ORIGINAL ARTICLE

Metagenomic profiling reveals dominance of gram-positive bacteria in the gut microbiome shifts associated with immunoglobulin A vasculitis (Henoch–Schönlein Purpura)

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Abstract

Objectives. Immunoglobulin A vasculitis (IgAV), previously known as Henoch–Schönlein purpura, is the most common vasculitis that has a classical skin manifestation of palpable purpuric rash. Factors pertinent to IgAV remain inadequately understood. Here, we aimed to examine the gut microbiome shifts associated with IgAV and its recovery. **Methods.** Stool samples were collected from 10 children with IgAV (6–14 years old) before and after a multi-drug therapy, along with 9 age-matched healthy children. The samples were subjected to metagenomic analyses to investigate the taxonomic and functional shifts of the gut microbiome. **Results.** The analyses revealed that compared with healthy controls, treatment-naïve patients exhibited substantial taxonomic and functional alterations of gut microbiota, including 104 IgAV-depleted species and 7 IgAV-elevated species (FDR < 0.05). After treatment, the IgAV patients displayed a partial restoration of the microbiota shifts, as the relative abundances of some biomarkers (e.g. 9 genera and 22 species) became comparable (FDR > 0.1) between the patients and healthy controls. The treatment-responsive features included *Weissella*, *Faecalibacterium prausnitzii* and *Bifidobacterium pseudocatenulatum* and three components of a putative glutamine transport system. Importantly, gram-positive bacteria accounted for over 85% of the numbers and total relative abundance of the species that were associated with IgAV and responsive to the treatment. In addition, of the 122 IgAV-depleted bacterial genes, 82 were mainly contributed by gram-positive bacteria and 12 by gram-negative bacteria. **Conclusions.** Gram-positive bacteria are the main drivers underlying the gut microbiome shifts of IgAV, which may assist rational management of the disease.

Keywords: gram-positive bacteria, gut microbiota, immunoglobulin A vasculitis (IgAV)

INTRODUCTION

Immunoglobulin A vasculitis (IgAV), previously known as Henoch–Schönlein purpura (HSP), is a rare, systemic disorder that is mainly found in children and damages the skin, joints, gastrointestinal tract or kidneys.[1–4] The disease is characterised by small vascular IgA deposition and inflammation, but its aetiology remains poorly understood. Although the symptoms of IgAV are generally mild and self-limiting, a substantial proportion of the patients may experience recurrent courses, as shown by reported relapse rates (14.4–66%) in multiple longitudinal studies.[5–9] This clinical problem of IgAV is accompanied by a long lack of an international therapeutic consensus[10,11] until recently published SHARE recommendations in Europe,[12] which have yet been widely adopted. As such, a better understanding of the factors related to IgAV occurrence is crucial. Whereas it has been known that preceding infections are common among IgAV patients and mostly found in the upper respiratory tract,[2–4,7,9] recent 16S rRNA gene-based analyses reveal substantial microbial abnormalities in the oral cavity and gut.[13,14] In addition, human and mouse studies on several diseases indicated that the enteric microbial community influences the medications and correlates with the treatment outcome.[15–17] The findings raise the question of whether some microbial elements are responsive to both occurrence and recovery of IgAV, which may provide an important clue to understand and manage the disease.

The primary goal of this study was to gain better knowledge for the gut microbiota shifts of IgAV. To this end, we performed metagenomic analyses to examine the stool samples of a group of IgAV patients before and after a therapy, which were compared with those of age-matched healthy controls. We hypothesise that by identifying the gut microbiome features that are associated with IgAV and its recovery, we may generate new insights into the understanding of IgAV pathogenesis and the experimental basis for developing a more effective therapy.

RESULTS

A multi-drug therapy and the treatment outcome

A multi-drug regimen was used to treat IgAV patients, which comprised three base drugs of

safflower yellow injection, *a*moxicillin/clavulanate potassium (substituted by azithromycin for penicillin skin test-positive or *Mycoplasma pneumoniae*-infected patients) and *v*itamin C as well as condition-specific agents such as prednisone, omeprazole, heparin or ganciclovir (Supplementary table 1). The therapy, referred hereafter as SACV, lasted between 5 and 21 days. Of the 33 patients who underwent SACV therapy, 32 were cured and one individual (male, 12 years old, hospital ID 1132280) showed recalcitrant renal symptoms and developed chronic purpuric nephritis later (Figure 1a and Supplementary table 1). Coincided with the resolution of symptoms in the patients (Figure 1b and Supplementary table 1), the therapy led to amelioration in three IgAV-related serum features (FDR < 0.05; Figure 1c), including two hypercoagulation indicators (i.e. fibrinogen degradation product and D-dimer) and one inflammatory indicator (i.e. erythrocyte sedimentation rate). Of the 32 cured patients, 4 individuals experienced relapse and all of them developed cutaneous rash and arthritis in the second half of the first post-therapeutic year, but their symptoms were all eventually cleared (Supplementary table 1). Although the treatment exhibited a relatively good efficacy, the absence of a randomised treatment control suggested a great caution in interpreting finding or assessing the efficacy. Nevertheless, the results allowed us to analyse gut microbiota shifts associated with recovery. Of note, the inflammatory signs in different sites or organs, particularly that in the gastrointestinal tract such as faecal occult blood (Supplementary table 1), suggested alterations in the gut environment.

