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RESEARCH ARTICLE

A systematic proteomic profiling and pathway analysis of protein biomarkers in diabetic retinopathy with subsequent validation of the IL-6 upstream regulator

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Abstract

Purpose Diabetic retinopathy (DR) is a leading cause of irreversible blindness worldwide. Identifying risk factors associated with DR development and progression is crucial for improving treatment efficacy. Although proteomic changes in DR have been extensively studied, the results remain equivocal. Hence, this study aims to summarize and identify potential diagnostic or prognostic markers for DR. In addition, the upstream regulator responsible for protein deregulation of this disease was also validated.

Methods We systematically analyzed the current literature on proteomic profile changes in DR, followed by pathway analysis identification. To validate the protein level changes, ELISA was performed from serum samples collected from 27 patients with DR and 25 healthy controls.

Results Our analysis revealed that 1 candidate marker (afamin [AFM]) distinguished non-proliferative diabetic retinopathy (NPDR) from type 2 diabetic patients with no diabetic retinopathy/controls, 65 candidate markers distinguished proliferative diabetic retinopathy (PDR) from NPDR, 1 candidate marker (thyroid receptor-interacting protein 11 [TRIP11]) distinguished PDR from PDR-DME/DME, and 3 candidate markers for therapeutic evaluation of PDR. Our results pinpoint that inflammatory response, which IL-6 mainly modulated, is responsible for the changes of proteomic profiles identified in DR. This was also validated by ELISA analysis, indicating that IL-6 could be potentially useful for diagnosing DR.

Conclusion We report a comprehensive patient-based proteomic approach to identify potential biomarkers for DR diagnosis, prognosis, and treatment evaluation.

Keywords Diabetic retinopathy · Proteomic profiles · Interleukin-6 · Biomarkers · Progression and evaluation

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Introduction

Diabetes mellitus (DM) remains one of the most challenging public health problems both in developed and developing worlds [1]. Models estimate that the number of people with DM will likely at least double by 2030 [2]. This is possibly related to population aging, the growing rates of obesity, and unhealthy lifestyle (e.g., smoking, excessive alcohol drinking, and low physical activity) [2, 3]. In one-third of patients diagnosed with DM, macro-and microvascular complications are often observed, particularly in older individuals [4–6]. In the eye, DR is well recognized as the most common microvascular complication of diabetes and is the leading cause of irreversible blindness in the middle-aged and elderly, particularly in the Asia-Pacific region [7–9].

DR can be classified into two main stages based on its severity, NPDR and PDR [10]. In the initial stage, patients may be asymptomatic, and thus they usually do not seek any medical assistance. However, in more advanced stages of the disease, patients may experience symptoms such as blurred vision, visual distortion, and floaters as a consequence of the blood-retinal barrier breakdown, which causes macular edema (ME) [7, 10]. Notably, ME that occurs during NPDR is closely associated with the degree of visual impairment [11, 12].

Although the pathophysiological mechanism underlying the development of DR, such as prolonged hyperglycemia, has been extensively studied [4], much remains unknown about the precise pathogenesis of the disease. Interestingly, however, a complex interplay between genetics, the immune system, inflammation, microvascular, and neurodegeneration is closely linked to the progression of DR [13]. Because initially latent or asymptomatic DR could lead to sudden vision loss, early detection and appropriate treatment modalities can however effectively decelerate its progress. Notwithstanding, no specific drugs are currently available to slow down DR [14, 15]. While the development of future diagnostics and therapies for DR will be based on the pathophysiology of the disease, early identification of predictive biomarkers is indisputable.

Proteomics analysis is a useful technique to identify protein profiles associated with various physiological and pathological states. The advanced development of the proteomic approach allows faster identification of proteins in a small number of samples from various types of tissues and body fluids [16]. To the best of our knowledge, there has not been an extensive review comparing and/or combining proteomics studies to gain more insight into the role of specific proteomic biomarkers in the pathogenesis of DR. Therefore, we aim to systematically review the currently published literature on the proteomic profile changes of DR patients in order to identify differentially expressed proteins that can be used for diagnostic purpose and pathway analyses in elucidating DR pathogenesis. Additionally, serum samples were also collected from patients with DR to assess the levels of the upstream regulator responsible for proteomic changes in DR.

Methods

Literature search

A literature search was conducted in PubMed and Scopus databases using the following search terms: "proteomic" (all fields), "diabetic retinopathy" (all fields), "proliferative diabetic retinopathy [PDR]" (all fields), "diabetic macular edema [DME]" (all fields), dated up to June, 2021. Titles and abstracts were screened to select articles in which protein profile was compared between DR/PDR/DME and control group or between PDR and PDR treated with anti-vascular endothelial growth factor (VEGF).

Group definition

The studies were stratified according to DR severity and sample type. We additionally divided PDR patients into 2 sub-groups: (1) preoperatively treated with intravitreal injection of anti-VEGF (intravitreal injection was performed 5–7 days before PPV) and (2) without intravitreal injection of anti-VEGF. The control groups in all included studies were healthy individual or non-diabetic patients with idiopathic macular hole (iMH), epimacular membrane (EM), rhegmatogenous retinal detachment (DDR), or corneal transplant.

Data extraction and analysis

Differentially expressed proteins reported in each study were included in the joint dataset with the following criteria: (1) Proteomic studies of adult patients with DR or DME; (2) proteins were only counted once for each study with multiple spot identifications or evaluated in a different proteomic technology; (3) proteins were included if the expression levels were changed at least 50% compared to control levels [17]. Data sets of proteins were excluded if proteomic results reported in the study exhibited fold changes between 0.8 and 1.2 unless the p-value was significant. We also did not include proteomic data derived from animal, in-vitro, in-silico, or ex-vivo studies.

A Venn diagram and an UpSet plot were generated to visualize the similarities and differences of the significant protein profiles among groups. Datasets representing genes repeatedly or commonly identified by differential proteomics were imported into QIAGEN's Ingenuity Pathway Analysis

(IPA) in order to evaluate genetic networks available in the Ingenuity database. In addition, datasets were also imported into PANTHER (http://www.pantherdb.org/) to evaluate GO annotation and PANTHER-enriched pathways. Fisher's exact test was used to statistically determine the overrepresentation of PANTHER classification categories. False discovery rate (FDR)-corrected *p*-values of 0.05 were considered significant.

Patients, sample collection, and ethical consideration

Blood samples were collected from 27 patients (9 male, 18 female) with DR (40–66 years old) and 25 healthy subjects (7 male, 18 female, 30–70 years old) at the Ophthalmology Clinic of Muhammadiyah Hospital, Malang, Indonesia. The study was conducted after approval from the Ethics Review Board of the faculty (Ref. No. 055/EC/KEPK-FKIK/2021) and was conducted in accordance with the tenets of the Declaration of Helsinki. Patients were fully informed and written consent was obtained at the beginning of the study. Patients were excluded if they had a history of intraocular ischemia, systemic vascular, inflammatory, and hematological disease, renal dysfunction, hepatic disorder, malignancy, and hyperlipidemia. These criteria were also applied to all control subjects. All participants underwent a complete ocular examination, such as visual field testing, slit-lamp biomicroscopy, and indirect ophthalmoscopy.

