# Changes and related factors of blood CCN1 levels in diabetic patients

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**Objective:** To study the differences in blood cellular communication network factor 1 (CCN1) levels between patients with diabetes mellitus (DM) and healthy individuals and to explore the relationship between CCN1 and diabetic retinopathy (DR).

**Methods:** Plasma CCN1 levels were detected using ELISA in 50 healthy controls, 74 patients with diabetes without diabetic retinopathy (DM group), and 69 patients with diabetic retinopathy (DR group). Correlations between CCN1 levels and age, body mass index, mean arterial pressure, hemoglobin A1c, and other factors were analyzed. The relationship between CCN1 expression and DR was explored using logistic regression after adjusting for confounding factors. Blood mRNA sequencing analysis was performed for all subjects, and the molecular changes that may be related to CCN1 were explored. The retinal vasculature of streptozotocin-induced diabetic rats was examined using fundus fluorescein angiography; in addition, retinal protein expression was examined using western blotting.

**Results:** Plasma CCN1 levels in patients with DR were significantly higher than in the control and DM groups; however, no significant differences were observed between healthy controls and patients with DM. CCN1 levels negatively correlated with body mass index and positively correlated with the duration of diabetes and urea levels. It was observed that high (OR 4.72, 95% CI: 1.10−20.25) and very high (OR 8.54, 95% CI: 2.00−36.51) levels of CCN1 were risk factors for DR. Blood mRNA sequencing analysis revealed that CCN1-related pathways were significantly altered in the DR group. The expression of hypoxia-, oxidative stress-, and dephosphorylation-related proteins were elevated, while that of tight junction proteins were reduced in the retinas of diabetic rats.

**Conclusion:** Blood CCN1 levels are significantly elevated in patients with DR. High and very high levels of plasma CCN1 are risk factors for DR. Blood CCN1 level may be a potential biomarker for diagnosis of DR. The effects of CCN1 on DR may be related to hypoxia, oxidative stress, and dephosphorylation.

KEYWORDS

diabetic Retinopathy, CCN1/Cyr61, diabetic complications, blood-retinal barrier, blood biomarker

# 1 Introduction

Diabetes is a metabolic disease characterized by hyperglycemia. Long-standing hyperglycemia chronically damages various organs, particularly the eyes, kidneys, heart, blood vessels, and nerves, leading to serious complications. Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes and one of the leading causes of incident blindness in working-age adults (1).

Cellular communication network factor 1 (CCN1) is a dynamically expressed extracellular matrix protein (2). CCN1, which is primarily secreted by endothelial cells, binds to the extracellular matrix and cell surface and controls the signal transduction between them (3). CCN1 is rapidly upregulated in response to various stimuli (e.g., mechanical/shear stress, hypoxia, and inflammation) and plays a key role in wound repair, cell growth, differentiation, adhesion, and angiogenesis (4). CCN1 is an essential regulator of vascular development and an important component of pathological neovascularization.

CCN1 is closely associated with diabetic microangiopathy (5–8), particularly diabetic retinopathy (5). You et al. reported the occurrence of higher levels of CCN1 in the vitreous humor of patients with proliferative diabetic retinopathy (PDR) compared to patients without diabetes (5). This observation has been consistently confirmed by Zhang et al. (9), Choi et al. (10), and Zhou et al. (11). Additionally, it was observed that the vascular activity in patients with PDR significantly affected the vitreous levels of CCN1 (11, 12). Zhou et al. observed that CCN1 levels were significantly higher in patients with active PDR than in patients with quiescent PDR (11); additionally, Zhang et al. found that vitreous CCN1 levels were higher in patients with PDR who had extensive neovascularization in the retina than in those with limited neovascularization (9). However, CCN1 levels were higher in the PDR group than in the control group, regardless of the PDR status (11, 12). Furthermore, CCN1 was upregulated in the neovascularized membranes of patients with PDR (12, 13) and was mostly expressed in endothelial cells and myofibroblasts (12). Additionally, the anti-vascular endothelial growth factor (VEGF) vitreous injection was able to reduce VEGF levels in the vitreous; however, it was unable to reduce CCN1 levels (12).

Animal and in vitro experiments have demonstrated the key role of CCN1 in DR (3–5, 9, 10, 13, 14). In animal models with streptozotocin (STZ)-induced diabetes, retinal CCN1 protein levels were significantly elevated (4, 9, 13, 15); additionally, Zhang et al. found that CCN1 was substantially concentrated in the ganglion cell and inner plexiform layers (9). Vitreous injections of exogenous advanced glycation end products and VEGF increased retinal CCN1 levels (4, 14). Depletion or knockdown of CCN1 significantly inhibited retinal neovascularization in mouse models with oxygen-induced retinopathy (3, 5, 10, 16, 17). Lee et al. reported that CCN1 produced by pericytes was capable of promoting choroidal neovascularization through Wnt5a (3). Di et al. found that the neoangiogenic effect of CCN1 was mediated by the PI3K/Akt-VEGF pathway (16, 17).

