Salivary Microbiome Differences in Prepubertal Children With and Without Adrenal Androgen Excess

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Abstract

**Background**—Premature adrenarche is a condition of childhood adrenal androgen excess (AAE) in the absence of gonadotropin-dependent puberty, and has been linked to insulin resistance and progression to metabolic syndrome. Microbial dysbiosis is associated with progression of inflammatory states and chronic diseases. Here, we aimed to examine the salivary microbiomes of children with adrenal androgen excess (AAE) and assess the relationship with adrenal androgens and metabolic parameters.

**Methods**—In a prospective cross-sectional study of children with AAE and healthy controls, adrenal and metabolic parameters were characterized and salivary microbiome was profiled using V3-V4 16S rDNA gene amplicon sequencing.

**Results**—There was increased \(\alpha\)-diversity in AAE (5 M, 15 F) compared to controls (3 M, 8 F), with positive correlation of 11OHA4, 11KA4, testosterone, androstenedione, DHEA, and DHEAS. Subanalyses showed increased \(\alpha\)-diversity in both overweight/obese AAE and normal weight AAE compared to normal weight controls. Genus *Peptostreptococcus*, *Veillonella*, and
Streptococcus salivarius were increased in normal weight AAE. Genus Prevotella, Abiotrophia, and Neisseria were increased in overweight/obese AAE.

Conclusion—These pilot data demonstrate differences in salivary microbiome profiles of children with and without AAE. Further studies are needed to assess the causal relationships between adrenal androgens, metabolic dysfunction, and salivary microbiome composition.

Introduction

Premature adrenarche (PA) is the early maturation of the adrenal zona reticularis and rise of 19-carbon steroids, predominantly dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). It is the most common cause of premature pubarche (PP), the early onset of pubic and/or axillary hair before the age of 8 years in girls and 9 years in boys. PA is a diagnosis of exclusion, and can be made after ruling out other causes of hyperandrogenism such as congenital adrenal hyperplasia, androgen-secreting tumors, and exogenous androgen exposure. Laboratory data usually reflect increased levels of DHEA, DHEAS, and androstenedione; recent studies have correlated 11-oxygenated 19-carbon steroids (11-oxygenated androgens [11oAs]) with the clinical signs of pubarche independent of adrenarche. PA has been associated with obesity, metabolic syndrome, and polycystic ovary syndrome (PCOS), and may represent an early hyperandrogenic state in girls who later present with PCOS, with hyperinsulinism playing a key role.

The human gut microbiome is closely linked to the host’s physiology and metabolism, and can contribute to metabolic disease states including obesity, insulin resistance, diabetes, and PCOS. There is a bidirectional relationship between steroid hormones and gut microbial communities, with studies showing that prenatal and neonatal exposure to androgens can lead to gut dysbiosis in rats. Several studies have shown that women with PCOS show taxonomical differences in their gut microbiomes and further signs of dysbiosis, including a reduced α-diversity that negatively correlates with serum testosterone levels in both healthy and obese PCOS subjects. Imbalances in the gut microbiome can lead to the compromise of intestinal epithelial cell connections and a “leaky” gut, increasing the permeability of the gut lining to inflammatory mediators such as lipopolysaccharides. This inflammation can subsequently induce a state of insulin resistance and hyperinsulinemia, which has been shown to play a role in the etiology of hyperandrogenic states like PCOS.

Associations between salivary microbiome dysbiosis, systemic inflammation, and metabolic disease states have been described. These relationships may also have predictive value in the diagnosis of systemic disease early in childhood. Studies have demonstrated unique salivary microbiome signatures in subjects (including children and adolescents) with obesity, nonalcoholic fatty liver disease, type 2 diabetes, and PCOS.

