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Alterations of the gut microbiome and plasma proteome in Chinese patients with adolescent idiopathic scoliosis

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ABSTRACT

The etiology of adolescent idiopathic scoliosis (AIS), the most common rotational deformity of the spine, is still unclear. Emerging evidence suggests that gut microbiota dysbiosis influences musculoskeletal diseases such as arthritis and osteoporosis. However, the alterations of the fecal microbiome in AIS remain unknown. Thus, the current study was conducted to explore the gut microbiota compositions of Chinese AIS patients. Microbiota communities in the feces of 51 AIS patients and 34 age- and sex-matched healthy individuals were investigated using 16S rRNA sequencing. Meanwhile, the changes in the plasma proteome were detected using tandem mass tag (TMT) labeling coupled with liquid chromatography-mass spectrometry (LC-MS). The relationship between gut microbiota and AIS clinical characteristics as well as the correlation between gut microbiota and the changes in plasma proteins were analyzed. The structure of the gut microbiota differed between the AIS and healthy groups, however, the richness was similar. The genera *Prevotella*, *Gelria*, and *Desulfovibrio* were enriched in the feces of AIS patients. In contrast, the abundance of *Parasutterella*, *Tyzzerella*, and *Phascolarctobacterium* was decreased in the AIS group. More remarkably, a positive correlation between the abundance of the fecal genera *Prevotella* and the Cobb angles of the AIS patients was observed. Moreover, the major differential plasma proteins related to AIS were Fibronectin 1 (FN1), voltage-dependent anion channel 1 (VDAC1), Ras homolog family member A (RHOA), and AHNAK nucleoprotein (AHNAK). Additionally, the positive correlations between fecal *Prevotella* and the expression of host plasma FN1 as well as the negative relationships between fecal *Prevotella* and the expression of host VDAC1 and AHNAK were confirmed. Elucidating these differences in the gut microbiota will provide a foundation to improve our understanding of the pathogenesis of AIS and to support potential therapeutic options based on modifying the gut microbiota.

1. Introduction

Adolescent idiopathic scoliosis (AIS) is the most common rotational deformity of the spine; it generally affects approximately 1–4% of adolescents worldwide [1]. Severe AIS has a poor prognosis, including respiratory failure, cardiovascular risk, and mortality [2]. The etiology of AIS remains unknown. Nonetheless, several hypotheses have been proposed to explain its pathogenesis, including genetic factors, central nervous system issues, skeletal spinal growth and bone metabolism impairment, metabolic pathways, biomechanics, and other factors [3]. Both the clinical symptoms and pathological changes of AIS indicate that the metabolic dysfunction and biochemical factors are involved in its pathogenesis prior to the aberrant growth of the spine [4–6]. Recently, evidence has accumulated that the gut microbiota is an important environmental factor that contributes to skeletal growth and bone formation by regulating metabolic and biochemical pathways [7,8]. The gut microbiota is currently of considerable interest as a potential factor in musculoskeletal diseases [9–11]. Investigating the profiles of gut microbiota in the progression of AIS would allow us to identify patients with rapid progressive AIS and improve our understanding of the pathogenesis of AIS.

Emerging data suggest that the gut microbiota, as an important

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regulator of nutrition and metabolism, is associated with many musculoskeletal diseases. Gut microbial communities provide nutritional support to their host by synthesizing essential amino acids and vitamins to maintain the bone health of the host. In addition, the gut microbiota has an important metabolic function, as exemplified by its unique enzymatic capability to degrade complex compositions derived from the diet, which is also a necessary condition for maintaining bone system function [12]. However, gut microbiota dysbiosis in different musculoskeletal diseases is diverse, and thus, there has been growing interest in the investigation of the bacterial profiles of gut microbiota in AIS patients. Recently, 16S rRNA sequencing has emerged as a typical approach for describing microbiota compositions. However, because the alteration of gut microbiota compositions does not necessarily indicate changes in host biological functions, the combination of mass spectrometry (MS)-based proteome analysis of host plasma and 16S rRNA sequencing of gut microbiota, not only provides more host and microbiota precise functional information, but also contributes to deciphering host-microbe interactions in complex intestinal eco-systems.

In this study, we focused on whether gut microbiota could play roles in the progression and the detailed mechanisms of AIS. The association between changes in the gut microbiota and alteration of the host plasma was also investigated. These results may provide a foundation to improve our understanding of the pathogenesis of AIS and to support potential therapeutic options for modifying the gut microbiota.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. All the participants provided a written informed consent to participate in this study.