Structural and taxonomic shifts of the gut microbiome in the IgAV patients

To examine the gut microbiota traits associated with IgAV, a metagenomic analysis was performed to analyse the stool samples in 10 randomly selected IgAV patients before and after the SACV therapy. In comparison with the healthy controls, treatment-naïve IgAV patients exhibited a reduced microbial diversity, as shown by the Shannon–Wiener index (Wilcoxon rank-sum test, adjusted $P < 0.05$; Figure 2a) and a different microbial structure, as shown by principal coordinates analysis (PCoA; ANOSIM test, adjusted $P < 0.01$; Figure 2b). After the treatment, the IgAV patients showed a partial recovery in the microbial diversity such that the Shannon–Wiener index of the post-therapeutic IgAV patients was
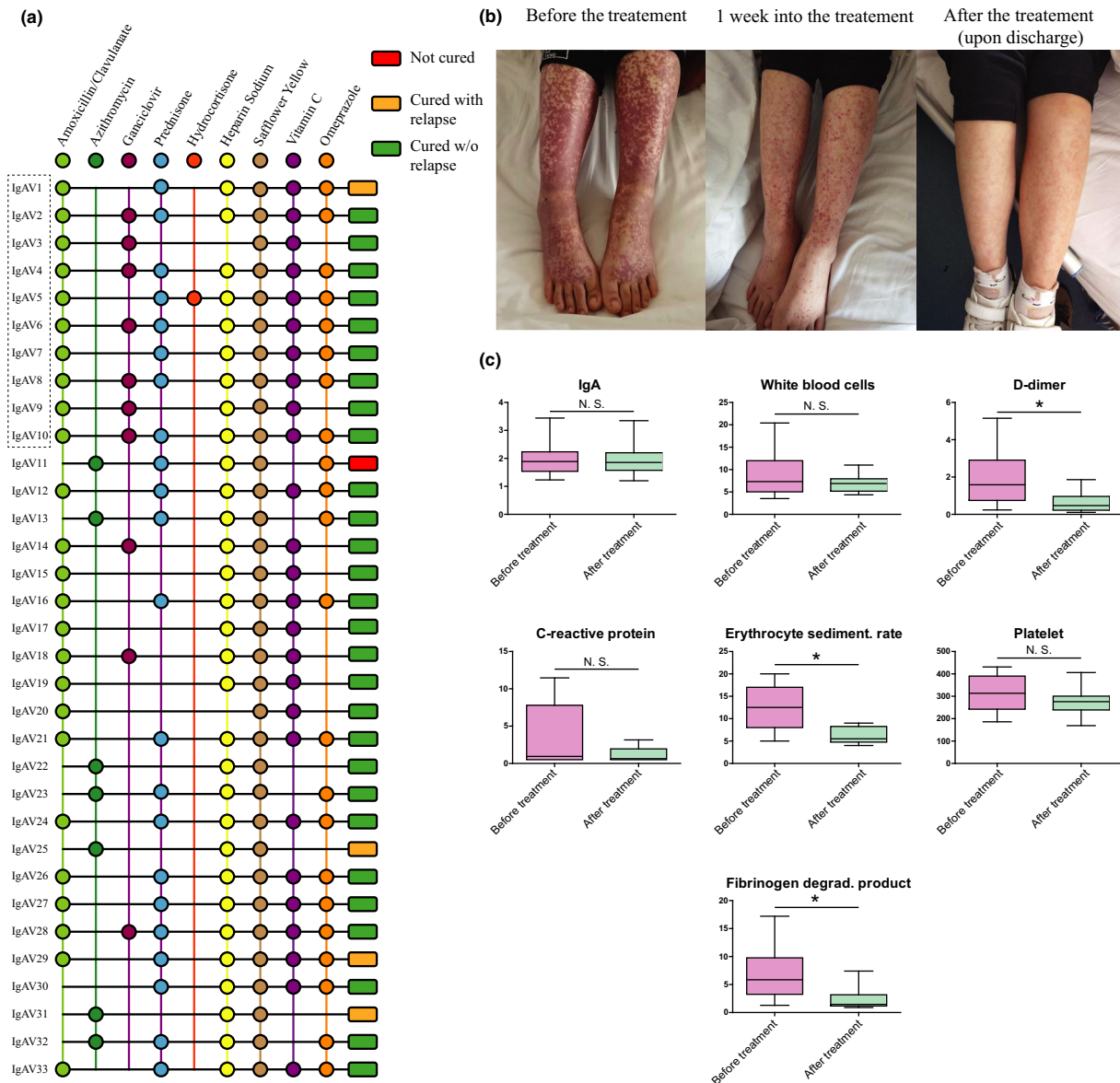
Figure 1. The outcome of SACV multi-drug regimen. **(a)** The summary of personalised medication and therapeutic outcomes of the patients. Participants in the dashed box were subjected to metagenomic analysis. **(b)** Skin manifestations of a representative patient during treatment. **(c)** Treatment responses of IgAV-related serum indicators in the patients. N. S., not significant.

comparable with that of the healthy controls (Wilcoxon rank-sum test, adjusted $P = 0.104$; Figure 2a), although there was no statistical difference between the treatment-naïve value and post-therapeutic value in the IgAV patients (Wilcoxon rank-sum test, adjusted $P = 0.684$). In addition, the difference in microbial structure between the healthy controls and patients (Wilcoxon rank-sum test, adjusted $P < 0.05$) was not appreciably affected by the treatment

(Figure 2b). Overall, our findings on the gut microbiota diversity and structure suggested that considerable compositional alterations were associated with IgAV and were partially restored after the SACV treatment.

We next examined the taxonomic characteristics in the participants and revealed considerable differences between the treatment-naïve IgAV patients and healthy controls. At the phylum level, the IgAV group exhibited an increase in the

relative abundance of Blastocladiomycota, a fungal taxon, and a decrease in that of Firmicutes (Wilcoxon rank-sum test, FDR < 0.05; Figure 2c and Supplementary table 2). At the genus level, the naïve IgAV patients were characterised by elevated presence of 5 taxa, namely *Allomyces* (a fungal genus within Blastocladiomycota), *Arsenophonus*, *Candidatus Azobacteroides*, Clostridia bacterium UC511A9 and Clostridiales bacterium VE202-07, and decreased proportions of 44 taxa, including *Roseburia*, *Fecalibacterium*, *Eubacterium*, Firmicutes bacterium CAG:65 and *Coprococcus* (Wilcoxon rank-sum test, FDR < 0.05; Figure 2c and Supplementary table 2). The highly skewed ratio between the over-represented taxa and under-represented ones in the IgAV patients was maintained at the species level such that 7 bacteria and 1 fungus showed increases in relative abundance, and 104 bacteria showed decreases in the IgAV group compared with those in the control group (Figure 2d and Supplementary table 2). This imbalanced pattern with far greater numbers of IgAV-decreased taxa was consistent with the reduced microbial diversity observed in the untreated patients (Figure 2a).