To our knowledge, there are currently no studies on the microbiome of children with adrenal androgen excess (AAE), including PA or PP. The collection of a salivary sample is a convenient, noninvasive way to evaluate microbial dysbiosis in pediatric populations, with virtually no discomfort to the patient. As children with PA are at elevated risk for progression to metabolic syndrome and related diseases such as PCOS, the study of their
microbiomes may reveal early signs of aberrant dysbiosis, and may provide insight into the connections between PA and metabolic disease states. The aims of this study were to describe the salivary microbiome in children with AAE (PA or PP), and to investigate the potential of specific taxa and measures of bacterial diversity to distinguish between children with AAE and controls. Additionally, we sought to determine if features of childhood AAE (elevated adrenal androgens including 11oAs) and common co-occurring features (elevated body mass index (BMI) and signs of insulin resistance and metabolic syndrome) are correlated with microbial dysbiosis in children with AAE compared to healthy controls.

Methods

Study subjects

This was a cross-sectional prospective cohort study of children (cases and controls) at a tertiary university hospital medical center. Approval was obtained from the Institutional Review Board at Columbia University Irving Medical Center (CUIMC). Prepubertal children ages 3 to 8 (girls) and 3 to 9 (boys) were recruited from the pediatric endocrinology practices of CUIMC, affiliated general pediatric practices, and in response to community flyers and the CUIMC RecruitMe website. Among the subjects recruited, inclusion criteria for the case group were clinical signs of adrenarche including axillary odor, axillary hair, and/or pubic hair, in the absence of true puberty (Tanner I breast in girls and testicular volume ≤3 mL in boys); the remaining children were in the control group. Exclusion criteria were a history of chronic illness or other known endocrinopathies, evidence of adrenal enzyme defect, elevated inflammatory marker (C-reactive protein), history of hormone exposure, chronic glucocorticoid use, psychostimulant use, use of antibiotics in the prior 3 months, or use of probiotics in the last 1 month. The case group was previously subdivided into PA and PP subgroups based on elevation of adrenal androgen serum DHEAS in a prior study; however, as described in our publication, there did not appear to be any significant anthropometric or biochemical differences in this population (notably in 11oAs). Therefore, the PA and PP subgroups were treated as one case cohort (referred to in this study as the AAE group) for analysis of salivary microbiome measures.

Study visit and measurements

After obtaining appropriate parental consent and subject assent (7 years of age and older), subjects underwent a one-day early morning study visit at the Clinical Research Resource at Columbia Irving Institute for Clinical and Translational Research (UL1TR001873) after an overnight fast of at least 8 hours (only water prior to saliva sampling). A complete medical history was obtained using a standardized history template, in the language each participant/parent preferred (English or Spanish). A complete physical examination by a pediatric endocrinologist was performed including Tanner staging of puberty and measurements of blood pressure, height, weight, and waist circumference. The subject was asked to chew a paraffin tablet (Orion Diagnostica) until sufficient accumulation of saliva in the oral cavity, and then spit into a 50 mL sterile DNA-free conical tube (Falcon) repeatedly until 5 mL of saliva was collected. Saliva samples were immediately cooled on ice and within 30 minutes were frozen at −80°C until analysis. Fasting blood samples were obtained, centrifuged for 20 minutes at 3000 rpm, and frozen at −80°C until analysis.