A total of 51 AIS patients and 34 age- and sex-matched healthy individuals were recruited from August 2017 to March 2018. The inclusion criteria for the patients were: a definitive diagnosis of AIS and an age of 12–16 years. The exclusion criteria were as follows: other types of scoliosis caused by congenital, or postural, neuromuscular factors; acute infectious disease within one month before recruitment; severe allergies; gastrointestinal disease; and abnormal liver and kidney function (recruitment flowchart provided in Fig. 1). Body height, weight, and body mass index (BMI) were calculated for all the subjects. Radiographic data were collected for each AIS patient.

2.2. Fecal sample collection and DNA extraction

Fecal samples were obtained from all recruited subjects after their breakfasts for 16S rRNA sequencing. The individuals were not given any antibiotic treatment within one month before fecal sample collection. Each fecal sample was briefly stored in a −20 °C freezer before transiting to the laboratory within 48 h. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA integrity and the sizes of the genomic DNA in each fecal sample were assessed using 1% agarose gel electrophoresis. The DNA was stored at −80 °C prior to sequencing.

2.3. 16S rRNA gene amplicon sequencing

The composition of the gut microbiome in the fecal samples was determined by 16S rRNA sequencing. In brief, the V4–V5 regions of the 16S rRNA gene were amplified and sequenced on the MiSeq platform (Illumina, San Diego, CA). Quality control, merging of pair ends, operational taxonomic unit (OTU) clustering, and taxonomic assignation were performed as described [13]. De novo OTU clustering was carried out across all reads using USEARCH software, and reads with a 97% identity threshold were grouped.

2.4. Proteomics profiling of the human plasma samples

Whole blood samples were drawn before fecal sample collection from 4 AIS patients and 4 matched healthy individuals using vacutainer tubes. The EDTA-anticoagulated plasma was immediately frozen at −80 °C until the proteomics analysis. The Multiple Affinity Removal Spin Cartridge System (Agilent Technologies, Santa Clara, CA) was used to remove > 98% of the most abundant proteins (such as albumin, IgM, IgG, IgA, and haptoglobin) according to the manufacturer's instructions. Protein digestion was performed using trypsin, and the resulting peptide mixture was labeled using the 10-plex tandem mass tag (TMT) reagent. TMT-labeled peptides were fractionated by SCX chromatography using the AKTA Purifier System (GE Healthcare, USA). Finally, the collected fractions (approximately 14 fractions) were combined into 10 pools and desalted. Each fraction was concentrated by vacuum centrifugation and stored at −80 °C until liquid chromatography-mass spectrometry (LC-MS) analysis. All LC-MS experiments were performed on a Q Exactive MS as previously described [14]. The LC-MS data of the selected differentially expressed proteins were retrieved in batches from the UniProtKB database in FASTA format.

2.5. Statistical analysis

SPSS (ver. 24.0) and R software (ver. 3.1.0) were used for the statistical analysis. Rarefied α diversity and β diversity indexes (Bray-Curtis) were calculated in Quantitative Insights Into Microbial Ecology (QIIME). Comparisons between the groups were performed with Student's *t*-tests or Wilcoxon signed-rank test in R, depending on whether the variable was normally distributed. The correlations between the fecal microbiota species and differential host plasma proteins in the AIS patients were calculated using Spearman's rank correlation analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The basic characteristics of the AIS and healthy groups

The demographic characteristics of the AIS group and the healthy group are summarized in Table 1. No differences in age, gender, and BMI were detected between the two groups. The AIS patients had an average Cobb angle of 23.87 ± 9.99° and an average Risser stage of 2.27 ± 1.26. The average blood-calcium levels of the AIS patients were 2.40 ± 0.05 mmol/L, blood-phosphate levels were 1.41 ± 0.19 mmol/L, vitamin D levels were 57.24 ± 21.08 mmol/L, alkaline phosphatase levels were 173.58 ± 69.51 U/L, and parathyroid hormone levels were 3.84 ± 1.05 pmol/L. Additionally, the AIS patients did not receive any surgery or rehabilitation therapy.

3.2. Alpha and beta diversity between the AIS and healthy groups

Regarding the gut microbiota, although the AIS individuals had slightly lower OTU counts than the controls (Fig. 2a), the mean community diversity indexes (i.e., α-diversity, including the Chao, Shannon, and Simpson indexes and based on OTU levels) of the AIS group were similar to those of the healthy group (Fig. 2b–d). Significant differences were observed in β-diversity based on the weighted UniFrac ($R = 0.115$, $P = 0.022$) but not the unweighted UniFrac ($R = 0.042$, $P = 0.174$) between the AIS and healthy groups (Fig. 2e, f). These results indicate that the gut microbial structure, but not the richness and diversity, in the AIS group was obviously different from that of the healthy controls in the presence of OTU.