ELISA measurement

Serum levels of interleukin (IL)-6 and its soluble receptor (Cat. No. E0090Hu and E0226Hu, Bioassay Technology Laboratory) were quantified according to the manufacturer's protocols. Serum levels of each parameter were calculated using iMark™ Microplate Absorbance Reader (Biorad) according to the standard linear plot obtained from the optical density readings of control wells.

Statistical analysis

Demographic and clinical features between the two groups were compared using the Mann-Whitney test and independent T-test for categorical and continuous variables, respectively. Pearson correlation was employed to analyze the association between two continuous variables. The values of the parameters measured were used to generate receiver operating characteristic (ROC) curves in order to determine the area under the curve (AUC). The optimal cut-off for each parameter was determined using Youden's index, and the corresponding sensitivity and specificity were also quantified. GraphPad Prism (Version 9.0) was used for statistical analysis and graph construction. All statistical analyses were 2-sided with a significance threshold of $p < 0.05$.

Results

Dataset description

A schematic representation of our literature search is shown in Fig. 1. A total of 300 articles were screened, among which 20 were reviewed [4, 5, 7, 10, 13, 15, 18–31]. According to DR severity, 8 and 14 studies enrolled NPDR [4, 5, 7, 10, 25, 27, 28, 31] and PDR patients [5, 10, 13, 15, 18–26, 30], respectively. While only 2 studies recruited DME patients [24, 29]. Of the eligible studies, 11 were proteomes studies using samples collected from vitreous humor (VH) [15, 18–24, 26, 29, 30]. The remaining studies were composed of serum/plasma (4 studies) [25, 27, 28], aqueous humor (AH) (2 studies) [4, 21], tear (2 studies) [5, 7], and saliva (1 study) [10]. Only 3 studies evaluated protein profile changes of PDR treated with anti-VEGF [13, 18, 22]. Proteomic analyses of the included studies were mainly evaluated by LC-MS/MS (50%), followed by 2-D DIGE (40%) and iTRAQ (10%). The complete lists of differentially expressed proteins extracted from the included studies are depicted in Supplementary Tables 1–4.

Proteomic profiles in DR

In order to identify proteins that were commonly deregulated in DR, first, we identify proteins that were differentially expressed between controls and cases in each study (see Supplementary Tables 1–3). Utilizing the Venn diagram categorized by DR severity and sample type, 465 and 464 unique proteins were observed. Among them, 13 and 10 proteins were commonly identified between groups (Fig. 2A-B).

To identify shared protein in the NPDR group, we compare the sets of differentially expressed proteins obtained from each study ($n = 8$ studies). Our analysis showed that 15 proteins were frequently observed between studies (three proteins, zinc-alpha-2-glycoprotein (AZGP1), Ig alpha-1 chain C region (IGHA1), and apolipoprotein A-I (APOA1) were on top of the lists, Table 1), with the largest intersection size was found between the study of Lu et al. [28] and Kim et al. [31] (Supplementary Fig. 1A). When we performed subgroup analysis according to sample type (Supplementary Fig. 1B), serum/plasma IGHA1, AZGP1, and albumin (ALB) were overlapped with the sample collected from tear, while APOA1 and serotransferrin (TF) were overlapped with the sample collected from AH.

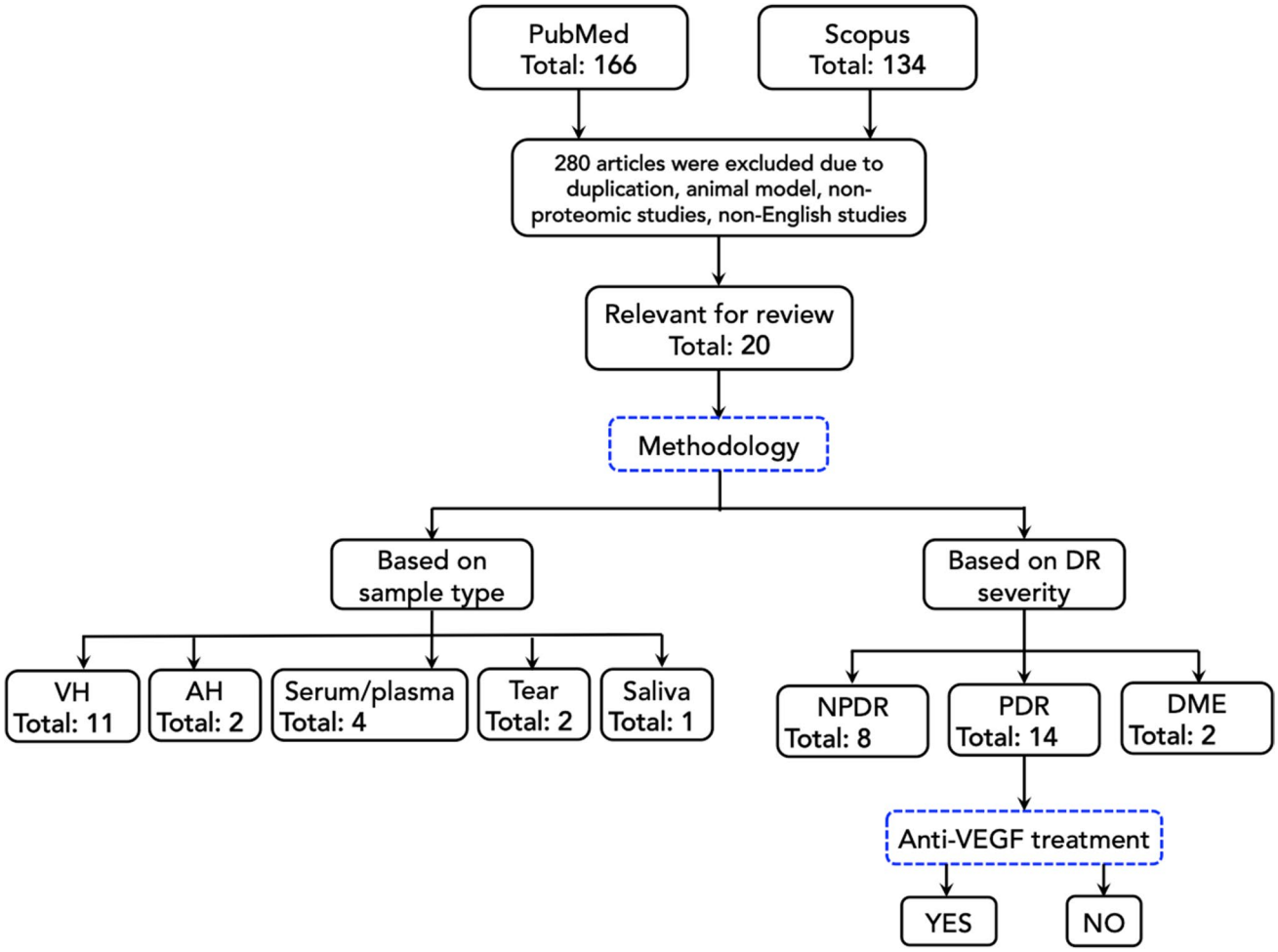


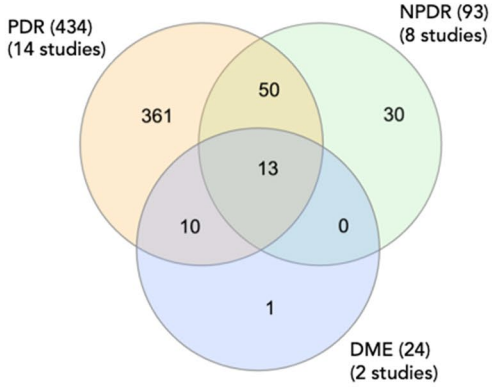
Fig. 1 Workflow of literature search, selection, and stratification. AH, aqueous humor; DR, diabetic retinopathy; DME, diabetic macular edema; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; VH, vitreous humor

