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Serum metabolomic profiles and semaphorin-3A as biomarkers of diabetic retinopathy progression

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Abstract

Diabetic retinopathy (DR) is a typical microvascular complication of diabetes mellitus (DM) and it remains one of the leading causes of vision loss worldwide. Studies postulated that a distinct metabolic signature of DR exists and can be resolved from that of diabetes alone. Serum Semaphorin3A (Sema3A) levels have also been found to be correlated with the phenotypes of diabetic retinopathy. We aimed to analyze and identify serum metabolites and serum Sema3A levels that could be useful biomarkers of DR progression. This cross-sectional study included 45 type 2 diabetes (T2D) patients. Diabetic patients were divided into three groups based on the status of their complications: non-DR (NDR, n=15), non-proliferative DR (NPDR, n=15), and proliferative DR (PDR, n=15) groups. Serum metabolomic profiles of these patients were determined by using high-performance liquid chromatography-mass spectrometry (HPLC-MS), and serum Sema3A levels measured by ELISA. Metabolomics analysis revealed a set of metabolites that were altered in the serum of PDR patients as compared with NPDR and NDR groups. Among these metabolites total asymmetric dimethylarginine (ADMA) and Kynurenine were potential predictors of PDR patients. Significantly higher serum levels of Sema3A in PDR patients as compared with NPDR and NDR groups (p<0.001), their serum levels were positively correlated with the central macular thickness (r= 0.952, p<0.001) and negatively correlated with the superficial macular density (r=-0.952, p<0.001). In conclusion, the metabolite signatures identified in this study and serum Sema3A levels could be proposed as biomarkers for DR development and progression in T2D patients. However, Sema3A was superior to metabolomics in the prediction of the severity of DR.

Keywords: Diabetic retinopathy, Metabolomic profiles, Semaphorin3A

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Introduction

Diabetic retinopathy (DR) is a major complication of diabetes mellitus (DM), and it is the consequence of microvascular retinal changes caused by high glucose levels over a long time. DR is the leading cause of blindness in working-age adults[1]. The prevalence of DR will likely increase as the global population with diabetes is projected to rise by 55% from 2013 to 2035. A large proportion of DR-related blindness is preventable with early diagnosis and treatment. Current treatment options for DR, including laser photocoagulation and intravitreal injections, are both invasive and costly.[2] It is generally established in type 2 diabetes mellitus (T2 DM), the prevalence of DR increases proportionally to the disease duration. Long-standing disease, combined with hyperglycemia, hypertension, hyperlipidemia, and genetic factors, is a major risk factor for DR.[3]

Depending on whether neovascularization is present, DR can be divided into non-proliferative DR (NPDR) and proliferative DR (PDR). NPDR is the early retinal disease without neovascularization, while PDR is the more advanced form of NPDR with the development and growth of new variable-sized vessels on the retinal surface.[4] NPDR is characterized by retinal microaneurysms and hemorrhages that may progress to PDR, which is defined by retinal neovascularization and has the potential to cause a significant vision loss. NPDR is often asymptomatic, while PDR is frequently associated with visual impairment.[5] Therefore, metabolomic profiling and early detection of DR are very important in preventing the progression of NPDR to PDR.[6]

Metabolomics is one of the newer “omics” fields and builds on the principles of genomics, transcriptomics, and proteomics.[7] The study of small molecules present in a biological sample may reveal metabolic differences that distinguish healthy and disease states apart. Metabolomics profiling is a rapidly developing method used to identify and quantify the low molecular weight metabolites in biological samples and investigate disease progression.[8] It is generally recognized that metabolic phenotype is the end product of the interaction between genetic and environmental factors, and it reflects the pathophysiological circumstances of various diseases. Thus, the association of these metabolites with the biological processes must be studied.[9]

Numerous metabolomic profiling studies were carried out to identify the metabolites linked to disease progression. [8] However, there are a small number of studies on metabolomics profiling associated with DR. [4,10] Metabolic differences between patients with PDR, NPDR and non-DR (NDR), identifying differences in these metabolic profiles could explain the molecular mechanisms of DR, facilitating the identification of new therapeutic targets. Therefore, metabolomic studies into DR are attractive proposition.[6]

Among these metabolites, plasma asymmetric dimethylarginine (ADMA) was found to be connected to both the early changes in retinal vessels in DR and the final status of DR progression. Other metabolites as cytidine, uridine, fumaric acid, and acetic acid, were identified as candidate biomarkers of PDR progression.[11]

Other studies identified some metabolites including carnitines, phosphatidylcholines, total ADMA, and several amino acids as potential biomakers of DR progression in T2D. The metabolite profiles “metabolite signatures” identified in these studies provided insight into the mechanisms underlying DR development and progression in T2D patients.[12]

Previous studies indicated that several semaphorins are upregulated in DR, contributing to the neurovascular pathophysiology of DR, and remained an intense investigation topic for DR. [13] Semaphorin3A (Sema3A) is secreted by retina glia cells and binds to neuropilin-1 (Nrp1) on endothelial cells to induce vascular permeability.[14] Sema3A is induced in ischemic retinal ganglion cells in response to interleukin 1 beta (IL-1β) after vascular injury and prevents revascularization of ischemic but salvageable neurons. Sema3A binds Nrp1 to mediate endothelial cell cytoskeleton collapse and prevent migration and promote endothelial apoptosis. It appears that Sema3A is secreted by

hypoxic neurons and inhibits the vascular regeneration of the retina while enhancing pathologic pre-retinal neovascularization.[15] Sema3A levels were significantly elevated in the vitreous of patients with PDR. Also, serum Sema3A levels were found to be correlated with the phenotypes of DR.[16] A study by Joyal et al., 2011[17] demonstrated that inhibition of Sema3A facilitated normal revascularization of the inner retina after vascular injury in oxygen-induced retinopathy. In the light of these data, biomarkers can provide early warning signs in patients with serious diseases as DR. Therefore, they help in the early diagnosis of the disease so that effective treatment can be made available to patients at the earliest stages. Consequently, the goal of our study was to analyze and determine serum metabolites and Sema3A levels in DR patients, that could be useful biomarkers of DR progression.