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Gestational size was calculated using the PediTools Electronic Growth Chart Calculator\textsuperscript{37}; small for gestational age status was defined as birth weight less than 10\textsuperscript{th} percentile for gestational age. BMI was calculated from the weight and height measurements. Subjects were classified as underweight (< 5\textsuperscript{th} percentile), normal weight (5\textsuperscript{th} to < 85\textsuperscript{th} percentile), overweight (85\textsuperscript{th} to < 95\textsuperscript{th} percentile), and obese (≥95\textsuperscript{th} percentile), using cutoff criteria based on the sex-specific 2000 Centers for Disease Control and Prevention (CDC) BMI-for-age growth curves. In light of unique salivary microbiome signatures reported in prior studies\textsuperscript{30,31} the study cohort was subdivided into four groups for microbiome subanalyses: cases and controls with BMI < 85\textsuperscript{th} percentile (“normal weight AAE” and “normal weight controls”) and with BMI ≥85\textsuperscript{th} percentile (“overweight/obese AAE” and “overweight/obese controls”). Height age was defined as the age at which the measured height plots at the 50\textsuperscript{th} percentile on the CDC stature-for-age sex-specific growth curves. Age-, sex-, and height-specific blood pressure percentiles and z-scores were determined using a pediatric blood pressure reference chart (Children’s Nutrition Research Center, Baylor College of Medicine, Houston, TX).\textsuperscript{38} Age- and sex-specific waist circumference percentiles were determined using data from the Third National Health and Nutrition Examination Survey (NHANES III).\textsuperscript{39}

**Laboratory analysis**

The following serum analytes were evaluated in the Biomarkers Core Laboratory of the Irving Institute for Clinical and Translational Research (Columbia University, New York, NY): insulin-like growth factor 1 (IGF-1), insulin (Immumite 1000, Siemens), blood glucose, hemoglobin A1c (HbA1c), total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and high-sensitivity C-reactive protein (Cobas Integra 400, Roche Diagnostics). Adrenal androgens including DHEA, DHEAS, androstenedione, and total testosterone were assessed by commercial laboratory (Esoterix, Inc, Calabasas Hills, CA) by high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). Sera for 11oAs including 11β-hydroxyandrostenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-ketoandrostenedione (11KA4), and 11-ketotestosterone (11KT) were analyzed by LC-MS/MS as previously described by Wright \textit{et al.}\textsuperscript{40} (University of Michigan, Ann Arbor, MI). The lower limit of quantification (LLOQ), defined as the minimum concentration achieving an extrapolated signal-to-noise ratio of 3, ranged from 3 ng/dL (0.1 nmol/L) (11OHT, 11KA4, 11KT) to 10 ng/dL (0.3 nmol/L) (11OHA4). Intra-assay coefficients of variability for the 11oAs were all less than 12%. Laboratory values that were below the LLOQ for the assay were recorded as the LLOQ cut-off value for the respective assay.

Low-density lipoprotein cholesterol (LDL-C) was calculated from total cholesterol, HDL-C, and triglycerides using the Friedewald formula. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula (fasting glucose [mmol/L] × fasting insulin [mU/L]) / 22.5. Subjects were classified as having childhood metabolic syndrome using de Ferranti \textit{et al.}\textsuperscript{41} definition of three or more of the following criteria: waist circumference > 75\textsuperscript{th} percentile for age and sex, fasting blood glucose ≥110 mg/dL (≥6.1 mmol/L), triglycerides ≥100 mg/dL (≥1.3 mmol/L), HDL-C ≤40 mg/dL (1.03 mmol/L), and blood pressure ≥90\textsuperscript{th} percentile for age, sex, and height.
Microbiome analysis

**DNA isolation and sequencing**—DNA was extracted using the Qiagen MagAttract PowerSoil DNA kit (Qiagen, Hilden, Germany). Samples were homogenized with garnet beads in 750 μl of Powerbead solution by bead-beating and further extracted on an Eppendorf epMotion 5705 automated system (Eppendorf, Hamburg, Germany) for extraction following the manufacturer’s protocol. Extracted DNA was stored in elution buffer at −20°C.

16S rRNA gene amplification for detection of the bacterial 16S rRNA gene, PCR amplification of the V3–V4 region was performed using gene-specific sequences with Illumina adapter overhang nucleotide sequences. Libraries were multiplexed by using Illumina Nextera XT Index kits, the calculated equimolar pools were sequenced on the Illumina MiSeq platform using paired-end 300 cycle MiSeq Reagent Kit V3 (Illumina).

