Metabolomic Analysis of Human Vitreous in Rhegmatogenous Retinal Detachment Associated With Choroidal Detachment

Mengxi Yu, Zhifeng Wu, Zhengwei Zhang, Xiaoli Huang, and Qing Zhang

Department of Ophthalmology, Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, Jiangsu Province, People’s Republic of China

Correspondence: Zhifeng Wu, Department of Ophthalmology, Nanjing Medical University Affiliated Wuxi Second Hospital, Wuxi, Jiangsu Province, People’s Republic of China; zhifengwug@126.com.

MY and ZW contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Rhegmatogenous retinal detachment associated with choroidal detachment (RRDCD) is a serious and unusual type of rhegmatogenous retinal detachment (RRD). The occurrence of RRDCD has been reported to be 2.0% to 4.5% in Western countries and 4.2% to 18.1% in China.1–3 Its typical pathological features are the presence of RRD accompanied by a detached choroid and/or ciliary body from the sclera. The onset of RRDCD develops rapidly and typically involves severe uveitis and hypotony. Without prompt treatment, the prognosis becomes worse because of rapid onset of massive vitreous and periretinal proliferation.4 The latest clinical multicenter study showed that choroidal detachment is an independent explanatory variable of retinal detachment repair failure.5 Most studies have focused on the treatment of RRDCD, and the underlying etiology and pathogenesis of RRDCD have not been elucidated because of its poor prognosis.6 Hypotony may be the most important factor in the pathogenesis of RRDCD.7,8 The vitreous humor enters into the subretina via a retinal tear, which may lead to hypotony. Hypotony provokes choroidal detachment by arteriolar dilation and ciliary body swelling and detachment, leading to the breakdown of the blood-retinal barrier and the exudation of excessive serum, containing fibronectin and platelet-derived growth factor. These factors cause the migration of the RPE into the vitreous cavity, which may cause an inflammatory reaction leading to proliferative vitreoretinopathy. Another theory suggests that the main mechanism of RRDCD is uveitis caused by rhegmatogenous retinal detachment,9 where the liquefied vitreous passes through a retinal tear and is absorbed by the RPE pump, stimulating the uvea to produce inflammatory mediators, such as 5-hydroxytryptamine, prostaglandin, and histamine. Due to arteriolar dilation and destruction of the blood-retinal barrier caused by ciliary body edema, the permeability of uveal vasculature increases and intravascular fluid and protein leak into the suprachoroidal space. These factors finally cause the detachment of the choroid and/or the ciliary body. As there are no conclusions about the pathogenesis or cause behind the poor prognosis of RRDCD, it may be useful to understand the metabolic changes occurring with RRDCD.

Metabolomics is an efficient technique to elucidate the metabolic pathways involved with physiological processes.10,11 Identification of metabolic products could be the key to a potential understanding behind the etiology of many diseases.12 By identifying endogenous metabolites, metabolomics can be used to monitor the intricate biochemical alternations that occur during pathogenesis.13 The research methods and analysis techniques associated with metabolomics are varied...
and the sensitivity and technology related to metabolomics are constantly improving.

Because of the existence of the blood-retinal barrier, the vitreous can reflect the intraocular environment effectively. Based on this principle, we designed a parallel comparative study by measuring vitreous metabolites to identify metabolic markers that may be useful to discriminate between RRD and RRDCD. In this study, we used a liquid chromatography-quadrupole-time-of-flight/mass spectrometry (LC-Q-TOF/MS)-based metabolomics strategy to explore the RRDCD mechanism. To calculate all the possible variations in the mass spectra, we used a technique called principal component analysis (PCA). Principal component analysis is chemometric algorithm. Types of chemometrics include principal component, partial least squares, and principal component regression analysis. The remarkable advantage of PCA is that subtle changes in mass spectra that often cannot be discerned by a trained spectroscopist and can be quantified and correlated to factors such as disease. The objective of this study was to discover different metabolites and metabolic pathways that could be significant to our understanding of the pathogenesis and the causes of the poor prognosis of RRDCD.

METHODS

Patients

The guidelines of the declaration of Helsinki were followed in this study. All recruited patients provided written informed consent for the retention and analysis of their tissues. All patients with RRD and RRDCD underwent a comprehensive system examination by two chief physicians of ophthalmology from Nanjing Medical University Affiliated Wuxi Second Hospital between 2013 and 2014. Patients with RRDCD who had choroidal detachment when RRD was onset were included in the study. Patients were excluded from the study if they had any systemic infections, diabetes, hypertension, or were unable to cooperate during the vitrectomy. Once a diagnosis was made, vitrectomy was performed before any hormonotherapy. A total of 29 vitreous samples (14 from RRD patients and 15 from RRDCD patients) were obtained from patients undergoing a 25-gauge vitrectomy. Undiluted vitreous samples (0.8–1.0 mL) were collected at the time of vitrectomy before opening the infusion line. Samples were collected in 2-mL Eppendorf tubes containing liquid nitrogen to maintain sample stability. Samples were processed within 2 hours of collection and centrifuged to remove cells before freezing at −80°C.

