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ORIGINAL RESEARCH

Associations of Gut and Circulating Microbiota with Circulating Vitamin D_3, Type I Interferon, and Systemic Inflammation in Chronic Spontaneous Urticaria Patients

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Objective: To analyze the associations of the gut and circulating microbiota with circulating vitamin D_3 (VD3), type I interferon (IFNI), systemic inflammation, and clinical profiles in chronic spontaneous urticaria (CSU) patients.

Methods: A total of 36 CSU patients with VD3 insufficiency (VDI; serum 25(OH)VD3 <30 ng/mL) and 36 sex-, age-, and body mass index–matched CSU patients with non-VDI were enrolled. Fecal and serum bacteria were identified through 16S rRNA sequencing, and serum 25(OH)VD3 and inflammation biomarkers were assessed using ELISA kits. IFNI response was determined by measuring the stimulatory activity of serum on IFNI-stimulated response element in HEK293 cells in vitro with luciferase assays.

Results: Higher urticarial activity score over 7 days (UAS7), higher frequency of levocetirizine resistance, and more severe proinflammation but weaker IFNI response were observed in VDI than non-VDI patients (all $P<0.05$). IFNI response was strongly positively associated with serum 25(OH)VD3 level in both groups ($P<0.001$). Compared to non-VDI patients, abundance of the fecal genera *Prevotella* 9, *Escherichia–Shigella*, and *Klebsiella* was significantly increased, while *Bacteroides*, *Faecalibacterium*, and *Agathobacter* were remarkably reduced in VDI patients (all $P<0.05$). *Burkholderia–Caballeronia–Paraburkholderia* (40.95%), *Acinetobacter* (3.05%), and *Aquabacterium* (2.37%) were the top three bacteria in sera from VDI patients. Both serum 25(OH)VD3 level and IFNI response were positively associated with fecal *Bacteroides* in the two groups ($P<0.05$). In non-VDI patients, there were moderately positive associations between IFNI response and fecal *Lachnoclostridium*, unclassified_f__Lachnospiraceae, and *Phascolarctobacterium* and between serum 25(OH)VD3 level and fecal *Lachnoclostridium* (all $P<0.01$). Circulating microbiota in VDI patients was closely related only to proinflammation and UAS7 (both $P<0.05$).

Conclusion: Changes in gut but not circulating microbiota composition are associated with serum 25(OH)VD3 insufficiency and impaired IFNI homeostasis, which points to greater disease severity (UAS7) and systemic proinflammation in CSU patients.

Keywords: chronic spontaneous urticaria, microbiota, vitamin D_3, type I interferon, inflammation

Introduction

Chronic spontaneous urticaria (CSU), a clinical subtype that accounts for about two thirds of cases of chronic urticaria, is generally regarded as a debilitating skin disorder characterized by recurrent episodes of intensely pruritic wheals with or without associated angioedema persisting ≥6 weeks. Although affecting up to 0.67% of the global population at some point during life, it is obviously self-limiting.[1] However, CSU patients still come to dermatology departments seeking help for the intolerable itching that significantly impairs sleep and daily activities. Regrettably, up to 40%–60% of patients receiving first-line treatment (second-generation nonsedating H_1 antihistamines [nsAHs] at licensed doses) still require higher doses of nsAHs or other approaches, including glucocorticoids, cyclosporine, or biologics, which inevitably brings substantial financial burden and drug-safety concerns for them.[2]

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The release of histamine and other inflammatory mediators upon mast-cell activation is widely recognized as central to CSU pathophysiology. We and others have shown that CSU is also involved other systemic abnormalities, including autoimmunity, imbalance of $T_h1/T_h2/T_h17$ cytokines, enteric dysbacteriosis, abnormal activation of the coagulation system, and vitamin D_3 (VD3) insufficiency (VDI).[3,4] Noteworthily, the above abnormalities may not concurrently present in one CSU patient, though one of them may predominate. Data concerning serum 25(OH)VD3 concentration and partial clinical benefit in CSU with VD3 supplementation indicate a need for dermatologists to focus on the possible causation and regulating effects of VD3 in the heterogeneity of CSU pathogenesis.[5]

To better manage this frustrating disease with VD3, efforts to explore potential interactions between VD3 and other systems should be sustained. In various diseases, including microbial infections, tumors, systemic lupus erythematosus, and multiple sclerosis, VDI and impaired type I interferon (IFNI; including IFNα and IFNβ) response are usually concurrently observed and reciprocal regulation may exist.[6] Plasmacytoid dendritic cells (pDC) in CSU fail to secrete IFNα upon TLR9 activation, implying the role of IFNIresponse impairment in CSU,[7] but its relationship to VDI remains unclear. More recent works have shown evidence that IFNI homeostasis depends on gut microbiota in viral and neoplastic diseases.[8,9] Although we have compared the diversity of gut microbiota and circulating bacterial DNA (btDNA) load between healthy and CSU patients with or without nsAH resistance,[10] the relationships among gut or circulating microbiota, IFNI response, and VD3 status in CSU have not been elucidated. These unresolved issues warrant exploration, as the cross talk among them will enhance the understanding of the contribution and mechanism of VD3 in CSU.

