Heliyon 9 (2023) e16109

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Azelaic acid and guanosine in tears improve discrimination of proliferative from non-proliferative diabetic retinopathy in type-2 diabetes patients: A tear metabolomics study

Xin Wen [a,b], Tsz Kin Ng [a,b,c], Qingping Liu [a], Zhenggen Wu [a], Guihua Zhang [a], Mingzhi Zhang [a,*]

[a] Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, Shantou, Guangdong, China
[b] Shantou University Medical College, Shantou, Guangdong, China
[c] Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Keywords:
Diabetic retinopathy
Tear biomarkers
Metabolomics
Azelaic acid
Guanosine

ABSTRACT

Background: Diabetic retinopathy (DR) is the microvascular ocular complication of diabetes mellitus (DM), which can lead to irreversible blindness and visual impairment if not properly treated. Tears can be collected non-invasively, and the compositions of tears could be the potential biomarkers for ocular diseases. Here we aimed to delineate the metabolomics signature in tears collected from Chinese type-2 DM patients with DR.

Methods: The metabolomics profiles of tear samples from 41 Chinese type-2 DM patients with DR and 21 non-diabetic subjects were determined by the untargeted liquid chromatography-mass spectrometry. The associated pathways of the differentially abundant metabolites were delineated, and the receiver operating characteristic curve analysis was conducted to identify the metabolites differentiating non-proliferative DR (NPDR) from proliferative DR (PDR).

Results: Total 14 differentially abundant metabolites were identified between total DR and non-diabetic subjects, and 17 differentially abundant metabolites were found between the NPDR and PDR subjects. Moreover, total 18 differentially abundant metabolites were identified between the NPDR and PDR subjects with stratification in DR duration and blood glucose level. D-Glutamine and D-glutamate metabolism was significantly highlighted in the PDR group as compared to the non-diabetic group. For the predictive performance, azelaic acid combined with guanosine achieved the area under receiver operating characteristic curve of 0.855 in the comparison between NPDR and PDR groups.

Conclusion: This study revealed the metabolomics changes in tear samples of DR patients. The metabolites in tears could be the potential biomarkers in the DR analysis.

Abbreviations: ANOVA, analysis of variance; AUC, area under receiver operating characteristic curve; Da, daltons; DEET, N,N-diethyl-meta-toluamide; DM, diabetes mellitus; DR, diabetic retinopathy; FDR, false discovery rate; FFA, fundus fluorescein angiography; Hb, hemoglobin; LC, liquid chromatography; MS, mass spectrometry; NPDR, non-proliferativediabetic retinopathy; OCT, optical coherence tomography; PDR, proliferative diabetic retinopathy; ROC, receiver operating characteristic; SD, standard deviation.

* Corresponding author. Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, North Dongxia Road, Shantou, Guangdong 515041, China.

E-mail address: zmz@jsiec.org (M. Zhang).

https://doi.org/10.1016/j.heliyon.2023.e16109
Received 22 November 2022; Received in revised form 2 May 2023; Accepted 5 May 2023
Available online 18 May 2023

1. Introduction

Diabetic retinopathy (DR) is the microvascular ocular complication of diabetes mellitus (DM) and the leading cause of irreversible blindness and visual impairment among the working-age individuals [1–3]. The global prevalence of overall DR is 34.6% [4]. In China, a meta-analysis reported the prevalence of 1.14% for overall DR, 0.90% for non-proliferative DR (NPDR), and 0.07% for proliferative DR (PDR) [5]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy showed that 75% individuals developed DR at 10 years after the initial diagnosis of diabetes, and approximately two-thirds developed more severe stages [6]. The advanced stage of DR, the PDR with the features of neovascularization and vitreous/pre-retinal hemorrhage, is the most vision-threatening form of the disease [7]. Poor glycemic control and hypertension are considered as the major risk factors for DR [8,9]. Yet, some DM patients would still develop DR even under tight glycemic and blood pressure control. Accurate diagnosis of neovascular form of DR is mainly relied on fundus fluorescein angiography (FFA) and optical coherence tomography (OCT) examinations upon the clinical presentations. Early identification of DR and its advanced forms, prior to the clinical presentation, could facilitate early treatment to prevent further retinal and microvasculature damage and the progression to PDR and severe visual impairment [10].

The metabolites are the intermediate or end products generated during metabolism, catalyzed by various enzymes that naturally occur within the cells [11]. Metabolomics aims to identify the levels of all metabolites with molecular mass <1500 Da (Da) present in the tissue, cellular or fluid samples [12,13]. A metabolomics study on vitreous samples identified the altered glucose metabolism, purine metabolism, and activation of the pentose phosphate pathway in the PDR subjects, as compared to the patients with rhegmatogenous retinal detachment [14]. Moreover, a metabolomics study on serum samples reported 62 differentially abundant metabolites between the non-diabetic and DR subjects, 53 between the non-diabetic and NPDR subjects, and 30 between the NPDR and PDR patients [15]. Similarly, 22 differentially abundant plasma metabolites were found to be associated with the occurrence of DR, which pseudouridine, glutamate, leucylleucine, and N-acetyltryptophan are specifically identified in the PDR patients [16]. Moreover, 8 differentially abundant metabolites from aqueous humor and 15 from vitreous humor were identified between the DR patients and non-diabetic individuals with highlighting the abnormal gluconeogenesis, ascorbate-aldarate and arginine-proline metabolism, and valine-leucine-isoleucine biosynthesis involved in the development of DR [17]. Although vitreous humor should better reflect the metabolism in the retina of the DR patients, it is invasive to collect the intra-ocular fluids. Instead, the collection of tears, as a non-invasive method, offers an alternative strategy for ocular disease biomarker identification [18–20]. Herein, we aimed to delineate the metabolomics profiles in the tear samples of Chinese type-2 DM patients with NPDR and PDR, and compared to that of the non-diabetic subjects. In addition, the predictive performances of the differentially abundant metabolites between NPDR and PDR groups were also evaluated.

2. Materials and methods

2.1. Study subjects

Total 62 study subjects were enrolled at Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong from June to September 2021, including 21 non-diabetic subjects, 21 type-2 DM patients with NPDR, and 20 type-2 DM patients with PDR. All study subjects received routine ophthalmic examinations, including visual acuity, intraocular pressure, slit-lamp examination, and indirect ophthalmoscopy. All PDR subjects and the NPDR subjects at the moderate and severe stages were further arranged to receive the FFA examination, which was confirmed by the retinal specialists (G.Z. and Z.W.) according to the severity scale in the Diabetic Retinopathy Preferred Practice Pattern in 2019 [21]. For the NPDR subjects at the mild stage, if the subjects did not show allergic response to fluorescein sodium, the subjects were also recommended to receive the FFA examination based on their preferences. The inclusion criteria for the PDR subjects was based on the fundus signs with the presence of neovascularization and the vitreous/pre-retinal hemorrhage, whereas that for the NPDR subjects was based on the fundus signs that: (1) mild NPDR: microaneurysms only; (2) moderate NPDR: more than just microaneurysms but less than severe NPDR; or (3) severe NPDR (any of the following and no signs of proliferative retinopathy): more than 20 intra-retinal hemorrhages in each of the 4 quadrants; definite venous beading in 2 quadrants; prominent intra-retinal microvascular abnormalities in 1 quadrant. The non-diabetic subjects were recruited from the senile cataract patients without previous medical history of DM and hypertension. The exclusion criteria included: (1) the subjects with a history of ocular surface diseases, such as conjunctivitis and meibomian gland dysfunction, or other fundus diseases, such as age-related macular degeneration; (2) the subjects with a history of system diseases, such as infections, cancers or immune disorders; (3) the subjects with a history of eye surgery; or (4) the subjects unable to cooperate with the tear collection. This study was approved by the Ethics Committee for Human Medical Research at the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval number: EC20200609 (6)-P24), which is in accordance with the tenets of the Declaration of Helsinki. Written informed consents were obtained from all study subjects after explanation of the nature and possible consequences of the study.

