• Clinical Research •

Gut microbiota induced abnormal amino acids and their correlation with diabetic retinopathy

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Abstract

● **AIM:** To explore the correlation of gut microbiota and the metabolites with the progression of diabetic retinopathy (DR) and provide a novel strategy to elucidate the pathological mechanism of DR.

● **METHODS:** The fecal samples from 32 type 2 diabetes patients with proliferative retinopathy (PDR), 23 with non-proliferative retinopathy (NPDR), 27 without retinopathy (DM), and 29 from the sex-, age- and BMI- matched healthy controls (29 HC) were analyzed by 16S rDNA gene sequencing. Sixty fecal samples from PDR, DM, and HC groups were assayed by untargeted metabolomics. Fecal metabolites were measured using liquid chromatography-mass spectrometry (LC-MS) analysis. Associations between gut microbiota and fecal metabolites were analyzed.

● **RESULTS:** A cluster of 2 microbiome and 12 metabolites accompanied with the severity of DR, and the close correlation of the disease progression with PDR-related microbiome and metabolites were found. To be specific, the structure of gut microbiota differed in four groups. Diversity and richness of gut microbiota were significantly lower in PDR and NPDR groups, than those in DM and HC groups. A cluster of microbiome enriched in PDR group, including *Pseudomonas*, *Ruminococcaceae-UCG-002*, *Ruminococcaceae-UCG-005*, *Christensenellaceae-R-7*, was observed. Functional analysis showed that the glucose and nicotinate degradations were significantly higher in PDR group than those in HC group. Arginine, serine, ornithine, and arachidonic acid were significantly enriched in PDR group, while proline was enriched in HC group. Functional analysis illustrated that arginine biosynthesis, lysine degradation, histidine catabolism, central carbon catabolism in cancer, D-arginine and D-ornithine catabolism were elevated in PDR group. Correlation analysis revealed that *Ruminococcaceae-UCG-002* and *Christensenellaceae-R-7* were positively associated with L-arginine, ornithine levels in fecal samples.

● **CONCLUSION:** This study elaborates the different microbiota structure in the gut from four groups. The relative abundance of *Ruminococcaceae-UCG-002* and *Parabacteroides* are associated with the severity of DR. Amino acid and fatty acid catabolism is especially disordered in PDR group. This may help provide a novel diagnostic parameter for DR, especially PDR.

● **KEYWORDS:** proliferative retinopathy; gut microbiota; *Ruminococcaceae*; amino acid metabolism; arginine
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INTRODUCTION

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes, typically characterized by microvascular damage and progressive retinal ischemia. It may lead to vision impairment or permanent visual loss due to vitreous hemorrhage, tractional detachment of retina and neovascular glaucoma. The prevalence rates of DR was 16.3% in 50 000 patients with diabetes from China DiaChronic Study, significantly higher in the northern than in the southern regions[1]. Furthermore, in a survey of 7233 people with diabetes, 16.97% of them were diagnosed with DR[2]. Therefore, DR will be an even significant health issue in the near future.

Mechanisms underlying the pathogenesis of DR are multifactorial and complicated. The gut dysbiosis plays a significance role in the development of ocular diseases including uveitis, glaucoma, and age-related macular degeneration[3-4]. Therefore, the concept of "gut-retina axis" has been proposed[5]. Dysregulation of intestinal microbiota by diet and antibiotics changes the progression of retinal disease[6-7]. Diabetic patients with delayed gastric emptying have a higher risk of retinopathy, while bariatric surgery can reduce the incidence of DR[8-9], *via* changing the microbiota structure and enhancing the intestinal barrier function, besides facilitating weight loss and improving glycemic control[10-11]. Recent studies reported that intestinal microbiota has emerged as a crucial regulator of DR[12]. In diabetic mice, dysbiosis of gut microbiota causes impaired intestinal barrier function, increases the numbers of acellular capillaries and inflammation cells in retina[13]. Age and gender matched DR patients had lower diversity of intestinal microbiome, such as *Escherichia-Shigella*, *Faecalibacterium*, *Eubacterium_hallii_group* and *Clostridium* than healthy controls (HC)[12]. In addition, short-chain fatty acids, produced by gut microbiota, can reduce the inflammatory reaction, β-cell autoimmunity and insulin resistance[12]. Moreover, significant increases in *Acidaminococcus*, *Escherichia* and *Enterobacter* were observed in the patients with DR compared to HC[14]. As some published studies reported, inflammation, oxidative stress and insulin resistance markedly contribute to the initiation and progression of proliferative retinopathy (PDR)[15-17], in which however DR patients were not divided into non-proliferative retinopathy (NPDR) and PDR based on the severity of DR. Therefore, we hypothesized that alternations of intestinal flora may play a crucial role in the progression of DR from NPDR to PDR.

Metabolomics is a powerful tool to screen the changes in metabolic profiles and to provide the mechanistic clues of pathological changes. It can identify and quantify endogenous small-molecule metabolites. Liquid chromatography-mass spectrometry (LC-MS) based-metabolomics has been applied to characterize specific metabolites of intraocular fluid such as anterior aqueous humor, vitreous humor or blood samples[18-19]. In DR patients, screening metabolic biomarkers from the abnormal changes of metabolites in their living organisms plays a great role in identifying the interaction between intestinal flora and diseases[18,20]. Targeting the biomarkers or the gut microbiota-derived metabolites such as tauroursodeoxycholate (TUDCA) *via* diet or probiotics might be a new therapeutic strategy[21]. The dysregulation of gut microbiota and related metabolites, such as arachidonic acid and linoleic acid have been reported to mediate DR in patients[22]. The changes of serum metabolites, such as glutamate, aspartate, glutamine, N-acetyl-l-glutamate, N-acetyl-l-aspartate dihomo-gamma-linolenate, docosahexaenoic, and icosapentaenoi can distinguish PDR and NPDR[18]. However, few metabolomic studies have explored the role of gut microbiota and its derived metabolites in the DR progression.

In the present study, we aimed to investigate gut microbiota and fecal metabolic characteristics in PDR patients, and to explore the correlation of gut microbiota and its derived metabolites with DR progression using multiomics technology of 16S rDNA gene sequencing and untargeted LC-MS based metabolomics.