Herein, we hypothesize that the intermodulations from microbiota, IFNI homeostasis, and VD3 contribute to the onset or progression of CSU. The current study aimed firstly to describe the clinical characteristics and gut or circulating microbiota profiles in CSU patients with or without VDI, and further to analyze the associations between the microbiota and clinical characteristics, including IFNI response and serum 25(OH)VD3 levels.

Methods

Study Population

This investigation was conducted in the dermatology clinic of the Third Affiliated Hospital of Chongqing Medical University, China from 2020 to 2022. Adult CSU patients (aged ≥18 years) who met the diagnostic criteria proposed by new European Academy of Allergology and Clinical Immunology (EAACI)–Global Allergy and Asthma European Network (GA^2LEN)–European Dermatology Forum (EDF)–World Allergy Organization (WAO) guidelines were voluntarily recruited.[11] None of the CSU patients had taken antihistamines for at least 1 month before being administered 2 weeks of levocetirizine (5 mg per day), and CSU patients resistant to treatment with levocetirizine (5 mg per day, 2 weeks) were defined as having refractory CSU. All participants had usual living and eating habits. Patients with other allergic or autoimmune diseases, other nonallergic dermatosis, or systemic diseases, with a history of gastrointestinal surgery, immunosuppressive medication in the last 6 months, smoking/alcohol abuse habits, or pregnant/lactating were excluded. The study complied with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Third Affiliated Hospital of Chongqing Medical University (2020–11), and written informed consent was obtained from each participant at the time of recruitment.

Patient Profiles

A structured questionnaire was used to collect general demographic variables (sex, ethnicity, and body mass index [BMI]), medical history, previous or current medications, smoking habits, and alcohol intake. Urticarial activity score over 7 days (UAS7) at the time of patient recruitment and response to levocetirizine at sample collection (adequate response or resistance) were assessed for each patient according to EAACI–GA^2LEN–WAO–EDF guidelines.[11]

Blood Collection and Related Assessment

Fasting venous blood (10 mL) was collected from each patient after 2 weeks of levocetirizine treatment, and sera obtained by centrifugation were stored at −80°C. About 3 mL serum was used to determine protein levels of total immunoglobulin E (IgE; 5613, Meimian, Jiangsu, China), IL6 (E000482, 3SBio, Shanghai, China), CRP (E007462,

3ABio, Shanghai, China), claudin 3 (abx250611, Abbexa, Cambridge, UK), and human 25(OH)VD3 (50347H1, Meimian) by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions from commercially available kits.

To determine endogenous IFNI levels, HEK293 cells (CL-0001, Procell, China) stably expressing IFNI receptor complex (IFNAR1/2) with lentivirus were used. Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (HyClone) with 100 U/mL penicillin and 100 μg/mL streptomycin were used to culture the cells. Briefly, HEK293 cells were first cotransfected with *p*-ISRE-FLuc (100 ng/well, 96-well plate) and *p*-RLuc (20 ng/well, 96-well plate) for 24 hours. This was followed by retreatment of the cells with serum (100 μL/well) for another 24 hours. Then, the intensity of Firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) was measured using a Luciferase Assay System Kit (Promega) according to the manufacturer's instructions. The mean ratio of Fluc/Rluc (ie, ISRE activity) from three independent experiments indicates the intensity of IFNI response. Theoretically, ISRE activity is positively correlated with IFNI levels in serum.[12]

In addition, genomic DNA was isolated from 1 mL serum with a QIAmp DNA blood mini kit (51104, Qiagen, Hilden, Germany) according to the working manual. Then, btDNA was acquired from the genomic DNA by running a broad-range PCR with forward primer 5'-AGAGTTTGATCATGGCTCAG-3' and reverse primer 5'-ACCGCGACTGCTGCTGGCAC-3', the universal eubacterial primers of a conserved V3–V4 region of the 16S rRNA gene. The amplified btDNA was then purified with a DNA product purification kit (DP203, Tiangen, China) and pooled in equimolar and paired-end sequencing (2×300) on the MiSeq platform (Illumina, San Diego, USA), as with the detection and analysis of fecal btDNA. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA1102712).

Fecal Sample Collection and Related Determination

Approximately 5 g of fresh stool sample from each patient were collected after 2 weeks of levocetirizine treatment in a sterile plastic cup and stored at −80°C immediately. Fresh fecal lysate was used to detect fecal calprotectin protein using an ELISA kit (13732, Meimian). Methods for fecal btDNA extraction and sequencing were described in our previous work.[3] The raw reads were deposited into the NCBI SRA database (accession number: PRJNA809140).

Bioinformatic Analysis

Sequencing-based bioinformatic analyses are described in our previous work.[10] Notably, linear discriminant analysis (LDA), α-diversity, and β-diversity analyses were not performed in circulating btDNA from the VDI group. Additionally, the x^2 or Student's *t* test was conducted to compare the continuous or categorical variables, respectively, derived from nonsequencing data between the two groups. To calculate the associations between serum 25(OH)VD3 level and clinical variables, partial Pearson correlation analysis controlling for age, sex, and BMI was applied. $P<0.05$ (two-tailed) was accepted as the cutoff for statistical significance.