Treatment responses of naïve IgAV-associated taxa

The partial recovery of gut microbial diversity after the treatment (Figure 2a) implied that the relative abundances of some IgAV-associated taxa correlated with the convalescence process. To investigate this question, we first defined a treatment-responsive feature as a microbiome component showing post-therapeutic disappearance of the statistical significance in relative abundance analysis (Wilcoxon rank-sum test, HC-pthIgAV FDR > 0.1) that was present between the control and IgAV groups (Wilcoxon rank-sum test, HC-IgAV FDR < 0.05). We showed that although most of the naïve IgAV-associated taxa were not affected by the treatment, a subset of them responded as their relative abundance values approached the corresponding levels in the healthy controls (Figures 2c and 3, Supplementary figure 1, Supplementary table 2). Of note, no responders displayed a statistical difference between the naïve and post-therapeutic values, further corroborating the observation (Figure 2a) that the post-therapeutic microbiota recovery was partial. At the genus level, 8 taxa displayed treatment responses, including 6 of the 44 naïve IgAV-decreased genera, namely *Weissella*, *Brachybacterium*,

Faecalibacterium, *Cryptobacterium*, *Brachyspira* and Lachnospiraceae_bacterium_5_1_57FAA, as well as 2 of the 5 naïve IgAV-increased genera, which were Clostridia bacterium UC511A9 and a fungal taxon *Allomyces*.

At the species level, 22 taxa exhibited treatment responses, including 21 of the 104 naïve IgAV-decreased taxa as well as 1 of the 7 naïve IgAV-increased taxa. These treatment-responsive microbes included *Brachybacterium muris*, *Butyrivibrio* sp. AD3002, *Clostridium* sp. CAG:43, *Clostridium* sp. CAG:7, *Brachyspira pilosicoli*, *Butyrivibrio crossotus* CAG:259, *Blautia* sp. Marseille-P2398, *Ruminococcus* sp. CAG:9, *Eubacterium eligens* and *Enterococcus casseliflavus*. Our results showed that the multi-drug therapy correlated with a partial recovery of IgAV-associated taxonomic shift (Figures 2c and 3, Supplementary figure 1, Supplementary table 2).

An interesting phenomenon of the taxonomic results was that gram-positive bacteria accounted for the majority of the species that were associated with IgAV (17/111; 84.7%) and that responded to the treatment (20/22; 90.9%; Figures 2d and 3e, Supplementary table 2). The actual ratio might be higher because 7 of the 8 IgAV-associated species of unknown gram stain traits were unclassified taxa in Firmicutes or Clostridiales of this phylum, in which most members are gram-positive. However, the greater numbers of the gram-positive taxa do not necessarily mean they being the major drivers of the IgAV-related shifts because of the following two reasons: (i) the relative abundances of the gram-positive biomarkers might be overall a lot lower than those of the gram-negative ones and (ii) there are controversy and constant changes in bacterial phylogenetics,[18–20] leading to scenarios that one species could be alternatively regarded as several. In the light of this, we calculated the combined relative abundance of the gram-positive species or gram-negative species that were associated with IgAV and that correlated with the SACV treatment. The results (Figure 4a, b) showed that in both healthy controls and IgAV patients, gram-positive bacteria accounted for over 85% in the total abundance of both types of biomarkers, whereas the shares of gram-negative bacteria were minimal. This was in clear contrast to the gram-positive shares of the biomarkers in all other diseases being examined here (Supplementary figure 2).

We next examined whether gram-negative bacteria or gram-positive bacteria as a whole
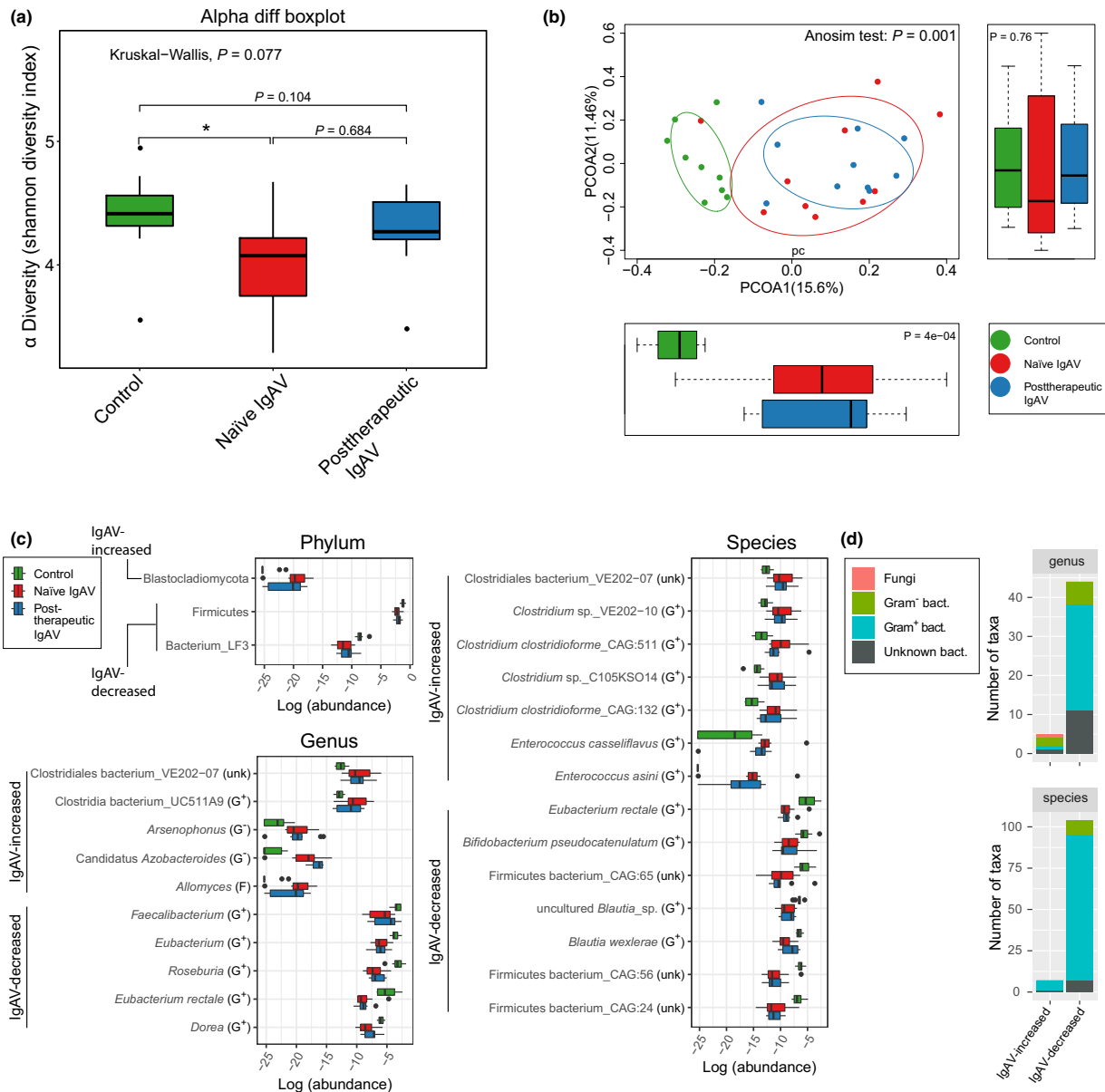
Figure 2. The taxonomic shift of gut microbiota in the IgAV patients. **(a)** Taxonomic Shannon indices of the samples. **(b)** PCoA based on the Bray–Curtis distance metric of species abundance. **(c)** Relative abundance of the most abundant taxa at levels of phylum, genus and species that were significantly different (FDR < 0.05) between the healthy controls and naïve IgAV patients. 'F', 'G−', 'G+' and 'unk' in the parentheses represent fungi, gram-negative bacteria, gram-positive bacteria and bacteria of unknown gram stain trait, respectively. **(d)** The numbers of genera and species showed differences in relative abundance (FDR < 0.05) between the healthy controls and naïve IgAV patients. Gram- bact., gram-negative bacteria; gram+ bact., gram-positive bacteria; unknown bact., bacterial taxa with an unknown gram stain trait; IgAV-increased, increased relative abundance in the naïve IgAV patients; IgAV-decreased, decreased relative abundance in the naïve IgAV patients.