For the PDR group, we compare the sets of differentially expressed proteins obtained from all studies (sample collected from VH and non-VH) ($n = 15$) or studies evaluating proteome profile changes collected from VH ($n = 11$). We detected a core of 80 and 77 proteins repeatedly identified from all studies and studies utilizing VH samples, respectively (Table 2). Among them, 19 and 3 proteins were identified in all sample types and non-VH only, respectively (Table 2), with fibrinogen beta chain (FGB) on top of the list (identified in 7 studies; 46.7%, Table 2). Because 73% of the PDR group consisted of proteome studies from VH, we then specifically analyzed this particular category (Table 2). We found the largest intersection size between the study of Loukovaara et al. [13] and Wang et al. [23] (Fig. 3A). Among differentially expressed proteins, retinol-binding protein 3 (RBP3) was listed at the top (detected in 6 studies; 55%, Table 2). For DME, only vitamin D-binding protein (GC) was commonly identified between the two studies (see Supplementary Table 3).

Proteomic profiles of PDR treated with anti-VEGF

To identify protein profile changes in response to anti-VEGF treatment, we compared the significantly deregulated protein of subgroup 1 (PDR) and subgroup 2 (PDR treated with anti-VEGF) (Supplementary Table 4). The combination of these groups yielded 442 unique proteins, of which 36 proteins were identified in both subgroups (Fig. 3B). Next, we investigated whether these proteins were commonly observed in DR by comparing them with 13 deregulated proteins identified in Fig. 1A. Notably, only 3 (fibrinogen beta chain (FGB), fibrinogen gamma chain (FGG), and beta2-glycoprotein-I (APOH)) of these 36 proteins were identified in all groups (Fig. 3B).

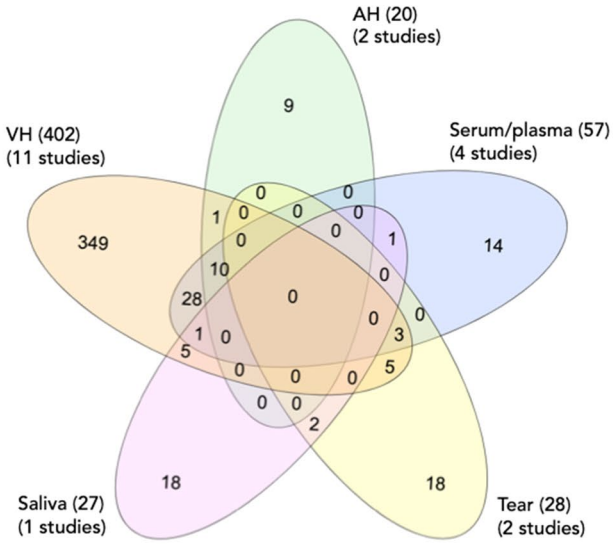
A



B

Commonly deregulated protein in DR individuals	
No.	Protein name
1	Pigment epithelium-derived factor [SERPINF1]
2	Apolipoprotein A-I [APOA1]
3	Vitamin D-binding protein [GC]
4	Gelsolin [GSN]
5	Alpha-1-antichymotrypsin [SERPINA3]
6	Complement C3 [C3]
7	Fibrinogen beta chain [FGB]
8	Fibrinogen gamma chain [FGG]
9	Serotransferrin [TF]
10	Ceruloplasmin [CP]
11	Apolipoprotein A-IV [APOA4]
12	Complement factor B [CFB]
13	Beta-2-glycoprotein-I [APOH]

C



D

Commonly deregulated protein identified in VH, AH, and serum/plasma of DR individuals	
No.	Protein name
1	Apolipoprotein A-IV [APOA4]
2	Apolipoprotein A-I [APOA1]
3	Ceruloplasmin [CP]
4	Fibrinogen beta chain [FGB]
5	Serotransferrin [TF]
6	Apolipoprotein A-II [APOA2]
7	Alpha-1-acid glycoprotein 2 [ORM2]
8	Fibrinogen alpha chain [FGA]
9	Alpha-2-macroglobulin [A2M]
10	Alpha-1-acid glycoprotein 1 [ORM1]

Fig. 2 Venn diagram of differentially expressed proteins in diabetic retinopathy (DR). **A**–**B** Stratified by the severity of disease; **C**–**D** Stratified by sample types. AH, aqueous humor; DME, diabetic macular edema; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; VH, vitreous humor

Pathways and interactions analysis among differentially expressed proteins

To investigate possible biological interactions of differently deregulated proteins, datasets representing genes corresponding to the altered protein expression derived from our analysis were imported into QIAGEN's IPA. The list of differentially expressed proteins commonly occurring in DR revealed that inflammatory response was identified on top of the biological networks and functions (Supplementary Fig. 2) with interleukin (IL)-6 as the main upstream regulator within this particular network (Table 3).

Seventy-seven repeatedly deregulated proteins in PDR were also evaluated, exhibiting 10 significant networks (Supplementary Table 5). Figure 4 A represents the list of the top 5 networks identified by IPA. Of these networks, metabolic disease, neurological disease, and organismal injury and abnormalities were the highest-rated network with 19 focus molecules and a significant score of 42

Table 1 Top lists of protein in NPDR that repeatedly identified in differential studies

Gene name	Protein name	Combine (8 studies)	
		Total	%
AZGP1	Zinc-alpha-2-glycoprotein	3	37.5
IGHA1	Ig alpha-1 chain C region	3	37.5
APOA1	Apolipoprotein A-I	3	37.5
ALB	Serum albumin	2	25
TF	Serotransferrin	2	25
AFM	Afamin	2	25
VTN	Vitronectin	2	25
SERPINA1	Alpha-1-antitrypsin	2	25
A2M	Alpha-2-macroglobulin	2	25
C3	Complement C3	2	25
APOH	Beta2-glycoprotein-I	2	25
LYZ	Lysozyme C	2	25
HP	Haptoglobin	2	25
CP	Ceruloplasmin	2	25
GSN	Gelsolin	2	25

Table 2 Top lists of protein in PDR that repeatedly identified in differential studies