Patients and Methods

Study design

Selection of study subjects

This cross-sectional study included 45 T2D patients. Their age ranged from 20 to 65 years. The patients were recruited from the outpatient clinics, and the Department of Ophthalmology of Al- Zahraa University Hospital, Faculty of Medicine (for Girls), Al- Azhar University. The study was carried out during the period from July 2021 to April 2022. Diabetic patients were divided into three groups based on the status of their complications: a non-diabetic retinopathy (NDR, n=15) control group, NPDR, (n=15), and PDR, (n=15). All patients enrolled in this study were diagnosed as T2D, made by endocrinologist or their primary care provider, based on the American Diabetes Association (ADA) criteria.[18]

DR patients were diagnosed by dilated fundus examination. The presence of DR was confirmed and documented by Topcon fundus camera (TOPCON, TRC.50EX 35° fundus camera, TOPCON, Tokyo, Japan). DR patients were included in the study group if they met the following criteria: T2D with PDR or NPDR, which were determined by the grading of color fundus photographs by a masked image grader, according to the modified Early Treatment DR Study (ETDRS);[19] a best-corrected visual acuity between 20/20 and 20/200; and a central subfield macular thickness of the retina of 300μm or more on spectral-domain optical coherence tomography. Diagnosis of NPDR was based on the presence of microaneurysms, blot hemorrhages, cotton-wool spots, or intraretinal microvascular abnormalities and no evidence of active PDR or history of treatment for PDR. Diagnosis of PDR was based on the presence of neovascularization of the retina or optic nerve. The diabetic control group (NDR) included patients with a diagnosis of T2D for at least 2 years and no clinical evidence of DR as determined by dilated fundus examination.

Patients with NPDR or PDR were excluded from the study if they met any of the following criteria: the presence of vitreous hemorrhage that obscured the fundus examination or severe lens opacity; previous treatments, including antiangiogenic drugs such as ranibizumab, bevacizumab, and pegaptanib, laser photocoagulation, or previous vitrectomy, the presence of infectious disease or prostate disease, patients with a history of liver, kidney diseases; patients with uncontrolled hypertension. Cases with nondiabetic retinopathy, asymmetric grades nondiabetic retinopathy, glaucoma, active ocular inflammation, and retinal degeneration, were also excluded from the study.

The macular thickness was calculated using macular cube (512 × 128 scan), and obtained with an optical coherence tomography-angiography (OCT-A) system (RTVue XR Avant Optovue−Angiovue Inc., Fremont, California, USA), in order to measure central foveal thickness (CFT). Macular vascular perfusion was measured using swept-source OCT-A (SS-OCT-A) system (RTVue XR Avant Optovue−Angiovue Inc., Fremont, California, USA), 6× 6 mm raster imaging and each scan pattern consisted of 500 A-scans per B-scan, at 500 B-scan positions. Slabs of the superficial capillary plexus (SCP) were automated and segmented by the built-in software. A minimum signal strength threshold of 7 of 10 was required for inclusion, and significant motion artifact or incorrect

segmentation, were excluded and repeated. A series of quantitative OCT-A metrics of SCP was defined as shown in Figure 1. To discover DR-specific metabolites, we excluded patients with other diabetic complications such as diabetic nephropathy.

The protocol of the study was reviewed and approved by the Research Ethics Committee, Faculty of Medicine (for Girls), Al- Azhar University, (approval number 917 dated 30/6/2021). An informed written consent was obtained from all subjects prior to enrollment in the study. Full history taking, complete general and ophthalmic examinations were done for all cases, and BMI was calculated, which was defined as weight divided by the square of height (Kg/m2).

Sample collection and Investigations

Under a complete aseptic condition, a venous blood sample (6 ml) was withdrawn from each patient by antecubital vein-puncture, using a sterile disposable syringe.The blood sample was divided into two parts: the first part (2 ml) was evacuated into an EDTA tube for measuring hemoglobin (Hb) A1c, using an automated analyzer (COBAS C 311 auto-analyzer, Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The second part (4 ml) was placed into a tube, allowed to clot and the serum was obtained after centrifugation at 1776 xg for 10 minutes. Serum samples were divided into three parts. The first part was used for measurement of kidney function tests, lipid profile, and fasting blood sugar (another blood sample was obtained after 2 hr for postprandial blood sugar estimation), using an automated blood chemistry analyzer (COBAS C 311 auto-analyzer, Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

The second and third serum parts were kept frozen at -20 °C until used for measurement of serum metabolomics by a high-performance liquid chromatography-mass spectrometry (HPLC-MS), and serum Sema-3A by ELISA. Serum Sema3A assessment was done by using commercially available human ELISA kits (Catalog number E2078Hu, Bioassay Technology Laboratory, China) according to the manufacturer's instructions. For the serum Sema3A assessment, an ELISA system which included a plate shaker-incubator (Thermo-Shaker from EU for Grant Instruments Ltd, Cambs, England), an ELISA washer (ELx50 Biokit, Italy), and a plate reader (AS 1851 from DAS, Italy), was used according to the manufacturer's instructions.

HPLC-MS Metabolomics Analysis

- *Sample preparation*

Sample preparation for metabolomics was performed according to the manufacturer's instructions. One hundred microliters of each sample were lysed slowly at 4°C, then 400 µL of precooled methanol was added, vortexed for 60s, incubated at -80°C for 8 hours, and the protein was precipitated by centrifugation at 16,000 xg for 10 min at 4°C. The resultant supernatant was then used for HPLC analysis.