(Supplementary table 3), not just the biomarkers, behaved differently between the healthy controls and IgAV patients. PCoA showed that in comparison with the gram-negative microbiota, the gram-positive microbiota exhibited a better separation between the healthy controls and IgAV

patients ($P = 0.014$ and R = 0.101 for the gram-negative microbiota; $P = 0.001$ and R = 0.380 for the gram-positive microbiota; Figure 4c, d), indicating that gram-positive bacteria were more responsive in IgAV-related changes than their counterparts with the different envelope
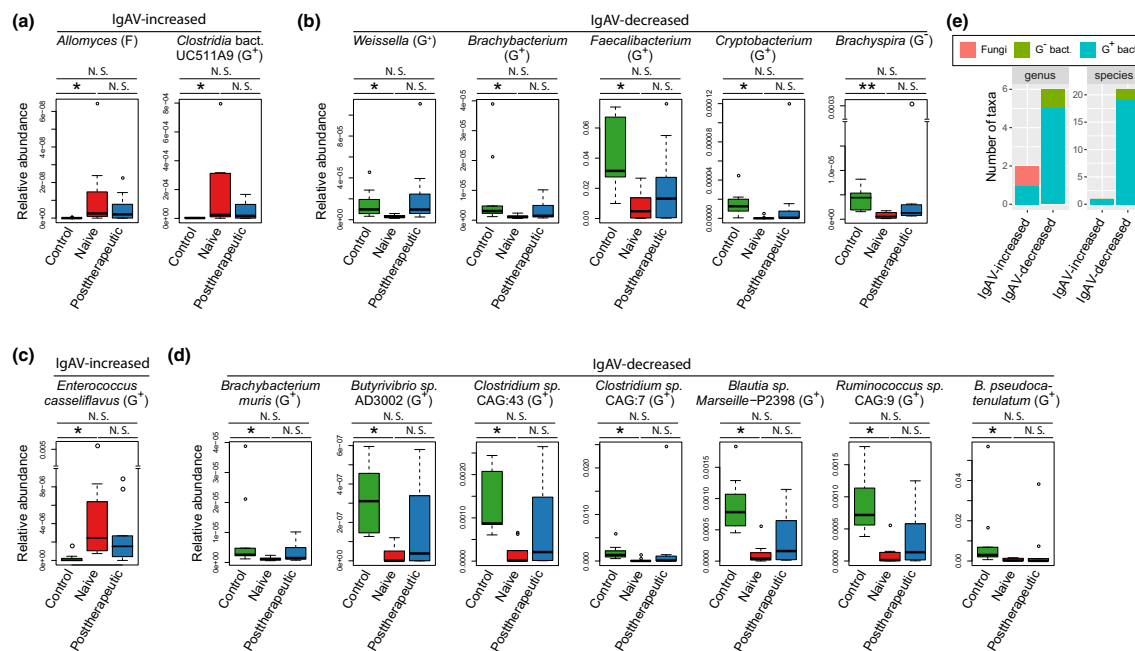
Figure 3. The microbial taxa that responded to the treatment. The treatment-responsive taxa (**a** and **b**, genus; **c** and **d**, species) were defined as those that showed a significant difference in relative abundance between the healthy controls and naïve patients (HC-IgAV FDR < 0.05) but lacked a significant difference between the healthy controls and post-therapeutic patients (HC-pthIgAV FDR > 0.1). Results of Wilcoxon rank-sum tests are shown at the top of each box (N.S., not significant; *, FDR < 0.05; **, FDR < 0.01). 'F', 'G−' and 'G+' in the parentheses denote fungus, gram-negative bacterium and gram-positive bacterium, respectively. **(e)** The numbers of treatment-responsive genera (upper panel) and species (lower panel). Gram- bact., gram-negative bacteria; gram+ bact., gram-positive bacteria.

ultrastructure. Hence, multiple lines of evidence indicated that gram-positive bacteria were the major drivers in IgAV-related taxonomic shift.

