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Original Article

Aqueous humor metabolomic profiles in association with diabetic mellitus

Yuerong Yao[1*], Hanmin Wang[2*], Beijing Zhu[1], Jun Hu[1], Jie Huang[1], Weimin Zhu[3], Wanhong Miao[3], Jianming Tang[1]

[1]Department of Ophthalmology, Baoshan District Traditional Chinese and Western Medicine Hospital, Shanghai, P. R. China; [2]Department of Ophthalmology, Putuo Hospital, Shanghai University of Traditional Chinese Medical, Shanghai, China; [3]Department of Ophthalmology, Eastern District of Shuguang Hospital, Shanghai, China. [*]Equal contributors.

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Abstract: Diabetic mellitus (DM), commonly referred to diabetes, is a worldwide metabolic disorder, which usually causes high morbidity and mortality rates. Especially, DM may result in serious macrovascular problems including cataract. To investigate the underlying molecular mechanism, here we for the first time employed gas chromatography-time-of-flight mass spectrometry (GC-TOF MS) for an untargeted metabolomics study. Totally 263 metabolites were determined in aqueous humor (AH) samples from 30 patients: 15 for the controls and 15 with DM. Both the heat map and principal component analysis (PCA) plot showed a significantly distinct metabolomics profiles between patients with DM and the controls. Moreover, 20 metabolites were determined to be significantly altered ($P \leq 0.05$) in DM patients, some of which were associated with oxidative stress. Metabolic pathway analysis of these significantly different metabolites identified ten most relevant pathways in the group of DM patients when compared with the control group. Among them, three pathways including fatty acid biosynthesis, fatty acid metabolism, and linoleic acid metabolism were the three most significantly influenced pathways ($P \leq 0.05$), which probably play key roles in the formation of DM and its complication, cataracts. Altogether, this work not only indicated a distinct AH metabolomic profile in association with DM, but presented novel insights into the molecular mechanisms of DM formation, as well as formation of cataracts.

Keywords: Aqueous humor (AH), cataract, diabetic mellitus (DM), metabolite-metabolite correlation, metabolomics

Introduction

Diabetic mellitus (DM), commonly referred to diabetes, is a chronic disease that affects millions of people worldwide [1]. The latest 2016 data from the WHO reported that an estimated 422 million adults are living with DM. Notably, DM usually causes complications such as kidney function loss, eye problems, heart problems, or other serious problems [2]. More importantly, DM remains a main cause of blindness. Especially in the long term, DM may result in serious macrovascular problems including vitreous hemorrhage, rubeosis, temporary blurring of vision, glaucoma, diabetic retinopathy (DR), and cataracts [3]. Among them, retinopathy is responsible for most of the sight-threatening complications of DM, while cataract is another major secondary complication [4].

So far, there have been already a great number of researches focusing on the underlying mechanism of DM and its complications, which aims to find out potential prevention and possible treatment strategies. For example, to determine the role of the cytokines, Demircan *et al* (2006) measured interleukin-1 beta and tumor necrosis factor-alpha in both serum and vitreous humor samples from patients with proliferative DR, which were attributed to the role of interleukins in the development of this disease [5]. So far, there have been several genome-wide association studies on the complications of diabetes including DR and cataract [6, 7]. Burdon *et al* (2015) employed a genome-wide association approach to determine novel contributors of sight-threatening DR, showing a strong connection to genetic variation close to the GRB2 gene [6]. Similarly, Chang *et al* (2016)

Table 1. Data of human AH samples

Group	Patient No.	Gender	Age (Years old)	Axial length	LOCSIII
Controls	A1_1	Female	50	21.91	C4N3P1
	A2_1	Female	66	21.75	C4N3P2
	A3_1	Female	68	23.88	C2N4P2
	A4_1	Female	63	24.3	C3N3P2
	A5_1	Female	68	23.54	C3N3P1
	A6_1	Male	70	23.63	C5N4P2
	A7_1	Male	66	24.01	C4N5
	A8_1	Female	65	23.94	C3N4P2
	A9_1	Female	53	24.63	C3N4P2
	A10_1	Male	62	23.2	C3N2P4
	A11_1	Male	76	21.48	C2N2P3
	A12_1	Female	57	22.59	C3N3P2
	A13_1	Male	78	23.19	C4N5P2
	A14_1	Female	53	24.75	C2N2P5
	A15_1	Male	78	22.97	C3N3P4
Patients with diabetic mellitus	D1_1	Male	70	25.9	C4N4P4
	D2_1	Female	48	21.98	C2N2P5
	D3_1	Female	63	24.47	C3N3P4
	D4_1	Male	68	24.95	C3N3P3
	D5_1	Female	61	26.22	C3N2P2
	D6_1	Male	40	22.96	C3N2P5
	D7_1	Male	50	23.51	C2N2P3
	D8_1	Male	58	22.86	C3N3P3
	D9_1	Male	60	24.8	C3N3P4
	D10_1	Female	76	24.17	C5N3P2
	D11_1	Female	55	27.45	C4N5
	D12_1	Female	57	22.56	C3N3P4
	D13_1	Male	68	23.29	C4N4P4
	D14_1	Female	52	21.75	C2N2P3
	D15_1	Female	72	23.11	C4N2P2

revealed genetic factors for diabetic cataract by using a genome-wide association method, indicating that the CACNA1C gene is connected to diabetic cataracts [7]. Moreover, another useful approach, proteomics analysis, has been employed in various ocular diseases associated with DM and its complications. For example, Chiang *et al* (2012) conducted a comparative study on proteomics between the controls and DM patients with the development of DR, which finally identified potential AH biomarkers, as well as susceptibility factors for predicting DR development [8]. Furthermore, by using two-dimensional differential in gel electrophoresis connected to MS, Su *et al* (2014) determined differential changes on proteomics and metabolomics between "slow" type 2 and "fast" type 1 diabetic cataracts in rats, which was helpful for identifying the shared and differential mechanisms [9].

