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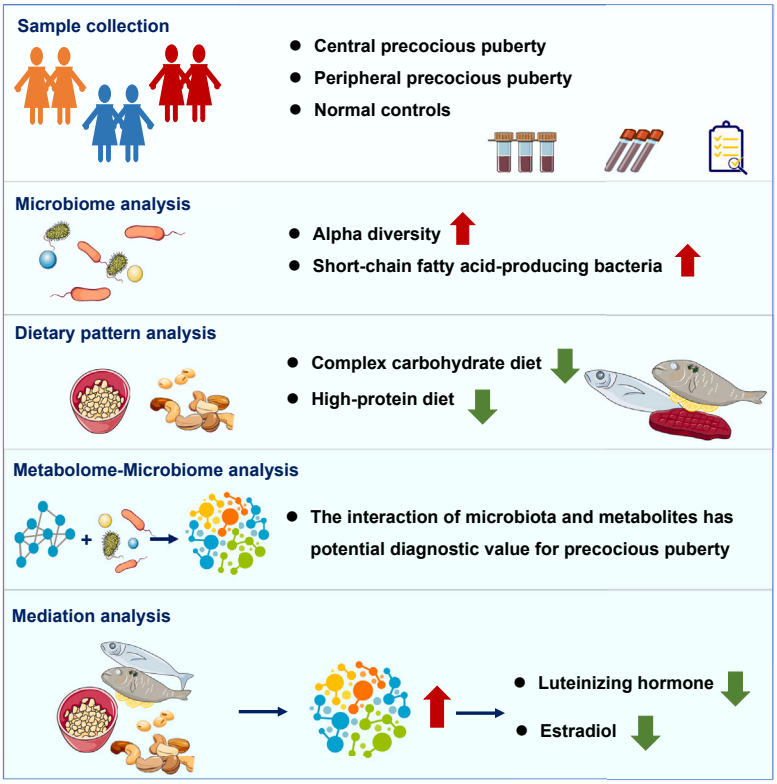

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Highlights

Precocious puberty girls have increased alpha-diversity and SCFA-producing bacteria

Complex carbohydrates and high-protein diets reduce the risk of precocious puberty

The microbiota-metabolites combination has diagnostic value for precocious puberty

Microbiota-metabolites interactions mediate the regulation of hormone levels by diet

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Gut microbiota-metabolite interactions meditate the effect of dietary patterns on precocious puberty

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SUMMARY

Precocious puberty, a pediatric endocrine disorder classified as central precocious puberty (CPP) or peripheral precocious puberty (PPP), is influenced by diet, gut microbiota, and metabolites, but the specific mechanisms remain unclear. Our study found that increased alpha-diversity and abundance of short-chain fatty acid–producing bacteria led to elevated levels of luteinizing hormone and follicle-stimulating hormone, contributing to precocious puberty. The integration of specific microbiota and metabolites has potential diagnostic value for precocious puberty. The *Prevotella* genus-controlled interaction factor, influenced by complex carbohydrate consumption, mediated a reduction in estradiol levels. Interactions between obesity-related bacteria and metabolites mediated the beneficial effect of seafood in reducing luteinizing hormone levels, reducing the risk of obesity-induced precocious puberty, and preventing progression from PPP to CPP. This study provides valuable insights into the complex interplay between diet, gut microbiota and metabolites in the onset, development and clinical classification of precocious puberty and warrants further investigation.

INTRODUCTION

Precocious puberty is a pediatric endocrine disorder characterized by the premature onset of puberty, marked by the early onset of secondary sexual characteristics and physical development.[1] Its incidence is predominantly higher in females[2] and is steadily increasing.[3] Precocious puberty can lead to early fusion of the epiphyseal plates, resulting in reduced final adult height and is strongly associated with mental disorders in affected children and elevated risks of adult-onset conditions such as hypertension, type 2 diabetes, ischemic heart disease, stroke, estrogen-dependent cancers, and cardiovascular mortality.[4]

Precocious puberty can be divided into two main types, namely central precocious puberty (CPP) and peripheral precocious puberty (PPP), depending on whether the hypothalamic-pituitary-gonadal (HPG) axis is activated.[5] In some cases, children with PPP can convert to CPP.[6] While a number of factors including genetics, environmental endocrine disruptors, and obesity have been identified as contributing to the development of precocious puberty, its complex pathogenic mechanism remains not fully understood.

Bidirectional interactions between the gut microbiome and the sex hormones have been observed in various endocrine-related diseases.[7–9] Recent studies have shown that the gut microbiota can influence sex hormones and obesity-dependent perturbation of mice and rats.[10,11] Changes in the gut microbiome and associated metabolome have also been observed in girls with PPP and CPP.[12,13] In addition, dietary patterns have a significant impact on estrogen metabolism and related hormone levels such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), leptin, and insulin, and influence the timing of puberty.[14–19] At the same time, dietary patterns play a critical role in shaping the composition of the human gut microbiota and the metabolome.[20,21] Therefore, precocious puberty is thought to be triggered by specific pathophysiological stimuli, including gut microbiota or dietary patterns.[22–24] However, the precise impact of the complex interactions

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Table 1. Patient characteristics

	Overall	CPP	PPP	Normal	*Pvalue*[a]	*Pvalue*[b]	*Pvalue*[c]
N	114	21	45	48			
Basic characteristics							
Age	7.12 (0.71)	7.30 (0.67)	7.10 (0.73)	7.05 (0.69)	0.18	0.77	0.326
BMI	16.17 (2.74)	15.89 (1.92)	16.49 (2.76)	15.98 (3.08)	0.579	0.05	0.397
BA	8.43 (1.30)	9.17 (1.09)	8.09 (1.25)	NA	NA	NA	0.004
Hormone							
LH	0.27 (1.21)	1.12 (2.72)	0.08 (0.04)	0.08 (0.04)	<0.001	0.99	<0.001
FSH	2.12 (1.45)	3.71 (2.54)	1.78 (0.78)	1.74 (0.59)	<0.001	0.94	<0.001
E2	20.12 (18.66)	32.25 (41.11)	18.25 (4.31)	16.56 (4.25)	0.003	0.001	0.023
TES	7.99 (3.97)	11.09 (3.92)	6.88 (3.42)	7.26 (3.62)	0.002	0.521	<0.001
PRL	12.81 (10.73)	12.84 (6.36)	11.08 (7.79)	14.42 (14.05)	0.958	0.072	0.118
PRG	0.16 (0.15)	0.12 (0.03)	0.15 (0.13)	0.18 (0.19)	0.281	0.087	0.982
Reproductive system							
Uterine volume	1.93 (2.48)	3.04 (4.13)	1.41 (0.71)	NA	NA	NA	0.024
Ovarian volume	2.25 (1.81)	2.58 (2.35)	2.10 (1.50)	NA	NA	NA	0.596
Breast volume	4.10 (5.45)	7.09 (8.15)	2.70 (2.72)	NA	NA	NA	<0.001
GnRH stimulation test result							
LH/FSH 30min ratio	0.90 (0.61)	1.18 (0.62)	0.42 (0.11)	NA	NA	NA	<0.001
LH/FSH 60min ratio	0.80 (0.54)	1.06 (0.51)	0.35 (0.10)	NA	NA	NA	<0.001
Trace element							
Cu	15.69 (3.36)	17.34 (2.86)	16.03 (2.81)	14.64 (3.71)	0.003	0.085	0.016
Zn	68.11 (8.14)	69.46 (6.48)	67.00 (5.98)	68.55 (10.28)	0.855	0.159	0.011
Ca	1.53 (0.12)	1.56 (0.04)	1.52 (0.08)	1.53 (0.16)	0.265	0.864	0.002
Mg	1.39 (0.16)	1.50 (0.06)	1.38 (0.07)	1.36 (0.23)	<0.001	0.52	<0.001
Fe	7.94 (0.47)	7.98 (0.20)	7.93 (0.28)	7.92 (0.66)	0.906	0.36	0.006
Pb	17.21 (6.18)	22.47 (7.40)	20.90 (10.51)	16.49 (5.41)	0.128	0.5	0.655

N, number; BMI, body mass index; BA, bone age; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; TES, testosterone; PRL, prolactin; PRG, Progestational hormone; GnRH, gonadotropin-releasing hormone; Cu, Copper; Zn, Zinc; Ca, Calcium; Mg, magnesium; Fe, iron; Pb, Lead. *Pvalue*[a] indicates the *p* value obtained from the Wilcoxon rank-sum test comparing the CPP and Normal groups. Pvalue[b] indicates the *p* value obtained from the Wilcoxon rank-sum test comparing the PPP and Normal groups. *Pvalue*[c] represents the *p* value from the Wilcoxon rank-sum test comparing the CPP and PPP groups.

between dietary patterns, gut microbiota, and metabolites on the regulation of hormone metabolism, progression, and different clinical types of precocious puberty remains largely unknown.

