

# Alterations of the Gut Microbiome and Metabolome in Patients with Proliferative Diabetic Retinopathy

Ke Yao (✉ [xlren@zju.edu.cn](mailto:xlren@zju.edu.cn))

Zhejiang University School of Medicine Second Affiliated Hospital Department of Ophthalmology

<https://orcid.org/0000-0002-6764-7365>

Panpan Ye

zhejiang university

Xueyou Zhang

zhejiang university

Yufeng Xu

Zhejiang University

Jia Xu

zhejiang university

Xiaoxiao Song

zhejiang university

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## Research

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# Abstract

**Background:** Diabetic retinopathy (DR) has been reported to associate with gut microbiota alterations in murine models and thus “gut-retina-axis” has been proposed. However, the role of gut microbiome and the associated metabolism in DR patients still need to be elucidated.

**Results:** Fecal samples from 45 patients with proliferative DR (PDR) and 90 matched diabetic patients (1:2 according to age, sex and duration of diabetes) without DR (NDR) were subjected to 16S rRNA gene sequencing and untargeted metabolomics. Significantly lower bacterial diversity was observed in PDR group than that in NDR group. Differential gut bacterial composition was found, with significant depletion of 22 families (e.g., *Coriobacteriaceae*, *Veillonellaceae* and *Streptococcaceae*) and enrichment of 2 families (*Burkholderiaceae* and *Burkholderiales\_unclassified*) in PDR group as compared to NDR group. There were significantly different fecal metabolic features, which were enriched in metabolic pathways such as arachidonic acid and microbial metabolism, between the two groups. Among 36 co-abundance metabolite clusters, 11 were positively/negatively contributed to PDR using logistic regression analysis. Fifteen gut microbial families were significantly correlated with the 11 metabolite clusters. Furthermore, a fecal metabolites-based classifier was constructed to distinguish PDR patients from NDR patients accurately.

**Conclusions:** PDR is associated with reduced diversity and altered composition of gut microbiota and specific microbe-metabolite interplay. Our findings help to better understand the disease pathogenesis and provide novel diagnostic biomarkers and therapeutic targets for PDR.

## Background

Diabetes mellitus is growing fast and estimated to affect 10.2% (578 million) of global population in 2030 [1]. Diabetic retinopathy (DR) is its most common microvascular complication and a major cause the vision impairment worldwide [2]. Proliferative diabetic retinopathy (PDR) is the advanced stage of DR and the leading cause of blindness. The pathological hallmarks of PDR include capillary occlusion and neovascularization [2]. Accordingly, intravitreal injection of anti-VEGF drugs, laser, and vitrectomy have been developed to treat PDR. Although these treatments seem to be effective, some of the treated eyes still deteriorate and lose vision finally [2], necessitating the development of novel therapeutic approaches.

Recent progresses in basic science greatly improve our knowledge about how diabetes impacts retina such as the proposed ‘gut-retina axis’. Gut microbiota is the most densely populated microbial ecosystem in human body and is integral to the maintenance of human health. Alterations in the gut microbiota have been implicated in the onset and development of various human diseases including diabetes [3] and ocular disorders [4]. Previous studies in murine models have demonstrated that type 2 diabetes-altered gut microbiota is associated with the exacerbation of DR, indicating a possible role of gut-retina axis [4]. Targeting the gut microbiota and their-associated metabolites such as tauroursodeoxycholate (TUDCA)

via diet or probiotics might be a new therapeutic strategy [4]. However, the specific gut microbiota composition and the related metabolic profiling in DR, particularly the highly blindness-causing PDR, is still unknown in human. We herein conducted the pioneering human study to identify the pathological mechanism of PDR from a deep understanding of gut-retina axis and to seek the novel predictive and therapeutic targets.

## Methods

### Study cohort and samples

We recruited patients with type 2 diabetes in Eye center, the Second Affiliated hospital, Zhejiang University School of Medicine between January 2020 and July 2020. This study was approved by the ethical committee of the Second Affiliated hospital, Zhejiang University School of Medicine (approval number 2020776) and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consents were obtained from all subjects. The inclusion criteria were all adult patients with age > 18 year, Han population, a history of type 2 diabetes, ECOG score 0–1, receiving visual acuity, dilated funduscopy, optical coherence tomography (OCT) and fundus fluorescein angiography (FFA). The exclusion criteria were as follows: sepsis in need of antibiotics within 12 weeks prior to enrollment, specifically infection such as hepatitis virus or tuberculosis, cancers, severe systemic diseases and hospitalized, unhealthy lifestyle such as alcohol abuse and smoking. The patients with the presentation of retinal neovascularization in FFA were diagnosed as PDR. During the study period, a total of 45 enrolled patients were diagnosed as PDR and received vitrectomy (PDR group, n = 45). Another 90 matched patients (1:2 according to age, sex and duration of diabetes) without DR were included using propensity score (NDR group, n = 90). Stool samples were obtained and immediately frozen in liquid nitrogen before storage at -80 °C. Clinical data including biochemistry were acquired and analyzed.

