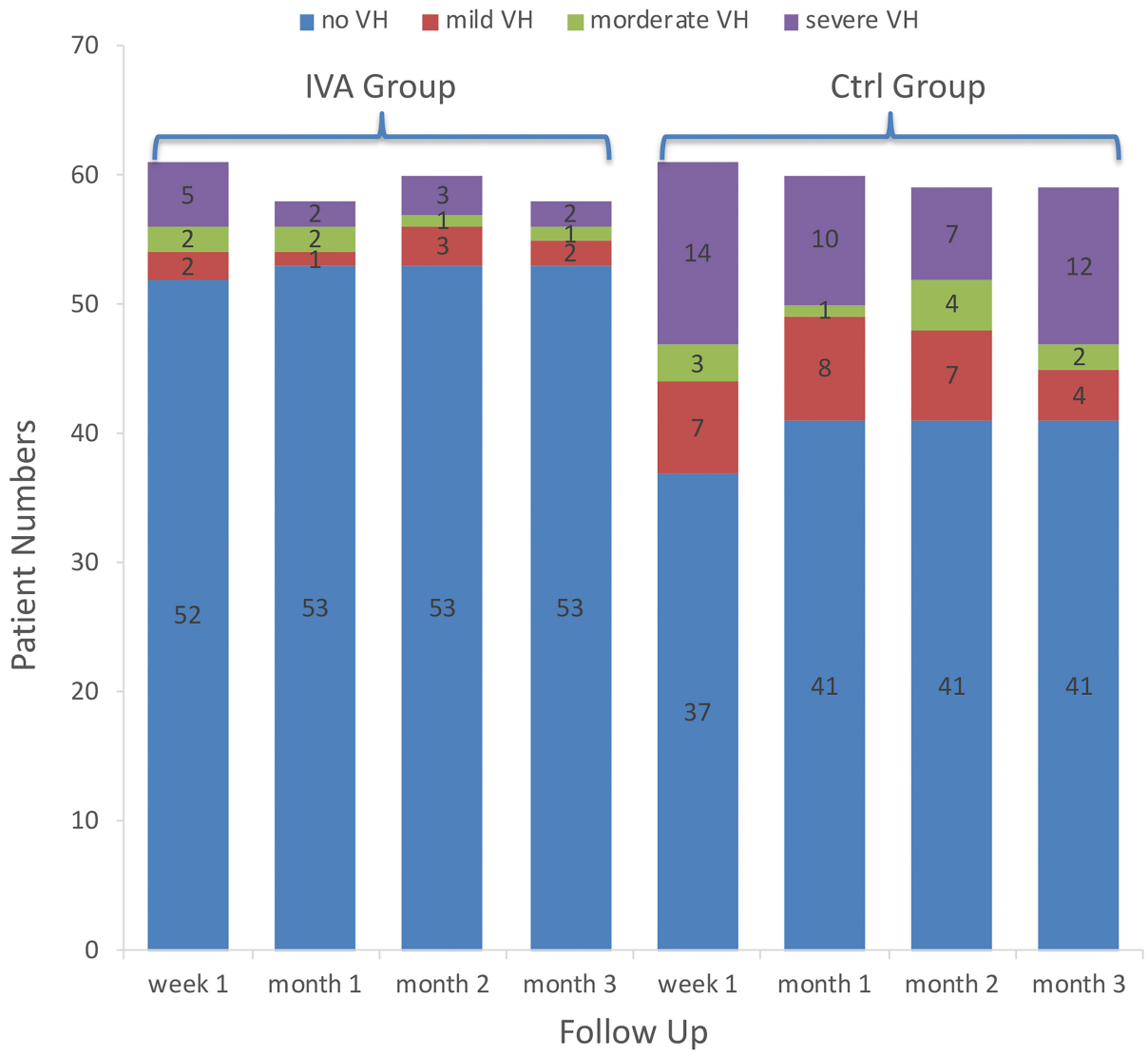

FIGURE 1
Vitreous hemorrhage grading in the two groups. The number of patients with different categories of vitreous hemorrhage grading in the preoperative intravitreal aflibercept (IVA) injection group and control group (bar with dotted pattern) during follow-up.

detachment. Postoperative data included BCVA at each visit and the number of episodes of complications. Postoperative VH was defined as a new episode of VH of grade 1 or above, occurring later than 3 days after the primary surgery and was evaluated according to the Diabetic Retinopathy Vitrectomy Study grading system. Incidences of VH at week 1, month 1, month 2, and month 3 were recorded. In the case of a gas-injected eye, complications were assessed in the region without the gas bubble. Outcome assessors were masked from the allocation of each study eye.

The present study compared baseline clinical data and postvitrectomy complications between the IVA group and the control group in patients with PDR. The chi-square test and the Mann–Whitney test were used. A *P*-value of <0.05 was considered statistically significant. All analyses were performed using SPSS 18.0.

Results

One hundred fifty-four eyes were enrolled in the study and allocated randomly into two groups: 78 eyes in the IVA group and 76 eyes in the control group. During follow-up, 26 eyes were excluded: 16 cases because of the loss of follow-up and 10 eyes because of the administration of intravitreal silicone

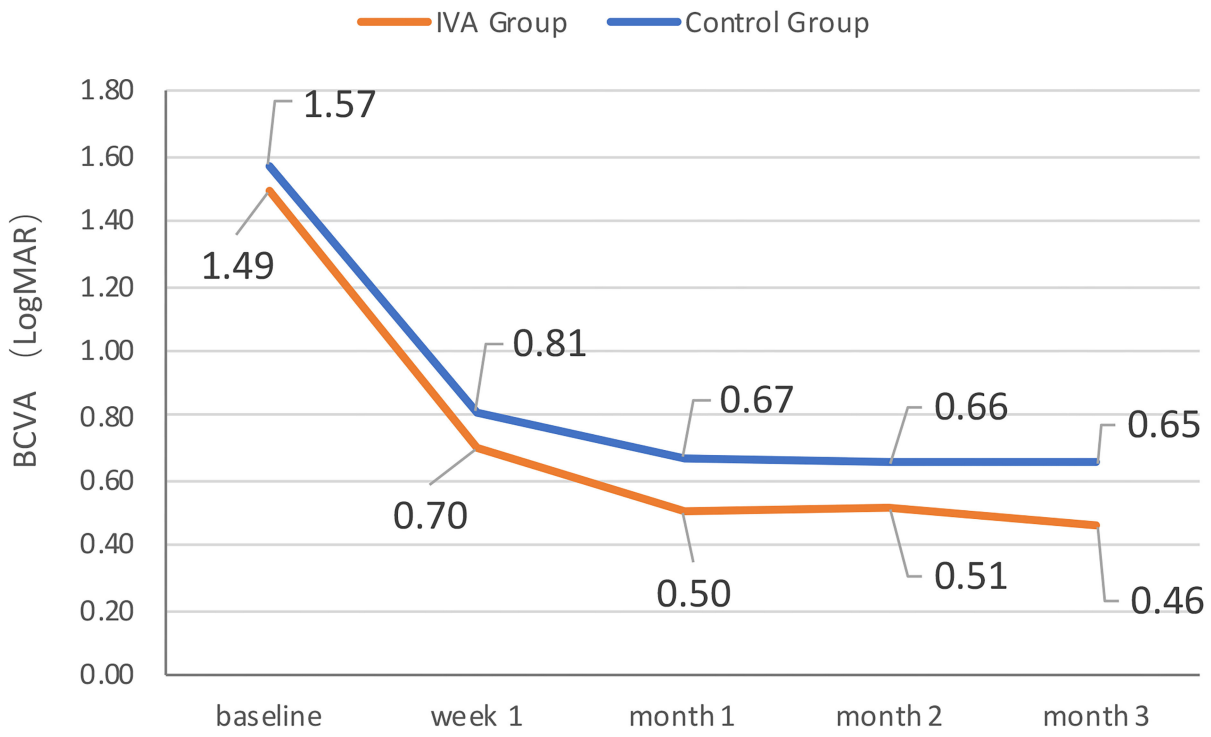

FIGURE 2
The mean best-corrected visual acuity (BCVA) at each visit in the two groups. The mean BCVA in logMAR (Logarithm of the Minimum Angle of Resolution) scale at baseline, week 1, month 1, month 2, and month 3 after surgery in the preoperative intravitreal aflibercept (IVA) injection group and control group.

oil injection. For the final data analysis, 64 eyes in each group were included.

Patient demographics and preoperative clinical findings of the two groups are summarized in Table 1. A total of 128 eyes of 128 patients (82 males and 46 females) who underwent vitrectomy for PDR were studied. Their median age was 54 years (range, 29–86 years). The median HbA1c level was 6.7% (range, 3.9–23%). Fifty-two patients (43%) had hypertension and 7 patients (6.3%) had renal dysfunction. There were no statistically significant differences in age, gender, duration of diabetes mellitus (DM), hypertension, hemoglobin A1c (HbA1c), renal dysfunction, previous history of PRP, baseline BCVA, lens status, the severity of VH, and size and location of retinal proliferation between the two groups at baseline. The mean number of laser shots added during the surgery was 514.4 ± 260.3 in the IVA group and 581.7 ± 355.0 in the control group, and there was no statistically significant difference between them ($p = 0.229$).

The VH incidences in the IVA group and the control group were 14.8 and 39.3% at week 1, 8.6 and 31.7% at month 1, 11.7 and 30.5% at month 2, and 8.6 and 30.5% at month 3, respectively. Intergroup differences showed a significantly decreased VH rate in the IVA group compared with that of the control group at week 1, month 1, and month 3 ($p = 0.021$, 0.006, and 0.047, respectively). The incidence of VH did not differ significantly between the two groups at month 2 ($p = 0.089$) (Figure 1).

The mean BCVA changes in the logMAR scale were better in the IVA group than in the control group at week 1, month 1, month 2, and month 3 after surgery (0.70 vs. 0.81, 0.50 vs. 0.67, 0.51 vs. 0.66, and 0.46 vs. 0.65, respectively), but the difference between the two groups was not statistically significant at each visit point ($p = 0.35$, 0.11, 0.22, and 0.09, respectively) (Figure 2). The mean BCVA changes in the logMAR scale (baseline-visit) at week 1, month 1, month 2, and month 3 after surgery compared to baseline in the IVA group were 0.78, 0.96, 0.96, and 0.98, respectively. The mean logMAR BCVA changes at week 1, month 1, month 2, and month 3 after surgery compared to baseline in the control group were 0.78, 0.90, 0.96, and 0.97, respectively. The mean logMAR BCVA changes did not differ significantly between the two groups at each visit point ($p = 0.84$, 0.58, 0.81, and 0.73, respectively) (Figure 3). There are a greater number of eyes with BCVA change of more than 2 logMAR in the IVA group than in the control group at week 1 (8 vs. 2, $p = 0.048$), but this difference between the IVA group and the control group was not statistically significant at month 1, month 2, and

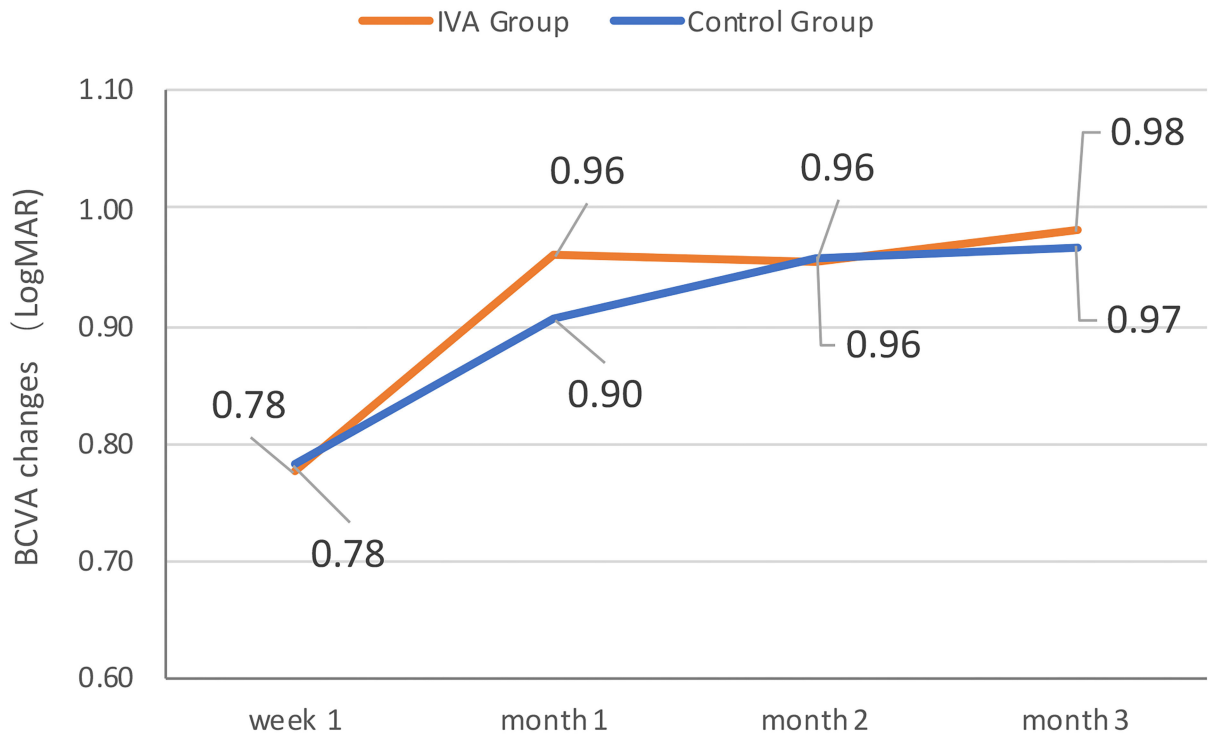

FIGURE 3
The mean best-corrected visual acuity (BCVA) change comparing to baseline during follow-up in the two groups. The mean BCVA change in logMAR (Logarithm of the Minimum Angle of Resolution) scale at week 1, month 1, month 2, and month 3 after surgery comparing to baseline in the preoperative intravitreal aflibercept (IVA) injection group and control group.

month 3 after surgery (9 vs. 5, 9 vs. 4, and 8 vs. 4, respectively) (Figure 4).

There were no incidences of neovascular glaucoma (NVG), endophthalmitis, or TRD progression in the cases included for the final analysis.

Discussion

Diabetic retinopathy (DR) is one of the leading causes of being legally blind in working-aged people and is responsible for up to 4.8% of blindness worldwide. Although PPV was the standard management for PDR with VH or TRD, they may suffer from postoperative VH. This complication will hinder fundus monitoring and/or additional retinal photocoagulation and delay their visual recovery.

The incidence of postoperative VH in PDR had been reported to be around 75% in the 1980s (1, 2), but with the development of surgical techniques and instruments, it has decreased to 12–40% in recent years. The risk factors of postoperative VH include younger age, later detection of DM, poor diabetic and hypertension control, higher serum creatinine, broader area of active neovascularization, increased extent of membrane peeling, postoperative hypotony, postoperative residual neovascularization membrane, unrelieved vitreous retinal contraction, and insufficient PRP (23, 24). In this study, baseline characters, including age, duration of DM, HbA1c, and patient proportion with renal dysfunction, were compared between the two groups, and vitrectomies were all performed by skillful surgeons with more than 15 years of experience to minimize the selective bias and heterogeneity in surgery.

Vascular endothelial growth factor has been proven to play an important role in the development of neovascularization in DR (7, 8). Preoperative vitreous injection of anti-VEGF may induce the regression of retinal neovascularization, decrease the intraoperative bleeding, make the dissection of fibrovascular membrane easier, and fasten vitreoretinal surgery. Previous studies showed that preoperative IVB can make vitrectomy easier and faster with less intraoperative bleeding. However, the effect of anti-VEGF intravitreal injection before vitrectomy on the incidence of postoperative recurrent VH had been reported in the literature with the controversial result. Ahmadieh et al. reported preoperative IVB was effective in reducing early ($\leq$4 weeks) VH compared with a control group in patients with PDR (12). Ahn et al. compared preoperative IVB with intraoperative IVB and no IVB and found that the adjunctive use of IVB

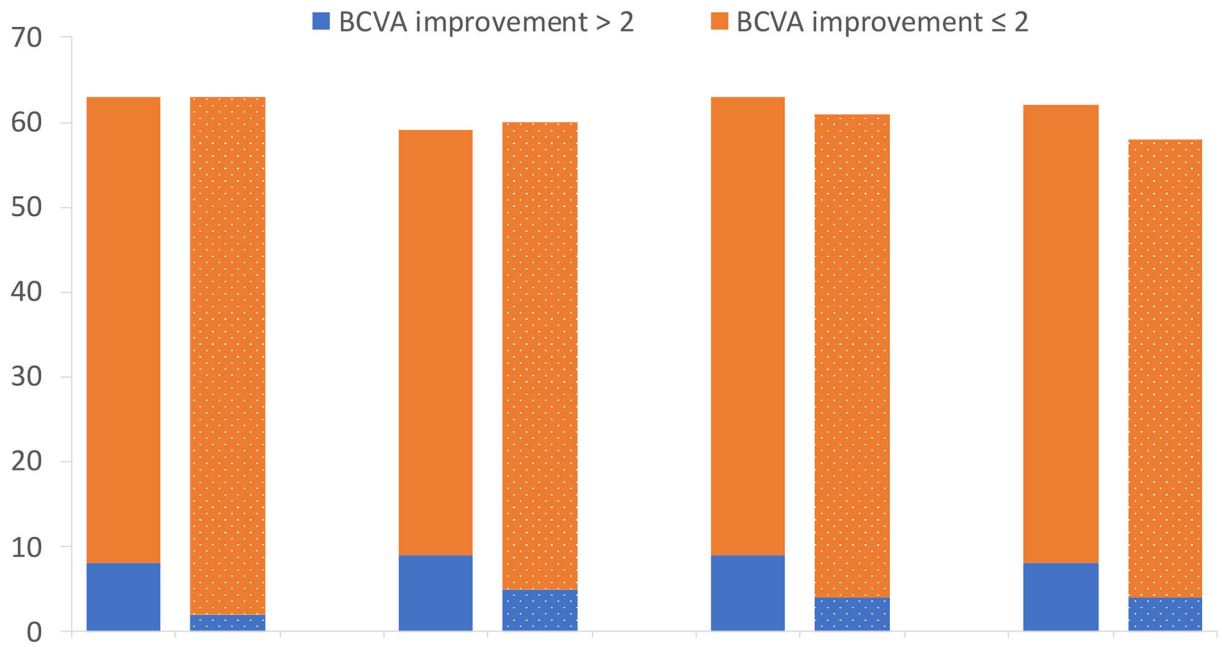

FIGURE 4
The number of patients with different categories of best-corrected visual acuity (BCVA) improvement in the two groups. The number of patients with different categories of BCVA improvement in logMAR (Logarithm of the Minimum Angle of Resolution) scale at week 1, month 1, month 2, and month 3 after surgery comparing to baseline in the preoperative intravitreal aflibercept (IVA) injection group and control group (bar with dotted pattern).

did not reduce postoperative VH incidence significantly (17). However, intraoperative IVB can significantly reduce early postoperative VH and fasten VH clearance compared with the control group. Lo et al. also found no significant differences in the postoperative VH rate between patients with PDR with and without preoperative IVB, but this study was limited by significant differences in the baseline characteristics between their groups (6).

These controversies exist because there are numerous differences in the detail of each strategy, such as the time point for the injection of anti-VEGF, the trocar gauge, and the suture of sclera wound. Heterogeneity of baseline characteristics among different studies also made it difficult to compare their results directly. Wang et al. conducted a network meta-analysis including 26 randomized controlled trials (RCTs) and 1,806 patients with PDR to compare preoperative anti-VEGF to the sham group. They found that injection at 6 to 14 days before vitrectomy could significantly reduce the duration of surgery, improve postoperative BCVA, and decrease the incidence of postoperative VH (25). Performing anti-VEGF injection at more than 14 days, 6 to 14 days, or 1 to 5 days before vitrectomy could significantly reduce the incidence of intraoperative bleeding, while there is no significant benefit for the incidence of postoperative VH. While in their studies, 19 RCTs used IVB, 4 used conbercept, and 1 used ranibizumab but none of them used aflibercept (25).

The ideal timing for pretreatment is controversial. Some authors suggested injecting more than 14 days before vitrectomy to make full use of anti-VEGF agents and induce the complete regression of neovascularization. However, Russo et al. studied the incidence of TRD following preoperative anti-VEGF injection and showed that the incidence of TRD after injection was 2.7% when the interval of injection and vitrectomy was less than 6 days, while it will be increased to 56% when the interval was prolonged to more than 10 days (25). A previous study showed that the timing of anti-VEGF therapy played an important role in the development of fibrosis, with longer lapses following IVB treatment resulting in increased levels of basic fibroblast growth factor (bFGF) (26). Another study showed that the expression of fibroblast cells and connective tissue growth factor (CTGF) increased in epiretinal fibrovascular membranes of the IVB group 21 days after treatment (27). Several investigators reported the progression to TRD after intravitreal injection of anti-VEGF in patients with PDR, which they call "anti-VEGF crunch syndrome" (28). It presents with the symptom of sudden vision loss after 1 to 6 weeks after intravitreal anti-VEGF injection in the affected eye. A higher dose of anti-VEGF may increase the severity of diabetic retinopathy and may be a risk factor for fibrosis. Tan et al. (28) reviewed these data and found that intravitreal anti-VEGF should be used with caution when treating patients with severe PDR and preexisting retinal fibrosis. They recommend close

monitoring of crunch symptoms and proceeding promptly with surgery if there is new TRD or progression of TRD if patients underwent anti-VEGF injection before a planned vitrectomy. We injected the patients 1 to 5 days before vitrectomy in our study and did not find any formation or aggravation of TRD during the surgery.

Postoperative BCVA might be associated with numerous factors, such as the history of TRD, macular edema, macular ischemia, and ellipsoid zone (EZ) band integrity recurrent VH and gas or silicone oil tamponade (25). Zhao et al. did a meta-analysis including 14 RCTs involving 613 patients with PDR and found that patients in the anti-VEGF group achieved significantly better postoperative BCVA than those in the control group (29). Dervenis did another systemic review including 13 RCTs involving 688 patients with PDR and reported that preoperative IVB provided better long-term visual acuity (30). In another meta-analysis including more number of RCTs, they reported that performing only anti-VEGF injection given at 6 to 14 days before vitrectomy could significantly improve postoperative BCVA compared with the sham group (25). The present study found a greater number of eyes with BCVA improvement of more than 2 logMAR in the IVA group than in the control group at week 1, but this benefit disappeared in further follow-up and neither the mean BCVA nor the BCVA change did differ significantly between two groups. Whether this different result was related to the timing of preoperative injection or other factors cannot be concluded in this study.

To our knowledge, this is the first RCT that compares the incidence of postoperative VH between the preoperative IVA and sham groups for PDR. However, it has the following limitations: (1) failure to compare the influence of different timings of preoperative anti-VEGF injection; (2) having a relatively shorter follow-up period; and (3) the lack of a group with different anti-VEGF agents.

In conclusion, this RCT demonstrated that injective IVA at 1 to 5 days before vitrectomy for patients with PDR could reduce the incidence of early and late postoperative VH.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of People's Hospital of Peking University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JQ and XL contributed to the interpretation of the data. The first draft of the manuscript was written by JQ and all authors commented on previous versions of the manuscript. All authors contributed to the study's conception and design. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A comparison between the therapeutic effects of Conbercept combined with panretinal photocoagulation and panretinal photocoagulation monotherapy for high-risk proliferative diabetic retinopathy

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Objective: To compare the therapeutic effects of the administration of intravitreal Conbercept (IVC) plus panretinal photocoagulation (PRP) to that of PRP monotherapy in patients with high-risk proliferative diabetic retinopathy (PDR).

Methods: In this retrospective consecutive case series, we analyzed the data on high-risk PDR patients followed up for 12 months. Patients were divided into two groups: the IVC+PRP group and the PRP monotherapy group. Patients in the IVC+PRP group were initially administered 3 IVC injections and PRP, while patients in the PRP monotherapy group received PRP only. Depending on the grouping criteria, patients in both groups were administered either IVC+PRP or PRP only if the neovascularization (NV) did not regress. From the initiation to month 12 of treatment, we recorded and compared the data on the NV regression rate, improvement in best-corrected visual acuity (BCVA), laser spots, changes in central macular thickness (CMT), complications, and the need for vitrectomy for all patients.

Results: In this study, 79 eyes of 58 patients in the IVC+PRP group and 86 eyes of 60 patients in the PRP monotherapy group were included. During the follow-up of 12 months, the number of eyes with complete regression, partial regression, and no regression or increase in NV were 56 (70.88%), 23 (29.12%), and 0 (0%) in the IVC+PRP group and 13 (15.12%), 50 (58.14%), and 23 (26.74%) in the PRP group ($p < 0.001$). The BCVA was significantly higher and CMT was lower in the patients of the IVC+PRP group than in the PRP monotherapy group at 3, 6, and 12 months of follow-up ($p < 0.05$). The mean number of laser spots was lower in the patients of the IVC+PRP group than in the PRP group (1,453 $\pm$ 87 spots vs. 2,267 $\pm$ 94 spots, $p < 0.05$). A

significantly lower percentage of patients in the IVC+PRP group underwent vitrectomy than that in the PRP group (7 (8.86%) vs. 27 (31.40%), $p < 0.001$).

Conclusion: High-risk PDR patients treated with IVC + PRP showed a higher rate of NV regression, more effective improvement in the BCVA, and lower vitrectomy rate compared to those who were administered PRP monotherapy.

KEYWORDS

Conbercept, panretinal photocoagulation, high-risk PDR, anti-VEGF (vascular endothelial growth factor), neovascularization

Introduction

Diabetic retinopathy (DR) is the main retinal complication of diabetes mellitus (DM) and the leading cause of loss of vision and blindness in working-age people (1–3). Proliferative diabetic retinopathy (PDR) is characterized by neovascularization (NV) of the optic disc or vitreous and pre-retinal hemorrhage, which finally develops into a tractional retinal detachment. A study found that the average percentage of PDR in all DM patients was 6.96% (6.87–7.04), suggesting that around 17 million PDR patients worldwide are at risk of losing their eyesight (2).

High-risk PDR occurs when NV is accompanied by vitreous hemorrhage or when NV of the disc (NVD) occupies more than or equal to one-quarter to one-third of the disc area, even in the absence of vitreous hemorrhage, indicating severe ischemia (4). Bressler et al. found that high-risk PDR had a higher probability of advancing PDR, e.g. more vitrectomies of vitreous hemorrhage or tractional retinal detachment were needed for patients with high-risk PDR than that required for patients with moderate PDR, even after intensive treatment, such as retinal photocoagulation (5).

Panretinal photocoagulation (PRP) has been used as a classical treatment for PDR for over 40 years. In this procedure, the ischemic regions of the peripheral retina are eliminated to decrease NV while maintaining central vision. PRP also significantly lowers the probability of severe loss of vision in patients with high-risk PDR by inducing retinal NV regression. In high-risk PDR individuals, PRP should be administered at the earliest to effectively reduce retinal NV and PDR progression (4, 6, 7). Anti-vascular endothelial growth factor (anti-VEGF) agents, including ranibizumab and aflibercept, can facilitate the regression of NV while eliminating diabetic macular edema (DME), and hence, are recommended for treating high-risk PDR patients (8, 9). By investigating different PDR treatment modalities, the RELATION study showed that PDR patients with DME benefited more from Ranibizumab+PRP combined therapy than from PRP monotherapy. The PROTEUS study found that Ranibizumab+PRP therapy was more effective than PRP monotherapy in preventing the recurrence of NV with fewer PRP treatment times over 12 months (10, 11).

Conbercept is a member of the recombinant VEGF decoy receptor class. It is a recombinant fusion protein consisting of the constant region and the third and fourth Ig domains of VEGFR2, as well as, the second Ig domain of VEGFR1 (12, 13). Intravitreal administration of Conbercept (IVC) is effective in treating PDR and DME cases. Treatment with IVC combined with PRP has a greater effect on functional outcomes than PRP monotherapy, including improvements in the visual acuity of the patients and reduction of macular edema (14). However, as studies on the therapeutic effects of IVC+PRP on high-risk PDR patients are limited, further research on this treatment method for high-risk PDR should be encouraged. We conducted a retrospective consecutive case series study to compare the therapeutic effects of the combined treatment using IVC plus PRP to those of PRP monotherapy in high-risk PDR patients.

Methods

Study design

This study had a retrospective consecutive case series design. Following the guidelines of the Declaration of Helsinki, informed consent forms were signed by all participants after they received information on the risks of IVC and PRP therapy. The study was approved by the Medical Ethics Committee of Peking University People's Hospital.

Patients

In total, 118 high-risk PDR patients (165 eyes) who visited the Department of Ophthalmology, Peking University People's

Hospital, from September 2016 to April 2021, were recruited in this study. All patients underwent a follow-up of 12 months. The patients were placed either in the IVC+PRP group (79 eyes) or the PRP monotherapy group (86 eyes). The inclusion criteria were as follows: 1) Patients primarily diagnosed with high-risk PDR and confirmed by color fundus photography (CFP) and/or fluorescein angiography (FA) (CFP and FA both conducted using the Optos PLC 200TX, Dunfermline; United Kingdom); 2) Those who were followed up for at least 12 months; 3) Patients who underwent IVC+PRP combined therapy or PRP monotherapy. The exclusion criteria were as follows: 1) Patients with other retinal disorders like rhegmatogenous retinal detachment, uveitis, epiretinal membrane, age-related macular degeneration, high myopia fundus changes, and ocular tumors; 2) Patients who were administered intraocular treatment other than IVC and PRP, such as intravitreal injections of other anti-VEGF agents or steroid components or macular grid pattern photocoagulation; 3) Patients who underwent any intraocular surgery within 6 months before participation; 4) Patients with a proliferative membrane because of PDR. The clinical data of the patients at 3, 6, 9, and 12 months were recorded and compared. The data collected 15 days before or after 3, 6, and 9 months and 30 days before or after 12 months were considered to be the data corresponding to 3, 6, 9, and 12 months, respectively.

Treatment

Panretinal photocoagulation (PRP) was conducted according to a previously described protocol (Lumenis Novus Omni, Lumenis Be, Inc. San Jose, USA) (15). A level II to level III reaction for retinal photocoagulation was identified; the exposure time and the spot size were 0.3 s and 300 μm, respectively. The photocoagulation scope was two papilla diameters (PD) away from the temporal side of the macula and from both the upper and lower vascular arcades on the retina to the peripheral retina, and 1 PD away from the nasal side of the optic disc to the peripheral retina. Conbercept (0.05 mL/0.5 mg; Chengdu Kanghong, China) was administered to all patients of the IVC+PRP group. Intravitreal injections were performed according to a previously reported method (16). In the IVC+PRP group, the initial treatment included the administration of three IVC injections, once every four weeks. PRP was performed simultaneously, following the diagnosis of high-risk PDR, and was completed within two weeks. The patients in the PRP monotherapy group, however, received PRP treatment only. Three months after the start of treatment, fundus fluorescein angiography (FFA) was performed in both groups. Patients in the PRP monotherapy group underwent re-treatment with photocoagulation if the NV did not regress. Similarly, for the IVC+PRP group, if NV persisted, IVC+PRP was administered again, regardless of the presence of DME.

Efficacy and safety assessments

The general and medical information of the patients was recorded at the beginning before eye treatment was started. The data on the age, gender, body mass index, blood pressure, and fasting glucose level of the patients were recorded. All treated patients received standard ophthalmological examinations and optical coherence tomography (OCT) (Zeiss Cirrus HD-OCT5000, Carl Zeiss Meditec AG; Jena, Germany) during every visit. On the first visit and months 3, 6, 9, and 12, the best-corrected visual acuity (BCVA) of both eyes was checked and recorded using the Early Treatment Diabetic Retinopathy Study [ETDRS] letters. Visual acuity improvement of ≥2 lines was considered to be improved vision, while a decrease in visual acuity by ≥2 lines was considered to be a deterioration of visual acuity. The rest was considered to be unchanged visual acuity. Compared to the status of NV at baseline, the complete absence of NV was considered to be complete NV regression. Persistent or increased NV was considered to be the absence of NV regression or increase in NV. NV regression partially was considered to be "partial NV regression".

Additionally, the intraocular pressure was evaluated at the first visit, as well as on months 3, 6, 9, and 12. Spectral domain-optical coherence tomography (SD-OCT) was performed on both eyes using an acquisition methodology for determining the macular thickness. Central macular thickness (CMT) was determined by SD-OCT examinations and was calculated as the combined thickness of the subretinal fluid and neurosensory retina. CMT increased ≥50 μm was considered to be increased CMT, while a decrease in CMT by ≥50 μm was considered to be decreased CMT. CMT change within 50 μm was considered an unchanged CMT. CFP was performed on all patients at each visit. FA was also performed at the first visit, as well as, after 3, 6, 9, and 12 months if the patient had no history of allergies and had normal hepatic and renal functions. From the beginning of treatment through month 12, the data on parameters, such as the NV regression status, improvement in BCVA, laser spots, changes in CMT, other complications, and the need for vitrectomy, for all patients were investigated and compared. The primary efficacy analysis was the NV regression rate. The number of eyes with complete regression, partial regression, no regression, or increased NV was divided by the number of total eyes treated and was calculated as as the NV regression rate. Other results were investigated as a secondary efficacy analysis. We also recorded systemic and ocular complications.

Statistical analysis

The data were analyzed using the SPSS software (version 12.0). The Shapiro-Wilk test was conducted to check whether the data

were normally distributed. Qualitative data were either analyzed by Chi-squared tests or Fisher's exact tests. Quantitative data that were normally distributed were tested by independent samples t-tests, whereas non-normally distributed data were tested by Mann-Whitney U tests. All differences were considered to be statistically significant at $p < 0.05$.

Results

Baseline information

From September 2016 to April 2021, data on 165 eyes (118 patients) were recorded. Among all participants, 71 (60.17%) were men, and 47 (39.83%) were women; the mean age of all participants was 57.09 years, respectively. The baseline information is shown in Table 1. The differences in age, gender, body mass index, blood pressure, fasting glucose, BCVA, IOP, CMT, and area of NV between the patients in the IVC+PRP and PRP groups were not statistically significant ($p > 0.05$; Table 1).

NV regression

The number of eyes with complete NV regression, partial regression, and no regression or increase was 56 (70.88%), 23 (29.12%), and 0 (0%), respectively, in the IVC+PRP group and 13 (15.12%), 50 (58.14%), and 23 (26.74%), respectively, in the PRP monotherapy group after 12 months of treatment compared to their corresponding values at baseline. The NV regression rate in the IVC+PRP group was significantly higher than that in the monotherapy group ($p < 0.001$; Figure 1).

Changes in the BCVA

At 12 months of follow-up, the number of eyes with improved, unchanged, and decreased BCVA was 68 (86.08%), 9 (11.39%), and 2 (2.53%), respectively, in the IVC+PRP group and 20 (23.26%), 48 (55.81%), and 18 (20.93%), respectively, in the PRP monotherapy group. The differences in the changes in the BCVA between the IVC +PRP and PRP groups were significant ($p < 0.001$; Figure 2). The average BCVA was significantly greater in the IVC+PRP group than in the PRP monotherapy group at each visit. Additionally, the differences were significant at months 6 ($p = 0.042$), 9 ($p = 0.049$), and 12 ($p = 0.011$; Figure 3).

Changes in the CMT

After 12 months of treatment, the numbers of eyes with decreased, unchanged, and increased CMT were 59 (74.68%), 20 (25.32%), and 0(0%), respectively, in the IVC+PRP group and 26 (30.23%), 34 (39.54%) and 26 (30.23%), respectively, in the PRP monotherapy group. Significant differences were observed in the CMT between the groups ($p < 0.001$; Figure 4). The average CMT was significantly lower in the IVC+PRP group than in the PRP monotherapy group at each visit. Additionally, significant differences were recorded at months 6 ($p = 0.07$), 9 ($p = 0.015$), and 12 ($p = 0.014$; Figure 5).

Other outcomes

The mean number of laser spots was significantly lower in the IVC+PRP group than in the PRP group (1,453 ± 87 spots vs. 2,267 ± 94 spots, $p < 0.05$). The difference in the total number of laser

TABLE 1 Demographic information for the two groups.

Group	IVC+PRP	PRP	p-value
Female, frequency (%)	24 (40.00)	23 (39.66)	0.56
Age, (mean ± SD), y	54.67 (13.3)	59.59 (16.9)	0.13
BMI, (mean ± SD), kg/m^2	27.87 (2.2)	29.01 (2.7)	0.44
Systolic blood pressure, (mean ± SD), mmHg	134.62 (11.9)	138.11 (16.0)	0.68
Diastolic blood pressure, (mean ± SD), mmHg	78.84 (8.3)	77.65 (9.2)	0.52
fasting glucose, mmol/L	7.61 (2.3)	7.07 (2.8)	0.28
IOP, (mean ± SD), mmHg	16.7 (3.0)	16.9 (2.4)	0.54
NV area (mean ± SD) Disc Area (DA)	2.57 (1.4)	2.87 (1.6)	0.35
BCVA, (mean ± SD)	54.25 (21.6)	51.95 (25.5)	0.76
CMT, (mean ± SD), μm	325.05 (106.93)	302.90 (100.90)	0.51
No. of eyes with DME (CMT ≥250um)	35 (44.30%)	41 (47.67%)	0.66

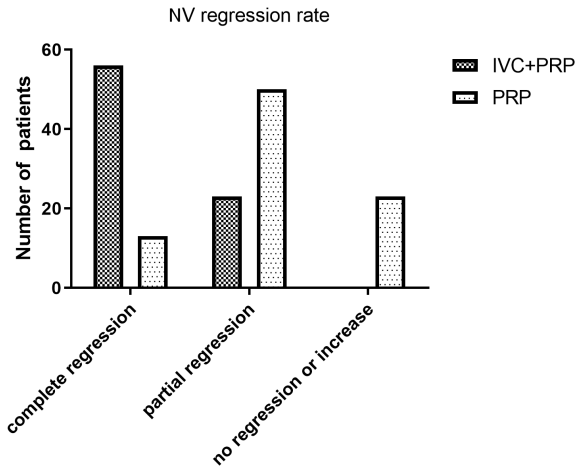

FIGURE 1
The NV regression rates of patients in both groups. Treatment with IVC combined with PRP showed a higher rate of NV regression at month 12.