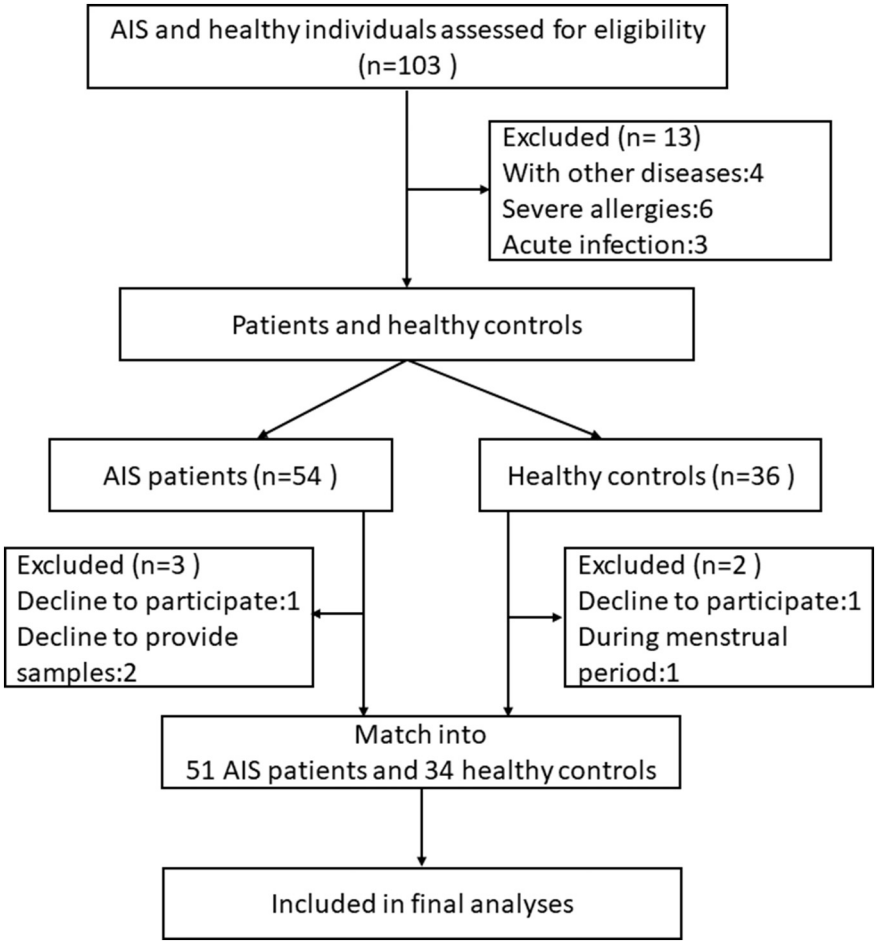

Fig. 1. Flowchart illustrating the recruitment of AIS patients and healthy volunteers based on the exclusion and inclusion criteria.

Table 1
Clinical characteristics of AIS and healthy subjects.

Characteristics	AIS group (n = 51)	Healthy group (n = 34)	P value
Age (years)	13.25 ± 1.63	13.26 ± 1.75	0.936
Female (n, %)	41, 80.39%	26, 76.47%	0.669
BMI (kg/m^2)	19.13 ± 3.55	18.76 ± 2.55	0.393
Cobb angles (°)	23.87 ± 9.99	–	–
Risser Stage	2.27 ± 1.26	–	–
Blood calcium (mmol/L)	2.40 ± 0.05	–	–
Blood phosphate (mmol/L)	1.41 ± 0.19	–	–
Blood vitamin D (mmol/L)	57.24 ± 21.08	–	–
Alkaline phosphatase (U/L)	173.58 ± 69.51	–	–
Parathyroid hormone (pmol/L)	3.84 ± 1.05		