2.2. Tear sample collection and pre-analytical procedures

All tear samples were collected with the Schirmer strips [22,23]. Briefly, the strips were placed at the conjunctival sac of the study subjects for 10 min and gently removed by a sterile forceps. To avoid tear evaporation, the strips were placed into a 2-mL tube and immediately stored at −80 °C before further processing. One strip was applied for each of the two eyes for each study subject, and the two strips from the two eyes for each subject were pooled together as one sample for the metabolomics profiling. Placing the Schirmer

strip on the conjunctiva could be irritable so that repeated collection for the same eye is not recommended. To minimize the inter-eye effect, only the subjects with bilaterally affected and with the same DR stage and similar disease duration were included in this study.

Before the metabolomics profiling, the tear samples were processed as follows: (1) The samples were unfrozen on ice; (2) 80% methanol was added directly to the sample, and the sample was grinded at 65 Hz for 90 s. Vortex oscillation was used to fully mix the samples, and ultrasound was applied at 4 °C for 30 min; (3) The samples were placed at −20 °C for 1 h and centrifuged at 12,000 rpm for 15 min at 4 °C; (4) The supernatant (200 μL) was removed, and 5 μL of internal standard (1 mg/mL dichlorophenylalanine) was added to the chromatographic bottle.

2.3. Metabolomics profiling and analytical procedures

The metabolites in the tear samples were determined by the untargeted liquid chromatography (LC)/MS (LC/MS) platforms (Waters, UPLC; Thermo, Q Exactive). All tear samples were assessed in the same single-run experiment. The conditions of the LC/MS experiment were shown as follows: the separation of all samples was performed on an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) with the temperature sustained at 40 °C. The flow rate was 0.3 mL/min, and the mobile phase was consisted of water +0.05% formic acid (A) and acetonitrile (B). The sample injection volume was 4 μL. The mass spectrometer was operated in both positive and negative ion modes. For the positive mode, the conditions were set as: heater temperature = 300 °C; sheath gas flow rate = 45 arb; auxiliary gas flow rate = 15 arb; sweep gas flow rate = 1 arb; spray voltage = 3.0 kV; capillary temperature = 350 °C; S-Lens RF level = 30%. For the negative mode, the conditions were set as: heater temperature = 300 °C, sheath gas flow rate = 45 arb; auxiliary gas flow rate = 15 arb; sweep gas flow rate = 1 arb; spray voltage = 3.2 kV; capillary temperature = 350 °C; S-Lens RF level = 60%.

The original file was obtained from the mass spectrometry detection for spectral processing, and a database search was performed to obtain the qualitative and quantitative results of metabolites. The data quality control was carried out to ensure the accuracy and reliability of the data. Partial least square discriminant analysis (OPLS-DA) was conducted to identify the differentially abundant metabolites among different groups based on the criteria of VIP (variable importance in the projection) > 1 and $P < 0.05$. Permutation test was conducted for further validation of the model. Commercially available databases, including KEGG (http://www.genome.jp/kegg/) and MetaboAnalyst (http://www.metaboanalyst.ca/), were utilized to search for the pathways of the differentially abundant metabolites.

2.4. Statistical analysis

The continuous variables were presented as mean ± standard deviation (SD) and compared using the independent T-test or one-way analysis of variance (ANOVA) with false discovery rate (FDR) correction. The categorical data was evaluated by the χ^2 test. Receiver operating characteristic (ROC) analysis was used to estimate the sensitivity and specificity of the predictive performance of the metabolites for PDR. All statistical analyses were conducted using the commercially available software (IBM SPSS Statistics 22; SPSS Inc., Chicago, IL). Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Demographics of the study subjects

The mean age of the PDR subjects (57.60 ± 8.56 years) was significantly lower than that of the non-diabetic (67.95 ± 7.49 years, $P < 0.001$) and NPDR subjects (64.76 ± 6.10 years, $P = 0.003$; Table 1). Moreover, there were more male subjects in the non-diabetic group (57.1%) than the PDR group (25%, $P = 0.037$). The blood glucose levels of NPDR (8.74 ± 2.57 mM, $P = 0.001$) and PDR patients (9.50 ± 3.21 mM, $P < 0.001$) were significantly higher than the non-diabetic subjects (6.10 ± 0.61 mM). Additionally, the levels of

Table 1
Demographics and clinical measurements of the study subjects.

Characteristics	Control (n = 21)	NPDR (n = 21)	P (NPDR vs Control)	PDR (n = 20)	P (PDR vs Control)	P (PDR vs NPDR)
Age (years; ±SD)	67.95 ± 7.49	64.76 ± 6.10	0.169	57.60 ± 8.56	<0.001	0.003
Gender (female; %)	9 (42.86)	11 (52.38)	0.537*	15 (75.00)	0.037*	0.133*
DM Duration (years; ±SD)	N/A	7.24 ± 4.56	/	8.40 ± 3.86	/	0.385
DR Duration (years; ±SD)	N/A	1.11 ± 0.93	/	1.37 ± 1.25	/	0.462
Fasting blood glucose (mM)	6.10 ± 0.61	8.74 ± 2.57	0.001	9.50 ± 3.21	<0.001	0.313
HbA1c (%)	N/A	8.13 ± 1.64	/	8.60 ± 1.60	/	0.410
Froctosamine (mM)	0.27 ± 0.03	0.30 ± 0.04	0.009	0.31 ± 0.05	0.005	0.865
Triglycerides (mM)	2.08 ± 2.19	1.59 ± 0.76	0.689	3.09 ± 6.16	0.396	0.223
Total cholesterol (mM)	5.56 ± 1.04	5.36 ± 0.96	0.683	6.21 ± 2.25	0.183	0.091
HDL (mM)	1.47 ± 0.38	1.53 ± 0.43	0.586	1.30 ± 0.25	0.150	0.054
LDL (mM)	3.36 ± 0.90	3.22 ± 0.95	0.700	3.70 ± 1.41	0.321	0.180
Urea (mM)	6.03 ± 1.80	7.40 ± 3.44	0.421	10.27 ± 8.75	0.016	0.098
Creatinine (mM)	77.11 ± 15.18	129.86 ± 175.98	0.174	125.95 ± 118.13	0.214	0.919

*χ^2 test; DM: diabetes mellitus; DR: diabetic retinopathy; HbA_{1C}: hemoglobin A_{1C}; HDL: high density lipoprotein-cholesterol; LDL: low density lipoprotein-cholesterol.

fructosamine among the NPDR (0.30 ± 0.04 mM, $P = 0.009$) and PDR patients (0.31 ± 0.05 mM, $P = 0.005$) were also significantly higher than that in the non-diabetic subjects (0.27 ± 0.03 mM). Yet, there were no significant differences in blood glucose, fructosamine, and hemoglobin A_{1C} (HbA_{1C}) levels as well as the DM and DR durations between the NPDR and PDR patients. Furthermore, the

