Metabolomics in Diabetic Retinopathy: A Systematic Review

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PURPOSE. Diabetic retinopathy (DR), a common microvascular complication of diabetes, is the leading cause of acquired blindness in the working-age population.1 It is well established that traditional risk factors for DR include long diabetes duration and poor glycemic and blood pressure control.2 However, several disadvantages of these methods should be acknowledged. First, imaging diagnosis is effective only if specific pathologic changes have been detected and confirmed.3 Second, genomic and proteomic studies may provide limited correlation with disease phenotypes, and only a small fraction of patients would benefit from these therapies.4

Metabetomics is a promising branch of omics for revealing the metabolic changes and underlying mechanisms involved in the pathogenesis of diseases.5 The retina is a highly metabolically active tissue, and the application of metabolomics in retinal diseases may achieve success in identifying useful biomarkers.6 Although quite a few studies have demonstrated that metabolomics is a promising strategy for identifying potential biomarkers, the number of samples is limited, and most studies are focused on specific aspects of DR.7 Therefore, identifying potential biomarkers that can be used to identify useful biomarkers for risk stratification is necessary to prevent irreversible retinal damage among diabetic patients.8

METHODS. We searched PubMed and Web of Science for relevant metabolomics studies on humans published before September 30, 2020. Information regarding authors, title, publication date, study subjects, analytical platforms, methods of statistical analysis, biological samples, directions of change of potential metabolic biomarkers, and predictive values of metabolomic biomarker panels was extracted, and the quality of the studies was assessed.

RESULTS. We found nine studies focused on the identification of potential biomarkers. Repeatedly identified metabolites including L-glutamine, L-lactic acid, pyruvic acid, acetic acid, L-glutamic acid, D-glucose, L-alanine, L-threonine, citrulline, L-lysine, and succinic acid were found to be potential biomarkers of DR. It was observed that L-glutamine and citrulline changed in all biological samples. Dysregulation of metabolic pathways involved amino acid and energy metabolism.

CONCLUSIONS. This review summarizes potential biomarkers and metabolic pathways, providing insights into new pathogenic pathways for this microvascular complication of diabetes.

Keywords: diabetic retinopathy, metabolomics, biomarkers, pathway analysis

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Metabolomic studies have reported possible metabolic biomarkers and pathways of DR, a systematic summary is lacking.

In this effort, we aimed to summarize the metabolites that are significantly altered in DR and explore the metabolic pathways involved in the onset and progression of DR. The findings might move the care of patients with DR toward a personalized approach and lead to new therapeutic options.

**METHODS**

**Search Strategy**

Publications on metabolomics studies on DR were independently searched and scanned by two authors (XWH and YW). Disagreements were solved by discussion with a senior author (CWP). We obtained relevant articles by searching PubMed and Web of Science before September 30, 2020, with the following search terms: (“metabolomics” or “metabonomics” or “metabolome” or “metabolic profiling”) AND “diabetic retinopathy.” Additional articles were identified by searching the reference lists of the included studies.

**Inclusion and Exclusion Criteria**

Studies assessing the application of metabolomics to identifying biomarkers of DR, as well as studies that applied mass spectrometry (MS)-based or nuclear magnetic resonance (NMR)-based metabolomics on humans, were considered in this systematic review. Studies with clear statistical methods and that met the criteria for differential metabolites were included in the review. Animal studies, drug-evaluation reports, review articles, and abstracts without full texts were excluded.

**Data Extraction and Quality Assessment**

QUADOMICS is a quality assessment tool specifically designed for omics-based diagnostic assessment studies to address specific challenges based on omics techniques in systematic reviews. We used this tool to assess the quality of the studies we reviewed. After reading the full articles and supplementary materials, we extracted information regarding authors, title, publication date, study subjects, detection and analytical platforms, methods of statistical analysis, and the frequency and directions of changes in potential metabolic biomarkers.