3.3. Alterations in taxa between the AIS and healthy groups

The microbiota of the AIS and healthy groups were compared by utilizing the linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify the specific OTUs (Fig. 3a). Our results suggested a remarkable difference in the gut microbiota between these two groups. We particularly considered differences in the taxa at the genus and species levels. At the genus level, the relative abundances of the genera *Parasutterella*, *Tyzzerella*, and *Phascolarctobacterium* were higher in the healthy group than in the AIS group, whereas the relative abundances of the genera *Gelria* and *Desulfovibrio* were higher in the AIS patients than in the healthy controls (Fig. 3b). Additionally, at the species level, the relative abundances of *Bacteroides fragilis*, [*Clostridium*] *lactatifermentans*, and [*Clostridium*] *scindens* were higher in the healthy controls than in the AIS patients, while the relative abundances of *uncultured rumen bacterium* and *bacterium YE57* were higher in the AIS patients than in the healthy controls (Fig. 3c). Krona, an interactive metagenomic visualization tool, was used to visually model the difference in microbial composition between the two groups (Fig. 3d, e). More remarkably, the average abundance of the genus *Prevotella* was approximately 13% in AIS patients, compared to only 1% in healthy volunteers ($P < 0.05$) according to Krona analysis. Furthermore, we also confirmed the positive correlation between the abundance of fecal *Prevotella* and Cobb angles in AIS patients ($R = 0.772$, $P < 0.01$) (Fig. 3f). Together, these data reveal microbial changes in the gut microbiome in AIS patients at the genus and species levels and indicate that *Prevotella* may be a potential biomarker linking gut microbiota and the severity of AIS.

3.4. Plasma proteome in the AIS and healthy groups

Forty-two proteins were differentially expressed in a total of 652 identified plasma proteins using proteomic techniques. Among them, 17 proteins that were differentially expressed between the AIS and healthy groups were confirmed (fold change > 1.5, $P < 0.05$) (Fig. 4a, b). The major molecular functional classes were cadherin binding involved in cell-cell adhesion, identical protein binding, poly(A) RNA binding, and serine-type endopeptidase activity (Fig. 4c). According to the cellular component, the largest proportion of proteins was distributed in extracellular exosome, followed by cytosol and extracellular space (Fig. 4d). The major differential proteins related to the AIS were Fibronectin 1 (FN1), voltage-dependent anion channel 1 (VDAC1), Ras homolog family member A (RHOA), and AHNAK nucleoprotein