### DNA extraction and 16S rRNA gene sequencing

DNA was extracted using a kit (TIANGEN Biotech, Beijing, China). The V3-V4 region of the bacterial 16S rRNA gene was amplified and sequenced using an Illumina HiSeq2500 platform (Illumina, California, USA) as we described previously [5]. Quality-filtered sequences were clustered into operational taxonomic units (OTUs) and displayed using R software (Version 2.15.3). Bacterial richness and diversity analyses were calculated. Principal component analysis (PCA) using weighted and UniFrac distance metrics were conducted. Wilcoxon rank-sum test was performed for comparison between the two groups. Linear discriminant analysis (LDA) effect size (LEfSe) method was used for distinguishing taxonomic types. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis was performed to predict functional composition profiles.

### Liquid chromatography-mass spectrometry-based metabolomics analysis

Fecal samples were used for liquid chromatography-mass spectrometry-based metabolomics (UPLC – MS) analysis as we described previously [6]. The raw data were analyzed with Compound Discoverer 3.0 (CD 3.0, Thermo Fisher). Peaks were matched with the database of mzCloud (<https://www.mzcloud.org/>) and ChemSpider (<http://www.chemspider.com/>). MetaboAnalyst 2.0 and SIMCA software were used for functional analysis [6].

## Unsupervised clustering of co-abundant metabolites

Clusters of co-abundant metabolites were identified using the R package WGCNA. Top 75% Median absolute deviation of identified MS1/MS2 features were filtered to cluster a signed and weighted metabolite co-abundance correlation network. The correlation method in all functions was bicor(biweight mid-correlation) (maxPOutliers = 0.05) because of its robustness [7]. The power value of our scale-free topology was 3.

## Cross-omics correlation network analysis

Spearman correlations between the gut microbiota and metabolite modules were performed using R 4.0.2. We also performed biweight mid-correlation [7] to calculate correlation between metabolite modules and clinical phenotype. Adjusted *P* values (FDR) were controlled by the Benjamini-Hochberg method. Logistic regression was performed using stats R package. Simulate Residuals test was performed using DHARMA R package. Multicollinearity test was performed using car R package.

## Support Vector Machines classification

We performed Support Vector Machines (SVM) with Radial Basis Function Kernel classification using the implementation of this method in caret R package. We considered SVM classifiers for predicting NDR/PDR status. SVM was trained on 70% of the samples and tested on the remaining 30%. Features were not selected in any way before training. Data were scaled and centered before training, and cross-validated (5-fold and repeated 100 times) for resampling. Parameter used for the model were sigma = 0.0002563017 and C = 0.25. The performance of models was measured by area under curve (AUC), which was calculated using pROC R package.

## Statistical analysis

The significance was compared using Student's *t* test, ANOVA or the Kruskal-Wallis test. Other statistical approach in dataset bioinformatics analyses has been described above. Data were analyzed using GraphPad Prism software version 6.1 (GraphPad software, Inc.), R version 4.0.2 (R foundation for Statistical Computing) and SPSS 13.0 (SPSS Inc.). A *P* value of < 0.05 was statistically significant.

## Results

### Patient characteristics

A total of 45 patients receiving operation for PDR were included. Another 90 propensity score-matched diabetic patients without PDR according to age, gender and history of diabetes were enrolled as control group. Patient characteristics are shown in Table 1. There was no significant difference between the two groups in body mass index (BMI), diabetic complications, metabolic comorbidities, therapeutic strategies and laboratory data.

Table 1  
Patient characteristics

	PDR (n = 45)	NDR (n = 90)	p
Age (yr)	59.9 ± 11.3	60.9 ± 9.9	0.599
Male (n)	25	50	1.000
BMI (kg/m <sup>2</sup> )	24.4 ± 2.7	24.9 ± 3.8	0.400
History of diabetes (yr)	10.0 (2.5, 16.7)	10.0 (2.0, 15.3)	0.277
Complications (n)			
Macrovascular	10	18	0.764
Kidney	7	13	0.864
Neuropathy	9	19	0.881
Comorbidities (n)			
Hypertension	17	29	0.521
Fatty liver	10	24	0.575
Dyslipidemia	12	19	0.469
Medication (n)			
Oral anti-diabetic drugs	30	68	0.275
Metformin	22	48	0.626
Glycosidase inhibitors	12	22	0.779
DPP4 inhibitors	9	18	1.000
Insulin secretagogues	9	15	0.633
Insulin injection	24	43	0.543
Laboratory data			
Fasting glucose (mmol/L)	6.6 ± 2.3	6.6 ± 2.9	0.992
HOMA-IR	2.6 (1.8, 4.7)	2.5 (1.5, 4.6)	0.244
HbA1c (%)	9.6 ± 2.2	8.8 ± 2.3	0.144
Triglyceride (mmol/L)	1.40 (0.99, 2.06)	1.44 (1.02, 2.18)	0.590

PDR, proliferative diabetic retinopathy; BMI, body mass index; DPP4, dipeptidyl peptidase-4; HOMA-IR, homeostasis model assessment of insulin resistance

	PDR (n = 45)	NDR (n = 90)	p
Cholesterol (mmol/L)	4.37 ± 1.23	4.35 ± 1.11	0.929
Creatinine (umol/L)	66.0 ± 30.4	69.9 ± 23.6	0.569
PDR, proliferative diabetic retinopathy; BMI, body mass index; DPP4, dipeptidyl peptidase-4; HOMA-IR, homeostasis model assessment of insulin resistance			