DR is a common microvascular complication of diabetes that can lead to vision loss and blindness; thus, the early detection and treatment of DR are crucial. The study of DR biomarkers is conducive to the early detection of DR and the exploration of its mechanism, as blood samples are easier to obtain than vitreous humor samples. Although CCN1 is closely associated with DR, there are few studies on blood CCN1 levels in patients with diabetes. Studies on the comparison of the blood CCN1 levels in patients with diabetes and healthy individuals and the relation between blood CCN1 levels and DR have not been reported. Additionally, the study of blood CCN1 levels, as a DR biomarker, could be a potential diagnostic tool. In this study, we analyzed plasma CCN1 levels and mRNA sequencing results in 50 healthy controls and 143 patients with type 2 diabetes (74 without DR and 69 with DR) to explore the predictive potential of plasma CCN1 in the diabetic population and the plausible mechanism through which systemic CCN1 affects DR.

# 2 Materials and methods

## 2.1 Subjects and examination

The participants included in this study were recruited from the Shanghai Cohort Study of Diabetic Eye Disease (SCODE; clinicaltrials.gov identifier: NCT03665090). This study adhered to the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of Shanghai First People's Hospital (2013KY023). In 2003, SCODE established a health record for diabetic residents and began conducting annual assessments (18). In 2008, SCODE built a community remote DR screening system that combined digital fundus photography with Internet technology, which improved the efficiency of DR diagnosis (19). This study selected cross-sectional data from the SCODE study conducted between 2017–2018.

The examination methods for the SCODE study have been previously described (20). The inclusion criteria for this study were as follows: (1) diabetes mellitus (DM) diagnosed in the endocrinology department and (2) intraocular pressure in both eyes in the range of 10–21 mmHg. The exclusion criteria were as follows: (1) history of eye diseases other than DR, such as glaucoma, optic neuropathy, and hereditary eye diseases, among others; (2) history of eye surgery; (3) severe systemic diseases; (4) inability to cooperate with the examination; and (5) fundus images of poor quality. The diagnoses of type 1 and type 2 diabetes in this study were based on the diagnostic criteria for diabetes proposed by the WHO in 1999 (21). The diagnosis of DR was based on the International Clinical Classification of DR proposed at the 2002 International Ophthalmology Conference (22).

The researchers recorded basic information such as the date of birth, sex, height, weight, diabetes type, diagnosis time, and medical history of the research subjects. Venous blood samples were drawn from each subject, and tested for the levels of blood glucose, hemoglobin A1c (HbA1c), total blood cholesterol, and blood urea, among others. The best-corrected visual acuity was measured using an international standard visual acuity chart. The eyelids, conjunctiva, cornea, anterior chamber, iris, pupil, and lens were examined using slit-lamp biomicroscopy (SL130, Zeiss, Germany). Body Mass Index and Mean Arterial Pressure were

calculated using the following equations:

$$Body\ Mass\ Index\ (BMI,\ kg/m^2) = weight\ (kg) \div height^2\ (m^2)$$

$$Mean\ Arterial\ Pressure\ (MAP,\ mmHg)$$
$$= systolic\ blood\ pressure\ (mmHg) \times \frac{1}{3}$$
$$+ diastolic\ blood\ pressure\ (mmHg) \times \frac{2}{3}$$

## 2.2 Plasma CCN1

CCN1 concentrations in human plasma samples were determined using a commercially available ELISA kit (DCYR10, R&D Systems, USA) according to the manufacturer's instructions (mean minimum detectable dose: 1.54 pg/mL, intra-assay precision: 2.3%, inter-assay precision: 6.4%).

## 2.3 mRNA sequencing

Total white blood cell RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany). Paired-end libraries were synthesized using the TruSeq™ RNA Sample Preparation Kit (Illumina, USA) following the TruSeq™ RNA Sample Preparation Guide and sequenced on an Illumina NovaSeq 6000 (Illumina, USA). Library construction and sequencing were performed by Sinotech Genomics Co. Ltd. (Shanghai, China).