No	Gene name	Protein name	Vitreous (11 studies)		Combine (15 studies)	
			Total	%	Total	%
1	RBP3	Retinol-binding protein 3	6	55	6	40.0
2	CFI	Complement factor I	5	45	5	33.3
3	FGB	Fibrinogen beta chain*	5	45	7	46.7
4	ORM2	Alpha-1-acid glycoprotein 2*	5	45	6	40.0
5	A2M	Alpha-2-macroglobulin*	4	36	5	33.3
6	ALB	Serum albumin*	4	36	5	33.3
7	APOA2	Apolipoprotein A-II	4	36	4	26.7
8	APOH	Beta-2-glycoprotein-I*	4	36	5	33.3
9	APP	Amyloid beta A4 protein	4	36	4	26.7
10	C3	Complement C3	4	36	4	26.7
11	C4B	Complement C4-B	4	36	4	26.7
12	F2	Prothrombin	4	36	4	26.7
13	FGA	Fibrinogen alpha chain*	4	36	5	33.3
14	GC	Vitamin D-binding protein	4	36	4	26.7
15	GSN	Gelsolin	4	36	4	26.7
16	SERPINF1	Pigment epithelium-derived factor	4	36	4	26.7
17	A1BG	Alpha-1B-glycoprotein	3	27	3	20.0
18	AHSG	Alpha-2-HS-glycoprotein	3	27	3	20.0
19	APOA1	Apolipoprotein A-I*	3	27	5	33.3
20	AZGP1	Zinc-alpha-2-glycoprotein*	3	27	4	26.7
21	C4A	Complement C4-A	3	27	3	20.0
22	CLSTN1	Calsyntenin-1	3	27	3	20.0
23	CLU	Clusterin*	3	27	4	26.7
24	CP	Ceruloplasmin*	3	27	5	33.3
25	CRYBA1	Beta-crystallin A1	3	27	3	20.0
26	CST3	Cystatin-C*	3	27	4	26.7
27	CTSD	Cathepsin D	3	27	3	20.0
28	FGG	Fibrinogen gamma chain*	3	27	4	26.7
29	HBB	Hemoglobin subunit beta	3	27	3	20.0
30	HBD	Hemoglobin subunit delta	3	27	3	20.0
31	HPX	Hemopexin*	3	27	4	26.7
32	HRG	Histidine-rich glycoprotein	3	27	3	20.0
33	PTGDS	Prostaglandin-H2 D-isomerase	3	27	3	20.0
34	SERPINA1	Alpha-1-antitrypsin*	3	27	4	26.7
35	SERPINA3	Alpha-1-antichymotrypsin	3	27	3	20.0
36	SERPINC1	Antithrombin III	3	27	3	20.0
37	TF	Serotransferrin*	3	27	4	26.7
38	ACTA2	Actin. aortic smooth muscle.	2	18	2	13.3
39	AGT	Angiotensinogen	2	18	2	13.3
40	AMBP	Protein AMBP	2	18	2	13.3
41	APLP2	Amyloid beta-like protein 2	2	18	2	13.3
42	APOA4	Apolipoprotein A-IV*	2	18	3	20.0
43	APOC1	Apolipoprotein C-I	2	18	2	13.3
44	APOC3	Apolipoprotein C-III	2	18	2	13.3
45	C1orf94	Uncharacterized protein C1orf94	2	18	2	13.3
46	C1QC	Complement C1q subcomponent subunit C	2	18	2	13.3
47	C9	Complement C9	2	18	2	13.3
48	CAT	Catalase*	2	18	3	20.0
49	CD14	Monocyte differentiation antigen CD14	2	18	2	13.3
50	CFB	Complement factor B	2	18	2	13.3
51	CPB2	Carboxypeptidase B2	2	18	2	13.3
52	CRYBA4	Beta-crystallin A4	2	18	2	13.3
53	CRYGS	Gamma-crystallin S	2	18	2	13.3
54	DKK3	Dickkopf-related protein 3	2	18	2	13.3

Table 2 (continued)

No	Gene name	Protein name	Vitreous (11 studies)		Combine (15 studies)	
			Total	%	Total	%
55	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	2	18	2	13.3
56	ENO2	Gamma-enolase	2	18	2	13.3
57	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2	18	2	13.3
58	GPX3	Glutathione peroxidase 3	2	18	2	13.3
59	HBA1	Hemoglobin subunit alpha	2	18	2	13.3
60	IGHA1	Ig alpha-1 chain C region*	2	18	3	20.0
61	IGHG2	Ig gamma-2 chain C region	2	18	2	13.3
62	IGHV3-74	Protein IGHV3-74	2	18	2	13.3
63	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	2	18	2	13.3
64	ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3	2	18	2	13.3
65	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	2	18	2	13.3
66	KNG1	Kininogen-1	2	18	2	13.3
67	OPTC	Opticin	2	18	2	13.3
68	ORM1	Alpha-1-acid glycoprotein 1*	2	18	4	26.7
69	PGK1	Phosphoglycerate kinase 1	2	18	2	13.3
70	PLG	Plasminogen	2	18	2	13.3
71	PROS1	Protein S	2	18	2	13.3
72	RTBDN	Retbindin	2	18	2	13.3
73	SERPING1	Plasma protease C1 inhibitor	2	18	2	13.3
74	TIMP2	Metalloproteinase inhibitor 2	2	18	2	13.3
75	TTR	Transthyretin	2	18	2	13.3
76	VEGFA	Vascular endothelial growth factor A	2	18	2	13.3
77	VTN	Vitronectin	2	18	2	13.3
78	IGLC1	Ig lambda-1 chain C regions**	-	-	2	13.3
79	LCZ	Lysozyme C**	-	-	2	13.3
80	HP	Haptoglobin**	-	-	2	13.3

*Identified in vitreous humor (VH) and non-VH; **Identified only in non-VH.

(Fig. 4B). In the canonical pathway analysis, acute phase response signaling, liver X receptor-retinoid X receptor (LXR/RXR) or farnesoid X receptor (FXR)/RXR activations, coagulation system, clathrin-mediated endocytosis signaling came out to be the top five most significant pathways ($p < 0.05$). The molecules associated with the top of canonical pathway lists are presented in Supplementary Table 6. We also analyzed the upstream regulators of target molecules in our PDR dataset, revealing HNF1 Homeobox A (HNF1A) and IL-6 to be the two most significant regulators (Table 4). Simultaneously, the differentially expressed protein lists were also analyzed by the PANTHER enrichment test (Table 5). Similarly, we found that response to external stimulus, blood coagulation, and complement activation were identified among the significantly enriched pathways.

Quantitative validation by ELISA

Because we aim to understand the upstream regulator responsible for proteomic profile changes in DR, and we found that IL-6 was identified on top of the network. We thus then validated the level of IL-6 in patients with DR and control subjects. Demographic and laboratory data of the included subjects are depicted in Table 6. No significant difference was observed between groups regarding age ($p = 0.440$) and gender ($p = 0.999$). We then focused on the level of IL-6 that was previously identified as the upstream agent responsible for protein deregulation in DR. We performed ELISA to confirm the changes in IL-6 levels and its soluble receptor in DR. The serum IL-6 level (246.1 ± 56.9 vs. 67.22 ± 33.35 pg/mL, $p = 0.01$, Fig. 5A) and IL-6/sIL6-R ratio (3.9 ± 1.4 vs. 0.6 ± 0.3 pg/mL, $p = 0.02$, Fig. 5C) were significantly higher in the DR group than in controls. No significant difference was observed in the level of sIL-6R between the two groups (Fig. 5B).

To demonstrate the diagnostic performance of trans-signaling IL-6, ROC analysis was performed for the IL-6 and IL-6/sIL-6R ratio parameters. Although both parameters exhibited an excellent capability of discriminating DR to controls (for IL-6, AUC = 0.81, cut-off of 13.99 pg/mL, 76% sensitivity and 74% specificity; for IL-6/sIL-6R ratio, AUC = 0.81, cut-off of 0.22, 84% sensitivity and 70% specificity, Fig. 5D), measurement of IL-6 level is sufficient

to determine the risk of DR. The levels of IL-6 and IL-6/sIL-6R ratio were positively correlated with random blood glucose (RBG) and hemoglobin A1C (HbA1C) levels (r = 0.678, p = 0.0001; r = 0.717, p = < 0.0001, r = 0.605, p = 0.0008; r = 0.628, p = 0.0005, respectively), but not with age (Table 7). The level of sIL-6R was not correlated with age, RBG, or HbA1C (Table 7).