Results

Characteristics of Patients

This study enrolled 36 CSU patients with VDI (serum 25(OH)VD3 13.86±6.04 ng/mL) and 36 (male:female ratio 24:12 vs 21:15, $P=0.626$) age (33.93±8.82 vs 32.61±9.16 years, $P=0.536$)– and BMI (22.40±1.81 vs 21.91±1.56, $P=0.219$)–matched non-VDI (serum 25(OH)VD3 46.20±15.08 ng/mL) patients. Compared with the non-VDI group, the VDI group showed a significantly higher rate of nsAH resistance (77.78% vs 36.11%, $P=<0.001$), remarkably longer course of disease (11.72±6.99 vs 7.94±4.03 months, $P=0.006$; Figure 1A), significantly higher UAS7 (18.58±9.85 vs 14.17±4.04, $P=0.025$; Figure 1B), serum CRP (9.22±4.21 vs 6.46±3.05 mg/L, $P=0.002$; Figure 1C), IL6 (9.35±4.20 vs 6.39±3.32 pg/mL; $P=0.001$, Figure 1D), claudin 3 (46.61±18.05 vs 35.10±17.06 ng/mL, $P=0.007$; Figure 1E), and stool calprotectin (166.39±81.19 vs 120.29±77.46 μg/g, $P=0.016$; Figure 1F), but significantly lower IFNI response intensity (0.92±0.58 vs 1.66±0.60, $P=<0.001$; Figure 1G). Total serum IgE did not differ between the two groups (3.34±1.03 vs 3.42±1.48 μg/mL, $P=0.769$; Figure 1H).

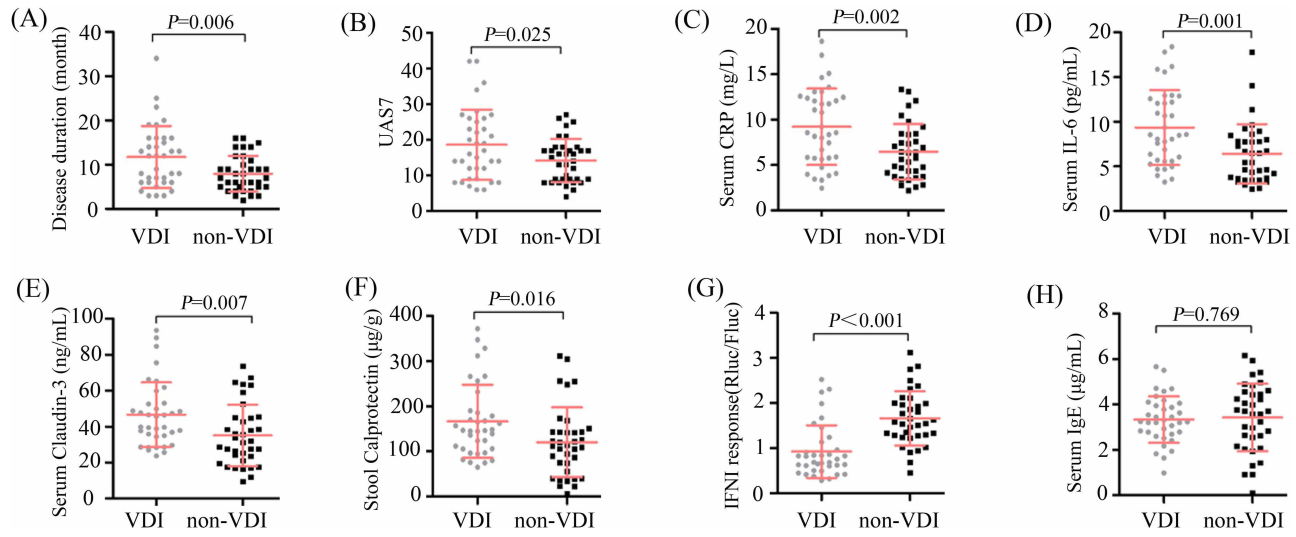

Figure 1 Comparisons of disease duration (**A**), UAS7 (**B**), serum CRP (**C**), serum IL6 (**D**), serum claudin 3 (**E**), stool calprotectin (**F**), IFNI response (**G**), and serum IgE (**H**) between the two groups. Calculation of IFNI response intensity specified in Methods section. Data are presented as scatter dot plots with means ± SD.
Abbreviation: UAS7, urticarial activity score over 7 days.

Further correlation analysis showed that serum 25(OH)VD3 in the VDI group demonstrated a strong positive correlation with IFNI response intensity (R=0.779, P=<0.001), mild negative correlations with CRP (R=−0.340, P=0.042), IL6 (R=−0.359, P=0.031), claudin 3 (R=−0.409, P=0.013), and UAS7 (R=−0.335, P=0.045), but no significant correlation with serum IgE (R=−0.029, P=0.867), stool calprotectin (R=−0.156, P=0.361), or disease duration (R=−0.190, P=0.265). No significant correlation between serum 25(OH)VD3 and other variables was observed in the non-VDI group, except for a strong positive correlation between 25(OH)VD3 and IFNI response intensity (R=0.668, P=<0.001).