Paired-end sequence files (FASTQ) were mapped to the reference genome (GRCh38.100) using Hisat2 (Hierarchical Indexing for Spliced Alignment of Transcripts, version 2.0.5). The output SAM (sequencing alignment/map) files were converted to BAM (binary alignment/map) files and sorted using SAMtools (version 1.3.1).

Gene abundance was expressed as fragments per kilobase of exons per million reads mapped (FPKM). StringTie software was used to count the fragments within each gene and the TMM algorithm was used for normalization.

Differential mRNA expression was analyzed using the edgeR package. Differentially expressed RNAs with |log1.5(FC)| values > 1 and q-value< 0.05, were considered significantly different and retained for further analysis. In this study, we specifically analyzed genes that differed in both DR vs. DM and DR vs. control comparisons that changed in the same direction.

## 2.4 Animals

Animals were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. A single intraperitoneal injection (40 mg/kg) of STZ was used to induce type 1 diabetes in adult Brown Norway rats weighing >200 g. Rats were fasted for 12 h prior to intraperitoneal injection. Rats of similar age and weight (n = 6) were used as the control group and were injected with equal amounts of sodium citrate buffer (0.1 mol/L). Body weight and blood glucose levels were measured before the injection and at 3, 4, and 8 weeks after the injection. Rats were considered diabetic if their blood glucose levels were >300 mg/dL three days after STZ injection (n = 6). Nine weeks after the injection, the control and STZ rats were sacrificed, and retinal tissue samples were collected from each eye (Supplementary Figure 1). Tissue samples were placed in liquid nitrogen and subsequently stored at −80°C for further experiments.

## 2.5 Fundus fluorescein angiography

The rats in each group were subjected to fluorescein fundus angiography (FFA) using Micron IV (Phoenix Research Labs, USA) 8 weeks after the induction of DM. The rats were intraperitoneally injected with sodium pentobarbital (40 mg/kg) for anesthesia, pupil dilation was performed with one drop of tropicamide (Santen Pharmaceutical Corporation, Japan), and the corneal surface was coated with methyl cellulose to keep it moist. During the FFA examination, the rats were intraperitoneally injected with 10% sodium fluorescein (0.001 mL/g, Fluorescite, Alcon, USA) for a quick examination.

## 2.6 Western blot analysis

Proteins were extracted from tissue homogenates. For Western blot analysis, protein samples were fractionated in 4–20% SurePAGE™ Precast Gels (M00657, GenScript Biotech Corporation, China) and transferred to nitrocellulose membranes. The analysis was performed with anti-CCN1 (ab228592, Abcam, UK), anti-CDC42 (2466T, Cell Signaling Technology, USA), anti-Claudin5 (4C3C2, Invitrogen, USA), anti-COX6c (ab150422, Abcam, UK), anti-CREB1 (R23983, ZEN- BIOSCIENCE, China), anti-HIF1α (ab179483, Abcam, UK), anti-NDUFα1 (15561-1-AP, Proteintech Group, USA), anti- SEPN1 (55333-1-AP, Proteintech Group, USA), anti-SHP1 (ab32559, Abcam, UK, anti-VEGFa (NB100-664, Novus Biologicals, USA), and anti-β-tubulin (2146S, Cell Signaling Technology, USA) antibodies. Immunoassays were performed using enhanced chemiluminescence (17046; ZEN-BIOSCIENCE, China), in accordance with the manufacturer's instructions. Protein levels were determined by densitometric scanning of protein bands. Six retinas were used in each group, and the results for each retina were averaged from three replicates.

## 2.7 Statistical analysis

The Kolmogorov–Smirnov U test was used to test the normality of all variables. Normally distributed descriptive data were

represented as the mean ± standard deviation, and non-normally distributed descriptive data were represented as the median (quartile 1, quartile 3). One-way analysis of variance was used to compare the between-group differences in normally distributed data. The Kruskal-Wallis test was used to compare the between-group differences for non-normally distributed data. The Mann-Whitney U test was used to compare differences in non-normally distributed data between the two groups. Spearman's correlation test was used to analyze the correlation between parameters when the data were normally distributed. A binary logistic regression model was established with DR as the dependent variable (DR = 1, without DR = 0) to evaluate the relationship between the variables and CCN1. CCN1 was stratified according to its quartiles (low CCN1< 127.72 pg/mL, 127.72 pg/mL ≤ moderate CCN1< 173.85 pg/mL, 173.85 pg/mL ≤ high CCN1< 250.32 pg/mL, very high CCN1 ≥ 250.32 pg/mL). Age, sex (male = 1, female = 0), BMI, duration of diabetes, urea, MAP, fasting glucose, HbA1c, low-density lipoprotein, and total cholesterol levels were included in the multivariate model for confounding factor correction. The Bonferroni correction was performed for multiple uncorrected comparisons. Differences were considered statistically significant at P< 0.05. Statistical analysis was performed using SPSS 22 software (International Business Machines Corporation, America).