Besides being an emerging and potentially powerful tool, metabolomics has been employed on studies of various diseases including DM and ophthalmology researches. It allows the simultaneous determination of numerous endogenous compounds including amino acids, organic acids, lipids, and nucleic acids in specific cells/tissues at a special time. Generally, two main analytical platforms for metabolomics studies includes nuclear magnetic resonance (NMR), and MS based metabolomic methods such as GC-MS, liquid chromatography connected to MS (LC-MS), and capillary electrophoresis connected to MS (CE-MS). By using high-resolution ^{1}H NMR, Mayordomo-Febrer *et al* (2015) profiled the AH in corresponding controls and in glaucoma-induced eyes, which showed that after a series of sodium hyaluronate injections, levels of certain metabolites were significantly different [10]. The metabolomic data played a very important role in glaucoma pathogenesis. Furthermore, Barbas-Bernardos *et al* (2016) employed both LC-MS and CE-MS to compare patients with various severities of myopia, which not only showed metabolic variation among various severities of myopia, but provided potential biomarkers and new targets [11].

Recently taking advantage of GC-TOF MS, Ji *et al* (2017) reported metabolic characterization of human AH referred to high myopia, showing significant variation not only in metabolite abundances but also in metabolite-metabolite correlations [12]. Likewise in the present study, we also employed GC-TOF MS to profile

30 AH samples including 15 for controls, and 15 with DM. We believed that our work may provide potential AH biomarkers for clinical diagnosis and monitoring DM. More importantly, it may present novel insights into the molecular mechanism of DM formation and its complication of cataracts.

Materials and methods

Subjects

Thirty subjects were recruited in the present study as shown in **Table 1**: 15 patients with DM and 15 for the controls. All of them met the inclusion criteria as the previous study [12]. Moreover, the mean age for DM patients was more or less 60, while the mean age in the control group was nearly 65. The statistical analysis showed no significance for both age and sex between these two groups. Additionally, other characters including axial length were also shown in **Table 1**. The Ethics Committee of Baoshan District Traditional Chinese and Western Medicine Hospital (Shanghai, China) has reviewed and approved the study protocol.

Sample collection and GC MS analysis

Sample collection and preparation of AH were done as per the previous report [12]. The supernatant after final centrifuging was immediately transferred in liquid nitrogen until GC MS analysis. Metabolic profiling of all the AH samples was performed similarly to that described in the previous study [12]. After the process of metabolite extraction, the supernatant (400 μL from the samples) was then collected and dried in a vacuum concentrator, followed by derivatization and injection into the GC system for metabolomic analysis as described before [12].

Metabolites identification and metabolomic data analysis

The mass spectrometry data for each sample were mapped to the databases for metabolites identification as previously reported [12]. After data normalization, the metabolomic data were input to Mev (MultiExperiment Viewer) 4.8 for hierarchical cluster analysis. And meanwhile, SIMCA-P 13.0 software (Umetrics, Malmö, Sweden) was employed for PCA and partial least squares discrimination analysis (PLS-DA), together with which independent t-tests were conducted for identifying the distinct metabolomics profiles and determining significant differences between the controls and the patients with DM [12].

Pathway analysis

All 20 differential metabolites between the controls and the patients with DM were imported into the website for pathway analysis (http://www.metaboanalyst.ca/). The pathway library of Homo sapiens was chosen, while hypergeometric test and relative-betweenness centrality were selected in the algorithms, respectively. Moreover, the reference metabolome was "used all compounds in the selected pathways".

Results

Metabolites profiling for human AH

To fully uncover human AH metabolome, we took advantage of GC-TOF MS for untargeted metabolites profiling. Totally 263 metabolites were determined in all 30 samples including 15 controls and 15 with DM (Supplementary Table 1). Moreover, these 263 metabolites covered the major and central metabolism pathways, which included 35 amino acids, 50 carbohydrates, 15 lipids, 5 nucleotides, and other 158 compounds (Supplementary Table 1). Among those 158 compounds, 39 biochemicals were named, while 59 biochemicals were identified as analytes and the left were defined to be unknown.

Distinct metabolomics profiles between the controls and patients with DM

To snapshot metabolic characterization between the controls and patients with DM, we inputed all the metabolic profiles into Mev software for hierarchical cluster analysis (**Figure 1**). The results indicated very distinct metabolomic profiles in these two groups. The 15 samples from patients with DM clustered together, which were clearly apart from the other 15 samples from the controls. We further performed PCA on all the 30 samples to provide an overview of the information hidden in the metabolomic data (**Figure 2**). Likewise, the 15 samples from patients with DM were clearly separated from those of the controls, which re-