**Statistical Analysis**

Pathway analysis, including enrichment analysis and topology analysis, was derived from integrating differential metabolites using MetaboAnalyst 3.0, based on the Kyoto Encyclopedia of Genes and Genomes and Human Metabolome Database. P values were established based on pathway enrichment analyses, and pathway impact values were derived from pathway topology analyses.

**RESULTS**

**Study Characteristics**

A total of 865 articles were identified by searching databases and adding 235 hand-searched references. After the removal of duplicates (n = 185), we screened titles and abstracts of the remaining 915 articles and excluded 837 articles that were not relevant to this research topic or did not meet the inclusion criteria. Finally, nine articles were included in the systematic review. A simplified flow diagram of study selection is shown in Figure 1.

A total of three types of biological tissues were involved in the study. Four used plasma, one used aqueous humor (AH), and one used vitreous samples. With regard to the analytical platforms, MS-based metabolomics studies were reported in seven articles, including liquid chromatography–mass spectrometry (LC-MS; n = 4), gas chromatography–mass spectrometry (GC-MS; n = 2), and a combination of LC-MS and GC-MS (n = 1). Two studies employed a NMR platform. The sample sizes ranged from 34 to 173 individuals. All of the included studies were untargeted metabolomics studies. Rhee et al. reported more complete data processing and analysis. The corresponding operating software included SAS software (SAS Institute, Cary, NC, USA). Raw data files were converted using Masslynx DataBridge software (Waters Corporation, Milford, MA, USA); peak detection, retention time correction, and alignment were processed using the MetAlign software package; and multivariate statistical analysis was conducted using SIMCA-P+. Three studies used MetaboAnalyst for pathway analysis. Characteristics of the studies are presented in Table 1, and demographic information is provided in Supplementary Table S1.

**Metabolic Biomarkers Identified for DR**

A large number of differentially expressed metabolites associated with DR were identified by these studies (Fig. 2). A total of 86 statistically significant differential metabolites were extracted from these studies. Table 2 summarizes the potential metabolic biomarkers of DR that had a frequency of two or more reports in the literature. Overlaps of differentially altered metabolites in different biological samples are shown in Figure 3. L-Glutamine and citrulline were observed to differ in all biological samples, but L-glutamine showed concordance among all of the biological samples. Citrulline showed downregulation in AH but upregulation in plasma and vitreous. The direction of change in the levels of some metabolite markers differed among the studies, including that of pyruvic acid, l-glutamic acid, D-glucose, and l-lactic acid. Citrulline, l-glutamine, and l-lactic acid were reported a total of three times.

**Quality Assessment of the Included Studies**

The quality of the included studies was assessed according to QUADOMICS, and detailed information is provided in Supplementary Table S2. The second and 14th items of QUADOMICS regarding metabolomics in practice did not apply to all of the included studies; therefore, they were not included. All of the studies met the evaluation criteria of 70%, and two of the studies met the criteria of 13 out of 14 items. With regard to item 16, five studies (55.5%) lacked measures to avoid overfitting. None of the studies met the 12th item in the tool; that is, the index test results were interpreted with knowledge of the results of the reference standard, rather than without.
FIGURE 1. Flow diagram of literature search and study selection for metabolite markers of DR.