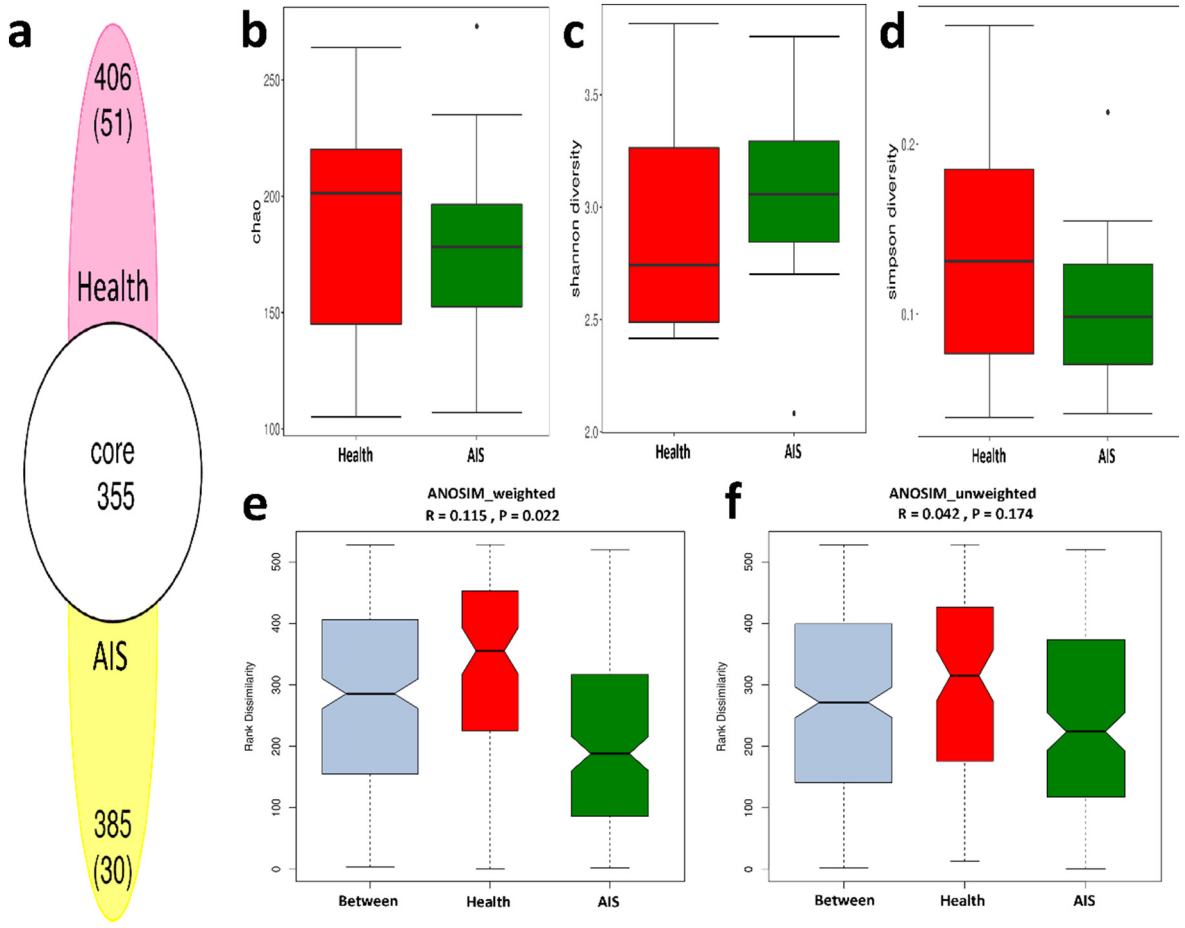

Fig. 2. The OTU counts, α-diversity and β-diversity indices of the gut microbiome in AIS patients and healthy subjects. (a) OTU counts in AIS and healthy groups separately. (b–d) Box plots indicate differences in the gut microbiome α-diversity between two groups according to Chao index, Shannon index and Simpson index. Each box plot represents the median, interquartile range, minimum, and maximum values. (e,f) Weighted and unweighted ANOSIMs based on the distance matrix of UniFrac dissimilarity of the gut microbial communities in AIS and healthy groups. ANOSIM R values show the community variation between two groups, and P values are indicated.

(AHNAK).

To gain more information about the nature of the 42 differentially expressed plasma proteins and the specific processes that are significantly modulated in the AIS pathogenesis, the proteomic data were further characterized using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway tool. The major biological pathways related to AIS were cell-cell adhesion, the Wnt signaling pathway, proteolysis, and Fc-epsilon receptor signaling. These data demonstrate that the plasma protein profiles of AIS patients are different from those of healthy individuals and that FN1, VDAC1, RHOA, and AHNAK may constitute potential candidates linking plasma proteins and AIS.

3.5. Associations of the gut microbial species with the host plasma proteome

Correlations between gut microbial species and differential host plasma proteins were observed in the AIS patients. For example, the abundance of fecal *Prevotella* was positively correlated with the expression of host plasma FN1; however, it was slightly negatively correlated with the expression of host VDAC1 and AHNAK ($P < 0.05$) (Fig. 5a–c). We also discovered correlations between the abundance of fecal *Prevotella* and differential host plasma proteins (Supplementary Fig. 1). These data suggest that the abundance of fecal *Prevotella* may modulate the levels of circulating plasma proteins associated with the development of AIS.

Taken together, these findings show an association between the gut microbiota and the progression of AIS. More remarkably, a positive correlation between the abundance of fecal *Prevotella* and the Cobb angles of AIS patients was observed. Moreover, the plasma protein profiles of the AIS patients were analyzed. FN1, VDAC1, RHOA, and AHNAK, as the major differential proteins, may contribute to the development of AIS. Additionally, we discovered the correlations between fecal *Prevotella* and the expression levels of host plasma FN1, VDAC1 and AHNAK proteins.

4. Discussion

Here, we provide the first evidence that the gut microbiota can contribute to the development of AIS, which may act on changes in host plasma proteins. The strength of our study relies on its relatively comprehensive studies of gut microbial communities and host plasma proteomics associated with AIS, especially the clinical characteristics of AIS.

In this study, we found that the richness of the gut microbiota (α-diversity) in AIS patients was similar to that of healthy controls. However, the structure of the gut microbiota (β-diversity) in the AIS group was different from that of the healthy group. Compared to previous studies investigating the gut microbiota in other musculoskeletal diseases, Zhang et al. reported that there was no difference in the gut microbial diversity and richness between the rheumatoid arthritis patients and healthy controls [15]. In the case of ankylosing spondylitis, the α-diversity indexes were obviously lower in patients with AIS than in the healthy volunteers [16]. Furthermore, although metabolic