Differences in Gut Microbiota Between the Two Groups

Sequencing Data and Bacterial Diversity Analysis

After quality filtering and trimming, we obtained 2,393,881 (ranging from 40,244 to 92,457) valid sequences with a mean length of 418 bp, and 2,031,248 (ranging from 34,319 to 74,995) with a mean length of 417 bp from 36 fecal samples in the VDI group and 36 in the non-VDI group, respectively. The reads involved 3340 operational taxonomic units (OTUs; including 17 phyla, 25 classes, 57 orders, 116 families, 330 genera, and 820 species) and 2681 OTUs (including 14 phyla, 22 classes, 52 orders, 105 families, 289 genera, and 714 species) from the two cohorts, respectively. Good's coverage estimator was 99.99%. At OTU level, the two cohorts had comparable index values for Ace (P=0.939), Sobs (P=0.939), Chao 1 (P=0.939), Shannon (P=0.423), Simpson (P=0.297), Shannoneven (P=0.297), and Simpsoneven (P=0.081), demonstrating no significant difference in fecal bacterial community richness, α-diversity, or evenness between the two groups. However, at OTU level, cohorts werevisualized using principal coordinate analysis (Figure 2A), and β-diversity analysis using the ANOSIM based on unweighted UniFrac metrics also showed significant distinct clusters between the two cohorts (R=0.159, P=0.001).

Microbiota Distribution and Structure Differences

There were 266 shared bacteria be-tween the two groups at genus level, with 64 and 23 unique ones in the VDI and non-VDI groups, respectively. More than 80% of fecal bacteria were of the phyla Firmicutes and Bacteroidota in the two groups. Regarding relative abundance at the genus level, the top five taxa identified in the VDI group were *Prevotella* 9 (25.15%), *Bacteroides* (8.90%), *Escherichia–Shigella* (8.01%), *Megamonas* (4.67%) and *Faecalibacterium* (4.65%), and those in the non-VDI group were assigned to *Bacteroides* (26.69%), *Faecalibacterium* (7.63%), *Prevotella* 9 (7.24%), and *Megamonas* (7.03%).

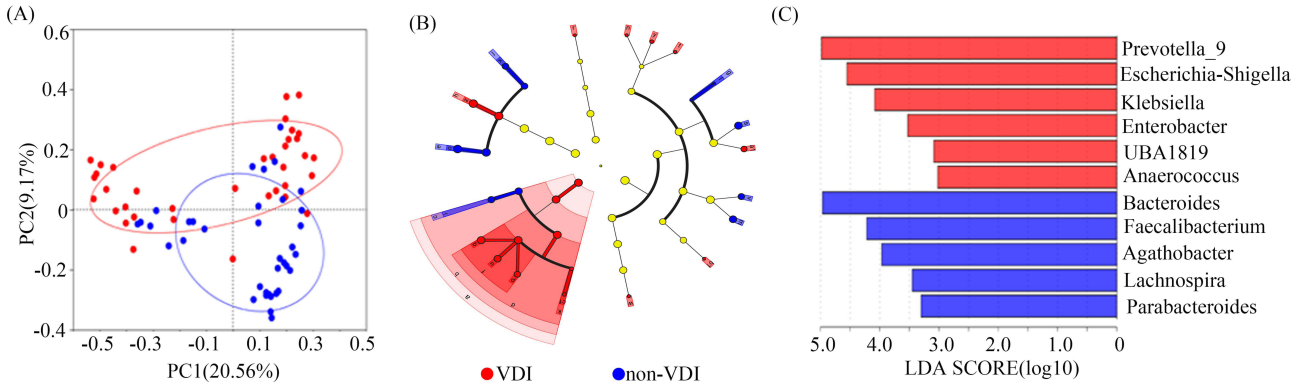

Figure 2 Differences in gut microbiota between the two groups: β-diversity analysis of gut microbial structure by principal coordinate analysis (**A**), distribution of differently abundant taxa from phylum to genus levels (**B**), and linear discriminant analysis (LDA) showing the impact of genera on the difference between the two groups (**C**; LDA ≥3).

At the phylum level, the relative abundance of Proteobacteria in the VDI group was much higher than that in the non-VDI group (12.17±15.30 vs 4.51±12.85, *P*=0.002). Differences in the relative abundance of the top 25 genera of fecal microflora in the two groups were further analyzed using the Wilcox rank-sum test. Table 1 showed that the VDI group displayed a significant increase in *Prevotella* 9 (25.15±31.56 vs 7.24±13.23, *P*=0.047), *Escherichia–Shigella* (8.01±10.64 vs 0.39±0.86, *P*=<0.001), and *Klebsiella* (2.54±10.01 vs 0.22±1.16, *P*=0.021), while members of the *Bacteroides* (8.91 ±12.16 vs.26.69±23.14, *P*=<0.001), *Faecalibacterium* (4.66±4.12 vs.7.64±5.27, *P*=0.020) and *Agathobacter* (2.12±3.54 vs.3.88±3.72, *P*=0.028) were relatively decreased compared to the non-VDI group. However, only *Bacteroides* and *Escherichia–Shigella* remained significantly altered after correcting the *P* values.