TABLE 1. Metabolomics Studies Analyzing Samples From Patients With DR

| References         | Biofluid | Comparison                                                                 | Metabolomic Technique Employed | Differential Metabolite Evaluation Standard |
|--------------------|----------|-----------------------------------------------------------------------------|--------------------------------|---------------------------------------------|
| Sumarriva et al.12 | Plasma   | DR cases (n = 83) and diabetic controls (n = 90)                             | LC-MS                         | PLS-DA                                      |
| Haines et al.19    | Vitreous | Rhegmatogenous RD (n = 25), PDR (n = 9), and controls without significant retinal disease (n = 8) | UHPLC-MS                      | ANOVA, Student’s t-test                     |
| Zhu et al.14       | Plasma   | NPDR (n = 21) and PDR (n = 21)                                              | UHPLC Q-TOF-MS                | t-test, PLS-DA                              |
| Chen et al.15      | Plasma   | NPDR (n = 40) and patients with diabetes without DR (n = 40; discovery set) | GC-MS                         | Mann–Whitney U test                         |
| Rhee et al.13      | Plasma   | DR cases (n = 32) and no-DR cases (n = 32)                                   | UHPLC Q-TOF-MS, GC-TOF-MS     | OPLS-DA, Welch’s t-test                     |
| Wang et al.20      | Vitreous and AH | Vitreous samples: PDR (n = 28) and non-diabetic patients with MH (n = 22) AH samples; PDR (n = 25) and non-diabetic patients with cataract (n = 25) | GC-TOF-MS                      | OPLS-DA, Mann–Whitney U test                |
| Paris et al.17     | Vitreous | PDR (n = 20) and non-diabetic controls (n = 31)                              | HILIC and RPLC Q-TOF-MS       | Welch’s t-test                              |
| Jin et al.16       | AH       | Patients with type 2 DM and cataract (n = 14), patients with DR and cataract (n = 13), and senile cataract patients (n = 7) | 1H-NMR                        | OPLS-DA, Pearson correlation                |
| Barba et al.18     | Vitreous | PDR (n = 22) and non-diabetic patients with MH (n = 22)                       | 1H-NMR                        | Unknown                                     |

All of the articles used untargeted metabolomic methods. PLS-DA, partial least squares discriminant analysis; RD, retinal detachment; UHPLC-MS, ultra-high-performance liquid chromatography–mass spectrometry; ANOVA, one-way analysis of variance; NPDR, non-proliferative diabetic retinopathy; Q-TOF-MS, quadrupole time-of-flight mass spectrometry; MH, macular hole; OPLS-DA, orthogonal projections to latent structures discriminant analysis; HILIC, hydrophilic interaction liquid chromatography; RPLC, reversed-phase liquid chromatography.
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FIGURE 2. Descriptions of differentially expressed metabolites in the included studies. (↑), positively altered metabolites in DR; (↓), negatively altered metabolites in DR.

TABLE 2. Repeated Metabolic Biomarkers Related to DR

| Metabolite Name | Human Metabolome Database ID | Hits | Biological Samples To Be Analyzed |
|----------------|-----------------------------|------|-----------------------------------|
| L-Glutamine    | HMDB0000641                 | 3    | Plasma (↑), vitreous (↑), AH (↑)   |
| Citrulline     | HMDB0000904                 | 3    | Plasma (↑), vitreous (↑), AH (↑)   |
| l-Lactic acid  | HMDB000190                  | 3    | AH (↑), vitreous (↑), AH (↑)       |
| l-Glutamic acid| HMDB000148                  | 2    | Vitreous (↑), vitreous and AH (↑)  |
| Pyruvic acid   | HMDB000243                  | 2    | Plasma (↑), vitreous (↑)           |
| Acetic acid    | HMDB000042                  | 2    | Vitreous and AH (↑), vitreous (↑)  |
| d-Glucose      | HMDB000122                  | 2    | Vitreous and AH (↑), vitreous (↑)  |
| l-Alanine      | HMDB000161                  | 2    | Vitreous and AH (↑), AH (↑)        |
| l-Threonine    | HMDB000167                  | 2    | Vitreous and AH (↑), AH (↑)        |
| l-Lysine       | HMDB000182                  | 2    | AH (↑), vitreous (↑)              |
| Succinic acid  | HMDB000254                  | 2    | AH (↑), vitreous (↑)              |

(↑), positively altered metabolites in DR; (↓), negatively altered metabolites in DR.