Materials and Reagents

Acetonitrile of HPLC grade and methanol were obtained from Honeywell (Morristown, NJ, USA). Formic acid of MS grade was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ultrapure water was prepared using a Milli-Q water purification system (Billerica City, MA, USA). Commercial standards used for different metabolite identification were purchased from Chemsky International Co., LTD (Shanghai, China).

Preparation of Vitreous Samples for Metabolomic Study

Frozen vitreous samples were thawed before analysis. Two preparation methods (water and chloroform extraction methods) were used for each sample. Methanol extraction method was a common method and was able to extract most of the metabolites in vitreous, but there were still some important hydrophobic moieties that were not extracted by methanol. According to our test, chloroform was able to efficiently extract hydrophobic metabolites, so we used chloroform for extraction in our study (Fig. 1). One method used chloroform extraction, where a 100-μL aliquot of vitreous was added to 400 μL chloroform. The mixture was vortexed for 30 seconds, and then equilibrated for 10 minutes at room temperature. The sublayer was then used for analysis. For the water extraction method, a 100-μL aliquot of vitreous was added to 400 μL chloroform. The mixture was vortexed for 30 seconds, and then centrifuged at 9838g for 10 minutes at 4°C. The supernatant was then used for analysis. A quality control sample was made by pooling the same volume (10 μL) of each vitreous sample that was then prepared in the same way as the samples. A blank sample of acetonitrile was treated exactly the same way as the vitreous samples and run every three sample injections to minimize carryover.

Liquid Chromatography–Q-TOF/MS Analysis

The metabolomic study was performed using an Agilent-1200 LC system coupled with an Agilent-6520 Q-TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Separation was performed on an Eclipse plus C18 column

FIGURE 1. Positive ion mode of the primary mass spectrogram from four extraction modes (from top to bottom: chloroform, ethyl acetate, water, and methanol).
The column oven was maintained at 45°C and the flow rate was set at 0.3 mL/min. The mobile phases were ultrapure water (A) with 0.1% formic acid and acetonitrile (B). The gradient program was: 0 to 15 minutes, 5%B to 95%B. The sample injection volume was 1 mL.

The spectrometer was operated in both positive and negative ion modes. The parameters of mass detection were set as follows: the drying gas (N2) flow rate was 8 L/min; the gas temperature was 330°C; the nebulizer gas pressure was 35 psi, Vcap was 3700 V; the fragmentor was 160 V; the skimmer was 65 V; the mass scan range was m/z 50 to 1000. The MS/MS analysis was acquired in targeted MS/MS mode with three collision energies of 5 eV, 10 eV, and 20 eV.

Method Validation

The large-scale sample analysis was highly repeatable and stable and could be used for metabolomics analysis (Table 1). The variation of peak areas and retention times of four ions, m/z 89.0453 (alanine, positive mode), m/z 179.0566 (hippuric acid, positive mode), m/z 131.0685 (creatine, positive mode), and m/z 129.0421 (4-oxoproline, negative mode) were selected to assess stability and repeatability.

To evaluate repeatability, six parallel samples obtained from a random vitreous sample were injected continuously to evaluate repeatability. The relative standard derivations (RSDs) of these four peaks were less than 8.99% for peak areas and 2.13% for retention times.

The stability of the LC-Q-TOF/MS system for large-scale sample analysis was determined by using pooled quality control samples. Retention times and m/z values were selected to evaluate system stability. The RSDs of four selected peaks were less than 1.5%. The retention time and mass data (m/z) were listed as an identifier of each peak. The ion intensities of detected peaks were normalized (linear function transformation) to control the MS response shift through sequence analysis. The PCA and partial least squares discriminant analysis (PLS-DA) were analyzed with SIMCA-P (Version 11.0; Umetrics, Umeå, Sweden) for multivariate analysis, and SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for significance analysis using a one-way ANOVA with Bonferroni correction. Differences were considered significant if P was less than 0.05.