Discussion

DR is a microvascular complication of DM and is one of the leading causes of visual disability and blindness worldwide [32]. Recently, several proteomic studies have been performed, providing new insight into DR pathogenesis. However, the results remained equivocal. This might be a result of biological (e.g. study population, genetic factors) and methodological differences (e.g. sample types, sample preparation, type of proteomic methods, data analysis). Hence, we aimed to comprehensively review proteomic studies of DR by combining and comparing based on disease severity and sample types, and subsequently validate the level of candidate protein that was identified on the top of the main regulator responsible for the proteomic changes in DR. We identified 13 proteins that were repeatedly deregulated in DR patients, among which 5 proteins (apolipoprotein A-IV (APOA4), APOA1, ceruloplasmin (CP), FGB, TF) were frequently found in AH, VH and serum/plasma of DR individuals. However, when we focused

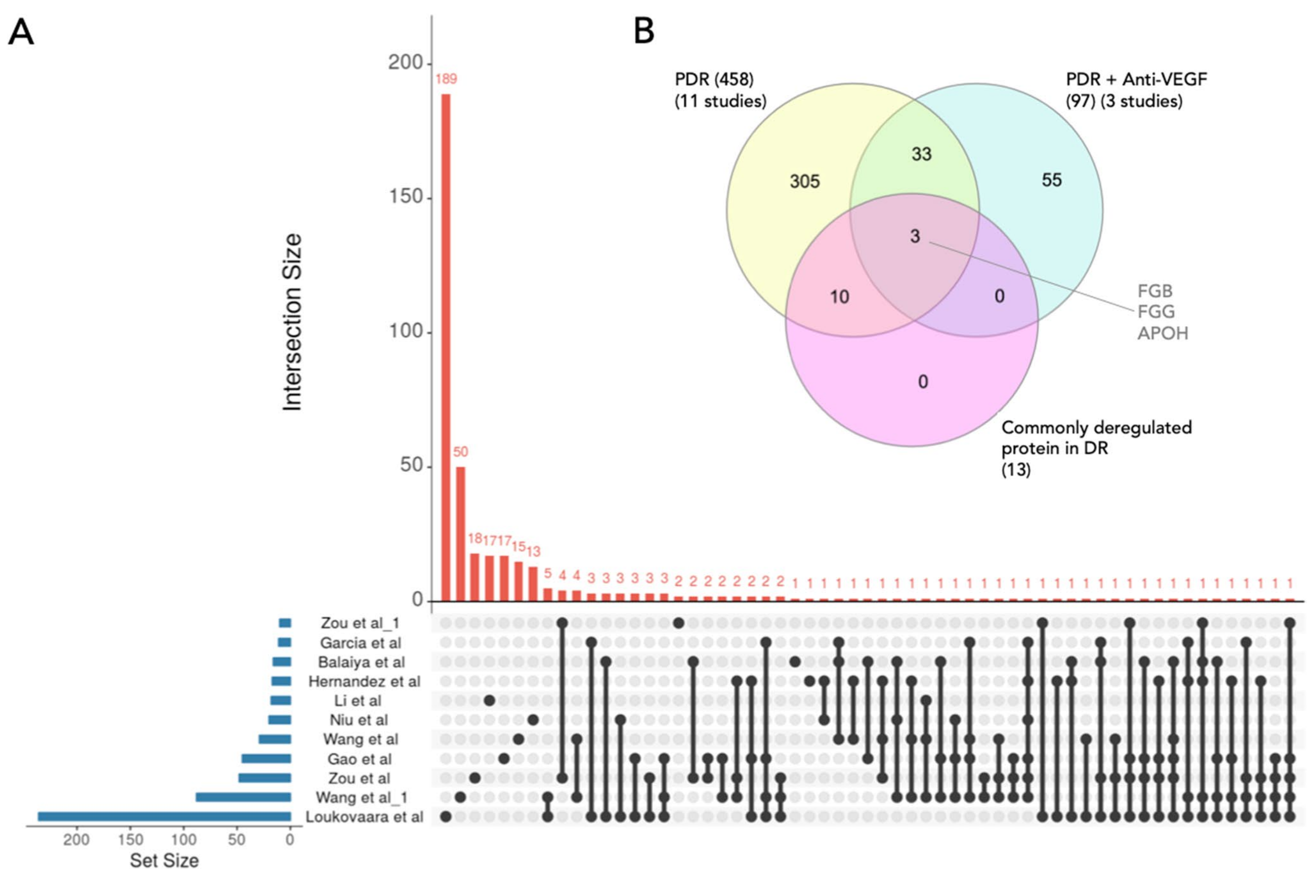


Fig. 3 Proteins were identified in the vitreous humor (VH) of proliferative diabetic retinopathy (PDR) from different proteomic studies. **A** The numbers and intersections of the identified proteins from different proteomic studies were visualized using an Upset plot. Connected dots display shared proteins between study datasets, and the total number of proteins identified in each study is indicated in the set size. **B** Venn diagram representing the intersection of commonly identified proteins in Fig. 1A and B with differentially expressed protein in PDR and PDR treated with vascular endothelial growth factor (VEGF). FGB, FGB, fibrinogen beta chain and gamma chains, respectively; APOH, beta2-glycoprotein-I

Table 3 Top 10 of 251 upstream regulators of target molecules in DR dataset

No.	Upstream regulator	Molecule type	*p*-Val of overlap
1	IL6	Cytokine	1.02E-07
2	HNF1A	Transcription regulator	3.62E-07
3	IL1B	Cytokine	6.40E-07
4	Stat3-Stat3	Complex	7.52E-07
5	TNF	Cytokine	2.38E-06
6	IL13	Cytokine	3.17E-06
7	PPARA	Ligand-dependent nuclear receptor	4.53E-06
8	FOXA2	Transcription regulator	8.98E-06
9	SMAD3	Transcription regulator	1.57E-05
10	ABCA4	Transporter	1.71E-05

on the studies of VH proteome in PDR patients, 77 proteins were commonly deregulated. Together, this may give hints to the potential biomarker identification and pathogenic pathways of DR and its severity.