The functional shift of gut microbiota in the IgAV patients

We next investigated the microbial functional characteristics of the IgAV patients in both the illness and convalescence phases. Of the 4826 genes functionally annotated to Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous groups (KOs), 143 were differentially abundant between the HCs and naïve IgAV patients, including 21 over-represented and 122 under-represented in the patients (Figure 5a, b, Supplementary table 4; Wilcoxon rank-sum test, FDR < 0.05). Hence, both taxonomic and functional shifts were characterised by greater numbers of IgAV-decreased features (Figure 2). The functional differences were corroborated by a clear separation of functional structure between the HCs and IgAV patients (Wilcoxon rank-sum test, $P = 0.002$; Figure 5c). Although the overall

functional shift was not appreciably affected by the SACV treatment (Wilcoxon rank-sum test, $P = 0.671$; Figure 5c), 10 KOs exhibited treatment responses, including 7 IgAV-decreased features and 3 IgAV-increased features (Wilcoxon rank-sum test, HC-pthIgAV FDR > 0.1). These treatment-responsive KOs included three genes of a putative glutamine transport system, namely ABC.GLN1.A (K10041; an ATP-binding protein), ABC.GLN1.S (K10039; a substrate-binding protein) and ABC.GLN1.P (K10040, a permease protein). The other treatment-responsive KOs included *dgt* (K01129; dGTPase), *hpsN* (K15509; sulfopropanediol 3-dehydrogenase), *atpA* (K02111; F-type H⁺-transporting ATPase subunit alpha), *mtnN* (K01243; adenosylhomocysteine nucleosidase), *pct* (K01026; propionate CoA-transferase), *bfr* (K03594; bacterioferritin) and *trbC* (K12059; conjugal transfer pilus assembly protein).

HUMAnN2[21] was used to identify the taxonomic contributors of the functional biomarkers (Figure 5d, Supplementary figures 3 and 4). Based on the contribution by the two types of bacteria,
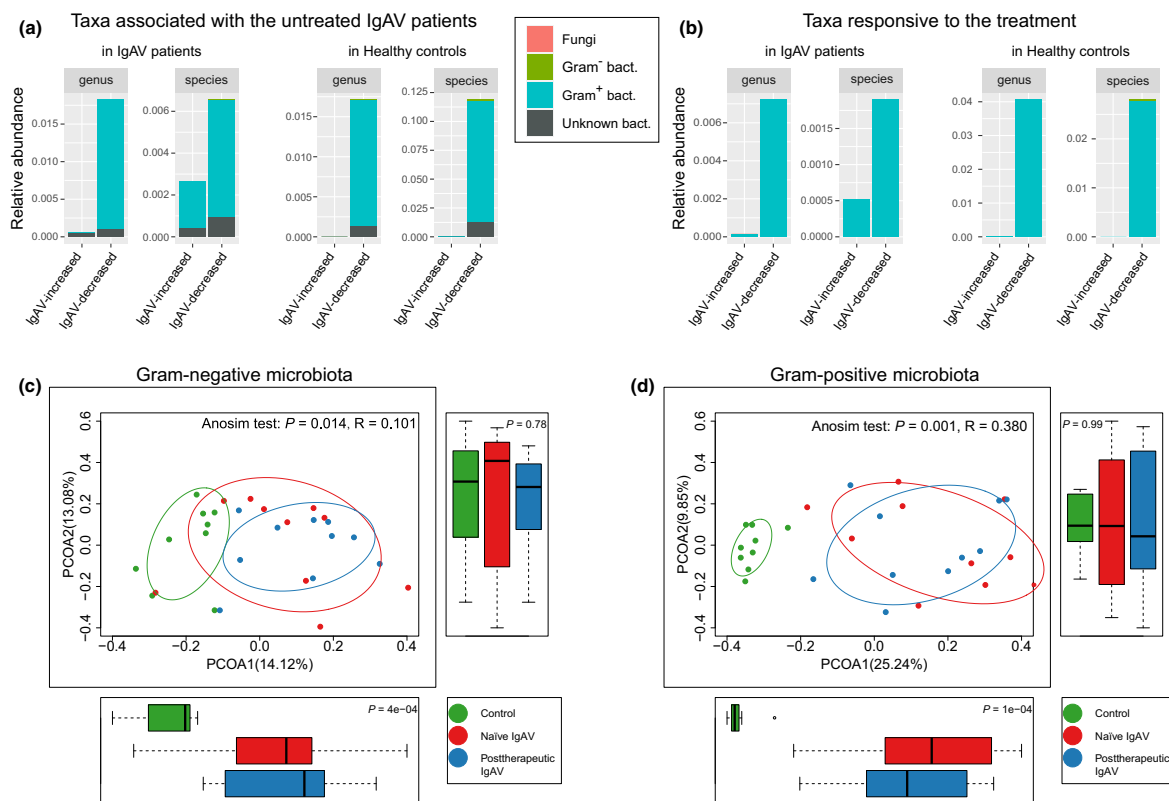
Figure 4. Global differences between gram-positive bacteria and gram-negative bacteria. **(a, b)** The combined relative abundance of genera and species that were associated with the untreated IgAV patients **(a)** and that responded to the treatment **(b)** in healthy controls and untreated IgAV patients, showing predominance of gram-negative bacteria in combined relative abundance. Gram- bact., gram-negative bacteria; gram⁺ bact., gram-positive bacteria; unknown bact., bacterial taxa with an unknown gram stain trait. **(c, d)** The shift of all gut gram-negative bacteria or all gut gram-positive bacteria in the IgAV patients based on the Bray–Curtis distance metric of species abundance.

we stratified the KOs into 5 categories: gram-positive leading (all top 7 identified contributors were gram-positive; among the top 7 identified contributors, there was only one gram-negative species and its ranking was no higher than the fourth), gram-positive majority (among top 7 identified contributors, there was only one gram-negative species and its ranking was the second or third), gram-negative leading (as gram-positive leading except gram-negative species were dominant), gram-negative majority (as gram-positive majority except gram-negative species were dominant) and comparable (the remaining cases). In both IgAV-increased and IgAV-decreased KOs, we observed an inverse connection between contribution by gram-positive bacteria and the relative abundance ranking of KO (in healthy controls) (Figure 5e). Specifically, the top 12 most abundant IgAV-increased KOs were exclusively gram-negative leading, whereas among the remaining 9 IgAV-increased KOs, there were 3 comparable, 1 gram-positive majority and 1 gram-positive leading, respectively. For the IgAV-decreased KOs, there were only 4 gram-positive leading and no gram-positive majority among the 30 most abundant ones (1 among the top 20), but the gram-positive leading and gram-positive majority features were dominant among the remaining KOs. The findings appeared to reflect two underlying phenomena: Many of the most abundant microbes in the human gut are gram-negative bacteria (e.g. species of *Bacteroides*, *Parabacteroides* and *Alistipes*), which may produce some functional alterations under IgAV conditions even though the relative abundances of themselves do not change; however, as gram-positive bacteria are the major drivers of IgAV-related taxonomic shift, they inevitably lead to substantial functional influences, especially in those genes that are not swayed by the dominant gram-negative taxa.