RESULTS

Fourteen eyes with RRD and 15 eyes with RRDCD were enrolled in this study. Patient demographics are presented in Table 2. The mean age of RRD patients was 50.5 (range, 39–66) and was 54.0 (range, 36–64) in RRDCD patients. There were 7 (50%) males and 7 (50%) females with RRD, and 8 (53.3%) males and 7 (46.7%) females with RRDCD. The median time of duration of detachment was 4 days (range, 2–8 days) for the RRD group and 4 days (range, 2–7 days) for the RRDCD group. There were no differences in the proliferative vitreoretinopathy (PVR) grade between the two groups.

Table 1. The Repeatability and Stability Data of the Established Method

<table>
<thead>
<tr>
<th>Mass</th>
<th>Compound</th>
<th>Repeatability, n = 10</th>
<th>Stability, n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT, min</td>
<td>Area</td>
<td>RT, min</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>RSD, %</td>
<td>Mean</td>
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<tr>
<td>89.0453</td>
<td>Alanine</td>
<td>1.08</td>
<td>2.13</td>
</tr>
<tr>
<td>179.0566</td>
<td>Hippuric acid</td>
<td>5.87</td>
<td>1.82</td>
</tr>
<tr>
<td>131.0685</td>
<td>Creatine</td>
<td>10.31</td>
<td>1.09</td>
</tr>
<tr>
<td>129.0421</td>
<td>4-Oxoproline</td>
<td>14.58</td>
<td>0.81</td>
</tr>
</tbody>
</table>

RT, retention time.
were less than 10.25% for peak areas and less than 3.99% for retention times.

**Metabolomic Study**

In the positive ion mode, there were 5682 and 1232 ion signals in the chloroform and water solutions, respectively. In the negative ion mode, there were 2423 and 780 ion signals in the chloroform and water solutions, respectively. Due to the difficulty in identification because of the high number of moieties, PCA was used to detect the differences in the levels of metabolites associated with RRD and RRDCD. Before PCA analysis, a table of molecular weight and associated intensity data was made from the MS data. After normalization, the table of data was imported into SIMCA-P software for multivariate statistical analysis. Samples were grouped in a classical elliptical Mahalanobis boundary (Figs. 2, 3). The separation was significant ($R^2_X = 0.79$, $Q^2 = 0.70$), which indicated a difference in the levels of metabolites between the RRD Group and RRDCD groups.

Similarly, a PLS-DA was used to further identify the ions that contributed to the discrimination of RRD and RRDCD groups (Fig. 3, $R^2_X = 0.79$, $R^2_Y = 0.80$, and $Q^2$ were 0.79, 0.80, and 0.79, respectively). A total of 265 (variable importance in the project $>1$) ions were significantly different between the RRD and RRDCD groups. When searching in the Scripps database (metlin.scripps.edu), the m/z ppm tolerance was set at 5 ppm. Among these ions, 68 ions did not match the database contents, 71 ions were peptide segments, and 60 ions were exogenous compounds from food. Then 56 ions were left for further analysis. We selected these ions in mixed sample using selected monitor (SIM) method for MS/MS analysis. For example, with using the SIM method, the retention time and molecular weight for these ions would be clarified. The collision energy from argon in MS was given to these appointed ions. If the energy was more than the bond energy of a molecule, the ion fragment would appear. Generally, the hydroxyl group in molecules easily led to lose 18 m/z weight and carboxyl group easily led to lose 44 m/z. According the fragment molecular and the molecular structures of the left 56 ions, it could tentatively ensure the name of the metabolites. If there were chemical standards, we also analyzed the standards and further ensured the metabolites according the retention time, m/z, and MS/MS spectrometry. Through comparing MS/MS and chemical standards, 24 metabolites (23 in positive mode, 1 in negative mode) were finally identified by searching MS and MS/MS fragments in the Biofluid Metabolites Database (http://metlin.scripps.edu, in the public domain) and the Human Metabolome Database (http://www.hmdb.ca, in the public domain), and finally confirmed by commercial standards. Table 3 shows different identified metabolites and their metabolic pathways found by searching the KEGG PATHWAY Database. The related pathways of the 24 different metabolites were classified into nine metabolic processes and their interaction networks are shown in Figure 4.

**DISCUSSION**

Our metabolomics/PCA/PLS/DA approach was able to distinguish between RRDCD and RRD by identifying metabolic differences. The metabolites that were found to be different may be potential biomarkers in the diagnosis and treatment of RRDCD.