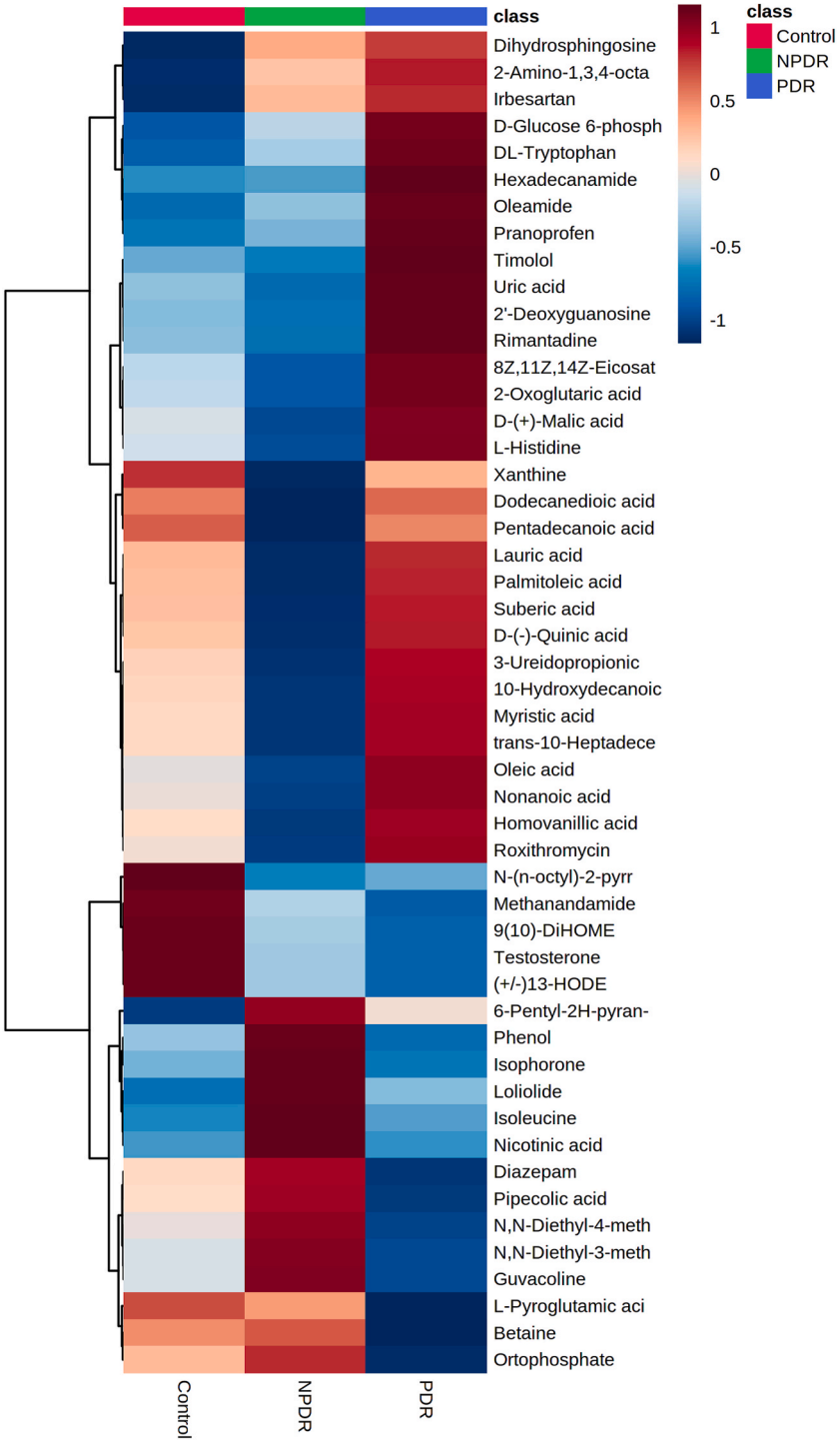

Fig. 1. Heatmap of tear metabolites among the three study groups. Tear samples were collected from total 62 study subjects, including 21 non-proliferative DR (NPDR), 20 proliferative DR (PDR), and 21 non-diabetic control subjects. Hierarchical clustering of the identified metabolites from the tear samples among the PDR, NPDR and control groups. Red: upregulation; Blue: downregulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

urea level of the PDR patients (10.27 ± 8.75 mM) was significantly higher than that of the non-diabetic subjects (6.03 ± 1.80 mM, $P = 0.016$). Other clinical parameters showed no statistically significant differences among the three study groups.

3.2. Metabolomics profiling of tear samples in non-proliferative and proliferative diabetic retinopathy patients

Total 3213 peaks were detected by LC-MS from all 62 tear samples. The score plots from the OPLS-DA models demonstrated cumulative R^2Y of 0.755 and Q^2 of 0.428 for the comparison between total DR (NPDR + PDR) and the non-diabetic groups (Supplementary Fig. 1A), cumulative R^2Y of 0.861 and Q^2 of 0.391 for the comparison between the NPDR and non-diabetic groups (Supplementary Fig. 1B), cumulative R^2Y of 0.877 and Q^2 of 0.704 for the comparison between PDR and the non-diabetic groups (Supplementary Fig. 1C), and cumulative R^2Y of 0.625 and Q^2 of 0.305 for the comparison between the NPDR and PDR groups (Supplementary Fig. 1D). Permutation test plots repeated 200 iterations of the OPLS-DA, indicating the robustness of the models and showing a low risk of over fitting and reliable (Supplementary Fig. 2). The heat map showed clear separations of the identified metabolites among the PDR, NPDR, and non-diabetic groups (Fig. 1).

The LC/MS platform detected 196 metabolites in the tear samples by the secondary mass spectrometry qualitative matching analysis (Supplementary Table 1). Upon data analysis, the volcano plot showed 14 significant differentially abundant metabolites between total DR (NPDR + PDR) and the non-diabetic groups, including 6 upregulated and 8 downregulated metabolites in the total DR group (Fig. 2A). 3-Indoxyl sulphate was the most upregulated metabolite (2.37 folds, $P_{corr} = 0.002$) in total DR group, whereas phenol was the most downregulated metabolite (−1.29 folds, $P_{corr} = 0.040$; Table 2). For the DR sub-group analysis, 9 metabolites showed significant differences between the NPDR and non-diabetic groups, including 1 upregulated and 8 downregulated metabolites in the NPDR group (Fig. 2B). Phenol was the only upregulated metabolite (1.40 folds, $P_{corr} = 0.023$) in the NPDR group, whereas benzoic acid was the most downregulated metabolite (−1.41 folds, $P_{corr} = 0.028$; Supplementary Table 2). For the PDR, 21 metabolites showed significant differences as compared to the non-diabetic group, including 8 upregulated and 13 downregulated metabolites in the PDR group (Fig. 2C). N-phenylacetylglutamine was the most upregulated metabolite (2.98 folds, $P_{corr} = 0.039$) in the PDR group, whereas (±)9 (10)-DiHOME was the most downregulated metabolite (−1.50 folds, $P_{corr} < 0.001$; Supplementary Table 3). To compare