Differentially deregulated proteins with overlapping expression provide a clue on the general mechanisms associated with DR pathogenesis. Notably, overlapping protein of serpin family F member 1 (SERPINF1 also known as pigment epithelium-derived factor (PEDF)), APOA1, GC, gelsolin (GSN), serpin family A member 3 (SERPINA3 also known as alpha-1-antichymotrypsin), complement C3 (C3), FGB, FGG, TF, CP, APOA4, complement factor B (CFB), and APOH (Fig. 1A–B) indicates inflammatory response. The activation of the immune system and inflammatory mediators have been extensively reviewed elsewhere [33, 34]. Interestingly, our analysis revealed that significantly

Table 4 Top 10 of 897 upstream regulators of target molecules in PDR dataset

No.	Upstream regulator	Molecule type	*p*-Val of overlap	Target molecules in dataset
1	HNF1A	Transcription regulator	1.75E-20	AGT, AHSG, ALB, AMBP, APOA2, APOC3, APOH, C9, CFI, CPB2, F2, FGA, FGB, GC, HPX, ITIH4, KNG1, PGK1, PLG, SERPINA1, SERPING1, TTR, VTN
2	IL6	Cytokine	4.28E-18	A2M, ACTA2, AGT, ALB, APOA1, APP, C3, CD14, CLU, CP, CPB2, CST3, ENO2, ENPP2, FGA, FGB, FGG, HPX, ORM1, PLG, SERPINA1, SERPINA3, TF, TTR, VEGFA
3	Hmgn3	Other	6.17E-16	AHSG, AMBP, APOA1, APOA2, AZGP1, FGA, KNG1, SERPINA1, TTR
4	HNF4A	Transcription regulator	1.18E-15	A1BG, ACTA2, AGT, AHSG, ALB, AMBP, APOA1, APOA2, APOA4, APOC1, APOC3, APOH, APP, C3, C4A/C4B, CAT, CP, CPB2, FGA, FGB, GSN, HPX,I TIH3, ITIH4, KNG1, ORM1, ORM2, PLG, PTGDS, SERPINA1, SERPINA3, TF, TTR, VTN
5	TGFB1	Growth factor	4.85E-13	ACTA2, ALB, APP, C1QC, C3, CAT, CD14, CFB, CFI, CLU, CRYGS, CST3, CTSD, DKK3, ENO2, F2, FGA, FGB, FGG, GSN, ITIH3, KNG1, PGK1, PTGDS, SERPINA1, SERPINA3, SERPINF1, TIMP2, VEGFA
6	Tcf 1/3/4	Group	2.21E-12	AHSG, ALB, AMBP, APOH, TTR
7	FOXA2	Transcription regulator	2.92E-11	A2M, ACTA2, ALB, APOA1, APOC3, C3, FGB, PROS1, PTGDS, SERPINA1, TF, TTR
8	CEBPB	Transcription regulator	1.4E-10	ACTA2, AGT, ALB, APOC3, APP, C1QC, C3, CD14, CP, CPB2, HBB, HPX, ORM1, PLG, SERPINA1, TF
9	APP	Other	2.43E-10	ACTA2, ALB, APLP2, APP, C3, C4A/C4B, CAT, CLU, CP, CTSD, ENO2, ENPP2, HBA1/HBA2, KNG1, PGK1, PTGDS, SERPINF1, TTR, VEGFA
10	STAT3	Transcription regulator	2.65E-10	A2M, ACTA2, AGT, AHSG, APOA4, CAT, CFB, ENPP2, FGA, FGB, FGG, HBB, PGK1, SERPINA1, SERPINA3, VEGFA

deregulated proteins identified in the DR dataset are mainly modulated by IL-6, and the ELISA results were generally consistent with an earlier report in animal models [32, 35]. Additionally, our previous finding also shows that the upregulation of IL-6 production is observed in DR (with IL-6 levels relatively higher in PDR individuals than those diagnosed with NPDR) and is closely associated with rs1800795 polymorphism [36]. In the present pathway analysis, our data also suggest that IL-6 trans-signaling may influence vascular permeability and remodeling through the activation of complements and complement-related components. Indeed, the addition of high concentrations of IL-6/sIL-6R stimulates VEGF-A release in human retinal endothelial cells (HREC) [37], thereby implicating that the IL-6 signaling pathway plays a critical role in diabetes-induced retinal vascular dysfunction possibly by modulating functional behavior of HREC.

In contrast with DR in general, significantly deregulated proteome profiles in PDR can be linked to pathologically relevant molecular networks specifically related to retinal and vascular injuries. Our data also clearly demonstrated distinctive proteome profiles between patients with PDR and NPDR (See Supplementary Fig. 3; Table 8). The upregulation of RBP3 has been known to suppress retinal degeneration in experimental diabetic mice and rats by modulating VEGF level and glucose uptake via glucose transporter 1 (GLUT1) [38]. Complement factor I (CFI) and complement C4-B (C4B) are involved in the complement system [39]. The local activation of the complement system within the eye has been proven to be responsible for the progression of retina degeneration in vitro and in vivo [39–41]. Indeed, our current study indicates that retina degeneration marked by thinning of the retinal nerve fiber layer (RNFL) was observed in PDR, but not in NPDR [42].

Although numerous studies consider alpha-1-acid glycoprotein (α1-AGP) as a non–specific reactive protein in the acute phase, the level of α1-AGP rises under chronic inflammation, including in PDR [25]. The overexpression of α1-AGP is implicated in accelerated wound healing and neural regeneration in the peripheral nerve injury model [43]. Additionally, the accumulation and altered metabolism of amyloid beta A4 protein (APP) have been linked to the development of retinal ganglion cell apoptosis [44, 45]. Localization of apolipoprotein A (ApoA) and PEDF are detected in the neural retina of developing mouse eyes [46, 47]. However, the expression levels of ApoA and PEDF are differentially regulated in the retina of diabetic patients [48, 49]. It is generally known that ApoA and PEDF are potent scavengers of oxygen-reactive species [48, 50]. Moreover, the administration of PEDF ameliorated the

Table 5 Biological process and enriched pathways from PANTHER analysis in PDR dataset

No.	PANTHER GO-Slim biological process	Homo sapiens genes (ref)	Differentially expressed gene list				
		#	#	Expected	+/−	*p*-Val	FE
1	Negative regulation of blood coagulation	4	4	0.02	+	1.8E-08	245.18
2	Positive regulation of peptide secretion	5	2	0.02	+	3.4E-04	98.07
3	Positive regulation of wound healing	5	2	0.02	+	3.4E-04	98.07
4	Platelet aggregation	7	2	0.03	+	5.8E-04	70.05
5	Cholesterol transport	9	2	0.04	+	8.8E-04	54.48
6	Lens development in camera-type eye	17	3	0.07	+	7.0E-05	43.27
7	Negative regulation of endopeptidase activity	62	10	0.25	+	3.1E-13	39.54
8	Iron ion transport	23	3	0.09	+	1.6E-04	31.98
9	Protein activation cascade	105	6	0.43	+	6.0E-06	14.01
10	Positive regulation of multicellular organismal process	118	5	0.48	+	1.5E-04	10.39
11	Complement activation	102	4	0.42	+	9.2E-04	9.61
12	Response to external stimulus	611	11	2.49	+	4.2E-05	4.41
13	Proteolysis	678	11	2.77	+	1.1E-04	3.98
14	Catabolic process	921	11	3.76	+	1.3E-03	2.93
No.	PANTHER pathways	Homo sapiens genes (ref)	Differentially expressed gene list				
		#	#	Expected	+/−	*p* -Val	FE
1	Blood coagulation	48	12	0.20	+	1.06E-17	61.29
2	Plasminogen activating cascade	21	5	0.09	+	6.03E-08	58.38

#, number of genes; expected, the number of genes expected in the list for this PANTHER category based on the reference list; +/- over representation of a category is denoted by a + sign and under representation by a – sign; FE, fold enrichment