**16S rRNA Microbiome Analysis**—16S rRNA sequences were processed and applied using the DADA2 pipeline and R v3.3.0. Taxonomic classification was performed using a Naïve Bayes classifier trained using the GreenGenes 97% clustered sequences (version 13.8), downloaded from [https://benjjneb.github.io/dada2/training.html](https://benjjneb.github.io/dada2/training.html). The table was imported into R v3.6.1 to analyze for α-diversity (Shannon, Chao) and β-diversity (UniFrac), and analyses were performed using a function of the phyloseq v1.28.0 package. Based on α-diversity rarefaction, we applied a minimum cutoff of 10,000 read counts for inclusion in the analysis.

Univariate linear regression models (lm in R) of α-diversity were used for testing with clinical and laboratory measurements. Case status (AAE or control) was applied for this analysis as well. β-diversity was analyzed using permutational multivariate analysis of variance (PERMANOVA): a non-parametric method to conduct multivariate ANOVA and determine if the centroids of sample clusters differ. The test statistic is calculated from the comparison of dissimilarities among inter-class objects to those among intra-class objects. Differential abundance testing of bacterial amplicon sequence variant (ASV)s between groups was also performed using DESeq2.

Remaining statistical calculations were conducted using SAS software (version 9.4, Cary, NC, USA) to compare case and control groups: nonparametric Mann Whitney U test for continuous data and Fischer’s exact test for categorical data. A stepdown bootstrap with replacement adjustment was used for multiple comparisons of between-group differences for variables of interest within laboratory metabolic data and steroid data. A P value of < 0.05 was considered statistically significant.

Results

**Description of cohort**

Over a 21-month period, 32 participants were enrolled into the study and completed screening with exam and laboratory work; one of these participants was subsequently excluded from analysis due to an abnormally elevated C-reactive protein laboratory value. Baseline characteristics and laboratory data were collected for 20 children with AAE (5 M,
15 F) and 11 healthy controls (3 M, 8 F) (Table 1). While children with AAE were older than controls ($P = 0.016$) and had greater height age ($P = 0.002$), there was no difference in height age to chronological age (HA/CA) ratio, and all children were confirmed on physical exam to be prepubertal. Preterm gestations were higher in AAE (7/20) compared to controls (0/11) ($P = 0.033$), but there was no difference in small for gestational age status between groups (0/20 cases and 2/11 controls; $P = 0.120$). There were no other significant differences between groups for sex, BMI, birth history, or other demographic or clinical data (Table 1). Criteria for childhood metabolic syndrome were met in 3 cases (15%) and 1 control (9%). Both serum insulin and HOMA-IR were elevated in the AAE cohort compared to controls, though these differences were not statistically significant after adjustment of metabolic data for multiple comparisons (Table 1). Serum blood glucose, HbA1c, HDL-C, triglycerides, cholesterol, LDL-C, and C-reactive protein were similar between groups.

Serum concentrations of testosterone and androstenedione were higher in subjects with AAE, though the laboratory value differences were not clinically significant and in the expected range for the subjects’ Tanner stage for pubic hair. Serum levels of IGF-1 ($P = 0.001$), DHEA ($P = 0.002$), and DHEAS ($P < 0.001$) were elevated in AAE compared to controls, and serum 11oAs were significantly higher in subjects with AAE including 11OHT ($P = 0.001$), 11KT, 11OHA4, and 11KA4 ($P < 0.001$).

Microbiome data
We then aimed to compare differences in saliva microbiota between AAE and controls. Compared to controls, the participants with AAE had significantly higher $\alpha$-diversity as measured by richness ($P = 0.021$, Chao1) and richness and evenness ($P = 0.0072$, Shannon) (Fig. 1). Increased $\alpha$-diversity was observed in overweight/obese AAE ($P = 0.088$, Chao1; $P = 0.05$, Shannon) and normal weight AAE ($P = 0.015$, Chao1; $P = 0.0069$, Shannon) compared to normal weight controls. $\alpha$-diversity was increased in preterm compared to term gestations ($P = 0.032$, Chao1; $P = 0.048$, Shannon). There were no differences in $\alpha$-diversity observed for age, sex, race, ethnicity, children meeting criteria for childhood metabolic syndrome, or children with acanthosis nigricans on exam.