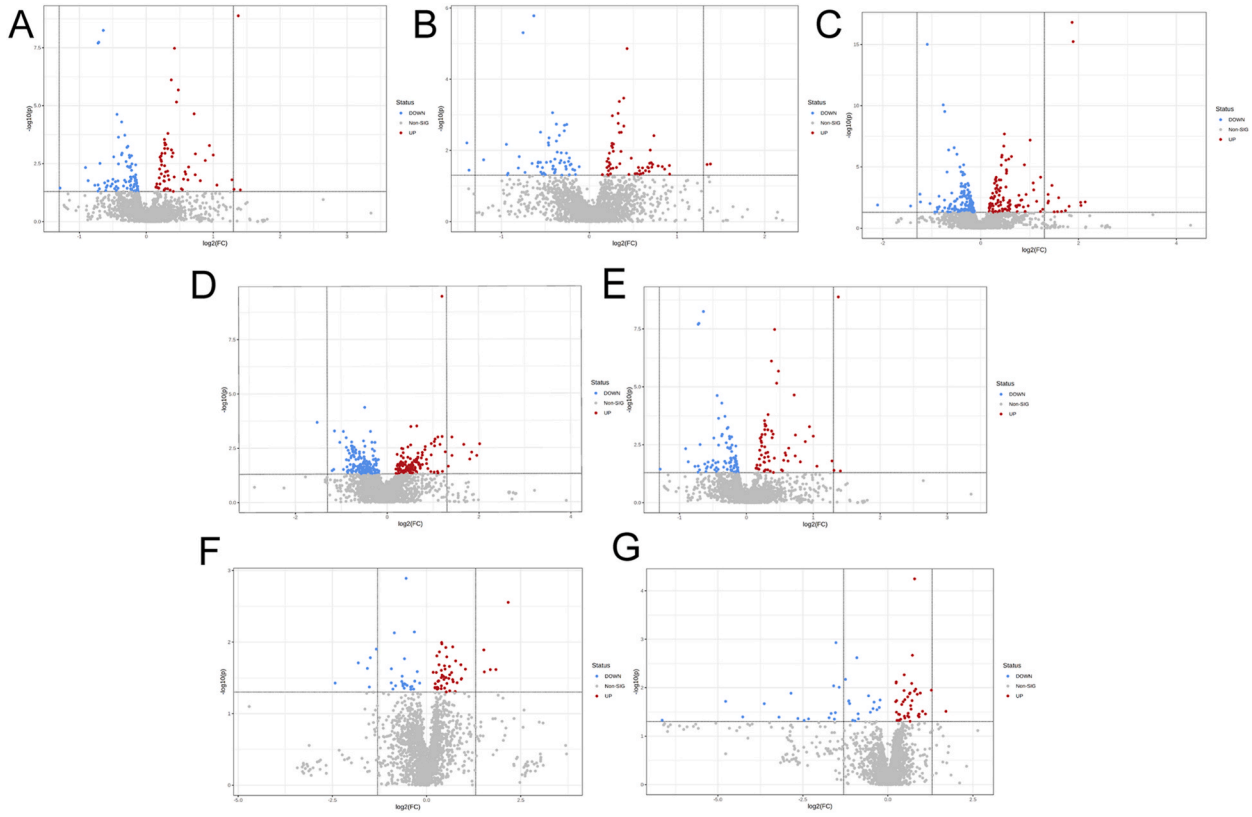

Fig. 2. Volcano plots of tear metabolite expression in patients with diabetic retinopathy. Metabolomics profiling of tear samples between (A) Total diabetic retinopathy (DR) (non-proliferative DR (NPDR) + proliferative DR (PDR)) versus control; (B) NPDR versus control; (C) PDR versus control; (D) PDR versus NPDR; (E) NPDR (duration >1 year) versus NPDR (duration ≤1 year); (F) PDR (duration >1 year) versus PDR (duration ≤1 year); (G) PDR (blood glucose level >9.0 mM) versus PDR (blood glucose level ≤9.0 mM). Red dot: significant upregulation; Blue dot: significant downregulation; Grey dot: no significant change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Differentially expressed metabolites in the tear samples of total diabetic retinopathy subjects compared to the control subjects.

Metabolites	VIP	P_{corr}	Fold Change	Type
3-Indoxyl sulphate	1.009	0.002	2.366	Up
2-Amino-1,3,4-octadecanetriol	1.855	0.003	1.880	Up
Hexadecanamide	1.370	0.007	1.496	Up
6-Pentyl-2H-pyran-2-one	1.382	0.036	1.302	Up
Dihydrosphingosine	1.125	0.031	1.290	Up
Andrastin D	1.639	0.024	1.263	Up
Phenol	1.673	0.040	1.287	Down
(±)9 (10)-DiHOME	1.989	0.033	1.272	Down
Testosterone	1.868	0.004	1.215	Down
DEET	2.179	0.007	1.181	Down
N-(n-octyl)-2-pyrrolidinone	1.327	0.019	1.117	Down
Indole	1.380	0.035	1.111	Down
Triptophenolide	1.229	0.037	1.103	Down
2-(Methylthio)benzothiazole	1.316	0.049	1.096	Down

Total diabetic retinopathy = non-proliferative diabetic retinopathy + proliferative diabetic retinopathy. DEET: N,N-diethyl-meta-toluamide; VIP: variable importance in the projection.

the metabolomics profile of PDR with that of NPDR, 17 metabolites showed significant differences in the PDR group as compared to the NPDR group, including 6 upregulated and 11 downregulated metabolites in the PDR group (Fig. 2D). Guanosine was the most upregulated metabolite (1.72 folds, P_{corr} = 0.019) in the PDR group, whereas N,N-diethyl-4-methoxybenzamide was the most downregulated metabolite (−1.73 folds, P_{corr} = 0.031; Table 3).

To better characterize the metabolic features with different DR-associated factors, the NPDR and PDR subjects were further divided into subgroups according to the DR duration (more than or below 1 year) and blood glucose level (more than or below 9.0 mM). The volcano plot showed 3 significantly downregulated metabolites in the NPDR subjects with DR duration >1 year as compared to those ≤1 year (Fig. 2E). 3-Ureidopropionic acid was the most downregulated metabolite (−2.22 folds, P_{corr} = 0.030) in the NPDR subjects with DR duration >1 year (Supplementary Table 4). For the PDR subjects, 9 metabolites showed significant differences in the PDR subjects with DR duration >1 year as compared to those ≤1 year, including 5 upregulated and 4 downregulated metabolites in the PDR subjects with DR duration >1 year (Fig. 2F). Hexadecanamide was the most upregulated metabolite (6.01 folds, P_{corr} = 0.023), whereas N-acetylneuraminic acid was the most downregulated metabolite (−1.58 folds, P_{corr} = 0.036; Supplementary Table 5). For the stratification with the blood glucose level, 6 metabolites showed significant differences in the PDR subjects with blood glucose level >9 mM as compared to those ≤9 mM, including 3 upregulated and 3 downregulated metabolites in the PDR subjects with blood glucose level >9 mM (Fig. 2G). All trans retinal was the most upregulated metabolite (1.77 folds, P_{corr} = 0.009), whereas skatole was the most downregulated metabolite (−3.18 folds, P_{corr} = 0.019; Supplementary Table 6). Yet, no differentially expressed metabolite was identified in the NPDR subjects with blood glucose level >9.0 mM as compared to those with blood glucose level ≤9.0 mM.

3.3. Pathway analysis on the differentially abundant metabolites

To delineate the potential biological functions of the differentially abundant metabolites, the pathway analysis was conducted.

Table 3
Differentially expressed metabolites in the tear samples of proliferative diabetic retinopathy subjects compared to the non-proliferative diabetic retinopathy subjects.