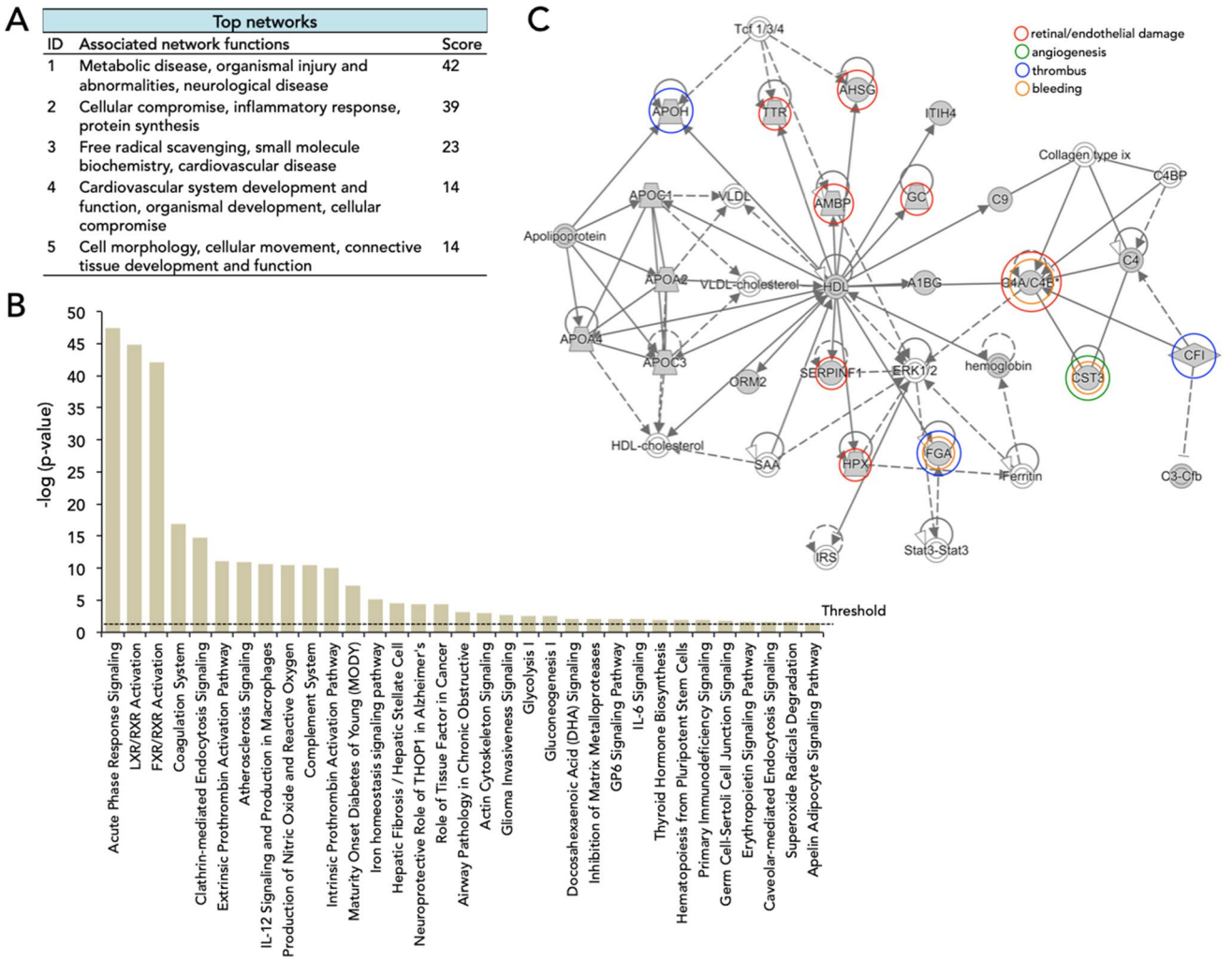


Fig. 4 Ingenuity pathways analysis (IPA) of PDR dataset. To investigate possible interactions of differently regulated proteins, datasets representing 77 genes of the altered expression profile observed in the vitreous humor (VH) of PDR were evaluated. **A** The list of top five networks with their respective scores obtained from IPA; **B** Representation of the most highly rated network (Metabolic disease, organismal injury and abnormalities, neurological disease). The genes that are shaded were differentially expressed according to our analysis. A solid line represents a direct interaction between the two genes, while a dotted line represents indirect interaction. Genes associated with retinal/endothelial damage, angiogenesis, thrombus, and bleeding are circled with red, green, blue, orange colors, respectively. **C** Canonical pathways analysis. The x-axis represents the top canonical pathways as calculated by IPA based on differentially expressed genes and the y-axis represents the ratio of the number of genes from the dataset that map to the pathway and the number of all known genes ascribed to the pathway

Table 6 Baseline characteristic of the subjects

	Control subjects	DR patients	*p*-value
n	25	27	
Age (years)	53.10 ± 13.58	57 ± 16.18	0.440
Gender (M:F)	7:18	9:18	0.999
RBG (mg/dL)	n.d.	302.1 ± 123.2	n.d.
HbA_{1C} (%)	n.d.	7.76 ± 1.87	n.d.

F, female; HbA_{1C}, hemoglobin A1c; M, male; RBG, random blood glucose

characteristic changes in DR by stimulating Müller cells activation [49]. Therefore, it could be postulated that the α1-AGP, APP, ApoA, and PDEF play an important role in protecting the retina from diabetes-related hyperglycemic stress. In addition to the above mentioned agents, hyper-functional coagulation factors, characterized by an increase in prothrombin and fibrinogen concentrations, mediate microglial activation that leads to neuronal and vascular damage [51–54]. Altogether, among the top 10 biomarkers identified in PDR, most of them are responsible for retinal neuroprotection. Hence, retina degeneration