## Reduced gut microbiota diversity in proliferative diabetic retinopathy

We performed 16S rRNA gene sequencing to assess the landscape of gut microbiome. A total of 8,569,316 high-quality valid tags (range: 46,269 to 80,826 per sample) were obtained. PDR group showed a decreased value of observed OTUs than NDR group, indicating reduced richness and bacterial diversity (Fig. 1a). We further evaluated the differences in gut microbiome diversity between the two groups using alpha diversity and beta diversity. There was significantly lower alpha diversity of gut microbiome in PDR group than that in NDR group according to four independent indices including species richness indices (Observed OTUs and Chao1) and species diversity indices (Shannon and Simpson) (Fig. 1b). PCA displayed segregation of the microbiota between the two groups (Fig. 1c). The score plot of principal coordinate analysis based on unweighted/weighted UniFrac distances showed differences in the structure and composition of bacterial community between the two groups (Fig. 1d).

## Alterations of gut microbiome signatures in proliferative diabetic retinopathy

To investigate the gut microbiome signatures, we assessed the relative abundance of gut bacterial composition by microbial taxon assignment at both phylum and family levels. At the phylum level, *Firmicutes*, *Actinobacteria* and *Proteobacteria* accounted for most microbial communities (Fig. 1e). However, no significant difference was found between the two groups including the *Firmicutes*/*Bacteroidetes* ratio. At the family level, PDR group showed significantly less abundant in 22 families including *Coriobacteriaceae*, *Veillonellaceae* and *Streptococcaceae*, and more abundant in 2 families (*Burkholderiaceae* and *Burkholderiales\_unclassified*) than NDR group (Fig. 1f). The representative high-abundance families are shown in Fig. 1g. At the genus levels, there were 66 significantly different taxa between the two groups (Additional file 1: Fig. S1).

To characterize the distinct microbial compositions, we further identified key discriminative OTUs in PDR group relative to NDR group using LEfSe analysis. There were 28 discriminative features with LDA scores > 3.5 level (Fig. 2a). At the family level, PDR group was enriched by *Burkholderiaceae*, whereas NDR group was enriched by *Coriobacteriaceae*, *Streptococcaceae*, *Veillonellaceae* (Fig. 2b). At the genus level, PDR group was enriched by *Morganella*, while NDR group was enriched by *Collinsella*, *Streptococcus*,

*Erysipelotrichaceae\_UCG\_003*, *Ruminococcaceae\_UCG\_002*, *Ruminococcaceae\_UCG\_014*, *Faecalibacterium*, *Eubacterium\_coprostanoligenes\_group*, *Agathobacter*, *Roseburia* and *Dorea*.

To predict the functional alteration of gut microbiota in PDR patients, we performed PICRUST analysis. PDR group showed significant difference in nucleotide metabolism, amino acid metabolism and lipid metabolism as compared to NDR group (Additional file 1: Fig. S2). There were 32 KEGG pathways were significantly differentially enriched between the two groups (all  $P < 0.05$ ) including secretion system, ribosome, peroxisome, purine metabolism and carbohydrate metabolism (Fig. 2c).

## Fecal metabolic profiles of proliferative diabetic retinopathy

To directly evaluate the influence of PDR on gut microbial metabolism, we performed UPLC-MS-based analysis of metabolomics using fecal samples. PCA score plots displayed that the quality control (QC) samples in both electrospray ionization positive ion (ESI+) mode and negative ion (ESI-) mode were clustered tightly together, indicating the analysis was stable and repeatable (Additional file 1: Fig. S3). The partial least squares discriminate analysis (PLS-DA) score plots showed apparent separation between PDR group and NDR group in both ESI+ and ESI- models (Fig. 3a). There were 133 and 129 features in ESI+ and ESI- models significantly altered between the two groups according to selection criteria of  $P < 0.05$ , the variable importance in the projection (VIP)  $> 1$  and fold change  $> 2$ . Most of the metabolites belonged to lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives and benzenoids superclasses (Fig. 3b). They were significantly enriched in metabolic pathways including arachidonic acid metabolism, microbial metabolism in diverse environments, linoleic acid metabolism and purine metabolism (Fig. 3c). Of interest, 34 metabolic features were significantly enriched in microbial metabolism in diverse environment, indicating a potential impact of gut microbiota on the metabolic profiling.

## Cross-omics correlation network analysis

Gut bacteria provide bioactive metabolites, which enter the bloodstream of host and affect the disease progression. We performed cross-omics analysis to show the link between gut bacteria, the associated metabolites and PDR. A total of 8208 identified MS1/MS2 features were filtered by top 75% Median absolute deviation from 10944 features. First, we classified the metabolites into 36 co-abundance clusters including 15 clusters that were significantly correlated with PDR ( $P < 0.05$ , Fig. 4a). Various kinds of mechanism can come up clusters of co-abundance metabolites such as: (1) metabolites correlated with the same biological process/pathway; (2) the same/similar precursor metabolite's chemical modification; (3) metabolites co-yielded by the specific gut microbiome. Second, we performed correlation analysis between gut microbiota families and the metabolite clusters. We found significant correlation between 19 families and 25 clusters (Fig. 4b). For instance, *Burkholderiaceae* was significantly positively correlated with cluster 5 and 19 but negatively correlated with cluster 12, 22, 29 and 36, while *Coriobacteriaceae* was significantly positively correlated with cluster 7 and 32. We further entered the gut microbiota-correlated 25 metabolite clusters into logistic regression and identified 11 clusters that significantly contribute to the risk of PDR (Fig. 4c). The detailed information of the 11 clusters including