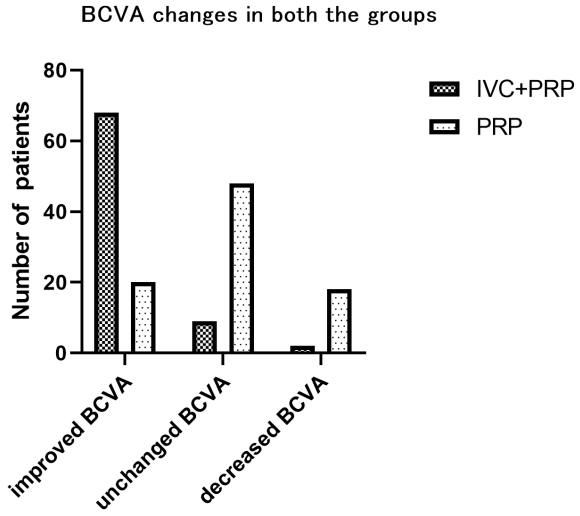

FIGURE 2
The BCVA changes in the patients of both groups. After 12 months, a significant difference in the BCVA changes was found between the groups for the number of eyes with improved BCVA, unchanged BCVA, and decreased BCVA.

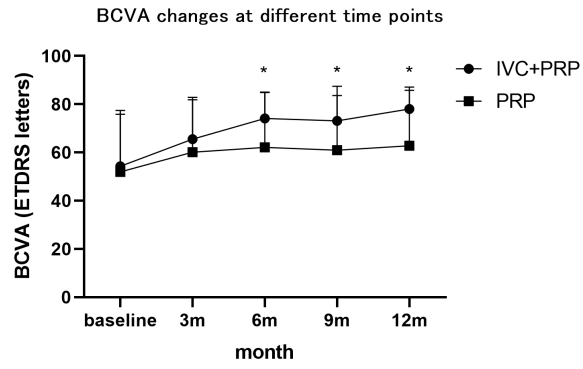

FIGURE 3
The BVCA changes at different time points. The average BCVA was greater in the patients of the IVC+PRP group than in those of the PRP monotherapy group at each visit, while significant differences were found at months 6, 9, and 12. *Demonstrates statistically significant difference.

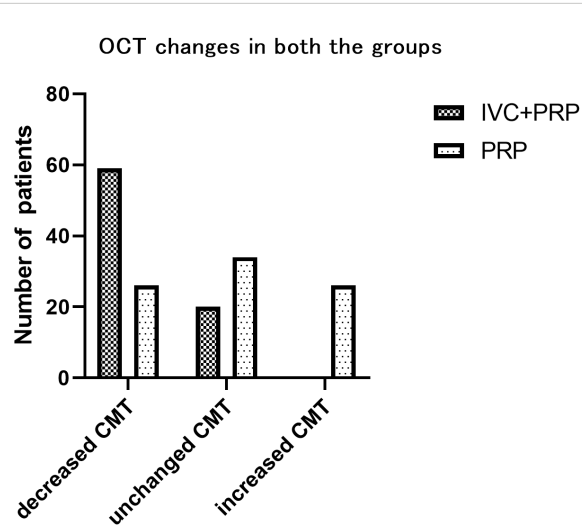

FIGURE 4
The changes based on OCT examinations in the patients of both groups. At month 12, a significant difference was found between the groups for the number of eyes with decreased CMT, unchanged CMT, and greater CMT.

treatments between the groups was not significant. Patients in the IVC group received 4.95 ± 0.90 injections. In the IVC+PRP group, vitrectomy due to disease progression to severe vitreous hemorrhage was conducted on 7 eyes (8.86%). In the PRP monotherapy group, vitrectomy was conducted on 27 eyes (31.40%). The percentage of patients who required vitrectomy was statistically different between the groups ($p < 0.001$; Figure 6). Four eyes in the PRP group developed neovascular glaucoma, while no case of neovascular glaucoma was reported in the IVC+PRP group. No endophthalmitis, retinal tear, or cataract exacerbation due to the treatment or serious systemic side events were reported in either group.

Discussion

Our results suggested that the treatment with IVC+PRP was more effective than PRP monotherapy in causing NV regression among high-risk PDR patients during a follow-up of 12 months. The effectiveness of an anti-VEGF agent combined with PRP in high-risk PDR was consistent with previously reported results.

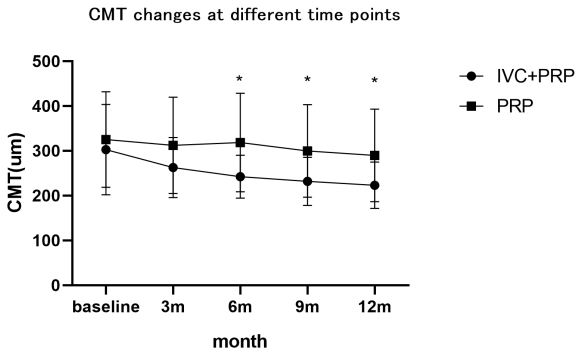

FIGURE 5
The changes in the CMT at different time points. The average CMT was lower in the patients of the IVC+PRP group than in those of the PRP monotherapy group during each visit, while significant differences were found at months 6, 9, and 12. *Demonstrates statistically significant difference.

This was the first study to investigate the effects of combined treatment with IVC and PRP in high-risk PDR cases (10, 17).

The findings of our study were similar to those of previous studies, which suggested that PRP and anti-VEGF combination therapy can achieve optimal efficacy in treating high-risk PDR patients by enhancing BCVA and NV regression while decreasing the risk of adverse effects (18). Although PRP is a standard therapeutic strategy for PDR, in some studies, it was effective in only 60% of PDR patients, and the remaining 40% of the patients either underwent surgery or developed poor vision (19, 20). Some studies have shown that an increase in VEGF expression in PDR is closely related to hypoxia and inflammatory responses (21, 22). By phosphorylating tight-junction proteins, VEGF increases capillary permeability, which causes macular edema and angiogenesis. Thus, VEGF inhibition is necessary for anti-vascularization therapy in PDR patients. Anti-VEGF

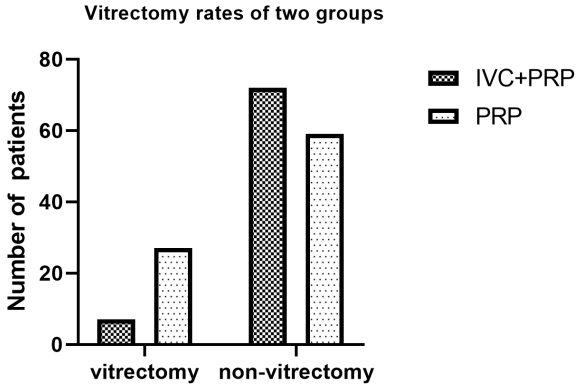

FIGURE 6
Vitrectomy rates of the patients in the two groups. The percentage of patients undergoing vitrectomy was significantly different between the groups.

medication might be administered to prevent NV in high-risk PDR cases before the completion of PRP within the effective action period of the drug. This can prevent disease progression before the patients receive PRP treatment. PRP takes over three weeks from operation to display its full effects, whereas anti-VEGF therapy acts fast. Thus, it can be administered to avoid disease progression to the point of requiring vitreous surgery before the effects of PRP treatment are expressed. In our study, a lower percentage of patients in the combined treatment group underwent vitrectomy, which was similar to the results reported in other studies (23, 24). Anti-VEGF rescue therapy can also be used to manage some cases of PRP-treated PDR patients with persistent NVs, even in cases where neovascular regression cannot be achieved (25). Matteo et al. found insufficient information to compare PRP treatment and combined treatment using anti-VEGF and PRP for NV regression in a meta-analysis due to high inconsistencies among the included studies. However, after adjustments by surface under the cumulative ranking curve (SUCRA) analysis in this meta-analysis, the combination treatment was recommended (26).

The results of the other tested parameters showed that patients in the IVC+PRP group had better vision outcomes with lower CMT values. In patients with combined macular edema, vision improved mainly due to the remission of macular edema. In patients without macular edema, visual acuity improved due to the absorption of pre-retinal or inter-retinal hemorrhage caused by the regression of neovascularization. Anti-VEGF plays an important role in macular edema treatment. VEGF is the most significant molecule that needs to be broken down in the retinal barrier. Pathologically, hyperglycemia, protein kinase C activation, and advanced glycation end-product protein synthesis during DR and DME affect the production of VEGF. VEGF inhibitors are used to prevent inner blood-retinal barrier disruption and control DME (27–29). The decrease in retinal edema also facilitated the implementation of PRP and its early effects. Fewer laser spots were observed in the IVC+PRP group in our study, which was consistent with the PROTEUS study (10). Although Bressler et al. had concerns regarding the long-term benefits of anti-VEGF in PDR, the differences in the loss of vision between the anti-VEGF and PRP groups vanished after five years of follow-up. However, fewer laser treatments were required to reduce retinal damage and patient pain.

In this study, Conbercept, a newly developed therapeutic agent in China, was used as an anti-VEGF agent. Its treatment effects are mostly attributable to the VEGF family of factors (VEGF-A, B, C, and PIGF) that prevent the growth of NV and the reduction of vascular permeability in the retina (30). Xia et al. found that Conbercept can strongly inhibit inflammation, angiogenesis, and oxidative response in the PDR model by reducing macrophage inflammatory protein-1 (MIP-1), intercellular cell adhesion molecule-1 (ICAM-1), IL-1β, IL-6, and TNF-α protein levels (31). Concerning the improvement of vision, a meta-analysis showed that Conbercept with PRP greatly increased the overall

effectiveness and decreased the central thickness of the macula and other complications compared to the condition of the patients in the control group (14). Previous studies concentrated more on improving visual acuity and reducing macular edema in patients. We found that treatment with IVC+PRP was more effective than treatment with PRP in facilitating the regression of NV in PDR patients. Thus, the administration of Conbercept should be continued in the clinical setting.

All VEGF inhibitors have relatively short half-lives, while PRP treatment has a permanent effect. Thus, PRP is the preferred and major method to treat PDR (32). Our study showed that PRP monotherapy caused the regression of NV in 73.26% of eyes (total and partial regression). The Diabetic Retinopathy Study showed that PRP significantly lowered the risk of severe visual loss in patients with high-risk PDR. PRP is regarded as the gold standard for treating PDR cases (8, 19) and is recommended as the first-line treatment for PDR when anti-VEGF therapy is not available due to difficulties in frequent follow-ups or financial reasons (33).

The main limitation of this study was that this was a single-center, retrospective study with a follow-up time of only 12 months. Thus, prospective, randomized, and multicenter studies with a longer follow-up are needed to comprehensively compare the effects of IVC+PRP treatment to those of PRP monotherapy in PDR. Also, as the study was a retrospective one, we could not obtain more information on various aspects of the patients, including visual changes, non-perfusion areas, etc.

To summarize, treatment with IVC combined with PRP caused a higher rate of NV regression, greater improvement in the BCVA, and also decreased the need to perform vitrectomy in patients with high-risk PDR, compared to monotherapy with PRP.

Typical cases

Case 1

A 40-year-old man presented with blurred vision in the left eye for two weeks. He had a history of diabetes for eight years. A

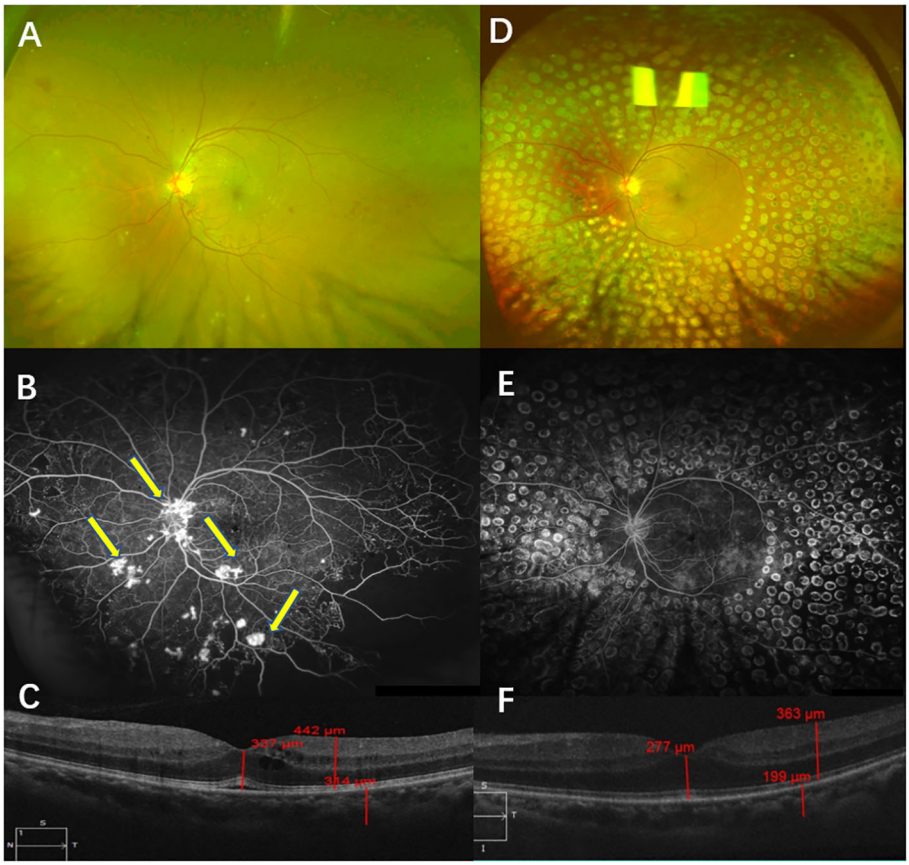

FIGURE 7
Typical case 1. Color fundus photography (CFP) **(A)** and fluorescein angiography (FA) **(B)** were performed before treatment and showed high-risk PDR in the left eye. CFP examinations showed NV, which was confirmed by FFA (**B**, arrow). OCT examinations showed macular edema **(C)**. CFP and FA examinations after a follow-up of 12 months showed that the retina had laser shots. No NV was found *via* either CFP or FA examinations **(D, E)**. OCT examinations of the left eye showed the transformation from edema to full recovery of the macula **(F)**.

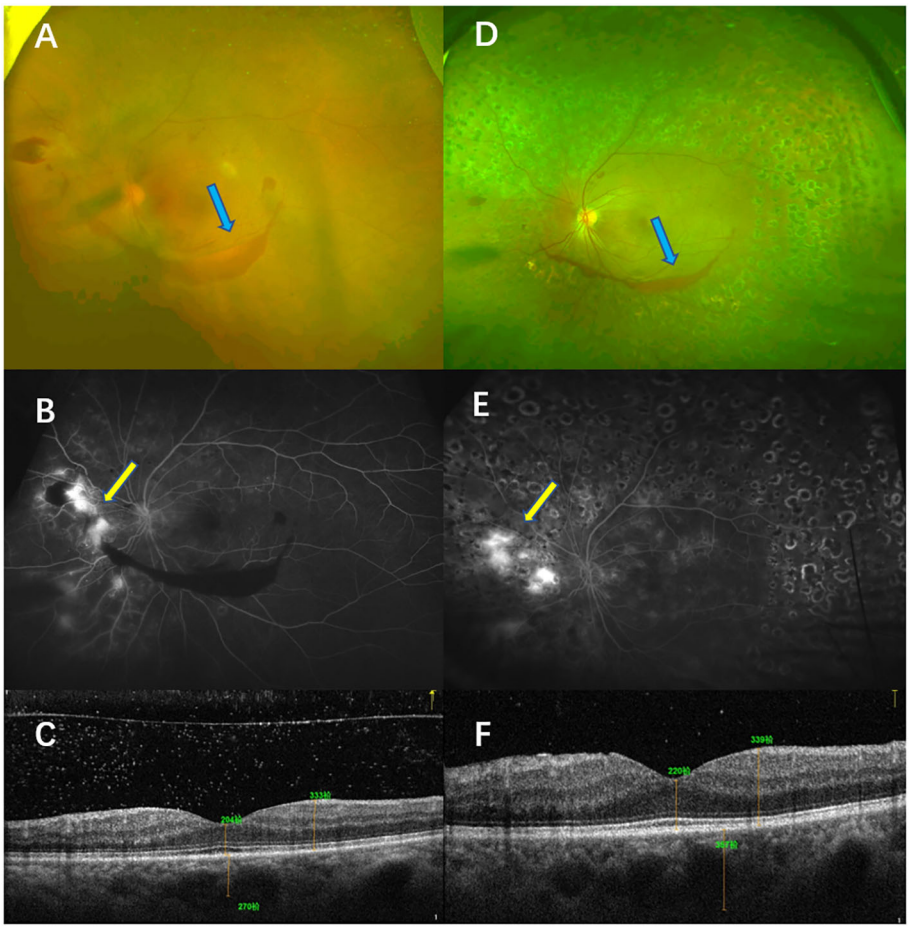

FIGURE 8
Typical case 2. The patient was diagnosed with high-risk PDR in the left eye and treated with PRP. Color fundus photography (CFP) **(A)** and fluorescein angiography (FA) **(B)** before treatment showed high-risk PDR in the left eye. The CFP examinations showed vitreous hemorrhage (blue arrow, **A**), and the FFA examinations showed NV [yellow arrow, **(B)**]. OCT showed that no macular edema was present **(C)**. CFP and FA examinations after a follow-up of 12 months showed that the retina had laser shots. The CFP examinations showed that the area of the vitreous was smaller (blue arrow, **D**), but the FA examinations showed NV leakage (yellow arrow, **E**). OCT examinations of the left eye showed that macular edema was absent **(F)**.

physical examination showed that the BCVA in his left eye was 45 letters. The CFP examinations showed NV, which was confirmed by FFA. The results of SD-OCT examinations indicated macular edema, and the CMT was 337 μm. He was diagnosed with high-risk PDR in the left eye and was administered IVC (five times) and PRP. After a follow-up of 12 months, complete NV regression in the left eye was recorded. Also, his BCVA was 85 letters, and his CMT was 277 μm after 12 months (Figure 7).

Case 2

A 55-year-old woman presented with blurred vision in the left eye for a month. She had a history of diabetes for 15 years. Her physical examination showed that the BCVA of her left eye was 40 letters. The CFP examinations showed NV and vitreous hemorrhage, which were confirmed by FFA. The CMT was 204 μm. She showed NV even after receiving PRP monotherapy. Although rescue photocoagulation was conducted, after a 12-month follow-up, only partial NV regression was recorded in the left eye. Her BCVA was 65 letters, and her CMT was 211 μm after 12 months (Figure 8).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was approved by Peking University People's Hospital Medical Ethics Committee. Written informed consent forms were signed by all of the patients.

Author contributions

All authors contributed to the study conception and design. Performing the screening diagnosis and treatment of high-risk PDR (HQ, YS). Collection and assembly of data, data analysis, and interpretation (YS). Manuscript writing: All authors. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Biomechanical homeostasis in ocular diseases: A mini-review

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Diabetes mellitus-induced hyperglycemia is responsible for multiple pathological ocular alternations from vasculopathy to biomechanical dyshomeostasis. Biomechanical homeostasis is crucial to maintain the normal physiological condition of the eyes. Biomechanical features vary in eye tissues regarding different anatomical positions, tissue components, and cellular functions. The disturbance in biomechanical homeostasis may result in different ocular diseases. In this review, we provide a preliminary sketch of the latest evidence on the mechano-environment of the eyeball and its possible influencing factors, thereby underscoring the relationship between the dyshomeostasis of ocular biomechanics and common eye diseases (e.g., diabetic retinopathy, keratoconus, glaucoma, spaceflight-associated neuro-ocular syndrome, retinal vein occlusion and myopia, etc.). Together with the reported evidence, we further discuss and postulate the potential role of biomechanical homeostasis in ophthalmic pathology. Some latest strategies to investigate the biomechanical properties in ocular diseases help unveil the pathological changes at multiple scales, offering references for making new diagnostic and treatment strategies targeting mechanobiology.

KEYWORDS

biomechanical homeostasis, keratoconus, glaucoma, diabetic retinopathy, myopia

Introduction

Diabetes mellitus (DM) imposes a heavy economic burden worldwide with a detrimental impact on ocular health. Chronic exposure to hyperglycemia exerts toxicity to cells and aggravates the metabolic dysfunction in ocular tissues at both physiological and pathophysiological scales. Glucose-rich ambiance is the major culprit of DM-related eye diseases, which could stimulate the polyol pathway, boost the production of advanced glycation end-products (AGEs), activate protein kinase C, increase oxidative stress, and upsurge inflammatory pathways (1). For instance, hyperglycemic conditions promote the activity of aldose reductase in the polyol pathway and induce chronic accumulation of sorbitol in the lens, which further raises the osmotic pressure along with the excessive oxidative stress, and eventually contributes to the onset of cataracts (2). Meanwhile, DM-induced hyperglycemia could trigger subsequent ocular changes that range from the impairment of vascular supply to the elevation of intraocular pressure (IOP). As reported, for every 10 mg/dL increase in fasting serum glucose, IOP increases by 0.09 mmHg in men and 0.11 mmHg in women (3). The IOP level was found to be lower in DM patients with adequate control of the blood sugar than in those without (4). Moreover, owing to the end-organ effect of uncontrolled glucose levels, DM has also been considered as a potential risk factor for other deleterious abnormalities such as glaucoma (5). Therefore, maintaining biomechanical homeostasis is vital for eye health in the context of oculopathy management including DM and glaucoma.

As the only visual sensation organ, the eye is physiologically subjected to multiple sources of pressure, which is referred to as ocular biomechanics (Figure 1). The term "biomechanics" defines the physical responses of biological tissues under different pressure influences (6).

Starting with the anterior compartment, the cornea is the outermost component of the eye globe. Exposed to the open air, the cornea directly bears the exterior stimuli generated by atmospheric pressure (7), eye movement (8), and tear film motion (9), etc. To counterbalance, the internal stresses were fostered by aqueous humor (10) and IOP (7). As the continuous tissue of the cornea, the anterior sclera also percepts comparable stresses and strains (11). However, the posterior sclera [peripapillary sclera and scleral canal connected to lamina cribrosa (LC)] is mainly affected by "external" stresses imparted by cerebrospinal fluid pressure (CSFP) from the back of the eye globe (12). Regarding the anterior chamber angle, the aqueous outflow is driven by the mechanical strain on the trabecular meshwork (TM) and the shear stress arising from the circumferential flow through the Schlemm's canal (SC), thereby modulating the IOP homeostasis (13). Additionally, SC and TM are also subjected to the ocular pulse generated from either cardiac pulsation in the retina and choroid or the pressure oscillations in the episcleral vessels (14). The lens capsule completely encloses the crystalline lens, with its thicker and more durable anterior capsule (toward aqueous humor) accommodating to the IOP (15), and the posterior capsule (toward vitreous cavity) facing the intravitreal pressure (16). The viscosity and elasticity properties of the capsule membrane exhibit high resistance to extrinsic mechanical strength and intrinsic deformative stress occurring in the lens shape alterations (16). Under physiological status, the anterior and posterior chamber is interconnected by the iris-lens channel allowing the aqueous flow. The pressure difference between these two chambers is relatively equilibrated, with only <1 mmHg difference in human eyes estimated by a mathematical model (17).

The posterior chamber of the eye is a spherical cavity filled with gel-like vitreous humor buffering the mechanical stimuli exerted on the lens or retina under both static and dynamic nature (18, 19). Specifically, the vitreous chamber serves as a torsionally oscillating sphere in the eye rotation process. The subsequent vitreous motion would result in a small shear stress on the retina in a radius manner (20). Bruch's membrane (BM) is a thin acellular lamina at the inner layer of the choroid, subjected to constant pressure-induced mechanical stress resulting from the choroidal flow changes resonating cardiac pulsation (21). As the physical and biochemical barrier between the retinal pigment epithelium and the choroid, BM facilitates metabolic transportation across tissues *via* the stress-strain (22). The biomechanics at the optic

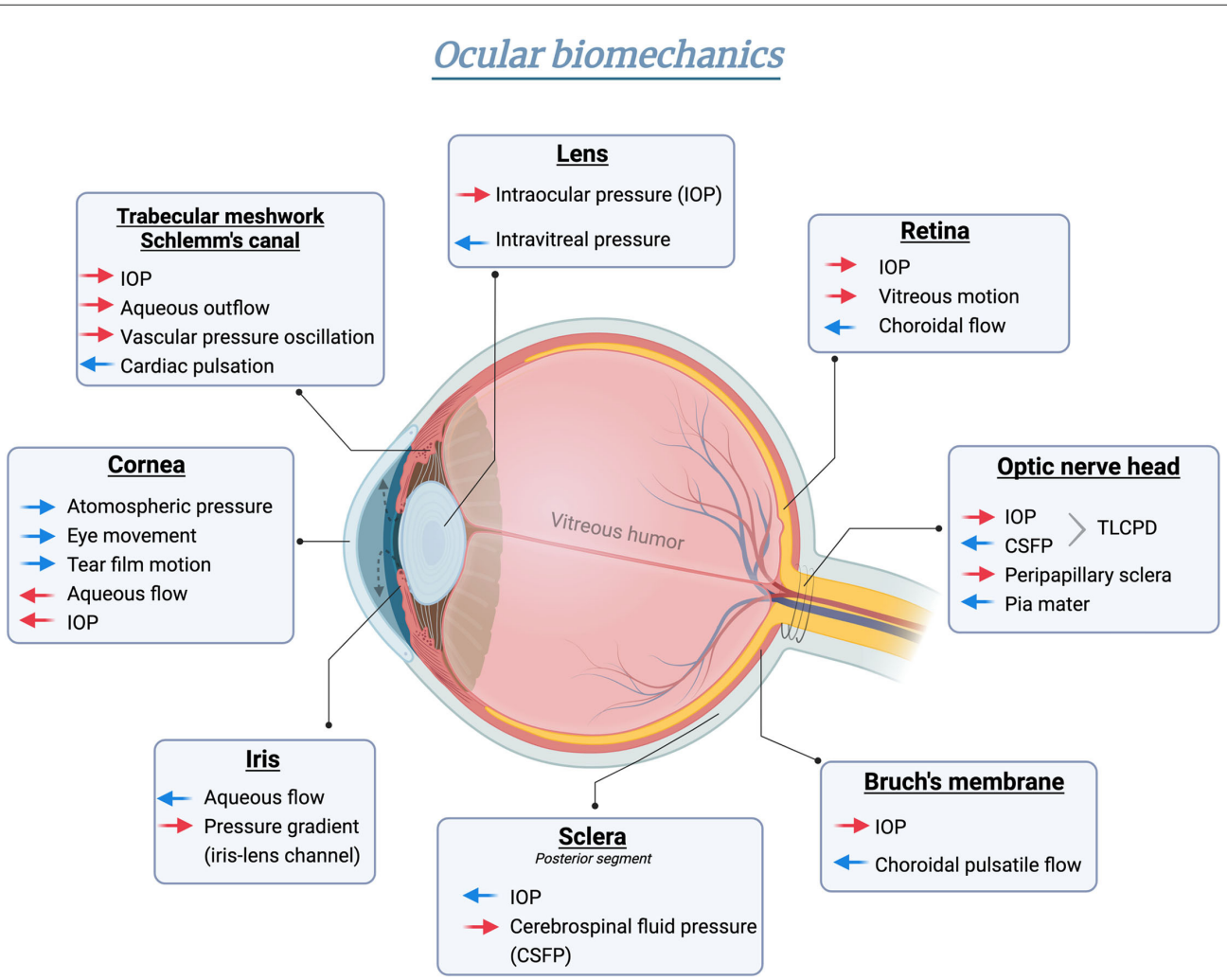

FIGURE 1
Schematic illustration of biomechanical homeostasis in different ocular tissues. Red arrows indicate intrinsic stress formed in the globe, whereas blue arrows represent the counterforce or "external" pressure outside the eye. Created in BioRender.com.

nerve head (ONH) of the posterior orbit are regulated in a more complex way. Anatomically, the eye and the brain are connected by the optic nerve passing through the translaminar region and the subarachnoid space. Biochemically, the translaminar cribrosa pressure difference (TLCPD), formed by IOP and intracranial pressure (ICP) across the ONH, establishes significant levels of pressure gradient along the nerve tract (23). Meanwhile, other properties, including orbital tissues and pia mater, are also involved in the mechanical features imposed on the optic nerve (12). Taken together, these features delicately manifest the regional specialization of ocular biomechanical dynamics.

Under normal physiological conditions, the ocular biomechanics is generally kept in a dynamic-balanced fashion, with temporary fluctuations in stresses and strains. As the predominant and solely modifiable risk factor in glaucoma, IOP has a clear circadian oscillation pattern. Thus, the 24-h IOP recording is better at reflecting the biological features of IOP (24). The normal IOP, lying between a range of 10–21 mmHg, can be termed as "normal resting IOP." The normal resting IOP is influenced by multiple extrinsic factors. Specifically, the IOP level alters with eye movement, generally increasing in the eye upgaze phase, and decreasing in the inferonasal gaze phase (25). Weekly and seasonal variations of IOP are also observed (26). Notably, eyelid-related maneuvers such as eyelid squeezing or rubbing can trigger a transient IOP spike excessing normal range on a time scale of less than a second (27). Here, we defined this specific type of IOP elevation as "transient IOP fluctuation." Under normal conditions, these transient "attacks" would not lead to any observable functional or structural damage. One possible speculation is that these short-term IOP spikes are managed by some mechanical response units which can help neutralize and prevent the potential damage caused by pressure insults.

At the cell level, the mechanical response unit mainly refers to the mechanosensitive channels, categorized into Na^+-permeable, K^+-permeable, and non-selective cation (TRP, Piezo) channel families. These channels serve as bandpass filters allowing transmission of certain types of mechanical loading pressure such as tension, stretch, shear flow, and compression at specific amplitude. Previous studies identified the expression of Piezo and TRP family channels in the cornea (28), TM (29), and retina (30), which exerts a vital impact on the regulation of inflammation, oxidative stress, cell apoptosis, and neurotransmission, etc. (31). Widely distributed mechanosensitive channels serve as the multi-functional mechanical transducer and play parts in maintaining ocular biomechanics. The disruption of mechanical homeostasis initiated by the dysfunction of mechanosensitive channels or other pathological stimuli may exacerbate the damage to the stressed tissues, thus leading to the occurrence and progression of ocular diseases.

Disorder of biomechanical homeostasis in ocular diseases

Diabetic retinopathy

Diabetic retinopathy (DR) is one of the most prevalent complications of DM. The progression of DR is associated with chronic DM status, hyperglycemia, hypertension, dyslipidemia, higher body mass index, and smoking (32). Recent studies demonstrated the potential association between glaucoma and DR, as they share several common risk factors (e.g., blood pressure, obesity, serum total cholesterol, etc.) and pathophysiological features (e.g., impairment of vascular supply, and neuroretina degeneration, etc.) (33, 34). We previously confirmed that the body mass index (BMI) is positively correlated with CSFP (35), and the latest meta-analysis demonstrated that obesity (BMI $>$ 30 kg/m^2) was a risk factor for non-proliferative DR. Collectively, we speculated that the high BMI induces the elevation of CSFP, which may lead to the dysfunction of capillary reflux and the upregulation of retinal venous pressure. Retinal venous pressure is reported to be increased in both DR and glaucoma (36). The elevated retinal venous pressure causes hypoxia and tissue edema, resulting in potential pathologic changes including microaneurysm and cotton wool spots at the early stage of DR. Moreover, the increased retinal venous pressure may trigger mechanosensitive channels such as TRPV4 in endothelial cells. TRPV4 activation is linked to higher BRB permeability, and the genetic ablation of TRPV4 could efficiently alleviate retinal edema and BRB compromise in diabetic mice (37, 38). Thus, targeting mechanosensitive channels like TRPV4 could be a promising therapeutic strategy for the treatment of DR.

Intriguingly, the possible association between DM and keratoconus (KC) was also reported. McKay et al. (39) proposed a similar collagen crosslinking mechanism in the development of both diseases, they hypothesized that DM is associated with increased ACEs that led to inter- and intramolecular crosslinking, thus increasing the corneal rigidity. On the contrary, KC is characterized by decreased mechanical stiffness and secondary corneal ectasia. Hence, excessive crosslinking in DM may protect against KC development, but further studies are required to verify this hypothesis.

Keratoconus

The cornea is the outermost transparent tissue of the eye, its biomechanical properties, such as strength and stiffness, are determined by its five composing layers, namely epithelium, bowman's layer, stroma, Descemet's membrane, and the endothelium. The imbalance of biomechanical homeostasis cross cornea contributes to the occurrence of corneal diseases such as KC. KC is a progressive corneal ectasia condition featured as a cone-shaped cornea with local thinning corneal stroma. Top risk factors of KC include family history, eye rubbing, eczema, asthma and allergy (40). Associated with disorganization and undulation of tissue structure, the alteration in ocular biomechanics plays an essential role in the pathogenesis of KC. Bettahar et al. (41) reported that eye rubbing is a considerable contributing factor in corneal degeneration of KC patients. Rubbing action triggers several mechanical insults, including IOP spikes, altered shear stress, and high hydrostatic tissue pressure. For instance, vigorous rubbing can skyrocket the IOP to more than 10 times of a normal resting IOP, generating more dramatic pressure strain on the cornea (7). Mechano-transducers like YAP in stromal cells and β-catenin in epithelial cells are associated with the regulation of substrate stiffness and protease production in KC (42, 43). A comprehensive understanding of the mechanobiology of corneal diseases may pave the way for new avenues for therapeutic approaches.

Glaucoma

Glaucoma is an irreversible visual impairment disease with substantial changes in ocular biomechanical properties. The main risk factors for glaucoma include aging, elevated IOP, family history of glaucoma, and high myopia (44). The biomechanical disturbance is indispensable in the pathogenesis of glaucoma. Moreover, the glaucomatous biomechanical stress is generated by several ocular tissues (e.g., TM, iris, peripapillary sclera, and ONH), which exert direct or indirect biomechanical roles in various subtypes of glaucoma. Here, we mainly discuss the biomechanical features of glaucoma in predominant clinical subtypes including primary congenital glaucoma (PCG), primary angle-closure glaucoma (PACG), malignant glaucoma and primary open-angle glaucoma (POAG).

PCG is characterized by the abnormal anatomical structure of the TM and anterior chamber angle, thus resulting in aqueous outflow resistance and IOP elevation in infancy (45). With the progression of the disease, the affected eye may display a larger cornea or eyeball size than normal individuals, which is named "hydrophthalmos" or "buphthalmos." Of note, these two terms involve different etiologies and clinical features. Hydrophthalmos mainly refers to the enlargement of the cornea, with or without the whole eyeball expansion. Here, we speculate that the vitreous biomechanics might be involved in the formation of hydrophthalmos. The vitreous cavity is full of intact, dense and regularly structured vitreous gel without vitreous liquefaction in infancy (46), which acts as a favorable mechanical buffer to counteract the anterior pressure derived from the elevated IOP. The posterior segment of the eye tissues is less susceptible to mechanical stimuli than the cornea, thus the primary ocular deformation occurs in the cornea. However, with the constant IOP elevation and chronic damage to the eye tissues, the biomechanical buffering role of the vitreous cannot fully offset the excessive pressure impacted on the still-elastic young eye, eventually forming "buphthalmos" featured by sclera distension and eyeball enlargement (47).

The relative pupillary block between the iris and lens is the common mechanism of PACG. The pathogenic structural changes include lens antedisplacement, plateau iris configuration and iris bombe, sequentially inducing the pupillary block accompanied by the obstruction of aqueous humor. These changes raise the pressure difference between the posterior chamber and anterior chamber, contributing to the angle closure and IOP elevation (48). Severe acute angle-closure glaucoma can lead to morphological changes of lens, known as the glaucomatous fleck, which is an irregular grayish-white spot in the anterior lens capsule at the pupillary area. It might relate to nutritional disorders, or direct contact between the iris and the anterior lens capsule under a high IOP attack (49).

Malignant glaucoma is featured with the progressive elevation of IOP and resistance to therapeutics. It is also termed as ciliary block glaucoma, vitreous displacement glaucoma, aqueous humor misdirection syndrome, or vitreociliary block glaucoma. Although the underlying etiology of malignant glaucoma is not well-elucidated, some widely-accepted theories indicate that it may result from the anterior displacement of irido-crystalline diaphragm elicited by the swelling, hypertrophy, or anterior displacement of the ciliary body, or by the laxity of zonular (50). The increasing pressure difference in these compartments blocks the normal forward passage of aqueous humor and traps the refluxed aqueous flow in the vitreous cavity. The excessive pressure difference between vitreous cavity and anterior chamber escalates the anterior displacement of irido-crystalline diaphragm, accompanied by a flattened anterior chamber (51). Recent research proposed potential risk factors such as choroidal expansion and anterior vitreous abnormalities in malignant glaucoma, subsequent confirmation still needs to be performed (52).