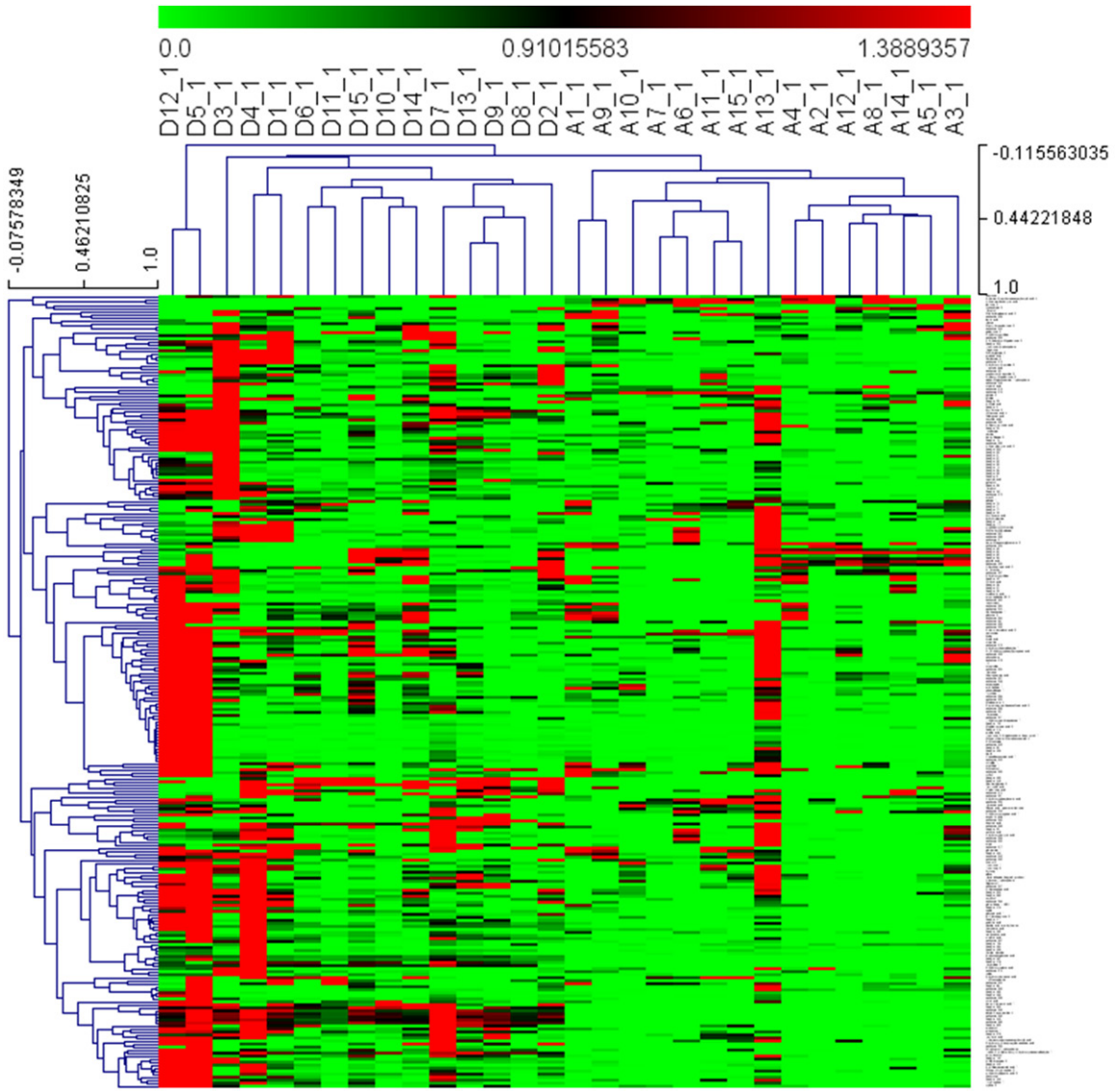

Figure 1. Hierarchical cluster analysis of 263 metabolites between controls and patients with DM.

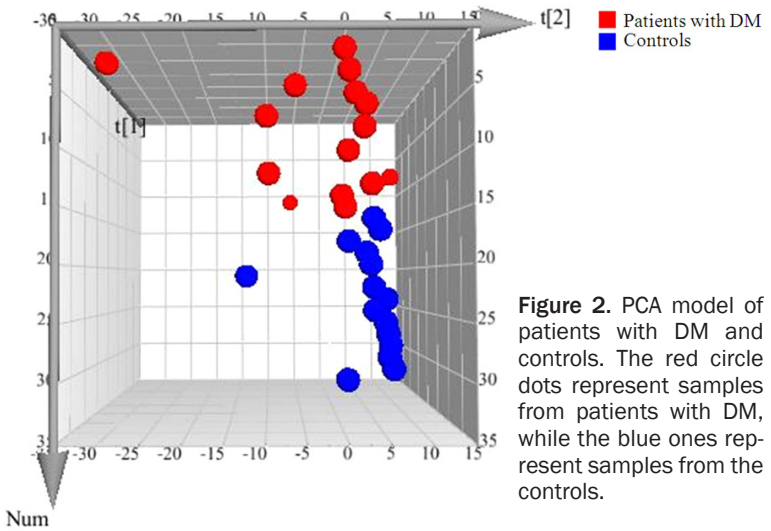

Figure 2. PCA model of patients with DM and controls. The red circle dots represent samples from patients with DM, while the blue ones represent samples from the controls.

confirmed that the metabolomic profile in patients with DM was significantly different from that in the control group.