MS2 metabolites, MS2 superclass and compassion significance between PDR group and NDR group are provided in Additional file 2: Table S1. According to the contribution power (risk ratio), we classified the 11 clusters into 5 PDR-positive metabotypes and 6 PDR-negative metabotypes. The most representative risk (cluster 8) and protective metabotypes (cluster 20) for PDR are shown in Fig. 4d. Cluster 8 contained 46 features including Desogestrel and Dehydrosalsolidine that were significantly more abundant in PDR group, whereas cluster 20 contained 37 features including N-Methyl-L-alanine and D-erythro-Sphinganine that were significantly less abundant in PDR group as compared to NDR group. Finally, we construct an interaction network for 15 gut microbiota families and the 11 PDR-positive/negative metabotypes, suggesting that gut microbiota might contribute to the development of PDR through interacting with metabolites (Fig. 4e).

## Predictive model for proliferative diabetic retinopathy

To assess the predictive role of fecal metabolites in PDR, we trained SVM classifiers on patient fecal metabolites profiles. We divided all patients into a train set and a test set according to a ratio of 7:3. Classification performance was evaluated by AUC. We found that the MS2 metabolites could well distinguish PDR patients from NDR patients, as shown by AUCs of 0.960 and 0.943 in train and test sets (Fig. 5a). The metabolites model presented high accuracy in predicting PDR with sensitivity of 0.846 and specificity of 0.936 (Fig. 5b). The most discriminate metabolites included Alantolactone, Desogestrel, Adenine, D-erythro-Sphinganine and Corosolic acid (Fig. 5c).

## Discussion

Gut-retina axis has been proposed according to the mice experiments [4] and the hypothesize that diabetic-associated microbiome could lead to increased inflammation and vascular permeability, which influence the development and progression of DR [8]. However, alterations of gut microbiota have not been directly linked to DR in human studies. A previous study using fecal colony culture and PCR strategy did not find significant difference in the abundance of *Bacteroides* between diabetic patients with and without DR [9]. This is a pioneering study to explore the DR-associated alterations of human gut microbiome and metabolome. We enrolled PDR patients as the DR group, which were compared with NDR group, to maximize the diversity between DR and NDR. We demonstrated sharply decreased bacteria abundance and gut microbiota diversity in diabetic patients with PDR as compared to those without DR. The loss of microbial taxonomic diversity is frequently observed in many human diseases such as diabetes and cancer and is recognized to be associated with systemic inflammation [10, 11], which play a clear role in the pathogenesis of DR [2]. Therefore, we suppose the microbial diversity might reflect the severity of DR.

Moreover, we showed the change of microbiota composition and specific population of bacterial species in diabetic patients with PDR as compared to those without DR. There was no significant difference in bacterial abundance at phylum level between the two groups. However, at family level, PDR was found to be associated with significantly decreased abundant of a series of bacteria including *Coriobacteriaceae*,

*Veillonellaceae* and *Streptococcaceae*. Several bacteria have known function particularly in metabolic diseases. For instance, *Coriobacteriaceae* may regulate host glucose homeostasis via liver energy metabolism and protect against hyperglycemia [12]. *Veillonellaceae* are the key organisms in human gut that metabolize lactate [13], thereby reducing the risk of developing diabetic complication including PDR [14]. In addition, *Clostridiales\_unclassified*, *Ruminococcaceae*, *Firmicutes\_unclassified*, *Clostridiaceae* and *Rikenellaceae* were the TOP bacterial taxa at the family level contributing to the ClpB-like gene function that leads to reduced fat mass [15]. In contrast to the bacteria with decreased abundance, the family *Burkholderiaceae* was the only bacterial taxa that enriched in the gut of PDR patients and the key discriminative microbial marker as identified by LEfSe analysis. *Burkholderiaceae* is a known heterotrophic bacteria that was reported to colonize in the gut of patients with immunosuppression [16] and positively correlate with chemokine IP-10, inducing systemic inflammation [17]. Moreover, *Burkholderiaceae*, along with *Coriobacteriaceae* and *Streptococcaceae*, were closely correlated with altered glutamate metabolism [18], which has been proven to be an early pathogenic event in the development of DR [19].

We further demonstrated that not only the abundance and composition of gut microbiome but also the gut-derived metabolites displayed PDR-specific biosignature. The significantly differential expressed metabolites were enriched in metabolic pathways such as linoleic acid metabolism, purine metabolism, tyrosine metabolism and carbohydrate metabolism. Some metabolic pathways including arachidonic acid metabolism and purine metabolism were predicted to be altered by microbiome and further proved by metabolome. We found that the arachidonic acid metabolites such as hydroxyeicosatetraenoic acids (HETEs) and leukotriene, which are known mediators for DR development [20, 21], were increased in the fecal of PDR patients, making them as the potential diagnostic markers and therapeutic targets. Our results were consistent with previous studies that HETEs were also increased in the serum of DR patients as compared to NDR patients [22]. On the other hand, there were 34 fecal metabolites including Vanillate, D-galactonate, D-gluconic acid and Aerobactin that were significantly enriched in microbial metabolism, showing an extensive interplay between the gut microbiota and the host through metabolic exchange and substrate co-metabolism [23].

We next revealed an integrated cross-omics framework to better understand the link between gut microbiome and metabolome particularly under the circumstance of PDR. We enriched coexpression-based clusters to classify metabolites with similar physicochemical properties. The method greatly shortened the numbers of metabolomics parameters from thousands of metabolite features to dozens of metabolite clusters, which made the risk analysis using logistic regression available. We then identified several metabolite clusters that were significantly associated with PDR risk. For instance, the metabolic pattern of cluster 8, including 33 up-regulated (e.g., Desogestrel and Acylcarnitine 21:2) and 13 down-regulated metabolite features (e.g., LysoPA 21:0 and Linoleic acid) in PDR patients as compared to NDR patients, sharply increased the risk of PDR by 18.5-fold in diabetic patients. In contrast, the metabolic pattern of cluster 20, including 12 up-regulated (e.g., Succinic anhydride) and 25 down-regulated metabolite features (e.g., Acylcarnitine 22:2 and (-)-Riboflavin) in PDR patients, dramatically reduced the PDR risk by 17.6-fold. The results not only confirmed the previously identified PDR-associated

metabolites such as the arginine and carnitine metabolites [24, 25] but also provided new candidates from unlabeled metabolites. Furthermore, we correlated the gut microbiota with the metabolic phenotype and found significant relationships between certain bacteria families and PDR-associated metabolite clusters. For example, cluster 3, the most microbial affected metabolite cluster containing 19 organic acids and derivatives, was significantly correlated with 9 microbial families including *Clostridiaceae*, *Lachnospiraceae*, *Lactobacillaceae* and *Bifidobacteriaceae*. The PDR-negative metabotype cluster 32 was only positively correlated with *Coriobacteriaceae*. The results shed light on the PDR-linked microbe-metabolite interaction.

There were limitations in this study. First, sample size was relatively small. Therefore, we used propensity-score matched cohort to minimize the selection bias. Second, there were some confounding factors. Although the diabetic complications and medications, which have impact on the gut microbiome and metabolomics, were not differ significantly between the two groups, the diet habit and lifestyle were not controlled and may also affect the results. Third, the casual relationship between gut microbe-metabolite and PDR could not been determined by this study. Last, most co-abundance metabolite clusters remained largely uncharacterized and need further exploration. The therapeutic significance of restoration of gut microbiota in PDR also needs to be proved.

## Conclusions

In summary, to the best of our knowledge, it is the first study to identify the alterations in both gut microbial community and metabolism in diabetic patients with PDR versus those without PDR. There was sharply reduced richness and bacterial diversity in PDR patients, making supplemental probiotics a potential therapeutic strategy through restoring the balance. Moreover, there was a specific interplay between gut microbiome and host metabolome under the circumstances of PDR. A fecal metabolites-derived classifier was constructed and could effectively discriminate PDR patients from NDR patients. Our results not only help to better understand the physiopathological mechanisms of PDR but also provide novel strategies in disease prevention, diagnosis and treatment.

## Abbreviations

DR, diabetic retinopathy; PDR, proliferative diabetic retinopathy; NDR, non-diabetic retinopathy; TUDCA, tauroursodeoxycholate; OCT, optical coherence tomography; OUT, operational taxonomic unit; PCA, principal component analysis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; UPLC – MS, liquid chromatography-mass spectrometry-based metabolomics; SVM, support vector machines; AUC, area under curve; BMI, body mass index; DPP4, dipeptidyl peptidase-4; HOMA-IR, homeostasis model assessment of insulin resistance; QC, quality control; ESI+, electrospray ionization positive ion; ESI-, electrospray ionization negative ion; PLS-DA, partial least squares discriminate analysis; VIP, variable importance in the projection; HETEs, hydroxyeicosatetraenoic acids

# Declarations

## FUNDINGS

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## AVAILABILITY OF DATA AND MATERIALS

The dataset supporting the conclusions of this article are available in the baiduyun repository with hyperlink to dataset in <https://pan.baidu.com/s/1U5ueGwvknVwhQtGgJSwcKg> under Key zvw b.

## AUTHOR'S CONTRIBUTION

PPY designed the study, obtained the samples and clinical records and wrote the manuscript. XYZ analyzed the data. YFX, JX and XXS obtained the samples and clinical records. KY supervised the study and revised the manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethical committee of the Second Affiliated hospital, Zhejiang University School of Medicine (approval number 2020776) and informed consent was obtained from all subjects.

## CONSENT FOR PUBLICATION

Not applicable.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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Not applicable.

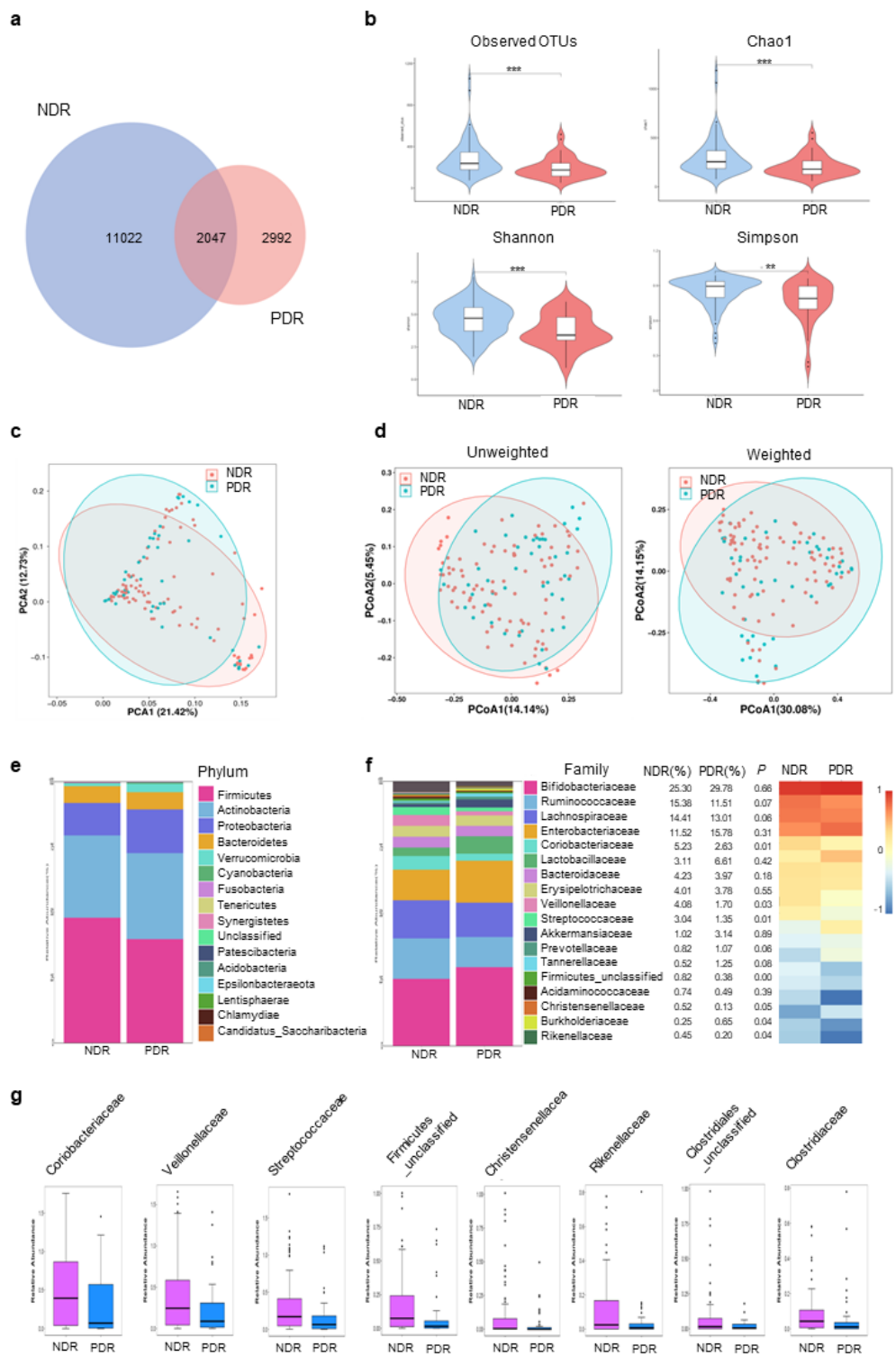
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## Figures



**Figure 1**

Gut microbiome diversity and structure comparison. a The OTUs in the two groups; b Alpha diversity comparison between the two groups; c PCA analysis based on the relative abundance of operational taxonomic units; d Unweighted/weighted Unifrac the score PCoA plot; e Component proportion of bacteria phylum in the two groups; f The comparison of bacteria at family level; g TOP8 altered bacteria at family level. \*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ; \*:  $P < 0.05$ ; PDR, proliferative diabetic retinopathy; NDR, non-

diabetic retinopathy; OTUs, operational taxonomic units; PCA, principal component analysis; PCoA, plot of principal coordinate analysis.

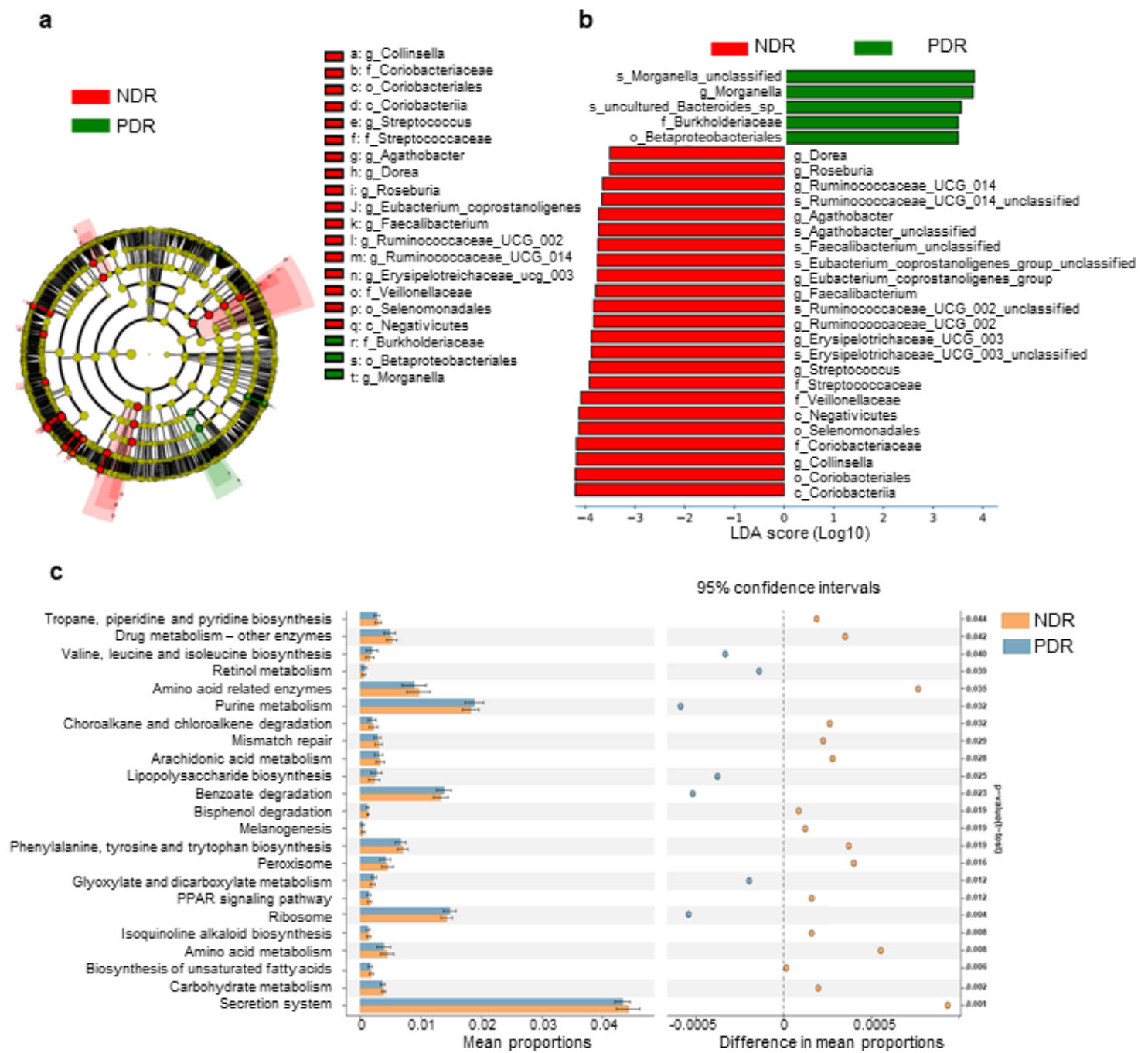
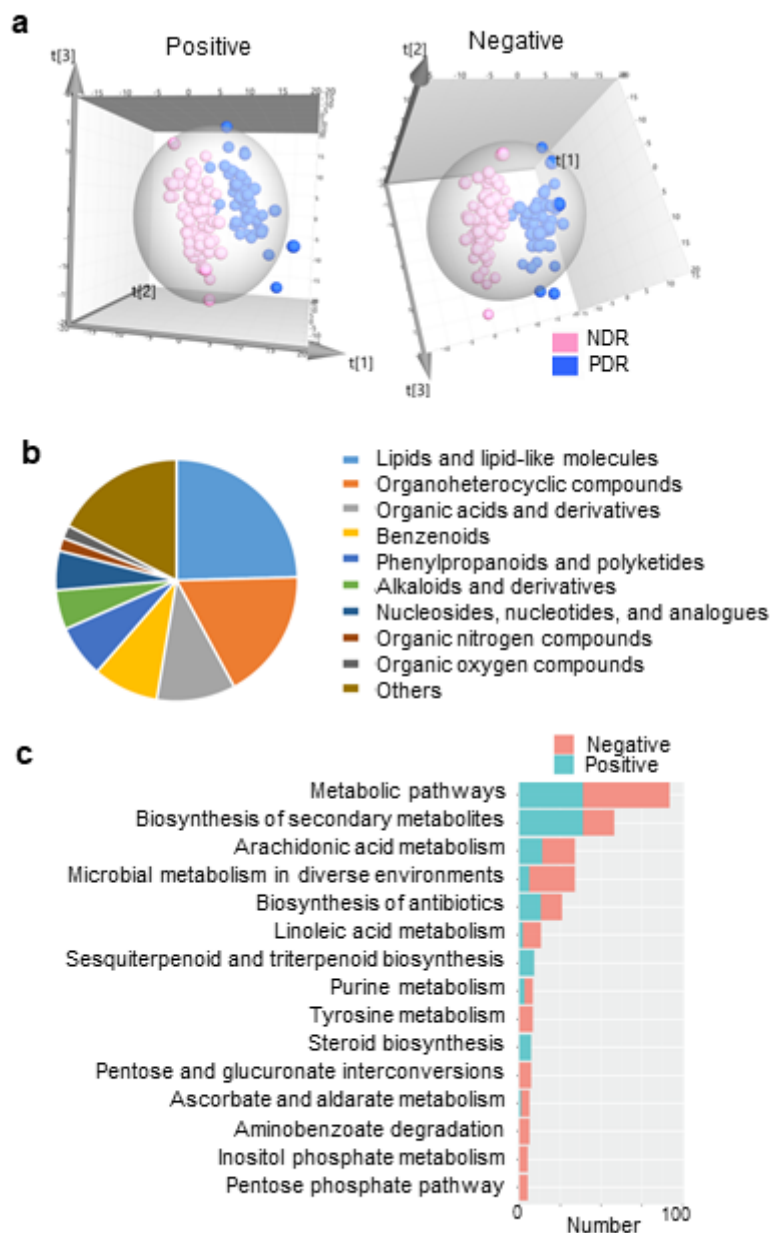


Figure 2

The discriminative operational taxonomic units and the predicted function. a The phylogenetic distribution of gut microbiota correlated with the two groups as shown by cladogram using LDA effect size analysis; b The differences of gut microbiota in abundance between the two groups (LDA >3.5); c KEGG pathways predicted by PICRUSt analysis. PDR, proliferative diabetic retinopathy; NDR, non-diabetic retinopathy; LDA, linear discriminant analysis; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.