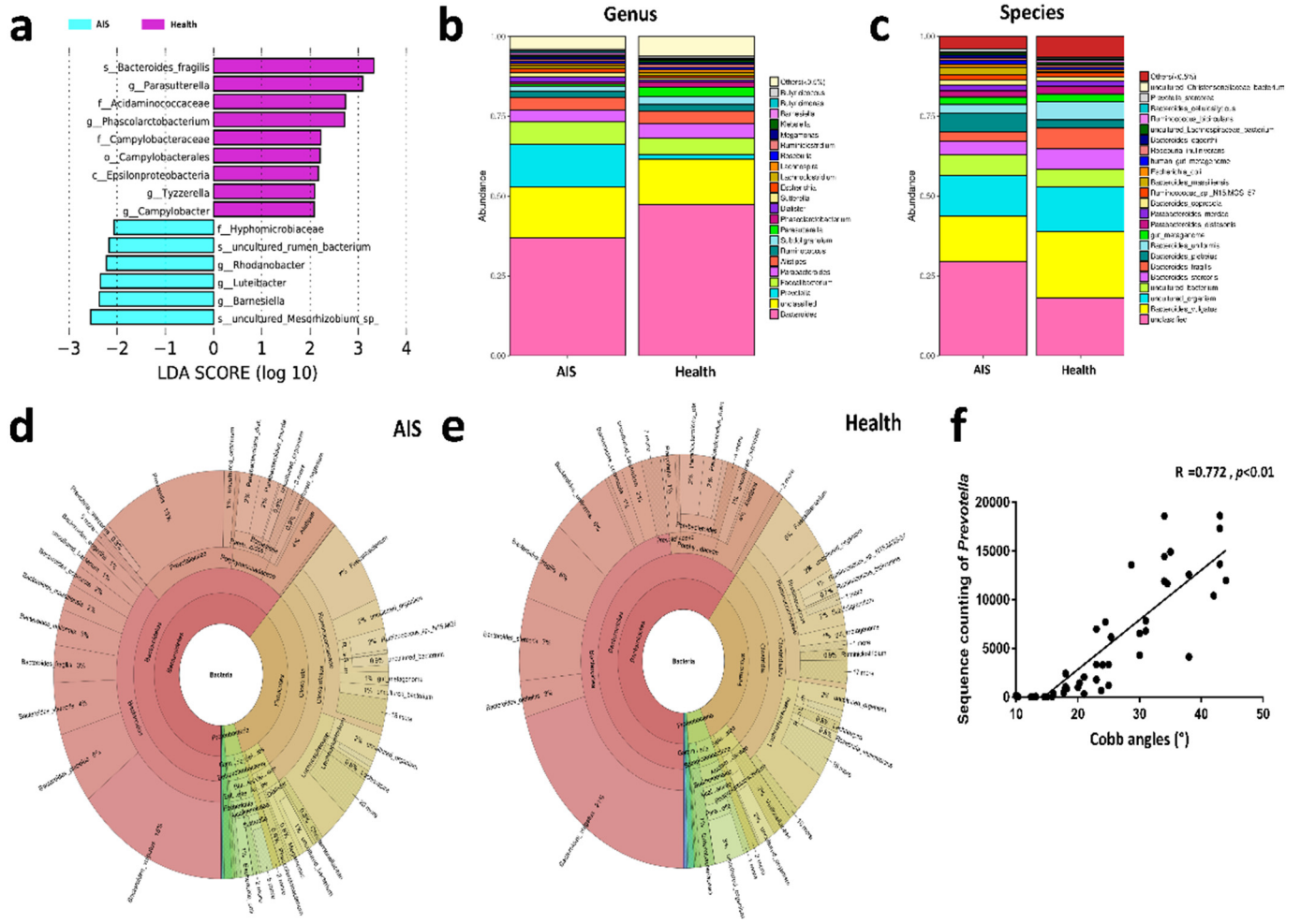

Fig. 3. Taxonomic differences of gut microbiota in AIS and healthy groups. (a) LEfSe analysis revealed significant differences in gut microbiota between the AIS (negative score) and healthy groups (positive score). The LDA scores (log10) > 2 and P < 0.05 are listed. (b, c) The phylogenetic abundance of gut microbiota that were changed in the AIS patients comparing to health controls at genus and species levels. (d, e) Krona software showed the difference of microbial composition between two groups. (f) The positive correlation between the abundance of fecal genera *Prevotella* and Cobb angles of AIS patients.

dysfunction and biochemical factors participate in the pathogenesis of AIS, they are mainly involved in the metabolism of calcium, phosphate, vitamin D, and various hormones, rather than in glucose or amino acid metabolism. Thus, there is no obvious difference in the diversity and richness of the gut microbiota in AIS compared to other metabolic diseases such as diabetes [17].

In the current study, we found that AIS patients may have a different gut microbiota profile than controls, even at the phylum level. In particular, we showed for the first time that the genus *Prevotella* exhibited a considerable increase in individuals with AIS, and was correlated with Cobb angles, the most important clinical characteristic of AIS. Previously, the genus *Prevotella* was only considered related to plant-rich diets; however, it has recently been associated with chronic inflammatory conditions [18]. Wen et al. reported an increase in the abundance of fecal *Prevotella* in ankylosing spondylitis patients [16]. A similar result was observed in the HLA (human leukocyte antigen)-B27 transgenic rats as well [19]. They hypothesized that fecal *Prevotella* might stimulate an immune reaction such as the activation of transcription factor NF-κB, which might then target joint tissues. In addition, the presence of *Prevotella* is strongly correlated with rheumatoid arthritis through changers in the host immune system and biochemical factors [20]. Thus, we speculated that chronic inflammation may be involved in the process of AIS via the change of in fecal *Prevotella*, however, this hypothesis needs to be tested.