Metabolic changes in patients with DM

We further employed both the supervised statistical method PLS-DA and t tests for determining significant metabolites responsible for the identified metabolic separations. The result showed that 20 metabolites were found to be significantly altered ($P \leq 0.05$), which

Table 2. Significantly different metabolites betweencontrols and patients with DM

Super Pathway	Compound Name	Retention time (minutes)	Ratio (DM/ARC)*	*p* value
Amino acid	2,6-Diaminopimelic acid 1	12.65	7.39	2.30E-02
Carbohydrate	beta-Mannosylglycerate 2	11.96	0.21	3.00E-03
	6-phosphogluconic acid	14.72	6.55	0.00E+00
	Lactose 2	16.00	0.09	1.20E-02
	Leucrose 1	16.67	3.73	9.00E-03
Lipids	Linoleic acid methyl ester	13.23	13.09	8.00E-03
	Palmitic acid	12.96	3.63	1.20E-02
	Stearic acid	13.86	3.21	3.40E-02
Others	3-Hydroxypyridine	7.50	2.93	2.70E-02
	Analyte 290	10.91	12.58	0.00E+00
	Analyte 411	12.86	5.93	1.70E-02
	Analyte 449	14.04	6.44	0.00E+00
	Analyte 467	14.64	9.4	1.00E-03
	Conduritol b epoxide 2	12.51	4.79	3.70E-02
	Indole-3-acetamide 4	13.98	5.83	0.00E+00
	Trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde 1	13.32	29.73	0.00E+00
	Unknown 058	13.93	4.52	0.00E+00
	Unknown 059	14.00	6.24	0.00E+00
	Unknown 060	14.03	5.78	0.00E+00
	Urea	8.21	2.61	6.00E-03

*DM represents patients with diabetes mellitus while ARC represents the controls.

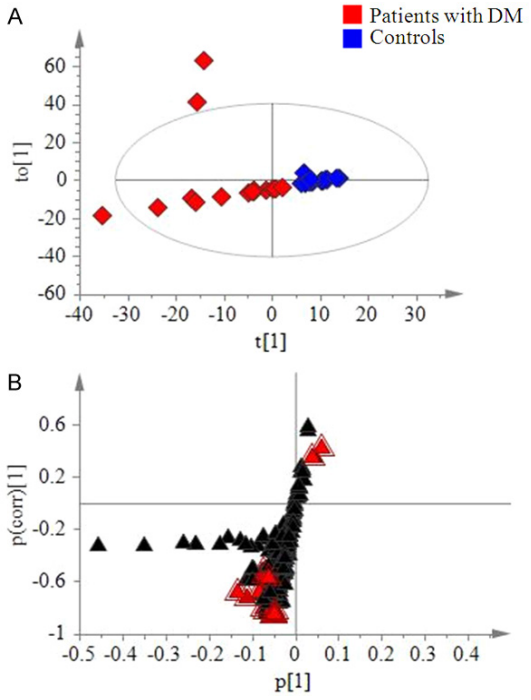

Figure 3. PLS-DA model of patients with DM and controls. A. Score plot. The red boxes represent samples from patients with DM, while the blue ones represent samples from the controls. B. S plot. Metabolites marked with red triangles play key roles for separation.

included 18 up-regulated and 2 down-regulated biochemicals participated in 4 super pathways (**Table 2** and **Figure 3**). The only two down-regulated biochemicals were beta-mannosylglycerate 2 and lactose 2, whose fold-change between patients with DM and controls were respectively 0.21 and 0.09. The 18 significantly up-regulated biochemicals included 2,6-diaminopimelic acid, 1,6-phosphogluconic acid, leucrose 1, linoleic acid methyl ester, palmitic acid, and stearic acid, ranged from 2.61 to 29.73 folds.

Metabolic pathway analysis

To further facilitate the biological interpretation, all 20 differential metabolites between patients with DM and the controls were imported into the MetaboAnalyst web server. The results indicated that nine metabolites mapped to HMDB/PubChem/KEGG were involved in ten most relevant pathways (**Figure 4** and Supplementary Table 2) including fatty acid biosynthesis, fatty acid metabolism, linoleic acid metabolism, fatty acid elongation in mitochondria, pentose phosphate pathway, and alpha-linolenic acid metabolism. Especially, fatty acid biosynthesis, fatty acid metabolism, and linoleic acid