In univariate regression analysis, several serum analytes were positively associated with $\alpha$-diversity: 11OHA4, 11KA4, testosterone, androstenedione (Chao1), DHEA, and DHEAS (Chao 1, Shannon) (Table 2). Markers of metabolic dysfunction (glucose, insulin, HOMA-IR) were not found to have statistically significant association with $\alpha$-diversity.

We then compared microbial community composition using $\beta$-diversity. There were no differences in clustering of UniFrac distance matrices for $\beta$-diversity between AAE and control groups (Fig. 1). To further determine if groups had differential abundance of ASVs, we performed differential abundance testing with DESeq2. There were no significant differential abundant taxa between AAE and control groups. However, in subgroup analyses for normal weight and overweight/obese AAE and control groups, we found multiple differentially abundant taxa that were increased (Fig. 2). This included increased genus Peptostreptococcus, Veillonella, and Streptococcus salivarius in the normal weight AAE group compared to normal weight controls. Prevotella nanceiensis, genus Abiotrophia,
and *Neisseria* were increased in normal weight controls compared to overweight/obese controls. These taxa were also increased in the overweight/obese AAE group compared to overweight/obese controls.

Taken together, these findings suggest that alterations in bacterial diversity may be mediated in part by elevated adrenal androgens, and this may be independent of other metabolic health factors associated with AAE. However, subanalyses suggest overweight/obese status may influence the differential abundance of taxa observed.

**Discussion**

To our knowledge, this is the first study to describe the salivary microbiome in children with AAE (either PA or PP). We found that our participants with AAE had a significantly higher $\alpha$-diversity as measured by richness and evenness. Additionally, there was increased $\alpha$-diversity in overweight/obese AAE and normal weight AAE compared to normal weight controls, suggesting that this increase in $\alpha$-diversity relates to AAE, not weight, status. This result affirms the knowledge that oral and gut communities are quite distinct: decreased $\alpha$-diversity is observed in studies of the gut microbiome in metabolic disease, including obesity and PCOS. Previous studies of the salivary microbiome in children demonstrate increased bacterial diversity in obesity; a recent study by Mameli *et al.* reported increased $\alpha$-diversity in the salivary microbiome of obese children and adolescents, and another pediatric study found that $\alpha$-diversity tended to be higher in obese children as compared to children with type 2 diabetes and controls – although not significantly so. Future studies in larger cohorts are needed to investigate the potential relationship between early androgen excess and salivary microbial diversity.

Finally, our study showed that several androgens, including 11OHA4, 11KA4, testosterone, androstenedione, DHEA, and DHEAS were positively associated with salivary microbiome $\alpha$-diversity as measured by richness and evenness. This finding suggests that correlations between androgen levels and salivary dysbiosis exist. In contrast, a pilot study by Lindheim *et al.* assessed the salivary microbiome profile of women with PCOS and showed no significant difference in $\alpha$-diversity between cases and controls. In this study population, the authors described that the study cohort had, on average, a mild PCOS diagnosis; this study should not rule out the possibility that more severe hyperandrogenic states may associate with significant differences in salivary microbial diversity.