In addition, the application of the LefSe method identified a total of 29 taxa with significantly different abundance from phylum to genus levels between the two groups (LDA score >2, *P*<0.05), and the distribution and weighting of each taxon on the distribution tree is visualized in Figure 2B. Specifically, the fecal microbiota of VDI patients was differently enriched with genera *Prevotella_9*, *Escherichia–Shigella*, *Klebsiella*, *Enterobacter*, *UBA1819*, and *Anaerococcus*, whereas the non-VDI group was enriched with genera *Bacteroides*, *Faecalibacterium*, *Agathobacter, Lachnospira*, and *Parabacteroides* (Figure 2C).

Table 1 Relative abundance of genera between the two groups

	VDI	Non-VDI	*P*
Bacteroides	8.91±12.16	26.69±23.14	<0.001
Prevotella 9	25.15±31.56	7.24±13.23	0.047
Faecalibacterium	4.66±4.12	7.64±5.27	0.020
Megamonas	4.67±10.29	7.03±13.05	0.411
Escherichia–Shigella	8.01±10.64	0.39±0.86	<0.001
Blautia	4.15±6.37	3.29±2.82	0.796
Agathobacter	2.12±3.54	3.88±3.72	0.028
Subdoligranulum	3.74±5.79	1.01±1.04	0.112
Bifidobacterium	1.65±3.23	2.77±4.18	0.123
Lachnoclostridium	2.46±9.17	1.48±2.16	0.065
Veillonella	1.84±7.05	1.48±6.62	0.860
Klebsiella	2.54±10.01	0.22±1.16	0.021
Dialister	1.11±2.13	1.01±2.14	0.806

Notes: Relative abundance of genera presented as means ± SD; differences assessed by Wilcoxon rank-sum test between the two groups. VDI, vitamin D_3 insufficiency.

Relationships with Clinical Characteristics

Then, the analyses of the relationship between observed genera and clinical characteristics were separately conducted in the two groups. The variance-inflation factor (VIF) of a clinical indicator >10 underwent subsequent correlation analysis based on the existence of multicollinearity among clinical factors. Upon inspection, the VIFs of disease duration, presence of nsAH resistance, UAS7, serum IgE, IL6, CRP, claudin 3, serum 25(OH)VD3 level, IFNI response intensity, and gut calprotectin were 2.82 and 2.61, 1.25 and 2.97, 3.47 and 1.45, 1.32 and 2.96, 5.42 and 2.70, 6.34 and 1.43, 1.73 and 2.50, 3.03 and 2.50, 2.82 and 2.41, and 2.44 and 1.43 in the VDI and non-VDI groups, respectively. As the correlation heat map graph shows (Figure 3A), in the VDI group, there were moderate positive correlations between abundance of the genera *Escherichia–Shigella* and CRP (R=0.432, P=0.008) and IL6 (R=0.474, P=0.003), between *Bacteroides* and 25(OH)VD3 level (R=0.470, P=0.003) and IFNI response intensity (R=0.506, P=0.001), and *Klebsiella* (R=0.516, P=0.001) or *Enterobacter* (R=0.533, P=<0.001) and claudin 3, and a moderate negative correlation between *Enterobacter* and 25(OH)VD3 (R=−0.492, P=0.002). Contrastingly, in the non-VDI group (Figure 3B), IFNI response intensity was moderately positively associated with *Lachnoclostridium* (R=0.429, P=0.009), unclassified_f__Lachnospiraceae (R=0.495, P=0.002), and *Phascolarctobacterium* (R=0.449, P=0.006); claudin 3 negatively with UCG-002 (R=−0.444, P=0.007) and unclassified coprostanoligenes (R=−0.468, P=0.004); 25(OH)VD3 moderately positively and negatively with *Lachnoclostridium* (R=0.468, P=0.004) and *Romboutsia* (R=−0.460, P=0.005), respectively; and presence of nsAH resistance moderately positively and negatively with *Megamonas* (R=514, P=0.001) and *Eubacterium_hallii_* (R=−0.465, P=0.004), respectively. Some weak correlations were also found in the two groups.

Circulating Microbiota and Its Clinical Significance in VDI Group

Finally, we tried to amplify btDNA fragments from sera of patients with V3–V4 16s PCR primers, and the results showed that there were 30 and four btDNA fragments meeting the quality for deep sequencing in the VDI and non-VDI groups, respectively. We further performed bioinformatic analysis of the circulating microbiota and analyzed its clinical significance in patients with VDI. In total, we obtained 3,487,171 (ranging from 84,036 to 130,848) valid sequences with a mean length of 458 bp involving 9400 OTUs (including 44 phyla, 123 classes, 317 orders, 553 families, 1140 genera, and 2291 species) from the VDI group. At the phylum level, the top three taxa identified were Proteobacteria (68.72%), Firmicutes (12.46%), and Bacteroidota (4.79%). Correspondingly, *Burkholderia–Caballeronia–Paraburkholderia* (40.95%), *Acinetobacter* (3.05%), and *Aquabacterium* (2.37%) were found to be the top three bacteria at the genus level (Figure 4A). Relationship analysis