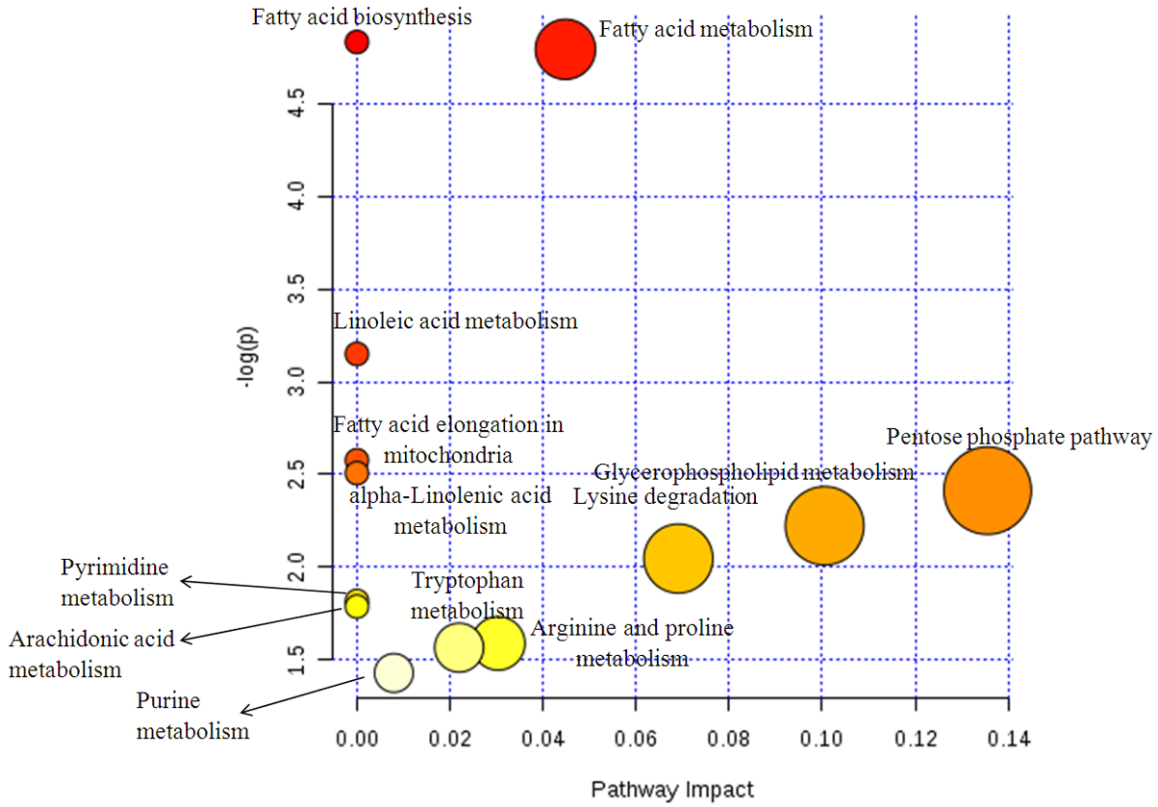

Figure 4. A systemic view of disordered metabolic pathways in association with DM.

metabolism were the most significantly influenced pathways ($P < 0.05$) in DM patients.

Discussion

There already have been many metabolomic studies focusing on DM and DM-related diseases including DR and diabetic kidney disease [13-15]. Most of these studies were conducted for serum samples and only one recent study was performed for aqueous and vitreous humors samples by metabolomic profiling of reactive persulfides and polysulfides. Here, we took advantage of GC-TOF MS for untargeted metabolite profiling for 30 AH samples from 15 control patients and 15 patients with DM. More importantly, the identified metabolites discovered here, for the first time disclosea much broader AH metabolome in association with diabetic mellitus [12-17].

Increasing evidence revealed oxidative stress plays critical roles in the formation of both types of DM and cataracts [4, 18]. Here the level of linoleic acid methyl ester was greatly increased in DM patients, which was reported to increase oxidative stress in patients with DM, further triggering and modulating the process of apoptosis [19, 20]. Likewise, high levels of stearic acid and palmitic acid in patients with DM may induce apoptosis and finally lead to DM and cataract [21]. The other 17 metabolites may also play critical roles and be involved in regulatory pathways in relation to DM. For example, a direct precursor of indole-3-acetic acid, indole-3-acetamide 4, triggered an increased tolerance to different toxic compounds and several stress conditions [22]. Moreover, both mannosylglycerate 2 and lactose in carbohydrate metabolism were reported to be involved in protecting functional protein (such as photoreceptor proteins) activities from denaturation [23, 24]. Another metabolite in carbohydrate metabolism, leucrose 1, was found to exhibit a very strong hydrophobic effect, which may also play pathophysiologic roles in DM and its complication cataract [25].

DM is a chronic progressive metabolic disorder that remains a growing and major global health problem. It is characterized by impaired carbohydrate (especially glucose) metabolism with

hyperglycemia, mainly due to deficiency of insulin. Recent studies have identified that tissue lipid accumulation and dysregulated fatty acid metabolism both participate in the formation of insulin resistance and DM. Likewise, fatty acids metabolism are reported to be contributed to cataractogenesis [26]. For example, some fatty acids including linoleic and linolenic acid are involved in the development of higher risk of nuclear cataract [12, 27]. In the current study, the levels of palmitic, stearic, and linoleic acid methyl ester were all significantly elevated in patients with DM, which was consistent with a previous study [28]. The metabolic pathway analysis here suggested pathways including fatty acid biosynthesis, fatty acid metabolism, and linoleic acid metabolism may be critical in the formation of DM and diabetic cataracts.

In summary, by using a non-targeted technology, GC-TOF MS, we comprehensively revealed AH metabolomic profiles from a serious of 30 patients (including 15 for controls and 15 with DM). Significantly different metabolomics were observed in association with diabetic mellitus. More importantly, significantly changed metabolites related to oxidative stress and their corresponding pathways including fatty acid biosynthesis, fatty acid metabolism, and linoleic acid metabolism may play key roles in the formation of DM and diabetic cataracts. Our effort may present potential biomarkers for predicting DM in AH, and also broaden our understanding of the underlying molecular mechanisms.

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Disclosure of conflict of interest

None.

Address correspondence to: Jianming Tang, Department of Ophthalmology, Baoshan District Traditional Chinese and Western Medicine Hospital, No. 181 Youyi Road, Shanghai 201900, P. R. China. Tel: +86-13916977039; E-mail: 13916977039@163.com

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Supplementary Table 1. List of 263 detected metabolites in 30 AH samples