To explore the potential mechanisms of gut microbiota in AIS patients, plasma proteomics was performed in this study. Plasma FN1, VDAC1, and AHNAK proteins were different between the AIS patients and healthy individuals. The *FN1* gene encodes fibronectin, which not only promotes the assembly of collagens, fibrillin-1, and other proteins but also plays roles in skeletal tissues through its secretion by osteoblasts, chondrocytes, and mesenchymal cells. It has been reported that mutations in FN1 can lead to severe scoliosis [21]. VDAC1 is a major component of the outer mitochondrial membrane, which facilitates the exchange of metabolites and ions across the outer mitochondrial membrane and may regulate mitochondrial functions. VDAC1 is mainly involved in the pathways of apoptosis and survival regulation of apoptosis by mitochondrial proteins [22]. It plays a vital role in the skeletal muscle function through the formation of tethers between the sarcoplasmic reticulum and the mitochondria [23]. Because skeletal muscle dysfunction is involved in the development of AIS, VDAC1 may participate in AIS by damaging skeletal muscle function. AHNAK plays a role in diverse biological processes such as cell structure and migration, cardiac calcium channel regulation, and tumor metastasis [24]. Several studies have demonstrated that AHNAK is correlated with myopathy, even in case of skeletal muscle regeneration [25,26]. Thus, we surmised that AHNAK, as a novel component of the dysferlin protein complex, contributes to skeletal muscle remodeling during the development of AIS. Although the cause of plasma protein imbalance in AIS was not assessed directly, correlations between fecal *Prevotella* and plasma FN1, VDAC1, and AHNAK proteins were detected. This suggests

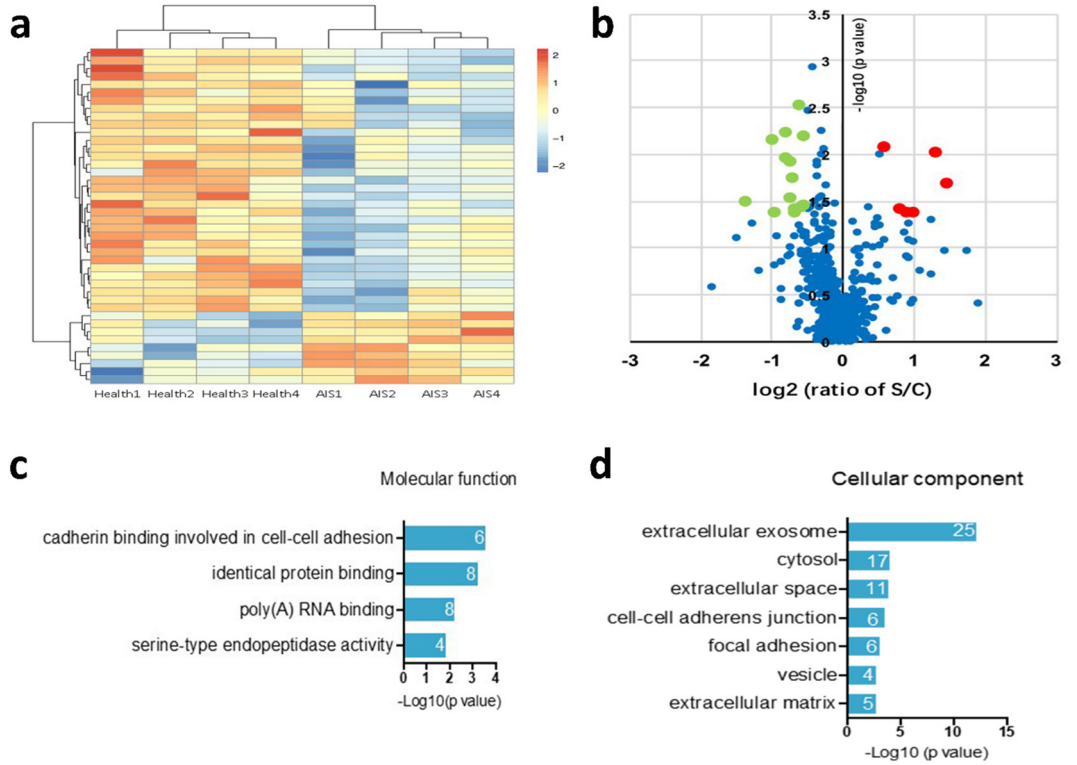

Fig. 4. The characteristics of plasma proteome in AIS and healthy groups. (a) Heatmap showed 42 proteins were differentially expressed in two groups. (b) Seventeen differentially proteins obviously associated with AIS were confirmed (Fold change > 1.5) (green and red). (c) The major molecular functional classes involved in these differential proteins. (d) The major cellular components involved in these differential proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that the gut microbiota may participate in the AIS process through the modulation of some plasma proteins associated with the development and function of skeletal tissues. These results may help to understand the mechanism of the gut-bone axis.