Potential Metabolite Marker Panels to Predict and Classify DR

Five studies conducted explicitly predictive analyses to assess the effect of models when using the information of metabolomics (Table 3). The area under the receiver operating characteristic (ROC) curve (AUC) or sensitivity and specificity, classification accuracy, and adjusted odds ratios were used to evaluate the model predictions. Sumarriva et al. did not use specific metabolites for evaluation, and Zhu et al. separately assessed the discriminability of four potential markers. Rhee et al. determined and evaluated the ratios of the level of glutamine to glutamic acid, whereas the others evaluated a panel of metabolites. Another two studies also assessed potential metabolic features to discriminate DR. Haines et al. separately evaluated the predictive power of xanthine, proline, citrulline, and pyruvate, but AUC values are not explicitly reported. Barba et al. utilized metabolic features to distinguish DR from controls, which exhibited a sensitivity of 86% and a specificity of 81%, but the report did not describe the methods of statistical analysis.

Pathway Analysis

We extracted 81 potential candidate biomarkers from the included studies and performed a pathway enrichment analysis. The results showed that the biomarkers were involved in 45 metabolic pathways, and 14 pathways were significantly associated with metabolites (P < 0.05). As shown in Figure 4, six amino acid metabolism-related pathways were reported in the studies: (1) arginine biosynthesis; (2) alanine, aspartate, and glutamate metabolism; (3) valine, leucine, and isoleucine biosynthesis; (4) arginine and proline metabolism; (5) D-glutamine and D-glutamate metabolism; and (6) histidine metabolism. The six carbohydrate-related metabolic pathways included (1) glyoxylate and dicarboxylate metabolism; (2) galactose metabolism; (3) starch and sucrose metabolism; (4) citric acid cycle (tricarboxylic acid [TCA] cycle); (5) pentose phosphate pathway; and (5) pyruvate metabolism. The one translation-related metabolic pathway identified was aminoacyl-tRNA biosynthesis, and the one energy-related metabolic pathway was nitrogen metabolism.

Starch and sucrose metabolism pathways had the largest impact (impact value = 0.512). Arginine biosynthesis was...
TABLE 3. Classification/Prediction Potential of the Information of Metabolomics

| References            | Discriminant Model          | Biomarker Panel                                                                 | Precision                      |
|-----------------------|-----------------------------|----------------------------------------------------------------------------------|--------------------------------|
| Sumarriva et al.12     | Support vector machine      | 236 differential features; Fumaric acid, uridine, acetic acid, and cytidine.    | Classification accuracy = 88.5% |
| Zhu et al.14           | Pattern recognition         | Erythritol; maltose/trehalose; mannose; ribose+1,5-anhydroglucitol; mannose;    | AUCs = 0.96, 0.95, 1.0, and 0.95, respectively |
| Chen et al.15          | Logistic regression (adjusted for HbA1c) | 1,5-anhydroglucitol; 1,5-gluconolactone; 2-deoxyribonuronic acid; gluconic acid; and lactose/cellobiose and urea. | Adjusted odds ratios for 1,5-anhydroglucitol; 1,5-gluconolactone; 2-deoxyribonuronic acid; gluconic acid; and lactose/cellobiose and urea remained significant |
| Rhee et al.13          | Logistic regression         | Ratio of the levels of glutamine to glutamic acid.                              | AUC = 0.742                     |
| Wang et al.20          | Logistic regression         | AH: D-2,3-dihydroxypropanoic acid, isocitric acid, fructose 6-phosphate, and L-lactic acid. Vitreous: pyroglutamic acid and pyruvic acid. | AH: AUC = 0.965, sensitivity = 88%, specificity = 95.7% |

FIGURE 4. Results of the pathway analysis of metabolic biomarkers of DR. The color of the circle indicates the significance level in the enrichment analysis; darker color (more red) indicates greater significance. The size of the circle reflects the pathway impact value in the topology analysis, such that the larger the circle, the larger the impact value. The x-axis is the pathway impact value calculated based on topology analysis.

the most prominent pathway derived from the selected biomarkers. Metabolites had the highest pathway matching status in arginine biosynthesis, matching eight out of 14 metabolites in the pathway, followed by alanine, aspartate, and glutamate metabolism. Detailed information regarding the enriched pathways is shown in Supplementary Table S3.