The prominent role of TLCPD, established by IOP and CSFP across the ONH, is well-acknowledged in the POAG etiology. Our previous studies identified that patients with normal-tension glaucoma had significantly lower CSFP and a higher TLCPD when compared with the normal subjects (53). The increased TLCPD may contribute to the LC deformation involving astrocyte migration, axonal bundle disorganization and extracellular matrix alternation (54, 55). Specifically, the individual role of these two forming ingredients is not equivalent, IOP-driven biomechanical effects display a more dominant role than CSFP (56). Multiple mechanosensitive channels such as Piezo, TRPV4, and TREK-1 are proven to have biomechanical effects in glaucoma on an experimental basis. The chemical inhibition or genetic ablation of these channels significantly ameliorates pathological phenotypes of optic nerve degeneration caused by IOP elevation, indicating the potential therapeutic roles of targeting mechanosensitive channels in glaucoma (57).

Spaceflight-associated neuro-ocular syndrome

After a long-term spaceflight, some astronauts were bothered by visual changes associated with ocular conditions, which were termed spaceflight-associated neuro-ocular syndrome (SANS) (58). The occurrence of SANS is primarily attributed to the chronic exposure of the astronauts to the unique microgravity environment during long-term spaceflight. Other associated risk factors include radiation exposure, inflated ambient CO_2 concentrations, high salt diets, intense resistance exercise, nutritional disturbance, and genetic variations in the one-carbon metabolism pathway (59, 60). Due to the prolonged microgravity exposure, SANS is generally characterized as fluid redistribution in the optic nerve sheath (ONS) and cerebrospinal fluid cavity (61). The cephalad fluid shifts occurring with weightlessness elevate the biomechanical strain transmitted to the ONH, as evidenced by progressive papilloedema and globe-flattening (62). To better distinguish the pathologies, a terrestrial analog called 6-degree head-down tilt bed rest (HDTBR) was established. After 30 days of examination, similar ocular changes of SANS were also identified in the HDTBR model with elevated ICP (63). Moreover, the alterations of TLCPD are also suspicious in the development of optic disc edema with increased ONS pressure protruding the LC anteriorly (64). Several countermeasures have been proposed to rebalance the biomechanical homeostasis at the site of ONH in SANS cases. A lower body negative pressure apparatus has been used to combat the cephalad fluid shift and showed a significant reduction of ICP in HDTBR testing (65). To rebuild the positive and posteriorly-directed pressure gradient, a swim goggles compression experiment was adopted to increase IOP and restore the normal TLCPD (64). These discoveries highlight the malignant impacts of imbalanced TLCPD induced by idiopathic intracranial hypertension, underscoring the fundamental role of biomechanical homeostasis in ocular health.

Retinal vein occlusion

Retinal vein occlusion (RVO) is a constellation of hypertensive retinopathies associated with multiple risk factors like aging, systemic hypertension, cardiovascular disorders, hyperlipidemia, diabetes, glaucoma, and thrombophilic mutations (e.g., antithrombin, protein C or protein S) (66, 67). The physical obstruction of the retinal venous system is generally induced by thrombosis, deformation of the vein wall, and external biomechanical compression secondary to glaucoma (68). Mechanically, it is postulated that the elevated IOP compresses the LC and optic disc, thereby leading to the stretching and weakening of the vessel wall, which further predisposes the retinal vein to occlusion (69). An excessive dropout of parapapillary choroidal microvasculature is also observed in RVO patients (70). Meanwhile, the direct biomechanical insult of IOP obstructs the retinal vein drainage and induces venous stasis, consequently exacerbating the intimal proliferation in the vein (71). Substantial evidence is required to further elucidate the underlying biomechanical changes in RVO etiology.

Myopia

As the most common refractive condition, myopia often starts in childhood and is manifested as short- or near-sightedness. Emerging evidence has supported the role of nature (genetics and inheritance) and nurture (environment and lifestyle) in the onset of myopia (72). Specifically, the major risk factors include higher education levels, prolonged near-work time, reduced outdoor activities, and inherited genetic predispositions (e.g., MYP1 family, ZNF644, SCO2, BSG, APLP2, etc.) (72–74). The biological deformation of myopia is typically characterized by an elongated posterior scleral shell. Severe scleral thinning in high myopia can lead to the biomechanical deformation of the posterior scleral wall, manifested as posterior staphyloma. It has been validated in several animal studies that the sclera thins during experimental myopia, suggesting the distinct role of scleral remodeling in the pathological axial elongation (75, 76). Scleral remodeling is a process of micro-deformation in a volume-conserving pattern, which results in the rearrangement of existing tissue materials. In highly myopic eyes, this mechanical adaption to the scleral tension is even greater than an equivalent IOP attack in the aspect of globe enlargement and posterior thinning of the eye wall (77). Besides, David et al. (78) have studied the impact of vitreous torsional oscillation stress on the retina secondary to regular ocular motion. They found that the high myopia eye is hypersensitive to this chronic mechanical torsional stress, speculating it as the underlying cause of rupture-induced retinal detachment occurred in pathological myopia.

Discussion

Emerging evidence has associated biomechanical homeostasis with ocular health. The biomechanical features of the anterior segment (cornea, sclera, drainage route, and lens capsule) and the posterior segment (vitreous, Bruch's membrane, choroid, retina, and optic nerve) of the eye have been documented with substantial evidence, whereas the understanding of inner homeostasis between different tissues remained unclear. Knowledge of these physical interactions is pivotal not only to clarify the underlying pathogenesis of a vast range of retinal and vitreoretinal diseases, such as DR, KC and glaucoma, but also to optimize the surgical handling of ocular tissues and the design of novel therapies.

Till now, the present studies of biomechanical analysis mainly focus on glaucoma (79), DR (80) and high myopia (81). The mainstream analytical methods of ocular biomechanics can be summarized into three subcategories, including (1) computational modeling (e.g., finite element modeling): a simplified model under ideal conditions with substantial variations from real-life situations (79); (2) microfluidic eye chips: a newly emerging 3D cell culture system providing novel insights for biomechanical studies *in vitro* (82); (3) the commercially available medical equipment (e.g., Corvis ST and wearable IOP biosensor) for clinical assessment (83, 84).

Novel treatment approaches and concepts have been proposed for the restoration of biomechanical homeostasis in ocular disorders. For the anterior segment, corneal cross-linking is widely utilized to increase corneal biomechanical resistance in treating ectasia and KC (85). Similarly, as collagen fiber crimping and re-alignment are observed in the development of myopia, collagen crosslinking has also been recommended as a potential therapeutic strategy for progressive myopia (86). For the posterior segment, LC stiffening is a common pathology in multiple ocular diseases like glaucoma and DR, which can be triggered by elevated IOP and increased AGEs, respectively. To alleviate the stresses and strains, collagenase treatment has been investigated in human cadaver eyes for the reduction of the biomechanical stiffness of LC (87). Besides, the posterior segment ring implantation (e.g., intrascleral or subarachnoid space ring) has been proposed as a potential countermeasure to delay the LC deformation in glaucoma at the conceptional level (12).

Altogether, biomechanical homeostasis is crucial to maintain the physiological function of the eye. In-depth acknowledgment of ocular biomechanics could help us better understand the underlying mechanical properties and molecular mechanisms in different ophthalmic conditions, further providing novel diagnostic methods and countermeasures from the perspective of mechanobiology.

Author contributions

The topic was devised and conceptualized by NW. YC and TR conducted the literature review and wrote the manuscript. All authors have read and agreed to the published final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The link between diabetic retinal and renal microvasculopathy is associated with dyslipidemia and upregulated circulating level of cytokines

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Purpose: To investigate the mechanisms underlying the correlations between diabetic retinopathy (DR) and diabetic nephropathy (DKD) and examine whether circulating cytokines and dyslipidemia contribute to both DR and DKD in patients with 2 diabetes mellitus (T2DM).

Methods: A total of 122 patients with T2DM were enrolled and categorized into the DM group (without no DR and DKD), DR group [non-proliferative DR (NPDR), and proliferative DR (PDR)] with no DKD), DR complicated with DKD groups (DR+DKD group). The biochemical profile, including fasting blood glucose (FBG), glycated hemoglobin (HbA1c), and lipid profile were estimated, and plasma inflammatory and angiogenic cytokines [monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF)-A, C, D, and placental growth factor (PlGF)] were analyzed by protein microarrays. The atherogenic plasma index (API) was defined as low-density lipoprotein cholesterol (LDL-C)/high-density lipoprotein-cholesterol (HDL-C); atherogenic index (AI) was calculated as [(total cholesterol (TC) -HDL-C)/HDL-C], and atherogenic index of plasma (AIP) was defined as log (TG/HDL-C).

Results: By multivariable disordered regression analysis, after controlling for duration of DM and hypertension, LDL-C ($p = 0.019$) and VEGF-D ($p = 0.029$) resulted as independent risk factors for DR. Albumin-to-creatinine ratio (uACR) ($p = 0.003$) was an independent risk factor for DR with DKD. In DR, NPDR, and PDR groups, grades of A1, A2, and A3 of albuminuria increased with the severity of DR. In A1, A2, and A3 grade groups, the severity of DR (DM, NPDR, and PDR) increased with higher albuminuria grades. Kendall's tau-b correlation coefficient analysis revealed that FBG ($p = 0.019$), circulating level of PlGF ($p = 0.002$), and VEGF-D ($p = 0.008$) were significantly positively correlated with the grades of uACR ($p < 0.001$), and uACR grades were significantly correlated with DR severity ($p < 0.001$).

Conclusions: The occurrence and severity of DR are closely correlated with kidney dysfunction. Among the three kidney functional parameters, uACR resulted as the better indicator of DR severity and progression than glomerular filtration (eGFR) and serum creatinine (Scr). Impaired FBG was associated with microalbuminuria, emphasizing that well-controlled FBG is important for both DR and DKD. The

link between diabetic retinal and renal microvasculopathy was associated with dyslipidemia and upregulated circulating level of angiogenic cytokines.

KEYWORDS

dyslipidemia, diabetes mellitus, diabetic retinopathy, fasting blood glucose, cytokines, diabetic kidney disease, Albumin-to-creatinine ratio

1. Introduction

Diabetes Mellitus (DM) is the most common noncommunicable epidemiological illness and a major public health problem that has been approaching epidemic proportions globally. China ranks number one, with the highest number of people with DM (1). The high mortality and disability rates caused by the various complications of diabetes impose a heavy economic burden on society. Thus, early diagnosis and time prevention of complication is of extreme importance.

Diabetic retinopathy (DR), diabetic kidney disease (DKD), and diabetic peripheral neuropathy are the most prevalent microvascular complications of type 2 diabetes (T2DM). DR and DKD have complicated interleaving relationships and are the main causes of death and disability in diabetic patients. In addition, DR is a leading cause of blindness in the working-age population (2). The META-EYE Study Group reported that the prevalence of DR is 34.6% worldwide, while the prevalence of vision-threatening proliferative DR that can lead to blindness is 10.2%, accounting for 51% of blindness cases worldwide (3).

DKD has an insidious onset and lacks distinctive clinical signs, and it is responsible for 20–40% of cases of DM (4–7). Its timeline is well characterized for type 1 diabetes mellitus (i.e., DKD develops within 10 years of the first onset of type 1 DM); in those with T2DM, it usually starts developing after the onset of hyperglycemia. Furthermore, DKD remains a leading cause of new-onset end-stage renal disease of DM (accounting for 50.1%) and is the leading cause of mortality (6, 8). Currently, there are still limited treatments for DR and DKD.

Numerous international large-scale epidemiological studies, including the UK Prospective Diabetes Study (UKPDS, follow up for 10 years) (9), The Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE, follow up for 4.3 years) (10), the Action to Control Cardiovascular Risk in Diabetes (ACCORD, follow up for 4 years) (11), and the Veteran Affairs Diabetes Trial (VADT, follow up for 5 years) (12) have demonstrated that even though the multiple risk factor interventions (hyperglycemia, blood pressure, and lipid regulation) can effectively reduce the risk of diabetic microvascular disease (DMVC), 51% of diabetic patients still develop DR (51% of those with DR) and DKD (25% of individuals with DKD) (13). DMVC (the residual risk of DMVC) that still exists after comprehensive management of diabetic patients is a major challenge for both ophthalmologists and endocrinologists.

The interrelationships between DR and DKD are currently receiving extensive attention. Several studies have suggested a close connection between the occurrence and progression of the two common diabetic microvasculopathies. According to a large-scale epidemiological investigation conducted by the Chinese Center for Disease Control and Prevention (CDC), the number of those suffering from both DR and DKD might exceed 2.5 million (14). According to the findings of a national cross-sectional study conducted by Jia's team in 2016, the prevalence of high level of proteinuria in patients with DR reached 47.8% among the 3,301 patients with T2DM (average age 59.34 ± 12.28 years, average DM course of 8.48 years) and the frequency of DR increased as urine albumin levels increased (15). The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) study also found that microalbuminuria was statistically significantly associated with proliferative diabetic retinopathy (PDR) and clinically significant macular in the younger group by multivariable analysis (4). Two prospective studies on the Singaporean population (N = 5,763, age ≥ 40 years) found that retinal microvasculopathy was associated with the risk of end-stage renal illness, and end-stage renal disease was 2.6 times more likely to occur in person with DR than in patients without retinopathy (16).

Although the complicated interrelationships between DR and DKD have been elucidated in numerous studies, the underlying mechanisms remain still uncertain. Our previous study suggested that homocysteine contributes to DR and is associated with increased urine microalbumin (17). In this study, we further investigated the correlations between DR and DKD in patients with T2DM, testing the hypothesis that known circulating angiogenic and inflammatory cytokines and dyslipidemia contribute to both DR and DKD.

Abbreviations: ACCORD, The Action to Control Cardiovascular Risk in Diabetes; ADA, American Diabetes Association; ADVANCE, The Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation; AI, atherogenic index; AIP, atherogenic index of plasma; API, atherogenic plasma index; AUC, Area Under Curve; BCVA, best-corrected visual acuity; CDC, Chinese Center for Disease Control and Prevention; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; DKD, diabetic kidney diseases; DMVC, diabetic microvascular disease; DR, diabetic retinopathy; eGFR, estimated glomerular filtration; FBG, fasting blood glucose; Fg, fibrinogen; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein-cholesterol; HIF-1α, hypoxia inducible factor-1; IGF-1, VEGF, insulin-like growth factors−1; IL, interleukin; IQR, interquartile range; KDIGO, the Kidney Disease: Improving Global Outcomes; LDL-C, low-density lipoprotein cholesterol; MCP-1, monocyte chemoattractant protein-1; NPDR, non-proliferative DR; PDR, proliferative DR; PlGF, placental growth factor; ROC, receiver operating characteristic curve; Scr, serum creatinine; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides; uACR, Albumin-to-creatinine ratio; UKPDS, the UK Prospective Diabetes Study; UMA, urine microalbumin; VADT, the Veteran Affairs Diabetes Trial; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor; WESDR, The Wisconsin Epidemiologic Study of Diabetic Retinopathy.

2. Materials and methods

2.1. Participants

This prospective study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University. All subjects signed an informed consent form before participation.

A total of 122 participants with T2DM, including 73 males and 49 females, aged 24–76 years old, were recruited from the outpatient department of Beijing Tongren Hospital from April 2016 to September 2020. Age, gender and duration of diabetes, and other related data were also collected.

2.2. Inclusion and exclusion criteria

Inclusion were the following: (1) patients diagnosed with T2DM and DR according to the 2016 American Diabetes Association (ADA) guidelines of DM (18) and 2002 A Position Statement of DR (19); those who were able to provide informed consent. Exclusion criteria were: those with T2DM with macular edema secondary to other retinal vascular diseases; co-existent other retinal diseases such as age-related macular degeneration, uveitis, and inherited retinal diseases; recent history of posterior segment or cataract surgery; ocular media opacity and unable to tolerate examinations due to severe system diseases. Also, T2DM with normal fundus but with abnormal estimated glomerular filtration (eGFR) or Albumin-to-creatinine ratio (uACR) were not considered. Patients with a history of other chronic kidney diseases were also excluded.

2.3. Extensive eye examinations

Best-corrected visual acuity (BCVA) and non-contact intraocular pressure (TX20 Automatic Non-contact Tonometer, Canon Co., Ltd, Tokyo, Japan) assessment, slit-lamp microscopic examination (SL-IE Slit Lamp Microscope, Topcon Co., Ltd, Tokyo, Japan), and fundus examination with mydriasis were applied for all the participants. Fundus photography (CR-1 non-mydriatic Fundus Camera, Canon Co., Ltd) was used to capture at least two fields centered on both eyes' optic disc and macula. Two independent ophthalmologists (Q.W. with 4 and B.Q. with 6 years of experience in the field) ascertained the DR status of the participants based on the International DR severity scale (19). Swept-source optical coherent tomography was applied (DRI OCT1 Atlantis scanner, Topcon Co., Ltd., Tokyo, Japan or Plex Elite 9000, Carl Zeiss Meditec, Inc, Oberkochen, German) for all the enrolled subjects. B-scan images were obtained by a 9 mm × 9 mm scanning range mode. The DR status of the worse eye was recorded as an individual's DR grade.

2.4. Definition and classification of diabetic kidney disease

DKD was defined as "a clinical diagnosis made based on the presence of albuminuria and/or reduced eGFR in the absence of signs or symptoms of other primary causes of kidney damage" according to the ADA "Standards of Medical Care in Diabetes 2022." uACR was used to evaluate the severity of albuminuria; high urinary albumin excretion was defined as ≥30 mg/g Cr. As recommended by ADA, eGFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (20); persistent >60 ml/min/1.73 m^2 was defined as normal (20).

Stages of DKD were graded according to the Kidney Disease: Improving Global Outcomes (KDIGO) classification criteria with incorporates albuminuria at all stages of eGFR. In this system, DKD is classified based on the cause (C), GFR (G), and albuminuria (A). GFR categories included (G1-G5): G1: normal to high (GFR≥90 ml/min/1.73 m^2), G2: mildly decreased 60–89 ml/min/1.73 m^2), G3: mildly to moderately decreased (45–59 ml/min/1.73 m^2), G4: moderately to severely decreased (30–44 ml/min/1.73 m^2), G5: severely decreased (15–29 ml/min/1.73m^2) and kidney failure (<15 ml/min/1.73 m^2); albuminuria categories included three classes (A1–A3): A1: normal to mildly (<30 mg/g), A2: moderately (30–299 mg/g) and A3: severely increased (≥300 mg/g) (20).

2.5. Determination of biochemistry profile and plasma cytokines

Blood biochemistry profile: fasting biochemical examination was performed for all the participants. These included low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides (TG), total cholesterol (TC), glycated hemoglobin (HbA1c), fasting blood glucose (FBG) etc. Atherogenic index (AI) was defined as (TC-HDL-C)/HDL-C; atherogenic plasma index (API) was calculated as LDL-C/HDL-C; atherogenic index of plasma (AIP) was calculated as log (TG/HDL-C).

To determine the plasma level of angiogenic and inflammatory cytokines, including vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, placental growth factor (PlGF), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6 and IL-8, the Luminex technology (Luminex 200^{TM} liquid chip detector, Millipore, Boston, Massachusetts, USA) was applied according to the manufacturer's instructions.

2.6. Subgrouping of the participants

According to the 2020 ADA guidelines of DM (18) and 2017 A Position Statement of DR (13), the participants were assigned to the DM group {no DR, 32 patients, aged 37–75 years, median [interquartile range (IQR)]: 56 (48–65) years}, non-proliferative diabetic retinopathy group [NPDR group) [56 patients, aged 29–76 years, 56 (51–61)] years], and PDR group [43 patients, aged 27–74 years, 55 (49–60) years].

According to the definition of DR and DKD, all participants were further grouped to DM (no DR and DKD), DR (no DKD), DKD (no DR), and DR+DKD groups. The DR participants were further categorized into "PDR" group if they had retinal and/or optic disc neovascularization in at least one eye. Those with any other DR grade were categorized as the NPDR group, and participants with no DR in both eyes were assigned to the "DM" group. DR and DKD were classified according to the criteria according to the 2020 ADA

guidelines of DM (18) and 2017 ADA: A Position Statement of DR (21) as described above.

2.7. Determination of the cutoff value of AIP, API, and AI by receiver operating characteristic (ROC) curve

API (LDL-C/HDL-C), AI [(TC-HDL-C)/HDL-C), and AIP (log(TG/HDL-C)] with high sensitivity and specificity at maximum Youden index were selected as the cutoff values on the ROC curve as described previously. Patients with API > 2.24 (AUC: 0.746; sensitivity = 0.708, specificity = 0.517), AI > 2.91 (AUC, 0.723; sensitivity = 0.629; specificity = 0.724) or AIP > 0.01 (AUC 0.564; sensitivity = 0.607, specificity = 0.552) were assigned to high API, high AI, and high AIP groups, respectively.

2.8. Sample size calculation

Power Analysis and Sample Size software (PASS 2022, NCSS LLC, Utah, USA) were used to determine the sample size as we previously described (17). The sample size was calculated at a 95% confidence level with a margin of error of +/− 5% and designed power (1-beta = 85%, the actual power was 86.59%). Based on our pilot study, as the representing parameter of the study group, the mean level of LDL-C was 3.17 and 2.46 in the control group (DM), respectively; the mean, the standard deviation was +−0.75, the minimum sample per arm (per group) was 22 subjects.

2.9. Statistical analysis

SPSS software (SPSS, Inc. 23.0, Chicago, IL, USA) was applied for statistical analysis. Kolmogorov-Smirnov test and Shapiro-Wilk test were used to assess data normality. Variance homogeneity was tested by Levene's test. Age of participants, duration of diabetes, and biochemical parameters were described as means ± standard deviation (mean ± SD) or median (IQR). One-way analysis of variance (ANOVA) or Kruskal-Wallis test were used for group comparisons. The circulating levels of cytokines VEGF-A, VEGF-C, VEGF-D, PlGF, MCP-1, IL-6, and IL-8 were described as mean ± SD or median (IQR); group comparisons (DM, NPDR, and PDR groups, or DM, DR, DR+DKD groups) were analyzed by independent sample *t*-test or Mann–Whitney *U*-test according to the data distribution. Bonferroni corrections were applied for comparison between the groups. The Kendall's Tau-b rank correlation coefficient was used for testing the correlations between the cytokines or chemical parameters, classification of DKD, and different grades of DR. Single ordinal logistic regression analysis was applied to assess the influence of the variables on DR or DKD. Multivariable ordinal logistic regression was applied to evaluate the effects of the variables on DR or uACR grading. Multivariable logistic regression analysis was used to evaluate the effects of the cytokines on different groups. A $p < 0.05$ indicated statistical significance.

3. Results

3.1. Baseline demographic and biochemical profile characteristics

A total of 122 participants with T2DM were included in the study. The participants were assigned to the DM group if they had no DR or DKD (23 patients, aged 24–76 years, median (IQR): 57 (53–65) years), DR group if they had DR but no DKD [35 patients, aged 27–71 years, 56 (49–60) years], and DR+DKD group if they had both DR and DKD [64 patients, aged 29–76 years, 55 (49–62.75) years]. 9 T2DM patients [aged 40–76 years, 49 (42–70) years] with normal fundus but with abnormal eGFR or uACR(the DKD group) were not considered in this study, because these patients cannot be excluded from other primary causes of kidney damage unless confirmed by a kidney biopsy, which was not accepted by those patients. Significant differences in the duration of DM ($p = 0.041$), LDL-C ($p = 0.022$), API ($p = 0.022$), FBG ($p = 0.031$), Scr ($p = 0.027$), eGFR ($p = 0.025$) and uACR ($p < 0.001$) were found among the three groups. There was no significant difference in age ($p = 0.453$), gender ($p = 0.720$) and duration of hypertension ($p = 0.955$) among the three groups (Table 1).

By multinomial logistic regression analysis, when DM was considered as the reference, there was a significant difference in AI ($p = 0.022$), AI grouping (when it is higher than 2.91, $p = 0.050$), TC ($p = 0.021$), LDL-C ($p = 0.007$), and API ($p = 0.007$) between the DR and DM groups. There was no significance in gender ($p = 0.621$), age ($p = 0.269$), duration of DM ($p = 0.055$) and hypertension ($p = 0.778$), TG ($p = 0.312$), HDL-C ($p = 0.862$), Hb1Ac ($p = 0.244$), FBG ($p = 0.271$) between the DR and DM groups. There was a significant difference in DM duration ($p = 0.008$), AI grouping (when it is higher than 2.91, $p = 0.038$), uACR (OR = 1.355, 95% CI 1.147–1.600, $p < 0.001$), LDL-C ($p = 0.020$), FBG ($p = 0.019$) and API ($p = 0.020$) between the DR+DKD and DM groups. uACR (OR = 1.375, 95% CI 1.165–1.623, $p < 0.001$), Scr (OR = 1.030, 95% CI 1.008–1.051, $p = 0.007$) and eGFR (OR = 0.991, 95% CI 0.983–0.998, $p = 0.017$) was significantly higher in DR+DKD group in comparison with DR group (Figure 1).

According to Kendall's tau-b correlation coefficient analysis, only FBG was significantly positively correlated with the grades of uACR (r = 0.157, $p = 0.019$). Other parameters including TC ($p = 0.339$), TG ($p = 0.253$), LDL-C ($p = 0.268$), HDL-C ($p = 0.339$), Hb1Ac ($p = 0.942$), AI ($p = 0.756$), API ($p = 0.630$), AIP ($p = 0.367$) were positively correlated with the grades of uACR, but there was no statistically difference (Figure 2).

3.2. Associations of a renal function profile among the DM, DR, and DR+DKD groups

To test the hypothesis that renal impairment is associated with the pathogenesis of DR and DKD, three renal function parameters were compared between the three groups by the Kruskal-Wallis H test in this study. There was significant differences in the most clinical used renal functional parameters serum creatinine (Scr, $p_{all} = 0.027$, $p_{DM\ vs.\ DR+DKD} = 0.023$), glomerular filtration (eGFR, $p_{all} = 0.025$, $p_{DR\ vs.\ DR+DKD} = 0.020$) and urine microalbumin-creatinine ratio (uACR, $p_{all} < 0.001$, $p_{DM\ vs.\ DR} < 0.001$, $p_{DM\ vs.\ DR+DKD} < 0.001$)

TABLE 1 Comparison of baseline demographic and clinical characteristics in subjects with DM, DR, and DR+DKD.

	DM	DR	DR+DKD	H/χ2/F	*p*
Number	23	35	64	/	/
Age, years	57.00 (53.00–65.00)	56.00 (49.00–60.00)	55.00 (49.00–62.75)	1.58[b]	0.453
Gender (Male/Female)	14/9	19/16	40/24	0.65[c]	0.720
Duration of DM, years	10.00 (2.00–15.00)	12.00 (10.00–16.00)	12.50 (8.00–19.50)	6.37[b]	0.041*
Duration of HBP, years	0 (0–5.00)	1.00 (0–7.00)	1.00 (0–7.00)	0.09[b]	0.955
TC, mmol/L	4.31 ± 0.76	5.03 ± 1.23	4.84 ± 1.20	2.86[a]	0.061
TG, mmol/L	1.42 ± 1.08	1.73 ± 1.20	1.52 ± 0.97	0.67[a]	0.513
LDL-C, mmol/L	2.45 (1.91–3.07)	3.15 (2.47–3.70)	2.88 (2.16–3.72)	7.63[b]	0.022*
HDL-C, mmol/L	1.24 (1.02–1.43)	1.13 (0.98–1.48)	1.15 (0.98–1.46)	0.38[b]	0.260
AI (TC-HDL-C)/HDL-C)	2.53 (1.80–3.11)	3.04 (2.20–4.27)	2.96 (2.44–3.67)	5.30[b]	0.071
API (LDL-C/HDL-C)	2.17 (1.54–2.42)	2.70 (1.97–3.33)	2.47 (1.89–3.03)	7.61[b]	0.022*
AIP (log (TG/HDL-C)	−0.02 (−0.27–0.07)	−0.01 (−0.22–0.39)	0.03 (−0.20–0.23)	1.32[b]	0.180
HbA1c, %	6.60 (6.10–8.90)	7.70 (7.00–8.90)	7.80 (6.70–9.08)	4.08[b]	0.130
FBG, mmol/L	6.48 (5.59–8.18)	8.10 (6.37–9.45)	8.34 (6.53–11.29)	6.95[b]	0.031*
Scr, μmol/L	66.00 (51.20–80.80)	59.90 (47.00–69.60)	71.45 (54.63–91.08)	7.21[b]	0.027*
eGFR, mL/min	103.85 (91.56–124.79)	123.79 (96.92–155.60)	101.47 (75.11–138.97)	7.41[b]	0.025*
uACR, mg/g Cr	13.14 (4.62–19.56)	10.31 (7.08–18.44)	214.99 (44.12–1173.91)	85.61[b]	<0.001*

*Statistically significant: $p < 0.05$. According to the type of data and the data distribution, [a]one-way ANOVA analysis, [b]Kruskal-Wallis analysis, [c]Chi-square test were applied. DM, Diabetes mellitus; DR, Diabetic retinopathy; DKD, Diabetic Kidney Disease; HBP, High blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, High-density lipoprotein Cholesterol; LDL-C, Low-density lipoprotein Cholesterol; AI, Atherosclerosis index [(TC-HDL-C)/HDL-C]; AIP, Atherogenic index of plasma [log (TG/HDL-C)]; API, Atherogenic plasma index (LDL-C/HDL-C); HbA1c, Hemoglobin; FBG, fasting blood glucose; Scr, Serum creatinine; eGFR, Estimated Glomerular Filtration Rate; uACR, Urinary albumin/creatinine ratio.

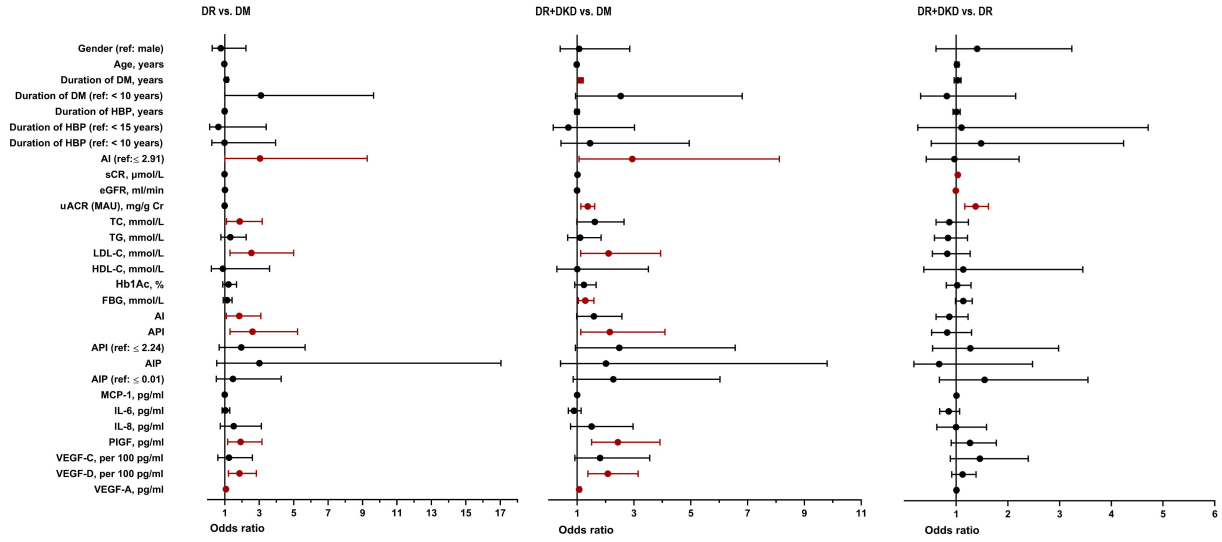

FIGURE 1
Univariate logistic regression analysis between DM, DR, and DR+DKD groups. DM, diabetes mellitus; DR, diabetic retinopathy; DKD, diabetic kidney disease; HBP, high blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AI, atherosclerosis index [(TC-HDL-C)/HDL-C]; AIP, atherogenic index of plasma [log (TG/HDL-C)]; API, atherogenic plasma index (LDL-C/HDL-C); HbA1c, hemoglobin; FBG, fasting blood glucose; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; uACR, urinary albumin/creatinine ratio; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; IL-8, interleukin-8; PlGF, placental growth factor; VEGF, vascular endothelial growth.

between the DM, DR and DR+DKD groups, indicating that uACR is more sensitive and closer renal dysfunctional parameters in the diabetic microvascular complications especially for patients with DR and DKD (Table 1).

When DR was considered as the reference, multinomial logistic regression analysis showed a significant difference in the three renal function parameters Scr ($p = 0.007$), eGFR ($p = 0.017$) and uACR ($p < 0.001$) between the DR+DKD and DR groups. There was no statistical significance in the lipid profile and other baseline parameters between the two groups (Figure 1).

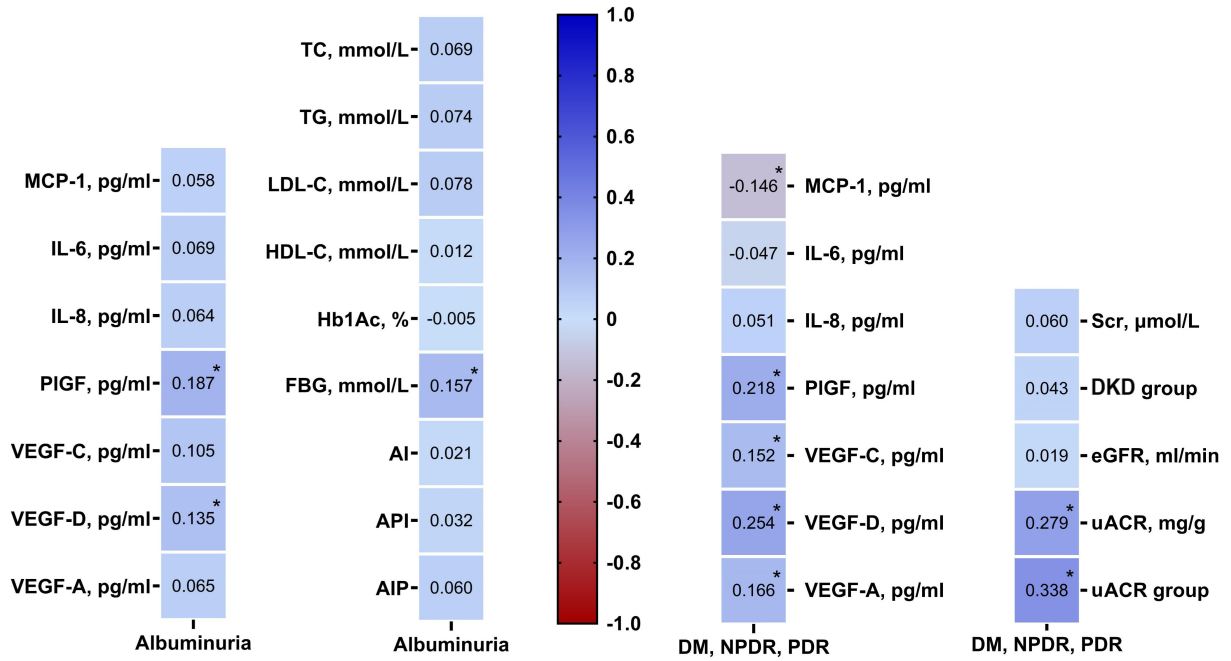

FIGURE 2
Kendall's Tau-b rank correlation coefficient analysis showing angiogenic cytokines were associated with DR and DKD grades, renal function profiles were related with DR grades. DM, diabetes mellitus; DR, diabetic retinopathy; DKD, diabetic kidney disease; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AI, atherosclerosis index [(TC-HDL-C)/HDL-C]; AIP, atherogenic index of plasma [log (TG/HDL-C)]; API, atherogenic plasma index (LDL-C/HDL-C); HbA1c, hemoglobin; FBG, fasting blood glucose; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; uACR, urinary albumin/creatinine ratio; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; IL-8, interleukin-8; PlGF, placental growth factor; VEGF, vascular endothelial growth.

TABLE 2 Comparison of plasma inflammatory and angiogenic cytokines in subjects with DM, DR, and DR+DKD.

	DM	DR	DR+DKD	H/χ2/F	*p*
MCP-1, pg/ml	250.59 (240.48–295.80)	247.61 (227.50–270.13)	249.94 (227.91–287.57)	1.23[b]	0.540
IL-6, pg/ml	0.70 (0.28–1.22)	0.55 (0.24–1.74)	0.63 (0.29–1.55)	0.19[b]	0.908
IL-8, pg/ml	0.91 (0.61–1.72)	1.37(1.02–1.99)	1.27 (0.98–1.65)	3.71[b]	0.157
PlGF, pg/ml	1.55 ± 1.27	2.40 ± 1.40	2.77 ± 1.10	8.47[a]	<0.001*
	$p_{DMvs.DR}$ <0.001; $p_{DMvs.DR+DKD}$ = 0.031; $p_{DRvs.DR+DKD}$ = 0.031				
VEGF-C, pg/ml	51.41 (8.58–148.15)	93.20 (55.69–122.65)	98.14 (53.81–182.03)	4.36[b]	0.113
VEGF-D, pg/ml	135.96 (14.73–248.50)	252.01 (131.06–395.90)	262.85 (164.43–435.87)	14.79[b]	0.001*
	$p_{DMvs.DR}$ = 0.012; $p_{DMvs.DR+DKD}$ <0.001				
VEGF-A, pg/ml	15.22 (9.62–26.27)	27.64 (19.99–32.91)	25.23 (19.65–35.98)	12.55[b]	0.002*
	$p_{DMvs.DR}$ = 0.002; $p_{DMvs.DR+DKD}$ = 0.006				

*Statistically significant: p < 0.05. According to the type of data and the data distribution, [a]one-way ANOVA analysis, [b]Kruskal-Wallis analysis. DM, Diabetes mellitus; DR, Diabetic retinopathy; DKD, Diabetic Kidney Disease; MCP-1, Monocyte chemoattractant protein-1; IL-6, Interleukin- 6; IL-8, Interleukin- 8; PlGF, Placental growth factor; VEGF, Vascular endothelial growth.