- *Metabolomics profiling*

Serum samples of patients with T2D were analyzed for metabolites on an HPLC-MS system (HPLC Agilent 1100 series, Waldborn, Germany), quaternary pump (G1311A), degasser (G1322A), thermostated autosamples (G1329A), variable wavelength detector (G1314A); and column (Zorbax 300SB C-18 column; 250 mmX4.5mmX 5um; Agilent Technologies, USA). The solvent system consisted of methanol with 0.1% formic acid (solvent A); acetonitrile with 0.1% of formic acid (solvent B). The gradient system was programmed as follows: starting at 30% solvent B, increasing to 60% over 10 min, increasing to 100% over 5 min, and then returning to 30% over 5 min. The injection was carried out under ambient temperature. Mass spectra were obtained in a positive ion mode in a mass range of m/z 100–800 amu at a fragmentor voltage of 70 eV. The drying gas used was nitrogen at a flow rate of 10 ml/min. Nebulizer pressure was set to 45 psi. Manual baseline subtraction for all spectra was performed. Quality control was performed for selection of metabolites for further analyses. Metabolites with significant differences in their concentrations were identified and analyzed in the different patients groups.

Statistical analysis

Data were collected, revised, coded, and entered into a computer using the Statistical Package for Social Science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations, and ranges when their distribution was found parametric. Also, qualitative variables were presented as numbers and percentages. Paired-wise comparison (post hock test) between groups was done by using a one-way analysis of variance (ANOVA) test. Correlation between two quantitative parameters in the same group was done by using Spearman correlation coefficients. The best cut-off point with sensitivity, specificity, positive and negative predictive value, and area under the curve (AUC) for the prediction of DR patients was done by using the receiver operating characteristic (ROC) curve. Univariate logistic regression analysis was used to assess the predictors of PDR with odds ratio and 95% confidence interval (CI). The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the *p*-value was considered significant at the level of < 0.05.

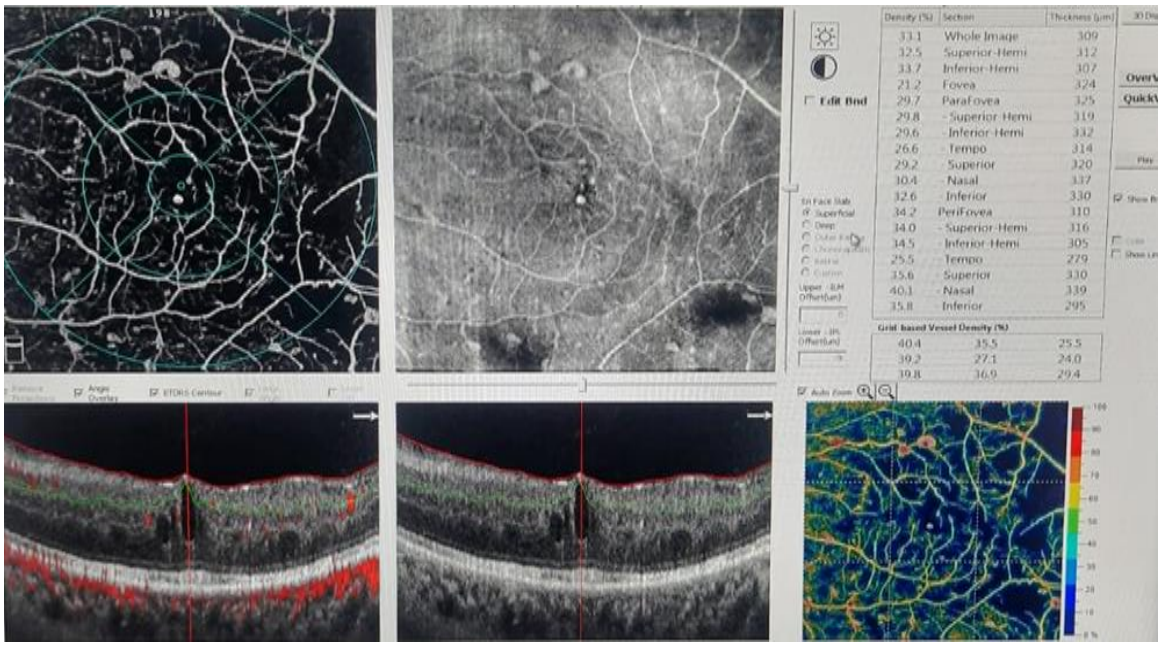

Figure 1. This shows Optical Coherence Tomography-Angiography (OCT-A) *en face* slab of the superficial capillary plexus of the macula of the retina with calculating the superficial vascular density table for case number 5 in the PDR group.

Results

There was no significant difference between the three groups (NPDR, PDR and NDR) in terms of age, sex or BMI. However, NPDR and PDR patients had a longer disease duration (p < 0.001). The mean T2D duration of each group was 8.5 years (NDR), 15.1 years (NPDR), and 19 years (PDR) (Table 1).

Significant difference was found between the three groups in blood pressure systolic blood pressure (SBP) and Diastolic blood pressure (DBP) (p<0.001 and p=0.002, respectively). Also, significantly higher central macular thickness and lower superficial macular density in PDR and NPDR patients as compared with the NDR group (p =0.047 and p =0.001, respectively). The percentage of patients who have macular ischemia was higher in PDR and NPDR patients as compared with NDR group (Table 1). As regard laboratory data, there were significant increase in laboratory parameters in PDR than in NPDR and NDR groups, included creatinine level (p =0.0003), blood glucose concentrations; fasting and post prandial (p <0.001 for both) and HbA1c (p <0.001) (Table 2).

Table 1. Comparison between study groups regarding demographic and clinical data.