# 3 Results

## 3.1 Characteristics of the participants

A total of 193 participants (95 female, 98 male) were included in the analysis. No significant differences (all p > 0.05) in age, BMI, MAP, low-density lipoprotein, and total cholesterol levels were observed between the groups (control, DM, and DR). However, levels of fasting glucose (H = 66.62, p< 0.001), HbA1c (H = 64.47, p< 0.001), high-density lipoprotein (F = 3.28, p = 0.04), and urea (F = 9.73, p = 0.008) were significantly different in the three groups. The levels of fasting glucose (H = 24.39, p = 0.027) and HbA1c (H = 31.48, p = 0.001) of the DR group were significantly higher than those of the DM group, whereas the levels of fasting glucose (H = 59.21, p< 0.001) and HbA1c (H = 52.34, p< 0.001) of the DM group were significantly higher than those of the control group. Levels of high-density lipoprotein were significantly higher in the control group than in the DM group (p = 0.036). Urea levels were significantly higher in the DR group than in the control group (H = 32.33, p = 0.005). Furthermore, the duration of diabetes was significantly longer in the DR group than in the DM group (H = 13.28, p< 0.001; Table 1).

## 3.2 Plasma CCN1

Blood CCN1 levels were significantly different in the three groups (H = 23.67, p< 0.001). CCN1 levels in the DR group were significantly higher than those in the DM (H = 38.44, p< 0.001) and control (H = 43.75, p< 0.001) groups. However, no significant difference in CCN1 expression was observed between the control

TABLE 1 Characteristics of the participants.

| | CCN1, pg/mL[†] | Age, y[†] | Duration of diabetes, y[†] | Fasting glucose, mmol/L[†] | HbA1c, %[†] | Urea, mmol/L[†] | BMI, kg/m²[‡] | MAP, mmHg[‡] | High-density lipoprotein, mmol/L[‡] | Low-density lipoprotein, mmol/L[‡] | Total cholesterol, mmol/L[‡] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 152.7 (104.9, 193.7) | 69 (67, 74) | N/A | 5.35 (5.15, 5.94) | 5.80 (5.70, 6.15) | 5.41 (4.31, 6.26) | 24.74 ± 3.62 | 96 ± 9 | 1.42 ± 0.39 | 3.05 ± 0.89 | 5.25 ± 1.02 |
| DM | 164.0 (99.3, 218.0) | 69 (66, 73) | 11.0 (4.0, 21.5) | 6.93 (6.05, 8.31) | 6.80 (6.25, 7.40) | 5.74 (4.93, 6.84) | 24.71 ± 3.56 | 95 ± 7 | 1.25 ± 0.33 | 2.90 ± 1.10 | 4.95 ± 1.26 |
| DR | 235.4 (157.4, 291.6) | 71 (66, 75) | 18.0 (11.6, 22.4) | 8.19 (7.11, 9.60) | 7.70 (6.90, 8.65) | 6.10 (4.98, 8.00) | 24.43 ± 2.47 | 97 ± 8 | 1.34 ± 0.35 | 2.90 ± 0.96 | 4.88 ± 1.05 |
| H/F | 23.67 | 2.35 | 13.28 | 66.62 | 64.47 | 9.73 | 0.18 | 0.97 | 3.28 | 0.4 | 1.73 |
| p-value | <0.001* | 0.31 | <0.001* | <0.001* | <0.001* | 0.008* | 0.83 | 0.38 | 0.04* | 0.67 | 0.18 |

CCN1, Cellular Communication Network Factor 1; BMI, Body Mass Index; MAP, Mean Arterial Pressure; HbA1c, Hemoglobin A1c; DM, Diabetes mellitus; DR, Diabetic Retinopathy; N/A, not applicable.
[†]skewed data analyzed using Kruskal-Wallis test; [‡] normal distributed data analyzed using one-way analysis of variance; * significant difference among groups, p< 0.05.

and DM groups (H = 5.32, p > 0.05; Table 1). In addition, similar levels of CCN1 were observed in both male and female subgroups (Supplementary Materials).