3.3. The correlations between a renal profile with DR severity

To further investigate if uACR is associated with DR severity, the same cohort was categorized into DM (without DR), NPDR, and PDR groups according to the criteria described above. By using Kendall's tau-b/c (Tb or Tc) correlation coefficient analysis, among all the indicators/parameters of renal function (Scr, eGFR, uACR, and grades of uACR), uACR ($r = 0.279$, $p < 0.001$) and uACR grades ($r = 0.338$, $p < 0.001$) were significantly correlated with DR severity. This result indicates that uACR is closely correlated with the DR severity and is a good indicator of DR progression (Figure 2).

3.4. Associations of plasma inflammatory and angiogenic cytokines with DM, DR, DR+DKD groups

We further investigated the effects of inflammatory and angiogenic cytokines on both DR and DKD from a global perspective. Interestingly, there was no statistical difference in the inflammatory cytokines between the DM, DR, and DR+DKD groups, but PlGF ($p_{all} < 0.001$, $p_{DM\ vs.\ DR} < 0.001$, $p_{DM\ vs.\ DR+DKD} = 0.031$, $p_{DR\ vs.\ DR+DKD} = 0.031$), VEGF-D ($p_{all} = 0.001$, $p_{DR\ vs.\ DM} = 0.012$, $p_{DR+DKD\ vs.\ DM} < 0.001$) and VEGF-A ($p_{all} = 0.002$, $p_{DR\ vs.\ DM} =$

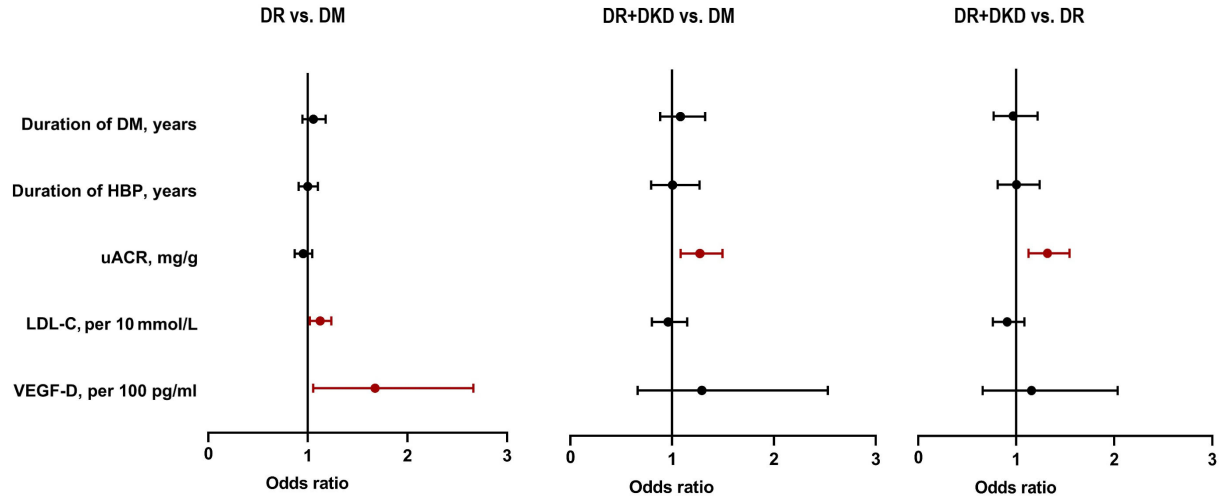

FIGURE 3
Multivariate logistic regression analysis showing uACR, LDL-C, and VEGF-D were risk factors for DR and DKD. DM, diabetes mellitus; DR, diabetic retinopathy; DKD, diabetic kidney disease; HBP, high blood pressure; LDL-C, low-density lipoprotein cholesterol; uACR, urinary albumin/creatinine ratio; VEGF, vascular endothelial growth.

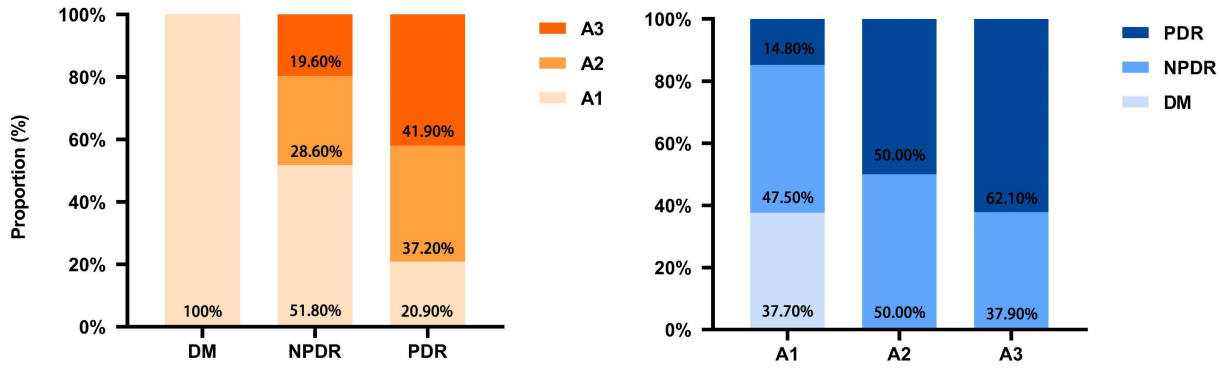

FIGURE 4
Correlation and proportion between DR and DKD grades. DM, diabetes mellitus; DR, diabetic retinopathy; DKD, diabetic kidney disease; A1, A2, A3, albuminuria categories DKD to three classes (A1–A3); A1, normal to mildly (<30 mg/g); A2, moderately (30–299 mg/g); A3, severely increased (≥300 mg/g).

0.006, $p_{DR+DKD\ vs.\ DM} = 0.002$) were significantly different among the three groups. The results indicated that angiogenic cytokines, not inflammatory cytokines, are differently regulated and contribute to diabetic microvasculopathy (Table 2).

When DM was considered as the reference, multinomial logistic regression analysis indicated that PlGF ($p = 0.010$), VEGF-D per 100 ($p = 0.004$) and VEGF-A ($p = 0.021$) were significantly different between the DR and DM groups; yet, there was no significant difference in the inflammatory cytokines between the two groups (Figure 1). When DM was considered as the reference, multinomial logistic regression analysis indicated that PlGF ($p < 0.001$), VEGF-D per 100 ($p = 0.001$) and VEGF-A ($p = 0.010$) were significantly different between the DR+DKD and DM groups. Also, there was no significant difference in the inflammatory or angiogenic cytokines between the DR+DKD and DR groups (Figure 1).

By Kendall's tau-b correlation coefficient analysis, PlGF (r = 0.187, $p = 0.005$) and VEGF-D (r = 0.135, $p = 0.048$) were significantly positively correlated with the grades of uACR. Other circulating cytokines, including MCP-1 ($p = 0.432$), IL-6 ($p = 0.313$), IL-8 ($p = 0.361$), VEGF-C ($p = 0.120$), and VEGF-A (r = 0.07, $p = 0.345$) were found to be positively correlated with DKD, but there was no statistical significance (Figure 2).

3.5. Associations of plasma inflammatory and angiogenic cytokines with DR severity

Kendall's tau-b correlation coefficient analysis showed that DR severity was significantly positively correlated with PlGF (r = 0.22, $p = 0.002$), VEGF-D (r = 0.25, $p < 0.001$), VEGF-C (r = 0.15, $p = 0.048$) and VEGF-A (r = 0.17, $p = 0.031$) but negatively correlated with MCP-1 (r = −0.15, $p = 0.043$). IL-6 (r = −0.05,

$p = 0.483$) and IL-8 ($r = 0.05$, $p = 0.498$) were also correlated with the development of DR, but the statistical difference was not significant (Figure 2).

3.6. Multivariable multinomial logistic regression analysis

By using multivariable multinomial logistic regression analysis, when DM was considered as the independent variable, LDL-C per 10 (OR = 1.122, 95%CI 1.019–1.235, $p = 0.019$) and VEGF-D per100 (OR = 1.674, 95%CI 1.053–2.661, $p = 0.029$) were significantly different between the DR and DM group (Figure 3). When DM was considered as the independent variable, uACR was significantly different in DR+DKD vs. DM group (OR = 1.273, 95%CI 1.083–1.495, $p = 0.003$) and in DR+DKD vs. DR group (OR = 1.318, 95%CI 1.125–1.544, $p = 0.001$) (Figure 3).

3.7. The relationship between retinal and renal microvasculopathy

Knowing that uACR (not eGFR) is closely correlated with the DR severity and a good indicator of DR progression, we further investigated the correlations between DR and DKD based on albuminuria (uACR) grades (according to the 2022 ADA grading criteria; albuminuria was graded to A1, A2, A3) (20). As shown in Figure 4, the proportion of albuminuria with A1, A2, and A3 in DM was 100%, 0% and 0%, respectively. In NPDR, the proportion of albuminuria with A1, A2, and A3 was 51.8, 28.6, and 19.6%, respectively. In PDR, the proportion of albuminuria with A1, A2, and A3 was 20.90, 37.20, and 41.90%, respectively; the difference between the three groups was statistically significant. Furthermore, in A1 albuminuria, the proportion of DM, NPDR, and PDR was 37.70, 47.50, and 14.80%, respectively; in A2 albuminuria, the proportion of DM, NPDR, and PDR was 0, 50, and 50%, respectively; in A3 albuminuria, the proportion of DM, NPDR, and PDR was 0, 37.90, and 62.10%, respectively. The difference was statistically significant. The results indicated that kidney impairment was significantly correlated with retinal microvasculopathy.

4. Discussion

In this study, we have shown that after controlling for the duration of DM and hypertension, LDL-C and circulating VEGF-D were independent risk factors for DR, while uACR was an independent risk factor for DR+DKD. DR severity was positively correlated with higher levels of albuminuria grades. These intriguing investigation results indicate that renal dysfunction is a strong pathological risk factor for DR and DKD, which is consistent with previous findings (22–27).

Blood retinal breakdown is a hallmark of DR, characterized by retinal endothelial dysfunction. Microalbuminuria is an early marker of generalized endothelial damage and is associated with an increased risk of DR (28). In a large cohort study, 10.7 mg/24 h of urine microalbumin (UMA) was shown to be a threshold that can predict the risk for the development of DR in T2DM, although it is in the traditionally accepted normal range (27). Won et al. showed that the presence of PDR is significantly associated with uACR (29). In their study, Cankurtaran et al. found that UMA was moderately correlated with the vessel density in the superficial retinal layer detected by the optical coherence tomography angiography, indicating that an elevated level of UMA could predict the early alterations in retinal microcirculation (28). Besides, a study demonstrated that remission of UMA is an independent protecting factor for the development of PDR and diabetic macular edema, and that aggressive treatment for DKD might help to prevent the progression of DR (30).

Numerous studies have shown that except UMA, eGFR (22, 24, 25, 31, 32), Scr (23, 29), AER (33, 34), and uACR (22, 32, 35), abnormal plasma phosphate (23) and renal biopsy parameters (22, 36–39) are also correlated with the occurrence and severity of DR and can further provide the pathological and mechanical evidence of the relationship between DR and DKD. The progressive narrowing and eventual occlusion of vascular lumina triggered by hyperglycemia lead to ischemia in both the retina and kidney (22). In the glomerulus, extensive capillary obstruction and podocyte loss have been found to induce urinary protein loss and decreased renal function (22). In the retina, ischemia could induce programmed cell death of endothelial, Muller and ganglion cells, leading to microvascular dysfunction, which further induces retinal hemorrhage, nicking, focal and generalized narrowing of arteriovenous (22). In this study, eGFR level was found to be in the normal range across the three groups, but lower in the DM group than the other two groups, leading to a statistically difference between the groups. This may be due to the relative shorter DM duration in the DM group, although we tried to match all the possible confounding factors that may produce bias. In the following logistic model analysis, we have controlled all the possible confounding variables, including the duration of DM. It is warranted to validate the current result by a well-designed cohort study in the near future. Additionally, uACR was abnormal in the three groups according to the DEIGO classification system. A statistically significant difference in the level of uACR was also found between the DM and DR+DKD groups ($p < 0.001$) and the DR and DR+DKD groups ($p < 0.001$). As eGFR was normal across the three groups, the difference between the groups did not mean the current results contradicted the hypothesis that DR and DKD are correlated. On the other hand, the results above confirmed our hypothesis that uACR is more sensitive than eGFR to predict the risk of DKD when the DM duration is not very long.

The similarity of the anatomical structure of the glomerulus and retina has been thought to be the pathological basis for DR and DKD. Microvessels are the common structural basis of DR and DKD Microvascular refers to the capillaries and microvascular network between tiny arteries and tiny veins with a lumen diameter $<100\,\mu m$. Microangiopathy mainly refers to the morphological changes and/or functional disorders of microvessels, microblood flow, and cells around microvessels at the microcirculation level under the action of various etiologies, resulting in corresponding clinical manifestations. Both pericytes and podocytes originate from mesenchymal cells and are important components of the outer barrier of microvessels. Pericytes are a class of pluripotent stem cells that have contractile, immune, hemostatic, phagocytic,

and hemostatic effects, participating in vascular development. Podocytes are a class of highly differentiated cells that wrap on the outside of glomerular capillaries. Preclinical studies have suggested that an early stage of the pathogenesis of DR and DKD is characterized by pericyte and/or podocyte loss, basement membrane thickening, and microvascular leakage. The numbers of pericytes and podocytes decrease as diabetes progress (40). Loss of pericytes and podocytes leads to increased microvascular permeability and vascular leakage (41, 42). It was also found that serum level of VEGF is significantly increased in DR and DKD patients (43, 44).

We further found that FBG is associated with microalbuminuria and that well-controlled FBG is important for both DR and DKD, which is supported by Kundu's findings that impaired glycemic control is associated with significant elevation of urinary UMA levels (45). Impaired FBG was also identified as a good indicator of chronic kidney disease, albuminuria, or worsening kidney function by the SPRINT study (46). Furthermore, circulating levels of PlGF and VEGF-D were found to be significantly and positively correlated with the grades of uACR, indicating that circulating PlGF and VEGF-D can be used as biomarkers for retinal and renal endothelial dysfunction (43, 47, 48). These data are consistent with a clinical study (49), which found that serum levels of hypoxia-inducible factor-1 (HIF-1α), VEGF, insulin-like growth factors −1 (IGF-1), von Willebrand factor (vWf), and fibrinogen (Fg) were positively correlated with uACR, but negatively correlated with 25(OH)VD3 and eGFR, further confirming that serum HIF-1α, VEGF, vWf, and IGF-1 are involved in DKD process through endothelial injury induced by inflammation, and angiogenesis under hyperglycemia. Circulating level of PlGF was also correlated with renal microvascular dysfunction (47), albuminuria, proteinuria in patients with DKD, and retinal microvascular dysfunction in patients with DR (43). The pathological changes of the glomerular endothelial cell surface layer, including glycocalyx, is a major cause of UMA. Serum or plasma level VEGF-D has been implicated in both the blood-retinal barrier and the glomerular filtration barrier breakdown, which are the early sign of DR and DKD (43, 50). In this study, angiogenic cytokines VEGF-D and PlGF were strong risk factors for DR severity which was consistent with our previous study (43), although the participants of the current study were different from our previous study population.

In this study, highest level of LDL-C and API was found in the DR group, lower level was shown in the DR+DKD group; but the levels were quite close and did not show statistically significance [LDL-C ($H = 0.745$, $p = 0.873$) and API ($H = 0.635$, $p = 0.431$)] between the two groups. The underlying mechanism may be that LDL-C and API have been found to contribute to the occurrence and severity of DR in several clinical studies (17, 43, 51, 52), but the correlations between LDL-C and API with DKD were not supported by trials (53, 54). This result further confirmed that LDL-C and API mainly contribute to the risk of DR, not DKD. A cohort study with a large sample size is warranted to validate the current results.

Chronic inflammation and oxidative stress have been implicated in the pathogenesis in both DR and DKD. Hyperglycemia and hypertension are the most common inducers of oxidative stress and inflammation (22, 55), which contribute to the occurrence and progression of DKD and DR. In this study, we showed angiogenetic cytokines, including PlGF, VEGF-A, VEGF-C, and VEGF-D were associated with both DR and DKD. Moreover, a recent study demonstrated that low doses of erythropoietin, which is mainly produced by the kidney, could inhibit oxidative stress and early vascular changes in the experimental diabetic retina (56). In this study, we did not find the correlations between inflammatory cytokines and DR and DKD due to the limited number of enrolled subjects.

Nayak et al. showed that the increased serum sialic acid and microalbumin were strongly related to DR and DKD (57). DR and DKD could be predictors for both by Kaplan-Meier and cox proportional hazards regression model (21, 32, 58, 59). However, the onset of DKD and DR remains unknown. Studies showed that DR precedes DKD in patients with type 1 DM (24, 28), but renal injury precedes retinal damage in patients with T2DM (24). It is warranted to further investigate the underlying mechanisms of the onset of DKD and DR in patients with T2DM in a large cohort study.

In this study, we did not consider those T2DM with normal fundus but with abnormal estimated glomerular filtration (eGFR) or Albumin-to-creatinine ratio (uACR), that is DKD patients without retinopathy. According to the guideline, retinopathy is one of the important diagnostic criteria for DKD, this phenotype in clinical practice occupied very small numbers. Furthermore, these patients cannot be excluded other primary causes of kidney damage unless confirmed by a kidney biopsy, which was not accepted by the patients.

This study has some limitations. This was a case-control study, which could not provide the causative effects of the angiogenetic cytokines on DR and DKD. Also, this study has a relative sample size, and some variables did not show a significant association between DR and DKD+DR. A well-designed large cohort study is warranted to further investigate the mechanisms of the associations between DKD and DR. Furthermore, the signal transduction pathway of VEGF-D, VEGF-A, and PlGF and their regulatory effects on lipid metabolism need to be further explored.

In summary, the novelty of this study we showed that the occurrence and severity of retinal microvasculopathy were closely correlated with kidney dysfunction. Among the three kidney functional parameters, uACR resulted as the better indicator of DR severity and progression than eGFR and Scr. Also, impaired FBG was associated with microalbuminuria, emphasizing that well-controlled FBG is important for both DR and DKD. Finally, we concluded that the link between diabetic retinal and renal microvasculopathy is associated with dyslipidemia and upregulated circulating level of angiogenic cytokines.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University. The patients/participants

provided their written informed consent to participate in this study.

Author contributions

XC: subjects enrollment, statistical analysis, and draft manuscript. XinZ: conceptualization, methodology, funding acquisition, statistical analysis, writing, and editing. ZG: statistical analysis. YY, XiaZ, QW, YW, and RX: subjects enrollment. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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The mechanism and therapeutic strategies for neovascular glaucoma secondary to diabetic retinopathy

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Neovascular glaucoma (NVG) is a devastating secondary glaucoma characterized by the appearance of neovascular over the iris and the proliferation of fibrovascular tissue in the anterior chamber angle. Proliferative diabetic retinopathy (PDR) is one of the leading causes of NVG. Currently increasing diabetes population drive the prevalence rate of NVG into a fast-rising lane. The pathogenesis underlying NVG makes it refractory to routine management for other types of glaucoma in clinical practice. The combination of panretinal photocoagulation (PRP), anti-vascular endothelial growth factor (VEGF) injections, anti-glaucoma drugs, surgical intervention as well as blood glucose control is needed. Early diagnosis and aggressive treatment in time are crucial in halting the neovascularization process and preserving vision. This review provides an overview of NVG secondary to diabetic retinopathy (DR), including the epidemiology, pathogenesis and management, so as to provide a better understanding as well as potential therapeutic strategies for future treatment.

KEYWORDS

neovascular glaucoma, diabetic retinopathy, pathogenesis, epidemiology, management

Introduction

Neovascular glaucoma (NVG) is a type of secondary glaucoma that potentially leads to irreversible vision loss and blindness. It was firstly reported by Weiss et al.z in 1963, who observed iris neovascularization in patients with central retinal vein occlusion (CRVO) and proposed the concept of NVG. Thus, NVG is characterized by progressive neovascularization in the iris (NVI) and angle (NVA). Patients usually suffer from sustained severe eye pain, photophobia, high intraocular pressure above 60 mmHg, accompanied by persistent hyperemia, corneal edema, mydriasis, and uveal ectropion. A large number of ocular and systemic disorders could cause NVG, including ischemic conditions, inflammatory conditions, retinal detachment, ocular tumor microenvironment, surgical effect and systemic diseases (1–3). The majority of NVG was secondary to proliferative diabetic retinopathy (PDR), retinal vein occlusion (RVO) and ocular ischemic syndrome (OIS), which causes retinal ischemia/hypoxia

and subsequent release of angiogenesis factors. These ischemia and angiogenesis factors drive neovascular growth in the iris and fibrovascular membranes proliferation in the anterior chamber angle, thus blocking the trabecular meshwork, and causing peripheral anterior iris adhesions and progressive closure of the anterior angle. The blockage of aqueous humor drainage eventually leads to a dramatic increase of intraocular pressure, which iteratively aggravates ischemia, destroys anterior chamber function and eventually leads to loss of vision (2). Based on its histological and clinical characteristics, NVG can be divided into three stages: rubeosis iridis, open-angle NVG, and angle-closure NVG. Although NVG can cause severe visual impairment and blindness, it could be controlled and the neovascularization process would be halted in the rubeosis iridis stage if treated promptly and appropriately. Once progressed to the second or third stage, the dysfunction of angle drainage occurs and the management becomes tough.

Proliferative diabetic retinopathy (PDR) is one of the leading causes of NVG, while the underlying pathogenesis of NVG secondary to PDR hasn't been fully elucidated. Therefore, its management has always been challenging for glaucoma, vitreoretinal and endocrinology specialists in clinical practice. The increasing diabetes population and prevalence of NVG make the situation even more urgent. Consequently, the present article will comprehensively review NVG secondary to diabetic retinopathy (DR) from the aspects of epidemiology, pathogenesis, and management so as to gain a better understanding of the disease and present potential therapeutic targets for future clinical treatment.

Etiology and epidemiology

Although the prevalence of NVG is relatively low, accounting for 0.7%-5.1% of the overall Asian glaucoma population (4, 5), 5.8% of glaucoma patients in China (6), and about 3.9% of glaucoma patients in Europe (7), it can cause sustained eye pain, devastating glaucomatous optic neuropathy and even blindness (2). It was estimated that the global prevalence of diabetes is about 10% of the total population and diabetes accounts for more than 30% of NVG cases (8). Based on that, proliferative diabetic retinopathy is the leading cause of NVG (9). The prevalence and composition of NVG are different among countries and races (10). In the United States, PDR is the primary cause of NVG, accounting for 52.38% of the population. Other factors are RVO accounting for 36.90%, and unknown factors accounting for 10.71%. In Korea, PDR, OIS, and RVO are the main reasons for NVG, with proportions of 67%, 17%, and 11%, respectively. In China, the reported data demonstrated that 39.7% of NVG was caused by PDR, 22.9% by RVO, and 2.3% by OIS (10).

The clinical feature of NVG due to diabetic retinopathy is also different from the others (11, 12). Patients with CRVO often display tortuous retinal veins, flame-shaped retinal hemorrhage, and a swollen optic disk. OIS patients are generally characterized by dilated but not tortuous retinal veins, dot and blot hemorrhages at the midperipheral retina and absence of hard exudates. While diabetic patients usually display beaded retinal veins, dot and blot hemorrhages at the posterior and midperiphery of the retina, scattered microaneurysms, and retinal exudates. Besides, The retinal arterial perfusion pressure is often decreased in OIS but not in CRVO and PDR.

The association of NVG with diabetic retinopathy is well-acknowledged (13–15). As a secondary systemic disease complication, the disease progression is often slow but irreversible if no early prevention and intervention are made. Studies have confirmed the association between long-term poorly controlled diabetes and the occurrence of NVG (16, 17). Thus NVG is often an advanced manifestation of DR. The reported prevalence of NVG was 2.1% in overall diabetic patients and rose to 21.3% in patients with PDR (13). Besides, NVG is more likely to occur after cataract surgery and vitreoretinal surgery due to surgery-induced inflammation cascade, retinal hypoxia, and the lack of anti-neovascular factors (14, 15). Furthermore, clinical studies have shown that posterior surgery might help the diffusion of vascular endothelial growth factor (VEGF) into the anterior chamber (18). Taking the above risk factors, the incidence of NVG in diabetic patients after ocular surgery raised to 80% (19).

What's worse, NVG is regarded as a terminal diabetic ocular complication with significant association with diabetic neuropathy/diabetic nephropathy (20, 21). A Logistic regression analysis revealed that HbAlc ($p < 0.001$) and diabetic nephropathy ($p < 0.001$) were two significant independent risk factors of NVG (22). Therefore, it's alert for NVG patients to be aware of poor glucose control and other severe diabetic complications.

Pathogenesis

In contrast to CRVO, in which the typical NVG occurs within 3 months since the onset of ischemic RVO (so-called '100-day glaucoma'), the establishment of hypoxia and ischemia from DR is relatively slow. The major factors causing vascular complications in diabetes are chronic hyperglycemia and ischemia-reperfusion. Studies have found that retinal hypoxia and ischemia lead to the production of a large number of neovascular-related factors (12), resulting in an imbalance between pro-angiogenesis and anti-angiogenesis processes. Normally, angiogenesis factor VEGF and angiopoietin-2 levels are in equilibrium (23). However, under hypoxia and ischemia microenvironment, this balance is broken, shifting to an imbalanced upregulation of VEGF, accompanied by the activation, proliferation, and migration of endothelial cells, pericytes and immune cells. The imbalance thereby stimulates angiogenesis and promotes the formation of neovasculature and neovascular membranes in the fundus, iris, and angle of the anterior chamber, thus blocking and stretching the anterior chamber angle, forcing iris trabecular meshwork adhesion, and eventually causing intraocular pressure elevation and visual impairment. The angiogenesis-related factors involved in the pathogenesis are VEGFs, hepatocyte growth factor (HGF), hypoxia-inducible factor 1-alpha (HIF1a), insulin-like growth factor (IGF), tumor necrosis factor (TNF), inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, etc), pigment epithelium-derived factor (PEDF), transforming growth factor-beta (TGF-β), thrombospondin, and somatostatin, etc (12, 24–26).

VEGF and angiogenesis. VEGF is the most widely studied factor implicated in the disease process of NVG (27, 28). It is produced by various cells in the retina (Muller cells, retinal pigment epithelium, pericytes, and ganglion cells) as well as the non-pigmented ciliary epithelium. Importantly, a small amount of VEGF is required in normal eyes to maintain normal ocular blood supply and normal

retinal development (29). However, overexpression of VEGF can induce devastating pathological neovascular genesis. Elevated levels of VEGF have been detected in the aqueous humor of patients with NVG secondary to diabetes (30), especially in eyes after ocular surgeries, which might help the diffusion of VEGF into the anterior chamber (18), indicating the critical role of VEGF in the pathogenesis of NVG. Experimental evidence also showed that the injection of human recombinant factor VEGF to primates is sufficient to generate iris neovascularization and NVG (30).

There are mainly five subtypes of VEGF, all of which can bind to specific subtype receptors and stimulate tissue-specific angiogenesis. Among them, VEGF-A is the isoform most closely associated with neovascularization, which inhibits cell apoptosis and capillary degeneration, and participates in the survival of endothelial cells. VEGF-A is markedly increased in the vitreous of PDR patients (31). Hyperglycemia and hypoxia condition activates downstream pathways, thus inducing an inflammation cascade and stimulating the expression of VEGF (32). Cells that produce HIF-1a could also stimulate the release of VEGF-A. Circulating VEGF-A then binds to VEGF receptors on endothelial cells, resulting in the activation of tyrosine kinase pathway and angiogenesis in the tissue (33).

Hyperglycemia and metabolic alteration. Studies based on a large population in Singapore and Japan showed a direct association between diabetes and long-term hyperglycemia with increased IOP after the adjustment for central corneal thickness (34, 35), indicating that diabetes might be a risk factor of elevated IOP. Hyperglycemia results in the loss of the pericytes, the apoptosis of the endothelial cells, the thickening of the basement membrane, and cell attachment impairment, which together lead to the breakdown of the blood retina barrier (BRB) (36). These morphological changes in tissue structure greatly strengthen the diffusion of angiogenesis and inflammatory factors, thus triggering subsequent biological processes. Hyperglycemia could also remodel glucose metabolism. The metabolic pathway includes polyol pathway, oxidative stress, protein kinase C (PKC) activation, and advanced glycation endproducts accumulation (37). Glucose is transformed to sorbitol by aldose reductase enzyme *via* the polyol pathway. The accumulation of impermeable sorbital results in pressure changes and osmotic damage to cells. Activation of PKC further accelerates the alteration of basement membrane and vascular permeability. In addition, the formation of advanced glycation endproducts causes the alteration of extracellular matrix proteins, thus exerting accumulated damage on retinal vessels as well as cell death.

Inflammation and immune response. Growing evidence suggests that inflammation is a key factor in the pathogenesis of NVG secondary to DR (38, 39), although the detailed molecular mechanism remains ambiguous. Chronic low-grade inflammation is a key driver of capillary occlusion and hypoxia, reinforcing VEGF expression and vascular abnormalities. Several processes, including oxidative stress, ischemia and hyperglycemia contribute to the inflammatory process. Evidence showed that patients with DR have higher levels of inflammatory cytokines (e.g. TNF-α, IL-6, IL-8, and IL-1β) and neurotrophins in their vitreous (40). Moreover, the levels of VEGF-A, IL-8 and EPO in the aqueous humor of NVG patients are significantly higher than that in control groups even received PRP and anti-VEGF therapy (39). Under the inflammatory microenvironment, Muller cells, microglia, astrocytes and T cells become activated, secreting TNF-α, IL-6, IFN-r, MCP-1 and VEGF, inducing endothelial damage and BRB impairment and neurodegeneration (32, 41). Moreover, the level of white blood cell, neutrophil, neutrophil/lymphocyte ratio (NLR), and lymphocyte/monocyte ratio (LMR) were latest found to be associated with NVG process, and NLR is significantly higher in NVG secondary to RVO or DR compared to healthy controls (42), which might present as a potential biomarker for NVG (43).

Studies show that anti-inflammatory drugs such as intravitreal triamcinolone acetonide and NSAIDs reduce VEGF expression and vascular permeability, inhibit retinal cell death, diminish leukostasis, and ultimately improve visual acuity and retinal function (44). Although the pathogenesis of NVG in eyes with uveitis is still unknown, studies indicated that anti-inflammatory treatment can be considered as the first choice for anterior uveitis-associated NVG (45). Targeting microglia for reprogramming of retinal microenvironment could also present a potential therapy for anti-inflammation therapy in the future (46, 47).

Management of neovascular glaucoma

The management of NVG secondary to DR is a real challenge with a high failure rate (48). NVG usually requires not only medication but also surgery to control the sustained elevated IOP. In adults, bilateral NVG is mostly due to DR (49). For diabetic patients, if NVG occurs in one eye, the other eye is almost inevitable to become NVG without prophylactic pan-retinal photocoagulation (PRP) treatment (49). Therefore, the prompt and intensive management of diabetes is of great importance. A study with long-term observation of 9 years reported that the rates for NVG were 24% in diabetic patients who received conventional treatment, and 8% for those who received intensive treatment (50), indicating that the management does make a difference in the prognosis of the refractory disease.

However, not all eyes with NVG caused by PDR can be directly treated with PRP, and patients with NVG often have significantly lower surgical success rates than other types of glaucoma (51). Previous study reported decreased successful rate of trabeculectomy in NVG secondary to PDR compared to CRVO and OIS (52), which indicated the progressive inflammation in the eyes with PDR as a contributing factor to postoperative scarring and failure. The reported failure rate of medical and surgical intervention of NVG is up to 62.8%, the majority of which suffer from blindness in the end (53). What's worse, the cost of the treatment is often high. A study in a tertiary hospital in Brazil showed that glaucoma treatment can cost up to 30% of the household income (54). Lower income was associated with worse visual acuity outcomes following NVG surgery (55).

Management guideline

Based on European Glaucoma Society Guidelines and the guideline for NVG in China, early detection of retinal ischemia and treatment of ischemic in time is the most essential and critical management, which minimize the progression of subsequent neovascularization process (56, 57). The treatment and management

of NVG secondary to PDR require careful and systematic work, with a team of glaucoma, vitreoretinal and endocrinology specialists to control the blood glucose, IOP and retinal ischemia condition etc at the same time. Management of NVG focus on mainly two aspects as shown in Figure 1: treatment of neovascularization and intraocular pressure. The final goal is to maximize the preservation of visual function with approaches including panretinal photocoagulation (PRP), anti-vascular endothelial growth factor (VEGF) therapy, anti-glaucoma therapy including drug therapy and surgical interventions, management of the systemic disease and intensive follow-up at the same time (12, 51, 58, 59).

Treatment of neovascularization

The treatment of retinal ischemia consists of pan-retinal photocoagulation (PRP) and intravitreal anti-VEGF injections (27). Drugs such as aflibercept, bevacizumab, ranibizumab, pegaptanib, and brolucizumab could suppress the expression of VEGF and therefore hinder the neovascularization process.

Anti-VEGF treatment. A case report showed that intravitreal aflibercept (2 mg into the vitreous body on the first day, 4 weeks, 8 weeks, and then every 8 weeks until 52 weeks) may be an effective treatment for the first and second stage of NVG, presenting rapid and sustained regression of NVI and NVA and well-controlled IOP (60). Periodic anti-VEGF treatment leads to more rapid regression of neovascularization than PRP and might be an appropriate therapy prior to any surgical treatment of NVG. However, each dose of anti-VEGF injection could only last for up to six weeks and the penetration distance limits its efficiency in working on the neovascular in the iridocorneal angle. Researchers are working on this problem by exploring novel agents. For instance, brolucizumab has the lightest molar mass (26 kD). Smaller molar mass enables it with higher delivery concentration to work on retinal tissue. Nevertheless, further studies are needed to optimize the delivery method, dose, timing, and agent for anti-VEGF administration.

The intravitreal injections of anti-VEGF should be administrated prior to PRP and/or surgical IOP control since the suppression iris and angle neovascularization only lasts for approximately 3-6 weeks with anti-VEGF injections, which preserves the time for adequate PRP and/or glaucoma surgery to be conducted (61). Furthermore, Somatostatin would inhibit the signal transduction pathway of IGF-1 which is upstream of VEGF, thus resulting in decreased VEGF production (62).

Pan-retinal photocoagulation (PRP). PRP is a well-acknowledged procedure for ischemic retinal conditions, and it is believed to reduce anterior segment neovascularization and prevent the development of NVG in diabetic retinopathy. It's recommended to create every possible condition to complete PRP as soon as possible. If PRP cannot be directly performed due to the opacity of the refractive medium, intravitreal anti-VEGF injections and surgeries to restore the transparency of the refractive medium should be performed (63) to create conditions for PRP, including cataract extraction or vitrectomy combined intraocular PRP. If treated promptly at the early stage, it's possible that the neovascular would regress and the neovascularization process would be halted. One study demonstrated that intravitreal triamcinolone prior to PRP improved the effect of PRP in eyes with PDR by alleviating NV and retinal thickening (64). Besides, topical steroids and cycloplegics can be used for PRP to control inflammation and improve comfort.

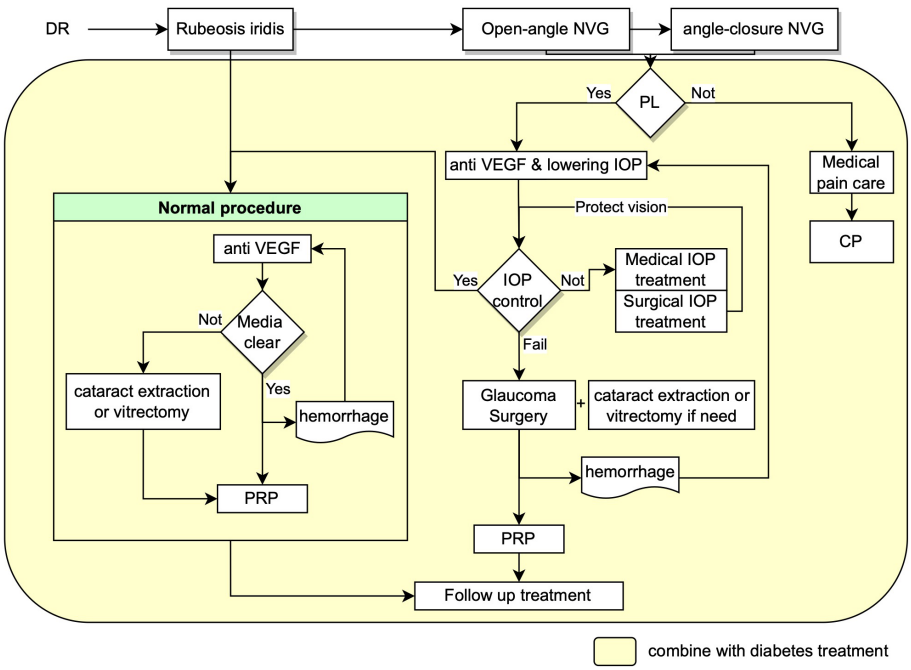

FIGURE 1
Flow chart showing the recommended management procedures for NVG secondary to DR. NVG: neovascular glaucoma, IOP: intraocular pressure, VEGF: Vascular endothelial growth factor, PRP: panretinal photocoagulation, PL: perception of light, CP: cyclodestructive procedures.