Demographic & clinical data		Group			*p*-value
		NDR (n= 15)	NPDR (n=15)	PDR (n= 15)	
Males Females	N (%) N (%)	7 (46.7%) 8 (53.3%)	8 (53.3%) 7 (46.7%)	8 (53.3%) 7 (46.7%)	NS
Age (year)	Mean ± SD	51.67±7.06 39-62	50.73±7.17 40-63	55.60±6.55 45-65	NS
Disease duration (year)	Mean ± SD	8.53±2.32 5-12	15.13±2.44 10-20	19.00±3.50 15-26	< 0.001*
SBP (mmHg)	Mean ± SD	127 ±9.12 110-140	135 ±5.97 120-140	138 ±4.55 130-145	< 0.001*
DBP (mmHg)	Mean ± SD	81.33±6.39 70-90	86.67±4.88 80-95	87.66±3.19 80-90	0.002*
BMI (kg/m^2)	Mean ± SD	31.86±2.41 27-35	31.26±2.68 26-35	31.33±2.55 27-35	NS
Central macular thickness	Mean ± SD	260.2± 17.2 224- 279	311.73 ± 82.90 226-471	317.33 ± 79.70 211-525	0.047*
Superficial macular density	Mean ± SD	49.40±2.32 45.80-55.00	42.59 ± 4.81 32.70- 49.00	42.41 ± 4.77 32.70- 49.00	< 0.001*
Macular	N (%)	0 (0)	11 (73.3)	15 (100)	< 0.0001*

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, BMI: Body mass index. *P* > 0.05 is not significant (NS).

Table 2. Comparison between study groups regarding laboratory data.

Laboratory data (Mean±SD)	NDR	NPDR	PDR	*P* value	*p*1	*p*2	*p*3
FBS (mg/dl)	145.80 ±7.04	162.3 ±15.8	171.9 ±8.6	< 0.001*	< 0.001*	NS	< 0.001*
PPS (mg/dl)	189.9 ±10.6	214.4 ±18.5	218.5±25.3	< 0.001*	< 0.001	NS	< 0.001
HbA1c %	7.36 ±0.30	7.76 ±0.63	8.57 ± 0.73	<0.001*	<0.0001*	0.0016*	NS
Urea (mg/dl)	38.86 ±5.35	42.26 ±7.96	39.7 ± 3.8	NS	NS	NS	NS
Creatinine (mg/dl)	1.00 ±0.13	1.18 ± 0.11	1.30 ± 0.08	< 0.001*	<0.0001*	0.0183*	0.0003*
Cholesterol (mg/dl)	216.9 ±22.4	219.3 ±22.9	225.1 ±18.2	NS	NS	NS	NS
Triglycerides (mg/dl)	174.33±21.4	183.7 ± 24.4	191.1 ± 10.3	NS	NS	NS	NS
HDL C (mg/dl)	35.00 ±3.58	34.06 ±3.39	34.9 ± 3.8	NS	NS	NS	NS
LDL C (mg/dl)	109.2 ±10.2	113.3 ±10.8	115.5 ±17.04	NS	NS	NS	NS

*p*1: NDR vs PDR, *p*2: NPDR vs PDR, *p*3: NDR vs NPDR, * *P* > 0.05 is not significant (NS).
FBS: Fasting blood sugar, PPS: Post prandial sugar, HbA1c: Hemoglobin A1c, HDL C: High density lipoprotein cholesterol, LDL C: Low density lipoprotein cholesterol.

Several metabolomics levels were significantly increased in the PDR group as compared with the NPDR and NDR groups, these included serum levels of total ADMA (p <0.0001), kynurenine (p <0.0001), carnitines (acyl carnitine and dehdroxy carnitine) (p <0.0001 for both), cytidine (p <0.0001), citrulline (p<0.0001), ornithine (p <0.0001), proline (p <0.0001), glutamic gamma semialdehyde (P <0.0001), lacate (p <0.0001), cytosine (p <0.0001), thymidine (p <0.0001), and arginine (p <0.0001) (Table 3). However, serum levels of other metabolomics were decreased in the PDR than in the NPDR and NDR groups, included tryptophan (p <0.0001), ascorbic acid (p <0.0001), arachidonic acid (p <0.0001) and linoleic acid (p<0.0001) (Table 3).

The receiver operating characteristic (ROC) curve analysis was done for determining the predicitve value of Sema 3A and other metabolomics in DR patients as shown in (Table 4) (Figures 2,3,4).

Univariate logistic regression analysis for Sema 3A and other metabolomics as predictors for PDR showed that serum levels of Sema 3A, total ADMA and Kynurenine were potential predictors of PDR patients (Table 5).

Table 3. Comparison between study groups regarding Sema3A and other metabolomics.