## 3.3 Influencing factors of CCN1

There were no sex differences in the CCN1 levels in the control (U = 304, p = 0.630), DM (U = 768, p = 0.201), or DR groups (U = 605, p = 0.895). CCN1 levels negatively correlated with BMI (r = −0.359, p< 0.001) and positively correlated with the duration of diabetes (r = 0.370, p< 0.001) and urea levels (r = 0.206, p = 0.041). CCN1 levels were not significantly correlated (all p > 0.05) with age (r = 0.103), MAP (r = −0.112), fasting glucose (r = 0.189), HbA1c (r = 0.190), high-density lipoprotein (r = 0.132), low-density lipoprotein (r = 0.141), or total cholesterol (r = 0.091) (Supplementary Figure 2).

## 3.4 DR and CCN1

The areas under the receiver operating characteristic curves for DR were 0.730 (95% CI: 0.644–0.817), 0.713 (95% CI: 0.625–0.802), and 0.684 (95% CI: 0.593–0.775) for CCN1 levels, HbA1c levels, and duration of diabetes, respectively, whereas no significant differences were observed for age, urea, and BMI (all p > 0.05; Table 2; Figure 1).

In patients with diabetes, very high blood CCN1 levels were associated with an increased risk of DR (OR 5.91, 95% CI: 2.12–16.49). Furthermore, after multifactorial correction, both high and very high blood CCN1 levels were found to be associated with an increased risk of DR (high: OR 4.72, 95% CI: 1.10–20.25; very high: OR 8.54, 95% CI: 2.00–36.51).

## 3.5 Changes in blood mRNA Associated with CCN1

Pairwise comparisons (DR vs. DM, DR vs. control) of genes obtained by mRNA sequencing analysis yielded 237 differentially expressed genes, of which 219 genes were upregulated and 18 genes were downregulated in the DR group. All the genes obtained from the screening met the |FC| value >1.5 and q-value<0.05. Among the

237 differential genes obtained by sequencing, those associated with CCN1 included *HIF1A-AS3, SELENON, MIF, CDC42-IT1, SRCAP, AC009927.1, AC116348.2, CDC42EP1, COX6CP1, AC112191.1, AL117336.1, NOG*, and *AL157871.3*.

Moreover, *HIF1A-AS3, SELENON, MIF, CDC42-IT1, SRCAP, AC009927.1, AC116348.2, CDC42EP1, COX6CP1, AC112191.1*, and *AL157871.3* were upregulated, while *AL117336.1* and *NOG* were downregulated in the DR group (Supplementary Tables 1, 2).

## 3.6 Animal examination of FFA

To analyze the changes in the retinal vascular system in diabetic rats, an FFA examination was performed. The optic disc is located at the center of the retina, and the retinal vessels radiate around it. Rats in the control group showed a clear fundus and fluorescence. However, eight weeks after DM induction, rats in the diabetic group exhibited retinal vascular tortuosity, dilation, filling defects, and leakage, which were consistent with DR (Figure 2A).

## 3.7 Changes in retinal protein associated with CCN1

Protein expression of CCN1 (U = 35, p = 0.004), as well as CCN1-related proteins (CDC42: U = 36, p = 0.002; COX6c: U = 34, p = 0.009; CREB1: U = 36, p = 0.002; HIF1α: U = 36, p = 0.002; NDUFα1: U = 36, p = 0.002; SEPN1: U = 34, p = 0.009; SHP1: U = 32, p = 0.026) were significantly upregulated in the retina of diabetic rats (all p value< 0.05). Additionally, the protein expression of VEGF was upregulated (U = 35, p = 0.004), while Claudin5 was downregulated (U = 34, p = 0.009) in the retina of diabetic rats (Figures 2B, C).
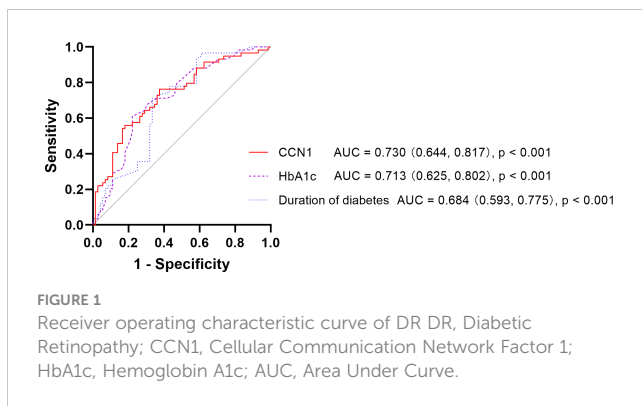
# 4 Discussion

DR is the leading cause of blindness in working-age adults (1). CCN1 is upregulated by various stimuli and exerts various biological effects by interacting with the extracellular matrix and cell surface. CCN1 has been shown to be closely related to DR. Moreover, CCN1 in the vitreous humor and neovascular membrane has received considerable attention; however, research on blood