Treatment of high intraocular pressure

Neovascular glaucoma requires aggressive intervention to lower intraocular pressure (65). Every possible measure should be taken to reduce intraocular pressure, including anterior chamber puncture, systemic or topical application of ocular hypotensive drugs and anti-glaucoma surgery (57, 58).

Medical treatment

The medications for NVG mainly include IOP-lowering drug such as carbonic anhydrase inhibitors, beta-blockers, and alpha-2 agonists. Prostaglandin agents are not recommended since they accelerate inflammation. Miotics should also be avoided because they may increase the permeability of the blood-aqueous barrier capillaries therefore aggravating inflammatory response. Moreover, topical steroids and cycloplegics can be used to alleviate inflammation and improve patients' comfort. Other medications such as hyperosmotic agents (mannitol) can be administered temporally to reduce IOP (49, 66, 67).

Surgical treatment

In most cases of angle-closure NVG, medical therapy would be insufficient to control IOP and prevent further visual loss. Once the dysfunction of angle drainage happens, neovascular glaucoma is refractory to medication intervention alone. The iridocorneal angle is altered by neovascularization. Surgery therapy for NVG includes trabeculectomy combined with antimetabolite, glaucoma drainage devices, cyclophotocoagulation and cyclocryotherapy.

Trabeculectomy, also known as glaucoma filtration surgery, is less efficient for NVG due to the severe inflammation of NVG, scar formation and unavoidable post-surgery complications. Importantly, VEGF does not only participate in angiogenesis but also involves in the process of wound healing and epithelialization. In addition, there are some evidence showing the high concentration of VEGF in the tenon tissue of patients with failed surgery, which may also account for the high failure rate of trabeculectomy for NVG (12).

Glaucoma drainage devices include valved and non-valved implants. Valved implants are recommended for NVG because of their high efficiency and safety in reducing IOP. Ahmed glaucoma valve (AGV), which was created by Mateen Ahmed and approved by FDA US in 1993, has a better mechanism to control IOP and is widely used in clinical practice. Ahmed valve consists of a plate, a drainage tube and a valve. Currently, there are at least eleven available models of Ahmed valves depending on single and double plate, pars plana or pars plana pediatric, and others (59). Numerous studies support that AGV implantation is efficient for refractory glaucoma like NVG (59). Some may worry about its postoperative complications such as cornea edema, damage of the corneal endothelial cells, exposure of the drainage tube, fibrosis around the plate, etc. However, with appropriate surgery procedures, these complications could be reduced to the minimum. We previously proposed modified procedures for AGV implantation and achieved decent clinical outcomes (68). The key point is the effective utilization of the posterior episcleral space and the minimum disturbance of the fascia around the drainage valve disc, thus avoiding the formation of fibrosis. Generally, a conjunctival incision was selected at 8mm behind the limbus and the disc was fixed at 10 mm behind limbus in the upper temporal region of the eyeball. The scleral flap and scleral tunnel are designed to ensure that at least 8 mm drainage tube is fully buried under scleral layers, which effectively reduces the possibility of drainage tube exposure and tube moving during eyeball movement, then reduce the incidence of corneal endothelial decompensation. Moreover, covering the drainage tube with an autologous scleral flap avoids possible rejection response, therefore results in fast postoperative recovery. In addition, the end of the drainage tube is cut into a bevel, which is convenient for the drainage tube to enter the eye through the channel. More importantly, it prevents the drainage tube from contacting the corneal endothelium and prohibits it from being blocked. Theoretically, a successful AGV implantation could keep a stable postoperative intraocular pressure below 12 mmHg. A meta-analysis comparing the efficacy of management for NVG has shown that Ahmed valves achieved better visual acuity as compared to the other devices (69), indicating AGV as an efficient surgical method of NVG. Similar to trabeculectomy, a higher concentration of VEGF in the tenon tissue may also account for the high failure rate of AGV implantation for NVG (12). While we should also be alert that the wound healing process would be slow in patients with diabetes after successful anti-VEGF treatment, especially in older people, which causes wound leakage and bleb-related complications.

Minimally invasive glaucoma surgery (MIGS). Recently, increasing attention has been drawn to MIGS, a revolution in glaucoma surgery with minimal incision and a faster recovery time. There are various categories of MIGS, including the aqueous shunt, Ex-PRESS shunt, XEN gel stent, etc. But their efficiency on NVG needs further validation.

Cyclodestructive procedures. Cyclodestructive procedures are the last resort of NVG patient resistant to medical and surgical treatment, which include cyclocryocoagulation, cyclodiathermy, and trans-scleral cyclophotocoagulation. These procedures would damage the ciliary epithelium and stroma by reducing aqueous humor production. It might also cause serious complications like inflammation and atrophia bulbi (70, 71). However, cyclophotocoagulation (CPC) is still another widely applied option for clinicians, which has been proved as an effective treatment for lowering IOP and relieving pain in advanced cases of NVG (72, 73). Recently, the micropulse transscleral cyclophotocoagulation (MP-TSCPC) has been developed declaring less damage to the ciliary body (74). Increasing studies support MP-TSCPC as a successful technique to reduce IOP in refractory glaucoma with substantially less severe complications compared to traditional cyclodestructive procedures (75–77).

Conclusion

In summary, intensive and aggressive monitoring of blood glucose and the primary disease should be of the highest priority for patients with NVG secondary to DR. Besides, the combination of intraocular anti-VEGF injection, PRP in time, and prompt IOP control offer routine management to halt NVG progression and preserve vision. Furthermore, unveiling the underlying pathology of NVG secondary to DR is of great significance to potential medical interventions. Novel cytokines towards anti-neovascularization and anti-inflammation processes need further investigation and validation.

Author contributions

YT did the literature review and drafted the manuscript. YS drafted and revised the manuscript. ZF designed and revised the manuscript, and provided financial support for the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Optimizing treatment for diabetic macular edema during cataract surgery

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Diabetic macular edema (DME) causes visual impairment in diabetic retinopathy (DR). Diabetes mellitus is a global epidemic and diabetic individuals are at risk of developing DR. Approximately 1 in 10 diabetic patients suffers from DME, which is the commonest cause of vision-threatening DR at primary-care screening. Furthermore, diabetes predisposes to a higher frequency and a younger onset of cataract, which further threatens vision in DME patients. Although cataract extraction is an effective cure, vision may still deteriorate following cataract surgery due to DME progression or recurrence, of which the risks are significantly higher than for patients without concurrent or previous history of DME at the time of operation. The management of pre-existing DME with visually significant cataract is a clinical conundrum. Deferring cataract surgery until DME is adequately treated is not ideal because of prolonged visual impairment and maturation of cataract jeopardizing surgical safety and monitoring of DR. On the other hand, the progression or recurrence of DME following prompt cataract surgery is a profound disappointment for patients and ophthalmic surgeons who had high expectations for postoperative visual improvement. Prescription of perioperative anti-inflammatory eye drops is effective in lowering the risk of new-onset DME after cataract surgery. However, management of concurrent DME at the time of cataract surgery is much more challenging because DME is unlikely to resolve spontaneously even with the aid of anti-inflammatory non-steroidal or steroid eye drops. A number of clinical trials using intravitreal injection of corticosteroids and anti-vascular endothelial growth factor (anti-VEGF) as first-line therapy have demonstrated safety and efficacy to treat DME. These drugs have also been administered perioperatively for the prevention of DME worsening in patients undergoing cataract surgery. This article reviews the scientific evidence to guide ophthalmologists on the efficacy and safety of various therapies for managing patients with DME who are particularly vulnerable to cataract surgery-induced inflammation, which disintegrates the blood–retinal barrier and egression of fluid in macular edema.

KEYWORDS

diabetic macular edema (DME), cataract surgery, diabetic mellitus (DM), corticosteriods, anti VEGF

Introduction

Epidemiology of diabetes mellitus

Diabetes mellitus (DM) is a chronic disease common worldwide, characterized by hyperglycemia due to impaired glucose regulation. People with type 1 diabetes mellitus (T1DM) are unable to produce sufficient insulin, whereas people with type 2 diabetes mellitus (T2DM) suffer from end-tissue resistance to the effects of insulin (1). DM is a serious public health issue that continues to place a high burden on patients and healthcare systems, thanks to a constant rise in its prevalence.

According to the International Diabetes Federation (IDF), the total number of people having DM (T1DM and T2DM combined) rose constantly from approximately 285 million people in 2009 to 366 million in 2011, 382 million in 2013, 415 million in 2015, and 425 million in 2017 (2–6). In 2019, 463 million people were estimated to live with DM globally, which accounted for 9.3% of the global adult population (20–79 years). Moreover, this number is expected to spring to 578 million (10.2%) in 2030 and 700 million (10.9%) in 2045 (7).

Research reported regional differences among the DM population. In terms of prevalence, Pacific Ocean Island nations maintained first place (8). For instance, Fiji, Mauritius, American Samoa, and Kiribati had prevalence rates of 20,277, 18,545, 18,312, and 17,432 per 100,000, respectively. In terms of the greatest total number of individuals with DM, China, India, and the US remained the top countries with 88.5 million, 65.9 million, and 28.9 million individuals with T2DM, respectively, due to their large population size. In terms of the greatest increase, the WHO reported that low- and middle-income countries, like Indonesia, Malaysia, Thailand, and Vietnam, had maintained their ranking in the last two decades.

Epidemiology of diabetic retinopathy

The inability to regulate blood sugar levels damages different body parts and leads to a multitude of complications, including but not limited to cardiovascular disease, neuropathy, and retinopathy. Here, we will first focus on how DM induces diabetic retinopathy (DR), as the eye is the organ where DM potentially first manifests and, hence, is a reflection of systemic diseases.

DR is recognized as the leading cause of vision loss in the working-age population in both developed and developing countries (9). DR is characterized by vascular abnormalities in the retina and is classified into two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR and PDR are also identified as vision-threatening diabetic retinopathy (VTDR).

Among the approximately 463 million DM population, approximately one-third exhibited signs of DR (10). The literature reported that up to 2020, the global prevalence of DR was 22.27%, among which 6.17% of patients are susceptible to vision loss from VTDR and 4.07% from clinically significant macular edema (CSME) (11). The global numbers of DR, VTDR, and CSME are expected to further escalate to 160.50 million, 44.82 million, and 28.61 million, respectively, by 2045. Africa had the highest rate of DR (35.90%), followed by North America and the Caribbean (33.30%), and finally South and Central America with the lowest rate (13.37%) (10). Hispanics and Middle Easterners who are diabetic showed higher susceptibility toward DR than Asians. In this regard, an Italian study group showed that among 745 diabetic patients undergoing phacoemulsification, NPDR, PDR, and laser-treated retinopathy were present in 101 (14.3%), 13 (1.7%), and 53 (7.5%) patients, respectively (12). Furthermore, there was a positive correlation between the duration of DM and the severity of DR (13).

Epidemiology of diabetic macular edema

Diabetic macular edema (DME) is defined by the breakdown of the blood–retinal barrier (BRB) causing swelling or thickening of the macula due to sub- and intraretinal accumulation of fluid (14). DME is the primary cause of vision loss in patients with DR (9). Elevated HbA1c is known to be a significant risk factor for diabetic retinopathy (15). Hence, the control of HbA1c levels is critical in DME. Among the one-third of the DM population who demonstrated signs of DR, a further one-third of them experienced VTDR, including DME (10). As there is a rising number of diabetes, DME is anticipated to pose a major threat to the public health system in the foreseeable future.

With the aid of various diagnostic modalities, such as slit lamp biomicroscopy, fundus photography, and optical coherence tomography (OCT), immense effort has been made to quantify DME. In particular, OCT outstands other tools with its supreme accuracy of measurement of retinal thickness and high resolution for monitoring of retinal changes on a microscopic level (16, 17). Therefore, OCT was considered as the gold standard for the diagnosis and prognosis monitoring of DME (18). While the prevalence of DME varied greatly among studies due to different diagnostic tools and criteria used, Im et al. focused on OCT-diagnosed DME and only included population-based studies to avoid skewed prevalence from hospital- and/or clinical-based samples (19). In that study, among diabetic patients, Im et al. proposed the overall pooled prevalence of DME was 5.47%, 5.81% for low-to-middle-income countries, and 5.14% for high-income countries. In contrast to DM or DR, the statistical difference in the prevalence of DME between high-income and low-to-middle-income countries was insignificant.

Epidemiology of cataract in DM patients

Cataract is the clouding of the crystalline lens and can be further differentiated according to types, such as nuclear, cortical, and posterior subcapsular cataract (20). The incidence of cataract formation was proved to be inflated among diabetic patients (21). With the advent of technological advancement, cataract surgery has gradually become a much safer procedure over the centuries to improve patients' vision. Despite this, postoperative complications are still inevitable and may lead to unsatisfactory visual outcomes. Examples include postoperative DME, DR progression, and posterior capsular opacification (22).

Research showed that diabetic patients are two to five times more prone to earlier onsets of cataract when compared with the control

group (23–26). In a study conducted based on the UK population, the incidence rates of cataract were 20.4 per 1,000 person-years (py) for the diabetic population, which almost doubled that of the general population, of which the baseline was 10.8 per 1,000 py (27). Furthermore, the incidence rate ratio peaked for patients 45 to 54 years old. Moreover, the longer the duration of having DM, the higher the risk of developing cataract.

Consistently, a community-based cross-sectional study done in Saudi Arabia showed that among 668 eyes from 334 patients with T2DM, 237 eyes (35.5%) had cataract (28). Similar to the findings of Becker et al., diabetic patients with cataract were associated with a longer duration of diabetes. Furthermore, DR was found in 215 diabetic cataract eyes (32.2%). Among them, 194 eyes (90.2%) were NPDR and 89 eyes (13.3%) were CSME.

Association of DME and cataract surgery

Pathophysiology (breakdown of the blood–retinal barrier)

Although the exact mechanism of the action of DR remains ambiguous, a considerable amount of prospective clinical studies have proved that hyperglycemia is the primary risk factor contributing to the pathogenesis of DME (29). Four major biochemical pathways were identified to be related to the hyperglycemia-induced pathogenesis of DR: 1) polyol pathway, 2) advanced glycation end products pathway, 3) protein kinase C (PKC) pathway, and 4) hexosamine pathway (30). These four pathways trigger heightened oxidative stress, inflammation, and vascular dysfunction. Oxidative stress and inflammation induce hypermodulation of growth factors and cytokines, which contribute to the breakdown of the BRB and the formation of DME. For instance, vascular endothelial growth factor (VEGF), angiopoietins, tumor necrosis factor (TNF), interleukins (ILs), and matrix metalloproteinases (MMPs) are the key modulators. The BRB plays a prominent role in maintaining the fluid electrolyte equilibrium in the retina. However, when the BRB is broken down, fluid accumulates in the different layers of the retina, leading to DME. Anatomically, the BRB is divided into outer and inner layers. The outer BRB is formed by retinal pigment epithelium (RPE) cells between the fenestrated choriocapillaris and the outer retina, whereas the inner BRB is composed of endothelial cells situated at the inner retinal capillaries. At the outer BRB, the RPE has been shown to eliminate water from the subretinal space toward the choroid *via* a mechanism driven by an active trans-epithelial Cl– gradient (31). At the inner BRB, the tight endothelial cell–cell junctions avoid molecular leakage from the retinal capillaries and, thus, play a critical role in the retinal hydro-ionic homeostasis. The cohesion of the cell–cell junctions is dynamically maintained by an intricate neuro-glio-vascular cross-talk between retinal Müller glial (RMG) cells and astrocytes, and their interactions with the surrounding smooth muscle cells and pericytes (32–34). With various ion and aqueous channels, the RMG cells contribute significantly to the regulation of fluid homeostasis (35). Together, an imbalance between fluid entry secondary to the breakdown of the BRB and dysfunctional fluid withdrawal of the RPE and RMG results in an upset fluid electrolyte equilibrium with a net gain of fluid and, hence, DME (36). In DME, the breakdown of the cell–cell junctions, pericyte loss, and thickening of the basement membrane are observed (37).

Incidence of new-onset DME after cataract surgery

As mentioned above, diabetic patients are more liable to develop cataracts. Research has shown that cataract surgery improves best-corrected visual acuity (BCVA) and vision-related quality of life in patients with DR (38). Meanwhile, patients with DR are more predisposed to poorer postoperative visual acuity and a higher risk of complications after cataract surgery when compared with those without DR (39–42). This is substantiated by a large database study of 81,984 eyes done in the UK that showed that there was an increased incidence of new-onset DME after cataract surgery (39). Among 4,485 diabetic eyes in the absence of preoperative maculopathy that underwent cataract surgery within 90 days, 2,807 (62.6%) of them did not have DR after surgery, while 1,678 (37.4%) of them suffered from postoperative DR. The data showed that diabetic patients, even with no retinopathy, had a higher relative risk of new DME onset of 1.80 after cataract surgery when compared with the control. The risk was even higher (6.23) in the presence of any pre-existing DR. The risk of developing postoperative DME is directly proportional to the severity of DR. Furthermore, the mean incidence of postoperative edema in the eyes of diabetic patients was found to be fourfold in comparison with non-diabetic patients (39).

Incidence of recurrent DME after cataract surgery and pre-existing DME progression after cataract surgery

A large cohort study done in Italy recruited a total of 3,657 patients who underwent cataract surgery in the past 3 months (12). Among the cohort, 745 (20.4%) patients were diabetic. Men had a significantly higher prevalence of DM (24.7%) than women (17%). Within the 745 diabetic patients, 205 (27.5%) patients showed signs of DME, among which 156 (20.9%) patients had non-clinically significant macular edema (N-CSME) and 49 (6.6%) patients had CSME. N-CSME was defined as the presence of intraretinal cysts associated with the center foveal thickness (CFT) of 257 μm, which was equivalent to 30% thicker than normal values. CSME was defined by the presence of intraretinal cysts associated with CFT of 598 μm, which was equivalent to >30% thicker than normal values. Patients with DME had a significantly longer history of DM, but no significant difference between gender or age groups was identified (13). More importantly, among the 3,657 patients, the prevalence of DME was 5.4%. Although this was not a population-based study, the prevalence of DME was consistent with the proposed general prevalence of DME of 5.4% in Im et al. as stated previously.

Apart from the incidence of DME after cataract surgery, it is also essential to understand how DME progresses, which is reflected by visual acuity after cataract surgery in patients with different degrees of

DR. Research evaluated diabetic patients' change in BCVA throughout a year after cataract surgery (43). Diabetic eyes without DR before surgery (n = 138) and eyes with NPDR (n = 125) gained a median of 11.0 and 10.0 Early Treatment Diabetic Retinopathy Study (ETDRS) letters from 65.0, respectively. Eyes with severe NPDR (n = 20) and PDR (n = 72) gained 20.5 and 15.0 letters from 55.0, respectively. Compared with eyes with severe NPDR or PDR, eyes without DR or mild/moderate NPDR had significantly greater improvements in VA when controlling for baseline VA. As a result, patients with a more severe degree of DR might result in poorer visual acuity even after cataract surgery. The conundrum of whether to offer cataract to diabetic patients remained controversial.

Management of DME and cataract surgery

In diabetic patients who underwent cataract surgery, macular edema can be resulted either from a new onset of pseudophakic cystoid macular edema (PCME) or the worsening of pre-existing DME. Both entities are characterized by fluid accumulation in the retinal tissues in the macular region, but these two diseases should be distinguished as they have different pathophysiologies and, hence, different treatment paradigms. DME often presents with an underlying DR, exudates, and macular edema (ME), while minimal DR and the absence of exudates point more toward PCME (44). To further differentiate between the two, OCT is an invaluable diagnostic tool. For DME, OCT shows such features as microaneurysms, hard exudates, and a higher parafoveal outer nuclear layer to inner nuclear layer thickness ratio, whereas for PCME, OCT demonstrates a high central macular thickness to retinal volume ratio and intact hyperreflective outer retinal bands (45).

As the pathophysiologies of DME and PCME are different, the treatments for DME and PCME differ. In this context, PCME is mostly managed with topical treatments, whereas DME is managed with more invasive treatments such as intravitreal injections and laser photocoagulation. Boscia et al. suggested that all diabetic patients undergoing cataract surgery should be treated with topical non-steroidal anti-inflammatory agents (NSAIDs) to prevent PCME. As for patients with pre-existing DME, intravitreal therapies, both with anti-VEGF drugs and steroids, can be considered (46). The perioperative treatment options for DME in patients with cataract have been summarized in Table 1.

NSAID eye drops

Given the incidence of new-onset DME after cataract surgery, the perioperative use of anti-inflammatory eye drops is recommended. Topical NSAIDs block cyclooxygenase enzymes, which in turn hinder prostaglandin production. This reduces vascular hyperpermeability and, hence, decreases the incidence and severity of macular edema.

Currently, the common options of NSAID eye drops include nepafenac, diclofenac, bromfenac, and ketorolac. Nepafenac is a prodrug that penetrates the cornea rapidly and forms the active metabolite, amfenac. Out of these four agents, nepafenac displays higher permeability, greater duration of action, and increased targeted activation. Topical nepafenac can be given in 0.1% formula three times a day or in 0.3% formula once a day. Both formulations have been proven to be effective against PCME development. In a randomized, double-masked study involving 263 adult patients, a significantly lower percentage of patients on 0.1% nepafenac developed ME compared with the vehicle group over 90 days (3.2% and 16.7%, respectively, $p < 0.001$). The central macular thickness

TABLE 1 Perioperative treatment options for DME in cataract patients.

Treatment options	Clinical pearls and recommendations	References
Topical non-steroidal anti-inflammatory drugs (NSAID)	➢Agents: nepafenac, diclofenac, bromfenac, and ketorolac ➢ Perioperative use is recommended in eyes without preoperative DME to reduce the risk of developing DME postoperatively	(45, 47–49)
Topical corticosteroids	➢ Lower penetration to the eye compared with NSAID ➢ Combined use of topical corticosteroid and NSAID was superior to either agent alone	(52–54)
Laser	➢ Lasers: focal, grid, subthreshold micropulse ➢ Considered as an adjunct treatment for refractory DME	(55–58)
Intravitreal corticosteroids	Triamcinolone acetonide (TA) ➢ Demonstrated longer duration of action than intravitreal bevacizumab for the control of DME ➢ Preoperative use may hasten cataract progression ➢ TA has a higher risk of increasing IOP	(62, 63)
	Fluocinolone acetonide (FA) implant ➢ The benefit of FA has been demonstrated in clinical trials ➢ Recommended for use in pseudophakic and chronic DME patients refractory to other therapies Intravitreal dexamethasone implant (Ozurdex) ➢ Intraoperative use is effective in the prevention of post-cataract surgery macular edema, with the effect lasting for up to 3 months ➢ Preoperative use also improved post-cataract surgery visual acuity significantly	(76–78) (83, 84)
Subtenon TA	➢ Decreased CMT significantly for the prevention of postoperative progression of DME ➢ A viable treatment option in cases of DME refractory to intravitreal anti-VEGF	(73, 74)
Intravitreal anti-VEGF	➢ First-line treatment to control preoperative DME ➢ Treatment still needs to be continued following surgery for the control of DME	(64, 65, 68, 69)

(CMT) increase and the change of macular volume from baseline were also significantly better in the nepafenac group over 14 days ($p < 0.005$) (47). Similar results were found in patients using 0.3% nepafenac, with the incidence of developing ME in the treatment and control groups being 4.1% and 15.9%, respectively ($p < 0.001$). No unanticipated safety events occurring in both trials were observed (48). Nepafenac has been approved in Europe and the Americas for the reduction of PCME development in diabetic patients (45).

The clinical benefits were also evident in the other types of NSAIDs as well. Alnagdy et al. stated that among diabetic patients undergoing cataract surgery, patients on topical NSAIDs, either ketorolac tromethamine 0.4% or nepafenac 0.1%, showed statistically significant improvement in BCVA ($p = 0.04$) and CMT over 3 months ($p = 0.004$) as compared with control without NSAIDs. There was no statistical difference in the efficacy between ketorolac and nepafenac (49). A retrospective analysis of 75 diabetics was also performed to investigate the effect of 0.1% bromfenac sodium hydrate. When compared with the control group over 6 months, bromfenac had better best-corrected visual acuity (0.12 ± 0.12 *vs.* 0.32 ± 0.42, $p = 0.142$), lower macular volume (8.46 ± 0.60 *vs.* 9.14 ± 1.53 mm^3, $p = 0.022$), and lower central macular thickness (265.58 ± 31.28 *vs.* 314.15 ± 76.11 µm, $p < 0.001$) (50).

NSAIDs are associated with side effects such as transient burning sensation and epithelial corneal defects (51). However, this side effect profile is relatively insignificant when compared with other treatment options, as there is no risk of endophthalmitis as in intravitreal injection and no risk of destruction of the foveal center as in laser surgery.

Topical corticosteroids

Corticosteroids suppress inflammation by inhibiting COX-2 and phospholipase A2 and, hence, lipoxygenase pathways. A study conducted in Croatia involving 55 patients has demonstrated that topical diclofenac effectively lowered intraocular IL-12 concentration, a marker for intra-ocular inflammation, and reduced ME formation (52).

Although the mechanism of action of topical steroids is similar to those of NSAIDs, a topical steroid is more inferior in the prevention of PCME, probably due to its lower penetration in the eye. Moreover, steroids exhibit more severe side effects when compared with NSAIDs, such as increased intra-ocular pressure (IOP). Hence, the prolonged use of topical steroids should be avoided.

Despite its inferior effect when used alone, steroid eye drops can be used in combination with other treatments. A meta-analysis involving seven trials showed that in diabetic patients with no pre-existing DME, combining topical NSAIDs with corticosteroids reduced the risk of developing PCME to a greater extent versus tropical corticosteroids alone (OR = 0.17) (53). Similar improvements were observed when topical NSAIDs and steroids, bromfenac and dexamethasone, were used in combination. A multicenter trial involving 12 European centers compared the incidence of developing PCME over 12 weeks postoperatively in patients treated with bromfenac, dexamethasone, or in combination. The incidence was 3.6%, 5.1%, and 1.5%, respectively (overall $p = 0.043$). Bromfenac had a lower incidence of PCME development than dexamethasone, and the combined treatment had the lowest incidence overall (54).

Laser

For patients with pre-existing DME, the treatment of DME is recommended preoperatively to reduce the risk of further progression.

The first prospective randomized clinical trial on laser photocoagulation—Early Treatment Diabetic Retinopathy Study (EDTRS)—examined 37,111 patients across 22 centers. It classified laser treatment into two techniques: focal and grid laser (55). Focal laser involved the treatment of focal lesions, such as microaneurysm, intraretinal microvascular abnormalities, and short capillary segment fluorescein leakage. Focal laser utilizes moderate intensity burns of 50 to 100 µm lasting 0.05 to 0.1 s in duration. Grid lasers are usually placed in the papillomacular bundle rather than the macular center or disc margin. The laser is of mild intensity with a sport size of 50 to 200 µm, lasting 0.05 to 0.5 s. There is also a modified ETDRS treatment approach, which uses a less intense laser with greater spacing.

The ETDRS concluded for clinically significant DME that focal photocoagulation should be considered (56). It defined clinically significant macular edema as retinal thickening at or within 500 microns from the macular center, or hard exudates at or within 500 microns of the macular center with adjacent retinal thickening, or retinal thickening greater than 1 disc diameter and within 1 disc diameter away from the macular center.

Mild macular laser photocoagulation (MMG) is a new modality of laser photocoagulation. Two hundred to 300 burns are applied to the entire area over the macular, both thickened and unthickened retina, and microaneurysms are directly photocoagulated. However, there was no evidence suggesting that MMG has a better outcome in terms of visual acuity or retinal thickening on follow-up after 12 months (57). Subthreshold diode micropulse laser photocoagulation is another technique to treat DME, with the aim to reduce laser damage to ocular tissues. The laser parameters are modified, such as decreased wavelength, retinal irradiance, and pulse duration, to reduce chorioretinal damage. The laser energy is given in pulses, lasting 300 ms each. In a study by Ulbig et al., 82% of patients treated with diode laser had completely or partially resolved DME (58). However, most trials on micropulse subthreshold diode therapy are non-randomized, uncontrolled, and retrospective and, hence, are of insufficient power for application in clinical practice. This relatively novel treatment modality still warrants further studies before its application in clinical settings.

There are also general complications of laser treatments which need to be considered. An important complication is the enlargement of a laser scar, which can threaten visual acuity. Maeshima et al. also reported that the expansion of laser scars was relentless and might continue over long time periods. The expansion rate was 8.8% during the first 4 years but then thereafter increased to 16.5% (59). Other complications include a transient increase of DME, accidental foveal burns, or choroidal neovascularization due to damage to Bruch's membrane (57).

Prior to the era of intravitreal injections, laser treatments were considered as the gold standard that improved long-term visual acuity outcomes for most patients. Although anti-VEGF shows better resolution of DME after the first year, in the EDTRS study, the best results were achieved on follow-up after 3 years (60). Therefore, laser treatments still play a role in the treatment of DME during cataract surgery, especially in the long term.

Triamcinolone acetonide versus anti-VEGF

Triamcinolone acetonide (TA) is a commonly used corticosteroid for intravitreal injections. The mechanism of TA is postulated to inhibit both inflammatory and angiogenic cytokines, hence an improvement in BCVA and a reduction in CMT.

When compared with anti-VEGF, TA has been shown to be more inferior. This is mostly due to the concerns raised by the side effect profile of TA. Intravitreal steroids pose a risk of transient increased IOP and endophthalmitis, with the prevalence of increased IOP up to 23.5% (60). In another study involving 12 patients, four patients showed increased IOP at 1 month after surgery. However, most of the IOP spike was manageable, as the IOP returned to normal without medication 6 months after the application of topical anti-glaucomatous drugs (61).

In comparison with anti-VEGF, intravitreal steroids may have a longer duration of action and possibly better control of macular thickness. In a prospective pilot study involving 41 DME patients, the visual outcomes between intravitreal bevacizumab (BVB) and TA administered intraoperatively were compared (62). After 6 months, there was no significant difference between the groups in terms of vision improvement. In the TA group, 69.9% of the patients were able to achieve visual acuity improvement of 15 letters or more at 6 months, as compared with 60.0% in the BVB group ($p = 0.728$). For a 10-letter improvement, the numbers were 82.6% and 73.3%, respectively ($p = 0.687$). However, only TA showed a sustained reduction in CMT. Three patients (12.5%) in the TA group experienced increased IOP compared with none in the BVB group. However, 70.6% of the participants in the BVB group required additional injections, compared with 16.7% in the TA group, suggesting that TA has less injection need in the long run.

This result was also supported by another randomized trial by Kandasamy et al. When TA and BVB were given in cataract surgery, both TA and BVB showed improved BCVA. TA and BVB patients had a letter gain of 21.4 and 17.3, respectively. However, only TA has sustained improvement in CMT, with only 24% of the patients requiring retreatment, when compared with 57% in the BVB group (63).

Other anti-VEGF agents were also investigated. Ranibizumab has been shown to be more effective when injected intraoperatively during cataract surgery than perioperatively and postoperatively in patients with DMR (64). Intraoperative aflibercept did not exert a significant effect on postoperative CMT or visual acuity at 3 months, probably due to a relatively shorter half-life (65). To date, there are no clinical trials yet examining the role of intraoperative injection of newer anti-VEGF agents, such as brolucizumab and faricimab on DMR, but their safety profiles and efficacies on DME were demonstrated in clinical trials (66, 67). Nevertheless, intravitreal anti-VEGF still remains the well-established first-line treatment for preoperative DME (68, 69). Further anti-VEGF treatment following cataract surgery still needs to be continued for the control of DME.

Despite anti-VEGF being more effective, not all patients demonstrate a response to anti-VEGF treatments. In a subanalysis of the DRCR.net Protocol I study, approximately 20% of patients had less than 20% reduction in CMT over a 1-year period. The study defined this as non-responders of ranibizumab therapy (70). Nunome et al. investigated the role of TA in DME treatment in ranibizumab non-responders (71). There was a significant improvement in visual acuity at 24 weeks, central retinal thickness (CRT) at 12 weeks, and retinal sensitivity threshold at 4 weeks in ranibizumab non-responders (71). This illustrates that TA combined with cataract surgery is useful for patients with anti-VEGF resistance.

Of note, TA can also be used in conjunction with other treatment modalities such as macular laser. Ozgur et al. reported that patients treated with IVTA and macular grid photocoagulation had a statistically significant increase in BCVA and a decrease in CMT at 6 months of follow-up, when compared with those who received macular laser alone ($p < 0.01$) (72). Furthermore, subtenon TA has been shown to decrease CMT significantly for the prevention of postoperative progression of DME (73). In this regard, subtenon TA is a viable treatment option in cases of DME refractory to intravitreal anti-VEGF (74).

Fluocinolone acetonide

Another corticosteroid alternative is intravitreal fluocinolone acetonide (FA). The Fluocinolone Acetonide in Diabetic Macular Edema (FAME) study is a landmark trial for FA. The trial demonstrated that after intravitreal injection of an FA implant which releases 0.2 µg FA per day, 34% of patients with DME over 3 years experienced a >15 letter gain compared with 13.4% in the sham group. There was a 140-µm reduction in CRT after 6 months of treatment (75).

Another study comparing the long-term benefits of high-dose versus low-dose FA also concluded that FA improved BCVA in patients with DME over 2 years. The mean improvement in BCVA score from baseline in the low-dose, high-dose, and sham groups was 4.4, 5.4, and 1.7, respectively ($p = 0.02$ and $p = 0.016$ compared with sham). The study concluded that FA could be administered to patients with benefits lasting for at least 2 years (76).

It should be noted that intravitreal corticosteroids favor cataract formation. Both trials failed to take into account the cataract status of the patients. Currently, the National Institute for Health and Care Excellence recommends that FA should be used in pseudophakic patients and chronic DME refractory to other therapies. In the US, FA is approved for treating refractory DME, provided that patients have been treated with a course of corticosteroids without clinically significant IOP rise (77).

In the context of IOP rise, studies suggested that the prevalence of IOP elevation was higher in FA (65.9%–79.0%) compared with TA (30.0%–45.9%) (71). Despite this, the findings from a *post-hoc* analysis of the FAME study supported the use of FA implants in both phakic and pseudophakic patients. For phakic patients with DME, cataracts developed at an expectedly high rate, and surgery was needed. However, the results suggested that the visual outcomes were not negatively affected by the cataract surgery. There was numerically a higher increase in BCVA scores and >15 letter improvement compared with those who were pseudophakic at baseline. Although more research is needed, the analysis suggests that FA may protect the patient against post-cataract surgical complications and is favorable for long-term visual outcomes (78).

Intravitreal dexamethasone implant (Ozurdex)

Ozurdex (Allergan, Inc., Irvine, CA, USA) is a single-use, biodegradable intravitreal dexamethasone drug-release system that

releases a total dose of 700 μg of dexamethasone to the human vitreous slowly and gradually over time (79–82). Composed of a biodegradable copolymer with polylactic-co-glycolic acid and micronized dexamethasone, Ozurdex was engineered to overcome drug delivery barriers by lengthening the effect of intravitreal dexamethasone. A study examining the pharmacokinetics of Ozurdex in monkey eyes demonstrated that the intravitreal concentrations of Ozurdex were characterized by two distinct phases, with peak concentration attained at day 60 and subsequent continuous release up to day 180 (79). As Ozurdex is administered intravitreally, the possible side effects brought upon by steroid administration *via* other routes of administration, such as systemic administration, could be reduced. Furthermore, the biodegradability of the implant eliminates the need for the removal of the implant, as the implant gradually degrades into water and carbon dioxide.

The effect of Ozurdex implant on diabetic macular edema after cataract surgery

For eyes with at least mild diabetic retinopathy and the absence of macular edema, an immediate intraoperative single Ozurdex injection after phacoemulsification was demonstrated to be effective in the prevention of macular edema by reducing the likelihood of CRT rise (83). Such an effect was observed to last for up to 3 months post-treatment, as evidenced by central retinal thickness, macular volume measurements with OCT, and improvement in best-corrected visual acuity (83). Furthermore, statistically significant improvement in visual acuity in groups of diabetic patients who received Ozurdex injection before phacoemulsification was also observed at 6, 12, and 24 weeks in comparison with the control (84). Meanwhile, there were no significant differences in intraocular pressure between the two groups.

The majority of adverse events associated with intravitreal dexamethasone implant injection are related to the injection per se and often resolve spontaneously (85). The common adverse effects include post-injection conjunctival hemorrhage, hyperemia, and chemosis, as well as raised intraocular pressure, and less commonly iritis, anterior chamber cell, and vitreous hemorrhage. The migration of the Ozurdex implant to the anterior chamber is a severe but rare complication. This could lead to corneal endothelial damage, corneal edema, and permanent decompensation, in which case corneal transplantation might be warranted. Immediate removal or repositioning of the implant should be performed urgently to avoid irreversible corneal endothelial damage. A study involving 640 eyes which received intravitreal dexamethasone implant injections revealed that anterior chamber implant migrations occurred in four eyes (0.63%) (86). The study identified the major risk factors for anterior chamber migration to be insufficient zonular support, defects or a non-intact posterior capsular membrane, and a history of vitrectomy. For patients with these risk factors, alternative treatments should be offered. Overall, Ozurdex was generally considered to be well-tolerated with a good safety profile (81, 85).

The mechanism of corticosteroids

The exact mechanism of how pseudophakic cystoid macular edema occurs still remains unclear. The literature suggested that such inflammatory mediators as VEGF could potentially play a pivotal role in breaking down the blood–aqueous and blood–retinal barriers, thus resulting in increased vascular permeability and cystoid macular edema (87). In this regard, intravitreal corticosteroid alleviates diabetic macular edema by targeting the inflammatory cascade *via* diminishing the production and release of VEGF and other pro-inflammatory mediators, thereby hindering the formation of diabetic macular edema among diabetic patients who received cataract surgeries.