This analysis was carried out on vitreous humor which was obtained by 25G vitrectomy, thus we were not able to ascribe the whole metabolic results to a specific cell type. Although the observed changes excluded the differences of sex, age, and course of the disease, there are likely other differences, such as race, that were not considered because of the limited number of samples.
Our clinical study was able to completely discriminate the different levels of metabolites in vitreous between RRDCD and RRD, finding a total of 265 metabolites significantly different between the groups (Fig. 3). Only 24 of the metabolites (23 in positive mode, 1 in negative mode) were identified by searching MS and MS/MS fragments in the Biofluid Metabolites Database and Human Metabolome Database. Thus, there will probably be metabolic changes and pathways that were not detected. Regardless, we were able to differentiate between RRD and RRDCD with repeatability and stability.

Of the 24 metabolites used to differentiate between the two diseases, 14 metabolites were at higher levels and 10 were at lower levels in vitreous from patients with RRDCD compared with patients with RRD. The metabolic processes of all the metabolites shown in Figure 4 were closely related, except for threonate. This implies that threonate is an exogenous metabolite in the vitreous. The urea and citrate acid cycles contained most of the metabolites and they are likely to play important roles during the pathological processes of RRD and RRDCD. As we know, the citric acid cycle is the central point of glucose metabolism that provides the energy for proliferation. Adenosine troposphere (ATP), which can provide the energy for the proliferation of cells, is a metabolite of the citric acid cycle. A change of citric acid cycle between the two groups might indicate a variation of proliferation. Succinate, lactate, and phenylpyruvic acid, among others directly related

<table>
<thead>
<tr>
<th>Mode</th>
<th>Name</th>
<th>Trend</th>
<th>RT</th>
<th>Molecular Weight</th>
<th>P</th>
<th>Fold Change</th>
<th>Pathway</th>
<th>Origin, Chloroform/Water</th>
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<tr>
<td>Positive</td>
<td>L-Carnitine</td>
<td>Higher in RRDCD</td>
<td>0.65</td>
<td>161.1048</td>
<td>7.75E-03</td>
<td>1.58</td>
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<td>Acetyl carnitine</td>
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<td>0.72</td>
<td>203.1125</td>
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<td>Water</td>
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<td>Succinate</td>
<td></td>
<td>0.76</td>
<td>118.0253</td>
<td>1.68E-03</td>
<td>2.05</td>
<td>Citrate cycle (tricarboxylic acid cycle), tyrosine metabolism, alanine, aspartate, and glutamate metabolism</td>
<td>Water</td>
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<td>Uric acid</td>
<td></td>
<td>0.89</td>
<td>168.0276</td>
<td>4.54E-03</td>
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<td>Phenylpyruvic acid</td>
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<td>0.90</td>
<td>164.0475</td>
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<td>Phenylalanine metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis</td>
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<td>L-Phenylalanine</td>
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<td>1.57</td>
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<td>Choline</td>
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<td>0.94</td>
<td>103.0978</td>
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<td>1.61</td>
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<td>2-Hydroxyglutarate</td>
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<td>5.67</td>
<td>148.0368</td>
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<td>1.39</td>
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<td></td>
<td>Allantoin</td>
<td></td>
<td>7.24</td>
<td>158.0423</td>
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<td>Purine metabolism</td>
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<td>Glutamine</td>
<td></td>
<td>7.74</td>
<td>146.0659</td>
<td>2.15E-11</td>
<td>2.11</td>
<td>D-Glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism</td>
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<td></td>
<td>Urea</td>
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<td>10.23</td>
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<td>L-Phenylalanine</td>
<td></td>
<td>11.47</td>
<td>282.2724</td>
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<td>Threonate</td>
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<td>11.50</td>
<td>136.0574</td>
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<td>Ascorbate and aldarate metabolism</td>
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<td>Glycero phosphocholine</td>
<td>Higher in RRD</td>
<td>0.66</td>
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<td>Alamine</td>
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<td>1.09</td>
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<td>302.2754</td>
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<td>4-Oxoproline</td>
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<td>14.59</td>
<td>129.0421</td>
<td>5.71E-07</td>
<td>2.04</td>
<td>Arginine and proline metabolism</td>
<td>Chloroform</td>
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</table>
to the citrate cycle, were found at higher levels in the RRDCD group of samples compared with the RRD group of samples, indicating a higher level of energy metabolism in RRDCD. This is perhaps due to differences in oxidative stress between the two groups.