TABLE 2 Association between CCN1 and DR: Binary and multivariable logistic regression analysis.

| | Unadjusted model | Adjusted model 1[†] | Adjusted model 2[‡] | Adjusted model 3[§] |
|---|---|---|---|---|
| CCN1 low | Reference | Reference | Reference | Reference |
| CCN1 moderate | 1.47 (0.51, 4.21) | 1.49 (0.51, 4.30) | 1.98 (0.53, 7.41) | 2.28 (0.56, 9.34) |
| CCN1 high* | 2.32 (0.86, 6.30) | 2.41 (0.87, 6.63) | 4.22 (1.10, 16.11) * | 4.72 (1.10, 20.25) * |
| CCN1 very high* | 5.91 (2.12, 16.49) * | 5.96 (2.11, 16.79) * | 8.99 (2.33, 34.62) * | 8.54 (2.00, 36.51) * |

CCN1, Cellular Communication Network Factor 1; OR, Odds Ratio; DR, Diabetic Retinopathy.
CCN1 was stratified according to its quartiles, low CCN1< 127.72 pg/mL, 127.72 pg/mL ≤ moderate CCN1< 173.85 pg/mL, 173.85 pg/mL ≤ high CCN1< 250.32 pg/mL, very high CCN1 ≥ 250.32 pg/mL
[†]adjusted for age and sex (male = 1, female = 0); [‡] adjusted for age, sex, BMI, duration of diabetes, and urea; [§]adjusted for age, sex, BMI, duration of diabetes, urea, MAP, fasting glucose, HbA1c, high-density lipoprotein, low-density lipoprotein, and total cholesterol; *significance of the logistic regression model, p< 0.05.

**FIGURE 1**
Receiver operating characteristic curve of DR DR, Diabetic Retinopathy; CCN1, Cellular Communication Network Factor 1; HbA1c, Hemoglobin A1c; AUC, Area Under Curve.

levels of CCN1 is limited. Since blood is a more accessible biological sample, the study of blood CCN1 levels could help us further understand the relationship between CCN1 and DR and its potential clinical application. We report, for the first time, the comparison of the blood CCN1 levels between patients with type 2 diabetes and healthy individuals. We observed that plasma CCN1 levels in patients with DR were significantly higher than those in diabetic patients without DR and healthy subjects. This finding is consistent with previous conclusions drawn from studies on vitreous humor.

Winzap et al. analyzed blood CCN1 levels in patients with acute coronary syndrome and established that diabetes does not affect CCN1 levels (23). This observation is consistent with our results, which revealed a non-significant difference in the CCN1 levels of the DM and control groups. HbA1c is an important indicator of long-term glycemic control. Neither the study conducted by Winzap et al. (23) nor our study showed a significant correlation between HbA1c and CCN1 levels. However, Winzap et al. found that baseline blood glucose levels were correlated with CCN1 levels (23), which was not observed in our study. This difference may be due to the inclusion of patients with acute coronary syndrome in their study; additionally, the blood glucose levels may have partially reflected the stress level in the body (23). However, the blood glucose level in our study reflected the subjects' glycemic control status.

We found that BMI, duration of diabetes, and urea levels were associated with CCN1 expression, but these correlations were weak. Considering the relation between CCN1 and DR, we believe that the correlation between CCN1 and these parameters may be due to their association with DR. CCN1 is closely associated with vascular injury. Feng et al. reported that blood CCN1 levels in diabetic subjects were positively correlated with severity of peripheral arterial disease (6). CCN1 levels is significantly elevated in patients with ST-segment elevation myocardial infarction compared to that in patients with non-ST-segment elevation myocardial infarction (24), and high levels of CCN1 can potentially predict the occurrence of cardiovascular risk events (23–25). We believe that the elevation of CCN1 levels may reflect the impairment of organ function, especially the function of blood vessels. DR is characterized by a series of fundus lesions caused by

leakage and occlusion of retinal microvessels. CCN1 may be involved in the development and progression of DR through its effects on the blood vessels.