Metabolites	VIP	P_{corr}	Fold Change	Type
Guanosine	2.395	0.019	1.724	Up
Uric acid	2.360	0.020	1.536	Up
D-(+)-malic acid	1.264	0.041	1.527	Up
Pimelic acid	1.990	0.014	1.399	Up
Azelaic acid	2.041	0.008	1.346	Up
2-Hydroxybenzothiazole	1.002	0.024	1.209	Up
N, N-Diethyl-4-methoxybenzamide	1.873	0.031	1.732	Down
Homovanillic acid	1.246	0.015	1.632	Down
Phenol	1.653	0.009	1.467	Down
Pipecolic acid	1.929	0.046	1.209	Down
Guvacoline	2.244	0.041	1.207	Down
Spironolactone	1.827	0.044	1.416	Down
N,N-Diethyl-3-methoxybenzamide	1.807	0.029	1.411	Down
Diazepam	2.126	0.009	1.350	Down
Prostaglandin F2α 1-11-lactone	1.339	0.044	1.248	Down
2-Methoxyestrone	2.201	0.014	1.176	Down
Tretinoin	1.447	0.015	1.127	Down

VIP: variable importance in the projection.

There was no significant pathway identified in the comparison of total DR or NPDR with the non-diabetic groups as well as PDR with NPDR groups (Supplementary Table 7). Instead, D-glutamine and D-glutamate metabolism was significantly highlighted in the comparison between the PDR and non-diabetic groups (impact: 0.000, $P = 0.046$; Fig. 3A). Moreover, pantothenate and CoA biosynthesis (impact: 0.029, $P = 0.024$), β-alanine metabolism (impact: 0.104, $P = 0.027$), arginine and proline metabolism (impact: 0.078, $P = 0.048$), and pyrimidine metabolism (impact: 0.013, $P = 0.050$) were significantly highlighted in the NPDR subjects with DR duration >1 year as compared to those ≤1 year (Fig. 3B). Additionally, phenylalanine metabolism was significantly highlighted in the PDR subjects with blood glucose level >9 mM as compared to those ≤9 mM (impact: 0.143, $P = 0.019$; Fig. 3C).

3.4. Predictive performance of the metabolites for PDR

To further evaluate the 17 differentially abundant metabolites for potential discrimination of PDR from NPDR, the ROC analysis was conducted (Supplementary Table 8). For the comparison between the NPDR and PDR groups, azelaic acid achieved an area under ROC curve (AUC) of 0.802 (95% C.I.: 0.639–0.925; Fig. 4A), uric acid achieved an AUC of 0.740 (95% C.I.: 0.583–0.886; Fig. 4B), and guanosine achieved an AUC of 0.738 (95% C.I.: 0.578–0.864; Fig. 4C). For the combination analysis, azelaic acid combined with uric acid achieved an AUC of 0.845 (95% C.I.: 0.687–0.964; Fig. 4D), whereas azelaic acid combined with guanosine achieved an AUC of 0.855 (95% C.I.: 0.665–0.987; Fig. 4E). Yet, combination of azelaic acid, uric acid, and guanosine did not further enhance the AUC (0.829, 95% C.I.: 0.660–0.964; Fig. 4F).

4. Discussion

Results from this study showed that: (1) 14 metabolites in tears showed significantly differential expression between total DR and the non-diabetic groups; (2) 17 metabolites showed significantly differential expression between the NPDR and PDR groups; (3) D-glutamine and D-glutamate metabolism was significantly highlighted in the PDR group as compared to the non-diabetic group; (4) azelaic acid combined with guanosine achieved the AUC of 0.855 in the comparison between the NPDR and PDR groups. Collectively, our results demonstrate the metabolomics profiling and potential biomarkers in the tear samples of the NPDR and PDR subjects.

4.1. Metabolism between DM and non-DM patients

In this study, total 44 differentially abundant metabolites were identified in total DR, NPDR, and PDR groups as compared to the non-diabetic subjects (Fig. 2, Table 2 and Supplementary Tables 2 and 3). With the matching analysis, phenol, DEET (N,N-diethyl-meta-toluamide), and testosterone were found to be the commonly downregulated metabolites among the 3 comparisons. Although there is no report on the relationship between phenol and DEET with DR, the lower level of testosterone has been frequently reported with type-2 DM [24]. Moreover, we found that the levels of uric acid and phenylacetylglutamine were elevated in the PDR subjects, whereas the levels of pyroglutamic acid and pipecolic acid were decreased (Fig. 2C and Supplementary Table 3). A systematic review and meta-analysis reported that uric acid levels are higher in the DR patients as compared to the controls [25]. Similarly, the concentrations of serum uric acid are associated with the severity of DR in the individuals with type-2 DM [26]. These metabolites could reflect the common dysregulation of the corresponding metabolic pathways in different biofluids of the DR patients.

For the pathway analysis, D-glutamine and D-glutamate metabolism was highlighted in the comparison between the PDR and non-diabetic groups (Fig. 3A and Supplementary Table 7), with 2-oxoglutarate (also known as α-ketoglutarate) significantly upregulated in the PDR subjects (Supplementary Table 3). α-Ketoglutarate is a metabolite of glutaminolysis, which plays a critical role in cell growth

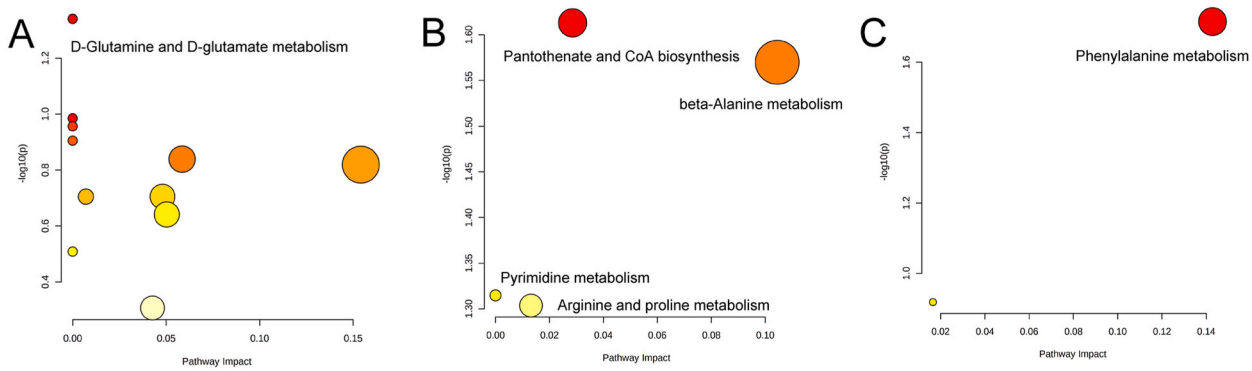

Fig. 3. Pathway analysis on the differentially abundant metabolites. The differentially expressed genes among the comparisons of (A) Proliferative diabetic retinopathy (PDR) vs control; (B) non-proliferative DR (NPDR) (duration >1 year) vs NPDR (duration ≤1 year); (C) PDR (blood glucose level >9.0 mM) vs PDR (blood glucose level ≤9.0 mM) were subjected to the pathway analysis. The size of the bubble and *x*-axis represents the impact of the pathway, and the color of the bubble and *y*-axis represents the negative of the logarithm of the *p*-value in the enrichment analysis ($-\log_{10} P > 1.3$ was considered as statistically significant). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