Biochemical Name	Super Pathway	Retention time (minutes)	CAS	KEGG	PubChem
beta-Glutamic acid 1	Amino Acid	10.68	56-86-0	C00025	611
1-Aminocyclopropanecarboxylic acid	Amino acid	8.02	22059-21-8	C01234	535
2,6-Diaminopimelic acid 1	Amino acid	12.65	583-93-7	C00680	865
4-Acetamidobutyric acid 1	Amino acid	9.96	3025-96-5	C02946	18189
4-Acetylbutyric acid 2	Amino acid	8.78	448671	C02129	18407
Alanine 1	Amino acid	7.13	56-41-7	C01401	602
beta-Alanine 2	Amino acid	9.55	107-95-9	C00099	239
Citrulline 1	Amino acid	11.81	372-75-8	C00327	833
Glutamine 1	Amino acid	11.55	56-85-9	C00064	5961
Glycine 2	Amino acid	8.68	56-40-6	C00037	750
Isoleucine	Amino acid	8.55	73-32-5	C00407	6306
Lysine	Amino acid	12.34	56-87-1	C00047	5962
Methionine 1	Amino acid	10.10	63-68-3	C01733	876
N-Acetyltryptophan 2	Amino acid	14.55	87-32-1		2002
N-Ethylglycine 2	Amino acid	7.79	627-01-0	C11735	316542
N-Methyl-DL-alanine	Amino acid	7.66	600-21-5		4377
Norleucine 2	Amino acid	7.87	327-57-1		9475
Ornithine 1	Amino acid	11.79	70-26-8	C00077	6262
Oxoproline	Amino acid	10.14	98-79-3	C02238	7405
Phenylalanine 1	Amino acid	10.78	63-91-2	C02057	994
Proline	Amino acid	8.63	147-85-3	C00148	614
Sarcosine	Amino acid	7.38	107-97-1	C00213	1088
Serine 1	Amino acid	8.98	56-45-1	C00716	617
Tryptophan 1	Amino acid	13.79	73-22-3	C00806	1148
Tyrosine 1	Amino acid	12.44	60-18-4	C01536	1153
Valine	Amino acid	7.99	72-18-4	C00183	1182
2-Amino-2-norbornanecarboxylic acid 4	Amino acid	9.31			
3-hydroxy-L-proline 2	Amino acid	9.76		C05147	7565
Asparagine 1	Amino acid	10.96	70-47-3	C00152	236
Aspartic acid 1	Amino acid	10.04	56-84-8	C00049	3351
Glutathione-H_2O	Amino acid	15.63	70-18-8	C00051	3353
L-Allothreonine 2	Amino acid	8.58	28954-12-3	C05519	99289
Nicotinoylglycine 2	Amino acid	12.25	583-08-4	C05380	68499
O-Hydroxyhippuric acid 2	Amino acid	13.12	487-54-7	C07588	10253
Threonine 1	Amino acid	9.16	72-19-5	C00188	205
2-Deoxy-D-galactose 2	Carbohydrate	11.69	1949-89-9		225612
2-Deoxytetronic acid	Carbohydrate	9.42	1518-61-2		150929
3,6-Anhydro-D-galactose 3	Carbohydrate	11.41	14122-18-0	C06474	441040
3-Hydroxypropionic acid 1	Carbohydrate	7.43	503-66-2	C01013	68152
6-phosphogluconic acid	Carbohydrate	14.72	921-62-0		
Allose 1	Carbohydrate	10.89	2595-97-3		102288
Alpha-D-glucosamine 1-phosphate	Carbohydrate	11.84	2152-75-2	C06156	740
beta-Mannosylglycerate 2	Carbohydrate	11.96	164324-35-0		5460194
Citraconic acid 4	Carbohydrate	8.93	498-23-7	C02226	5291
Citric acid	Carbohydrate	11.75	5949-29-1	C00158	311

D-galacturonic acid 2	Carbohydrate	12.57	685-73-4	C00333	3627
D-Glucoheptose 2	Carbohydrate	13.51	62475-58-5		219662
D-Glyceric acid	Carbohydrate	8.76	6000-40-4	C00258	752
Erythrose 2	Carbohydrate	9.57	583-50-6		94176
Fructose 1	Carbohydrate	12.02	57-48-7	C10906	5984
Fructose 2	Carbohydrate	12.07	57-48-7	C10906	5984
Fructose-6-phosphate	Carbohydrate	14.12	643-13-0		69507
Galactonic acid	Carbohydrate	12.75	576-36-3	C00880	4136
Glucoheptonic acid 3	Carbohydrate	13.37	23351-51-1		
Glucose-1-phosphate	Carbohydrate	11.48	59-56-3	C11450	65533
Guanidinosuccinic acid 1	Carbohydrate	11.04	6133-30-8		97856
Lactic acid	Carbohydrate	6.79	50-21-5	C01432	612
Lactose 2	Carbohydrate	16.00	63-42-3		493593
L-Malic acid	Carbohydrate	9.82	97-67-6	C00149	222656
Lyxose 1	Carbohydrate	10.79	1114-34-7	C00476	3759
Mannose 1	Carbohydrate	12.11	3458-28-4	C01662	24749
Myo-inositol	Carbohydrate	13.17	87-89-8	C06153	892
N-Acetyl-beta-D-mannosamine 4	Carbohydrate	13.24	3615-17-6	C00645	3918
Oxalic acid	Carbohydrate	7.42	144-62-7	C00209	971
Pyruvic acid	Carbohydrate	6.70	127-17-3	C00022	1060
Ribose	Carbohydrate	11.01	24259-59-4	C00121	993
Succinic acid	Carbohydrate	8.68	110-15-6	C00042	1110
Tagatose 1	Carbohydrate	11.92	87-81-0		92092
Threitol	Carbohydrate	9.92	7493-90-5		169019
Xylitol	Carbohydrate	11.20	87-99-0	C00379	3669
D-Arabitol	Carbohydrate	11.23	488-82-4		94154
Fructose 2,6-biphosphate degr prod 1	Carbohydrate	13.64	79082-92-1	C00665	105021
Galactose 2	Carbohydrate	12.34	59-23-4	C00124	3424
Gluconic acid 1	Carbohydrate	12.70	526-95-4	C00257	3556
Glucose 2	Carbohydrate	12.28	478529-49-6	C01662	24749
Itaconic acid	Carbohydrate	8.87	97-65-4	C00490	811
Leucrose 1	Carbohydrate	16.67	7158-70-5		
L-Threose 1	Carbohydrate	9.61	95-44-3		101562
Maleic acid	Carbohydrate	8.55	110-16-7	C01384	444266
Mannitol	Carbohydrate	12.43	69-65-8	C00392	3682
N-ethylmaleamic acid 3	Carbohydrate	9.80	4166-67-0		5369191
Oxalacetic acid	Carbohydrate	9.73	328-42-7	C00036	970
Sorbitol	Carbohydrate	12.39	50-70-4	C00794	5780
Sucrose	Carbohydrate	15.71	57-50-1	C00089	5988
Xylose 2	Carbohydrate	10.86	6763-34-4		644160
1-Monopalmitin	Lipids	15.45	542-44-9		
2-hydroxybutanoic acid	Lipids	7.30	600-15-7	C05984	8262
Caprylic acid	Lipids	8.36	124-07-2	C06423	379
D-(glycerol 1-phosphate)	Lipids	11.42	34363-28-5	C00093	439162
Diglycerol 1	Lipids	11.45	627-82-7		42953
Glycerol	Lipids	8.35	56-81-5	C00116	753
Linoleic acid methyl ester	Lipids	13.23	112-63-0		5284421
Malonic acid 1	Lipids	7.86	141-82-2	C04025	867
Palmitic acid	Lipids	12.96	21096	C00249	985