As an early response gene, CCN1 is induced by various factors, such as inflammation, hypoxia, and mechanical stimulation (2). Changes in *HIF1A-AS3, CDC42-IT1, SRCAP, AC116348.2, CDC42EP1*, and *AL117336.1* in blood mRNA sequencing analysis may reflect the induction of CCN1. Previous studies have shown that hypoxia significantly induced CCN1 expression in endothelial cells (5, 26) and HIF-1α promoted CCN1 transcription under hypoxia (26, 27). In our study, *HIF1A-AS3* was found to be upregulated in DR. Previous studies have revealed that *HIF1A-AS1* and *HIF1A-AS2* regulate the expression of HIF-1α mRNA (28, 29). We observed an increase in HIF1α in diabetic rats, which reflects the hypoxic state of the retina. In addition, changes in the expression of *CDC42-IT1, SRCAP, CDC42EP1*, and *AL117336.1* may indicate changes in CREB and small GTPase-related pathways, which have been shown to play a key role in the regulation of CCN1 (30–32). The phosphorylation of CREB and its kinases play a vital role in RhoA-mediated regulation (30, 31, 33). The *SRCAP*, which is upregulated in the DR group, is a transcriptional activator of CREB-mediated transcription (34). *CDC42EP1* is a CDC42-binding protein that promotes angiogenesis through cytoskeletal regulation (35). Additionally, *CDC42-IT1* and *AL117336.1* may be involved in the regulation of CREB-related pathways; however, their specific roles remain unclear. CDC42 and CREB1 levels were higher in the retina of diabetic rats. CREB regulates the transcription of CCN1 by directly binding to its promoter (30, 31). *AC116348.2* encodes an integrin-related molecule, and it has been reported that secreted CCN1 exerts its biological effects by binding to integrins (36).

High plasma levels of CCN1 in patients might indicate an impaired blood-retinal barrier. Changes in *SELENON, MIF, AC009927.1, COX6CP1, AC112191.1, NOG*, and *AL157871.3* in blood mRNA sequencing analysis indicated changes in systemic oxidative stress or phosphorylation levels, suggesting the possibility of blood-retinal barrier injury. Diabetic rats showed vascular tortuosity and leakage upon FFA examination. VEGF expression increased in the retinas of diabetic rats, while Claudin5 expression decreased. The retinal vessels of diabetic rats were damaged and the blood-retinal barrier was compromised. CCN1 is mainly expressed in endothelial cells and mediates angiogenic effects (36). Previous studies have suggested that increased CCN1 expression stimulates oxidative stress and disrupts tight junction integrity in endothelial cells (13). In our study, alterations in the expression of *SELENON, COX6CP1, NOG*, and *AL157871.3* suggest elevated levels of oxidative stress. We examined the relevant target molecules and found that the target molecules COX6c, NDUFα1, and SEPN1 levels were elevated in the retinas of diabetic rats. In addition, CCN1 can inhibit pericyte adhesion and cause anoikis by regulating the phosphorylation of focal adhesions (37). *AC009927.1* and *AC112191.1* are associated with the regulation of phosphorylation, particularly tyrosine phosphorylation. We examined the levels of SHP1, which promotes dephosphorylation,
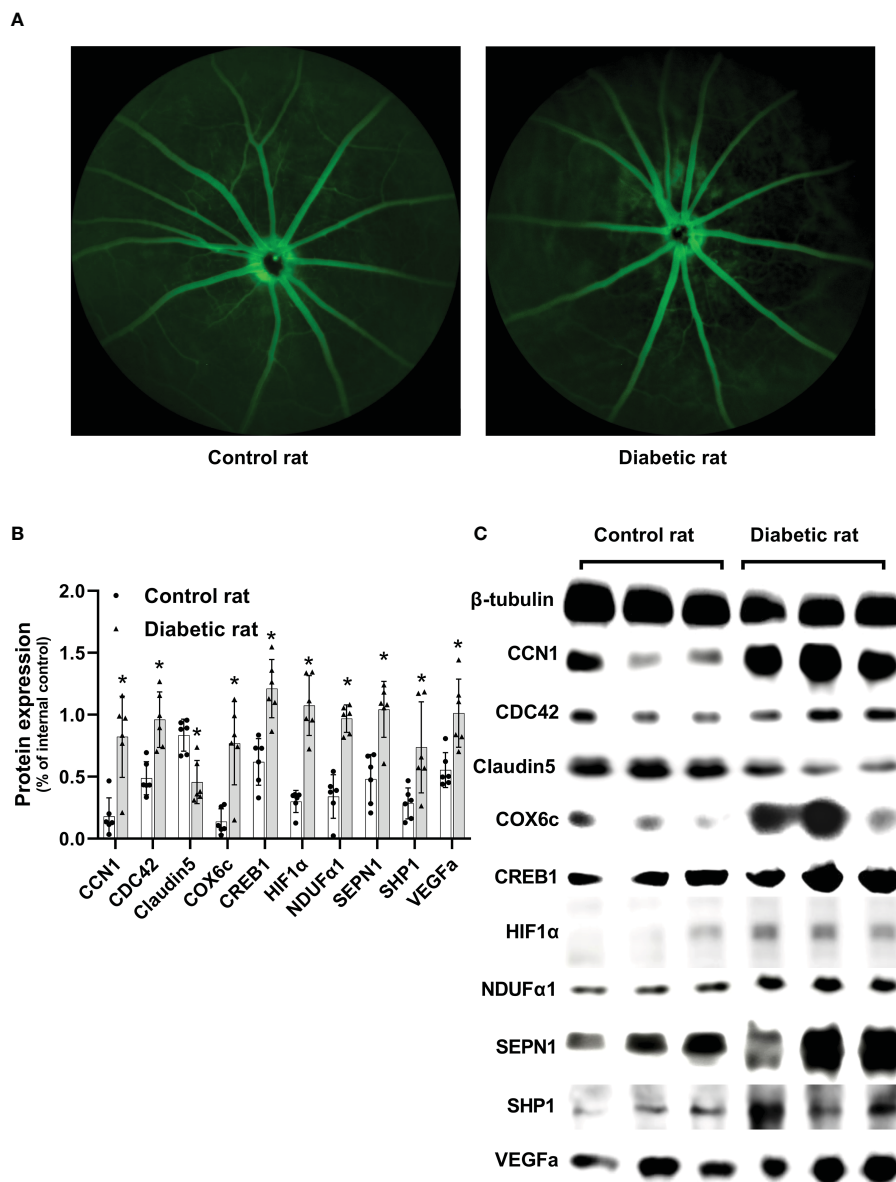
**FIGURE 2**
Differences in the retina between diabetic and non-diabetic rats. **(A)** Fundus fluorescein angiography of diabetic and non-diabetic rats. **(B)** Differences in protein expression levels between diabetic and non-diabetic rats. **(C)** Western blotting strip images of diabetic and non-diabetic rats. CCN1, Cellular Communication Network Factor 1; COX6c, cytochrome c oxidase subunit 6c; CREB1, Cyclic AMP-responsive element-binding protein1; HIF1α, Hypoxia-inducible factor 1α; NDUFα1, NADH dehydrogenase 1 alpha subcomplex; SEPN1, Selenoprotein N 1; SHP1, Protein-tyrosine phosphatase 1C; VEGFa, vascular endothelial growth factor a; Differences between control and diabetic rats were compared using the Mann–Whitney test, and the values of protein expression were analyzed using β-tubulin as the internal control; *p< 0.05, compared with controls.