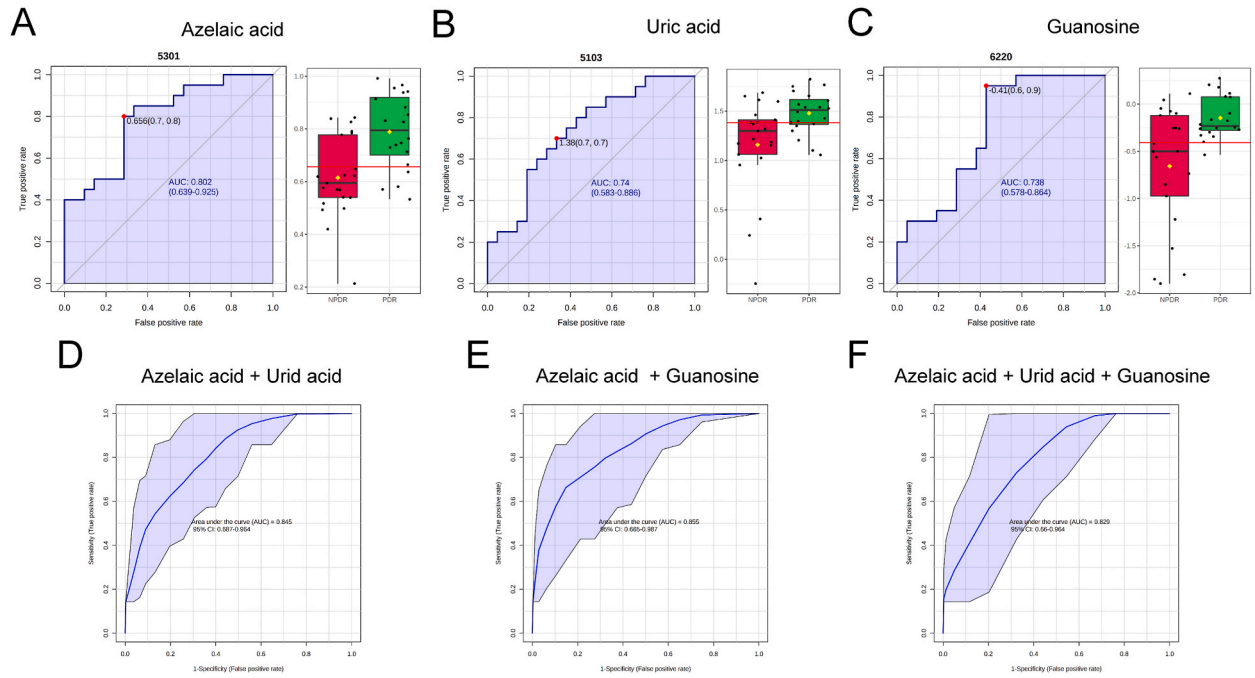

Fig. 4. Receiver operating characteristic analysis for PDR and NPDR discrimination. Total 17 differentially abundant metabolites between non-proliferative diabetic retinopathy (NPDR) and proliferative DR (PDR) were subjected to the receiver operating characteristic (ROC) analysis, and top 3 metabolites with highest area under ROC curve (AUC) were shown: (A) Azelaic acid; (B) Uric acid; (C) Guanosine. (D) Azelaic acid combined with uric acid; (E) Azelaic acid combined with guanosine; (F) Combination of azelaic acid, uric acid, and guanosine. X-axis: the false positive rate; Y-axis: the true positive rate.

and proliferation, skeletal development, immune system homeostasis, and cancer metabolism [27,28]. Genome-wide association studies have identified the α-ketoglutarate-dependent dioxygenase (*FTO*) gene variants as an important genetic determinant for DM [29]. In contrast, a metabolomics study reported an intracellular reduction of α-ketoglutarate synthesis in diabetic human cardiac mesenchymal cells and in the whole heart of high-fat diet-fed mice [30].

4.2. Metabolism between NPDR and PDR patients

This study, for the first time, demonstrated the association of azelaic acid with DR (Table 3). We also showed that azelaic acid alone or combined with guanosine showed a considerable AUC in the comparison of NPDR with PDR that their levels are higher in PDR subjects than the NPDR subjects (Fig. 4E), indicating that they could be the potential biomarkers in tears for PDR analysis. Azelaic acid has been reported to be one of the differentially abundant metabolites between diabetic nephropathy with macroalbuminuria and diabetic patients without albuminuria [31]. The level of azelaic acid is highly correlated with the carotid intima-media thickness [32], which shows thicker in the patients with DR [33]. Yet, azelaic acid treatment could restore the levels of plasma glucose, insulin, HbA_{1C}, blood hemoglobin, liver glycogen, and carbohydrate metabolic key enzymes to nearly normal level in diabetic mice [34]. How azelaic acid is involved in the development of PDR requires further investigations.

For the pathway analysis, arginine-proline metabolism, β-alanine metabolism, and pyrimidine metabolism were highlighted among the NPDR subjects with different DR durations (Fig. 3B), with D-proline as the matched metabolite for the arginine-proline metabolism and 3-ureidopropionate as the matched metabolite for the β-alanine metabolism and pyrimidine metabolism (Supplementary Table 7). Free D-proline has been found in human plasma and saliva [35]. D-proline has been found to be significantly decreased in the rats with diabetic neuropathic pain [36]. In contrast, the patients with mild-cognitive-impairment show higher D-proline proportion in the blood than the healthy control subjects [37]. 3-Ureidopropionate, a physiological metabolite in pyrimidine degradation, could act as endogenous neurotoxin via inhibiting the mitochondrial energy metabolism [38]. Plasma 3-ureidopropionate level was found to be increased in the subjects with high liver and kidney disease severity [39]. Moreover, higher 3-ureidopropionate level was also identified in the serum of coronary heart disease patients treated with a classical traditional Chinese medication (Taohong Siwu Decoction) [40]. Yet, 3-ureidopropionate has not been reported to be associated with DM or DR.

4.3. Comparisons of metabolome with other matrices

Compared to previous metabolomics studies on the vitreous, serum, and plasma samples [17,41–43], differential expressions in pyroglutamic acid, uric acid, pipecolic acid, and phenylacetylglutamine were observed in our tear samples in this study. Increased

expression of N-phenylacetylglutamine has been reported in the vitreous and plasma samples of the DR patients as compared to the non-diabetic subjects, while the expression of pipecolic acid was decreased [43]. Moreover, the α-ketoglutarate-dependent dioxygenase has been shown to be upregulated in the vitreous humor of DR patients [44].

4.4. Limitations

There were several limitations in this study. First, the non-diabetic subjects were adopted in this study. The DM patients without DR should better be the control subjects in order to understand the metabolic changes from DM to DR. Second, the metabolomics profiling was not stratified with age and sex due to small number of recruited study subjects. Larger sample size is needed to delineate the metabolic profiles in the patients with age and sex stratifications. Third, the DR patients were under medication for DM, which could influence the metabolite levels in the body. Besides, we did not evaluate the microbiomes on the ocular surface of the study subjects, which could also contribute to the metabolite changes in tear samples [45]. For the collection of the tear samples, microcapillary tube might be superior to the Schirmer strip with fewer variability for metabolites detection, whereas, for the extraction from the tear samples, methanol could yield more variability than 1:1 methanol/water [46]. Furthermore, this study was based on the non-targeted metabolomics analysis. Further analyses are needed to determine specific classes of metabolites using the targeted metabolomics.

5. Conclusion

This study revealed the metabolomics profiles of tear samples in Chinese type-2 DM patients with NPDR and PDR. Azelaic acid and guanosine could be the potential biomarkers in tears to distinguish PDR from NPDR in the DR assessment.