A high percentage of identified metabolites associated with cell proliferation were detected. Proliferation contributes critically to PVR. Proliferative vitreoretinopathy, a scarring process that is more serious in RRDCD, is the most common cause of surgical failure in the treatment of RRDCD. Proliferative vitreoretinopathy can also be considered as the proliferation of cellular membranes within the vitreous cavity. When a retinal tear occurs, RPE migrates to the vitreous cavity, which is considered to be a trigger of the PVR process. But, PVR must be identified as a result of an interaction between various cellular types, extracellular substances, and factors entering the vitreous cavity after the breakdown of the blood-retinal barrier. Therefore, we speculate that metabolic changes may be associated with this disease. It has been reported that uric acid can stimulate vascular smooth muscle cell proliferation by increasing platelet-derived growth factor (PDGF) A-chain expression. The expression level of uric acid is dramatically higher in RRDCD samples compared with RRD samples. As discussed earlier, the blood-retinal barrier is broken down in RRDCD which causes the exudation of excessive PDGF. The higher level of uric acid in RRDCD is efficient to recruit PDGF, which might be helpful for the proliferation of pathological cells.

Uric acid, which was also high in the vitreous of patients with RRDCD compared with the level in patients with RRD, can induce macrophage proliferation by inhibition of inducible nitric oxide synthesis. Urea is produced in the metabolism of arginine to ornithine, a process that has already been confirmed in both activated endothelium and macrophages at the expense of nitric oxide production. Phytosphingosine, sphingosine, and sphinganine levels, which were lower in RRDCD compared with RRD, are all involved in sphingolipid metabolism. Although there are a variety of reasons for this observation, we speculate that sphingolipids synthesis could be lower with RRDCD. As a central molecule in this pathway, ceramide serves as a second messenger for cellular functions, ranging from apoptosis to proliferation. Generally, ceramide inhibits growth and survival. In this study, the levels of both sphinganine, a ceramide precursor, and sphingosine, a ceramide product, were significantly different between RRD and RRDCD. We did not detect ceramide in any of our samples, perhaps because it may accumulate transiently and then be transformed into various other metabolites, such as sphingosine.

We also identified different metabolites related to inflammation in our study. Arachidonic acid is oxygenated and transformed into a variety of products that mediate or modulate inflammatory reactions. The main sources of inflammatory mediators, such as Prostaglandin E2 (PGE2), Leukotriene (LTx), and epoxyeicosatrienoic acid (EET), are produced by metabolizing arachidonic acid. Three inflammatory pathways (cyclooxygenase, lipoxygenase, cytochrome P450) related to arachidonic acid are known. A lower level
of arachidonic acid in RRDCD compared with RRD might indicate a greater consumption of arachidonic acid that would be stimulated by inflammation. L-carnitine may suppress proinflammatory cytokines and control the inflammatory and immune response.\textsuperscript{29,30} Therefore, a higher level of L-carnitine may be a response to inhibit inflammation. In other words, there may be more inflammation associated with RRDCD compared with RRD.

The levels of lactate in the RRDCD group of samples were higher compared with the RRD group of samples. Lactate is considered to be a major metabolite in the human vitreous,\textsuperscript{31,32} which is produced by anaerobic glycolysis in most tissues and can also be produced under aerobic conditions in the retina.\textsuperscript{33} Elevating the level of lactate by systemic administration or by some microinjection has been shown to stimulate intracellular blood flow by increasing blood velocity.\textsuperscript{34} Articular dilation and ciliary body swelling, which cause aqueous humor production, is lower in RRDCD, perhaps because of higher levels of lactate. The significant difference in the level of lactate in the vitreous suggests that RRDCD and RRD may involve hemodynamic differences. Meanwhile, in most tissues, aerobic metabolism is the major type of metabolism, but in some activated cells, such as lymphocytes and macrophages, the increased demand for energy is met by an accelerated metabolism, which is largely met by the production of lactate through anaerobic glycolysis. A higher level of lactate in RRDCD might imply a loss of immuno-inflammatory responses during the pathological process.

Although some of the significant metabolites were identified and discussed, there were still many moieties that remained to be identified. Due to the limited number of reliable spectral reference databases for metabolite identification, our interpretation of the results was limited. Moreover, like most metabolic studies associated with PCA analysis, the larger the sample size, the smaller the error. We discovered metabolites that differentiate RRDCD from RRD and should be studied first because they have already been implicated in the disease process and their role needs to be elucidated in future experiments.

**Conclusions**

A metabolomics method based on LC-Q-TOF/MS and multivariate data analysis has been used to successfully identify metabolites that were present in vitreous at significantly different levels between RRDCD and RRD. The levels of 24 metabolites were significantly different (23 in positive ion mode, 1 in the negative ion mode) in vitreous from patients with RRDCD and RRD. Of the 24 metabolites, 14 metabolites were higher and 10 lower in vitreous from patients with RRDCD compared with patients with RRD. The metabolic pathways and metabolites of the markers were discussed. The obvious different metabolites may be important to identify prognostic factors or therapeutic targets for RRDCD.

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**References**