**DISCUSSION**

In this review, nine metabolomics studies on DR were comprehensively reviewed, and the data of these studies were analyzed to identify valuable metabolic biomarkers and pathways involved in the pathology of DR. Several potential metabolites were summarized (observed in at least two studies), including L-glutamine, L-lactic acid, pyruvic acid, acetic acid, L-glutamic acid, D-glucose, L-alanine, L-threonine, citrulline, L-lysine, and succinic acid. Both L-glutamine and citrulline were especially observed to differ in all biological samples (plasma, vitreous, and AH). After analyzing the metabolic pathways of differential metabolites, we found that amino acid and energy metabolism significantly differed between patients with DR and controls.

**Amino Acid Metabolism in DR**

More and more studies have emphasized the protective effect of amino acid metabolism on microvascular endothelial cells. Welsh et al.21 reported a reduced risk of diabetic microvascular disease in people with higher levels of tyrosine and alanine. A study on patients with PDR showed a significant increase in vitreous amino acid levels, suggesting a beneficial effect of amino acids on DR.22 Lipocalin has been reported to improve mitochondrial function, insulin sensitivity, and antiinflammatory effects, whereas amino acids such as lysine and alanine were reported to be positively correlated with adiponectin.22,23 The effect of threonine on DR might be produced by serine/threonine kinase–activated non-protein coding RNA (BRAF-activated non-coding RNA [BANCR]), which is involved in the development of DR through its regulatory role in cell apoptosis.24

D-Glutamine and D-glutamate metabolism and the arginine biosynthesis pathway had higher impact values. As one of the major excitatory neurotransmitters, glutamate plays an important role in maintaining the normal structure and function of neurons. It has been found that increased glutamate levels or an imbalance between excitation and inhibition can exacerbate ischemic damage to the retina and promote neovascularization.25 Stimulated by the release of large amounts of glutamate, excitatory neurons continue to depolarize by activating glutamate receptors. Osmotic pressure and the electrochemical properties of ions have been shown to change due to the impaired neuronal regulatory function, leading to metabolism breakdown and nerve cell death.25 In addition, high levels of oxidative stress in diabetics has been proven to be associated with decreased glutamate synthesis, which in turn directly contributes to lower levels of glutamine and aggravates insufficient insulin secretion.26 Rhee et al.13 showed that plasma glutamate and glutamine were decreased and increased, respectively, in
patients with DR compared with the control group, and ROC curve analysis was performed using the glutamine/glutamic acid ratios of subjects, resulting in an AUC of 0.742. However, the reverse was found for glutamine levels, possibly due to the differences in the selection range of controls. Arginine, an important step in the ornithine cycle, is involved in arginine biosynthesis and the arginine and proline metabolism pathways. Arginase metabolizes arginine to form proline, polyamines, and glutamate. High arginase activity will reduce arginine levels, leading to the uncoupling of nitric oxide synthase and inducing polyamine oxidation and glutamate formation. The produced superoxide and nitric oxide react rapidly to form the toxic oxidant pernitrite, resulting in nerve vascular injury in the process of retinopathy.