Ethics statement

This study was approved by the Ethics Committee for Human Medical Research at the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval number: EC20200609 (6)-P24), which is in accordance with the tenets of the Declaration of Helsinki. Written informed consents were obtained from all study subjects after explanation of the nature and possible consequences of the study.

Author contribution statement

Xin Wen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Tsz Kin Ng: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Qingping Liu: Conceived and designed the experiments.
Zhenggen Wu, Guihua Zhang, Mingzhi Zhang: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by 2020 Li Ka Shing Foundation Cross-Disciplinary Research Grant {2020LKSFG14B}.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare no potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16109.

References

[1] R. Klein, K.E. Lee, R.E. Gangnon, B.E. Klein, The 25-year incidence of visual impairment in type 1 diabetes mellitus the Wisconsin epidemiologic study of diabetic retinopathy, Ophthalmology 117 (2010) 63–70.
[2] D.A. Antonetti, R. Klein, T.W. Gardner, Diabetic retinopathy, N. Engl. J. Med. 366 (2012) 1227–1239.
[3] N. Cheung, P. Mitchell, T.Y. Wong, Diabetic retinopathy, Lancet 376 (2010) 124–136.
[4] J.W. Yau, S.L. Rogers, R. Kawasaki, E.L. Lamoureux, J.W. Kowalski, T. Bek, S.J. Chen, J.M. Dekker, A. Fletcher, J. Grauslund, S. Haffner, R.F. Hamman, M. K. Ikram, T. Kayama, B.E. Klein, R. Klein, S. Krishnaiah, K. Mayurasakorn, J.P. O'Hare, T.J. Orchard, M. Porta, M. Rema, M.S. Roy, T. Sharma, J. Shaw,