Therefore, we performed a comprehensive analysis of the gut microbiome and serum metabolome in relation to dietary patterns and clinical features in girls with PPP, CPP, and normal controls. We investigated the potential impact of microbiota-metabolite interactions on clinical outcomes related to precocious puberty, as well as the ability of these interactions to serve as a diagnostic tool for precocious puberty. The mediating role of microbiota-metabolite interactions on diet-influenced precocious puberty risk was also investigated. This study provides new insights into the mechanism and development of diagnostic and intervention strategies for precocious puberty.

RESULTS

Baseline characteristics of participants

A total of 114 female participants were enrolled in this study, including 21 girls with CPP, 45 girls with PPP, and 48 normal controls (Table 1). Age and BMI did not differ significantly between the groups. Both girls with PPP and CPP showed advanced bone age and increased ovarian volume (>1mL). Baseline levels of LH, FSH, estradiol (E2), and testosterone (TES) were significantly higher in girls with CPP than that in PPP and normal controls ($p < 0.05$). A higher level of E2 was also observed in the PPP group compared to the normal control group. CPP girls also showed upregulation in bone age, gonadotropin-releasing hormone (GnRH) stimulation test results, breast volume, and uterine volume compared to PPP girls ($p < 0.05$). Serum levels of copper (Cu) and magnesium (Mg) were found to be increased in the CPP group compared to both the PPP and normal control groups ($p < 0.05$).

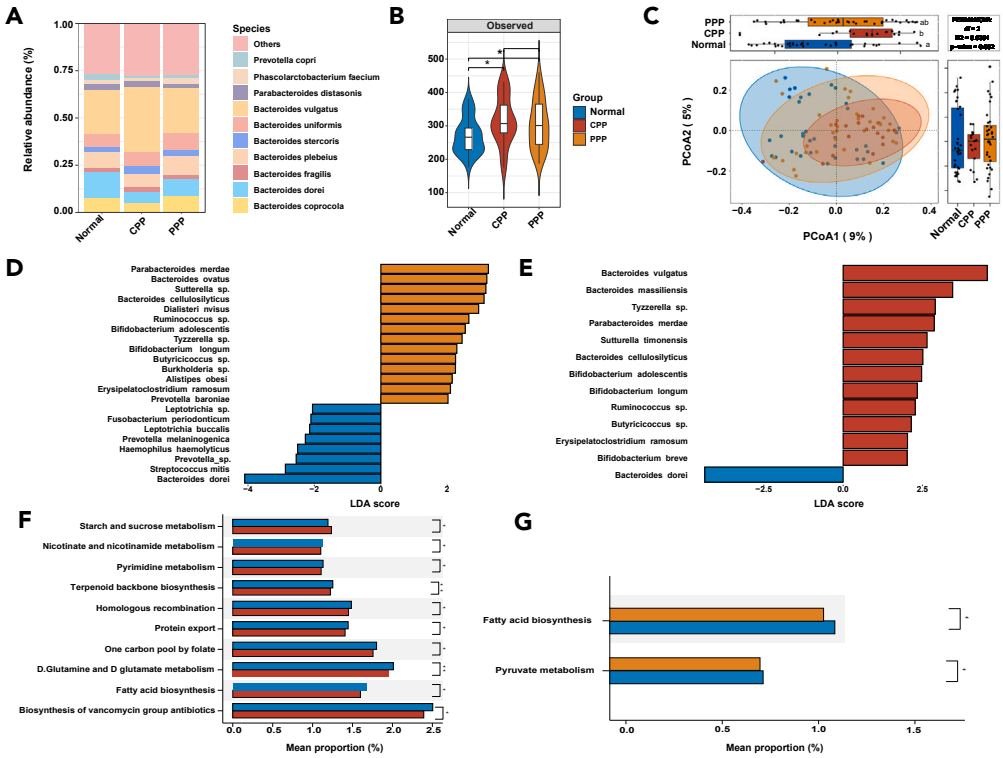


Figure 1. Gut microbiome dysbiosis in girls suffering from CPP and PPP

(A) Stack bar plot showing the bacterial composition at the species level in the PPP, CPP, and normal groups. Relative abundance of the top 10 most abundant bacterial species within each group were displayed. The category "Others" represents the combined abundance of all remaining species.

(B) Alpha diversity of the gut microbiota (measured by the observed metric) between groups.

(C–G) PCoA of beta diversity based on the Bray-Curtis distance between groups with the PERMANOVA test. Linear discriminant analysis Effect Size analysis results showed the differential abundance of species between PPP and normal groups (D) as well as CPP and normal groups (E). The significant differential KEGG pathways between CPP and normal groups (F) as well as PPP and normal groups (G). Statistical significance was indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$). CPP, central precocious puberty; PPP, peripheral precocious puberty; PCoA, principal co-ordinates analysis; PERMANOVA, permutational multivariate analysis of variance; LDA, Linear discriminant analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

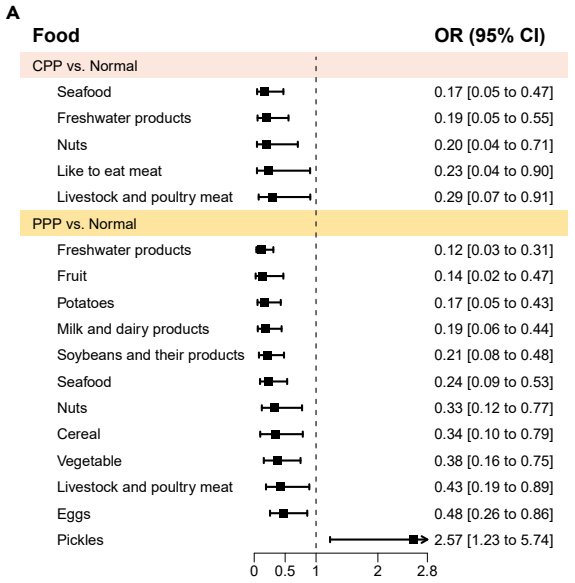
See also Tables S1 and S2.

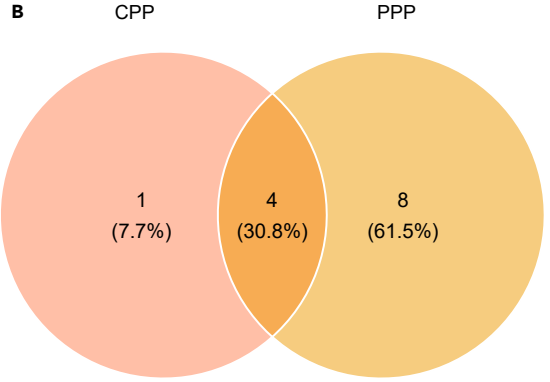
Gut microbiome dysbiosis in girls suffering from CPP and PPP

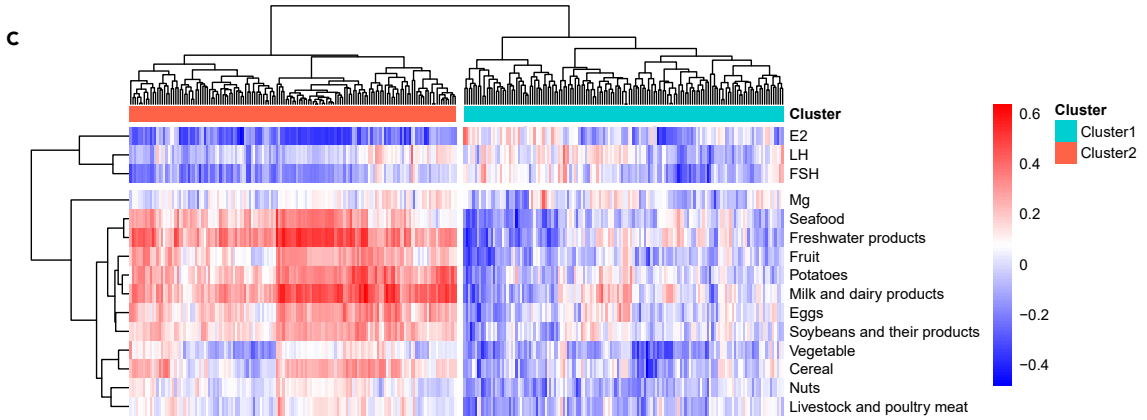
The taxonomic information and sequence of ASVs identified was displayed in Table S1. The bacterial composition at the species level in each group was displayed in Figure 1A, where the majority of the top 10 most abundant bacterial species belonged to the *Bacteroides* genus. The gut microbiome profile displayed a large variation among girls with CPP, PPP, and normal controls. Compared to the normal girls (Figure 1B), significantly increased bacterial alpha diversity (measured by the observed metric) was found in both CPP and PPP groups, while no difference in alpha diversity was detected between girls with CPP and PPP. Meanwhile, there were significant differences in beta diversity across the groups ($R^2 = 0.0364$, $p = 0.002$, Figure 1C). Specifically, the bacterial composition in both the CPP group ($R^2 = 0.0383$, $p = 0.001$) and the PPP group ($R^2 = 0.0255$, $p < 0.05$) showed notable distinctions compared to the normal group. Although there was difference in beta diversity between the PPP and CPP group, this difference did not reach statistical significance ($R^2 = 0.0184$, $p = 0.528$). The differentially abundant taxa were presented in Table S2. Among them, the species that showed differential abundance and were associated with PPP and CPP mainly belonged to the *Bacteroides* genus (Figures 1D and 1E). Specifically, *Bacteroides dorei* was a common beneficial species for both PPP and CPP while *Bacteroides ovatus*, *Bacteroides cellulosilyticus*, *Bacteroides vulgatus*, *and Bacteroides massiliensis* were risk species for the development of precocious puberty. Like the *Bacteroides* genus, other short chain fatty acids (SCFAs)-producing bacteria including *Tyzzerella*, *Butyricicoccus*, *Ruminococcus*, and *Erysipelatoclostridium ramosum* were also upregulated in girls with both CPP and PPP compared to normal controls. Furthermore, alterations on microbiome-mediated functional potentials (Figures 1F and 1G) indicated the fatty acid biosynthesis was associated with both CPP and PPP. Taken together, increased alpha diversity and the abundance of SCFA-producing bacteria in the gut served as the common risk factor for both CPP and PPP.

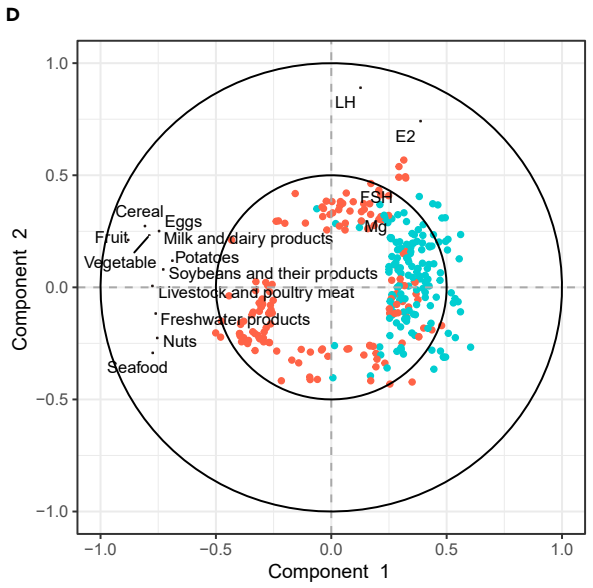
Microbiome clusters based on dietary patterns correlated with PPP and CPP

Dietary patterns were found to be closely associated with the risk of precocious puberty (Figure 2A; Table S3), among which, complex carbohydrate and high-protein diet showed potential protective effect for girls with PPP and CPP. Additionally, nuts, seafood, freshwater


A
Food
OR (95% CI)
CPP vs. Normal
Seafood
0.17 [0.05 to 0.47]
Freshwater products
0.19 [0.05 to 0.55]
Nuts
0.20 [0.04 to 0.71]
Like to eat meat
0.23 [0.04 to 0.90]
Livestock and poultry meat
0.29 [0.07 to 0.91]
PPP vs. Normal
Freshwater products
0.12 [0.03 to 0.31]
Fruit
0.14 [0.02 to 0.47]
Potatoes
0.17 [0.05 to 0.43]
Milk and dairy products
0.19 [0.06 to 0.44]
Soybeans and their products
0.21 [0.08 to 0.48]
Seafood
0.24 [0.09 to 0.53]
Nuts
0.33 [0.12 to 0.77]
Cereal
0.34 [0.10 to 0.79]
Vegetable
0.38 [0.16 to 0.75]
Livestock and poultry meat
0.43 [0.19 to 0.89]
Eggs
0.48 [0.26 to 0.86]
Pickles
2.57 [1.23 to 5.74]
0
0.5
1
2
2.8


B
CPP
PPP
1
(7.7%)
4
(30.8%)
8
(61.5%)


C
Cluster
E2
LH
FSH
Mg
Seafood
Freshwater products
Fruit
Potatoes
Milk and dairy products
Eggs
Soybeans and their products
Vegetable
Cereal
Nuts
Livestock and poultry meat
0.6
0.4
0.2
0
−0.2
−0.4
Cluster
Cluster1
Cluster2


D
LH
E2
FSH
Mg
Cereal
Eggs
Fruit
Milk and dairy products
Vegetable
Potatoes
Soybeans and their products
Livestock and poultry meat
Freshwater products
Nuts
Seafood
Component 2
Component 1
1.0
0.5
0.0
−0.5
−1.0

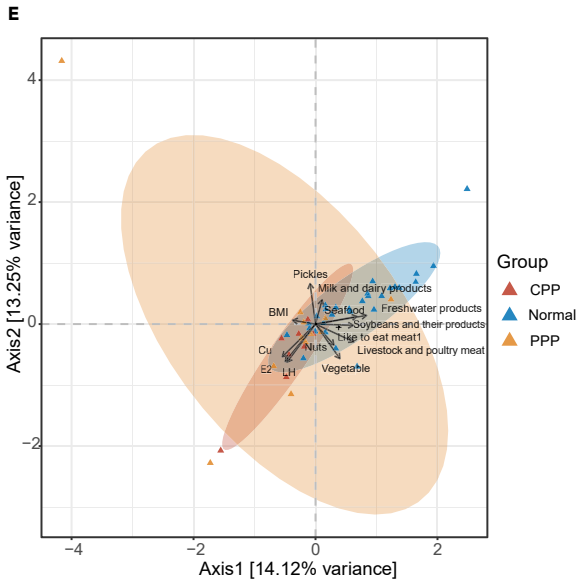

E
Pickles
Milk and dairy products
BMI
Seafood
Freshwater products
Soybeans and their products
Like to eat meat1
Cu
Nuts
Livestock and poultry meat
E2
LH
Vegetable
Axis2 [13.25% variance]
Axis1 [14.12% variance]
4
2
0
−2
−4
Group
CPP
Normal
PPP

Figure 2. Microbiome clusters based on dietary patterns correlated with PPP and CPP

(A) Forest plot displayed the significance and OR value of latent dietary patterns associated with CPP and PPP.

(B) Venn diagram of common latent dietary patterns associated with CPP and PPP.

(C) Hierarchical clustering map of the first two Sparse partial least squares (sPLS) dimensions, displaying pairwise correlations between 287 amplicon sequence variants (ASVs) and 11 dietary patterns and 4 clinical variables. Correlations greater than 0.25 or less than −0.25 were shown in red and blue, respectively.

(D) The correlation circle plot for the first two sPLS dimensions displayed correlations greater than 0.25 or less than −0.25. The two black circles indicate correlation coefficient radii at 0.5 and 1.0. Bacterial ASVs are displayed as circles, and are colored based on their affiliated cluster (cluster 1: blue; cluster 2: red).

(E) Canonical correspondence analysis (CCpnA) of gut microbiota (data not shown), dietary patterns and clinical variables. It showed dimension 1 and 2 of the CCpnA, which included continuous clinical variables (arrows), categorical variables (+) and samples (triangles). The samples in the CCpnA plot were colored based on their groups, and the ellipses presented an 80% confidence interval. CPP, central precocious puberty; PPP, peripheral precocious puberty; OR, odd ratios; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; Mg, magnesium.

See also Figure S1, Tables S3 and S4.

products, and livestock and poultry meat were common beneficial dietary factors that contribute to reducing the risk of both PPP and CPP (Figure 2B). The correlation between gut microbiota and these precocious puberty-related dietary factors led to the separation of the gut microbiota into two clusters using sparse partial least-squares regression (sPLS)-based hierarchical clustering (Figure 2C). A total of 142 and 145 ASVs were included in cluster 1 and cluster 2, respectively (Table S4). Notably, microbiota assigned to cluster 2 exhibited negative associations with several dietary pattern characteristics (such as reduced intake of complex carbohydrates and high-protein diets, Figure 2A) and clinical variables (including elevated levels of E2, LH, and FSH, Table 1) associated with precocious puberty. The sPLS analysis supported these consistent findings (Figure 2D). Consistent with this, girls in the normal group exhibited stronger correlations with most microbiota of cluster 2 (Figure S1A). These findings suggested that microbiota belonging to cluster 2 may have a potential beneficial effect in reducing the risk of CPP and PPP. In addition to *Bacteroides* and *Faecalibacterium* being common predominant genera in both clusters, the *Prevotella* genus accounted for a significant proportion of cluster 2 (Table S4). Loading plots of the ASVs showed that *Faecalibacterium* and *Lachnospiraceae_UCG−001* genus contributed most to the sPLS clustering (Figures S1B and S1C). Furthermore, the canonical correspondence analysis (CCpnA) suggested that the interaction between microbiome clusters and dietary patterns was involved with PPP and CPP risk (Figure 2E). Normal girls exhibited lower levels of LH and E2 and tend to have a high-intake of complex carbohydrates (e.g., nuts and vegetable) and high-protein diets (e.g., seafood, freshwater products, livestock and poultry meat, like to eat meat). In contrast, girls with PPP and CPP have higher levels of E2 and LH, which were associated with low-intake of the same complex carbohydrates and high-protein diets (Figure 2E). These results suggested that complex carbohydrate and high-protein diet have the potential to reduce the risk of precocious puberty by regulating related hormone levels, and emphasized the involvement roles of the diet-shaped microbiome.

Microbiome-metabolome interaction involve in the development and progression of precocious puberty

Untargeted metabolomic profiles revealed the metabolite changes in girls with PPP and CPP compared to normal girls. The majority of differential metabolites in PPP and CPP ($p < 0.05$, |log2FoldChange|>1, Figure 3A; Table S5) belonged to food-related metabolites or metabolites involved in microbiota-human co-metabolism. Among them, adenosine, (+)-4,11-eudesmadien-3-one, and 17beta-Nitro-5alpha-androstane were common metabolites that significantly up-regulated in both PPP and CPP girls. After integrating the microbiome and metabolome using MOFA 2, seven combinations of microbiota and metabolites were identified and clustered (Figure S2A). These factors together accounted for 79.32% of the variance in both omics, with 55.29% explained by microbiome and 24.03% by metabolite (Figure S2A). Of these, Factor 1 which accounted for the majority of the variance (38.44%, Figure S2B), showed no significant difference between the CPP, PPP, and normal groups (Figure S2C). This suggested that Factor 1 may have a fundamental effect on children rather than being a driving force behind precocious puberty. Notably, Factor 2, Factor 4, and Factor 5 (with explained variances of 13.10%, 7.27%, and 4.39%, respectively) exhibited notable differences between the CPP, PPP, and normal groups (Figures 3B and 3C). Factor 4 was upregulated in normal girls compared to the CPP and PPP groups, suggesting a potentially beneficial influence in reducing the risk of precocious puberty. In contrast, Factor 5 appeared to be a potential risk factor for precocious puberty, given its up-regulation in the CPP and PPP groups. Interestingly, Factor 2 showed a gradual decrease from the normal group to the PPP group, and further to the CPP group, suggesting its potential role in reducing the risk of precocious puberty and in the transition from PPP to CPP (Figure 3C). The top 10 metabolites and microbiota that contributed most to Factor 1, Factor 2, Factor 4, and Factor 5 were shown in Figures S3A–S3D, respectively. Overall, diet-shaped microbiota may interact with metabolites and play roles in the development and progression of precocious puberty.

Microbiota-metabolite combination signatures act as no-invasive tools for precocious puberty diagnosis

The proposed prediction framework indicated that microbiota-metabolite combination factors (Factor 2, Factor 4, and Factor 5) showed good performance in the diagnosis of PPP and CPP with high discrimination obtained with the random forest classification model, achieving a macro- and micro-average area under the receiver operating characteristics curve (AUC) greater than 0.75 (Figures 3D–3F). The weights of and microbiota metabolites in each factor were displayed in Tables S6 and S7, respectively. Furthermore, the top 10 microbiota and metabolites (ranked by weight) in each factor (Factor 2, Factor 4, and Factor 5) also showed promising diagnostic potential for PPP and CPP (Figures S2D–S2F).

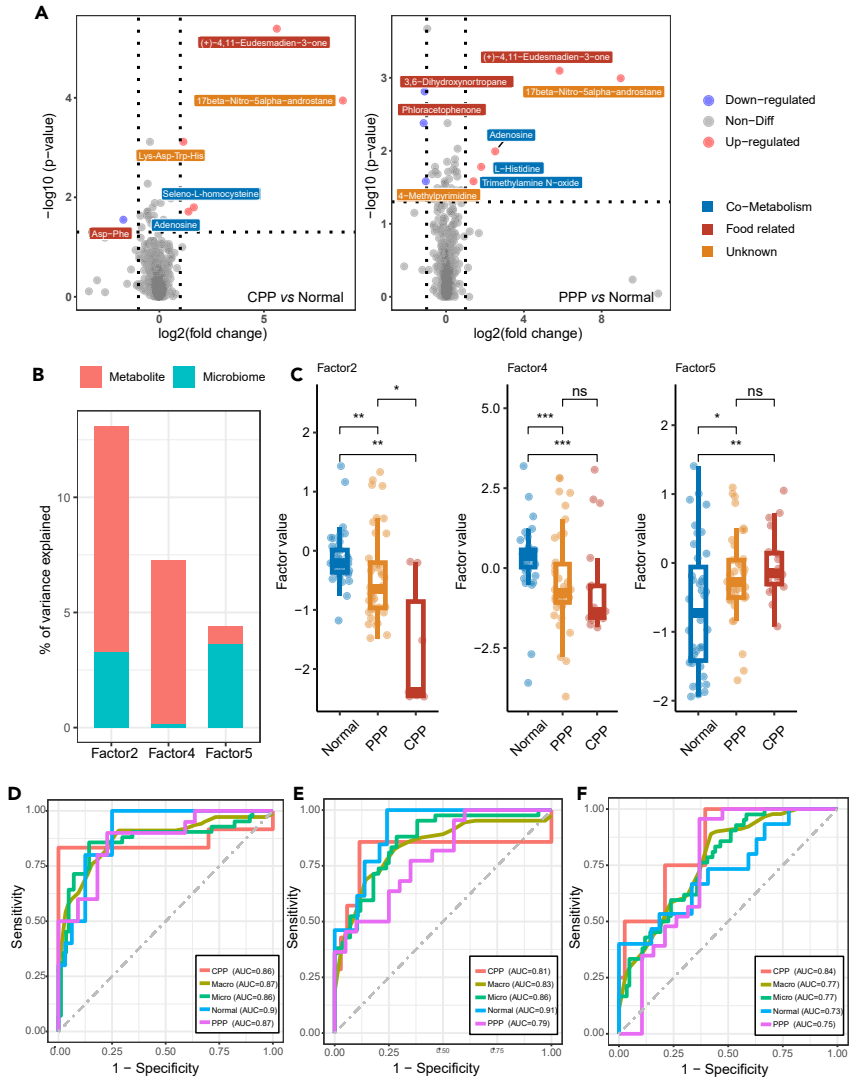


Figure 3. Effect and diagnostic potential of microbiota-metabolite factors for PPP and CPP

(A) Volcano plots were used to visualize the up-regulated (represented by red dots) and down-regulated metabolites (represented by blue dots) in CPP and PPP compared to the normal groups, with statistical significance represented by $p < 0.05$ and |log2FoldChange|>1. Metabolites from different sources were labeled with different colors.

(B) The proportion of variance explained by microbiome and metabolome in microbiota-metabolite factors including Factor 2, Factor 4, and Factor 5.

(C–E) Boxplots were used to display the factor values of Factor 2, Factor 4, and Factor 5 in CPP, PPP, and normal groups. The macro- and micro-average area under the receiver operating characteristic curve (AUC) for values of Factor 2 (D), Factor 4 (E), and Factor 5 (F) demonstrated excellent diagnostic value for PPP and CPP. Statistical significance was indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). CPP, central precocious puberty; PPP, peripheral precocious puberty; Non-Diff, non-differential; AUC, area under the curve.

See also Figures S2 and S3, Tables S5–S7.

Microbiota-metabolite interactions play a mediating role in diet-related precocious puberty

Supported by the above findings, potential relationships between gut microbiota, metabolites, dietary patterns, and disease characteristics were investigated. The Spearman correlation showed that the combined microbiota-metabolite factors were not only associated with the consumption of complex carbohydrates and high-protein diets, but also closely related to the clinical variables of girls with precocious puberty (Figure 4A; Table S8). As a risk factor for precocious puberty, Factor 5 was associated with lower fruit intake and higher levels of LH and FSH. The genus *Bacteroides* contributed most to Factor 5 (Figure S3C). Factor 4 emerged as a beneficial combination of microbiota and metabolites for both PPP and CPP due to its ability to reduce E2 levels and bone age, which tend to increase with a higher intake of nuts, cereal, seafood, freshwater products, and soybeans and their products. Importantly, Factor 4 acted as a mediator for the effect of nuts, cereal, seafood, and soybeans and their products on E2 levels. The genus *Prevotella* accounted for a significant proportion of the bacteria contributing

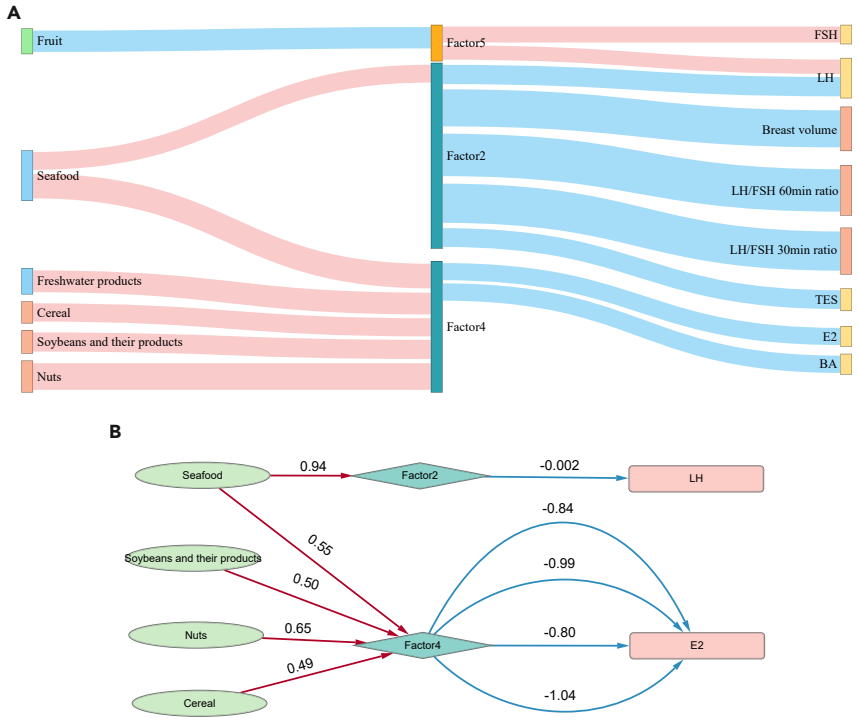


Figure 4. Dietary pattern-microbiota-metabolite interaction associated with precocious puberty

(A) Sankey plot was used to illustrate the Spearman correlation between dietary patterns, microbiota-metabolite factors, and clinical variables associated with precocious puberty. In this plot, the nodes represent dietary patterns, microbiota-metabolite factors, and clinical variables related to precocious puberty. The red and blue links indicate positive and negative correlations between the nodes, respectively.

(B) Results of the meditation analysis: Nodes represented by ellipses, diamonds and rectangles correspond to dietary patterns, microbiota-metabolite factors and clinical variables, respectively. Edges between nodes are color coded in red and blue to represent positive and negative effects. BA, bone age; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; TES, testosterone.

See also Table S8.

to Factor 4 (Figure S3B). Factor 2 has a potential benefit in the development of precocious puberty and prevention of PPP to CPP by reducing LH levels, TES levels, GnRH stimulation test results and breast volume. The obesity-related microbiota and metabolites contribute most to Factor 2 (Figure S3A). Increased seafood consumption could increase Factor 2 levels, and mediation analysis confirmed that Factor 2 partially mediated the reduced effect of seafood on LH levels (Figures 4A and 4B).

DISCUSSION

There is increasing evidence suggested that dietary patterns, gut microbiota, and metabolism play significant roles in the growth of children.[25,26] However, a clear understanding of the effects of dietary patterns, gut microbiota, serum metabolism, and their interactions in the pathogenesis and different clinical types of precocious puberty is still lacking. In this study, we performed a comprehensive analysis and found that girls with precocious puberty exhibited altered dietary patterns and significant dysbiosis of the gut microbiome and serum metabolome. Furthermore, the disruption of microbiota-metabolite interactions may directly influence the development of precocious puberty and potentially mediate hormonal imbalances associated with dietary patterns, thereby influencing the progression and classification of precocious puberty.

Dietary habits have the ability to modify gut microbiota and play a crucial role in the development of various metabolic diseases.[27,28] This study found that high-protein and complex carbohydrate diets may potentially have a protective effect on girls with PPP and CPP. Among these, certain beneficial dietary factors, such as nuts, seafood, freshwater products, and livestock and poultry meat, were associated with a reduced risk of both conditions.

Correspondingly, a significant shift in the microbial composition was detected in girls with precocious puberty, suggesting a gut microbial dysbiosis that may play a role in the development of this condition. Notably, the gut microbiota of girls with PPP and CPP showed adult-like characteristics such as species diversity, dominant species, and key species. Typically, children have lower levels of gut microbiome diversity than adults.[29] However, this study found that girls with PPP and CPP had higher alpha diversity. Previous research has shown that *Bacteroides* becomes one of the dominant bacteria during the transition from infancy to adulthood, and that enterotypes controlled by *Bacteroides* exhibit a more sophisticated pattern of increased diversity and stronger connections within the gut microbial

community.[30] Consistent with these findings, the dominant and significantly upregulated ASVs in girls with PPP (*Bacteroides ovatus* and *Bacteroides cellulosilyticus*) and CPP (*Bacteroides vulgatus*, *Bacteroides massiliensis*, and *Bacteroides cellulosilyticus*) in this study were predominantly of the *Bacteroides* genus. In addition to the *Bacteroides genus*, other SCFAs-producing bacteria such as *Parabacteroides merdae*, *Butyricicoccus*, *Ruminococcus*, and *Erysipelatoclostridium ramosum* were also increased in the girls with precocious puberty. SCFA-producing bacteria have been found to gradually increase in number during childhood and develop a composition similar to that of adults.[31,32] These bacteria can produce glycoside hydrolases, which catalyze the breakdown of glycosidic bonds within carbohydrates, including complex carbohydrates found in dietary fiber and yielding SCFAs (e.g., butyrate and propionate, etc.).[33] Accumulating evidence suggests that SCFAs-producing bacteria, as well as SCFAs themselves, play a role in the process of precocious puberty, although their specific functions remain controversial. Wang et al. found a reduction in primary SCFAs production in the colon of female rats with high-fat-induced early puberty, and the addition of SCFAs effectively reversed this condition by reducing hypothalamic GnRH release and delaying the development of the gonadal axis via the Kiss1–GPR54–PKC–ERK1/2 pathway.[11] In contrast, increased abundance of SCFAs-producing bacteria have been found in girls with precocious puberty,[12,34] while decreased levels of SCFAs have been found in fecal samples of female rats with aspartame-induced delayed puberty onset.[35] SCFAs have the ability to activate endogenous free fatty acid receptors (FFARs) such as FFAR2 and FFAR3, leading to enhanced leptin expression. Consequently, high levels of microbiota-derived SCFAs have the potential to induce early puberty onset by enhancing the leptin-stimulating activity of Kiss1 and GnRH.[36,37] Additionally, butyrate has been shown to promote LH and FSH levels in *in vitro* experiments.[38,39] It is worth noting that, Factor 5, which represents a microbiota-metabolite interaction cluster in this study, contributed mainly by the *Bacteroides* genus and another SCFA-producing genus (*Faecalibacterium*) were also significantly up-regulated in girls with CPP and PPP. Moreover, Factor 5 also had a positive effect on the serum levels of LH and FSH. These findings potentially provided support for the promoting effect of SCFA-producing bacteria on risk of precocious puberty. Taken together, the transition of the gut microbiome toward an adult-like composition, including increased alpha diversity and elevated abundance of SCFAs-producing bacteria, may serve as a potential trigger for precocious puberty. However, further research is needed to validate and explore the underlying mechanisms. Considering the primary metabolites of Factor 5, a number of small peptides, including Ile-Phe, Glu-Gln-Ala-Ala, Asp-Phe, and Phe-Phe, have been identified as having a negative effect on the value of Factor 5. Additionally, metabolites associated with nucleic acid metabolism, such as hypoxanthine, 5-Acetylamino-6-formylamino-3-methyluracil and pseudouridine were found to influence Factor 5 value as well. These findings emphasized the potential influence of amino acid and nucleic acid metabolism on the process of precocious puberty, consistent with previous studies.[40,41]

In contrast, another microbiota-metabolite interaction cluster, Factor 4 was found to be beneficial for girls with precocious puberty by reducing serum E2 levels and bone age. Additionally, Factor 4 mediated the complex carbohydrate and seafood-induced reduction in E2 levels. The bacterial strains that contributed most to Factor 4 were predominantly from the *Prevotella* genus (e.g., *Prevotella copri*, etc.), and possess complex carbohydrate-degrading enzymes. These bacterial strains have been reported to be enriched in individuals with a complex carbohydrate diet.[42,43] Recent studies suggested that certain species within the *Prevotella* genus, such as *Prevotella intermedia,* have the ability to synthesize steroid-metabolizing enzymes for steroid synthesis and catabolism, and to metabolize estradiol and progesterone.[44,45] The prevalence of the *Prevotella* genus has been found to be higher in males than in females,[46] as well as in postmenopausal women than in premenopausal women.[47] Correspondingly, anoestrous sows presenting with lower plasma estradiol levels also show increased abundance of the *Prevotella* genus.[44] The beneficial effect of consuming complex carbohydrate and seafood consumption on Factor 4, together with the negative effect of Factor 4 on E2 levels in the present study, supports previous research. In addition, the reduction of several lipoperoxidation products, including 13-KODE, 9(R)-HODE, (alpha)13-HpODE, (alpha)12(13)-EpOME and 13-HpOTrE(r), was found to be associated with Factor 4. An increase in these lipid peroxidation products is often considered to be an indication of increased oxidative stress, which results from an imbalance between reactive oxygen species (ROS) and antioxidants, leading to the accumulation of excess ROS such as free radicals and peroxides.[48] Research suggests that oxidative stress may escalate during puberty due to an increase in the release of lipoperoxidation products.[49] Similarly, elevated levels of several lipoperoxidation-related metabolites have been found in children with CPP, suggesting a link between early puberty and increased oxidative stress.[50] These findings highlight the potential benefit of Factor 4 in reducing the risk of CPP and PPP by reducing oxidative stress. Furthermore, our mediation analysis suggests that the *Prevotella* genus may increase as complex carbohydrate and seafood consumption increases, leading to a reduction in E2 levels due to estradiol metabolism, and therefore may have potential to reduce the risk of both PPP and CPP.

Factor 2 also acts as an important beneficial microbe-metabolite cluster for the risk of precocious puberty and plays a role in the prevention of PPP to CPP. This is achieved by reducing levels of LH and TES, GnRH stimulation test results, and breast volume. The primary components of the Factor 2 microbiota are associated with childhood obesity. Among these, *Eubacterium rectale*, *Erysipelotrichaceae*, *Fusicatenibacter*, and *Intestinibacter* have shown a positive association with the obesity risk.[51–53] Conversely, *Dialister invisus*, *Romboutsia*, *Subdoligranulum*, *Blautia*, and *Faecalibacterium* have been shown to have an anti-obesity effect. Reductions in *Dialister invisus*, *Faecalibacterium*, and *Subdoligranulum* have been observed in obese individuals.[54–56] Furthermore, the prevalence of *Subdoligranulum* was found to be negatively correlated with various obesity-related indices such as fat mass, BMI, adipocyte diameter, insulin resistance, leptin and insulin levels.[56,57] Mendelian randomization analyses confirmed the causal relationship between increased *Romboutsia* and decreased risk of childhood obesity.[58] *Blautia* has the potential to reduce HFD-induced obesity and insulin resistance among rats.[59,60] Among the major metabolites contributing to Factor 2, *p*-coumaric acid and 2-amino-8-oxo-9,10-epoxy-decanoic acid are also involved in maintaining a healthy weight. P-coumaric acid, being a phenolic acid, has anti-obesity effects by promoting fat metabolism and reducing inflammation.[61,62] And 2-amino-8-oxo-9,10-epoxy-decanoic acid is a sphingolipid-like compound that is structurally related to sphingosine, and alterations in

sphingolipid metabolism can potentially lead to insulin resistance, inflammation, and adipose tissue dysfunction, all of which are associated with obesity.[63,64] As childhood obesity is a considerable risk factor for precocious puberty,[5] this study proposes that the prevention of obesity-induced precocious puberty by reducing LH levels is mediated by the interplay between microbiota and metabolites within Factor 2. Additionally, seafood is a nutritious source of high-quality protein and essential nutrients such as vitamin D, vitamin B12, niacin, pantothenic acid, and omega-3 fatty acids. Seafood consumption has been shown to be negatively associated with BMI and obesity.[65] Our results highlight the mediation role of the microbiota-metabolite interplay in Factor 2 in the reducing effect for seafood on body weight and the associated reduction in LH levels.

Furthermore, the interplay between the microbiota and metabolites including Factor 2, 4, and 5 may prove to be non-invasive and reliable diagnostic biomarkers for the early detection of precocious puberty, contributing to avoid the time-consuming and painful GnRH stimulation test.

In conclusion, significant alterations of diet patterns, and dysbiosis of the gut microbiome and metabolome were observed in girls with precocious puberty. The shift of the gut microbiome toward a more adult-like profile, characterized by increased alpha diversity and an abundance of SCFA-producing bacteria, may potentially trigger precocious puberty through its involvement in the activation of the HPG axis and the elevation of serum LH and FSH levels. *Prevotella* plays a crucial role in microbiota-metabolite interactions that may be influenced by dietary factors such as increased intake of complex carbohydrates and seafood. These interactions could lead to a reduction in E2 levels through estradiol metabolism, potentially reducing the risk of precocious puberty. Furthermore, the interaction between obesity-related bacteria and metabolites may mediate the beneficial effect of seafood in reducing the risk of obesity-induced precocious puberty and the prevention of PPP to CPP. Overall, this study provides valuable insights into the complex interactions between dietary patterns, gut microbiota, and metabolites in the onset, development, and clinical classification of precocious puberty, which may provide clues for prospective diagnosis and therapeutic strategies for precocious puberty.

Limitations of the study

Limitations of this study must be stated. As a cross-sectional study, we were unable to track the longitudinal changes in the microbiome and metabolome during the processes of precocious puberty.

Moreover, it is challenging to determine whether the alterations in the microbiome and metabolome were triggers or consequences of precocious puberty. Therefore, the causality of this association remains uncertain. Further functional experiments and clinical validation are needed to explore the potential causal links and mechanisms between dietary patterns, gut microbiota, and metabolites in the context of precocious puberty.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Fast DNA SPIN Kit for feces	MP Biomedicals	Cat# 116570200
Deposited data		
Sequencing data	This study	NODE: OEP000731
Software and algorithms		
QIIME2 version 2020.2	Bolyen et al., 2019[66]	https://docs.qiime2.org/2020.2/install/
ProteoWizard version 3.0.7414	Chambers et al., 2012[67]	http://proteowizard.sourceforge.net/
phyloseq version 1.34.0	McMurdie and Holmes, 2013[68]	http://www.bioconductor.org/packages/release/bioc/html/phyloseq.html
PICRUSt2 version 2.3.0	Douglas et al., 2020[69]	https://github.com/picrust/picrust2
mixOmics version 6.22.0	Rohart et al., 2017[70]	https://bioconductor.org/packages/release/bioc/html/mixOmics.html
vegan version 2.5.7	Dixon, 2003[71]	https://mirrors.tuna.tsinghua.edu.cn/CRAN/web/packages/vegan/index.html
MOFA2 version 0.99.5	Argelaguet et al., 2018[72]	https://bioconductor.org/packages/release/bioc/html/MOFA2.html
multiROC version 1.1.1	Wei et al., 2018[73]	https://mirrors.tuna.tsinghua.edu.cn/CRAN/web/packages/multiROC/index.html
networkD3 version 0.4.	Allaire et al., 2017[74]	http://christophergandrud.github.io/networkD3/
mediation version 4.5.0	Tingley et al., 2014[75]	https://cran.ms.unimelb.edu.au/web/packages/mediation/index.html
R version 4.2.2	https://cran.r-project.org/doc/manuals/fullrefman.pdf	https://www.r-project.org

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhongtao Gai (gzt@etyy.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The datasets generated and analyzed during the current study have been deposited at NODE (http://www.biosino.org/node) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional data required to reanalyzed the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study participants

In this study, girls with CPP and PPP were diagnosed and recruited at Children's Hospital Affiliated to Shandong University (Table 1). The inclusion criteria for study participants were strictly limited to female individuals from 5 to 8 years of age. The case group consisted of girls who presented to the hospital for examination due to the development of secondary sexual characteristics and were eventually diagnosed with precocious puberty. This study contained 21 girls who were diagnosed with CPP in accordance with the following criteria[12,13,76]: (1) Secondary sexual characteristics (e.g., breast development, pubic hair and axillary hair growth) before the chronological age (CA) of 8 years; (2) Baseline LH levels >0.2 IU/L; (3) LH peak levels >5 IU/L and peak ratio of LH/FSH ratio >0.6 in the GnRH stimulation test And one of the following criteria (i) ovarian volume >1 mL, and multiple follicles >4 mm; (ii) advanced bone age ≥1 year above that for CA. This study also included 45 girls

diagnosed with PPP, which was distinguished from CPP by low baseline LH levels and suppressed levels on GnRH stimulation. Exclusion criteria for girls with CPP and PPP included (1) other organic etiologies with the presence of an isointense tumor on magnetic resonance imaging (MRI); (2) usage of antibiotics, probiotics, or prebiotics within 3 months prior to enrollment; (3) associated endocrine, gastrointestinal, metabolic disease (including obesity and diabetes, among others), mental disease, or hepatobiliary disease. In total, 48 age-matched girls who were generally in good health and without any self-reported disease and had no secondary sexual development were recruited as controls. The study protocol was maintained in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Children's Hospital Affiliated to Shandong University (ETYY-2016-202). Written informed consents and questionnaires were obtained from the children's parents.

METHOD DETAILS

Clinical laboratory tests

Clinical parameters were determined at the clinical laboratory of Children's Hospital Affiliated to Shandong University. The body weight and height of all participants were measured by a well-trained nurse. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. The levels of trace element including Cu, zinc (Zn), calcium (Ca), Mg, iron (Fe), and lead (Pb) from serum samples were measured using the flame atomic absorption method (BH5100, Bohui, China). The hormone test was conducted by analyzing the serum levels of FSH, LH, E2, TES; prolactin (PRL) and pregestational hormone (PRG); employing the chemi-luminescence immunoassay methods (Abbott, Architect I2000, US). GnRH stimulation test was conducted for the LH and FSH utilizing the chemi-luminescence immunoassay methods (Abbott, Architect I2000, US). The size of the uterus, breasts, and ovaries, and the number of ovarian follicles were determined by employing B-ultrasonic examination (EPIQ5, L12-5, Philips, Holland). The hand-wrist radiographs were used for bone-age assessment through nuclear magnetic resonance (MRI) examination (Digital Dianost3, Philips, Holland).

Sample collection

Stool and blood samples were collected from each participant and stored at −80°C before analysis. 200 mg stool was preserved in sterile 2 mL tubes containing pure ethanol, aliquoted (Tinygene Biological Company, China) and stored at −80°C for 16S rRNA sequencing. Blood samples were thawed at 4°C, 3000 rpm, and centrifuged at 4°C for 10 min. Serum aliquots were immediately frozen at −80°C for further untargeted metabolomics analysis.

DNA extraction and Illumina sequencing

Total DNA extraction from fecal samples (250 mg, wet weight) was performed using a Fast DNA SPIN Kit for feces (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions. The V1-V2 hypervariable region was amplified with the universal primer pair F27 (5′ -AGAGTTTGATCMTGGCTCAG-3′) and R355 (5′- GCTGCCTCCCGTAGGAGT -3′). Sequencing was conducted on Illumina HiSeq 2500 System (Illumina Inc., San Diego, CA, USA) using the 2 × 250 paired-end mode following the standard Illumina platform protocols. All sequencing data is available at NODE (http://www.biosino.org/node) with the accession number OEP000731).

16S rRNA gene sequencing data analysis

The 16S rRNA sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME2 version 2020.2).[66] In brief, raw sequence data were demultiplexed and DADA2[77] was employed to denoise sequencing reads for quality control, chimera detection and removal and the identification of ASVs via q2-dada2 plugin. Taxonomy classification was performed by utilizing classify-sklearn based on a Naive Bayes classifier against the Silva-138-99 reference sequences.[78] Samples with more than 10,000 ASVs were retained for the following analysis. ASVs with a total abundance (summed over all samples) of less than 20 were filtered out. Additionally, the abundance data at the taxonomic level was generated by aggregating ASVs according to their taxonomic classifications. Relative taxonomic abundance data was then calculated by dividing the count of each taxon by the total count.

Untargeted metabolomics and analysis

For each sample, 100 μL of serum was transferred to an eppendorf tube. After the addition of 300 μL of methanol (containing internal standard 1 μg/mL), the samples were vortexed for 30 s, followed by sonication for 10 min in ice-water bath, and incubation for 1 h at −20°C to precipitate the proteins. The sample was then centrifuged at 12000 rpm for 15 min at 4°C. The resulting supernatants were then transferred to LC-MS vials and stored at −80°C until the UHPLC-QE Orbitrap/MS analysis. The quality control sample was prepared by mixing an equal aliquot of the supernatants from all the samples collected.

LC-MS/MS analyses were performed using an UHPLC system (1290, Agilent Technologies) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to Q Exactive (Orbitrap MS, Thermo). The mobile phase A was 0.1% formic acid in water for positive, and 5 mmol/L ammonium acetate in water for negative, and the mobile phase B was acetonitrile. The elution gradient was set as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; 12 min, 1% B. The flow rate was 0.5 mL/min. The injection volume was 2 μL. The QE mass spectrometer was utilized due to its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Xcalibur 4.0.27, Thermo) continuously examines the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. ESI source conditions were set as follows: Sheath gas flow rate as 45

Arb, Aux gas flow rate as 15Arb, Capillary temperature at 400°C, Full ms resolution as 70000, MS/MS resolution as 17500, Collision energy as 20/40/60 eV in NCE model, Spray Voltage as 4.0 kV (positive) or −3.6 kV (negative), respectively.

Raw data was converted to mzXML format and processed using ProteoWizard (version 3.0.7414).[67] Preprocessed results were employed to generate a data matrix which consisted of the retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. In-house MS2 database was applied for metabolites identification.

Associations analysis between gut microbiome and girls with CPP and PPP

We first performed the microbiome analysis based on the relative taxonomic abundance data. Briefly, the distribution of the top 10 most abundant bacterial species in CPP, PPP and normal control groups were analyzed and displayed by stack bar plot; the differential taxa between PPP, CPP and normal groups at all taxonomic levels were further identified by using the Linear discriminant analysis Effect Size (LEfSe) analysis. Then the alpha diversity, beta diversity (Principal coordinate analysis based on Bray–Curtis distance) and the gut microbiota functions (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2, PICRUSt2) were compared among PPP, CPP and normal groups, using the ASVs data. Except for the PICRUSt2 analysis, which was performed using the PICRUSt2 (version 2.3.0) software,[69] all other analyses were performed using the phyloseq package (version 1.34.0) in R.[68]

Identification of latent dietary patterns associated with PPP and CPP

The dietary questionnaire used in this study was developed based on the Chinese Dietary Guidelines for School-Age Children (2022)[79] and the Dietary Guidelines for Chinese Residents (2022),[80] taking into account the most commonly consumed foods that are clinically considered to be closely related to precocious puberty. It consisted of 37 questions, including 24 questions on a 4-level food intake frequency scale and 13 questions on personal dietary preferences with binary variables. Each question has a unique meaning, and the type and detailed definition of each question were shown in Table S1. The questionnaires were administered to the participants and their parents in the presence of trained doctors, who provided professional guidance throughout the whole process. Univariate logistic regression analysis was employed to filter the latent dietary patterns contributing to the risk of PPP and CPP, which were retained for the following research. In which, dietary patterns that exhibited no variability within specific groups were excluded from this analysis to ensure its validity and effectiveness. The common latent dietary patterns associated with both PPP and CPP were further analyzed using Venn analysis.

Microbiome clustering based on precocious puberty-related dietary patterns

To gain insight into how dietary patterns and gut microbial communities may interact with precocious puberty, we performed a series of multivariate analyses. First, the microbial community data matrix was calculated based on Bray–Curtis distance by using the R package phyloseq (version 1.34.0). Then, using the R package mixOmics (version 6.22.0),[70] sPLS analysis was conducted based on the obtained data matrix of the microbial community and clinical factors associated with precocious puberty (latent dietary patterns, hormones, trace elements, etc.) followed by hierarchical clustering analysis using Pearson's correlation. Finally, ASVs with an absolute correlation coefficient value of >0.25 in sPLS were retained for subsequent CCpnA to assess possible bidirectional associations between patient characteristics, dietary patterns, hormones and microbiota using the R package vegan (version 2.5.7).[71] A variance inflation factor (VIF) analysis was performed to address collinearity issues in this process. Variables with VIF values greater than 10 generally indicate high multicollinearity, suggesting that the predictor variable is highly correlated with other predictors in the model. Therefore, only variables with VIF values less than 10 were retained for the CCpnA analysis.

Association between gut microbiota-metabolite interaction and precocious puberty risk

To explore the alteration of the metabolome in precocious puberty, differentially abundant metabolites in PPP and CPP compared to normal controls were identified by *Wilcoxon* test and log2 fold change analysis. Volcano plots was generated to display these differentially abundant metabolites. The origins of these identified metabolites were identified using the website, MetOrigin website (https://metorigin.met-bioinformatics.cn/home/).[81] To further investigate the function of microbiota-metabolite interactions in precocious puberty, we first performed the integration of metabolite abundance data and the ASVs data of microbiome by using the MOFA2 R package (v0.99.5)[72] and microbiota-metabolite combination factors were generated. The variance explained per factor in both omics and the total variance explained by microbiome and metabolome, respectively, were calculated. Then, the microbiota and metabolites contribute to each factor with the top 10 weights were analyzed and identified. After comparing of the factor value between PPP, CPP and normal controls, the latent microbiota-metabolite combination factors related PPP and CPP were identified.

Diagnostic power of microbiota-metabolite factors in PPP and CPP

The potential for identifying latent microbiota-metabolite factors useful for predicting PPP and CPP was tested and evaluated by using the multiROC R package (version 1.1.1).[73] We first randomized all participants into a 60%/40% split group for training and validation sets. A random forest model was constructed from the training sets to predict the discriminative effect of latent microbiota-metabolite factors in PPP, CPP and normal groups. The performance of the model was then evaluated on the validation set. Finally, the receiver operating characteristics (ROC) curve was used to illustrate the diagnostic ability to predict each group. The area under the curve (AUC) was used as a measure of the overall ability of the classification model to predict different groups. The micro-average ROC curve and macro-average ROC curve

were also calculated to evaluate the performance of the multi-class classification model. A greater AUC indicates a more useful and effective classification model.

Diet-microbiota-metabolite interaction in PPP and CPP

Spearman correlations were calculated between latent dietary patterns, microbiota-metabolite factors and clinical variables associated with PPP and CPP. Correlations with $p \leq 0.05$ were retained and visualized by Sankey diagram using the networkD3 R package (version 0.4).[74] To test and estimate how microbiota-metabolite interactions exert their influence on dietary-driven clinical variables associated with PPP and CPP, mediation analysis was conducted between latent dietary patterns, microbiota-metabolite factors and clinical variables using the mediation R package (version 4.5.0).[75]

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using R (4.2.2). Measurement data are represented as mean $\pm$ standard deviation. The two-sided Wilcoxon rank-sum test and the Kruskal-Wallis test were employed to compare the continuous variables between two groups and three groups, respectively. Differences in distance matrix between groups were tested using permutational multivariate analysis of variance (PERMANOVA) test. The Benjamini-Hochberg test was used to adjust p values for multiple comparisons. Differences were considered statistically significant when $p < 0.05$.