SUBJECTS AND METHODS

Ethical Approval Approval was granted by the Ethics Committee of the AIER Hospital Group (Ethics No.AIER2018IRB21). This study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College. This study was conformed to the tenets of Helsinki for research. The informed consents were written by all participants.

Participants and Fecal Sample Collection The 111 participants were included in this study. All of them were from the same geographical area (northern Anhui Province). The requirements of enrolling patients in this study were as follows: 1) participants aged 18y and above; 2) a similar history of type 2 diabetes (T2D); 3) no other eye disease; 4) no other diabetic complications; The exclusion requirements were as follows: 1) administration of any antibiotics, probiotics or prebiotics within 3mo; 2) history of abdominal or ocular surgery; 3) severe systemic diseases; 4) malignant tumors. According to the international clinical classification standards, the diabetic patients with occurrence of retinal neovascularization, vitreous hemorrhage or preretinal hemorrhage was classified into PDR group (n=32), or with the occurrences of microoaneurysms, intraretinal hemorrhages, beaded changes in vessels or moderate retinal microvascular abnormality were classified into NPDR group (n=23). T2D patients without apparent retinopathy were enrolled into DM group (n=27). Age- and gender-matched healthy individuals without significant ocular and systemic diseases were enrolled into HC group (n=29). The fecal samples of all participants were obtained at The First Affiliated Hospital of Bengbu Medical University. All samples were kept cold on dry ice and sub-packaged into two cryotubes. All fecal samples were snap-frozen in nitrogen, then kept at -80°C until analysis.

Microbial DNA Extraction and 16S rDNA Gene Sequence The DNA were extracted from fecal samples (about 150 mg) using SPINeasy DNA kit (ZEPING Biotech, Beijing, China) following the manufacturer's instructions. The quality and quantity of extracted DNA were measured by a NanoDrop one spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR was conducted with the bacterial universal primers

(F5'-ACTCCTACGGGAGGCAGCA-3' and R5'-GGACTACHVGGGTWTCTAAT-3') to amplify the variable V3-V4 region of 16S rRNA to obtain an amplicon library. PCR amplicon was quantified on Microplate reader (BioTek, FLx800) and purified using Vazyme VAHTSTM DNA Clean Beads (Vazyme biotech co., Nanjing, Jiangsu Province, China). The library was pooled in equal amounts, and pair-end 2×150 bp sequencing was performed on an Illumin Mise platform (Illumina Inc., San Diego, CA, USA) following the standard protocols from the BioNovoGene (Suzhou, China).

Bioinformatics and Statistical Analysis In general, sequencing data were processed with open-source bioinformatics pipeline Quantitative Insights into Microbial Ecology (QIIME), as previously described[23]. Raw reads were assigned to respective samples according to the adaptors, and trimmed to be clean data. Amplicon sequence variants (ASVs) identified by DADA2 were merged. Contingency-based filtering was performed to remove any singletons of ASVs, and consensus method was conducted to remove chimeric ASVs. Alpha diversity indices, such as Shannon diversity index and Simpson index were calculated based on the operational taxonomic unit (OTU) table from Quantitative Insights Into Microbial Ecology (QIIME). Beta diversity analysis, such as pairwise anosim analysis, was performed to evaluate the structural variation of microbial communities using weighted UniFrac distance metrics and unweighted UniFrac distance metrics. ASVs were assigned to taxonomy using a Navier Bayers classifier that was assigned on a Sliva database. Taxonomic abundance was conducted using the R package "Vegan" at phylum, class, order, family, genus, and specie level. LEfSe (Linear discriminant analysis effect size) was performed to analyze statistically different taxa across all groups using the default parameters.

The potential bacterial biomarkers were explored to discriminate the groups using the R package "randomForest" with 1000 trees and all default settings. The accuracy of algorithm was tested using 10-fold cross-validation, including the expected "baseline" error, which was evaluated by a classifier that predicted the most common category label. Function profiles of taxa were predicted by PICRUSt and KEGG.

Untargeted Metabolomics of Fecal Samples LC-MS were conducted to analyze fecal metabolites. Briefly, 100 mg feces were weighted from each sample, dissolved into 600 μL MeOH containing 2-amino-3-(2-chloro-phenyl)-propionic acid (4 ppm), and mixed by vortex for 30s. The 100 mg glass beads were then added into the mixture that was ground for 90s, further handled by ultrasound for 10min, then centrifuged for 10min at 12 000 rpm. The supernatants were filtered by 0.22 μL membrane, then transferred into detection bottles until subsequent experiments. The 20 μL fraction from each sample was extracted for quality control.

The Vanquish UHPLC System (Thermo Fisher Scientific, USA) coupled with ACQUITY UPLC HSS T3 (150 mm×2.1 mm, 1.8 μm; Waters, Milford, MA, USA) at 40℃ was applied for LC analysis. The flow rate of injection volume was set at 0.25 μL/min. For LC-ESI(+)-MS analysis, the mobile phases consisted of C [0.1% formic acid in acetonitrile (v/v)] and D [0.1% formic acid in water (v/v)]. The process was as follows: 0–1min, 2% C; 1–9min, 2%–50% C; 9–12min, 50%–98% C; 12–13.5min, 98% C; 13.5–14min, 98%–2% C; 14–20min, 2% C. For LC-ESI(-)-MS analysis, the analytes were carried out with A (acetonitrile, ≥99.9%) and B (ammonium formate, 5 mmol/L). The process was as follows: 0–1min, 2% A; 1–9min, 2%–50% A; 9–12min, 50%–98% A; 12–13.5min, 98% A; 13.5–14min, 98%–2% A; 14–17min, 2% A[24].

Mass spectrometric detection of metabolites was conducted on Q Exactive HF-X (Thermo Fisher Scientific, USA) with ESI ion source. The parameter settings were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and -2.50 kV for ESI(+) and ESI(-), respectively; capillary temperature, 325℃; MS1 range, m/z 81-1000; MS1 resolving power, 60 000 FWHM; number of data dependant scans per cycle, 8; MS/MS resolving power, 15 000 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic[25].