DISCUSSION

Currently, there are several problems in therapeutics of IgAV, including diagnosis for patients with skin rash only and a substantial rate of relapse. Paediatric patients with the single sign of non-thrombocytopenic purpura, typically on the legs and buttocks, may be diagnosed with IgAV,[22,23] but this manifestation is similar to that of other diseases of vasculitis, such as isolated cutaneous leukocytoclastic vasculitis. These rash-only IgAV patients typically have an excellent prognosis, but a few may progress into more severe forms such as IgA nephropathy. In other words, the diagnostic ambiguity has the possibility of serious consequences. In addition, multiple longitudinal studies revealed substantial recurrence rates (14.4–66%),[5–9] although a lower range (i.e. 2.7%, 4.8% and 16.4%) was reported by other studies.[23–25] The discrepancy appears to correlate with the two different approaches of calculation such that one is based on follow-up of a group of patients[5–9] and the other on recurring diagnosis in a retrospective analysis.[23–25] We argue that the recurrence rates based on longitudinal monitoring are more reliable. The lower rates derived from the alternative approach may be explained by possibilities that recurrent patients might forgo treatment or seek medical attention elsewhere. In the light of the diagnostic and treatment problems, a unique gut microbiota signature has a significant clinical potential for managing the disease.

Immunoglobulin A vasculitis is characterised by microbial abnormalities including preceding infections in the upper respiratory tract[2,3,9] and microbiota shifts in the oral cavity and gut.[13,14] In this study, metagenomic analyses were performed to characterise the gut microbiome shifts that were associated with IgAV and that responded to the multi-drug treatment. A crucial finding from the current study is the predominance of gram-positive bacteria within the gut microbiota that are responsive to IgAV and a therapy. This is congruent with the result that Firmicutes, a major enteric phylum where most members are gram-positive, exhibited over 50% reduction in the IgAV patients (Firmicutes was also negatively associated with IgAV in the 16S rRNA gene-based study[14]). On their surface, gram-positive bacteria are characterised by a lack of lipopolysaccharide, fully exposed peptidoglycan layer and presence of lipoteichoic acids in the cell wall. These building blocks of

envelope can all affect host immunity.[26–28] Studies are needed to investigate whether and how these components (or lack of which) in faecal gram-positive bacteria are implicated in the pathogenesis of IgAV. Among various taxa, *Weissella* exhibited the most sensitive treatment response (HC-pthIgAV FDR = 1). It is a genus of lactic acid bacteria that is closely related to *Lactobacillus* and has been used in food fermentation.[29] In addition, whereas most treatment-responsive taxa at genus or species level had a relative abundance below 0.01% in the healthy controls, *Faecalibacterium* and *Bifidobacterium pseudocatenulatum* both accounted for over 1% of gut microbiota in the healthy controls. *Faecalibacterium*, in which *F. prausnitzii* is the only known species, produces short-chain fatty acids[30] and anti-inflammatory peptides[31] through fermentation of dietary fibre, thereby modulating host inflammation. Interestingly, the genus was also diminished in infants at risk of asthma,[32] another immune-mediated inflammatory disease commonly found in children. *Bifidobacterium pseudocatenulatum*, along with over a dozen members within the same parent genus, is a common probiotic species that confers multiple health benefits including metabolic abilities and suppression of pathobionts.[33] Of note, all three genera are used as dietary supplements, raising the possibility of probiotic-assisted therapeutics for IgAV.