in the retina and found that SHP1 was upregulated in diabetic rats, consistent with previous studies (37).

This study has some limitations. First, this was a cross-sectional study, and longitudinal cohorts are needed to demonstrate the predictive effect of CCN1 on DR. Second, we did not differentiate the severity of DR and, therefore, could not clarify the relation between CCN1 levels and the severity of DR. Third, our study included only subjects from Shanghai, China, and the generalizability of the findings remains to be proven. Fourth, we discussed the mechanism of CCN1 based only on sequencing and

retinal protein results, which provides information for subsequent mechanistic studies that need to be verified in the future. Fifth, we did not test the level of CCN1 in the vitreous fluid and could not analyze the relationship between CCN1 vitreous levels and CCN1 blood levels.

In conclusion, we measured plasma CCN1 levels in the healthy controls, DM, and DR groups and found that CCN1 expression was significantly elevated in patients with DR, whereas there was no difference between healthy controls and DM groups. Additionally, it was observed that elevated CCN1 levels were risk factors for DR,

even after multifactorial correction. CCN1 was negatively correlated with BMI and positively correlated with the duration of diabetes and urea levels. Thus, CCN1 levels may reflect vascular damage caused by chronic hyperglycemia, rather than glycemic control. The results of mRNA sequencing analysis suggested that CCN1 levels in the blood could be upregulated due to hypoxia and may damage the blood-retinal barrier through oxidative stress and phosphorylation after interaction with integrin. Blood CCN1 levels may be a potential biomarker for DR and may be involved in the occurrence and development of DR.

## 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 below: https://www.ncbi.nlm.nih.gov/geo/, GSE221521.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai First People's Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Shanghai First People's Hospital Animal Ethics Committee.

## Author contributions

ZX contributes to the acquisition of data, analysis, and interpretation of data, and drafting the manuscript. SC, XQ, SL, YX and LL contributes to the acquisition of data and interpretation of data. HZ contributes to the conception, design, and final approval. All authors read and approved the manuscript.

## 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.2023.1131993/full#supplementary-material

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