**Energy Metabolism in DR**

The starch and sucrose metabolism pathway showed the greatest impact in the pathway analysis, suggesting the vital role of glycolysis and gluconeogenesis in DR. Glucose, pyruvic acid, and lactic acid in this pathway are differential metabolites that have been found repeatedly. Hyperglycemia is the well-known prominent feature of diabetes. Pyruvate is an end-product of glycolysis. Pyruvate can produce lactic acid through anaerobic metabolism and can also enter mitochondria to participate in oxidative phosphorylation. Therefore, abnormal pyruvate and lactic acid levels might indicate glucose metabolism and mitochondrial dysfunction. Also, pyruvate influences acetate levels through acetyl-CoA, so plasma acetate levels in diabetic patients might synchronously change with pyruvate. The citrate (TCA) cycle is the final metabolic pathway for decomposition of the three major nutrients. Jin et al. and Barba et al. reported decreased succinic acid levels in AH and vitreous, respectively. As an integral part of the TCA cycle, succinic acid could be used as a marker of energy metabolism disorder in DR. In addition, chronic hyperglycemia has also been reported to cause extensive oxidative stress and neuroinflammation in diabetes. Extensive oxidative stress products and inflammatory factors cause microvascular and neurological dysfunction, resulting in retinal hypoxia and metabolic dysfunction, ultimately leading to irreversible vision loss.

**Metabolomics Research Complementary to the Understanding of Disease**

The Early Treatment for Diabetic Retinopathy Study (ETDRS) disease severity scale is based on the Airlie House classification system, which refines the characterization of DR and expands the description and classification of DME. But, with progress in scientific research, especially the development of anti-vascular endothelial growth factor drugs, use of the ETDRS scale to guide clinical treatment is not sufficient. In addition, the emergence of ultra-wide-angle imaging technology could accelerate the diagnosis process by providing automatic real-time assessment while challenging the accuracy of standard assessment. New disease assessment methods for DR are urgently required that take advantage of the emergence of new technologies and research results. Traditional risk factors for DR include the course of diabetes mellitus and hemoglobin A1c levels, among others, but these factors do not accurately describe the progression of DR. As the embodiment of interactions between an organism and its environment, metabolomics has the potential to supplement current information regarding disease changes. Current studies have proposed that metabolomics could present well-discriminated information about DR, with AUC values reaching ≥0.95, 20

**Limitations and Future Directions**

To our knowledge, this is the first systematic review of DR metabolomics using bioinformatics methods. The pathway analysis revealed a series of metabolic dysregulations related to DR, which could become potential targets for drug therapy. However, there are still several limitations to current metabolomics research that should be acknowledged. First, the identification of biomarkers promotes our understanding of the complex mechanisms involved, but the accurate prediction of DR cannot be realized at present. Second, keeping with the DR reviews by Ting et al. and Liew et al., we have found 10 markers that can be identified repeatedly, but the research in this field still must be further developed. Finally, strict quality control is required in metabolomics research, and the influence of dietary, environmental, and physiological factors among the control groups should be considered. Experimental studies should add measures to avoid overfitting, such as independent external validation sets. Future studies on metabolomics could be carried out by blind tests. Ideally, intergroup analysis should be performed among between the healthy control group, the non-DR group with diabetes, and patients with DR, and the complete experimental process should be studied and verified.

Exploring the pathogenesis of complex diseases through metabolomics is an attractive field of study. Some studies on the metabolomics of DR that have provided metabolic information on DR were not included in this study because they did not focus on discriminant biomarkers of patients with DR. For example, Young et al. performed nuclear magnetic resonance spectroscopy on the vitreous of patients with PDR in an eye disease study. They pointed out that DR had unique metabolic characteristics that could distinguish it from other eye diseases. A study on DR metabolomics based on GC-MS analyzed the metabolic changes of diseases according to different disease classification methods (international classification systems and Chinese medicine classification of DR) to investigate research progress in traditional Chinese medicine. Another study, in conjunction with metabolomics and lipidomics, estimated the association between multiple metabolites and DR grading to identify risk markers for DR progression. The ultimate goal of metabolomics research is to realize the integration of research results and previous research results to develop a powerful tool for in-depth study of diseases. In future research, DR biomarkers should be non-invasive, rapid, and economical, helping us better understand the complex pathogenesis of diseases.

**Conclusions**

Several metabolites and metabolic pathways related to DR were revealed in our review, and the reported predictive value of metabolic biomarker panels suggests potential applications of metabolomics in the clinical management of DR. However, metabolomics research into DR is still in the preliminary stages of development, and the number of stud-
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