Although evidence that the gut microbiota contributes to the development of AIS is accumulating, the complexity of the microbiota and host system means that our understanding of the functional route of the gut microbiota is evolving slowly. We once hypothesized that the gut microbiota was a regulator of nutritional and metabolic processes associated with the AIS. However, similar nutritional and metabolic conditions can be observed even in the AIS patients with very different compositions of the gut microbiota (Table 1 and Fig. 2). Recently, it has been reported that extracellular vesicles derived from the gut microbiota contribute to the pathogenesis of inflammatory bowel disease [27]. In our study, the major cellular component of differential plasma proteins was exosome, which is a class of extracellular vesicle (Fig. 4d). Therefore, we speculated that the gut microbiota in the AIS patients might affect the AIS process by secreting exosomes containing functional proteins or RNAs and then targeting the host circulating proteins.

There are some limitations to the current study that need to be considered. First, in this study, only correlation research was carried out; the specific mechanism needs to be further explored. Second, this study is a cross-sectional study. Longitudinal studies focusing on the different periods of AIS will have an important role in terms of disease progression. Third, the sample size was limited, so larger research studies from different populations need to be conducted to confirm the findings. Thus, more solid and direct evidence regarding the relationship between the gut microbiota and AIS will be provided in future studies. A larger sample study with a longer follow-up is needed. In addition, we plan to transplant fecal samples from AIS patients into germ-free mice and observe the changes of host plasma and AIS-related phenotypes to investigate the detailed mechanisms of gut microbiota in

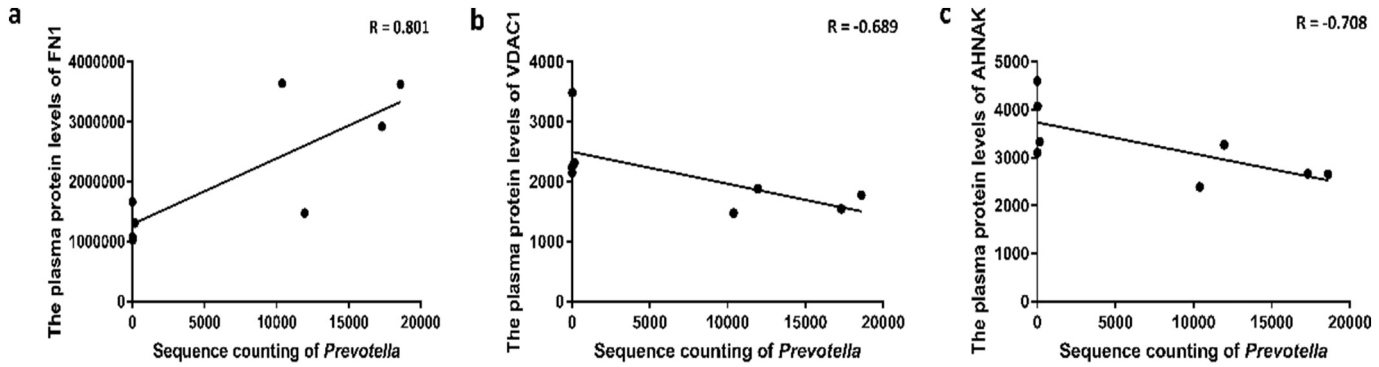

Fig. 5. Associations of the abundance of fecal *Prevotella* with host plasma differential proteins. (a) The positive correlation between the abundance of fecal *Prevotella* and host plasma FN1 proteins. (b, c) The negative correlation between the abundance of fecal *Prevotella* and host plasma VDAC1 and AHNAK proteins.

AIS.

5. Conclusion

In summary, we show the first time that the composition of the gut microbiome and changes in the plasma proteome are correlated with the AIS. In particular, we found that the fecal abundance of the genus *Prevotella* was closely related to the clinical characteristics of AIS and may play roles through host plasma FN1, VDAC1, and AHNAK proteins. These findings improve our understanding of the pathogenesis of AIS and could potentially support novel therapeutic options aimed at modifying the gut microbiota of AIS patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.11.017.

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