Discussion

Cataract surgery helps patients restore their vision and improves their quality of life. The increased risk of postoperative macular edema in diabetic patients, especially in the presence of pre-existing DME, often leads to suboptimal vision gain and patient dissatisfaction. The perioperative control of the systemic cardiovascular risk factors, such as diabetes, blood pressure, and lipids, is critical to reduce the risk of postoperative DME and postoperative endophthalmitis, as well as to promote corneal wound healing and hasten vision recovery. Intraoperative factors including a non-intact posterior capsule, prolapse or incarceration of vitreous causing macular traction, iris chafing secondary to a malpositioned intraocular lens, retained lens matter, and prolonged operation time with extensive surgical manipulations should also be noted, as these may increase the risk of postoperative macular edema.

The options of prophylaxis for postoperative macular edema include topical NSAID, topical/periorbital/intravitreal steroids, or intravitreal anti-VEGF injections. For diabetic patients without a history of DME, the preoperative use of topical NSAID for 1 week reduces the risk of new-onset DME during the early postoperative period. The addition of a topical steroid did not have a significant effect in lowering the chance of postoperative DME but should be prescribed to suppress other forms of intraocular inflammation during the postoperative period.

For patients with pre-existing DME, if the cataract is not jeopardizing the patients' activity of daily living (ADL) and there is an adequate fundal view, it is preferable to defer cataract surgery and control DME first, by achieving a static central foveal thickness on OCT on two consecutive monthly visits. Because of the short half-life of intravitreal anti-VEGF, injection within 14 days before cataract surgery is most efficacious in reducing macular thickness during the first postoperative month. Subtenon injection of triamcinolone acetonide has a longer half-life and should be given earlier. If the cataract is visually debilitating, affecting the ADL, or precludes fundal examination, then prompt cataract surgery is recommended with intravitreal anti-VEGF injection or intravitreal/subtenon injection of steroids. Of note, intravitreal dexamethasone implant has the risk of migrating into the anterior chamber if the posterior capsule is non-intact.

In the postoperative period, macular edema is the end result contributed by a combination of factors including postoperative inflammation and diabetes and often requires additional treatments, such as intravitreal or periocular steroids and intravitreal anti-VEGF injections. Patients who received anti-VEGF injections before cataract surgery can still experience improvements in vision postoperatively and can continue to receive anti-VEGF injections in the perioperative period.

Author contributions

All authors wrote sections of the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Corneal dendritic cells in diabetes mellitus: A narrative review

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Diabetes mellitus is a global public health problem with both macrovascular and microvascular complications, such as diabetic corneal neuropathy (DCN). Using *in-vivo* confocal microscopy, corneal nerve changes in DCN patients can be examined. Additionally, changes in the morphology and quantity of corneal dendritic cells (DCs) in diabetic corneas have also been observed. DCs are bone marrow-derived antigen-presenting cells that serve both immunological and non-immunological roles in human corneas. However, the role and pathogenesis of corneal DC in diabetic corneas have not been well understood. In this article, we provide a comprehensive review of both animal and clinical studies that report changes in DCs, including the DC density, maturation stages, as well as relationships between the corneal DCs, corneal nerves, and corneal epithelium, in diabetic corneas. We have also discussed the associations between the changes in corneal DCs and various clinical or imaging parameters, including age, corneal nerve status, and blood metabolic parameters. Such information would provide valuable insight into the development of diagnostic, preventive, and therapeutic strategies for DM-associated ocular surface complications.

KEYWORDS

corneal dendritic cell, diabetic mellitus, corneal nerves, corneal epithelial cells, *in vivo* confocal microscopy, diabetic corneal neuropathy, diabetic microvascular complications, ocular surface

1 Diabetes mellitus and diabetic corneal neuropathy

Diabetes mellitus (DM), characterised by elevated levels of blood glucose resulting from defective insulin secretion and/or action, has emerged to become a major global public health problem (1). In 2021, 537 million adults were living with diabetes, and estimably 6.7 million adults have died because of DM or its complications (2). The estimated global cost of diabetes was projected to increase from US$1.31 trillion in 2015 to $2.1 trillion in 2030 (3). DM is associated with both macrovascular complications, such as cardiovascular disorders, and

microvascular complications, including diabetic peripheral neuropathy (DPN) (4, 5). The manifestation of DPN in the cornea is referred to as diabetic corneal neuropathy, leading to diabetic keratopathy.

DCN is characterized by changes in corneal nerve fibres and occurs in 47-64% of patients during their clinical course of DM (6, 7). When evaluating corneal nerve changes in DCN, *in-vivo* confocal microscopy (IVCM) has been considered the gold standard. *In vivo* cell imaging uses light reflected from within the tissue, gathering information to aid the recognition of inter- and intracellular details (8). Different from conventional microscopy where the image can be observed directly, confocal microscopes obtain increased resolution by limiting the illumination and observation systems to a single point. Hence, to reconstruct a full field of view and allow for "real-time" viewing, rapid scanning is used for IVCM (8, 9). IVCM produces high-resolution images at a cellular level with a magnification of 600-800 times, a lateral image resolution of 1-2µm, and an axial resolution of 5-10 µm (10). Post-imaging quantitative evaluations of corneal nerve plexus can be done manually, in a semi-automated manner, or a completely automated manner using certain analytic software (5, 11). Numerous studies have reported IVCM findings of reduced corneal nerve fibre density (CNFD), corneal nerve fibre length (CNFL), and corneal nerve branch density (CNBD) in patients with type 1 diabetes mellitus (T1D) or type 2 diabetes mellitus (T2D) (Figures 1A, B) (5). A reduction in nerve beading frequency is also observed, indicating a decrease in nerve metabolic activity and an increase in the risk of neuronal damage (7). In addition, patients with T1D or T2D present with an increase in nerve fibre tortuosity, reflecting a degenerative and subsequent attempted regenerative nerve response (Figure 1C) (12, 13). Besides nerve changes in the central and peripheral cornea, an earlier reduction in CNFL and CNBD of the subbasal inferior whorl of the corneal nerves, located in the inferonasal cornea, is also reported, serving as an imaging site for early detection of DCN (Figures 1D, E) (5, 7, 14). Moreover, patients with T1D have a lower corneal nerve fractal dimension (CNFrD) compared to control subjects, suggesting a less healthy and less evenly-distributed nerve fibre network in patients with T1D (7). Changes in the morphology and quantity of corneal dendritic cells (DCs) in diabetic corneas were also observed in several studies

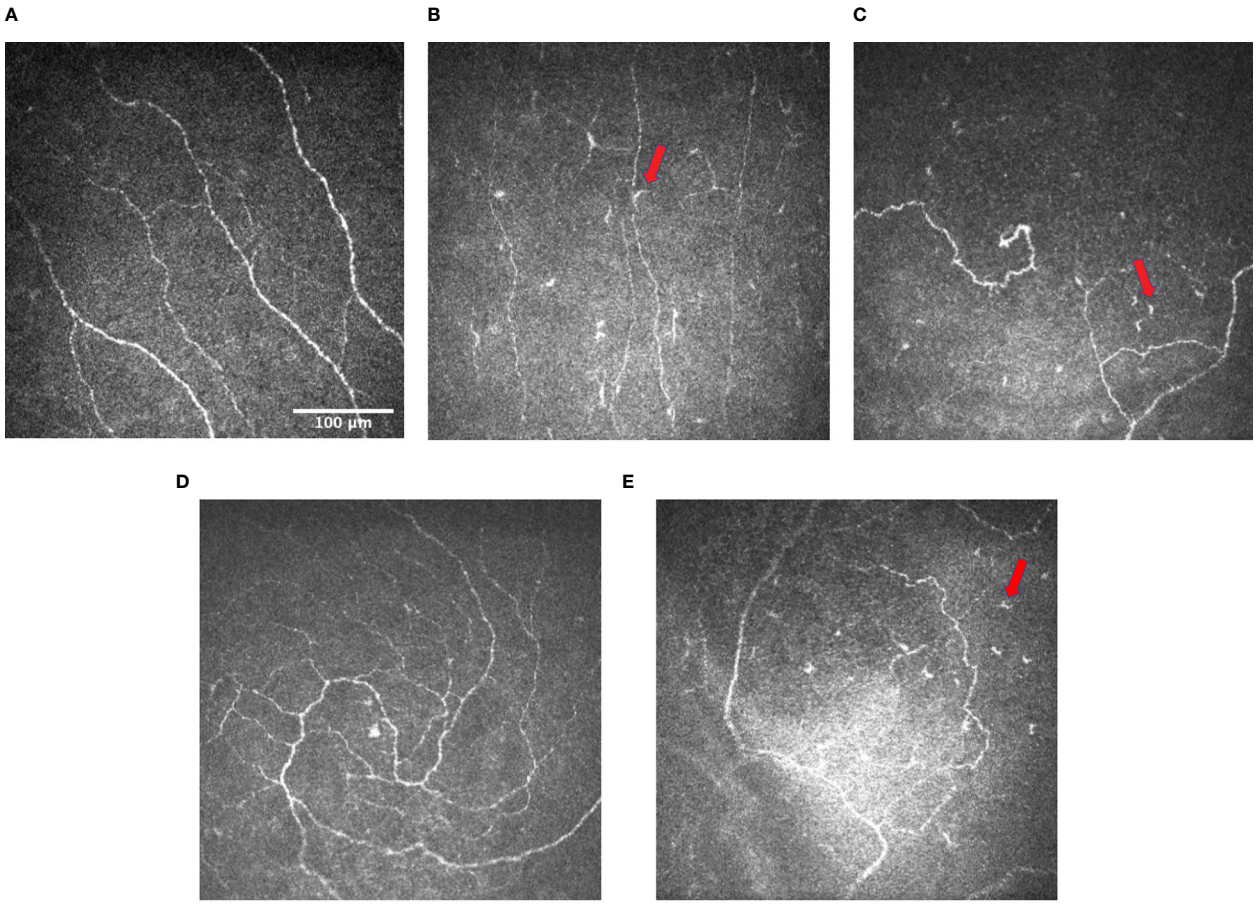

FIGURE 1
Representative IVCM images of **(A)** subbasal nerve plexus in normal controls (image taken from the subtemporal quadrant of the cornea of a middle-aged patient) (CNBD: 12.4992 no./mm^2); **(B)** subbasal nerve plexus with decreased corneal nerve fiber length and density in patients with DM, and the presence of dendritic cells (arrows) (CNBD: 0 no./mm^2) **(C)** subbasal nerve plexus with increased tortuosity and the presence of dendritic cells (arrows) in patients with DM (CNBD: 18.7488 no./mm^2) **(D)** inferior whorl of corneal nerves in normal controls (CNBD: 43.7472 no./mm^2); and **(E)** inferior whorl of corneal nerves in patients with DM showing the reduction in corneal nerve fiber length and density and the presence of dendritic cells (arrows) at the inferior whorl (CNBD: 6.2496 no./mm^2). Images were produced *via* the Heidelberg retina tomograph (HRT) Corneal Module (Heidelberg Engineering, Heidelberg, Germany), laser scanning confocal microscopy.

(15). However, the role and pathogenesis of the accumulation of the DCs have been not well understood.

2 Dendritic cells in normal corneas

DCs are bone marrow-derived antigen-presenting cells (APCs) that act as the initiator and modulator of immune responses (16). They are distinguished from other immune cell types through their cytoplasmic extensions (the dendrites), poor phagocytic activity, and the scarcity of their intracellular organelles (16). The most notable function of the DC family is to initiate primary T-lymphocyte-mediated immunity in response to an antigenic stimulus (17). This is achieved mainly through three functions of DCs: (a) capturing and presentation of antigens as sentinel cells; (b) migrating and binding to the antigen-specific T cells in lymphoid organs, and (c) activating T-cells and inducing their growth and proliferation (17).

2.1 Distributions of corneal dendritic cells

Naïve corneas were originally considered to lack the antigen-presenting system of DCs, contributing to their immune-privileged nature (18). However, more recent studies have shown a significant population of different subtypes of DCs residing in the cornea, with the number of which decreasing from the periphery towards the centre (19–22). Among the peripheral regions of the cornea, the inferior region has the highest density of DCs, followed by the superior region and the nasal region, while the temporal region has the lowest (22). In general, DCs can be subdivided into three main groups: the conventional DCs (cDCs), the plasmacytoid DCs (pDCs), and the monocyte-derived DCs (moDCs) (23). Such DCs subpopulations are defined based on their ontogeny, functional specialisation, and the requirement of specific transcription factors (TF) for the development (24). Different subtypes of corneal DCs are found in corneal epithelium and anterior stroma respectively (25, 26). Langerhans Cells (LCs), historically considered a subtype of conventional DCs (cDC), are observed in the periphery and centre of both human and murine corneal epithelium (25–29). However, the classification of LCs remains a topic with ongoing debate, since LCs were found to share properties with both DCs and macrophages. It has been argued by some that LCs may be considered a pecialized subset of tissue-resident macrophages based on their shared developmental origin (30, 31). Indeed, common DC precursors were found not to give rise to epidermal LCs. However, LCs share a remarkable number of functions with DCs, including migration to lymph nodes, and T-cell stimulation (31). The use of the term “LCs” has not been consistent across IVCM studies, and some other terms, such as APCs, dendritiform cells, or immune cells have also been used (28). Besides corneal epithelium, the anterior corneal stroma is also endowed with a different population of cDCs, namely the interstitial DCs. The interstitial DCs are primarily located in peripheral and paracentral regions of the anterior stroma with some toward the central anterior stroma in both murine and human cornea (25, 26, 29, 32). More recently, plasmacytoid dendritic cells (pDCs) have also been observed in the anterior stroma as well as epithelium in both the central and peripheral cornea of mice and human cadaver (29, 33–35).

2.2 Functions of corneal dendritic cells

Normally, mature DCs have developed dendrites that are absent in immature DCs (28). Unlike mature DCs, immature DCs lack the requisite accessory signals for T-cell activation, such as CD40, CD80, and CD86. To induce maturation of the dormant immature DCs, signals in the extracellular milieu through inflammatory mediators are needed (32). The distribution of corneal DCs at different maturation stages in the human cornea remains an issue of ongoing discussion. Some are consistent with the murine studies, which reported immature LCs in the centre of corneal epithelium, and both mature and immature LCs in the peripheral corneal epithelium (22, 27). Others demonstrated few mature LCs and interstitial DCs in epithelium and stroma respectively in both the peripheral and central cornea (25). The differences may have arisen from several reasons, potentially including different maturation markers and different models (*in-vivo* or ex-vivo) used (25, 27).

DCs serve both immunological and non-immunological roles in human cornea. The primary function of DCs in the cornea is to induce and amplify immunoinflammatory responses (18, 36). During the inflammatory process triggered by infection or allergy, the release of pro-inflammatory cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF)-α, CD40L, and lipopolysaccharide, or heat-shock proteins from dying cells, facilitates the activation of LCs/DCs in the cornea (20, 21). Resultingly, surface expression of co-stimulatory molecules (CD80/CD86) and CD40 is increased by DCs/LCs in the peripheral cornea, as well as acquired *de novo* by immature DCs/LCs in the central cornea (37). The activated corneal LCs/DCs function as APCs by transporting the antigens to lymphoid organs and presenting them to effector or memory T cells, priming the T cells for the antigen-specific adaptive immune response (18, 36, 37). Resident corneal DCs are considered long-lived, though it is still uncertain whether during the steady state, the corneal DCs self-regenerate through mitosis, emerge from tissue-resident precursors, or are recruited from the circulating blood (38–40). Nonetheless, in the presence of inflammatory stimuli and increased chemokine/cytokine levels in the cornea, corneal DCs are increased, at least partially through the recruitment of DC precursors from the blood (26, 38).

The non-immunological function of LCs/DCs is associated with tissue repair, through partnering with surrounding corneal epithelial cells. Upon injury, corneal intraepithelial LCs/DCs are activated either directly through recognition of danger signals, or indirectly from cytokines and chemokines secreted by epithelial cells in the injury site. The activated LCs/DCs modulate the migration, proliferation, and survival of epithelial cells in the wounding area *via* either cell-to-cell contact or the release of survival and growth factors (41). The epithelial cells, in turn, further activate corneal LCs/DCs and recruit them into the wound bed *via* epithelia-generated mediators (41).

It Is worth noting that although DCs and macrophages were historically regarded as two distinct types of immune cells, the classifications of DCs and macrophages have recently been challenged and remain a topic of ongoing discussion (42, 43). Due

to some shared surface markers and functional parameters between renal DCs and macrophages in both acute renal injury and chronic immune-mediated kidney disease (42–46). It was argued that the functional and phenotypic definitions of these two cell types, especially in the kidney, overlap greatly (42). Therefore, an improved classification system may be needed to better facilitate future research work (42, 44).

3 *In-vivo* confocal microscopy (IVCM) evaluation on corneal DCs

As IVCM can provide images at the cellular level, it has been used to evaluate the DC morphology and distribution (27, 47). Using IVCM, changes in corneal DCs have been observed in ocular surface diseases including dry eye disease and infectious keratitis, as well as systemic disorders including DM, multiple sclerosis, rheumatoid arthritis, ankylosing spondylitis, and systemic lupus erythematosus (15, 48–51). On IVCM evaluation, corneal epithelial DCs present as bright corpuscular particles and a diameter of up to 15μm (27). The presence of Birbeck granules, a type of cytoplasmic marker granules, distinguishes LCs from other DCs (27). Currently, phenotypic classification of corneal epithelial DCs is achieved mainly through morphological differences (49). The DCs morphology can be evaluated according to a 0-3 scale based on the size of the dendrites compared to the largest diameter of the cell body (Figure 2): A score 0 indicates an absence of DCs; a score 1 indicates the presence of DCs without processes; a score 2 indicates the presence of DCs with small processes, the length of which does not exceed the largest diameter of the cell body; a score 3 indicates the presence of DCs with long processes, the length of which exceeds the largest diameter of the cell body (48, 52). Longer processes and smaller cell bodies in DCs indicate a higher level of maturation and potential activity (48, 52).

4 Search strategy and selection criteria

The authors conducted a search on the online database PubMed Central, Google Scholar, and Science Direct for relevant articles that describe the changes in corneal dendritic cells in subjects with T1D/T2D or the relationship between corneal DCs and clinical or corneal imaging parameters in patients with T1D/T2D.

Articles were included up to May 2022. Keywords included but were not limited to "diabetes" AND "hyperglycaemia" AND "corneal dendritic cells" OR "corneal Langerhans cells" AND "corneal nerve" AND "corneal neuropathy" AND "corneal epithelial cells" AND "ag"" AND "diabetes duration" AND "blood metabolic profile". Our review only examined papers written in English, and we restricted the date of publication to the most recent ten years as much as possible. We have

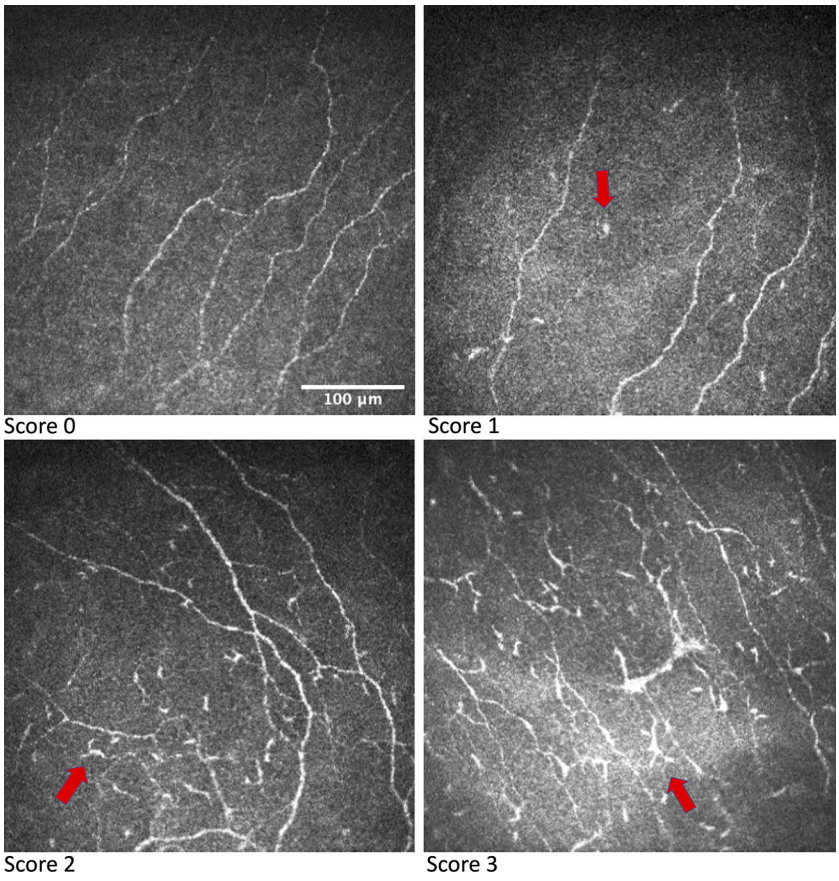

FIGURE 2
IVCM images showing grading of DC morphology. DCs are indicated by the arrow in respective IVCM images. Images were produced *via* the Heidelberg retina tomograph (HRT) Corneal Module (Heidelberg Engineering, Heidelberg, Germany), laser scanning confocal microscopy.

also extracted relevant articles from the bibliographies of the existing articles. The authors then manually screened the abstracts and shortlisted papers based on our inclusion criteria. The full-text version of all selected articles was further examined.

5 Changes in corneal dendritic cells in patients with DM

DM affects multiple ocular tissues, including the cornea (49). Studies have examined the changes in DCs, including the DC density, maturation stages, as well as relationships between the corneal DCs, corneal nerves, and corneal epithelium, in subjects with T1D/T2D (15, 53–60). Corneal DCs may serve as a biomarker for DM-associated ocular surface complications, such as diabetic corneal neuropathy (54).

5.1 Changes of corneal DC density in DM

Changes in DCs density in subjects with T1D/T2D have been investigated and remain a topic of ongoing discussion. The majority of animal and clinical studies reported an increase in DCs density in subjects with T1D/T2D, while a few presented the opposite (15, 53–59). The literature review on this topic is summarised in Table 1.

When mice models are used to investigate T1D, streptozotocin (STZ) is commonly used to induce hyperglycemia through the destruction of pancreatic β-cells (15, 59, 62). *Via* corneal IVCM and corneal *in vitro* whole-mounts confocal microscopy (WMCM), a constant increase of corneal DC density over 9 weeks upon diabetes induction was observed in one murine study (15). Contrastingly, another study using WMCM observed a significantly lower number of DCs in corneas from STZ-induced mice with T1D compared to controls (59). On the other hand, $Lep^{ob/ob}$ mice are often used as mouse models of T2D (62). One murine study investigating mice with T2D reported that the dendritic cell density in type 2 diabetic mice was 3-fold higher than in non-diabetic mice (15).

In clinical studies, the DC density was quantified in patients with T1D or T2D using IVCM. Compared to healthy controls, significantly higher DC density was observed in patients with T1D or T2D, as well as in patients with T1D/T2D and peripheral/somatic neuropathy or corneal punctate epitheliopathy (53–58). Among patients with T1D/T2D and peripheral/somatic neuropathy, DC density was significantly higher in patients with no or mild peripheral/somatic neuropathy compared to non-diabetic controls. However, with the progression of peripheral neuropathy, the DC density was reduced in patients with

TABLE 1 Studies reporting the changes in DC density in DM.

Authors	Study population	DC quantification technique	Findings
Literature reporting increased DC density in DM:			
Leppin et al. (15)	Streptozotocin (STZ)-induced T1D mice and $Lep^{ob/ob}$ mice with T2D	IVCM and *in-vitro* corneal whole-mounts confocal microscopy (WMCM)	Both STZ-induced mice and $Lep^{ob/ob}$ mice experienced increased corneal DC density.
Colorado et al. (53)	Patients with T1D	Time-lapsed IVCM	A higher density of DC without dendrites was observed in subjects with T1D compared to healthy controls.
D'Onofrio et al. (54)	Patients with T1D, T2D, or latent autoimmune diabetes of adults (LADA)	Laser scanning IVCM	A higher DC density was observed in patients with T1D, T2D, and LADA compared to controls.
Tavakoli et al. (55)	Patients with T1D/T2D and varying severities of diabetic peripheral neuropathy	IVCM	A significant increase in DC density was observed in patients with T1D/T2D and no or mild peripheral neuropathy A decrease in DC density was reported in patients with T1D/T2D and moderate or severe peripheral neuropathy, yet DC density still remained higher than control values.
Ferdousi et al. (56)	Children with T1D	IVCM	A significantly higher total DC density was observed in individuals with T1D compared to controls.
Qu et al. (58)	Patients with T2D diagnosed with corneal punctate epitheliopathy	IVCM	A significantly higher LC density was reported in punctate epitheliopathy patients with T2D compared to punctate epitheliopathy resulting from other causes.
Qu et al. (57)	Patients with T2D without and with cornea fluorescein staining	IVCM	A significantly higher LC density existed in T2D patients compared to healthy controls in all corneal areas. A significantly higher LC density was reported in T2D patients with punctate epitheliopathy compared to those without in the central and inferior zones of the cornea.
Literature reporting decreased DC density in DM:			
Gao et al. (59)	STZ-induced T1D mice	Whole-mount confocal microscopy (WMCM)	A reduced number of intraepithelial DCs was reported in diabetic corneas compared to non-diabetic corneas.
Literature reporting no significant change in DC density in DM:			
Chao et al. (61)	Patients with prediabetes or T2D	IVCM	No significant difference in DC density among patients with prediabetes, T2D, and healthy controls was observed.

T1D/T2D and moderate or severe neuropathy, though remained above control values. The authors proposed that DCs might be only involved in the early phases of nerve degeneration whereas the later phase of nerve damage in DM may be maintained by other factors, including glucose neurotoxicity (55, 63). The DC density in patients with T2D and corneal punctate epitheliopathy was also investigated (57, 58). A significantly higher DC density in all corneal areas was observed in both groups of type 2 diabetic patients with and without punctate epitheliopathy compared to healthy controls. In another study where the authors compared the DC density in patients with punctate epitheliopathy resulting from T2D or other causes, a significantly higher DC density was found in the former group, suggesting an association between T2D status and DC density (58).

There are several mechanisms proposed for the increase of DC density in DM populations. Though DM is characterised by elevated levels of blood glucose, it is suggested that the increase in DC might be unrelated to hyperglycaemia as no correlation between DC density and glycaemic control was observed (57). Instead, the increase of DCs in patients with T1D/T2D may be deemed as a cellular response to inflammation. Diabetes, especially T2D, has been suggested to be a pro-inflammatory cytokine-associated disease, involving both the innate and adaptive immune systems (60). There are several pathogeneses involved in the inflammatory state of T2D, including tissue hypoxia, cell death of expanding adipose tissue, activation of interleukins, and nuclear factor (NF)-κB pathways, contributing to the recruitment and activation of immune cells (5, 7, 64). For example, the NF-κB signaling pathway may be activated *via* the interaction of advanced glycation end-products (AGEs) and its cognate receptor for advanced glycation end-products (RAGE), subsequently promoting the secretion of TNF-α, IL-1β, IL-6, and other pro-inflammatory cytokines (65–68). Significantly increased levels of various AGEs compounds have been reported in both type 1 and type 2 diabetic patients, resulting from non-enzymatic glycation and oxidation of proteins and lipids (65, 69–75). The mechanism for the increased DC density is supported by the observations that corneal DC infiltration and maturation are induced when inflammatory stimuli like electric cautery, lipopolysaccharide, and tumour necrosis factor-α are applied to the ocular surface (26, 76). Findings by another study are also in line with this proposed mechanism where the DC density in the cornea increased by a factor of approximately eight during immune-mediated corneal inflammation secondary to an infection, allergy, or corneal graft rejection (77).

On the contrary, some literature showed the opposite findings in which decreased corneal DCs were observed in both animal models and patients with T1D/T2D (59, 78, 79). One proposed explanation is that prolonged exposure to hyperglycaemia may cause DC apoptosis, reducing the DCs density (80). Similar observations in other immune cells, such as increased apoptosis in neutrophils as well as impaired antigen presentation by monocytes, have also been reported under chronic hyperglycaemic conditions (81). Such observation might also be attributed to the different imaging techniques used.

5.2 Changes in maturation stages of corneal DCs in DM

Besides the density changes, changes in the maturation stages of DCs are reported in DM. Through wide-area three-dimensional mosaic projections of the corneal subbasal nerve plexus, a doubling in mature DCs (mDCs) proportion, as well as a proportional decrease in immature DCs (imDCs), were observed in patients with T2D (60). This finding suggests that the maturation of corneal DCs occurs as T2D develops. This is also supported by another study where the authors reported a higher percentage of patients with T1D/T2D/latent autoimmune diabetes of adults (LADA) (95%) with mature DCs in their central cornea compared to healthy controls (65%), while immature DCs can be found in all participants, including patients with T1D/T2D/LADA and controls (54).

It is proposed that tumour necrosis factor receptor super family member 9 (TNFRSF9) acts as a key contributor to the changes in the maturation stages of corneal DCs, by promoting the maturation and survival of DCs (60). Out of 92 plasma proteins analysed in a clinical study, TNFRSF9 was associated with the observed maturation of DCs from an immature to mature antigen-presenting phenotype. There was a significant association between TNFRSF9 and the proportion of mDC, and TNFRSF9 was also inversely correlated with the imDC proportion (60). TNFRSF9 is found to be expressed on immune cells including activated and regulatory T-cells and activated natural killer (NK) cells (82–84). Hence, when T cells are activated with the onset of T2D, it subsequently induces the expression of TNFRSF9, which further promotes the maturation of the DCs (60). Besides TNFRSF9, the involvement of AGEs in regulating the maturation of DCs has also been reported in both *in vitro* studies of human tissue and *in vivo* studies of diabetic mice with myocardial infarction (85, 86). The maturation of DCs in patients with T1D/T2D may be induced by the increased level of AGE through promoting the expressions of scavenger receptor-A (SR-A) and RAGE, *via* the Jnk pathway. Such a mechanism has been proposed in patients with atherosclerosis (86).

6 Relationship between corneal DCs and clinical or corneal imaging parameters

Studies have reported the associations between the changes in corneal DCs and various clinical or corneal nerve imaging parameters, including age, corneal nerve status, and blood metabolic parameters (15, 53–58, 87). Such associations may contribute to the current understanding of DM, further helping the development of diagnostic measures and biomarkers, as well as preventive and therapeutic strategies (54, 88, 89).

6.1 Relationship between corneal DCs and age

In healthy individuals, corneal DC density was reported to be independent of age by a meta-regression analysis (90). On the contrary, a significant and positive correlation between the DC density and age was observed in patients with T1D/T2D (55) (56). Moreover, specific to children with T1D, a significant positive correlation was found between the pubertal stage and the mature DC density, immature DC density, as well as to total DC density (56). These findings indicate that age may be a potential differential risk of DM and DM-associated ocular surface complications (56).

Besides the DC density, the age of the patients with T1D was also reported to be inversely correlated with the displacement of DCs without dendrites (woDCs), as well as the woDCs' persistence ratio (53). DC displacement was calculated as the straight-line distance between the start and end positions of a DC divided by the total time of movement. The authors further proposed that faster DC movements represent healthier DC behaviour and that reduced DC migration in older patients may contribute to age-associated immune dysfunction (53, 91, 92).

However, it is not entirely known how or whether the observed correlations between age and the corneal DC parameters in patients with T1D/T2D are involved or if they are influenced by the pathogenesis of diabetes. Aging has been linked to diabetes through several mechanisms, including age-associated insulin resistance and age-dependent disruption of insulin production (93, 94). Given that both the prevalence and incidence of T2D have been reported to increase dramatically as a function of age, further understanding of the mechanisms underpinning this differential risk is of great importance in the development of age-appropriate preventive and therapeutic strategies (88, 89).

6.2 Associations between corneal DCs and corneal nerves

Diabetes may perturb the interaction between DCs and other structures, especially corneal nerves (80). In the confocal images of mouse corneas stained with CD11c (inflammatory marker) and β-tubulin 3 (neuronal marker), intimate contacts between the DC body and its processes with sensory nerve endings were observed (59). It has also been demonstrated that DCs may be involved in diabetic nerve degeneration, yet whether DCs are neuroprotective or neurotoxic remains unclear with contrasting findings (49, 55).

Corneal nerve degeneration in DM may be associated with an increased DCs density (15). In STZ-induced type 1 diabetic mice, a significant negative correlation was reported between the corneal nerve fiber length and DC density (15). It was also observed that the density of DCs was higher in patients with T1D/T2D and no or mild peripheral neuropathy compared to those with moderate and severe peripheral neuropathy. The authors then proposed that DCs might be involved in the initial phase of nerve damage (55). This theory was further evidenced by the findings of other clinical studies. In patients with T1D/T2D, a significant negative correlation between increased DC density and corneal nerve fibre density, branch density, as well as fibre length was observed, suggesting a potential interaction between activated DCs and corneal nerve fibre degeneration (54, 58). Moreover, in adults with T1D or T2D with or without punctate epitheliopathy, a significant negative correlation was reported between the corneal nerve fiber length and DC density, specifically immature DC density for type 1 diabetic patients (15, 54, 57–59). An inverse correlation between the total DC density and corneal nerve total branch density was also reported in patients with T1D (54). In the immune-neuron crosstalk between nerves and DCs, cells from the neuroendocrine systems recognise the cytokines produced by immune cells. Reciprocally, the immune cells recognise the neurotransmitters and neuropeptides produced by the corneal nerves (95). It is speculated that the DC-nerve interaction in the cornea may be analogous to the neuro-immune axis in the skin and the gut (49, 80). For example, it was demonstrated that calcitonin gene-related peptide (CGRP)-containing nerve fibres were intimately associated with DCs and that CGRP could inhibit antigen presentation by epidermal DCs (96). The tolerogenic and immunomodulatory effects of many neuropeptides have also been previously indicated, which, in their absence due to damages to corneal nerves could lead to enhanced immune response, including increased DC density (97).

Contrary to the previous discussion, some studies found that nerve degeneration in DM may be associated with reduced DCs density (59). In STZ-induced type 1 diabetic mice with corneal epithelial debridement wounds, a reduced number of infiltrating DCs, as well as delayed sensory nerve regeneration, were observed (59). Though these observations were opposite from the findings of most other studies reporting on the same matter, the authors suggested a possible explanation (15, 54, 58). It is postulated that DCs may mediate corneal nerve innervation and regeneration through ciliary neurotrophic factor (CNTF). In the cornea, DCs are the major source of CNTF (59). It was demonstrated in mice with T1D that injection of CNTF-neutralising antibodies delayed nerve-ending regeneration, while exogenous CNTF accelerated nerve regeneration in corneas with local DCs depleted (59). Moreover, blocking the CNTF-specific receptor, CNTFα, induced corneal sensory nerve degeneration and delayed nerve regeneration, demonstrating the importance of CNTFα in the maintenance and regeneration of subbasal nerve plexus (59). Hence, in the case of the STZ-induced type 1 diabetic mice with corneal epithelial debridement wounds, decreased number of DCs on the cornea would lead to a decreased CNTF level, impairing corneal sensory nerve innervation and regeneration (59). Besides the involvement of CNTF, DCs may also be involved in the regeneration of neurons through the clearance of axonal debris. It was suggested that the clearance of axonal debris is a critical process in axonal regeneration in the peripheral nervous system (98). Besides murine studies, a clinical study has also reported similar observations where in children with T1D, a significant positive correlation was observed between the density of mature DCs and the corneal nerve fiber density (56).

However, the relationship between the DC density and corneal nerve parameters in subjects with T1D/T2D remains a topic for more investigation. There were also studies reporting no significant correlation to exist between the DC density and corneal nerve morphology in either T1D or T2D (54, 55). The analysis of the relationship may be confounded by several factors, such as variations in the type, stage, and duration of DM. It is also possible that corneal nerve fibre changes and DC density are different and independent phenomena that occur coincidentally at the same time, and other cells also play a role (15). For example, vascularisation that develops after denervation may also lead to the influx of DCs (15). Moreover, in patients with T1D/T2D, increased levels of AGE/RAGE signaling in neurons may induce the activation of inflammatory and oxidative stress pathways, including the NF-κB pathway, potentially causing damage and death of neuronal cells (99–101).

The associations reported may further help explore surrogate imaging markers for diabetic corneal neuropathy (54). For example, a significant correlation between DC density and the severity of diabetic peripheral neuropathy has been described (55). Moreover, the

reported associations between the DC density and various nerve parameters may provide evidence for a potential therapeutic strategy to promote corneal nerve regeneration. For example, given the fact that DCs are the major source of CNTF, using DCs as therapeutic targets for the repair of injured corneal nerves in patients with T1D/T2D may open a new avenue for treatment (49, 59).

The literature review on the association between corneal DCs and corneal nerve parameters is summarised in Table 2.

6.3 Interaction between corneal DCs and corneal epithelium in DM

Besides the interaction with the nerve, the interaction between DCs and epithelial cells may be perturbed in subjects with T1D/T2D, potentially affecting the corneal wound healing (80).

In both murine and clinical studies, it was observed that subjects with T1D/T2D had delayed corneal wound healing compared to healthy controls (59, 102–105). A decrease in basal epithelial cell (BEC) density in patients with T2D has also been reported by several clinical studies (57, 106, 107). Furthermore, a negative correlation between the BEC density and DC density in the cornea was observed in patients with T2D (57). Hence, it was speculated that DCs may be involved in the early stages of BEC proliferation and differentiation in DM (57). Epithelial wound closure requires cell reverse differentiation of wing cells to basal cell like cells, cell migration, and cell proliferation to replenish the lost cells (108, 109). Besides epithelial cells, immune cells, such as DCs, were also directly involved in accelerating epithelial wound healing (80). The anatomical proximity and structural intertwinements between DCs and epithelial cells have led to the suggestion that corneal epithelial cells and corneal intra-epithelial DCs interact with each other to form coordinated actions against adverse challenges, such as tissue injury and infection (41, 80). It was demonstrated in non-diabetic corneas that migratory epithelial cells during wound healing would express an elevated level of DC-targeting cytokines, to activate DCs around the injury site (41). Reciprocally, DCs would secrete growth factors, cytokines, and/or through cell-to-cell contact to facilitate migration and proliferation of epithelial cells,

TABLE 2 Studies reporting on the correlation between DC density and various corneal nerve imaging parameters.