One major limitation of this study is the modest sample size, which is in part due to the low prevalence of IgAV along with the time frame needed to monitor recurrence. However, there is considerable concordance between the microbial traits of IgAV generated in this metagenomics-based study and those derived from 16S rRNA gene sequencing[14]; these comprise a reduction of microbial diversity as well as multiple taxonomic shifts, including the phylum of Firmicutes, order of Clostridiales (along with its parent class of Clostridia), genera of *Roseburia* and *Lachnospira* and species of *Eubacterium eligens* and *Eubacterium rectale* (both identified as genera in the 16S study). In addition, the 16S rRNA gene sequencing-based evidence revealed that the family of Lachnospiraceae, genus of *Ruminococcus* or genus of *Streptococcus* showed a decreased relative abundance in the IgAV patients,[14] coincided with our results that there were 7 genera or 6 species within each corresponding family or genus with diminished proportions in
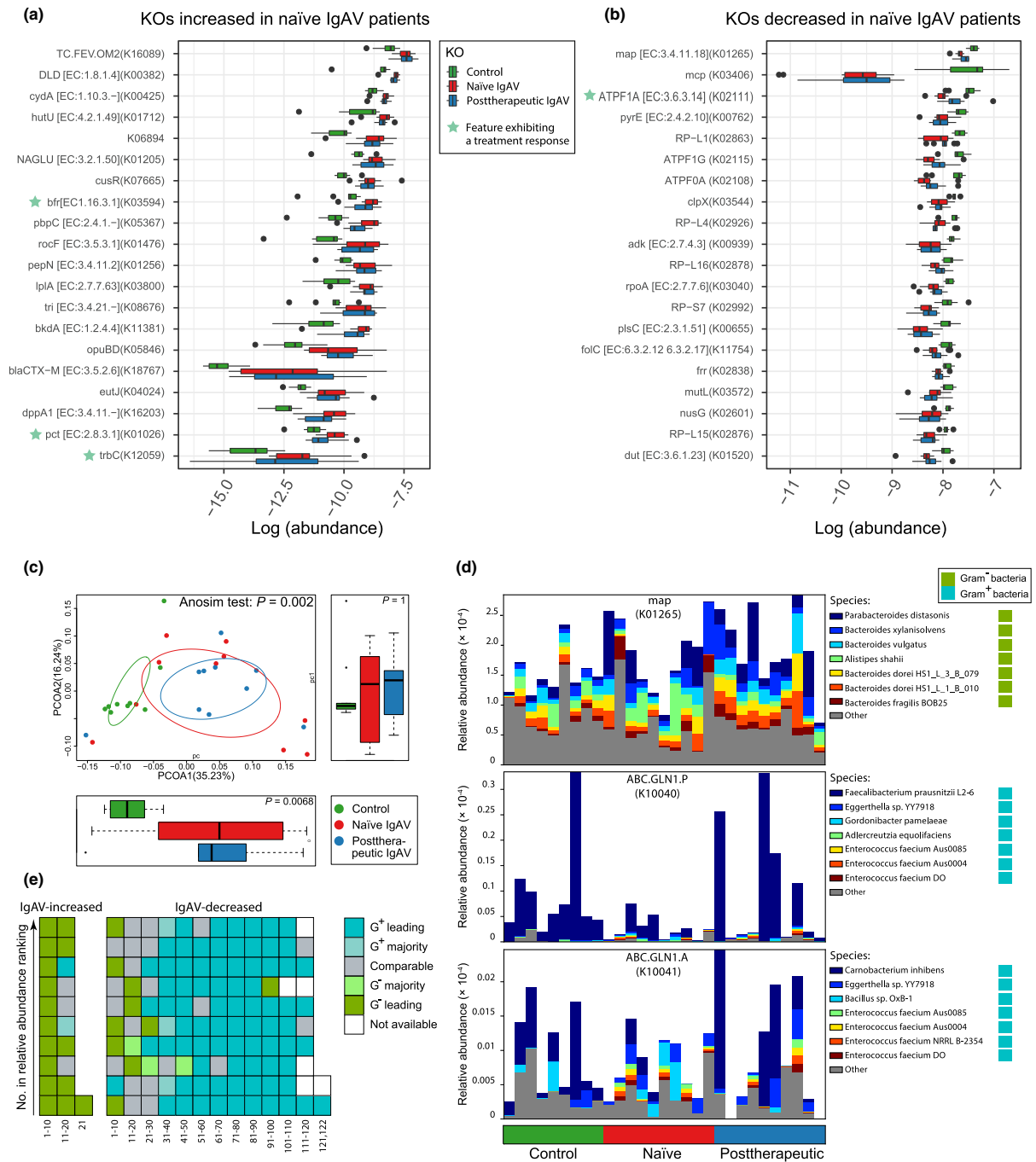
Figure 5. The functional shift of gut microbiota in the IgAV patients. **(a, b)** The relative abundance of top 20 KOs that were over-represented **(a)** and under-represented **(b)** in the untreated IgAV patients. **(c)** PCoA based on the Bray–Curtis distance metric of KO abundance. **(d)** The major microbes contributing to K01265 (map), K10040 (ABC.GLN1.P) and K10041 (ABC.GLN1.A); K01265 was the most abundant KO marker of IgAV, whereas K10040 and K10041 belong to a putative glutamine transport system and were among the 10 KOs responding to the SACV treatment. **(e)** Contribution of gram-positive and gram-negative bacteria to the IgAV-associated KOs. The KOs were arranged according to their relative abundance ranking in healthy controls and were colour-coded according to the contributions by gram-positive and gram-negative bacteria. Gram-positive leading, all 7 top identified contributors were gram-positive or only one top contributor was gram-negative and its ranking was no higher than the fourth; gram-positive majority, all except one top identified contributors were gram-positive, and the ranking of the only gram-negative species was the second or third; gram-negative leading/majority, as gram-positive leading/majority except most or all top contributors were gram-negative; not available, contributing species were identified in less than four participants; comparable, all remaining cases.

the disease group but none in the control group (Supplementary table 2); congruence in the opposite direction was also observed for the genera of *Enterococcus* (along with its parent family Enterococcaceae) in the 16S study[14] and 2 species of this genus in our results (Supplementary table 2). Hence, there was considerable conservation in the gut microbiota markers of IgAV between the two studies of different methodologies.

There are two caveats related to the SACV treatment. One is that the condition-specific agents (varied between the patients) within the regimen could be a compounding factor for gut microbiota analysis, although the number of patients in this study is too low to investigate this matter. Another issue is ascribed to the inclusion of antimicrobial agents or other drugs in the regimen that may suppress various bacteria to skew the taxonomic changes. It is, however, noteworthy that decreased abundance was the major taxonomic shift in the IgAV patients and that, among the taxa exhibiting such changes, barely any decreased further after the treatment and a subset of them instead displayed a rise in relative abundance (i.e. treatment responsiveness). In other words, the taxonomic changes after the treatment were the opposite of the anticipated antimicrobial effect. Moreover, the post-therapeutic microbiota shifts, like those associated with the disease, were dominant with alterations of gram-positive bacteria, which would not be expected if the antimicrobial effects stemming from any agent in the regimen played a major role.[34–38] As such, we conclude that post-therapeutic microbiota shifts indicate microbial responses to the recovery.

CONCLUSIONS

We show that gram-positive bacteria are dominant in the IgAV-associated gut microbiome shifts, which provides insights for better understanding the pathogenesis of IgAV and offers an experimental basis for the development of gram-positive diagnostic panels and probiotic-based therapeutics.

METHODS

Recruitment of the study participants

Between May 2014 and March 2015, 96 children who were between 6 and 14 years of age were diagnosed with IgAV

in the Department of Pediatrics, the First Affiliated Hospital of Kunming Medical University. Among them, 33 individuals were recruited for the clinical study based on the following inclusion criteria (Supplementary table 1): under 14 years of age; the presence of two or more additional IgAV-relevant clinical signs (e.g. gastrointestinal, joint and kidney symptoms) besides cutaneous lesion; no prior treatment for IgAV; no administration of antibiotics within latest 2 months before entry; and participated in a 5-year follow-up to monitor recurrence. All the IgAV patients received treatment that lasted between 5 and 21 days. For metagenomic analyses, 10 individuals were randomly selected from the 33 IgAV patients along with 9 healthy controls with comparable ages, body weights and gender ratios.