Stearic acid	Lipids	13.86	21128	C01530	5281
2-Hydroxyvaleric acid	Lipids	7.94	617-31-2		
2-Monoolein	Lipids	16.26	3443-84-3		5319879
Oxamic acid	Lipids	8.31	471-47-6	C01444	4622
Prostaglandin A2 3	Lipids	16.23	8237	C05953	27820
Thymol	Lipids	8.74	89-83-8	C09908	12094
Flavin adenine degrad product	Nucleotide	11.38	146-14-5		444188
Purine riboside	Nucleotide	14.52	550-33-4	C01736	68368
Ribonic acid, gamma-lactone	Nucleotide	11.09	1255190		111064
Thymidine 3	Nucleotide	14.61	50-89-5	C00214	5789
Uracil	Nucleotide	8.88	66-22-8	C00106	1174
1-Hydroxyanthraquinone 1	Others	14.23	129-43-1	C02980	5890
2-hydroxypyridine	Others	6.69	142-08-5	C02502	8871
2-mercaptoethanesulfonic acid 2	Others	10.64	3375-50-6	C03576	598
3-(2-Hydroxyphenyl) propionic acid	Others	11.16	495-78-3	C01198	873
3-Hexenedioic acid	Others	10.02	4436-74-2		107550
3-hydroxybutyric acid	Others	7.55	306-31-0		441
3-Hydroxypyridine	Others	7.50	109-00-2		7971
4-hydroxybenzaldehyde 1	Others	9.98	123-08-0	C00633	126
4-HYDROXYPYRIDINE	Others	7.64	626-64-2		12290
Analyte 10	Others	6.24			
Analyte 153	Others	8.34			
Analyte 22	Others	6.35			
Analyte 23	Others	6.39			
Analyte 230	Others	9.84			
Analyte 26	Others	6.45			
Analyte 27	Others	6.47			
Analyte 30	Others	6.49			
Analyte 32	Others	6.52			
Analyte 37	Others	6.58			
Analyte 38	Others	6.59			
Analyte 5	Others	6.20			
Analyte 52	Others	6.82			
Analyte 6	Others	6.23			
Benzoic acid	Others	8.33	65-85-0	C03096	243
Biotin	Others	15.10	58-85-5	C00120	3420
Cis-gondoic acid	Others	14.68	5561-99-9		5282768
Conduritol b epoxide 2	Others	12.51	6090-95-5		2859
Creatine	Others	10.35	57-00-1	C00300	586
D-erythronolactone 2	Others	10.56	15667-21-7		5325915
DL-Anabasine 1	others	9.34	13078-04-1	C06180	8431
Glycolic acid	Others	6.91	79-14-1	C03547	757
Homogentisic acid	others	11.86	451-13-8	C00544	3825
Indole-3-acetamide 4	Others	13.98	879-37-8	C02693	397
IS	Others	11.61			
Pelargonic acid	Others	9.05	112-05-0	C01601	8158
Phosphate	Others	8.39	7664-38-2	C00009	1004
Resorcinol	Others	9.20	108-46-3	C01751	5054
Saccharic acid	Others	12.84	576-42-1		33037