Authors	Study population	Nerve imaging parameters assessed	Findings
Ferdousi et al. (56)	Children with T1D (Age: 14.6 ± 2.5; Diabetes duration: 9.1 ± 2.7 years)	Corneal nerve fibre density	↑ density of mature DCs density, ↑ corneal nerve fibre density (r = 0.2, P = 0.01) in patients with T1D
D'Onofrio et al. (54)	Patients with T1D (Age: 53.3 ± 11.7; Diabetes duration: 19.4 ± 7.6 years), T2D (Age: 57.7 ± 7.5; Diabetes duration: 15.1 ± 4.9 years), or latent autoimmune diabetes of adults (LADA) (Age: 50.5 ± 11.5; Diabetes duration: 11.6 ± 9.6 years)	Corneal nerve fibre density	No significant correlation between DC density and corneal nerve fibre density in patients with T1D, T2D, or LADA.
D'Onofrio et al. (54)	Patients with T1D (Age: 53.3 ± 11.7; Diabetes duration: 19.4 ± 7.6 years), T2D (Age: 57.7 ± 7.5; Diabetes duration: 15.1 ± 4.9 years), or latent autoimmune diabetes of adults (LADA) (Age: 50.5 ± 11.5; Diabetes duration: 11.6 ± 9.6 years)	Corneal nerve branch density	↑ mature DC density, ↓ corneal nerve branch density (r = –0.5; P = 0.008); ↑ immature DC density, ↓ corneal nerve branch density (r = –0.4; P = 0.02); ↑ total DC density, ↓ corneal nerve branch density (r = –0.5; P = 0.01) DC density in patients with T1D but not in patients with T2D and LADA.
Ferdousi et al. (56)	Children with T1D (Age: 14.6 ± 2.5; Diabetes duration: 9.1 ± 2.7 years)	Corneal nerve branch density	No significant correlation between DC density and corneal nerve branch density in children with T1D.
D'Onofrio et al. (54)	Patients with T1D (Age: 53.3 ± 11.7; Diabetes duration: 19.4 ± 7.6 years), T2D (Age: 57.7 ± 7.5; Diabetes duration: 15.1 ± 4.9 years), or latent autoimmune diabetes of adults (LADA) (Age: 50.5 ± 11.5; Diabetes duration: 11.6 ± 9.6 years)	Corneal nerve fibre length	↑ immature DC density, ↓ corneal nerve fibre length (r = –0.4; P = 0.03) in patients with T1D but not in patients with T2D and LADA.
Qu et al. (58)	Patients with T2D diagnosed with corneal punctate epitheliopathy (Age: 59.8 ± 11.6; Diabetes duration: 13.4 ± 8.30 years)	Corneal nerve fibre length	↑ DC density, ↓ corneal nerve fibre length (r = 0.350; R2 = 0.1225; P = 0.034) in patients with T2D diagnosed with corneal punctate epitheliopathy
Qu et al. (57)	Patients with T2D without (Age: 60.51 ± 8.37; Diabetes duration: 13.40 ± 8.30 years) and with (Age: 63.75 ± 10.91; Diabetes duration: 13.90 ± 5.20 years) cornea fluorescein staining	Corneal nerve fibre length	↑DC density, ↓ corneal nerve fibre length in all corneal zones except the superior zone in patients with T2D.
Leppin et al. (15)	Streptozotocin (STZ)-induced T1D mice	Corneal nerve fibre length	↑ DC density, ↓ corneal nerve fibre length existed in STZ-induced diabetic mice. No such correlation was observed in non-diabetic controls.
Ferdousi et al. (56)	Children with T1D (Age: 14.6 ± 2.5; Diabetes duration: 9.1 ± 2.7 years)	Corneal nerve fibre length	No significant correlation between DC density and corneal nerve fibre length in children with T1D

modulating wound healing (41, 59). However, the specific role of DCs in delayed epithelial wound healing in patients T1D/T2D remains unclear, and several explanations have been proposed (41).

One explanation is that prolonged corneal wound healing response in subjects with T1D/T2D may lead to increased recruitment of DCs to the corneal wound through chemokines released by the injured site (41, 103). Several factors were reported to contribute to the delayed recovery of corneal epithelial wounds in DM, including nerve degeneration, accumulation of AGEs, and direct damage caused by hyperglycaemia to the corneal epithelial basement membrane (110). In particular, it was suggested that AGEs may delay corneal epithelial wound healing through the production of reactive oxygen species (111). In a wounded cornea, the corneal epithelial cells can further facilitate wound healing through the release of various cytokines, including the C-X-C motif chemokine ligand 10 (CXCL10) (41). CXCL10 acts as a chemokine to DCs, activated T cells, and NK cells, and it was reported to be highly expressed in migrating epithelial during corneal wound healing (41, 112). Hence, it was suggested that epithelia-released CXCL10 may facilitate the recruitment of resident corneal epithelial DCs and even the circulating DCs to the wound bed in the cornea (41). It is possible that, in diabetic cornea where the wound healing process is altered and prolonged, more chemokines may be released by the epithelia, resulting in increased recruitment of DCs into the cornea (103, 113). Similarly, clinical studies on the epidermis of diabetic foot ulcers have reported an accumulation of DCs at the edge of diabetic foot ulcers (113, 114).

Contrary to the aforementioned explanation of increased DCs in diabetic wound healing, a murine study examining subjects with T1D has reported a decreased number of infiltrating DCs in diabetic healing cornea compared to healthy controls. It was proposed that such a decrease in DCs population may hinder the proliferation of the epithelial cells, contributing to the impaired wound healing process (59, 115). As discussed previously, CNTF originates from DCs and is involved in sensory nerve survival and regeneration (59). Recently, CNTF was also discovered to be able to promote epithelial wound healing by stimulating the mitogenic activation of corneal epithelial stem/progenitor cells (115). In the corneas of mice with T1D, the level of CNTF was significantly downregulated, potentially due to the decreased infiltrating DCs population, contributing to the impaired proliferation of epithelial cells during wound healing in diabetic corneas (59, 115).

6.4 Correlations between corneal DCs and blood metabolomic profiles

Associations between the DC density and several metabolic parameters, including lipid profiles, glycaemic control, as well as renal function, have also been assessed in patients with T1D/T2D, as shown in Table 3 (55, 56, 87).

In patients with T1D, significant associations were found between the DC density and lipid parameters (87). The density of corneal DCs without dendrites (woDCs) was positively correlated with the HDL cholesterol level and was inversely correlated with the triglycerides level (87). Such observations suggest that woDC may be associated with better health since both a higher HDL level and a lower triglyceride level potentially indicate lower cardiovascular risks (116, 117). However, another clinical study demonstrated that rounded corneal DC density was correlated inversely with the HDL level in patients with T1D (87). Such disparity reported may signal that different DC subsets exert different immune activities on the cornea in patients with T1D (87).

For the renal function of patients with T1D, a significant positive correlation was detected between the eGFR and the displacement, trajectory, and persistency of corneal DCs in patients with T1D (87). These three parameters are indicative of DCs' mobilisation capacities which are critical for the role of the DCs in activating and mediating immune responses (87, 118). It has been proposed that the resident DCs in the cornea may function similarly to those in the kidney. eGFR was also negatively correlated with the number of DCs in the kidney for both healthy individuals and those with chronic kidney disease (119).

TABLE 3 Studies reporting on the correlation between corneal DC parameters and blood metabolic parameters.

Author (year)	Study population	Blood metabolic parameters assessed	Findings
Colorado et al. (87)	Patients with T1D (Age: 55.0 ± 11.0; Diabetes duration: 29 ± 14 years)	Lipid profile	↑ corneal DCs without dendrites (woDCs) density, ↑ the HDL level ($r = 0.59$, $p = 0.007$); ↑ corneal DCs without dendrites (woDCs) density, ↓ the triglyceride level ($r = -0.61$, $p = 0.005$); ↑ rounded corneal DC density, ↓ the HDL level ($r = -0.54$, $p = 0.007$) in patients with T1D.
Colorado et al. (87)	Patients with T1D (Age: 55.0 ± 11.0; Diabetes duration: 29 ± 14 years)	Glycaemic control	No significant association between HbA1c and corneal DC density as well as DC dynamics in patients with T1D.
Tavakoli et al. (55)	Patients with T1D/T2D and varying severities of peripheral neuropathy (Age: 58 ± 1; Diabetes duration: 15 ± 1 years)	Glycaemic control	No significant correlation between DC density and HbA1c.
Ferdousi et al. (56)	Children with T1D (Age: 14.6 ± 2.5; Diabetes duration: 9.1 ± 2.7 years)	Glycaemic control	No significant correlation between DC density and HbA1c
Colorado et al. (87)	Patients with T1D (Age: 55.0 ± 11.0; Diabetes duration: 29 ± 14 years)	Renal function	↑ displacement of corneal DCs, ↑ eGFR ($r = 0.74$, $p < 0.001$); ↑ trajectory of corneal DCs, ↑ eGFR ($r = 0.48$, $p = 0.031$); ↑ persistency of corneal DCs, ↑ eGFR ($r = 0.58$, $p = 0.008$) in patients with T1D.

Contrasting to lipid parameters and renal function, glycaemic control has not been reported to be significantly associated with DCs parameters (density and dynamics) (55, 56, 87). The increase in DC density observed in patients with T1D/T2D may be independent of hyperglycaemia (55).

7 Future work

The changes in corneal DCs in patients with T1D/T2D have attracted much attention and discussion in recent years. Despite the current progress toward understanding the DC changes and underlying mechanisms, many questions remain and are to be addressed.

Firstly, continued improvement in imaging technologies, as well as the identification and quantification techniques used for corneal DCs on IVCM images are required, to ensure accurate analysis across the studies. Secondly, it is possible that the identification and characterisation of corneal DCs *in vivo* may be further refined, to contribute to a deeper understanding of corneal DCs changes, as well as the roles played by corneal DCs in the diabetic corneas (49). Thirdly, DCs, corneal nerves, and corneal epithelium were previously considered to form an "epineuroimmune" function unit (80). However, it remains unclear which of the three components is the initial "sentinel" that detects the physiological changes in patients with T1D/T2D and subsequently induces the changes in the other two components of the "epineuroimmune" function unit. Furthermore, the initial "trigger" (e.g. hyperglycaemia, intracellular reactive oxygen species, or extracellular AGEs) in the diabetic cornea that causes the physiological and functional changes in the "epineuroimmune" function unit also requires elucidation (80). Hence, further mechanistic studies are needed to define the basis of the changes in the "epineuroimmune" function unit in the diabetic cornea, potentially adding value to the development of preventive and treatment strategies for DM-associated ocular surface complications (55).

8 Conclusions

This article has reviewed current clinical and animal studies reporting the changes in corneal DCs in diabetic corneas, as well as the potential mechanisms underlying the changes. For the changes in DC density, the majority of animal and clinical studies reported an increase in corneal DCs density in DM, while a few presented the opposite (15, 53–59). The increase in DC density may be explained as a cellular response to inflammation while the decreased density may be explained as a result of apoptosis caused by prolonged exposure to hyperglycaemia (60, 80). The maturation of corneal DCs in tandem with the disease course of T2D was indicated (60). DCs were also found to be involved in diabetic nerve degeneration, yet whether DCs are neuroprotective or neurotoxic remains unclear with contrasting findings (49, 55). The association between increased DCs density and corneal nerve degeneration in DM may be explained by an enhanced immune response caused by the absence of tolerogenic and immunomodulatory neuropeptides following corneal nerve damage (95). On the other hand, the association between decreased DCs density and corneal nerve degeneration in diabetic corneas may be explained by the decreased CNTF level expressed by the corneal DCs (59). Corneal DCs are also involved in delayed epithelial wound healing in diabetic corneas (80). One suggested mechanism is that prolonged corneal wound healing response leads to increased recruitment of DCs to the corneal wound bed through chemokines released by epithelia around the injury site (41, 103). We also further reviewed the association between the changes in the corneal DCs and various clinical or corneal nerve imaging parameters, including age, corneal nerve status, and metabolic parameters (15, 53–58, 87). Such associations contribute to our current understanding of DM-associated ocular surface complications, potentially further assisting the development of diagnostic, preventive, and therapeutic strategies.

Author contributions

FL and LYC were responsible for conducting the search, screening potentially eligible studies, and writing the manuscript. CL was responsible for the synthesis of images. IL and ML were involved in conducting the search. LYC provided the direction of the review and overall supervision of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Intravitreal anti-vascular endothelial growth factor, laser photocoagulation, or combined therapy for diabetic macular edema: A systematic review and network meta-analysis

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Purpose: To conduct a network meta-analysis (NMA) comparing the efficacy of anti-vascular endothelial growth factor (VEGF) therapy alone versus laser photocoagulation (LP) therapy alone or anti-VEGF therapy combined with LP therapy for diabetic macular edema (DME).

Methods: PubMed, Embase, Web of Science, and Cochrane Central Register of Controlled Trials were systematically searched for studies comparing anti-VEGF therapy alone versus LP therapy alone or anti-VEGF therapy combined with LP therapy for DME. Primary outcomes were mean best-corrected visual acuity (BCVA) and central macular thickness (CMT) change. Relevant data were collected and pooled using NMA.

Results: A total of 13 randomized controlled trials were included in our NMA. Anti-VEGF therapy significantly improved BCVA the most compared to the combined (mean difference [MD] = 1.5; 95% confidence interval [CI]: 0.084, 2.7) and LP (MD = 6.3; 95% CI: 5.1, 7.6) therapies at six months, while there was no difference in reducing CMT at six months between the anti-VEGF and combined therapies (MD = -16; 95% CI: -46, 13). At 12 months, no significant difference was found between the anti-VEGF and combined therapy in terms of BCVA (MD = 0.1; 95% CI: -1.7, 1.5) and CMT (MD = 21; 95% CI: -3.0, 44).

Conclusion: There was no significant difference between the anti-VEGF therapy and combined therapy. For the long-term treatment of patients with DME, combined therapy is recommended.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022376401.

KEYWORDS

diabetic macular edema, anti-vascular endothelial growth factor, laser photocoagulation, network meta-analysis, combined therapy

Introduction

Diabetic macular edema (DME), a manifestation of diabetic retinopathy (DR) that is diagnosed at any stage of the disease, is defined as retinal oedema and/or thickening, involving, or threatening the fovea. Although the management of diabetes mellitus (DM) has advanced tremendously over the last few decades, DME still accounts for a significant cause of vision loss among patients with DM, and if untreated, can result to blindness. DME affects approximately 7% of patients with DM (1) and represents a substantial public health concern worldwide (2, 3). The prevalence of DME is related to the duration of DM and stage of DR (4).

In recent years, with further understanding of the pathophysiological mechanisms of DME, treatment options for DME have shifted gradually. Laser photocoagulation (LP) was the gold standard treatment for DME prior to the availability of anti-vascular endothelial growth factor (VEGF) treatment (5). The mechanisms of LP include increased oxygen tension and phagocytosis of glial cells and retinal pigment epithelial cells, together with decreased production of vasoactive cytokines (mainly VEGF). LP provides vision stabilization in DME, while the efficacy of providing clinical improvement in patients' vision seems to be limited (5, 6). Currently, anti-VEGF agents are the first-line treatment option for DME. Ranibizumab, aflibercept, bevacizumab, and pegaptanib have shown significant efficacy in visual improvement in patients with DME in phase II/III clinical trials (7–10). However, anti-VEGF agents cannot treat macular hypoxia; thus, their efficacy is transitory. Additionally, the short half-life of anti-VEGF agents, such as ranibizumab and bevacizumab, in the eyes of 2.75 and 9.8 days, respectively, results in a limited duration of action with consequent high rate of recurrence; thus, requiring frequent injections (11, 12) and imposing a large burden on patients with DME. A combination of anti-VEGF and LP may be more effective than either monotherapy and may reduce the frequency of injections. Additionally, the effectiveness of LP may be improved by LP becoming easier because of the reduction in macular edema caused by anti-VEGF injections. Several studies have evaluated LP as an adjunctive treatment for anti-VEGF agents; however, their conclusions are inconsistent (13–18).

Network meta-analysis (NMA) is a novel data synthesis method that combines direct and indirect evidence from randomized controlled trials (RCTs) using statistical techniques to derive estimates of comparative efficacy (19). Therefore, this study compared the efficacy of anti-VEGF therapy alone, LP therapy alone, or anti-VEGF therapy combined with LP therapy in the treatment of patients with DME within an NMA framework, primarily aimed at assessing the mean best-corrected visual acuity (BCVA) and central macular thickness (CMT) changes.

Methods

The NMA was strictly conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (20) and the Cochrane Handbook guidelines (21).

Search strategy

RCTs evaluating the efficacy of anti-VEGF therapy alone, LP therapy alone, or anti-VEGF therapy combined with LP therapy in the treatment of DME were systematically searched in PubMed, Embase, Web of Science, and the Cochrane Central Register of Controlled Trials from inception to September 11, 2022. The search strategy (Table S1) was conducted corresponding to the following terms: "diabetic macular edema," "anti," "vascular endothelial growth factor," "vegf," "ranibizumab," "bevacizumab," "aflibercept," "pegaptanib," "laser," and "photocogulation," which were connected by and/or in different combinations. The search was restricted to human studies. No publication date or language limitation was imposed when searching for the RCTs. Additionally, reference lists of relevant articles were manually examined to identify potentially relevant studies.

Inclusion and exclusion criteria

Studies were eligible if they met the following inclusion criteria: (1) RCT; (2) patients/participants with DME; (3) comparison of at least two of the following comparators: anti-VEGF therapy alone, LP therapy alone, anti-VEGF therapy combined with LP therapy; (4) outcome measures, including the mean BCVA and/or CMT change; and (5) follow-up >6 months.

Exclusion criteria were as follows: (1) review articles, case reports, non-RCTs, meta-analyses, and redundant publications; and (2) studies with insufficient data.

Two authors (J C and H W) independently screened the titles and abstracts of the identified articles. All potentially eligible articles were full-text reviewed to evaluate whether they met the inclusion criteria. Any discrepancies were resolved *via* discussion. Unsettled discrepancies were arbitrated by a senior reviewer, Prof. Qiu.

Data extraction and quality assessment

Two authors (J C and H W) independently extracted data from all the included studies. The extracted data included the first author, publication year, geographic location, study design, interventions (including specific injection plan), follow-up time, and total number of eyes of different interventions, together with the details of outcomes, which included the mean BCVA and CMT change from baseline to 6 and 12 months. If any essential information was required for eligibility assessment or data extraction, the corresponding authors of the included studies were contacted. Logarithm of the minimal angle of resolution (logMAR) was converted into the ETDRS letter form when extracting BCVA data. The Cochrane collaboration tool was used to assess risk of bias (21).

Statistical analysis

The NMA was performed within a Bayesian framework to synthesize the mean BCVA and CMT changes from baseline to 6 and 12 months across the RCTs. We used R software (version 4.2.1) with gemtc and rjag packages to create forest plots. Statistical heterogeneity was evaluated using the I^2 statistic: <25%, no heterogeneity; 25–50%, low heterogeneity; 50–75%, moderate heterogeneity; and >75%, high heterogeneity (22). The node-splitting method was used to assess the inconsistency between direct and indirect comparisons in NMA (23). Significant heterogeneity was at $p < 0.05$. Efficacy of the interventions was evaluated using mean difference (MD) with 95% confidence interval (CI). Additionally, we conducted a ranking analysis based on simulations and calculated the rank's possibility of establishing a hierarchy of different interventions. We also assessed the potential publication bias by creating the funnel plot and conducting the Egger's test in the traditional meta-analysis.

Result

Study characteristics

A total of 1,727 articles (PubMed, 244; Embase, 692; Web of Science, 512; and Cochrane Central Register of Controlled Trials, 279) were retrieved from the electronic databases in the primary search, among which 604 articles were removed for duplicates. After screening the titles and abstracts, 1,093 articles were removed. Thirty full-text articles were reviewed to determine whether they met the inclusion criteria. Eventually, 13 articles were included in the NMA (Figure 1) (14, 18, 24–34). Characteristics of all the included RCTs are summarized in Table 1. All the RCTs compared two or more interventions and included a total of 2,432 eyes. The mean BCVA and CMT changes from baseline to 6 and 12 months were recorded for the NMA.

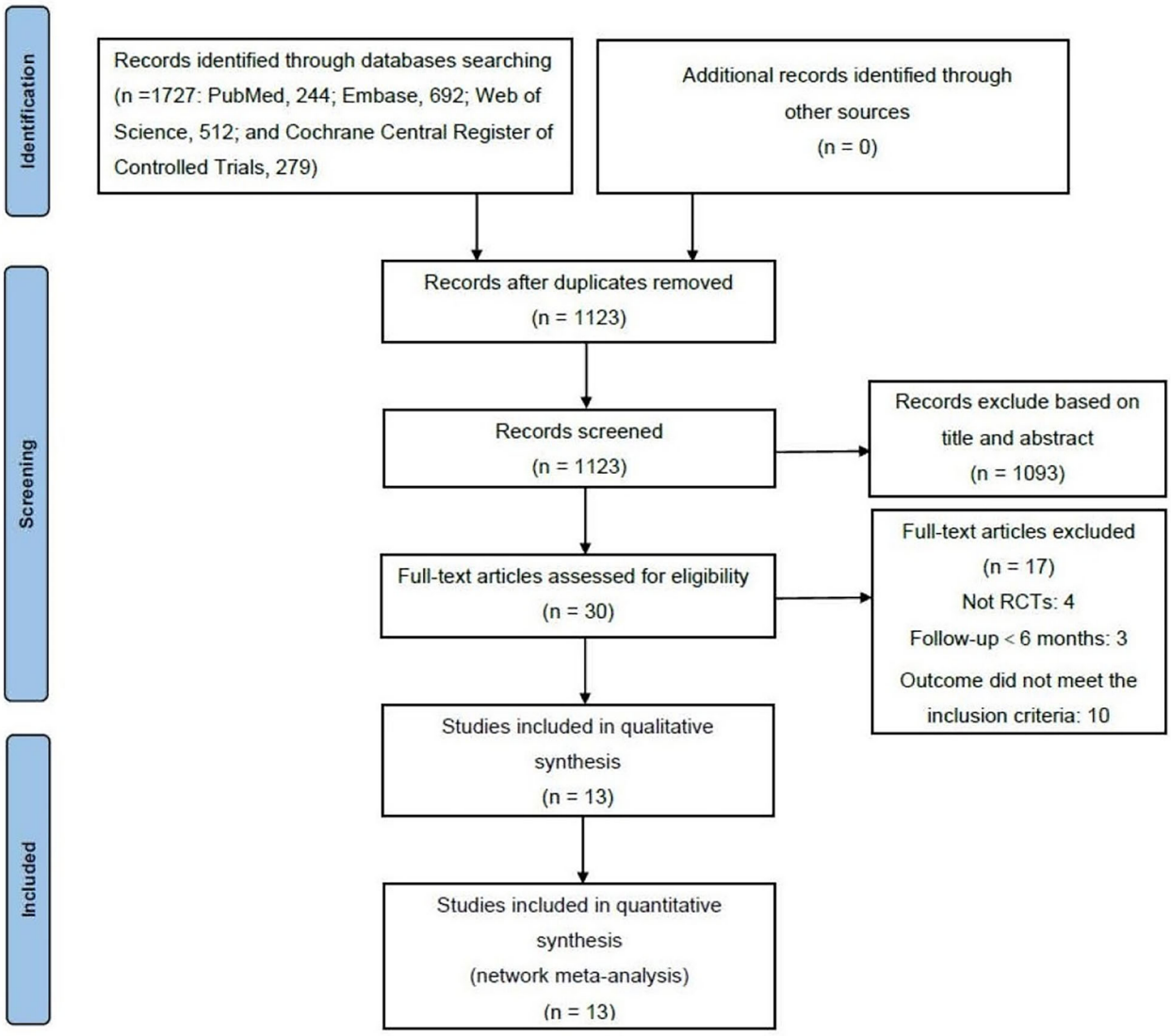

FIGURE 1
Flow chart depicting the selecting process of included studies.

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TABLE 1 Summary of the characteristics of included studies.

First author	Geographic location	Year	Study design	Intervention	Injection	Follow-up	Total eyes	BCVA-6m	BCVA-12m	CMT-6m	CMT-12m
Tatsumi	Multi-center	2022	RCT	IVA	monthly injection for 3 months, followed by monthly injection based on pro re nata (PRN) regimen	96weeks	25	+4.80 ± 8.45	+7.15 ± 6.9	-83.0 ± 105	-93.0 ± 94.9
				IVA+LASER	ditto		26	+8.65 ± 9.95	+7.55 ± 10.75	-106 ± 158	-115.0 ± 134.7
Li	China	2019	RCT	IVR	3 initial monthly injection, followed by monthly injection based on pro re nata (PRN) regimen until stable vison activity was achieved.	12months	307	+6.7 ± 7.88	+7.8 ± 8.72	-145.1 ± 157.69	-146.5 ± 157.61
				LASER			77	+0.3 ± 11.01	+2.5 ± 8.78	-72.2 ± 153.56	-85.9 ± 166.60
Baker	USA	2019	RCT	IVA	1 injection every 4 weeks	24months	226	NA	+2.1 ± 5.0	NA	-50 ± 55
				LASER			240	NA	+0.1 ± 5.5	NA	-30 ± 69
Lang	Multi-center	2018	RCT	IVR+LASER	4 initial monthly injections followed by pro re nata (PRN) injections	12months	85	+6.4 ± 4.0	+6.5 ± 4.3	NA	-96.7 ± 120.9
				LASER			43	+2.0 ± 3.3	+1.3 ± 3.7	NA	-54.0 ± 89.9
Yang	China	2017	RCT	IVR	1 injection every month. As of month 3, monthly reinjection according to patients' condition	12months	25	+7.5 ± 6.4	+6.5 ± 6.3	-96 ± 117	-101 ± 112
				IVR+LASER	ditto		28	+7.2 ± 6.1	+7.9 ± 7.1	-108 ± 131	-126 ± 157
Ishibashi	Multi-Center	2015	RCT	IVR	1 injections every month. As of month 3, continue monthly injections if stable vision was not reached.	12months	133	+6.2 ± 7.73	+6.6 ± 7.68	-118.8 ± 161.55	-134.6 ± 131.17
				IVR+LASER	ditto		132	+5.5 ± 8.06	+6.4 ± 10.67	-144.8 ± 166.22	-171.8 ± 160.85
				LASER			131	+0.9 ± 7.81	+1.8 ± 8.27	-35.0 ± 121.98	-57.2 ± 118.60
Berger	Canda	2015	RCT	IVR	3 monthly injections followed by as-needed therapy	12months	75	+7.1 ± 7.83	+8.9 ± 7.83	-129.3 ± 118.69	-143.5 ± 148.25
				IVR+LASER	ditto		73	+5.6 ± 8.58	+8.2 ± 9.44	-114.2 ± 113.29	-152.2 ± 142.47
				LASER			72	+0.9 ± 7.68	+0.3 ± 13.64	-64.4 ± 117.26	-107.1 ± 157.3

(Continued)

TABLE 1 Continued

First author	Geographic location	Year	Study design	Intervention	Injection	Follow-up	Total eyes	BCVA-6m	BCVA-12m	CMT-6m	CMT-12m
Comyn	England	2014	RCT	IVR	3 loading doses of ranibizumab then reinjection every 4 weeks as required	48weeks	22	NA	NA	NA	-131.5 ± 98.0
				LASER			11	NA	NA	NA	-102.9 ± 88.4
Liegl	Germany	2014	RCT	IVR	3 monthly injections and additional injections	12months	32	+7.6 ± 6.7	+6.3 ± 6.5	-88 ± 109	-105 ± 107
				IVR+LASER	ditto		34	+7.2 ± 7.1	+8.4 ± 8.3	-98 ± 197	-129 ± 170
Soheilian	Iran	2012	RCT	IVB	1 injection every 3 months	24months	50	+10.5 ± 10	+10.5 ± 13.5	-36 ± 119	-40 ± 133
				LASER		50	-1 ± 16.5	-1 ± 17	-11 ± 78	+6 ± 86	
Mitchell	Multi-Center	2011	RCT	IVR	3 monthly injections at months 0–2, further treatment according to retreatment criteria	12months	116	NA	+6.1 ± 6.43	NA	-118.7 ± 115.07
				LASER			111	NA	+0.8 ± 8.56	NA	-128.3 ± 114.34
				LVR+LASER	ditto		118	NA	+5.9 ± 7.92	NA	-61.3 ± 132.29
Nguyen	USA	2010	RCT	IVR	4 injections at baseline and months 1, 3, and 5	24months	33	+7.24 ± 4.46	+6.61 ± 5.58	NA	NA
				LASER			33	-0.43 ± 4.45	+2.39 ± 4.0	NA	NA
				IVR+LASER	1 injection at month 5		34	+3.8 ± 4.04	+4.81 ± 5.16	NA	NA
Michaelides	England	2010	RCT	IVB	3-9 injections in the first 12 months	12months	42	NA	NA	NA	-130 ± 122
				LASER			38	NA	NA	NA	-68 ± 171

IVA, intravitreal aflibercept; IVB, intravitreal bevacizumab; IVR, intravitreal ranibizumab; LASER, laser, micropulse laser, macular laser, grid laser and focal/grid laser; BCVA: mean change in best corrected visual acuity; CMT: mean change in central macular thickness; NA, Not available.

Risk of bias assessment

A summary of the risk of bias assessment for the included studies is shown in Figures 2, 3. One of the studies did not mention the method of generating the random allocation sequence, seven did not mention allocation concealment, six did not mention blinding of participants and personnel, and three did not mention blinding of outcome assessment; therefore, the risk of bias assessment was considered unclear. Additionally, one study had a high risk of bias in the random allocation sequence, one featured a high risk of blinding of participants and personnel, and three featured a high risk of blinding outcome assessment. Overall, quality of the included studies was considered high, although the risk of bias in several studies was high or unclear under some conditions.

Network meta-analysis

Mean BCVA change

Nine RCTs were included to conduct a NMA for mean BCVA change at six months and 11 RCTs for 12 months. A network of eligible comparisons for the mean BCVA change from baseline to 6 and 12 months is shown in Figure S1. Results of the mean BCVA change at six months from baseline suggested that the anti-VEGF group yielded a better vision improvement compared to the combined (MD = 1.5; 95% CI: 0.084, 2.7) and LP (MD = 6.3; 95% CI: 5.1, 7.6) therapies (Figure 4A). Likewise, the combined therapy yielded better vision improvement compared to the LP therapy (MD = 4.8; 95% CI: 3.7, 6.3). The results of ranking based on simulations suggested that anti-VEGF therapy (97.715%) was the best, followed by combined

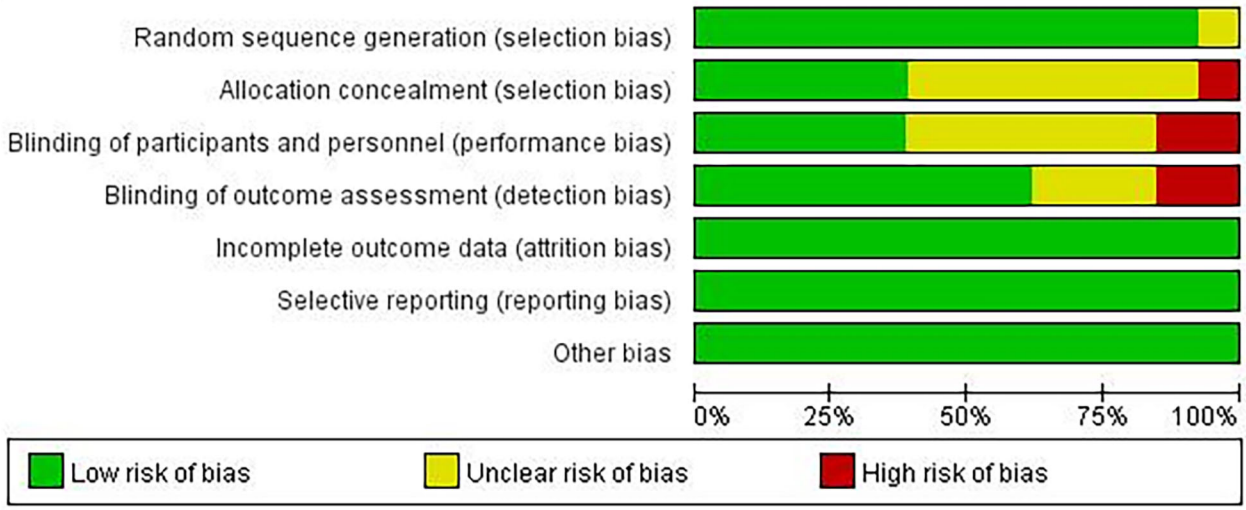

FIGURE 2
Risk of bias graph.

(2.285%) and LP (0.000%) therapies (Figure S7). However, at 12 months, there was no significant difference between the anti-VEGF and combined therapies (MD = 0.1; 95% CI: -1.7, 1.5). The anti-VEGF (MD = 4.7; 95% CI: 3.3, 6.5) and combined (MD = 4.8; 95% CI: 3.3, 6.7) therapies were significantly superior to the LP therapy (Figure 4B). Ranking based on simulations suggested that combined therapy (56.215%) was the best, followed by anti-VEGF (43.785%) and LP (0.000%) therapies (Figure S8). All the comparisons showed no significant heterogeneity (p>0.05). However, the I2 statistic showed high heterogeneity when comparing the mean BCVA change at 12 months between the anti-VEGF and LP therapies (Figures S3, 4). Funnel plots on the mean BCVA changes at 6 and 12 months were presented in Figures S11, S12. Visual inspection showed no significant asymmetry in plots, while Egger's tests also suggested that no potential threat of publication bias on the mean BCVA changes at 6 months (p=0.935) and 12 months (p=0.532).

Mean CMT change

Eight RCTs were included to conduct NMA for the mean BCVA change at six months and 12 RCTs at 12 months. A network of eligible comparisons for the mean CMT change from baseline to 6 and 12 months is shown in Figure S2. The NMA comparing the combined therapy versus anti-VEGF therapy showed no difference in the mean CMT change between the two therapies (MD = -16; 95% CI: -46, -13). Both anti-VEGF (MD = -65; 95% CI: -93, -37) and combined (MD = -81; 95% CI: -0.011, -50) therapies had a better outcome with a significant change in terms of reduced CMT compared to the LP therapy (Figure 5A). Ranking based on simulations suggested that the combined therapy (86.4%) was the best, followed by the anti-VEGF (13.6%) and LP (0.0%) therapies (Figure S9). The NMA of mean CMT change at 12 months showed similar results. There was no difference in the mean CMT change between the anti-VEGF and combined therapies (MD = 21; 95% CI: -3.0, -44). Efficacy of the anti-VEGF (MD = -44; 95% CI: -65, -25) and combined (MD = -65; 95% CI: -90, -41) therapies was better than that of the LP therapy (Figure 5B). Ranking based on simulations suggested that the combined therapy (95.61%) was the best, followed by the anti-VEGF (4.39%) and LP (0.00%) therapies (Figure S10). All the comparisons showed no significant heterogeneity (p>0.05) (Figures S5, 6). Funnel plots on the mean CMT changes at 6 and 12 months were showed in Figures S13, S14. Visual inspection showed little asymmetry in plots, and Egger's tests suggested the absence of substantial publication bias on the mean CMT changes at 6 months (p=0.739) and 12 months (p=0.680).

Discussion

In this NMA, which included 13 studies and a total of 2,422 eyes, we systematically reviewed the published literature and compared the efficacy of three different interventions in patients with DME. It was indicated that compared with the LP therapy, both the anti-VEGF therapy alone and combined anti-VEGF therapy with LP therapy were the most efficacious treatments, with no statistical significance based on the mean CMT change at six and 12 months, as well as the mean BCVA change at 12 months. We found that anti-VEGF therapy alone was better than the combined and LP therapies based on the mean BCVA change at six months. One possible reason is that compared with anti-VEGF therapy alone, the combined therapy may have a stronger anti-angiogenic and anti-inflammatory effect in the early stage after injection, which only affects the decrease in CMT, but has no significant improvement in BCVA (35). Additionally, the adverse effects of LP therapy may provide an explanation for the result that the anti-VEGF therapy alone was better than the combined therapy based on the mean BCVA change at six months. Regarding heterogeneity, we suspect that the high heterogeneity of the mean BCVA change at 12 months was mainly due to the large sample size, but limited therapeutic efficacy of the study by Backer et al. (25).