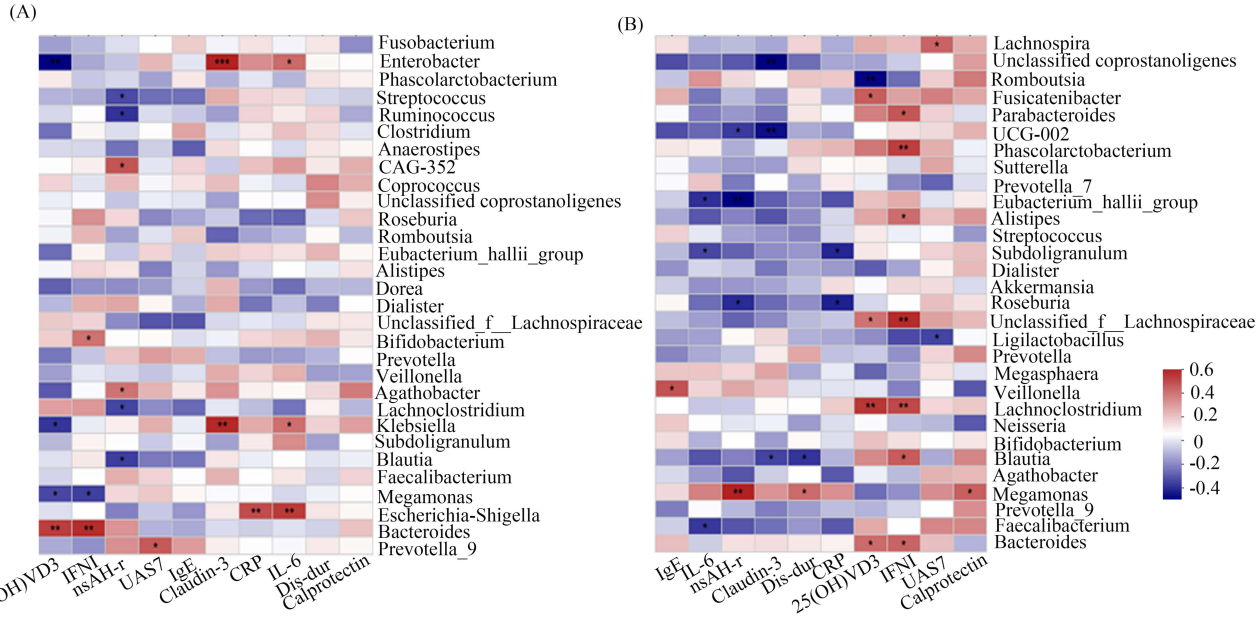

Figure 3 Correlation analysis of gut bacteria and related clinical indicators in VDI group (**A**) and non-VDI group (**B**). VDI, vitamin D_3 insufficiency. *P<0.05; **P<0.01; ***P<0.001.

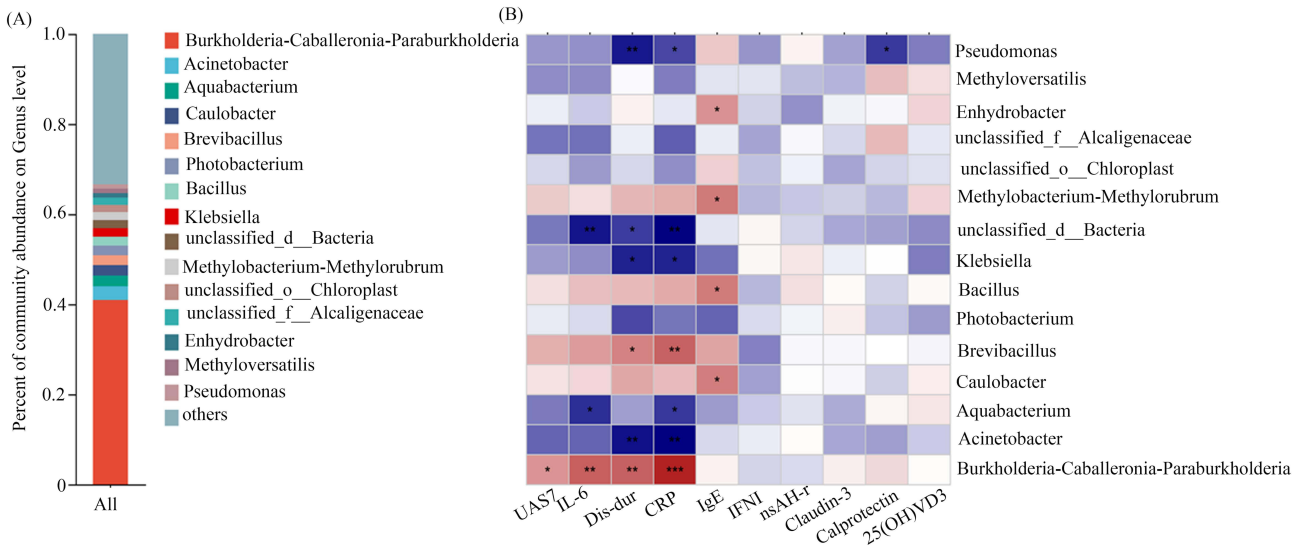

Figure 4 Circulating microbiota composition (**A**) and clinical relationships (**B**) in VDI group. VDI, vitamin D_3 insufficiency. *P<0.05; **P<0.01; ***P<0.01.

showed that the abundance of *Burkholderia–Caballeronia–Paraburkholderia* was weakly to strongly positively correlated with UAS7 (R=0.371, P=0.043), disease duration (R=0.517, P=0.003), serum IL6 (R=0.518, P=0.003), and serum CRP (R=0.688, P=<0.001); *Acinetobacter* moderately negatively associated with disease duration (R=−0.496, P=0.005) and serum CRP (R=−0.534, P=0.002); unclassified_d__bacteria moderately negatively associated with serum IL6 (R=−0.483, P=0.006) and serum CRP (R=−0.530, P=0.002); and *Pseudomonas* and *Brevibacillus* moderately negatively and positively associated with disease duration (R=−0.478, P=0.007) and serum CRP (R=0.509, P=0.004), respectively. However, no significant correlation between 25(OH)VD3 or IFNI response intensity with any observed circulating microbiota was found (Figure 4B).

Discussion

IgE/IgG-mediated mast-cell activation in skin together with other changes in systemic biology are suggested to be the dominant biological events in CSU pathophysiology. Of importance, the mechanistic background underlying the interrelationships between immunological and nonimmunological responses (such as neuroimmunopsychological factors) has not yet been fully elucidated.[13] Therefore, determining the multifaceted nature of CSU (especially with nsAH resistance) pathophysiology as much as possible is a continuing need, and efforts are in progress.[14] The current study tried to assess the correlations among CSU clinical characteristics, systemic aspecific inflammation, levels of serum 25 (OH)VD3 and IFNI homeostasis, and gut or circulating microbiota.

Clinically, more than a dozen case–control and retrospective studies have shown that the severity or disease duration of CSU is inversely associated with serum 25(OH)VD3 concentration, and a much higher prevalence of 25(OH)VD3 insufficiency is observed in CSU than the healthy.[15] There are also meta-analyses confirming the relationships in adult rather than pediatric CSU patients.[16,17] The fact that some CSU patients benefit from 4–12 weeks of VD3 supplementation further underlines the contribution of sufficient VD3 signaling in promoting the remission or preventing the onset of CSU.[17,18] No significant correlation between serum 25(OH)VD3 and CRP concentrations was observed,[19] but Mohamed et al found that alfacalcidol (a precursor of active VD3, 0.25 μg/daily, 12 weeks) significantly decreased serum levels of aspecific inflammation biomarkers, including IL6, CRP, and TNFα, in CSU.[18] We found longer disease duration and higher UAS7 and inflammation response in CSU patients with VDI (Figure 1), suggesting VD3 may alleviate CSU severity by suppressing aspecific inflammation responses. Of course, its regulation of T cell–mediated inflammation may also work.[15] Experiments in vitro have proved that mast cells can convert 25(OH)VD3 to 1α,25(OH)2D3 through CYP27B1 activity, and that active VD3 can further increase expression of the VD3 receptor, thus enhancing the stability of mast cells in the case of IgE challenge.[15] More recently, Zhao et al discovered that VD3-binding protein was increased and 25(OH)VD3 directly suppressed the production of VEGF in mast cells induced by serum from CSU patients through

the PI3K–Akt–p38MAPK–HIF1α axis.[20] All these data collectively highlight the need for adequate VD3 in the prevention and control of CSU.

In addition to sun exposure, diet, and liver and kidney functions, steady-state IFNI may also be a systemic factor influencing VD3 metabolism. Newmark et al comprehensively analyzed the evolutionary origin of the link between IFNI and VD3.[21] Recently, we also summarized the associations and found extremely complex cross talk depending on endogenous response, exogenous clinical application and disease specificity.[6] Currently, there are no available clinical studies determined the relationship in healthy or chronic inflammatory dermatoses due to the very low level of endogenous IFNI limiting its detection by conventional ELISA kits, though Suzuki et al uncovered that a VD3 analogue (calcipotriol) can interfere with IFNα expression by inhibiting the CpG–TLR9–MYD88 pathway and thereby alleviate psoriatic dermatitis in mice.[22] We found a strong positive correlation between IFNI response intensity and 25(OH)VD3 levels in CSU with or without VDI, but failed to prove causation. It remains unclear whether steady-state IFNI is involved in inflammatory dermatoses other than systemic lupus erythematosus.[23] pDC is capable of releasing up to 1000 times the IFNI of any other types of cell. Futata et al found that IFNα secretion by TLR9-mediated activation of pDC from CSU patients was much impaired due to the downregulation of TLR9 expression.[7] In addition, Tversky et al demonstrated a similar scenario in non-CSU patients with allergies.[24] These data collectively imply that inefficient basal IFNI response may be an unfavorable internal environmental factor in allergic diseases, including CSU. Recently, Kobayashi et al undertook an in vivo experimental verification of this hypothesis and found exacerbated systemic anaphylaxis after sensitization in mice with IFNI-receptor deficiency.[25] Therefore, impaired steady-state IFNI signaling is definitely involved in CSU pathogenesis, perhaps by affecting VD3 metabolism.

In recent years, studies have profiled gut microbiota between CSU patients and healthy controls.[26] However, both similarities and inconsistencies in the composition of gut microbiota among the studies were exhibited, which may be attributable to differences in sample size, subject characteristics (locale, living and eating habits), and urticaria-related therapeutics. We previously paid particular attention to nsAH resistance in CSU individuals (44 cases), and subgroup analysis showed that a greater abundance of *Escherichia* associating with systemic aspecific inflammation features the presence of nsAH resistance,[3] suggesting the necessity of differentiating CSU patients according to certain clinical characteristics. After that, we consistently recruited CSU cases and selected 36 patients with VDI and 36 age-, sex-, and BMI-matched patients without VDI for further analyses. The results showed that the VDI group was enriched in genera *Prevotella* 9, *Escherichia–Shigella*, and *Klebsiella*, but that there was scant *Bacteroides, Faecalibacterium*, and *Agathobacter* (Table 1). The data on the abundance of *Escherichia–Shigella* being positively linked to systemic inflammation in the VDI group may imply the presence of a VD3–microbiota–inflammation axis.

The maintenance of intestinal microbiota and mucosa permeability homeostasis requires appropriate VD3 signaling,[27] Both enteric dysbacteriosis and increased mucosa permeability have also been observed in CSU patients with VDI. In healthy populations, Bashir et al found that a high dose of VD3 supplementation can reduce the abundance of many intestinal microbiota, including *Escherichia–Shigella*,[28] which reinforces the possible profitability of VD3 supplementation in our VDI patients we included, as they showed enriched gut *Escherichia–Shigella*. Vice versa, the way that gut microbiota regulates VD3 metabolism remain largely unknown, but the proof that probiotics enhance circulating 25(OH)VD3 level and promote VDR expression suggests that gut microbiota is required for VD3 homeostasis,[29,30] and IFNI response is hypothesized to be the messenger. Increasing and convincing experimental evidence points to gut microbiota activity in the modulation of IFNI response locally and remotely in viral and neoplastic diseases, and microbiota-derived PRR ligands or metabolites controlling homeostatic IFNI may be the key mechanisms underpinning these interactions.[8,9] Vice versa, blocking IFNI signaling is bound to contribute to gut microbial ecology.[31] We found that the lower the abundance of *Bacteroides*, the less likely a CSU patient with VDI was to experience IFNI impairment, while *Lachnoclostridium*, unclassified_f__Lachnospiraceae, and *Phascolarctobacterium* were found to be positively associated with IFNI in CSU patients without VDI, which is partially consistent with findings from other studies on non-CSU diseases.[32,33] Unfortunately, there is no research elucidating how gut microbiota affectVD3 metabolism by modulating steady-state IFNI.

Additionally, emerging evidence has preliminarily demonstrated the presences of circulating microbiota or bacterial DNA in healthy controls and non-bacteremia diseases, including liver cirrhosis, COVID-19, colorectal cancer,

myocardial infarction, chronic kidney disease, type 2 diabetes, and psoriasis.[34–36] A much higher load of circulating bacterial DNA was detected by PCR from CSU patients with nsAH resistance than those without or healthy subjects in our previous work.[10] The current study successfully identified the composition of circulating microbiota by 16s rDNA sequencing in the VDI group and found a remarkably high rate of nsAH resistance. We also found that Proteobacteria accounted for 68.72% of the total abundance, which is significantly lower than what other research teams have found (>90%) in healthy populations.[34] To date, no studies have confirmed the exact mechanisms underlying the presumed clinical significance of circulating microbiota due to so many unanswered questions, including how they exist and how to intervened. In this study, no significant correlation between circulating microbiota and serum 25(OH)VD3 level or IFNI response intensity was detected, except for a few positive and negative correlations with inflammation, disease duration, and severity. We thus hypothesize that unlike gut microbiota, circulating microbiota contribute to CSU pathogenesis independently of VD3 metabolism or IFNI response, which is possibly related to aspecific systemic inflammation.

Several limitations exist in the current study. Firstly, specific gut or circulating bacteria, systemic inflammation, and VD3–IFNI cross talk may act simultaneously and/or follow each other. We did not establish causations in CSU, as with other case–control clinical studies. Secondly, if further correlation analyses in pooled CSU patients yield different analytical results from these in subgroup analysis according to serum 25(OH)VD3 levels, patient screening should be performed prior to the application of VD3 or IFNI for CSU treatment. Thirdly, multicenter within-subject verifications of associations in larger samples are lacking, and further validating the existence of commonalities in other IgE-mediated allergic diseases is warranted. Lastly, the biological activity of circulating microbiota and whether it originates from the gut deserve further exploration.

Conclusion

The present study clearly demonstrates the positive association between VDI and impairment of IFNI homeostasis, and IFNI response intensity is shown to be positively correlated with different gut bacteria in CSU with VDI (genera *Bacteroides*) and without VDI (*Bacteroides*, *Lachnoclostridium*, unclassified_f__Lachnospiraceae, and *Phascolarctobacterium*). Positive and negative associations between systemic inflammation and bacteria from the gut or circulation were also observed in CSU patients with VDI. Future therapeutics with microbiota or IFNI correction varying with VD3 status in CSU patients are expected.

Data Sharing

All data involved in this study are available upon reasonable request from the corresponding author.

Ethics Approval

This study was approved by the Medical Ethics Committee of the Third Affiliated Hospital of Chongqing Medical University.

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Disclosure

The authors report no conflicts of interest in this work.

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