Biomarkers	NDR	NPDR	PDR	*P* value	*p*1	*p*2	*p*3
Sema 3A (ng/ml)	275.5 ±33.1	398.2 ± 44.9	685 ± 139.2	<0.001*	<0.0001*	<0.0001*	0.0011*
Total ADMA (µM)	1.9±0.4	3.6 ± 0.6	5.7 ± 0.6	<0.001*	<0.0001*	<0.0001*	<0.0001*
Kynurenine (µM)	1.5 ± 0.6	3.6 ± 0.6	5.2 ± 0.7	<0.001*	<0.0001*	<0.0001*	<0.0001*
Tryptophan (µM)	3.7± 0.7	1.8 ± 0.3	0.5 ± 0.2	<0.001*	<0.0001*	<0.0001*	<0.0001*
Acyl Carnitine (µM)	0.6 ±0.2	1.8 ± 0.6	3.1 ± 0.5	<0.001*	<0.0001*	<0.0001*	<0.0001*
Cytidine (µM)	3.6 ± 1.2	8.3 ± 1.0	14.8 ± 2.1	<0.001*	<0.0001*	<0.0001*	<0.0001*
Citrulline (µM)	3.0 ± 0.7	4.9 ± 0.8	6.8 ± 0.6	<0.001*	<0.0001*	<0.0001*	<0.0001*
Ornithine (µM)	0.8 ± 0.3	1.7 ± 0.3	2.9 ± 0.5	<0.001*	<0.0001*	<0.0001*	<0.0001*
Proline (µM)	1.9 ± 0.5	3.0 ± 0.4	3.9 ± 0.2	<0.001*	<0.0001*	<0.0001*	<0.0001*
Glutamic gamma Semialdehyde (µM)	2.6 ± 0.7	5.1 ± 0.6	8.8 ± 0.9	<0.001*	<0.0001*	<0.0001*	<0.0001*
Dehdroxy Carnitine (µM)	2.0 ± 0.5	4.9 ± 1.0	7.3 ± 0.7	<0.001*	<0.0001*	<0.0001*	<0.0001*
Lacate (µM)	6.2 ± 0.8	8.7 ± 0.8	13.5 ± 1.2	<0.001*	<0.0001*	<0.0001*	<0.0001*
Cytosine (µM)	20.5 ± 1.8	26.5 ± 1.3	33.7 ± 1.4	<0.001*	<0.0001*	<0.0001*	<0.0001*
Thymidine (µM)	2.3± 0.8	7.2 ± 1.0	15.5 ± 2.4	<0.001*	<0.0001*	<0.0001*	<0.0001*
Ascorbic acid (µM)	15.9 ± 1.6	8.4 ± 1.5	2.6 ± 0.9	<0.001*	<0.0001*	<0.0001*	<0.0001*
Arachidonic Acid (µM)	115.7 ± 4.0	94.1 ± 7.1	62.6 ± 9.5	<0.001*	<0.0001*	<0.0001*	<0.0001*
Linoleic Acid (µM)	55.0 ± 7.4	33.2 ± 3.5	21.1 ± 3.4	<0.001*	<0.0001*	<0.0001*	<0.0001*
Arginine (µM)	2.2±0.6	4.4±0.6	6.7±0.8	< 0.001*	<0.0001*	<0.0001*	<0.0001*

*p*1: NDR vs PDR, *p*2: NPDR vs PDR, *p*3: NDR vs NPDR, *p ≤ 0.05 is considered significant.

Sema 3A: Semaphorin-3A, ADMA: Asymmetric dimethylarginine.

Table 4. Predicitve value of Sema 3A and other metabolomics for DR patients by ROC curve analysis.

Biomarkers	Cut off	AUC	Sensitivity	Specificity	PPV	NPV	*p* value
Sema 3A (ng/ml)	> 320	1	100	100	100	100	<0.0001*
Total ADMA (µM)	>2.5	0.985	99	98	98	99	<0.0001*
Kynurenine (µM)	>2.5	0.975	98	97	79	98	<0.0001*
Tryptophan (µM)	≤2.3	0.960	97	95	95.1	96.9	<0.0001*
Acyl Carnitine (µM)	>0.9	0.945	96	93	93.2	95.9	<0.0001*
Cytidine (µM)	>5.6	0.935	95	92	92.2	94.8	<0.0001*
Citrulline (µM)	>4.2	0.920	93	91	91.2	92.9	<0.0001*
Ornithine (µM)	>1.2	0.910	92	90	90.2	91.8	<0.0001*
Proline (µM)	0.983	0.800	91	89	89.2	90.8	<0.0001*
Glutamic gamma Semialdehyde(µM)	>3.5	0.780	90	88	88.2	89.8	<0.0001*
Dehdroxy Carnitine (µM)	>2.9	0.880	89	87	87.3	88.8	<0.0001*
Lactate (µM)	>7.5	0.870	88	86	86.3	87.8	<0.0001*
Cytosine (µM)	>23.1	0.860	87	85	85.3	86.7	<0.0001*
Thymidine (µM)	>3.9	0.850	86	84	84.3	85.7	<0.0001*
Ascorbic acid (µM)	>4.2	0.835	85	82	82.5	84.5	<0.0001*
Arachidonic Acid (µM)	≤105.7	0.815	83	80	80.6	82.5	<0.0001*
Linoleic Acid (µM)	≤40.1	0.800	81	79	79.4	80.6	<0.0001*
Arginine (µM)	>3.3	0.790	80	78	78.4	79.6	<0.0001*

AUC: Area under curve, PPV: Positive predictive value, NPV: Negative predictive value
*$p \leq 0.05$ is considered significant. Sema 3A: Semaphorin-3A, ADMA: Asymmetric dimethylarginine.

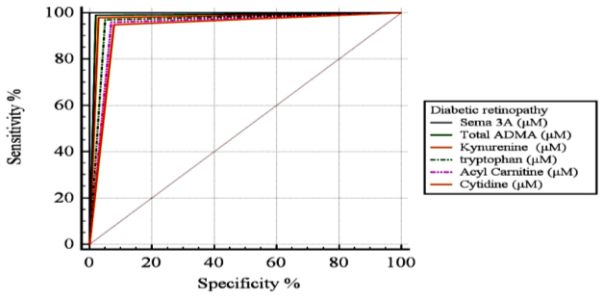

Figure 2. ROC plot for Sema3A and markers of metabolomics profile in DR patients.

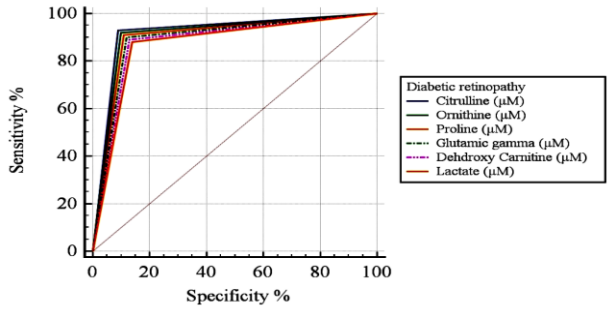

Figure 3. ROC plot for markers of metabolomics profile in DR patients.

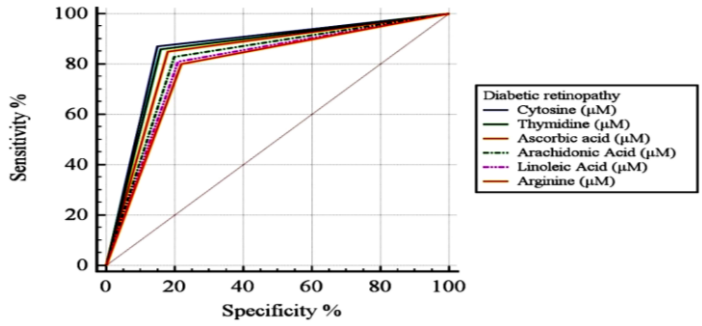

Figure 4. ROC plot for markers of metabolomics profile in DR patients.

Table 5. Univariate logistic regression analysis for SemaA3 and other metabolomics as predictors for PDR patients.

Biomarkers	R	S.E	Odds ratio	95% CI	*p* value
Sema 3A (ng/ml)	0.94	17033.693	2.16	0.87 to 0.97	<0.001*
Total ADMA (µM)	0.92	13284.127	2.16	0.83 to 0.96	<0.001*
Kynurenine (µM)	0.91	25282.927	1.77	0.84 to 0.96	<0.001*
Tryptophan (µM)	-0.92	34122.364	0.25	-0.96 to -0.84	<0.001*
Acyl Carnitine (µM)	0.89	10807.377	1.68	0.78 to 0.94	<0.001*
Cytidine (µM)	0.88	24509.941	1.58	0.75 to 0.94	<0.001*
Citrulline (µM)	0.83	24063.779	1.47	0.66 to 0.91	<0.001*
Ornithine (µM)	0.83	67122.129	1.43	3.3 to 4248.5	<0.001*
Proline (µM)	0.82	24644.017	1.40	0.64 to 0.91	<0.001*
Glutamic gamma Semialdehyde (µM)	0.82	44622.033	1.30	0.65 to 0.91	<0.001*
Dehdroxy Carnitine (µM)	0.82	206.012	1.27	0.99 to 1.10	<0.001*
Lactate (µM)	0.79	25690.826	1.21	2.8 to 364.48	<0.001*
Cytosine (µM)	0.76	28328.883	1.20	0.54 to 0.87	<0.001*
Thymidine (µM)	0.75	10046.543	1.18	0.85 to 0.964	<0.001*
Ascorbic acid (µM)	0.72	10342.534	1.11	0.81 to 0.903	<0.001*
Arachidonic Acid (µM)	-0.89	2366.238	0.66	-0.94 to -0.77	<0.001*
Linoleic Acid (µM)	-0.87	8187.942	0.63	-0.93 to -0.74	<0.001*
Arginine (µM)	0.69	57014.126	1.05	0.77 to 0.813	<0.001*

*$p \leq 0.05$ is considered significant. Sema 3A: Semaphorin-3A, ADMA: Asymmetric dimethylarginine.

Serum levels of Sema 3A were significantly increased in PDR as compared with NPDR and NDR groups (p <0.0001) (Table 3). Additionally, in DR patients, there was a positive correlation between Sema 3A serum levels and disease duration (r= 0.453, p= 0.012) and central macular thickness (r= 0.95, p= 0.0001) (Table 6, Figure 5). However, a negative correlation was observed between Sema 3A serum levels and superficial macular density (r=-0.952, p= 0.0001) (Table 6, Figure 6).

Table 6. Pearson correlation between serum Sema 3A levels and clinico-demographic data in DR patients.

Clinico-demographic data	Diabetic retinopathy (n=30)	
	Sema 3A	
	r	*p* value
Age (year)	0.272	NS
Disease duration (year)	0.453	0.0120*
SBP (mmHg)	0.173	NS
DBP (mmHg)	0.041	NS
BMI (kg/m^2)	0.101	NS
Central macular thickness	0.9524	<0.001*
Superficial macular vascular density	-0.9520	<0.001*

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, BMI: Body mass index. *P* > 0.05 is not significant (NS).

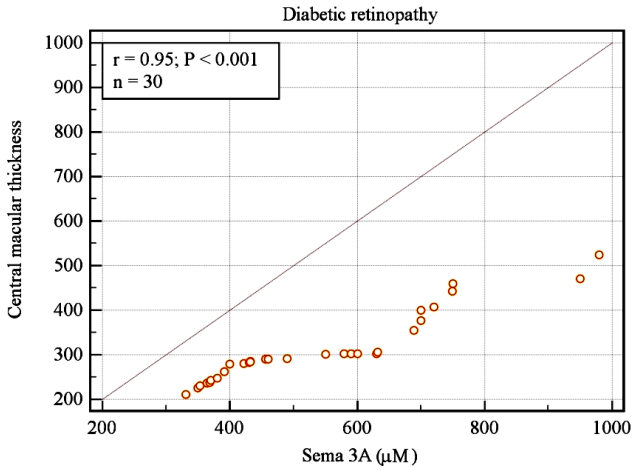

Figure 5. Correlation between serum Sema 3A levels and central macular thickness in DR patients.

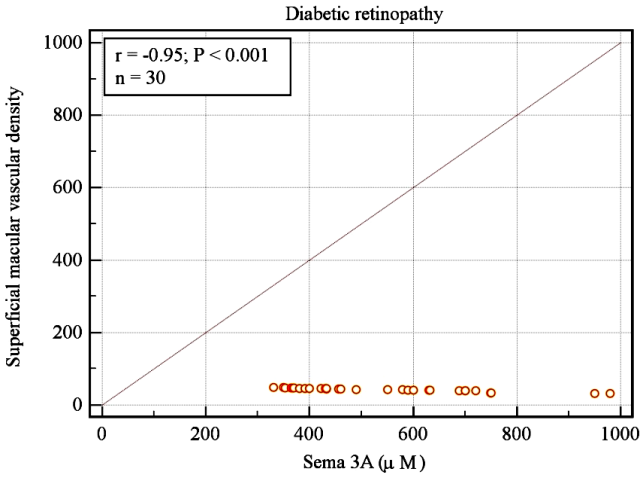

Figure 6. Correlation between serum Sema 3A levels and superficial macular vascular density in DR patients.

Discussion

Diabetic retinopathy is one of the serious complications of diabetes, it is difficult to cure. Early diagnosis and timely intervention can effectively prevent or slow down the progression of the disease. Therefore, the identification of biomarkers associated with disease progression can be very helpful.[20] Metabolomics is a branch of systems biology and a powerful tool for studying pathophysiological processes, which can aid in identifying the characteristic metabolic changes indicative of DR progression.[21] Therefore, this study intended to analyze and identify serum metabolites and Sema3A levels in DR patients, that could be useful biomarkers of DR progression.

In the present study, 17 differentially expressed metabolites that belonged to different metabolic pathways were identified and their levels associated with the occurrence of DR. We found that serum levels of circulating metabolites were significantly altered between the PDR, NPDR, and NDR groups. We also found that serum total ADMA levels were significantly higher in PDR patients as compared with NPDR and NDR groups. This result agreed with that reported by Yun et al., 2020[12] who showed that total ADMA levels were higher in both serum of PDR and NPDR than in NDR patients. According to previous reports, ADMA inhibits nitric oxide synthase (NOS), resulting in a decrease of nitric oxide (NO). NO deficiency is known to accelerate DR by increasing the production of oxygen and nitrogen reactive species. As a result, it was suggested that increased circulating ADMA levels were associated with DR in T2D patients.[22,6] Based on such results of our own and of previous studies, total ADMA may be suggestive metabolites of DR, linked to the regulation of NO synthesis in the blood of T2D patients.[23]

In accordance with our results regarding increased serum total ADMA levels with DN progression, findings by Malecki et al., 2007 demonstrated that plasma ADMA levels were associated with both early changes in retinal vessels in DR and also with the progression of the disease[24].

We also found significantly higher serum levels of kynurenine and lower levels of tryptophan in both PDR and NPDR patients, compared with those in NDR group, which matched with findings of Yun et al., 2020[12] who reported similar result. This could be explained by findings of Fiedorowicz et al., 2019[25] who showed that indoleamine 2,2-dioxygenase converts tryptophan to kynurenine. The tryptophan-kynurenine pathway has been implicated in several diseases; however, the reports of this pathway in DR are rare. Of note, tryptophan was metabolized to kynurenine, resulting in a decrease of tryptophan concentration, while the level of kynurenine, a product of tryptophan metabolism, was reciprocally increased.[26]

In our study, creatinine level was significantly higher in PDR patients than in NPDR, and NDR groups, which matched those reported by Yun et al., 2020[12]. Furthermore, Hsieh et al., 2018 [27] found that higher serum creatinine level was associated with PDR development. Renal dysfunction was associated with DR development and deterioration.[28] Additionally, it has been shown that DR is a prognostic factor in the progression of chronic kidney disease in T2D patients. These results could suggest that serum creatinine is a candidate marker of DR development and progression in diabetic patients.[29] On the other hand, Tomita et al., 2021[30] found lower creatine levels in the vitreous humor of PDR patients as compared with control participants. This discrepancy between results could be explained by small sample size of our study and different sample type.

Our study identified increased serum levels of arginine, citrulline, ornithine, proline, and glutamic γ-semialdehyde in patients with PDR than in NPDR and NDR groups. These agreed with findings of prior studies done by Paris et al., 2016[31], and Peters et al., 2021[32] in which they reported increased levels of ornithine, proline, citrulline, and arginine in the vitreous of patients with PDR,[31] and the elevations in serum arginine levels were found in patients with severe DR.[32] Arginine is metabolized through two different pathways in the retina: the NOS pathway, which generates citrulline and NO,

and the arginase pathway that produces ornithine and urea.[33]

In the urea cycle, arginine is converted by arginase into urea and ornithine. For completing the cycle, ornithine is then transformed back to arginine. Alternatively, ornithine through the intermediate glutamic γ-semialdehyde may be metabolized to proline[20,34]. Our findings add to the growing body of evidence that alterations in urea cycle metabolites, especially arginine and citrulline, are associated with DR. Arginine also acts as a substrate for the enzyme NOS, which catalyzes a process that results in citrulline and NO; a vasodilator that is essential for maintaining the health of the vascular endothelium. NOS is inhibited by ADMA, which has been found to be increased in serum, plasma, and aqueous humor of DR patients.[35] Given our findings and those of previous studies, it is possible that dysregulated metabolism of arginine may be linked not only through urea cycle metabolites, but also through ADMA and NO.