A possible confounder to our findings was that $\alpha$-diversity was also significantly higher in preterm gestations compared to term gestations. As we found that preterm gestations were higher in AAE, a larger cohort is needed to perform subanalyses and further describe the relationship between AAE, preterm gestational status, and salivary microbial diversity. The finding that $\alpha$-diversity correlates positively with several adrenal androgens as described above suggests that $\alpha$-diversity may be more dependent on adrenal androgen levels than preterm gestational status, and that the increase in $\alpha$-diversity in the preterm group is in part due to the overlap of this group with the AAE cohort.
In this study, genus *Peptostreptococcus*, *Veillonella*, and *Streptococcus salivarius* were significantly increased in the normal weight children with AAE compared to normal weight controls. *Peptostreptococcus* has been found to be significantly increased in an obese adult cohort and associated with periodontal infections. *Veillonella* and *Streptococcus salivarius* have been shown in several studies to be associated with dental caries in both adults and children. We also found that compared to overweight/obese controls, overweight/obese children with AAE showed significantly increased *Prevotella nanceiensis*, genus *Abiotrophia*, and *Neisseria*. *Prevotella* have been shown to predominate in periodontal diseases, systemic inflammation, and metabolic disease. One study found that in women with PCOS and gingivitis, *Prevotella intermedia* levels were significantly higher compared to systemically or periodontally healthy women. Overall, several of the taxa found to be elevated in our case cohort, compared to weight equivalent controls, have been associated with periodontal disease and oral inflammation.

A significant limitation of our study was lack of oral health histories for the study participants. Further studies are needed in a larger cohort that accounts for oral hygiene and disease to determine whether there are any associations between childhood androgen excess and oral inflammation. As the prevalence of periodontal disease has been shown to be higher in PCOS, it would be of interest to explore whether children with AAE are more likely to exhibit a poorer oral health profile. This study was further limited in its cross-sectional nature; future longitudinal studies that monitor children before and after onset of AAE could better assess the presence of causal relationships between adrenal androgens and salivary microbiome composition.

A major strength of our study was that our cohort was a well-defined group of both boys and girls with AAE, diagnosed by biochemical and clinical signs and based on exclusion of other causes of androgen excess. Additionally, we applied strict criteria to exclude any participants who had taken antibiotics within three months or probiotics within one month prior to saliva sample collection, greatly reducing the possible impact of antibiotics on their oral microbiome profiles. Furthermore, we collected samples at a consistent time of day, early in the morning after a midnight fast, controlling for the possible impact of eating in the hours before saliva sample collection. With the small sample size, this study serves as a pilot to initially investigate and describe the salivary microbiome in children with AAE; further understanding of the relationship between early androgen excess and the salivary microbiome warrants additional investigation with larger population studies that control for age and oral health status.

In summary, this study uniquely describes the salivary microbiome of children with AAE. α-diversity was increased in children with AAE, independent of weight status. Genus *Peptostreptococcus*, *Veillonella*, and *Streptococcus salivarius* were significantly increased in normal weight AAE compared to normal weight controls, and overweight/obese AAE showed significantly increased *Prevotella nanceiensis*, genus *Abiotrophia*, and *Neisseria* compared to overweight/obese controls; several of these taxa are associated with periodontal disease and inflammation. Studies with a larger cohort of AAE children with defined oral health status are necessary to illuminate the possible correlations between early androgenic states and oral microbial dysbiosis in children with AAE.
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Impact Statement:

- This study is the first to report the salivary microbiome of prepubertal children with adrenal androgen excess (AAE).
- α-diversity is increased in the salivary microbiome of children with AAE independent of weight status, and in this study cohort several serum androgens are positively associated with α-diversity.
- Several taxa that have been associated with periodontal disease and inflammation are found to be significantly increased in AAE.
Figure 1. Bacterial diversity in AAE and controls.

α-diversity in AAE and controls by (A) Chao 1 estimator and (B) Shannon index. (C) Principal Coordinates Analysis (PCoA) shows β-diversity based on the UniFrac distance metrics in AAE.
Figure 2. Differential abundant ASV in children with AAE and controls by weight status. Comparisons are shown between (a) Normal weight control and normal weight AAE, (b) Normal weight control and overweight/obese AAE, (c) Normal weight control and overweight/obese control, and (d) Overweight/obese control and overweight/obese AAE in differential abundance of ASV. ASV in blue indicate increased abundance in the control group whereas ASV in red show increased ASV in the respective case groups. The depicted taxa had a minimum differential abundance magnitude of change (fold change) > 0 with a $P$ value of <0.05.
Table 1.
AAE and control subject characteristics.