Data and Multivariate Analysis The raw data were converted into mzXML format using ProteoWizard. The peak detection, retention time correction, integration and alignment were conducted by the R-package XCMS. After quality control, the metabolite annotations were recognized using database including LipidMaps (http://www.lipidmaps.org), massbank (http://www.massbank.jp/), Human Metabolome Database (HMDB) (http://www.hmdb.ca), Metlin (http://metlin.scripps.edu), mzclound (https://www.mzcloud.org), as well as the metabolome database, which was constructed by the BioNovoGene (Suzhou, China) to avoid missing any important metabolite. The multivariate analysis was conducted by Ropls software. $P<0.05$ and variable importance projection (VIP) value >1 were considered as statistical significance. The biological pathways of key metabolites that manifested significant differences between groups were annotated. Biological pathway analysis was performed through metabolite set enrichment analysis using the MetaboAnalyst tool suite. Spearman rank correlation analysis was conducted to correlate the significantly different metabolites and microbes.

RESULTS

Characteristics of the Study Participants The study enrolled 111 participants based on the inclusion and exclusion criteria, including 32 PDR, 23 NPDR and 27 DM patients, and 29 age-, gender-, and body mass index (BMI) matched healthy

Table 1 The characteristics of participants *n*=111

Characteristic	PDR (*n*=32)	NPDR (*n*=23)	DM (*n*=27)	HC (*n*=29)	*P*
Age (y)	54.6±8.80	58.65±10.76	58.89±10.31	59.59±13.98	0.198
Gender (M/F)	12/20	6/17	15/12	10/19	0.171
BMI (kg/m^2)	25.78±3.86	27.17±4.15	25.50±3.40	24.99±3.40	0.19
Hypertension, *n* (%)	20 (62.5)	17 (73.91)	19 (70.37)	16 (55.17)	1.0
FPG (mmol/L)	9.9 (6.35–15.63)[a]	9.59 (7.24–13.26)[a]	8.53 (7.69–13.60)[a]	5.54 (5.01–6.35)	<0.001
HbA1c, %	7.25 (5.30–8.75)[a]	9.10 (6.20–10.30)[a]	8.4 (7.1–9.6)[a]	4.65 (4.24–5.13)	<0.001

[a]*P*<0.05 compared with HC. PDR: Type 2 diabetes patients with proliferative retinopathy; NPDR: Type 2 diabetes patients with non-proliferative retinopathy; DM: Type 2 diabetes patients retinopathy; HC: healthy controls; FPG: Fasting plasma glucose; HbA1c: Glycated hemoglobin.

individuals. The demographic and clinical characteristics of all subjects were listed in Table 1. In total, no significant difference was found in age, gender, BMI, and hypertension, among four groups (*P*>0.05). Fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) was significantly higher in PDR, NPDR, and DM than that in HC group.

Taxonomic and Functional Profiles of the Gut Microbiota in Four Groups Across all 111 fecal samples, 5 061 235 effective tags were generated and clustered into 75 449 ASVs. To investigate the overall level of difference in four groups, we performed diversity analysis. A total of 22 021 unique ASVs were found in HC group, accounted for the highest number. Meanwhile, the number of unique ASVs were 19 770 in DM group, 14 415 in PDR group and 8306 in NPDR group (Figure 1A). This indicated that structures of gut microbiota in HC and DM groups were more abundant than those in PDR and NPDR. Then, α and β diversity analysis also illustrated the difference among four groups. Moreover, Chao1 indices of DM and HC groups were significantly higher than those in PDR and NPDR groups, which was similar with the observed species among four groups (Figure 1B; *P*<0.05). Shannon indices were significantly higher in NPDR and DM groups (*P*<0.05). β diversity using pairwise anosim analysis showed significant difference among four groups. These data indicated that the diversity and richness of the gut micorbiota in PDR and NPDR groups were relatively poor, compared with HC and DM groups.

We further analyzed the composition of gut microbioba at all taxonomic levels to clarify which microbes were responsible for these differences. First, at phylum level, *Firmicutes, Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were the most possible components of microbiome in all groups, accounting for 98%. The PDR group had the relatively lowest abundance of *Firmicutes*, and highest abundance of *Actinobacteria* (Figure 1C). Second, at genus level, the structures of gut mirobiota in four groups were different. *Faecalibacterium*, *Subdoligranulum*, and *Bacteroides* were the main microbes in all groups. In addition, *Bifidobacterium* and *Ruminococcaceae_UCG-002* were enriched in PDR group, while *Agathobacter* enriched in HC group (Figure 1D). Finally, a heatmap of 50 microbes were conducted (Figure 1E).

LEfSe analysis by pairwise groups comparison was often used to select statistically different microbes. We found that 51 microbes were significantly higher in HC group, and that 74 microbes were significantly higher in PDR group (Figure 2A). Specifically, *Ruminococcaceae* (14 genus; including *UBA1819*, *Ruminiclostridium_5*, *UCG_002*, *Anaerotruncus*, *GCA_900066225*, *UCG_005*, *NK4A214_group*, *Eubacterium__coprostanoligenes_group*, *UCG_014*, *UCG_009*, *Flavonifractor*, *UCG_004, Phocea*, *UCG_003*), *Lachnospiraceae* (8 genus; *Eubacterium_fissicatena_group*, *Eubacterium_hallii_group*, *Ruminococcus_torques_group*, *Blautia*, *Hungatella*, *Marvinbryantia*, *Sellimonas*) *Pseudomonas*, *Eisenbergiella* were significantly enriched in PDR. Considering numerous different microbes, randomforest analysis was performed to verify the microbes for distinguishing PDR and HC groups, among which *Lachnospiraceae_NC2004_group* (enriched in HC group), *UBA1819* and *Blautia* (both enriched in PDR group) were the top three. To investigate the role of microbes in the progression of DR, we further analyzed the function of microbiota. Our current data suggested that glucose degradation, nicotinate degradation I and sesquiterpenoid biosynthesis were significantly enriched in PDR group (Figure 2B, 2C), and that O-glycan biosynthesis was enriched in HC group.

To investigate whether microbes would be associated with the severity of DR we further performed LEfSe analysis for all groups. The 33 microbes were significantly altered in PDR and NPDR groups; 77 microbes altered in PDR and DM groups; 74 microbes altered in NPDR and DM groups; 52 microbes altered in NPDR and HC groups; 71 microbes altered in DM and HC groups (*P*<0.05). We found that the abundance of *Ruminococcaceae_UCG-002*, *Parabacteroides* were significantly higher in PDR group than those in NPDR, DM and HC groups (*P*<0.05), which is in line with the progression of DR (Figure 2D).

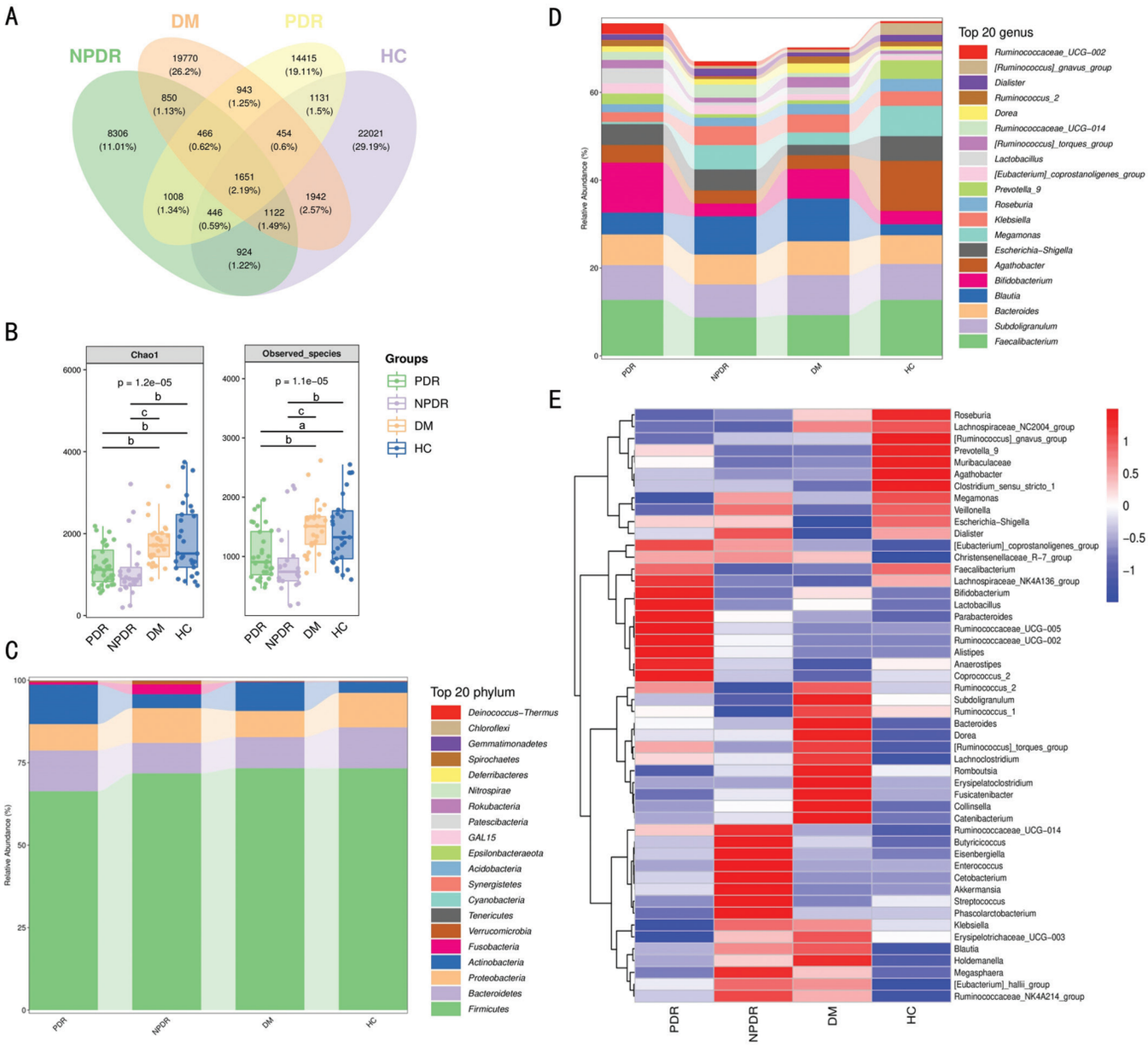

Figure 1 The composition of gut microbiota in PDR, NPDR, DM and HC groups A: Consistent and unique ASVs of venn diagram in four groups. B: Chao1 and Observed_species index of *α* diversity in four groups. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. C: Microbial composition structure at phylum level. D: Microbial composition structure at genus level. E: Enriched bacteria of Heatmap in Four groups. Red represents high levels and blue represents low levels. PDR: Type 2 diabetes patients with proliferative retinopathy; NPDR: Type 2 diabetes patients with non-proliferative retinopathy; DM: Type 2 diabetes patients without retinopathy; HC: Healthy controls; ASV: Amplicon sequence variants.

Fecal Metabolic Alternations of PDR, DM, and HC Groups Gut microbiota usually affects the human physiology by secreting various metabolites. Therefore, we performed untargeted LC-MS/MS on fecal samples from PDR, DM, and HC groups. After quality control, data filtering and normalisation, 31 414 MS (mass spectrometry) features were identified across 60 samples, in LC-MS positive and negative ionization mode. Further analysis of metabolites was performed, identifying a total of 1183 MSMS features by database. Compared with the HC group, 155 MSMS features in DM group were significantly higher, such as arachidonic acid, L-serine, L-cystine, and 20 MSMS features were significantly lower, such as L-proline, lithocholyltaurine. Compared with the HC group, 302 MSMS features in PDR group were significantly higher, such as L-arginine, ornithine, O-acetylserine, L-histidine, and 122 MSMS features were significantly lower, such as all-trans-retinoic acid (RA), L-proline, 7-dehydrocholesterol. Compared with the DM group, 123 MSMS features in PDR group were significantly higher, such as ornithine, L-histidine, L-arginine, uridine, and 130 MSMS features were significantly lower, such as lithocholic acid, L-proline, respectively (*P*<0.05; Figure 3A). We found that 12 core metabolites were different in three groups, including 1-oleoylglycerophosphocholine, uridine,

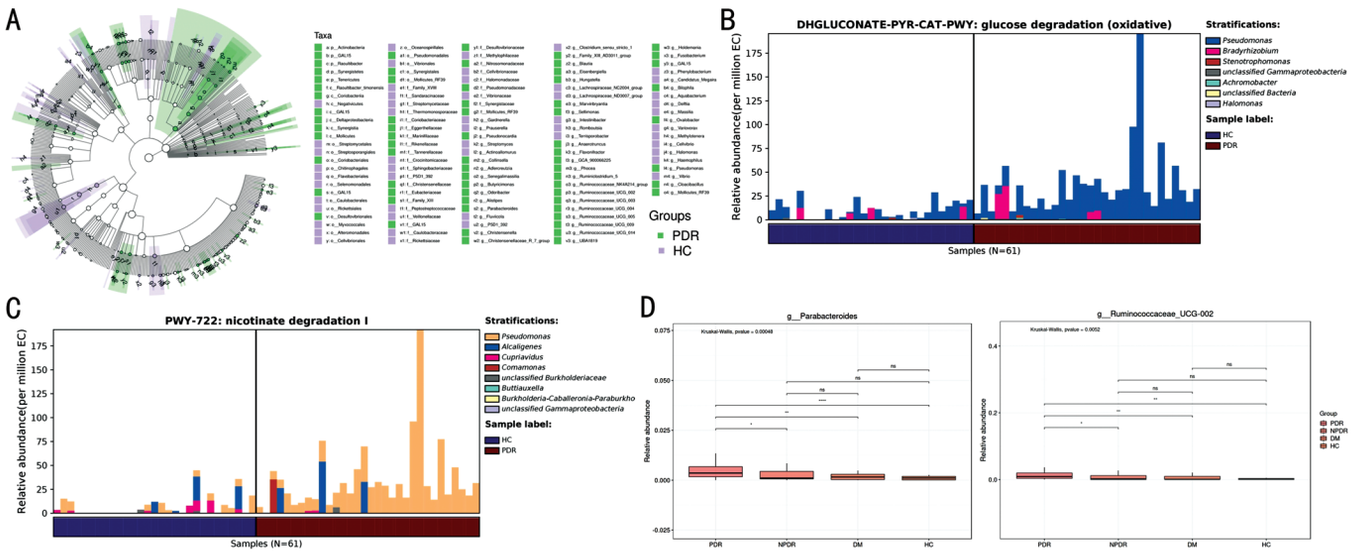

Figure 2 Gut microbiota was associated with PDR A: Statistically significant bacteria (P<0.05) of the lefse_cladogram analysis between PDR and HC; B: Glucose degradation (oxidative) mainly from *Pseudomonas* was enriched in PDR group compared to HC group (P<0.05); C: Nicotinate degradation I mainly from *Pseudomonas* was enriched in PDR group compared to HC group (P<0.05); D: Bacteria (*Parabacteroides*, *Ruminococcaceae_UCG-002*) were associated with severity of DR. PDR: Type 2 diabetes patients with proliferative retinopathy; NPDR: Type 2 diabetes patients with non-proliferative retinopathy; DM: Type 2 diabetes patients without retinopathy; HC: Healthy controls.

17a-estradiol, 6-amino-6-deoxyfutalosine, L-proline, ubiquinone-1, menthyl pyrrolidone carboxylate, N-formyl-L-methionine, undecanoic acid, N,N-diethyl-m-toluamide, pelletierine and pseudorhodomyrtoxin. These different metabolites were all parallel to the severity of DR (Figure 3B). These differential metabolites revealed an apparent disparity in PDR, DM, and HC groups. To characterize the similarity and inconsistency among three groups, we conducted PLS-DA analysis and verified the above-conclusions.

To clarify the potential function of PDR-related metabolites, we annotated metabolic features by using KEGG database. By comparing PDR group with HC group, the abnormal metabolism of amino acids and fatty acid was observed (Figure 3C). arginine biosynthesis, lysine degradation, histidine, D-Arginine and D-ornithine catabolism, linoleic acid and arachidonic acid, pyrimidine catabolism, insulin resistance, central carbon metabolism in cancer were enriched in PDR group, while intestinal immune network for IgA production was enriched in HC group.

Likewise, arginine biosynthesis, lysine degradation, central carbon metabolism in cancer, histidine, D-Arginine and D-ornithine catabolism, arachidonic acid metabolism were also enriched in PDR group compared with DM group. More interestingly, we found that lithocholic acid and steroid hormone biosynthesis were enriched in DM group (Figure 3D).

Moreover, these metabolic pathways also accompanied the severity of the disease. According the data, we found that the biochemical levels of some metabolites (such as amino acid and fatty acid catabolism) were parallel to the severity of the disease.

Correlations of the Gut Microbiota with Metabolic Phenotype of DR Considering vast amount of data in microbiome and metabolites from intestine, we intended to identify the relationship among microbiota, metabolites and disease. Based on receiver operating characteristic analysis, metabolites were found to better-distinguish PDR and DM groups [metabolites area under curve (AUC)=0.597; microbiota, AUC=0.57], PDR and HC groups (metabolites AUC=0.855; microbiota, AUC=0.778), or DM and HC groups (metabolites AUC=0.888; microbiota, AUC=0.768). Therefore, we used differential metabolites as indicators for association analysis.

Based on the data from PDR and HC groups, we found a cluster of metabolites (benfuracarb, imidazolelactic acid, cis-9,10-epoxystearic acid, L-arginine, dUMP, arabidiol, ornithine, pipecolic acid) that were positively related to *Ruminococcaceae_UCG-002* (P<0.01), and (4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoic acid ethyl ester that was negatively associated with *Ruminococcaceae_UCG-002* (P<0.01; Figure 4A). Likewise, dUMP was positively associated with *Ruminococcaceae_UCG-005*, and 9-OxoODE positively with *Ruminococcaceae_UCG-009* (P<0.01). *Christensenellaceae_R-7_group* was correlated with imidazolelactic acid, ornithine, dUMP, benfuracarb. These data highlighted that a cluster of metabolites might be associated with PDR-related microbiome, such as *Ruminococcaceae* and *Christensenellaceae_R-7_group.*

Another noteworthy finding about correlation between metabolites and microbiota was observed in PDR and DM

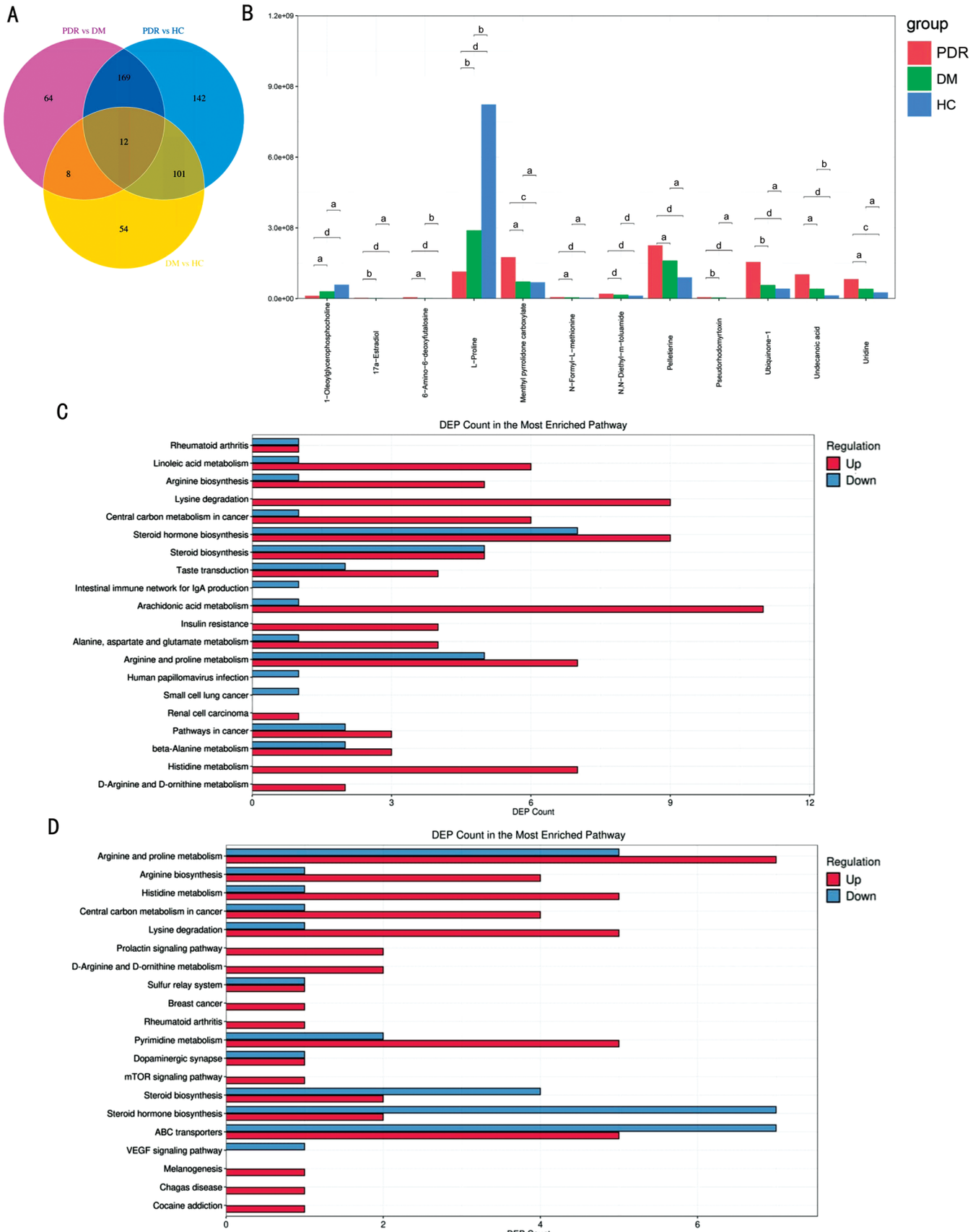

Figure 3 Altered metabolites in PDR, DM, and HC groups A: Common and unique features of statistically significant metabolites in three groups (P<0.05); B: Twelve metabolites were significantly different and parallel to severity of disease (^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001, ^{d}P<0.0001); C: Altered metabolism pathway between PDR and HC groups; D: Altered metabolism pathway between PDR and DM groups. PDR: Type 2 diabetes patients with proliferative retinopathy; NPDR: Type 2 diabetes patients with non-proliferative retinopathy; DM: Type 2 diabetes patients without retinopathy; HC: Healthy controls.

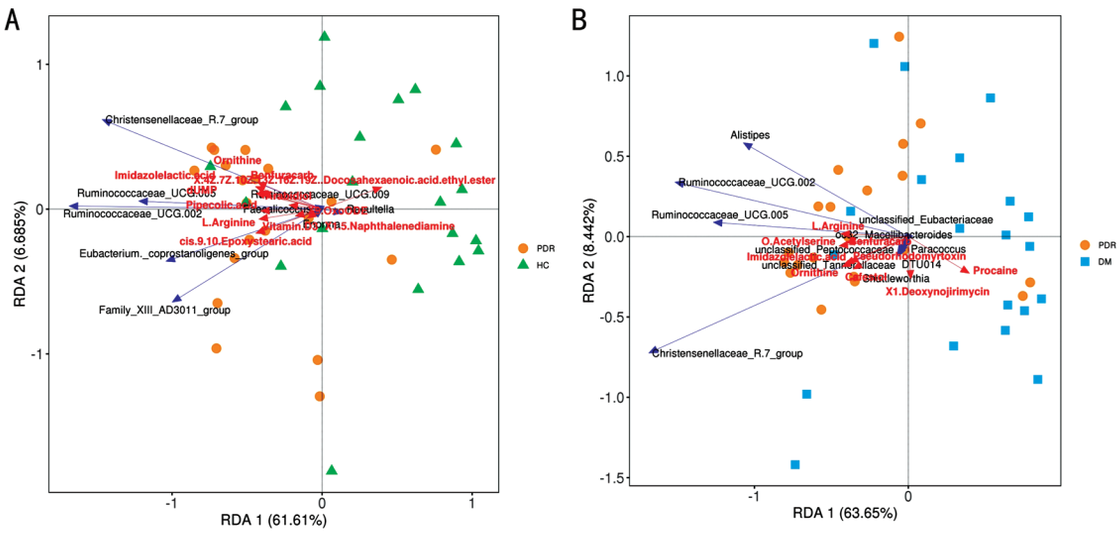

Figure 4 Associations between gut microbiota and their derived metabolites A: Redundancy analysis of metabolites and microbiota in PDR and HC groups (P<0.05); B: Redundancy analysis of metabolites and microbiota in PDR and DM groups (P<0.05). PDR: Type 2 diabetes patients with proliferative retinopathy; DM: Type 2 diabetes patients without retinopathy; HC: Healthy controls; RDA: Redundancy analysis.

groups (Figure 4B). We identified a group of bacteria that were significant and positively correlated with 1-deoxynojirimycin, including *Macellibacteroides*, *unclassified_Peptococcaceae*, *Shuttleworthia*, *oc32*, *unclassified_Eubacteriaceae*, *Paracoccus*, *unclassified_Tannerellaceae* and *DTU014* (P<0.01). *Christensenellaceae_R-7_group* were significantly and positively associated with imidazolelactic acid, ornithine, O-acetylserine, cafestol, pseudorhodomyrtoxin and benfuracarb (P<0.01). *Ruminococcaceae_UCG-005* was significantly and positively linked with O-acetylserine, ornithine and L-arginine, while negatively with procaine (P<0.01). *Ruminococcaceae_UCG-002* was also positively correlated with L-arginine (P<0.01).

DISCUSSION

Accumulating evidence suggested that gut microbiota was closely associated with diabetes and related retinopathy, yet the underlying mechanism remains unresolved[12,14,22,26]. Here, we applied 16S rRNA gene sequencing for PDR, NPDR, DM and HC groups, and metabolomics for PDR, DM, and HC groups, to investigate the difference of microbiota and its derived metabolites. Moreover, this is the first study regarding the correlation of intestinal microbiome with the progression of DR. We illustrated the dynamic microbiota and metabolite changes that linked with potential functions in four groups. We found the great alternations in microbial composition, metabolic pathway and functional profile among four groups. The diversity and richness of microbiota in DM and HC groups were significantly higher than those in PDR and NPDR groups. We found that metabolite and microbiome changed as the DR progressed, and that the altered microbiome (a group of *Ruminococcaceae*, a group of *Christensenellaceae_R-7_group*, *Pseudomonas*) linked with the dysregulation of metabolite (proline, arginine, lysine, histidine, ornithine). These data have contributed to the current evidence base by elaborating PDR-specific microbiome and metabolites, involving in the metabolic pathways.

We found a significant gradual increase of *Ruminococcaceae-UCG-002* and *Parabacteroides* from HC to DM, then to NPDR and PDR. The relative abundance of *Ruminococcaceae-UCG-002* in HC, DM, NPDR, PDR groups were 0.42%, 0.43%, 0.98%, 2.37% respectively, and the relative abundance of *Parabacteroides* in HC, T2D, NPDR, PDR groups were 0.14%, 0.21%, 0.29%, 0.51%, respectively. Consistent with our observations in the current study, *Ruminococcus* showed a higher abundance in DM patients compared to HC, leading to pro-inflammatory effects and also promoting pathogenesis in type 1 diabetes (T1D)[27-28]. Suppressing the growth of *Ruminococcaceae* has exerted hypoglycemic effects in diabetic animal models[29]. Another study showed that *Ruminococcaceae* has been considered to be significantly increased in patients with insulin resistance, T2D and inflammatory disease[30-31]. Additionally, *Ruminococcaceae_UCG-002* was reported to be positively associated with very-low-density lipoprotein (VLDL) particles and triglycerides levels, which were clinical features in T2D[32] Studies had suggested that increased *Parabacteroides* were found in T1D and T2D patients, compared to HC[33-36]. The colonization of *Parabacteroides* in the gut microbiota would accelerate diabetes onset in mice[37]. Another study reported that higher abundance of *Parabacteroides* coincided with elevation in blood glucose[38]. However, little studies had dissected the role of *Parabacteroides* in promoting T2D, and further studies were needed in order to explore the underlying mechanisms.

Furthermore, some of the microbes involved in T2D were also significantly elevated, but not in an gradient pattern,

including *Blautia*, *Alistipes*, *Christensenellaceae R-7, Ruminococcaceae_UCG-002*. In our current study, *Blautia* was more enriched in the participants with the diabetes than in HC individuals, especially in DM group. Studies reported that *Blautia* was elevated in diabetic patients[39-41]. *Blautia* was reported to have a positive correlation with TUDCA levels[41]. TUDCA is a farnesoid X receptor (FXR) antagonist that can help up-regulate glucose and lipid metabolism through its FXR receptor and bile acid G protein-coupled membrane receptor (TGR5). TGR5 can prevent retinopathy and prolong survival of *db/db* mice[42]. This may explain why *Blautia*-riched diabetic patients are less likely to develop retinopathy. The relative abundance of *Alistipes* was higher in T2D[43], which was consistent with our current study. The *Alistipes* play a potential role in epithelial changes and inflammation formation in hypertension[44]. It was believed that the hypotensive phenotype of inflammation was caused by the lipopolysaccharide (LPS) released by *Alistipes*, which was known to be pro-inflammatory, leading to an increase in Th17 cells expressing CD161 and CCR6/integrin beta7[45], as well as to the decrease in butyrate-producing bacteria, which were known to be anti-inflammatory[46]. Therefore, *Alistipes* may be a detrimental factor in PDR. Higher abundance of *Christensenellaceae_R-7_group* was positively correlated with the concentrations of reactire oxygen species (ROS) due to its imparing mitochondrial function, protein carboxyl, and interleukin-6 (IL-6) production[47]. Several studies reported that the abundance of *Christenseniaceae* was higher in patients with T1D and early renal function decline than in HC[48-49]. *Christensenellaceae_R-7_group* and *Alistipes* enriched in PDR group were thought to be positively associated with low-grade inflammation. In addition, mounting evidence suggested that *Lachnospiraceae*[50], *Pseudomonas*[51] and *Eisenbergiella*[52] were positively associated with T2D and flora disorder, consistent with our current study. The above results highlight the potential association between the gut microbiota and PDR.

The 12 metabolites were associated with DR and varied with the severity of the DR. We found a significantly gradual reduction of plasma proline from HC to DM, then to NPDR and PDR. Proline was reported to be associated with an increased risk of T2D[53-54] which was inconsistent with our current study. The reason is that proline is produced by a series of metabolic pathways. First, arginine is catabolized to ornithine by arginase 1 (ARG 1), and its relative abundance was higher in PDR in our current study. Then, ornithine is catabolized to L-glutamate 5-semialdehyde by ornithine aminotransferase (OAT). L-glutamate 5-semialdehyde is catabolized to 1-pyrroline-5-carboxylate, which is the precursor of proline and can be catalyzed by pyrroline-5-carboxylate reductase 2 (PYCR2). Meanwhile, L-glutamate 5-semialdehyde is also involved in the arginine biosynthesis, thus blocking proline biosynthesis. This phenomenon has been found in our current study, including an increase in arginine biosynthesis and a reduction in proline. One study showed that the proline-riched small protein 2A (SPRR2A) is an intestinal antimicrobial protein[55]. Besides, Proline is also an important component of collagen, which plays a key role in repairing damaged intestinal walls and the lining of the digestive tract[56]. Moreover, proline is a nutrient for the retinal pigment epithelium (RPE) and supports the metabolic needs of the RPE and retina[57]. On the other hand, proline prevented the cytotoxicity of ornithine in human RPE cells[58]. Another study showed that proline analogue (cis-hydroxyproline) can inhibit RPE proliferation, collagen synthesis and migration, thus inhibiting the progression of proliferative vitreoretinopathy[59]. According to the published papers, 1-Oleoylglycerophosphocholine was inversely associated with the risk of T2D[60-61]. Uridine was catabolized to uracil and ribose by commensal bacteria, which could promote quorum sensing and virulence gene expression, thus making bacteria from symbiotic to pathogenic[62].

Therefore, the dysregulation of amino acid metabolism was especially concerned in our current study. We found that the biosynthesis of valine, leucine and isoleucine belonging to branched chain amino acids (BCAAs), and of phenylalanine, tyrosine and tryptophan belonging to aromatic amino acids (AAAs) was enriched in PDR group, compared with HC group. Increasing evidence reported that BCAAs and AAAs were predictors of occurrence and development of T2D[63-65], which was consist with previous study. Of note, more data pointed that arginine biosynthesis, lysine degradation, histidine and D-ornithine catabolism were enriched in PDR group. Previous studies revealed that the level of arginine was increased in vitreous and plasma samples of PDR patients compared to diabetic or non-diabetic controls[66-67]. Numerous studies on diabetic patients and experimental animal models have demonstrated the importance of arginine metabolism in diabetes-induced oxidative stress, inflammation, and vascular dysfunction[68-70]. Under a hyperglycemic state, arginine produces ornithine and urea by arginine II enzyme (Arg-II enzyme). The elevation of ornithine level indicates the increased activity of the Arg-II enzyme, involved in microglia and macrophage-mediated chronic inflammation injury in T2D[71]. Meanwhile, the increased activity of Arg-II enzyme leaded to the declined activity of nitric oxide synthase (NOS), that mainly produces nitric oxide. The deficiency of nitric oxide and elevated level of polyamine could cause endothelial cell dysfunction, impaired vasodilation function, and induction of cell proliferation and fibrosis[72]. In addition, a few studies suggested that linoleic acid and arachidonic acid could

alleviate the development of T2D[73-74], while others showed that they were associated with T2D[75]. Our current data supported the latter. The large amounts of arachidonic acid would promote the entry of inflammatory substances into the bloodstream, including tumor necrosis factor (TNF) and IL-6, which could accelerate insulin resistance[76]. Arachidonic acid was stored at cell membrane phospholipids and enzymatically released from these phospholipids by phospholipases. Recent report suggested that more arachidonic acid was stored in membrane phospholipids of diabetic subjects than that of control subjects[77]. Thromboxane A2 (TXA2), a product of arachidonic acid, could enhance platelet aggregation, and it was found to be significantly elevated in diabetic subjects, compared to controls[78-79]. A diet containing higher level of linoleic acid increased the plasma level of IL-6. On the other hand, all-trans-RA and its metabolic pathway of intestinal immune network for IgA production were enriched in the HC group. IgA+B cell was facilitated by the secretion of RA from dendritic cell (DC). In another study, RA+transforming growth factor beta 1 (TGFβ1) - based micro/nanoparticle, as a "vaccines", was used to recover tolerogenic activity of DC, thus preventing and treating T1D[80]. We also found that ornithine was more enriched in PDR group than in HC group, but the underlying mechanism has not been examined so far. Our current data confirmed the presence of abnormal amino acid and fatty acid metabolism in the PDR patients, which may be used as biomarkers and drug targets for treatment.

More interestingly, we found that lithocholic acid was enriched in DM group compared with PDR group. In addition, bile acids were amphipathic steroid molecules, which not only promote the absorption or excretion of lipids in the small intestine, but also affect the metabolism and immunity of individuals by acting on multiple organs. Mounting literature reported that bile acids could suppress the growth of pathogenic bacteria[81-82]. Pathogenic bacteria could grow arbitrarily and impair the synthesis and consumption of amino acids, if lack of the inhibition of bile acid and human immune system[83].

There were some limitations in our current study. First, the sample size was relatively small. Second, there were some confounding factors that were not excluded, such as the environmental factors, genetic factors, lifestyle and dietary habit which may affect the results. Third, our current results do not show a casual relationship between the identified gut microbiota composition and PDR, which is an inherent limitation of case-control study. Lastly, due to low quality sample in NPDR, metabolomics was not performed.

In conclusion, we have comprehensively demonstrated the interplays among the gut microbiota, metabolites and the progression of PDR, and proposed new insights into novel strategies in disease prevention, diagnosis, and treatment.

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