Our study also showed that lactate, citrulline, ornithine and proline levels were increased in PDR and NPDR as compared with the NDR group. These observations partially matched with those of Tomita et al., 2021[30] who demonstrated that lactate, citrulline, ornithine, proline, pyruvate, allantoin, and urate levels were increased in vitreous humor of PDR patients as compared with control subjects. Also, our results were partially in accordance with those of Bringmann et al., 2006,[36] who observed elevated levels of arginine, proline, citrulline, ornithine, methionine, allantoin, decanoylcarnitine, and octanoylcarnitine in patients with PDR. These authors concluded that such findings implicated a compromised mueller glial cell metabolism in disrupting neurovascular crosstalk within the retina, potentially promoting DR progression.[37]

Our study demonstrated higher serum levels of lactate and lower levels of ascorbic acid in both PDR and NPDR patients as compared with those in the NDR group. This agreed with Barba et al., 2010[38] who found that lactate level was increased while ascorbic acid was decreased in the vitreous humor of PDR patients compared with control subjects. The high levels of lactate in DR patients most likely reflect increased tissue acidosis and anaerobic glycolysis, especially as the retina is one of the most metabolically active tissues. Barba et al., 2010[38] showed that after removal of lactate peak at 1.35 ppm, they found lower levels of galactitol attributed to activation of the polyol pathway, a metabolic pathway involved in the pathogenesis of DR.[39]

Ascorbic acid is a cofactor in the biosynthesis of catecholamines, collagen, neurohormones, and important antioxidant free radical scavenger. This last role takes on special significance in the retina where there is considerable light-induced free radical formation.[40] Hence, in chronic persistent hyperglycemia, ascorbic acid delivery into the retinal cells is impaired with consequent increased photo-oxidative damage.[41]

Additionally, ascorbic acid is a cofactor for several hydroxylases necessary for the production of neuropeptide. Thus, ascorbic acid deficiency may contribute to the early neurodegeneration observed in DR.[42] Finally, ascorbic acid may inhibit angiogenesis, which is a central event in DR.[43] This possibility is supported by a study showing that ascorbic acid metabolism was impaired in diabetic patients who developed DR compared to those who did not develop.[6]

Carnitine is a key molecule in the oxidation and metabolism of fatty acids.[44] This study identified increased serum levels of acylcarnitine, and de-hydroxy carnitine in patients with PDR compared with NPDR and NDR groups. Our results are consistent with a previous study done by Sumarriva et al., 2019[45] which compared PDR patients with nondiabetic control and found elevated acylcarnitine levels in vitreous samples of PDR patients. Carnitine is essential for the transport of long-chain fatty acids into mitochondria via acylcarnitine intermediates prior to β-oxidation.[45] However, a study by Bene et al., 2018[46] reported decreased carnitine levels in T2D and in various diabetic complications. It is likely that differences in the study design and clinical phenotyping, as well as small sample sizes in our study, contribute to these inconsistencies.

We found increased concentrations of cytosine, cytidine, and thymidine in PDR patients as compared with NPDR and NDR groups. These were in accordance with those of Chen et al., 2016[9] who observed that these compounds were associated with DR compared to those without the disease. Of these compounds, they found that cytidine had the highest area under the curve of 0.849 ± 0.048, and at an optimal cutoff point of 0.076 mg/ L; the sensitivity and specificity of cytidine as a biomarker for DR were 73.7 and 91.9%, respectively, suggesting potential value as a biomarker for DR.

We found lower serum levels of two omega-6 poly-unsaturated fatty acids (PUFAs) (arachidonic acid and linoleic acid) in PDR patients when compared with NPDR and NDR groups. These agreed with those of Li et al., 2011[47] who reported that lower levels of the two PUFAs in PDR patients may be associated with higher levels of circulating pro-inflammatory markers such as IL-1ra and IL-6 and lower anti-inflammatory marker TGFb.[48] Diabetic retinopathy is associated with pro-inflammatory cytokines, and downregulation of omega-6 PUFAs may potentiate these effects and increase the risk of developing the condition.[6]

As regard Sema3A levels, we found that both PDR and NPDR groups had significantly higher serum levels than NDR group. This was supported by a study of Kwon et al., 2016[49] who demonstrated that PDR and NPDR had significantly higher plasma Sema3A levels than the control subjects. It appears that in ischemic retinopathy as DR, Sema3A is secreted by hypoxic neurons and inhibits the vascular regeneration of the retina while enhancing pathologic preretinal neovascularization.[15]

Our results also suggested that serum Sema3A levels could serve as a biomarker for DR severity. This possibility is supported by our findings that patients with PDR, who were characterized by the formation of new blood vessels on the retina [4], had higher serum Sema3A levels than patients with NPDR.

Moreover, in diabetic retinopathy patients, we found a positive correlation between serum Sema3A levels and disease duration (r= 0.453, *p*= 0.012) and central macular thickness (r= 0.95, *p* =0.0001), and a negative correlation between their serum levels and superficial macular density (r=-0.952, *p* = 0.0001). Such findings partially agreed with those demonstrated by Kwon et al., 2016[49] who found that plasma Sema3A levels were correlated positively with the retinal nonperfusion area size and total macular volume. This was also consistent with the observation that in ischemic retinopathies as in diabetes, retinal ganglion neurons significantly increased their production of Sema3A.[50]

In conclusion, our study highlighted the possible use of serum metabolites in the diagnosis of DR. Determining variations in these metabolic profiles could reveal molecular mechanisms of DR and PDR, which would help in the identification of new therapeutic targets. Sema3A may be a potential biomarker of diabetic microvascular complications as DR and could serve as a biomarker for DR severity. It is more applicable to use Sema 3A instead of metabolic profile as assessment of Sema3A is simpler and relatively inexpensive.

Author Contributions

ERM, GEE; performed the laboratory work. MGA, NMS, and MMH; made the statistical analysis. DAM; examined the patients. DAM, ERM; collected samples. All authors participated in writing and reviewing the paper.

Declaration of Conflicting Interests

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Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee, Faculty of Medicine (for Girls), Al- Azhar University, (approval number 917 dated 30/6/2021).

Informed consent

An informed written consent was obtained from all subjects prior to enrollment in the study.

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