Treatment using multi-drug therapy and follow-up visits

Based on the general therapeutic guideline described in Pediatrics (in Chinese; People's Medical Publishing House, Beijing)[39] and our clinical experience, we developed a personalised SACV multi-drug therapy for IgAV. The therapy (Supplementary table 1) comprised the three base drugs and several condition-specific drugs; the base drugs were safflower yellow injection (50 mg daily; intravenous), amoxicillin/clavulanate potassium (1 mg per kg per day; intravenous; for *Mycoplasma pneumoniae*-negative participants), or azithromycin (10 mg per kg per day in the first 3 days of a week for 3 weeks; oral; for participants with a positive penicillin skin test or *Mycoplasma pneumoniae* infection) and vitamin C (100 mg per kg per day; intravenous). For individuals with abdominal pain, prednisone (1 mg per kg per day; oral) and omeprazole (0.6 mg per kg per day; intravenous) were provided; for individuals with hypercoagulation, heparin sodium (125 IU per kg per day; intravenous) was administered; for individuals with a viral infection, ganciclovir (5.0 mg kg^{-1}, twice daily; intravenous) was used.

Standard laboratory tests were performed for blood, urine and faecal measures including IgA, white blood count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet (Plt), fibrinogen degradation product (FDP), D-dimer (D-D) and occult gastrointestinal bleeding, whereby the treatment outcome was monitored. The treatment was withdrawn once a patient was cured, defined by resolution of IgAV-related symptoms including cutaneous rash, joint swelling and pain, abdominal pain and haematochezia. Of note, upon discharge some individuals might manifest mild nephropathy; in such cases, they were prescribed with oral administration of prednisone (1 mg per kg per day) at home until proteinuria turned negative, as determined by review urine tests. After being discharged, patients received follow-up phone calls every 6 months for 5 years to monitor relapse.

Sample collection, DNA extraction and metagenomic sequencing

For metagenomic analyses, fresh stool samples were collected and immediately stored at −80°C until laboratory

analyses. DNA was extracted from the samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity and size of the extracted DNA were confirmed with electrophoresis on 1% agarose gel containing 0.5 μg per mL ethidium bromide.

Following the Illumina TruSeq DNA Sample Prep v2 Guide (Illumina, Inc.; San Diego, CA, USA), we constructed the DNA paired-end libraries with an insert size of 500 base pairs (bp) for the 29 faecal samples (i.e. 10 from the IgAV patients before the SACV treatment, 10 from the patients after the treatment and 9 from the healthy controls). The quality of all libraries was evaluated using an Agilent bioanalyser (Agilent Technologies, Wokingham, UK) and the DNA LabChip 1000 kit. All samples were subject to 150-bp paired-end sequencing on an Illumina Hiseq 4000 platform (Illumina, Inc.; San Diego, CA, USA).

Raw reads were filtered to trim nucleotides from the 3′ end using a quality threshold of 30 and remove adaptor contamination and low-quality reads (e.g. reads containing more than 50% nucleotides with quality below 30, reads short than 70 bp or reads mapped to the human genome based on alignment with SOAPaligner 2.2120). An average of 95.82% high-quality reads was obtained from all samples.

De novo assembly and construction of the gene catalog

To construct the gut gene catalog, SOAPdenovo 21(version 2.04) was used to assemble the high-quality reads from each sample into contigs. MetaGeneMark 22 (version 3.26) was used to predict open reading frames (ORFs) in contigs. To obtain a non-redundant gene set, a pairwise comparison of predicted ORFs (filtered with a length of 100 bp) was performed using CD-HIT 23 (version 4.5.7) at 95% identity and 90% coverage. The final non-redundant gene catalog contained 1 127 116 microbial genes, which had an average length of 831 bp. Functional annotations were carried out by BLASTP search against the KEGG database 24 (e-value ≤ 1e-5 and high-scoring segment pair scoring > 60). For each functional feature (e.g. KO in the KEGG database), we estimated its abundance by aggregating the relative abundance of all affiliating genes.

Taxonomic and gene analyses

SOAPalign2.21 was used to align clean reads to the microbial reference genomes downloaded from the National Center for Biological Information (NCBI, http://www.ncbi.nlm.nih.gov) (74 201 genomes, including 65 770 bacterial, 898 archaeal, 1508 fungal and 6025 viral genomes). The taxonomic and gene relative abundance profiles were generated following the procedure described by Qin *et al*.[40] Reads aligned to multiple taxa were allocated proportionally to read counts uniquely mapped to these taxonomical units (normalised by genome length). The shotgun reads were annotated with Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology group (KO) assignments. Functional profiling was performed using HUMAnN2 version 0.9.4 in UniRef90 mode (http://huttenhower.sph.harvard.edu/humann2).[21]

A differential taxonomic or functional feature between the naïve IgAV patients and healthy controls was established by a false discovery rate (FDR) < 0.05. In addition, a differential feature was considered recovered if it met the following two criteria: (i) its mean relative abundances in post-therapeutic IgAV patients and HCs were not significantly different (FDR > 0.1); (ii) its mean relative abundance in naïve IgAV patients was numerically lower (or higher) than that in both the HCs and post-therapeutic IgAV patients (there was typically no statistical significance between naïve IgAV patients and post-therapeutic IgAV patients). Gram-positive bacteria and gram-negative bacteria were determined based on their envelope ultrastructure and/or phylogenetic relationship instead of the actual staining results. As such, the gram-variable bacteria (having a gram-positive cell wall structure but appearing negative in standard gram staining), such as *Butyrivibrio*, *Paenibacillus* and *Cellulosilyticum*, were classified here as gram-positive.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Jia Cao: Conceptualization; Data curation; Formal analysis; Writing-original draft. **Chunyan Wu:** Data curation; Formal analysis; Software. **Kunhua Wang:** Supervision; Visualization. **Hongwei Hu:** Methodology; Resources. **Jiang Duan:** Supervision. **Bo Zhao:** Supervision. **Jingjing Xiong:** Methodology. **Mei Liu:** Supervision. **Jingjing Cui:** Visualization. **Xiaofei Ji:** Methodology. **Tingting Zhang:** Methodology. **Nan Qin:** Software; Supervision. **Yongkun Huang:** Funding acquisition; Writing-review & editing. **Qian Xu:** Conceptualization; Software; Writing-review & editing.

ETHICS APPROVAL

This study was approved by the Ethics Committee of Kunming Medical University. Signed informed consent was obtained from the guardians of all participants upon their enrolment. All procedures were conducted in compliance with the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The sequencing dataset was deposited to the NCBI Sequence Read Archive under BioProject accession number PRJNA658422.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.