Although intravitreal injection of anti-VEGF agents has been the standard therapy for DME, LP treatment is still often used (2). LP therapy is associated with severe vision loss (36). With the

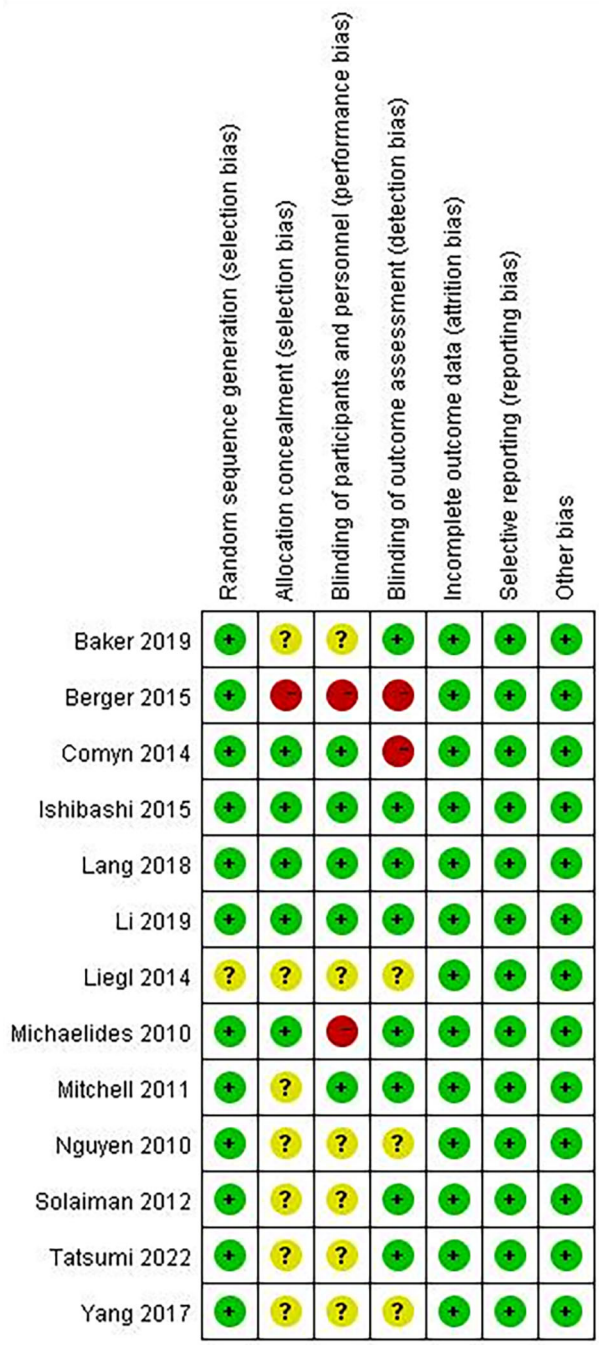

FIGURE 3
Risk of bias summary.

development of novel LP technologies, these adverse effects have reduced (37). This study involved conventional LP (such as grid LP) and novel LP (such as subthreshold LP) therapies. The NMA was based on the assumption that all LP therapies were same and clinicians should pay attention. However, it is also worth mentioning that conventional LP therapy was reported at least as effective as subthreshold LP therapy in the treatment of DME in the previous meta-analysis (38, 39). Moreover, LP therapy has a significant advantage as a long-lasting treatment compared with anti-VEGF therapy, the latter of which is a short-term treatment (40). Patients need to be followed-up for a long time to monitor therapeutic efficacy, and more long-term outcomes are needed to perform analysis and comparison. Owing to repeated injections, anti-VEGF therapy has complications, including intraocular pressure spikes (41) and endophthalmitis (42), to which attention should be paid during treatment. Therefore, anti-VEGF therapy may not be a good treatment option for all patients. A combination of anti-VEGF and LP can reduce the frequency of injections and thus, may solve this problem.

Previous studies have shown that the combined therapy is more effective (39). However, a recent study indicated that anti-VEGF therapy was the most efficacious based on the mean BCVA and CMT

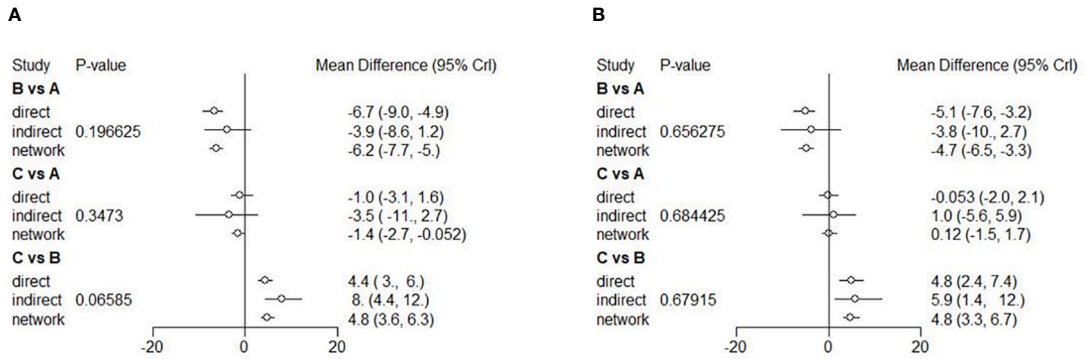

FIGURE 4
Forest plots of NMA showing mean BCVA change from baseline to 6 **(A)**, and 12 **(B)** months. Different treatments are indicated with capital letters A, B and C in the forest plots. Treatments are indicated as A [anti-VEGF therapy], B [LP therapy], and C [the combined therapy], respectively.

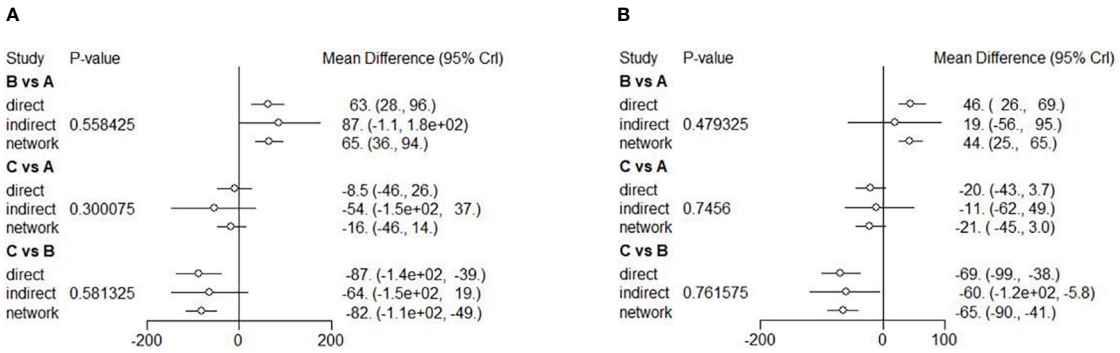

FIGURE 5
Forest plots of NMA showing mean CMT change from baseline to 6 **(A)**, and 12 **(B)** months. Different treatments are indicated with capital letters A, B and C in the forest plots. Treatments are indicated as A [anti-VEGF therapy], B [LP therapy], and C [the combined therapy], respectively.

changes at 12 months, while anti-VEGF and combined therapies had no significant difference in the decrease of CMT at six months (43). Further studies are required to provide more evidence. According to the present study, anti-VEGF therapy alone and combined therapy are both worth considering. The choice of treatments should consider the patient's tolerance, adherence, economic situation, and so on.

These therapies in our network meta-analysis are commonly used for the treatment of patients with DME; therefore, the results of our study will be instructive for clinical treatment. However, this study has several limitations. First, the number of included studies was relatively small, although they were generally high-quality studies. Second, the baseline characteristics of the patients in different studies were not balanced, but they were not included in the NMA models. The intervals between anti-VEGF and LP therapies and the types of LP therapy were also inconsistent. This might have potentially influenced the validity of the results of the mean BCVA and CMT changes. Finally, we did not compare the effects of the different anti-VEGF agents. To evaluate the efficacy of these therapies more accurately, more high-quality RCTs are necessary.

In conclusion, this NMA showed evidence of comparable efficacy in terms of BCVA and CMT between anti-VEGF therapy alone and anti-VEGF combined with LP therapy, with no overall significant difference. Considering the results of the forest plots and ranking based on simulations of treatments and need for long-term treatment, combined therapy is recommended for the treatment of patients with DME.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

WQ designed the study and revised the manuscript. JC and HW collected and analyzed the data. JC and HW drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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The causal effect of obesity on diabetic retinopathy: A two-sample Mendelian randomization study

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Background: The causal effect of obesity on diabetic retinopathy (DR) remains controversial. The aim of this study was to assess the causal association of generalized obesity evaluated by body mass index (BMI) and abdominal obesity evaluated by waist or hip circumference with DR, background DR, and proliferative DR using a two-sample Mendelian randomization (MR) analysis.

Methods: Genetic variants associated with obesity at the genome-wide significance ($P<5\times10^{-8}$) level were derived using GWAS summary statistics from the UK Biobank (UKB) with a sample size of 461 460 individuals for BMI, 462 166 individuals for waist circumference, and 462 117 individuals for hip circumference. We obtained genetic predictors of DR (14 584 cases and 202 082 controls), background DR (2026 cases and 204 208 controls), and proliferative DR (8681 cases and 204 208 controls) from FinnGen. Univariable and multivariable Mendelian randomization analyses were conducted. Inverse variance weighted (IVW) was the main method used to analyze causality, accompanied by several sensitivity MR analyses.

Results: Genetically predicted increased BMI [OR=1.239; 95% CI=(1.134, 1.353); $P=1.94\times10^{-06}$], waist circumference [OR=1.402; 95% CI=(1.242, 1.584); $P=5.12\times10^{-08}$], and hip circumference [OR=1.107; 95% CI=(1.003, 1.221); P=0.042] were associated with increased risk of DR. BMI [OR=1.625; 95% CI=(1.285, 2.057); $P=5.24\times10^{-05}$], waist circumference [OR=2.085; 95% CI=(1.54, 2.823); $P=2.01\times10^{-06}$], and hip circumference [OR=1.394; 95% CI=(1.085, 1.791); P=0.009] were correlated with the risk of background DR. MR analysis also supported a causal association between BMI [OR=1.401; 95% CI=(1.247, 1.575); $P=1.46\times10^{-08}$], waist circumference [OR=1.696; 95% CI=(1.455, 1.977); $P=1.47\times10^{-11}$], and hip circumference [OR=1.221; 95% CI=(1.076, 1.385); P=0.002] and proliferative DR. The association of obesity with DR continued to be significant after adjustment for type 2 diabetes.

Conclusion: This study using two-sample MR analysis indicated that generalized obesity and abdominal obesity might increase the risk of any DR. These results suggested that controlling obesity may be effective in DR development.

KEYWORDS

obesity, diabetic retinopathy, body mass index, Mendelian randomization, waist circumference

1 Introduction

Diabetic retinopathy (DR), which is a microvascular diabetic complication, remains one of the leading preventable causes of visual impairment and blindness worldwide. Almost all type 1 diabetes patients and 60% of type 2 diabetes patients develop retinopathy within 20 years (1). It is estimated that the number of DR cases will reach 191 million, and without timely intervention and treatment, 56.6 million patients will develop vision-threatening DR by 2030 (2). Moreover, even with strict glucose regulation, some patients with type 2 diabetes still develop DR after 6.5–13.3 years (3). Therefore, studies to identify other modifiable risk factors for DR are essential to guide clinical practice to prevent DR occurrence and progression (4).

Obesity (defined as a body mass index (BMI) ≥30 kg/m^2) is an emerging public health problem and a widely accepted risk factor for many diseases, such as type 2 diabetes, cardiovascular diseases (CVD), and cancer (5–7). Various studies have reported the effects of obesity on the risk of DR (8), but the causal association between obesity and DR remains controversial. According to the World Health Organization (WHO) classification, there are two types of obesity: general obesity assessed by BMI and abdominal obesity assessed by waist circumference, hip circumference, or waist-to-hip ratio (WHR) (7). Western studies have reported a significant association between higher BMI and any stage of DR (9, 10). In contrast, some studies conducted in Asian populations demonstrated no significant BMI-DR associations (11) and even inverse BMI-DR associations (12, 13). Therefore, there still seems to be an "obesity paradox" between obesity and DR (14). The term "obesity paradox" was originally used to describe the finding that being overweight or even obese is "protective" of or has no impact on CVD and mortality (15). Similarly, equivocal results have been obtained for the association between abdominal obesity and DR. WHR was reported to be positively associated with any stage of DR (13, 16) or to have no significant association (17). In a recent longitudinal cohort study, WHR was also connected with an increased risk of incident DR in a 2-year follow-up (18). Therefore, whether obesity causes protective or detrimental effects on DR needs to be further clarified. Furthermore, it is of critical importance to determine whether obesity is an independent risk factor for DR, as it is potentially modifiable.

Compared to traditional retrospective studies, Mendelian randomization (MR) studies are less affected by confounding factors, and the causal sequence is more reasonable (19). This approach treats genetic variations as a "natural" randomized controlled trial in which individuals are randomly assigned to different exposure levels over their lifetime, which has achieved great success in finding risk factors for many diseases (20). However, to our knowledge, no MR study has been used to evaluate the effects of obesity on the risk of DR. The present study used a two-sample MR approach to explore the causal relationship between obesity and DR, which may provide guidance on the prevention and treatment of DR.

2 Methods

2.1 Study design and instrumental variable extraction

We reported the MR study in adherence to the Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) (21). The MR study was analyzed using recent genome-wide association study (GWAS) summary statistics, and ethical approval was obtained from the respective institutions. A two-sample MR analysis was used to explore the causal relationships between obesity and DR. Type 2 diabetes is the most important risk factor for DR. Meanwhile, the genetic overlap between obesity and diabetes is widespread (22), and we used multivariable MR (MVMR) to mitigate potential pleiotropic effects *via* diabetes.

To evaluate the causal relationship between obesity (BMI, waist circumference, and hip circumference) and DR, single-nucleotide variations (SNVs) were selected according to the following criteria (Figure 1): (1) SNVs were closely associated with exposure and reached genome-wide significance ($p < 5 \times 10^{-8}$); (2) SNVs were not

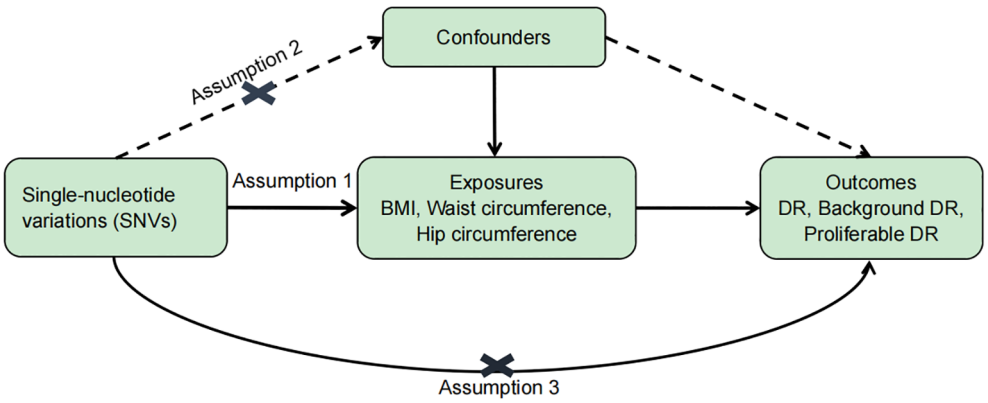

FIGURE 1
Basic assumptions of Mendelian randomization. Assumption 1: SNVs were closely associated with exposure. Assumption 2: SNVs were not associated with any potential confounders. Assumption 3: SNVs are only linked to the outcome through exposure.

associated with any potential confounders and were independent of each other to avoid biases caused by linkage disequilibrium (r^2 < 0.0001, clumping distance = 10,000 kb); and (3) SNVs are only linked to the outcome through exposure. An *F* statistic (*F* = beta^2/se^2; beta for the SNV-exposure association (beta); variance (se)) was calculated for each SNV (23). Since an empirical threshold of more than 10 indicates that the SNV has sufficient validity, SNVs with an *F* statistic of less than 10 were removed. MR–Steiger filtering was used to remove variations that were more strongly correlated with DR than with obesity (24). Information on the *F* statistic, SNVs, and MR–Steiger is provided in Supplementary Datasets 1–9.

2.2 Data sources

In the present work, we chose obesity-associated indices (BMI, waist, and hip circumference) from the UK Biobank (UKB) as exposures. UKB was a UK-based cohort study that recruited about 500,000 participants aged 40–69 years between 2006 and 2010, from whom a series of medical and physical information was collected (25). BMI is the ratio of weight in kilograms divided by the squared height in meters. The natural indent was measured for the waist circumference. The widest part of the hip was recorded for the hip circumference. To reduce confounding by race, we only used summary statistics from individuals of European descent with a sample size of 461,460 individuals for BMI, 462,166 individuals for waist circumference, and 462,117 individuals for hip circumference, and it is available for download (https://gwas.mrcieu.ac.uk/). Different stages of DR (DR, background DR, and proliferative DR) were chosen as outcomes. The GWAS summary statistics of DR were extracted from the FinnGen (https://r5.finngen.fi/). Participants in the DR (GWAS ID: finn-b-DM_RETINOPATHY) analysis included 14,584 cases and 202,082 controls; participants in the background DR (GWAS ID: finn-b-DM_BCKGRND_RETINA) analysis included 2,026 cases and 204,208 controls; and participants in the proliferative DR (GWAS ID: finn-b-DM_RETINA_PROLIF) analysis included 8,681 cases and 204,208 controls. Cases of different stages of DR were identified based on the International Classification of Diseases-Revision 9/10 criteria from the hospital discharge registry (https://r5.risteys.finngen.fi/). We obtained genetic predictors of type 2 diabetes from Mahajan et al. (26).

2.3 Statistical analyses

All statistical and MR analyses were performed using R software (version 4.1.1) using the R packages "TwoSampleMR" and "MR-PRESSO". $p < 0.05$ was considered to be statistically significant as evidence for a potential causal association.

The inverse variance-weighted (IVW) method was used as the primary method for calculating the causal effect. Given that the validity of the MR method is strictly dependent on the absence of pleiotropy, we used a series of MR analytical approaches to account for pleiotropy. First, we used MR–Egger (27) and weighted-median (WM) (28) methods as supplements. The better method between IVW and MR–Egger was selected *via* Ruecker's framework. $p < 0.05$ of Cochran's *Q* and Rucker's *Q* (*Q*–*Q*) indicates the MR–Egger analysis with the least heterogeneity (29), which is reported in Supplementary Table S1. Second, we determined the heterogeneity of different genetic variants using Cochrane's *Q* test and I^2. $p < 0.05$ of Cochrane's *Q* (30) and $I^2 > 25\%$ (31) were considered to indicate significant heterogeneity. Next, the pleiotropic effect of the genetic variants was assessed using the MR–Egger intercepts (32) and MR-PRESSO global test (33). In addition to these methods, MR–Steiger filtering was used to remove variations that were more strongly correlated with DR than with obesity. Finally, the SNV leave-one-out method was used to further verify the robustness of the data (Supplementary Figures S1–S9).

3 Results

Our results indicated that 305, 252, and 275 SNVs in DR were associated with BMI and waist and hip circumference, respectively. A total of 306, 252, and 275 SNVs in background DR were associated with BMI, waist circumference, and hip circumference, while 305, 252, and 274 SNVs in proliferative DR were associated with BMI, waist circumference, and hip circumference, respectively (Table 1). The *F* statistic of each SNV was greater than the empirical threshold of 10, and the minimum *F* statistics in subgroups are shown in Table 1. The explained variances ranged from 2.51% to 4.21% for different stages of DR. The main results of the MR analysis are presented in Figures 2–4, and more details are provided in Supplementary Table S2.

3.1 Causal effect of obesity on DR

First, we explored the causal relationship between obesity and DR, as shown in Figure 2. Genetically predicted higher BMI [OR = 1.239; 95% CI = (1.134, 1.353); $p = 1.94 \times 10^{-06}$] and waist circumference [OR = 1.402; 95% CI = (1.242, 1.584); $p = 5.12 \times 10^{-08}$] by the IVW method were significantly associated with a higher risk of DR, consistent with results obtained by WM. Nonsignificant pleiotropy in BMI was detected by Cochrane's *Q* test ($p = 0.525$), $I^2 = 0$, MR–Egger intercept ($p = 0.708$), or MR-PRESSO global test ($p = 0.535$). Slight heterogeneity was present in waist circumference ($Q = 292.95$; $p = 0.036$), but no significant outlier ($p < 0.05$) was identified by MR-PRESSO. Significant heterogeneity in hip circumference was detected by Cochrane's *Q* test ($p = 2.62 \times 10^{-04}$), and a significant outlier (SNV: rs7903146) was detected by MR-PRESSO. Higher hip circumference was also suggestively associated with the risk of DR using the IVW method [OR = 1.107; 95% CI = (1.003, 1.221); $p = 0.042$] after deleting the outlier. Moreover, our MVMR analysis suggested that the causal association between obesity and DR existed apart from diabetes. Using the MR–Steiger test, none of the variants were removed, and the results remained unchanged. Finally, the leave-one-out analysis found that no single SNV strongly drove the overall effect of obesity on DR.

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TABLE 1 Mendelian randomization results of obesity traits on DR.

Exposures	Outcomes	NSNVs	*F* statistic	R^2 (%)	I^2 (%)	Cochrane's *Q*		MR–Egger test		MR-PRESSO
						Q	*p*-value	Intercept	*p*-value	*p*-value
BMI	DR	305	29.88	4.2	0	301.82	5.25E−01	8.16E−04	0.708	5.35E−01
Waist circumference		252	29.76	2.51	14.23	292.95	3.60E−02	−1.12E−03	0.679	4.20E−02
Hip circumference		276	29.85	3.99	24.4	363.76	2.62E−04	−1.84E−03	0.489	1.67E−04
Hip circumference[a]		275	29.85	3.98	16.52	328.21	1.40E−02	−1.87E−03	0.458	7.98E−01
BMI	Background DR	306	29.88	4.21	8.84	334.57	1.18E−01	4.71E−03	0.429	1.25E−01
Waist circumference		252	29.76	2.51	10.01	278.93	1.09E−01	−4.66E−03	0.489	1.09E−01
Hip circumference		276	29.85	3.99	21.01	348.17	2.00E−03	−2.54E−03	0.702	2.00E−03
Hip circumference[b]		275	29.85	3.98	16.69	328.89	1.30E−02	−2.45E−03	0.704	8.40E−01
BMI	Proliferative DR	305	29.88	4.2	6.99	326.84	1.76E−01	2.35E−03	0.417	1.71E−01
Waist circumference		252	29.76	2.51	12.06	285.42	6.70E−02	−3.82E−04	0.911	6.90E−02
Hip circumference		276	29.85	3.99	27.5	379.33	3.00E−05	−1.87E−03	0.589	1.67E−04
Hip circumference[c]		274	29.85	3.96	17.85	332.31	8.00E−03	−1.88E−03	0.564	6.92E−01

DR, diabetic retinopathy; NSNVs, number of single-nucleotide variations; BMI, body mass index; R^2, phenotype variance explained by genetics. [a]One significant outlier (SNV:rs7903146) was deleted. [b]One significant outlier (SNV:rs35506085) was deleted. [c]Two significant outliers (SNV:rs35506085; SNV:rs7903146) were deleted.

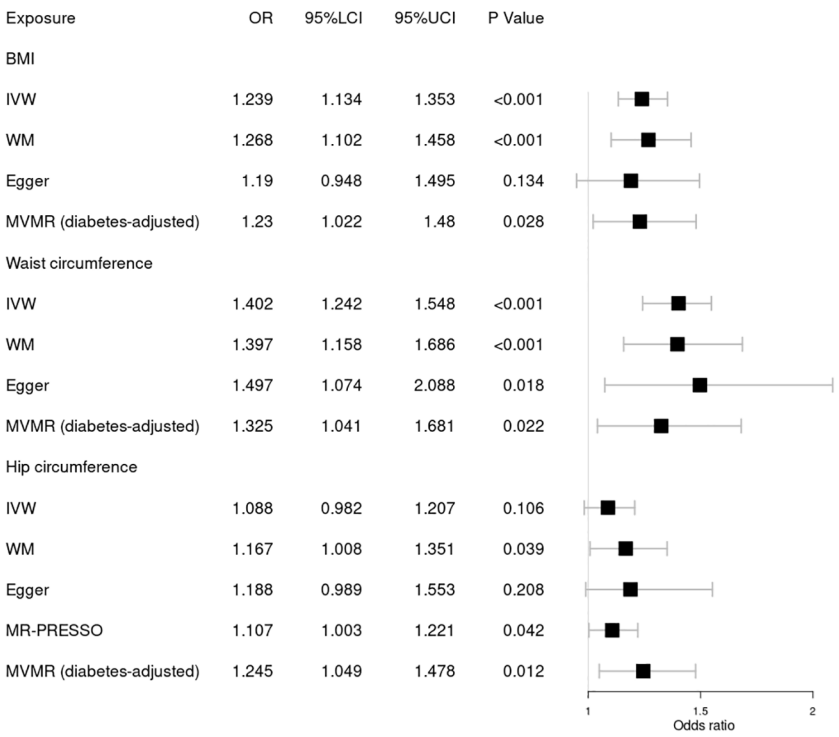

FIGURE 2
Forest plot of Mendelian randomization results of obesity effect on DR. BMI, body mass index; IVW, inverse variance weighted; Egger, MR–Egger; WM, weighted-median; MR-PRESSO, the outlier-corrected MR pleiotropy residual sum and outlier results (one significant outlier; SNV:rs7903146); MVMR, multivariable Mendelian randomization; 95% LCI, lower limit of 95% CI; 95% UCI, upper limit of 95% CI.

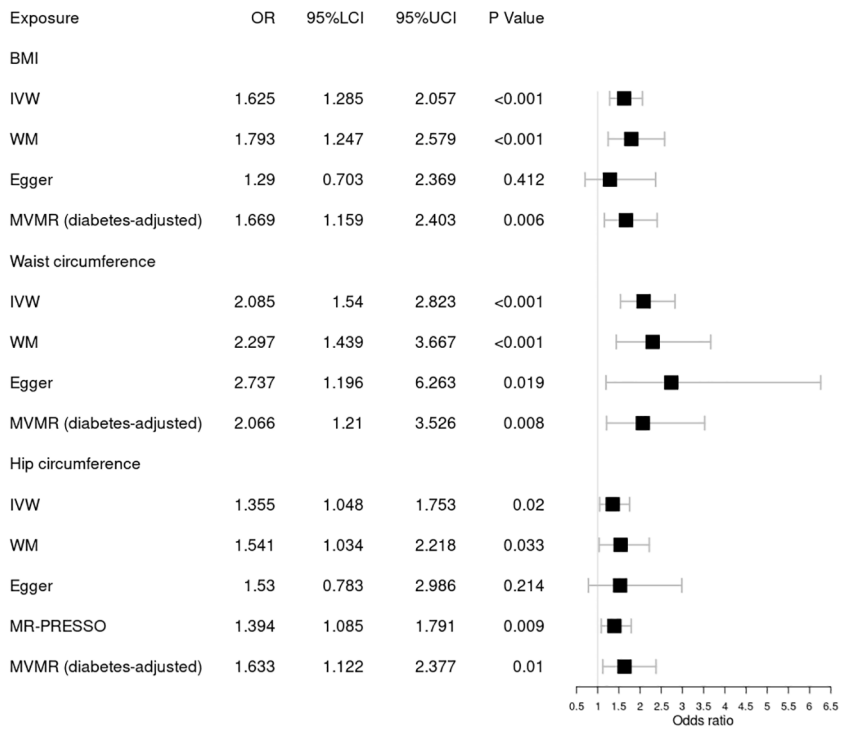

FIGURE 3
Forest plot of Mendelian randomization results of obesity effect on background DR. BMI, body mass index; IVW, inverse variance weighted; Egger, MR–Egger; WM, weighted-median; MR-PRESSO, the outlier-corrected MR pleiotropy residual sum and outlier results (one significant outlier, SNV: rs35506085); MVMR, multivariable Mendelian randomization; 95% LCI, lower limit of 95% CI; 95% UCI, upper limit of 95% CI.

3.2 Causal effect of obesity on background DR

Next, we assessed the causal relationship between obesity and background DR, as shown in Figure 3. IVW analysis indicated that genetically predicted increased BMI [OR = 1.625; 95% CI = (1.285, 2.057); $p = 5.24 \times 10^{-05}$], waist circumference [OR = 2.085; 95% CI = (1.54, 2.823); $p = 2.00 \times 10^{-06}$], and hip circumference [OR = 1.394; 95% CI = (1.085, 1.791); $p = 0.009$] were associated with a higher risk of background DR. The WM method showed similar results. Pleiotropy in BMI and waist circumference identified by Cochrane's Q test, I^2, MR–Egger intercept, and MR-PRESSO did not reach statistical significance (all $p > 0.05$ or $I^2 < 25\%$). However, there was significant pleiotropy (MR-PRESSO, $p = 0.002$) in hip circumference, and MR-PRESSO detected one significant outlier (SNV:rs35506085). The IVW and WM methods still suggested a causal association between the hip circumference and background DR after deleting the outlier. The associations for obesity and background DR remained significant after adjustment for type 2 diabetes through MVMR. No one SNV was excluded by MR–Steiger. Additionally, the leave-one-out test showed that the MR results were not significantly affected by a single SNV.

3.3 Causal effect of obesity on proliferative DR

Finally, we further investigated the relationship between obesity and the risk of proliferative DR using MR analysis, as shown in Figure 4. Higher BMI [OR = 1.401; 95% CI = (1.247, 1.575); $p = 1.46 \times 10^{-08}$], waist circumference [OR = 1.696; 95% CI = (1.455, 1.977); $p = 1.47 \times 10^{-11}$], and hip circumference [OR = 1.221; 95% CI = (1.076, 1.385); $p = 0.002$] were suggestively associated with the increasing risk of proliferative DR using the IVW method. These results were supported by those of the WM method. No significant pleiotropy in BMI or waist circumference was detected by several sensitivity MR analyses. However, significant pleiotropy in hip circumference was found by Cochrane's Q test ($p = 3.00 \times 10^{-05}$) or $I^2 = 27.5\%$. MR-PRESSO found two significant outliers (SNV: rs35506085; SNV:rs7903146). After deleting the two outliers, the causal relationship still persisted. Meanwhile, MVMR analysis indicated a causal relationship between obesity and proliferative DR aside from diabetes. Finally, MR–Steiger and the SNV leave-one-out method were used to further validate the data robustness, and no SNV was excluded.

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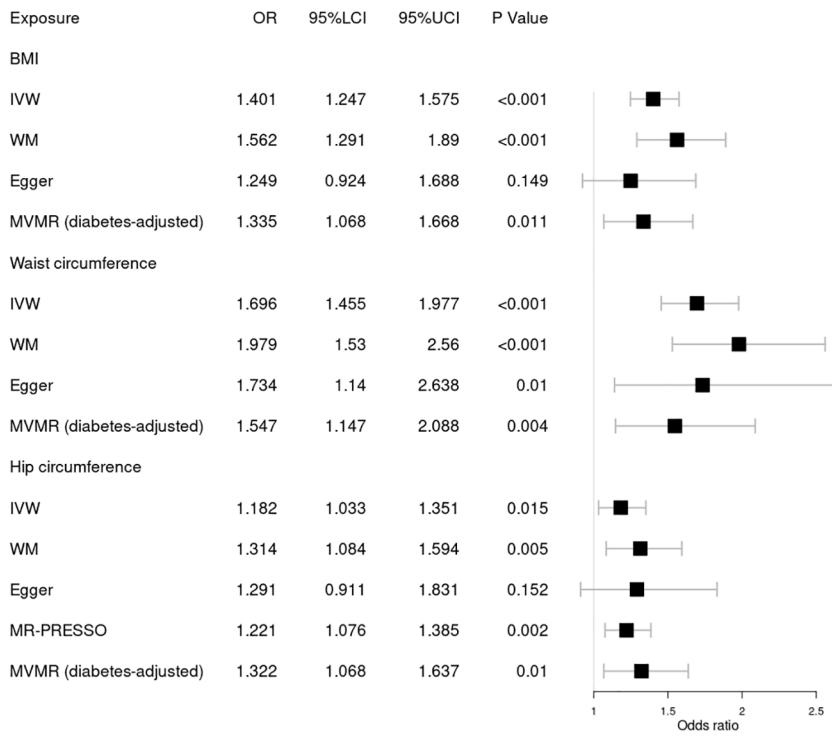

FIGURE 4
Forest plot of Mendelian randomization results of obesity effect on proliferative DR. BMI, body mass index; IVW, inverse variance weighted; Egger, MR–Egger; WM, weighted-median; MR-PRESSO, the outlier-corrected MR pleiotropy residual sum and outlier results (two significant outliers, SNV: rs35506085; SNV:rs7903146); MVMR, multivariable Mendelian randomization; 95% LCI, lower limit of 95% CI; 95% UCI, upper limit of 95% CI.

4 Discussion

The present study investigated the causal association between generalized obesity (evaluated by BMI) and abdominal obesity (evaluated by waist and hip circumference) with different stages of DR using MR analysis. This study corroborated the conclusion that both generalized obesity and abdominal obesity are risk factors for DR (including background and proliferative DR). After adjusting for diabetes by MVMR, the causal relationship between obesity and DR still exists, suggesting that obesity may be an independent risk factor for DR.

Obesity and diabetes are recognized as major public health problems worldwide. A series of scientific studies have indicated that obesity is involved not only in the pathogenesis of diabetes but also in the development of its complications (34). However, inconsistent conclusions on the association between generalized obesity or abdominal obesity and DR were reported in previous studies. In particular, some studies have shown an inverse BMI-DR association. In fact, BMI may be more susceptible to the impact of disease (35) than other obesity-associated indices. The "obesity paradox" is widely discussed in the association between obesity and CVD. Stamatina et al. strongly reaffirmed that being overweight heightens the risk of CVD and pointed out that the "obesity paradox" is mainly due to the effect of confounding on BMI (5). Therefore, the inverse BMI-DR association may be due to confounding BMI in these traditional studies.

A recent meta-analysis pooled only prospective cohort studies, providing a high level of evidence that a higher BMI significantly increases the risk of DR incidence (36). Our MR results supplied genetic evidence to support the notion that a higher BMI is a potential risk factor for any DR in individuals of European descent. To date, a few mechanisms may account for the deleterious effect of BMI on DR. First, an increase in BMI increases linearly with the risk of type 2 diabetes (37), which plays a key role in the pathogenesis of DR. Moreover, an elevated BMI is often correlated with hypertension and dyslipidemia, both of which are risk factors for DR (38). Second, high BMI exaggerates hyperglycemia-induced epigenetic modifications, leading to mitochondrial damage (39) and the development of DR (40). Additionally, ethnic differences should not be ignored when interpreting the relationship between BMI and DR (41).

BMI has limited value in accounting for fat distribution, as abdominal fat (i.e., waist circumference) is more strongly correlated with visceral fat than BMI (42). Abdominal obesity may be a more critical factor of DR and was positively associated with all stages of DR (13), which was supported by our MR results. Increasing waist circumference was causally associated with a higher risk of any DR. The consistent results of the MR analyses (IVW, WM, and MR–Egger) indicated that the conclusion was robust and reliable. A recent longitudinal cohort study also supported this relationship and indicated that abdominal obesity increased the risk of 2-year incident DR (18). The pathophysiological mechanisms underlying the detrimental effect of abdominal obesity on DR are unclear. In fact, abdominal obesity may be mediated through the impact of

visceral fat on adverse metabolic profiles, including insulin resistance and inflammation (43), which have been implicated in the pathogenesis of DR (44). Moreover, excess abdominal fat may disrupt the secretion of growth hormone (43), implicating pathological neovascularization in DR.

To the best of our knowledge, this is the first study that has applied MR analysis to investigate the potential causal association between obesity and the risk of DR. The first advantage of the study is the MR design, which mitigates bias from reverse causation and confounding. The second advantage is that our MR study strictly utilized European subjects, thus minimizing bias due to population stratification. This study also has several limitations. The greatest concern in MR studies is horizontal pleiotropy, which occurs when genetic variants influence the outcome of more than one pathway. We designed a series of MR analytical approaches to minimize this bias. However, it is not possible to completely rule out residual pleiotropy. Moreover, MR analysis only made the assumption of a linear relationship (20) between obesity and DR; thus, additional studies are needed to determine the underlying mechanism.

5 Conclusion

In conclusion, these findings demonstrated a causal relationship between obesity and DR. Our MR analysis showed that obesity may be an independent risk factor for different stages of DR, which suggested that controlling obesity may be effective in DR development.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

CZ and XW performed the main data analysis and wrote the draft of the manuscript. XC supervised the whole research and is responsible for the integrity of the data analysis. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1108731/full#supplementary-material

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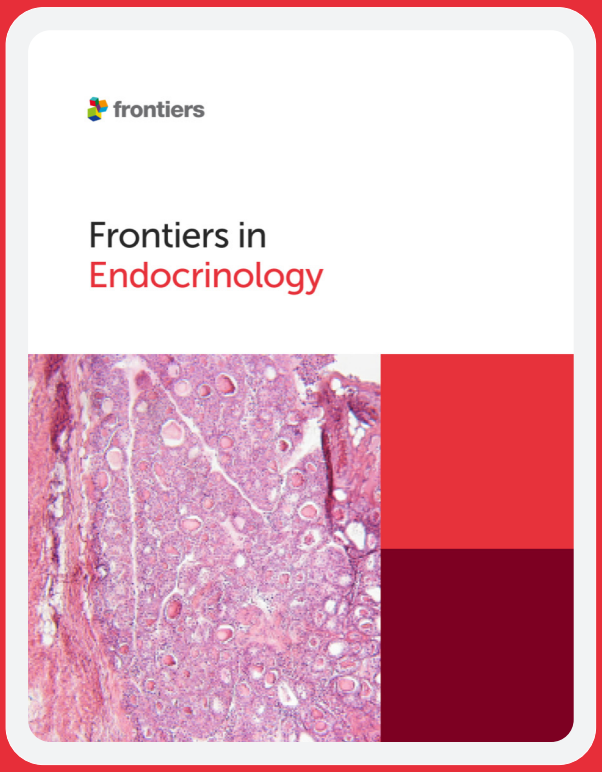

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