Data are medians [IQR] or counts (percentage). Statistical significance was determined by non-parametric Mann-Whitney U test for continuous data and Fischer’s exact test for categorical data. Bold values indicate statistical significance P value <0.05.

| Demographics and History | Control cohort (n=11) | AAE cohort (n=20) | P value |
|--------------------------|-----------------------|-------------------|---------|
| Chronological age (CA), y| 6.3 [3.9 - 7.0]       | 7.4 [6.5 - 7.8]   | 0.016   |
| Height age (HA), y       | 5.8 [4.2 - 7.4]       | 7.8 [7.4 - 8.8]   | 0.002   |
| HA/CA ratio              | 1.04 [0.86 - 1.16]    | 1.13 [1.05 - 1.19]| ns      |
| Sex, n (%)               | 8 F; 3 M (73%; 27%)   | 15 F; 5 M (75%; 25%)| ns      |
| Race (Asian; Black; Caucasian; Other), % | 18%; 18%; 45%, 18% | 10%, 25%, 60%, 5% | ns |
| Hispanic ethnicity, %    | 45%                   | 35%               | ns      |
| Family history of T2DM, %| 64%                   | 60%               | ns      |
| Family history of cardiovascular disease, % | 82%                   | 85%               | ns      |
| Preterm gestation, %     | 0%                    | 35%               | 0.033   |
| SGA status, %            | 18%                   | 0%                | ns      |

| Clinical data             |                      |                   |         |
|---------------------------|-----------------------|-------------------|---------|
| BMI percentile            | 71 [68 - 78]          | 60 [31 - 89]      | ns      |
| BMI z-score               | 0.55 [0.47 - 0.77]    | 0.25 [−0.51 - 1.22]| ns      |
| BMI class, %              |                       |                   |         |
| Underweight               | 0%                    | 5%                | ns      |
| Normal                    | 82%                   | 65%               | ns      |
| Overweight                | 9%                    | 15%               | ns      |
| Obese                     | 9%                    | 15%               | ns      |
| Waist circumference > 75%ile, % | 36%                   | 45%               | ns      |
| Acanthosis nigricans, %   | 9%                    | 40%               | ns      |
| Systolic BP percentile    | 79 [60 - 80]          | 74 [50 - 92]      | ns      |
| Diastolic BP percentile   | 80 [73 - 91]          | 74 [47 - 88]      | ns      |
| Childhood MetS, %         | 9%                    | 15%               | ns      |

| Laboratory data           |                      |                   |         |
|---------------------------|-----------------------|-------------------|---------|
| Glucose, mg/dL            | 83 [80 - 88]          | 88 [84 - 91]      | ns      |
| Insulin, uIU/mL           | 2.0 [2.0 - 3.6]       | 8.1 [3.6 - 10.0]  | ns      |
| HOMA-IR                   | 0.4 [0.4 - 0.8]       | 1.7 [0.8 - 2.1]   | ns      |
| HbA1c, %                  | 5.4 [5.2 - 5.5]       | 5.3 [5.2 - 5.4]   | ns      |
| IGF-1, ng/mL              | 85 [61 - 137]         | 179 [148 - 223]   | 0.001   |
| HDL-C, mg/dL              | 56 [50 - 60]          | 59 [51 - 63]      | ns      |
| Triglycerides, mg/dL      | 51 [44 - 61]          | 53 [41 - 57]      | ns      |
| Cholesterol, mg/dL        | 155 [142 - 165]       | 144 [127 - 169]   | ns      |
| LDL-C, mg/dL              | 82 [79 - 94]          | 80 [66 - 88]      | ns      |
| C-reactive protein, mg/L  | 0.30 [0.30 - 0.75]    | 0.30 [0.30 - 0.56]| ns      |
| Testosterone, ng/dL       | 2.5 [2.5 - 2.5]       | 5.6 [4.2 - 7.2]   | <0.001  |