**Figure 3**

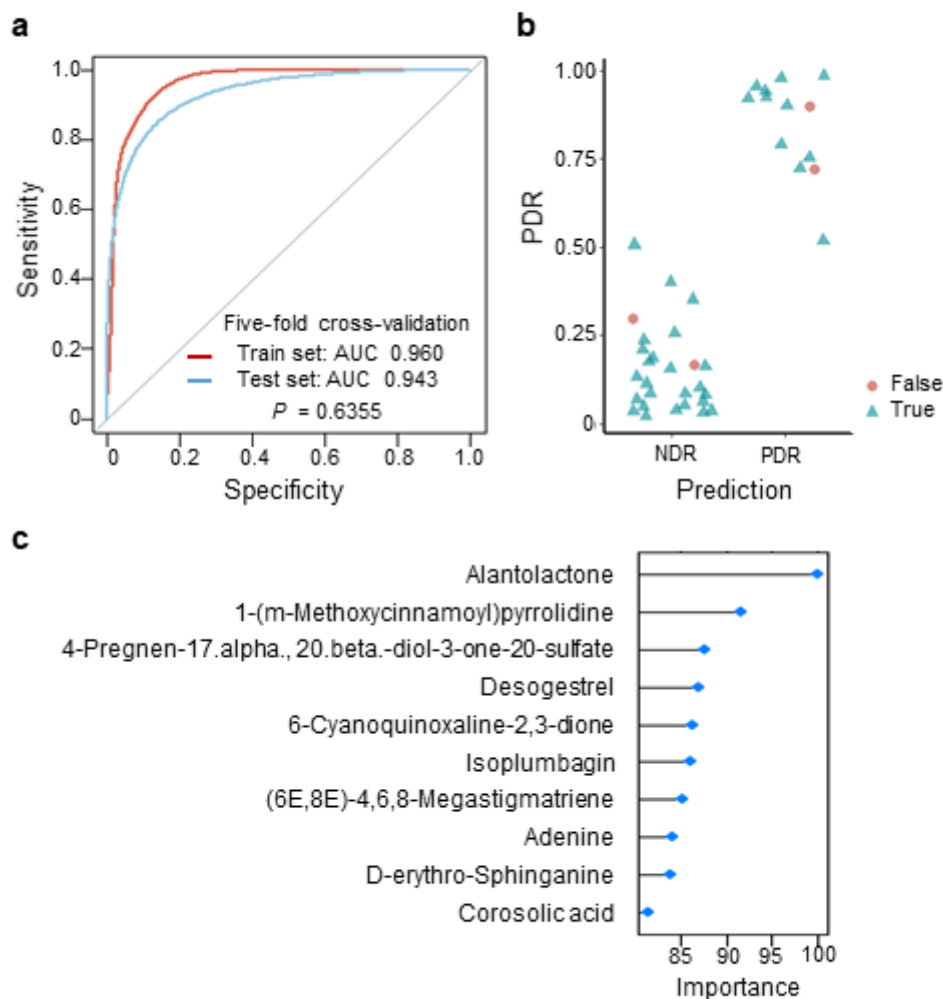
Fecal metabolic profiles in patients with and without proliferative diabetic retinopathy. a PCA score plots of the two groups; b The 3-Dimensional PLS-DA score plots of the two groups; c The classification of significantly altered metabolic features; d The enriched KEGG pathways of altered metabolic features. PDR, proliferative diabetic retinopathy; NDR, non-diabetic retinopathy; PCA, principal component analysis; PLS-DA, partial least squares discriminate analysis.



**Figure 4**

Relationship network between proliferative diabetic retinopathy-linked microbes and metabolites. a PDR-associated co-abundance clusters as revealed by biweight mid-correlation analysis; b Significantly correlated gut microbiota families with co-abundance clusters as shown by Spearman correlation analysis (\*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ; \*:  $P < 0.05$ ); c The positive/negative metabolotypes of PDR as identified by logistic regression analysis; d The detailed description of representative positive/negative

metabotypes of PDR as analyzed by co-abundance correlation network; e Visualization of the gut microbiota-metabolites network according to Spearman correlation analysis (Curve on the left) and logistic regression (Curve on the right). PDR, proliferative diabetic retinopathy; NDR, non-diabetic retinopathy.



**Figure 5**

Predicting proliferative diabetic retinopathy based on fecal metabolites. a Classification performance as shown by AUC; b Predicted probabilities for each sample; c TOP10 strongest discriminant metabolites in the classifier. PDR, proliferative diabetic retinopathy; NDR, non-diabetic retinopathy; AUC, area under curves.

## Supplementary Files

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- [Additionalfile1.docx](#)
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