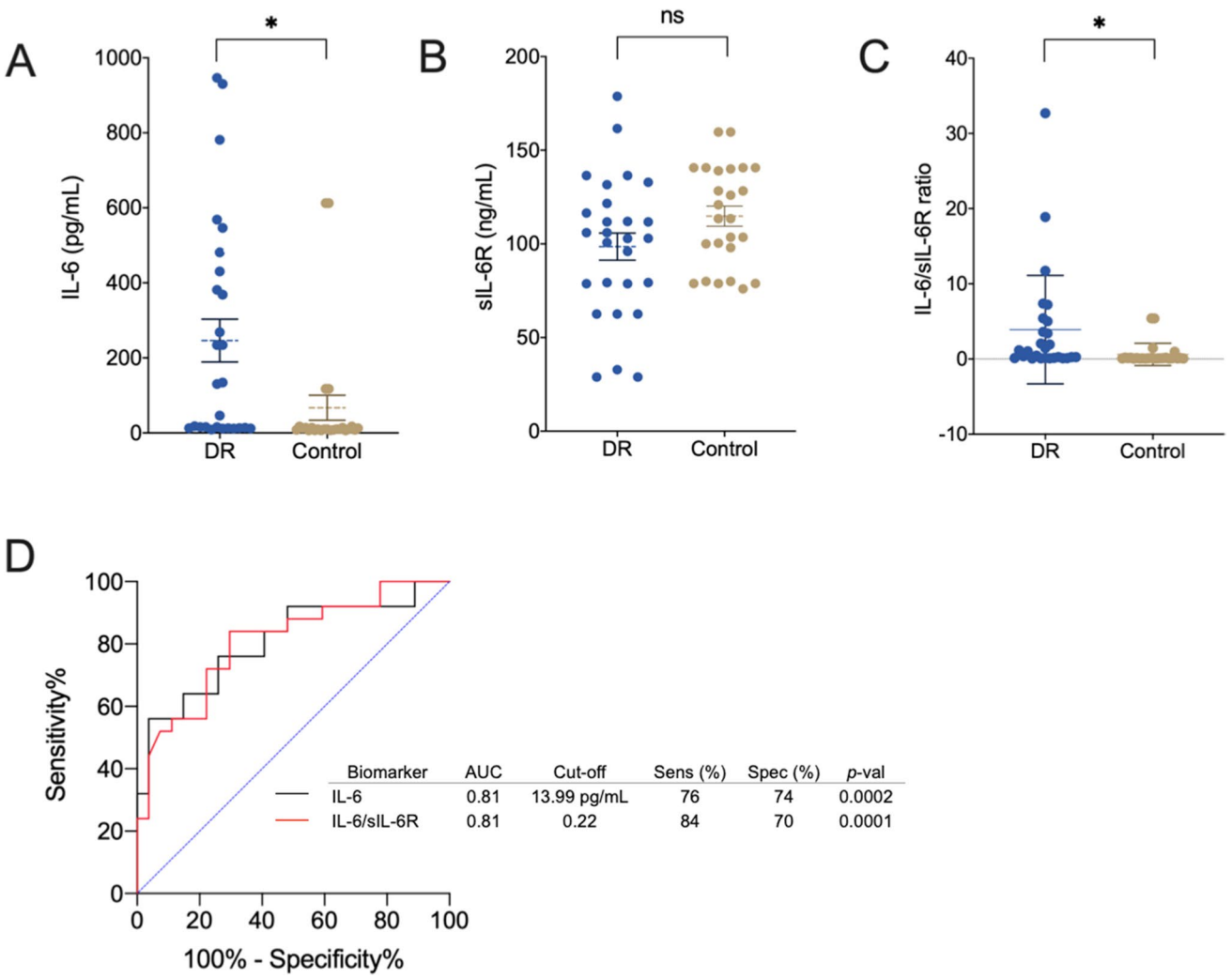


Fig. 5 The levels of IL-6 and its soluble receptor in patients with DR and controls. **A** IL-6 levels; **B** sIL-6R levels; and **C** IL-6/sIL-6R ratio. Data presented as mean ± SEM. *indicates $p < 0.05$; ns, not significant

Table 7 Correlation analysis between serum IL-6, sIL-6R, or IL-6/sIL-6R ratio and other clinical parameters in patients with DR

	IL-6		sIL-6R		IL-6/sIL-6R	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Age	0.340	0.082	– 0.295	0.134	0.314	0.110
RBG	0.678	**0.0001**	– 0.362	0.063	0.605	**0.0008**
HbA_{1C}	0.717	**< 0.0001**	– 0.252	0.203	0.628	**0.0005**

r, Pearson's correlation coefficient. Bold indicates statistically significant $p < 0.05$

should be considered a crucial factor in determining DR severity.

While overlapping proteins were observed between NPDR and PDR, only one protein, AFM, was specifically detected in NPDR. Thus, this protein could be used as a potential biomarker to differentiate between diabetic patients with and without DR. On the other hand, nearly all proteome profiles in DME overlapped with PDR, except TRIP11, which may be associated with angiogenesis [55] (Table 4). Based on the findings above, our observations thus indicate the possibility of a shared mechanism between DME and PDR, although both diseases could represent two distinct clinical-pathological entities [56]. Notwithstanding, it should be noted that only a limited number of DME studies were included in our analysis, and thus the result will require further validation. Additionally, it is interesting to note that

Table 8 Proposed biomarkers for DR and treatment evaluation

NPDR (see supplemantary Fig. 3)	PDR (Top 10/65) (see supplemantary Fig. 3)	DME (see Fig. 1A)	Therapeutic evaluation of PDR treated with anti-VEGF (see Fig. 3B)
Afamin	Retinol-binding protein 3	Thyroid receptor-interacting protein 11	Fibrinogen beta chain
	Complement factor I		Fibrinogen gamma chain
	Fibrinogen beta chain		Apolipoprotein A-IV
	Alpha-1-acid glycoprotein 2		
	Apolipoprotein A-II		
	Amyloid beta A4 protein		
	Complement C4-B		
	Prothrombin		
	Fibrinogen alpha chain		
	Pigment epithelium-derived factor		

FGB, FGG, and ApoA are not only useful for the assessment of DR progression but also can be implemented for therapeutic evaluation of VEGF therapy (Table 4). Thus, our data clearly illustrates that specific characteristics of deregulated proteins are linked to disease stage and thus could be used as prospective biomarkers for diagnosis, prognosis, and treatment evaluation of DR (See Table 6).

The strength of this study is in the cumulative evidence of proteomic data collected for our analysis, deeming the results presented as more conclusive. Several promising biomarkers, including trans-signaling IL-6, should be considered as an alternative marker to predict the proliferative stage of DR. This would be beneficial for the prevention of DR-induced vision loss. Several limitations were identified in our study. Firstly, the results of different proteomic approaches are inconsistent. This is possibly some of the studies' lack of true controls (normal individuals). Secondly, analysis of complex protein mixtures may not be accurate due to the intrinsic limitation of 2-DE. Additionally, proteins with low and high molecular weight might be precluded from the analysis. Thirdly, the LC-MS/MS method has some limitations including low resolution and less accuracy in identifying differential protein expression levels. And finally, further validation of proteomic profiling in patients with DR based on our meta-analysis is required. Despite said techinical limitations, it should be noted that proteomic profiling is a complex task, and no single technology will provide the complete proteome. Fourthly, although the levels of IL-6 were further validated by ELISA, this may not reflect the real condition of the eye because our study measured IL-6 from DR sera. Finally, we propose that future studies should examine which cell types of the retina contribute to the observed proteomic changes.

In summary, based on our findings, inflammation, neuronal injury, and complement/coagulation cascade are remarkable pathways in DR pathogenesis, highlighting the complexity and interconnection of multiple signaling pathways in DR. Importantly, we identified lists of differential protein expression that may serve as potential biomarkers for DR diagnosis, prognosis, and therapeutic evaluation. We also confirmed IL-6 as a major upstream regulator in modulating proteomic changes in DR. Neuroretina degeneration seems to be a hallmark of DR progression in addition to the retinal capillary changes, and there is a possibility of shared mechanisms observed in the brain neurodegeneration. Altogether, this study provides insight into the molecular mechanism of the disease and the possible development of new therapeutic avenues for DR.

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Author contributions Z.S.U. conceived, designed, and performed the study; Z.S.U., Y.T.N.H., L.A.I, and S.A.P. sample collection; Z.S.U. and N.K.R. performed ELISA experiments; Z.S.U. G.V.S analyzed the data; Z.S.U. and G.V.S wrote the main draft of the manuscript. Z.S.U. revised the manuscript. Z.S.U is the main contributor to this manuscript.

Data availability All data analysed in this study are included in this published article and its supplementary information files.

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Declarations

Ethical statement The study was conducted after approval from the Ethics Review Board of the faculty (Ref. No. 055/EC/KEPK-FKIK/2021) and was conducted in accordance with the tenets of the Declaration of Helsinki.

Competing interests The authors declare no competing interests.

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