Salicin	Others	15.02	138-52-3		5145
Sulfuric acid	Others	7.58	7664-93-9	C00059	1118
Synephrine 2	Others	11.07	34520	C04548	7172
Tartaric acid	Others	10.76	133-37-9	C00898	875
Tartronic acid	Others	9.16	80-69-3	C02500	45
Threonic acid	Others	10.25	7306-96-9		151152
Unknown 001	Others	6.67			
Unknown 002	Others	6.84			
Unknown 003	Others	7.07			
Unknown 004	Others	7.14			
Unknown 005	Others	7.49			
Unknown 006	Others	7.59			
Unknown 007	Others	7.63			
Unknown 008	Others	7.67			
Unknown 009	Others	7.70			
Unknown 010	Others	7.82			
Unknown 011	Others	7.81			
Unknown 012	Others	7.90			
Unknown 013	Others	7.98			
Unknown 014	Others	8.22			
Unknown 015	Others	8.29			
Unknown 016	Others	8.39			
Unknown 017	Others	8.76			
Unknown 018	Others	8.86			
Unknown 019	Others	9.00			
Unknown 020	Others	9.06			
Unknown 021	Others	9.07			
Unknown 022	Others	9.13			
Unknown 023	Others	9.24			
Unknown 024	Others	9.46			
Unknown 025	Others	9.47			
Unknown 026	Others	9.64			
Unknown 027	Others	9.67			
Unknown 028	Others	9.72			
Unknown 029	Others	9.87			
Unknown 030	Others	9.88			
Unknown 031	Others	9.97			
Unknown 032	Others	10.01			
Unknown 033	Others	10.09			
Unknown 034	Others	10.15			
Unknown 035	Others	10.20			
Unknown 036	Others	10.24			
Unknown 037	Others	10.39			
Unknown 038	Others	10.50			
Unknown 039	Others	10.53			
Unknown 040	Others	10.55			
Unknown 041	Others	10.61			
Unknown 042	Others	10.73			
Unknown 043	Others	10.82			

Unknown 044	Others	11.14			
Unknown 045	Others	11.23			
Unknown 046	Others	11.36			
Unknown 047	Others	11.52			
Unknown 048	Others	11.67			
Unknown 049	Others	11.85			
Unknown 050	Others	12.20			
Unknown 051	Others	12.24			
Unknown 052	Others	12.30			
Unknown 053	Others	12.37			
Unknown 054	Others	12.41			
Unknown 055	Others	12.47			
Unknown 056	Others	13.16			
Unknown 057	Others	13.78			
Unknown 058	Others	13.93			
Unknown 059	Others	14.00			
Unknown 060	Others	14.03			
Urea	Others	8.21	57-13-6	C00086	1176
2-hydroxy-3-isopropylbutanedioic acid	Others	10.49	3237-44-3		
2-keto-isovaleric acid 2	Others	7.41			
4-hydroxyphenylacetic acid	Others	10.87	156-38-7	C00642	3915
Acetol 3	Others	11.09	116-09-6	C05235	8299
Fluorene	Others	10.77	86-73-7	C07715	9917
Hydroxylamine	Others	7.29	7803-49-8	C00192	787
Maleamate 4	Others	9.60	557-24-4	C01596	4751
Trans-3,5-Dimethoxy-4-hydroxycinnamaldehyde 1	Others	13.32	4206-58-0		
Analyte 115	Others	7.72			
Analyte 117	Others	7.74			
Analyte 125	Others	7.85			
Analyte 14	Others	6.28			
Analyte 149	Others	8.27			
Analyte 201	Others	9.27			
Analyte 204	Others	9.32			
Analyte 25	Others	6.41			
Analyte 262	Others	10.45			
Analyte 276	Others	10.67			
Analyte 28	Others	6.49			
Analyte 290	Others	10.91			
Analyte 31	Others	6.51			
Analyte 39	Others	6.63			
Analyte 411	Others	12.86			
Analyte 415	Others	13.02			
Analyte 43	Others	6.71			
Analyte 442	Others	13.85			
Analyte 446	Others	13.99			
Analyte 449	Others	14.04			
Analyte 45	Others	6.73			
Analyte 451	Others	14.11			
Analyte 454	Others	14.16			

Analyte 455	Others	14.17
Analyte 460	Others	14.46
Analyte 464	Others	14.55
Analyte 466	Others	14.62
Analyte 467	Others	14.64
Analyte 469	Others	14.70
Analyte 475	Others	14.89
Analyte 476	Others	15.00
Analyte 480	Others	15.13
Analyte 489	Others	15.50
Analyte 495	Others	15.82
Analyte 503	Others	16.89
Analyte 508	Others	17.58
Analyte 60	Others	6.93
Analyte 64	Others	6.99
Analyte 65	Others	7.01
Analyte 66	Others	7.04
Analyte 71	Others	7.16
Analyte 74	Others	7.19
Analyte 77	Others	7.25
Analyte 83	Others	7.35
Analyte 93	Others	7.47

Supplementary Table 2. Pathway analysis results from MetaboAnalyst web server

Pathway Name	Match Status	p	-log (p)	Holm p	FDR	Impact
Fatty acid biosynthesis	2/49	0.0079889	4.8297	0.63911	0.33241	0.0
Fatty acid metabolism	2/50	0.0083101	4.7903	0.6565	0.33241	0.04482
Linoleic acid metabolism	1/15	0.042868	3.1496	1.0	1.0	0.0
Fatty acid elongation in mitochondria	1/27	0.076019	2.5768	1.0	1.0	0.0
Alpha-Linolenic acid metabolism	1/29	0.081447	2.5078	1.0	1.0	0.0
Pentose phosphate pathway	1/32	0.089539	2.4131	1.0	1.0	0.13556
Glycerophospholipid metabolism	1/39	0.10818	2.2239	1.0	1.0	0.10053
Lysine degradation	1/47	0.12908	2.0473	1.0	1.0	0.06909
Pyrimidine metabolism	1/60	0.16216	1.8192	1.0	1.0	0.0
Arachidonic acid metabolism	1/62	0.16715	1.7889	1.0	1.0	0.0
Arginine and proline metabolism	1/77	0.20378	1.5907	1.0	1.0	0.03036
Tryptophan metabolism	1/79	0.20856	1.5675	1.0	1.0	0.02196
Purine metabolism	1/92	0.23902	1.4312	1.0	1.0	0.00794