H. Taylor, J.M. Tielsch, R. Varma, J.J. Wang, N. Wang, S. West, L. Xu, M. Yasuda, X. Zhang, P. Mitchell, T.Y.G. Wong, Meta-Analysis for Eye Disease Study, Global prevalence and major risk factors of diabetic retinopathy, Diabetes Care 35 (2012) 556–564.
[5] P. Song, J. Yu, K.Y. Chan, E. Theodoratou, I. Rudan, Prevalence, risk factors and burden of diabetic retinopathy in China: a systematic review and meta-analysis, J. Glob. Health 8 (2018), 010803.
[6] R. Klein, B.E. Klein, S.E. Moss, K.J. Cruickshanks, The Wisconsin Epidemiologic Study of Diabetic Retinopathy. XV. The long-term incidence of macular edema, Ophthalmology 102 (1995) 7–16.
[7] T.Y. Wong, C.M. Cheung, M. Larsen, S. Sharma, R. Simo, Diabetic retinopathy, Nat. Rev. Dis. Prim. 2 (2016), 16012.
[8] J. Ding, T.Y. Wong, Current epidemiology of diabetic retinopathy and diabetic macular edema, Curr. Diabetes Rep. 12 (2012) 346–354.
[9] G.S. Tan, M.K. Ikram, T.Y. Wong, Traditional and novel risk factors of diabetic retinopathy and research challenges, Curr. Med. Chem. 20 (2013) 3189–3199.
[10] S. Hagan, E. Martin, A. Enriquez-de-Salamanca, Tear fluid biomarkers in ocular and systemic disease: potential use for predictive, preventive and personalised medicine, EPMA J. 7 (2016) 15.
[11] J.L. McCarville, G.Y. Chen, V.D. Cuevas, K. Troha, J.S. Ayres, Microbiota metabolites in health and disease, Annu. Rev. Immunol. 38 (2020) 147–170.
[12] J.K. Nicholson, J.C. Lindon, Systems biology: metabonomics, Nature 455 (2008) 1054–1056.
[13] L.A. Filla, J.L. Edwards, Metabolomics in diabetic complications, Mol. Biosyst. 12 (2016) 1090–1105.
[14] N.R. Haines, N. Manoharan, J.L. Olson, A. D'Alessandro, J.A. Reisz, Metabolomics analysis of human vitreous in diabetic retinopathy and rhegmatogenous retinal detachment, J. Proteome Res. 17 (2018) 2421–2427.
[15] J.H. Yun, J.M. Kim, H.J. Jeon, T. Oh, H.J. Choi, B.J. Kim, Metabolomics profiles associated with diabetic retinopathy in type 2 diabetes patients, PLoS One 15 (2020), e0241365.
[16] Y. Sun, H. Zou, X. Li, S. Xu, C. Liu, Plasma metabolomics reveals metabolic profiling for diabetic retinopathy and disease progression, Front. Endocrinol. 12 (2021), 757088.
[17] H. Wang, J. Fang, F. Chen, Q. Sun, X. Xu, S.H. Lin, K. Liu, Metabolomic profile of diabetic retinopathy: a GC-TOFMS-based approach using vitreous and aqueous humor, Acta Diabetol. 57 (2020) 41–51.
[18] C.N. Brown, B.D. Green, R.B. Thompson, A.I. den Hollander, I. Lengyel, E.-R. consortium, Metabolomics and age-related macular degeneration, Metabolites 9 (2018).
[19] L. Zhou, R.W. Beuerman, Tear analysis in ocular surface diseases, Prog. Retin. Eye Res. 31 (2012) 527–550.
[20] M. Yazdani, K.B.P. Elgstoen, H. Rootwelt, A. Shahdadfar, O.A. Utheim, T.P. Utheim, Tear metabolomics in dry eye disease: a review, Int. J. Mol. Sci. 20 (2019).
[21] C.J. Flaxel, R.A. Adelman, S.T. Bailey, A. Fawzi, J.I. Lim, G.A. Vemulakonda, G.S. Ying, Diabetic retinopathy preferred Practice pattern(R), Ophthalmology 127 (2020) P66–P145.
[22] A. Posa, L. Brauer, M. Schicht, F. Garreis, S. Beileke, F. Paulsen, Schirmer strip vs. capillary tube method: non-invasive methods of obtaining proteins from tear fluid, Ann. Anat. 195 (2013) 137–142.
[23] G. Urbanski, S. Assad, F. Chabrun, J.M. Chao de la Barca, O. Blanchet, G. Simard, G. Lenaers, D. Prunier-Mirebeau, P. Gohier, C. Lavigne, P. Reynier, Tear metabolomics highlights new potential biomarkers for differentiating between Sjogren's syndrome and other causes of dry eye, Ocul. Surf. 22 (2021) 110–116.
[24] J.T. George, J.D. Veldhuis, M. Tena-Sempere, R.P. Millar, R.A. Anderson, Exploring the pathophysiology of hypogonadism in men with type 2 diabetes: kisspeptin-10 stimulates serum testosterone and LH secretion in men with type 2 diabetes and mild biochemical hypogonadism, Clin. Endocrinol. 79 (2013) 100–104.
[25] Y. Guo, S. Liu, H. Xu, Uric acid and diabetic retinopathy: a systematic review and meta-analysis, Front. Public Health 10 (2022), 906760.
[26] L. Hou, Y. Shi, S. Wang, Q. Chen, Q. Li, M. Zhao, X. Zhou, Associations of serum uric acid level with diabetic retinopathy and albuminuria in patients with type 2 diabetes mellitus, J. Int. Med. Res. 48 (2020), 300060520963980.
[27] N. Wu, M. Yang, U. Gaur, H. Xu, Y. Yao, D. Li, Alpha-ketoglutarate: physiological functions and applications, Biomol. Ther. 24 (2016) 1–8.
[28] D. Xiao, L. Zeng, K. Yao, X. Kong, G. Wu, Y. Yin, The glutamine-alpha-ketoglutarate (AKG) metabolism and its nutritional implications, Amino Acids 48 (2016) 2067–2080.
[29] L.J. Scott, K.L. Mohlke, L.L. Bonnycastle, C.J. Willer, Y. Li, W.L. Duren, M.R. Erdos, H.M. Stringham, P.S. Chines, A.U. Jackson, L. Prokunina-Olsson, C.J. Ding, A.J. Swift, N. Narisu, T. Hu, R. Pruim, R. Xiao, X.Y. Li, K.N. Conneely, N.L. Riebow, A.G. Sprau, M. Tong, P.P. White, K.N. Hetrick, M.W. Barnhart, C.W. Bark, J. L. Goldstein, L. Watkins, F. Xiang, J. Saramies, T.A. Buchanan, R.M. Watanabe, T.T. Valle, L. Kinnunen, G.R. Abecasis, E.W. Pugh, K.F. Doheny, R.N. Bergman, J. Tuomilehto, F.S. Collins, M. Boehnke, A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants, Science 316 (2007) 1341–1345.
[30] F. Spallotta, C. Cencioni, S. Atlante, D. Garella, M. Cocco, M. Mori, R. Mastrocola, C. Kuenne, S. Guenther, S. Nanni, V. Azzimato, S. Zukunft, A. Kornberger, D. Surun, F. Schnutgen, H. von Melchner, A. Di Stilo, M. Aragno, M. Braspenning, W. van Criekinge, M.J. De Blasio, R.H. Ritchie, G. Zaccagnini, F. Martelli, A. Farsetti, I. Fleming, T. Braun, A. Beiras-Fernandez, B. Botta, M. Collino, M. Bertinaria, A.M. Zeiher, C. Gaetano, Stable oxidative cytosine modifications accumulate in cardiac mesenchymal cells from Type2 diabetes patients: rescue by alpha-ketoglutarate and TET-TDG functional reactivation, Circ. Res. 122 (2018) 31–46.
[31] A. Hirayama, E. Nakashima, M. Sugimoto, S. Akiyama, W. Sato, S. Maruyama, S. Matsuo, M. Tomita, Y. Yuzawa, T. Soga, Metabolic profiling reveals new serum biomarkers for differentiating diabetic nephropathy, Anal. Bioanal. Chem. 404 (2012) 3101–3109.
[32] M. Vinaixa, M.A. Rodriguez, S. Samino, M. Diaz, A. Beltran, R. Mallol, C. Blade, L. Ibanez, X. Correig, O. Yanes, Metabolomics reveals reduction of metabolic oxidation in women with polycystic ovary syndrome after pioglitazone-flutamide-metformin polytherapy, PLoS One 6 (2011), e29052.
[33] A.S. Talpur, Z. Amar, S. Zafar, A. Memon, A.H. Eimal Latif, F. Hafizyar, S. Hashim, K. Nazary, Association between diabetic retinopathy and carotid intima-media thickness, Cureus 13 (2021), e15575.
[34] S. Muthulakshmi, R. Saravanan, Efficacy of azelaic acid on hepatic key enzymes of carbohydrate metabolism in high fat diet induced type 2 diabetic mice, Biochimie 95 (2013) 1239–1244.
[35] Y. Nagata, R. Masui, T. Akino, The presence of free D-serine, D-alanine and D-proline in human plasma, Experientia 48 (1992) 986–988.
[36] Q. Zhang, Q. Li, S. Liu, H. Zheng, L. Ji, N. Yi, X. Zhu, W. Sun, X. Liu, S. Zhang, Y. Li, Q. Xiong, B. Lu, Decreased amino acids in the brain might contribute to the progression of diabetic neuropathic pain, Diabetes Res. Clin. Pract. 176 (2021), 108790.
[37] R. Kimura, H. Tsujimura, M. Tsuchiya, S. Soga, N. Ota, A. Tanaka, H. Kim, Development of a cognitive function marker based on D-amino acid proportions using new chiral tandem LC-MS/MS systems, Sci. Rep. 10 (2020) 804.
[38] S. Kolker, J.G. Okun, F. Horster, B. Assmann, B. Ahlemeyer, D. Kohlmuller, S. Exner-Camps, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, 3-Ureidopropionate contributes to the neuropathology of 3-ureidopropionase deficiency and severe propionic aciduria: a hypothesis, J. Neurosci. Res. 66 (2001) 666–673.
[39] A.L. Mindikoglu, A.R. Opekun, N. Putluri, S. Devaraj, D. Sheikh-Hamad, J.M. Vierling, J.A. Goss, A. Rana, G.K. Sood, P.K. Jalal, L.A. Inker, R.P. Mohney, H. Tighiouart, R.H. Christenson, T.C. Dowling, M.R. Weir, S.L. Seliger, W.R. Hutson, C.D. Howell, J.P. Raufman, L.S. Magder, C. Coarfa, Unique metabolomic signature associated with hepatorenal dysfunction and mortality in cirrhosis, Transl. Res. 195 (2018) 25–47.
[40] T. Tao, T. He, H. Mao, X. Wu, X. Liu, Non-targeted metabolomic profiling of coronary heart disease patients with Taohong Siwu decoction treatment, Front. Pharmacol. 11 (2020) 651.
[41] J. Zuo, Y. Lan, H. Hu, X. Hou, J. Li, T. Wang, H. Zhang, N. Zhang, C. Guo, F. Peng, S. Zhao, Y. Wei, C. Jia, C. Zheng, G. Mao, Metabolomics-based multidimensional network biomarkers for diabetic retinopathy identification in patients with type 2 diabetes mellitus, BMJ Open Diabetes Res. Care 9 (2021).
[42] Q. Xuan, Y. Ouyang, Y. Wang, L. Wu, H. Li, Y. Luo, X. Zhao, D. Feng, W. Qin, C. Hu, L. Zhou, X. Liu, H. Zou, C. Cai, J. Wu, W. Jia, G. Xu, Multiplatform metabolomics reveals novel serum metabolite biomarkers in diabetic retinopathy subjects, Adv. Sci. 7 (2020), 2001714.
[43] H. Wang, S. Li, C. Wang, Y. Wang, J. Fang, K. Liu, Plasma and vitreous metabolomics profiling of proliferative diabetic retinopathy, Invest. Ophthalmol. Vis. Sci. 63 (2022) 17.

[44] M. He, W. Wang, H. Yu, D. Wang, D. Cao, Y. Zeng, Q. Wu, P. Zhong, Z. Cheng, Y. Hu, L. Zhang, Comparison of expression profiling of circular RNAs in vitreous humour between diabetic retinopathy and non-diabetes mellitus patients, Acta Diabetol. 57 (2020) 479–489.
[45] P. Aragona, C. Baudouin, J.M. Benitez Del Castillo, E. Messmer, S. Barabino, J. Merayo-Lloves, F. Brignole-Baudouin, L. Inferrera, M. Rolando, R. Mencucci, M. Rescigno, S. Bonini, M. Labetoulle, The ocular microbiome and microbiota and their effects on ocular surface pathophysiology and disorders, Surv. Ophthalmol. 66 (2021) 907–925.
[46] S. Catanese, R.K. Khanna, A. Lefevre, H. Alarcan, P.J. Pisella, P. Emond, H. Blasco, Validation of metabolomic and lipidomic analyses of human tears using ultra-high-performance liquid chromatography tandem mass spectrometry, Talanta 253 (2023), 123932.