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## Table

| Androgen | Control cohort (n=11) | AAE cohort (n=20) | P value |
|----------|-----------------------|-------------------|---------|
| Androstenedione, ng/dL | 10 [10 - 10] | 22 [13 - 30] | 0.002 |
| DHEA, ng/dL | 20 [20 - 31] | 128 [78 - 244] | 0.002 |
| DHEAS, ug/dL | 10 [10 - 12] | 58 [44 - 94] | <0.001 |
| 11OHT, ng/dL | 3.0 [3.0 - 3.8] | 5.7 [4.8 - 6.5] | 0.001 |
| 11KT, ng/dL | 9.1 [5.8 - 10.4] | 24.2 [18.8 - 29.2] | <0.001 |
| 11OHA4, ng/dL | 16.1 [13.4 - 25.7] | 58.2 [51.4 - 94.4] | <0.001 |
| 11KA4, ng/dL | 5.8 [5.1 - 8.3] | 17.4 [14.0 - 20.2] | <0.001 |

AAE, adrenal androgen excess; IQR, interquartile range; HA, height age; CA, chronological age; T2DM, type 2 diabetes mellitus; SGA, small for gestational age; BMI, body mass index; BP, blood pressure; MetS, metabolic syndrome; HOMA-IR, homeostatic model of insulin resistance; HbA1c, hemoglobin A1c; IGF-1, insulin-like growth factor 1; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone; 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione.
Table 2.
Univariable linear mixed-effect regression models of salivary microbiome α-diversity with clinical and laboratory parameters.

| Variation       | Chao1 (LM) pval | Chao1 (LM) padj | Shannon (LM) pval | Shannon (LM) padj |
|-----------------|-----------------|-----------------|-------------------|-------------------|
| Age             | 0.859           | 0.902           | 0.971             | 0.971             |
| Systolic BP     | 0.324           | 0.494           | 0.458             | 0.641             |
| BMI z-score     | 0.938           | 0.938           | 0.955             | 0.971             |
| Glucose         | 0.434           | 0.608           | 0.345             | 0.641             |
| Insulin         | 0.687           | 0.837           | 0.404             | 0.641             |
| HOMA-IR         | 0.702           | 0.837           | 0.430             | 0.641             |
| Cholesterol     | 0.052           | 0.135           | 0.235             | 0.509             |
| Triglycerides   | 0.329           | 0.494           | 0.609             | 0.699             |
| HDL-C           | 0.155           | 0.362           | 0.208             | 0.509             |
| LDL-C           | 0.248           | 0.473           | 0.632             | 0.699             |
| CRP             | 0.315           | 0.494           | 0.425             | 0.641             |
| 11OHT           | 0.193           | 0.405           | 0.243             | 0.509             |
| 11KT            | 0.035           | 0.105           | 0.028             | 0.084             |
| 11OHA4          | 0.000           | 0.005           | 0.013             | 0.083             |
| 11KA4           | 0.006           | 0.021           | 0.019             | 0.083             |
| Testosterone    | 0.003           | 0.012           | 0.022             | 0.083             |
| Androstenedione | 0.002           | 0.009           | 0.024             | 0.083             |
| DHEA            | 0.001           | 0.006           | 0.002             | 0.018             |
| DHEAS           | 0.000           | 0.005           | 0.000             | 0.009             |

Bold values indicate statistical significance P value <0.05. pval, P value; padj, adjusted P value; BP, blood pressure; BMI, body mass index; HOMA-IR, homeostatic model of insulin resistance; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone; 11OHA4, 11β-hydroxyandrostenedione; 11KA4, 11-ketoandrostenedione; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate.