FABAD J. Pharm. Sci., 48, 3, 523-538, 2023
Doi: 10.55262/fabadeczacilik.1344851
RESEARCH ARTICLE

Metabolomic Studies in Girls With Central and Peripheral Precocious Puberty

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SUMMARY

Precocious puberty (PP) is the beginning of secondary sexual characteristics before eight years of age in girls. "Central precocious puberty (CPP)" occurs due to early activation of the hypothalamus-pituitary-gonad axis. "Peripheral precocious puberty (PPP)" is a rarer, and different condition that sidelines the hypothalamus-pituitary-gonad (HPG) axis, and it depends on the peripheral causes. Metabolomics is the identification, and quantitation small molecule metabolites (<1000 Da) in a certain period. This study aimed to determine the plasma, and urinary metabolic profiles of girls, who were diagnosed with CPP (n=50) or PPP (n=47), and compare their results to control group (n=50). Metabolomics analysis was performed by using gas chromatography-mass spectrometry. After the complex chromatograms were deconvoluted, and aligned, the metabolites were identified using retention index libraries. The results were evaluated statistically using univariate, and multivariate analysis. Binary compressions were performed between groups, and metabolites from amino acids were found to be significantly different between the groups. These alterations in metabolites are suggested for potential biomarkers of PP; however, more comprehensive studies are needed to verify these data, and for validation. In the future, the metabolic alterations underlying different diseases, particularly those of endocrine origin, should be evaluated with mechanistic toxicological studies. This will help the researchers to develop new therapy options, particularly for PP.

Key Words: *Central precocious puberty, gas chromatography-mass spectrometry, metabolomics, peripheral precocious puberty.*

Merkezi ve Periferik Erken Ergenliği Olan Kız Çocuklarında Metabolomik Çalışmalar

ÖZ

Puberte prekoks (PP), kızlarda sekonder cinsel özelliklerin sekiz yaşından önce başlamasıdır. Hipotalamus hipofiz-gonad ekseninin erken aktivasyonu nedeniyle "merkezi puberte prekoks (CPP)" meydana gelir. "Periferik puberte prekoks (PPP)" hipotalamus-hipofiz-gonad (HPG) aksına bağlı olmayan daha nadir görülen farklı bir durumdur ve periferik nedenlere bağlıdır. Metabolomiks, küçük molekül metabolitlerinin (<1000 Da) belirli bir süre içinde tanımlanması ve nicelendirilmesidir. Bu çalışmada CPP (n=50) ve PPP (n=47) tanısı alan kız çocukların plazma ve idrar metabolik profillerinin belirlenmesi ve sonuçlarının kontrol grubu (n=50) ile karşılaştırılması amaçlanmıştır. Metabolomik analiz, gaz kromatografisi-kütle spektrometresi kullanılarak yapılmıştır. Karmaşık kromatogramlar ayrıştırıldıktan ve düzenlendiken sonra, metabolitler tutunma indeksi kitaplıkları kullanılarak tanımlanmıştır. Sonuçlar, tek değişkenli ve çok değişkenli analiz kullanılarak istatistiksel olarak değerlendirilmiştir. Gruplar arasında ikili karşılaştırmalar yapılmış ve amino asitlerin metabolitlerinin gruplar arasında önemli ölçüde farklı olduğu bulunmuştur. Bu metabolitlerdeki değişiklikler potansiyel PP biyobelirteçleri olarak önerilebilir; ancak bu verileri doğrulamak ve validasyon için daha kapsamlı çalışmalara ihtiyaç vardır. Gelecekte, farklı hastalıkların, özellikle endokrin kaynaklı olanların altında yatan metabolik değişikliklerin, mekanistik toksikolojik çalışmalarla değerlendirilmesi gerekmektedir. Bu, araştırmacıların özellikle PP için yeni tedavi seçenekleri geliştirmelerine yardımcı olacaktır.

Anahtar Kelimeler: *Merkezi puberte prekoks, gaz kromatografisi gaz kromatografisi-kütle spektrometrisi, metabolomik, periferal puberte prekoks.*

Received: 17.08.2023
Revised: 28.09.2023
Accepted: 29.09.2023

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INTRODUCTION

Endocrine disorders are observed with increasing frequency in children. They may lead to physiological, pathological, metabolic, and psychological problems in childhood, and adulthood (Shin 2016, Schoelwer &Eugster 2016).

Puberty development starts with breast development, and pubic hair growth (Shin, 2016; Schoelwer &Eugster). Precocious puberty (PP) is the development of secondary sex characters before the age of eight in girls. In PP, breast growth is observed <8 years (Berberoğlu 2009). Increases in sex steroids are observed in both central precocious puberty (CPP), and peripheral precocious puberty (PPP). As a result, growth rate, somatic development, and skeletal maturation increase (Kiess et al., 2016).

Primary mechanism of CPP is early activation of the hypothalamus-pituitary-gonad (HPG) axis. The most common type is idiopathic CPP (ICPP), and its underlying mechanism is unknown. Genetic factors, neurotransmitter, and hormone interactions might lead to ICPP (Kletter et al., 2015, Fuqua, 2013). CPP is diagnosed by hormonal evaluation (Ritzén 2003). HPG axis is disabled due to peripheral reasons in PP. PPP may occur due to several pathologies (Ritzén 2003). In PPP, somatic development accelerates, and there is a significant improvement in bone age (Schoelwer &Eugster 2016; Berberoğlu, 2009; Fuqua, 2013).

There is no early biomarker for CPP, and PPP. It is crucial to detect, and treat symptoms of PP before it causes later life effects (Berberoğlu, 2009). Measurement of gonadotrophin releasing hormone (GnRH) stimulation for diagnosis is time-consuming, and costly (Poomthavorn et al., 2009). There is a need for easily measured biomarkers that can be used routinely for early diagnosis of PP.

Metabolomics is the identification, and quantification of small molecule metabolites in biological samples over a period. Each biological specimen has its own metabolomic profile even collected from same individual, and these differences can be used for identifying a biomarker. Therefore, to obtain a more comprehensive profile, different biological specimens from one individual must be used (Zeki et al., 2020). It is necessary to determine new biomarkers that can be used for the calculation of disease risks with untargeted analyses at the first step, and then to prove their validity with targeted studies in the second step (Liu & Locasale, 2017; Newgard ,2017; Haug et al., 2017; Zampieri et al., 2017).

In a study performed by Yang et al., (2012), urine samples were taken from patients with CPP (n=86), and healthy girls (n=144) of the same age, and their urine metabolites were compared. The presence of abnormal amino acid metabolism in the CPP group suggested an abnormal activity of the neuroendocrine system. Alterations in amino acid metabolism were suggested to contribute to the pathogenesis of CPP by activating the HPG axis, and suppressing the hypothalamus-pituitary-adrenal axis (HPA) axis (Yang et al., 2012).

Qi et al., (2012) collected urine samples from healthy children (n=57), PPP patients (n=49) CPP patients (n=57). Researchers observed differences in amino acid metabolism of children with CPP vs. PPP, and control group. Alterations in metabolic profiles of children with CPP who used the drug "triptorelin (a GnRH agonist)" in their treatment was normalized (Qi et al., 2012).

The aim of this study was to determine the metabolic plasma, and urinary profiles of girls diagnosed with CPP, and PPP. We targeted to determine the metabolic biomarkers/indicators that might be used to diagnose of both CPP, and PPP.

MATERIAL, AND METHODS

Chemicals, reagents, and preparation

Urease buffer reagent (Sigma-Merck, Manheim, Germany) was purchased, and prepared as 15U in ultrapure water. Myristic acid D27 (Sigma-Merck,

Manheim, Germany) was weighed in the amount to be used, and dissolved in methanol: water (8:1, v/v) mixture as 1 µg/mL. Methoxyamine hydrochloride (MeOx, 98%) was also purchased from Sigma-Merck. N-methyl-N-trimethylsilyltrifluoroacetamide+trimethylchlorosilane (MSTFA+1% TMCS) was obtained from Thermo-Scientific (TS48915; Waltham, MA) as ready-to-use ampoules. All other chemicals were also purchased from Sigma-Merck.

Ethical Approval

The ethical approval was received from Hacettepe University Non-Interventional Clinical Research Ethics Committee in 2017 (Ethics approval no: GO 17/613-06).

Study groups

The study groups were as follows:

I. CPP Group (n=50) consisted of girls who applied to Keçiören Training, and Research Hospital, Clinic of Pediatric Endocrinology Unit (age range: 8-10). These girls were diagnosed by a pediatric endocrinologist. Breast development before the age of eight, pubic, and/or axillary hair growth, bone age 1 year older than the calendar age, body mass index (BMI) between 25-85% according to age, and gender, peak luteinizing hormone (LH) in GnRH stimulation test response above 5 mIU/mL, and routine pituitary MR imaging were the diagnostic criteria.

II. PPP Group (n=47) consisted of girls who applied to Keçiören Training, and Research Hospital, Clinic of Pediatric Endocrinology Unit (age range: 8-10). These girls were diagnosed by a pediatric endocrinologist. Breast development before the age of 8, pubic, and/or axillary hair growth, bone age 1 year older than the calendar age, BMI between 25-85% according to age, and gender, peak LH in GnRH stimulation test response under 5 mIU/mL were the diagnostic criteria.

III. Control Group (n=50) consisted of healthy volunteers, who were matched with the study groups in terms of age (age range: 8-10).

Girls in all of the study groups did not have any chronic, endocrine or genetic disease. They were not obese, and they did not use drugs or supplements continuously.

Samples from the study groups were collected simultaneously August 2012- July 2013. Using the biological materials, another study was published before, and the endocrine disrupting chemical (EDC) levels were measured in that study. A questionnaire was obtained from both study groups and the control group. In that questionnaire, we asked the possible routes of EDC exposure, and tried to evaluate whether EDC exposure had a role in PPP or CPP (Buluş et al., 2016). However, we found no significant relationship between EDC exposure, and the metabolomics data we obtained herein. Therefore, we did not include those findings to the current work.

Collection, and storage of biological material

Blood, and spot urine samples taken from the CPP, and PPP patients, and healthy volunteers in the Keçioren Training, and Research Hospital, Clinic of Pediatric Endocrinology were brought to the Hacettepe University Faculty of Pharmacy Pharmaceutical Toxicology Laboratory on dry ice. Urine samples aliquoted, and stored at -80°C until analysis. Plasma samples were obtained from the whole blood samples following the centrifugation steps. Plasma samples were aliquoted, and stored at -80°C until analyses.

Metabolomics assays

Metabolomics analyses of urine, and plasma samples performed using GC-MS have been adopted from our previous studies (Eylem et al., 2020; Recber et al., 2020).

Plasma samples, and derivatization

Plasma samples (100 µL) were taken into the small capped locked centrifuge tubes. Later, 900 µL of methanol-water mixture (8:1, v/v) including 1 µg/mL of myristic acid D27 was added to each tube. The mixtures were centrifuged at 3000 rpm for 10 min.

400 µL of the supernatants were taken into other centrifuge tubes, and evaporated to dryness in a vacuum dryer concentrator. 20 µL of MeOx (20 µg/mL in pyridine) was added on residues, and tubes were incubated at 30°C for 90 min with the taps closed. Samples taken were then cooled to room temperature, and derivatized with 80 µL of MSTFA+1% TMCS. The tubes were incubated at 37°C for 30 min. 80 µL from each sample was taken into the silanized inserts that were placed in screw-capped vials.

Urine samples, and derivatization

Urine samples (35 µL) were taken into the small capped locked centrifuge tubes. Later, 70 µL of urease (15 U) was added to each tube, and the mixtures were incubated at 37°C for 15 min. After incubation, the sample preparation was performed same as in plasma samples.

GC-MS analysis

GC-MS (QP2010 Ultra, Shimadzu, Kyoto, Japan) was used for metabolomics analyses. The DB-5MS stationary phase column (30-m + 10-m DuraGuard × 0.25 mm i.d., and 0.25-µm film thickness) was used throughout the experiments.

Statistical analysis

Considering the size of the data obtained, multivariate statistical analysis was used for data evaluation. For this purpose, data matrices were created using MS-DIAL, and transferred to an Excel file. Then, data matrices were normalized using different normalization techniques; Local Polynomial Regression (LOESS), and mean centering for plasma samples, and LOESS, and MS total useful signal (MSTUS) for urine samples. The missing values in the data were filled with the half-value of the smallest concentration in the metabolite group. By transferring this data matrix to statistical method for supervised classification of data (SIMCA-P +) (Umetrics, Sweden) program, the principle component analysis (PCA), and partial least squares, discriminant analysis (PLS-DA) methods were used to determine the metabolites, and regression coefficients that were crucial in separating groups. All data obtained in the PCA analysis were used, and the differences between groups for each metabolite were evaluated. This analysis shows examples of outliers, and possible trends in the data. In the PLS-DA analysis, a more detailed analysis was performed. Which group to analyze was supervised, and specified by the investigator, and the metabolite(s) that were effective in the separation were found. At the end of the analysis, a Variable Important in Project (VIP) chart was obtained. The metabolite that had the most essential role in the distinction between metabolite groups with the highest score in the VIP graph was determined. Some data were specified as unknown (UN). These data showed the metabolites not yet identified in available data libraries. Biomarker c, andidates for early diagnosis of PPP, and CPP were determined by using the data in VIP charts. In VIP charts, the cut-off point was taken as>1.25. The goal of setting cutoffs is to minimize uncertainty in the match results while limiting the number of false categorizations. Other sources have used different cutoff points in metabolomic studies. In general cut-off points are selected by the researcher. In this study, cutoff point was selected according to the detailed information given in a review by Schiffman et al. (2019). As a result, the statistical difference of metabolites was determined by the Student t-test.

RESULTS, AND DISCUSSION

Using MS combined with GC or LC in metabolomics analysis provides both high resolution of the chromatographic system, and the high sensitivity of MS detectors by taking advantage of the strengths of both methods. In both GC-MS, and LC-MS, biological samples can be analyzed for thous, ands of metabolites. These metabolites are separated from each other as much as possible using chromatographic system depending on their different chromatographic properties. Thus, MS provides both an increase in number of metabolites to be analyzed,

and also high sensitivity. Otherwise, different factors like ion suppression or overloading of the MS system may cause a loss in sensitivity. The interactions between metabolites, and chromatographic methos are based on both physicochemical properties of metabolites, and the type of the stationary phase. Such interactions can determine the order of elution in the chromatographic system. It can be stated that analysis of volatile organic compounds, lipids, and derivatizable molecules can be carried out using GC–MS while majority of semi-polar metabolites can be analyzed by using LC–MS. Therefore, the type, and number of metabolites that can be analyzed by GC-MS, and LC-MS are different (Zeki et al., 2020). Considering this data, GC-MS was used in the current work.

When the PLS-DA graphs were examined, the PPP, CPP, and control groups showed different metabolomic profiles (Figure 1a, and 2a). To evaluate the validity studies of PLS-DA models, after r, andom probability analysis of the data matrix (n=100), classification statistics of the models were calculated.

R2 or R2X (for PCA) or R2Y (for PLS-DA) indicates the proportion of the data variance or variance that can be explained by the current model, showing the goodness of fit (the fit). Q2 indicates the proportion of the data variance that can be predicted by the current model, i.e., the prediction rate, indicating the predictive power of the current model (Creative Proteomics Metabolomics. 2023). The fact that the R^2, and Q^2 values produced as a result of the probability analyses are smaller than the values of the method (rightmost points) shows that the validity, stability, and predictive power of the PLS-DA model are sufficient. Permutation analysis results suggest that applied PLS-DA models are valid, and highly predictive, especially for plasma samples. A relatively low Q^2 value was noticed for the PLS-DA model of urine samples. However, low Q^2 values can be observed in clinical metabolomics studies (Chan et al., 2011).

Anthropometric characteristics

The anthropometric characteristics of control, PPP, and CPP groups are given in Table 1. There were no significant differences between the ages, and BMI of the study groups. However, heights of both PPP, and CPP groups were significantly different from control. Height standard deviation (height SDS) was only significant in CPP group. Body weight (BW) of both PPP, and CPP groups were significantly higher than control. Body weight standard deviation (BWSDS) was also higher in both of the PP groups vs. control. Bone age of both PPP, and CPP groups were markedly higher than the control group.

Table 1. Comparison of anthropometric values of three groups.

	Control (n=50)	PPP (n=42)	CPP (n=42)
Chronological age (year)	7.4±0.64	7.4±0.68	7.4±0.61
Height (cm)	122.7±5.0	131.9±5.3[a]	132.6±6.7[a]
Height SDS	-0.21±0.5	1.36±0.8	1.58±0.6[a]
BW (kg)	24.3±3.2	30.0±5.2[a]	31.6±8.0[a]
BWSDS	0.7±0.4	1.2±0.7[a]	1.1±0.7[a]
BMI (kg/m²)	15.9±1.0	17.1±4.0	17.8±2.8
Bone age	6.9±0.6	9.6±0.7[a]	9.9±1.0[a]

BMI: body mass index; BW: body weight; BWSDS: body weight st, andard deviation score; Height SDS: height st, andard deviation score; PPP: peripheral precocious puberty; CPP: central puberty precocious puberty

[a]$p<0.05$ vs. control.

Metabolomics analyses

After the plasma, and urinary metabolomic profiling studies were carried out, the number of metabolites defined in plasma, and urine samples were 130, and 169, respectively (excluding unknown metabolites-UN)

The differentiation between groups was examined with the PLS-DA graph. We observed that the control group showed a different metabolic profile than the patient groups in both plasma, and urine samples (Figure 1a for plasma; Figure 2a for urine). Metabolites leading to differentiation in the PLS-DA graph are shown in VIP plots (Figure 1b for plasma; Figure 2b for urine).

Later, binary comparisons were made between paired groups for both plasma, and urine. VIP charts are drawn for each (Figure 1c, 1e, 1g, and Figure 2c, 2e, 2g). The metabolite with the highest VIP value in the VIP graph is the metabolite that is the most effective in separating the groups, and has the highest statistical change. The level of metabolites in each VIP graph (decreased or increased) is shown with coefficient plots (Figure 1d, 1f, 1h, and Figure 2 d, 2f, 2h).

In plasma samples, metabolites such as lauric acid, serotonin, maltose, pyrophosphate, phenylalanine, and methionine decreased in PPP group compared to control; in contrast metabolites such as caprylic acid, capric acid, inosine, and glucuronic acid showed increases (Figure 1d). When the plasma samples of the control group, and the CPP group were compared, a decrease in metabolites, such as P-cresol, adenosine, 3-aminopropionitrile, alpha ketoglutaric acid, pyrophosphate, malic acid, and an increase in metabolites such as palatinose, malonic acid, palmitoleic acid, methyl oleate, and methyl linoleate were observed (Figure 1f). When we compared PPP, and the CPP groups, reduction in metabolites such as palatinose, proline, β-alanine, lauric acid, maltose, tryptophan were observed in the PPP group, while increases in 3-amino propionitrile, caprylic acid, adenosine, inosine, capric acid, and α-ketoglutaric acid metabolites were determined (Figure 1h).

In the PPP group, metabolites such as adenine, thymine, norepinephrine, glyceric acid, pyruvic acid decreased, neohesperidin, 3-methylcathechol, glycolic acid, lactic acid, and tyrosine were higher in urine samples vs. control (Figure 2d). While reduction in metabolites such as 1-methylhydantoin, guaiacol, N-acetyl-5-hydroxytyriptamine, and maltitol were observed in urine samples of CPP group vs. control, increases were found in metabolites such as cycloleucine, lactic acid, oxalic acid, glyceric acid, lactose, and glycine (Figure 2f). Decreases were observed in metabolites such as cycloleucine, glyceric acid, norepinephrine, tartaric acid in the PPP group compared to the CPP group; however, increases in methystearate, tyramine, proline, stearic acid metabolites were determined (Figure 2h).

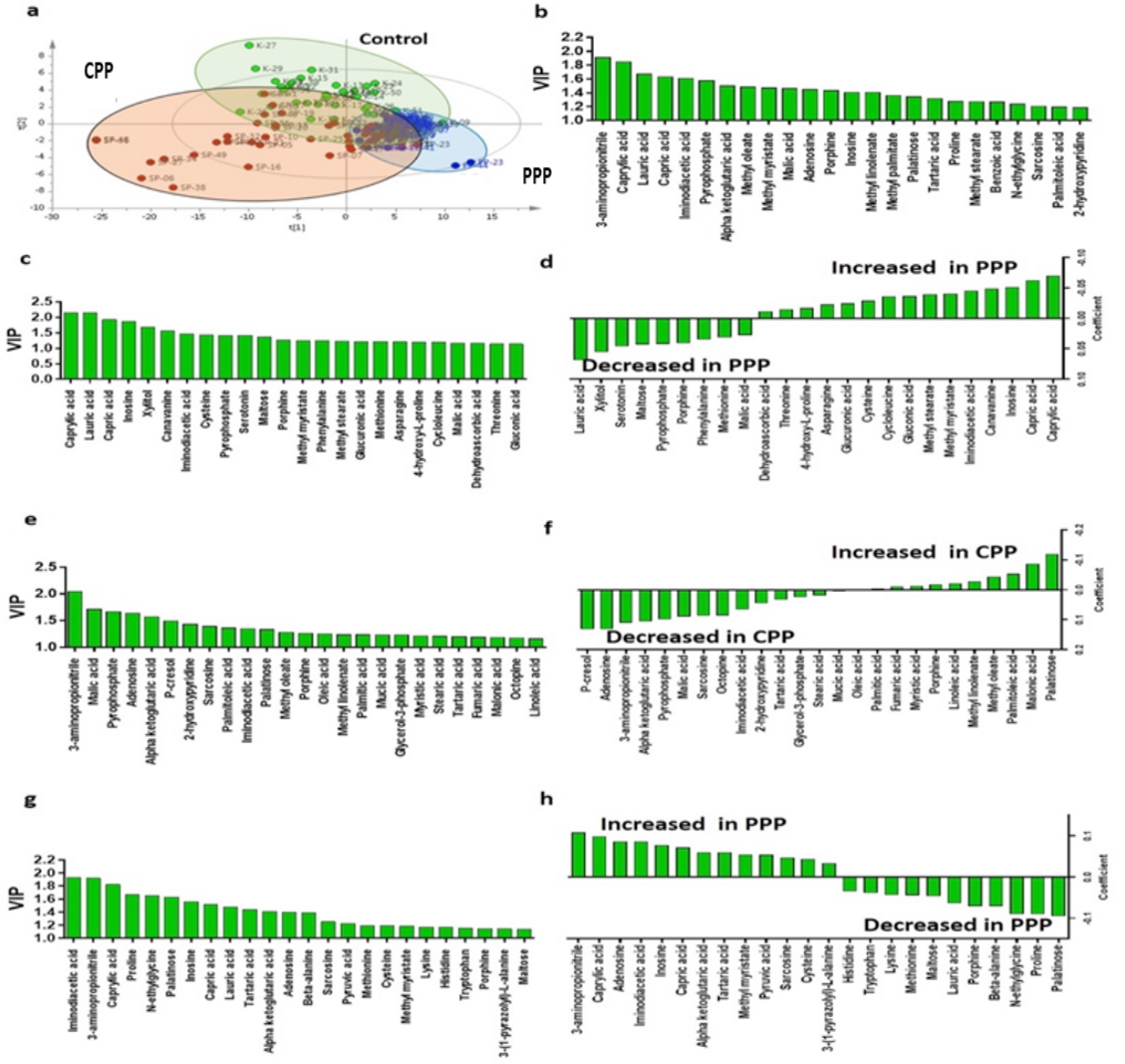

Figure 1. Plasma samples.

a) Partial least squares-discriminant analysis (PLS-DA) score plot: Control, PPP, and CPP.

b) Changes in variable importance in projection (VIP) values for 24 metabolites in plasma samples.

c) VIP plot: Control, and PPP.

d) Increase, and decrease in several metabolites: Control, and PPP (*p<0.05)

e) VIP plot: Control, and CPP.

f) Increase, and decrease in several metabolites: Control, and CPP (*p<0.05)

g) VIP plot: PPP, and CPP.

h) Increase, and decrease in several metabolites: PPP, and CPP (*p<0.05).

*In graph a, red circle shows group CPP, while light green circle shows control group, and blue circle shows PPP group.

**The PLS-DA VIP is used to screen out what may be the within-group difference variable, which is easily misleading.

***The comparative analysis can be made only two by two because in the search for differentials it is determined whether the substance is a differential based on the difference in content. The amount of a substance in one group rises/declines relative to the other group while the amount of change relative to the other two groups cannot be calculated simultaneously.

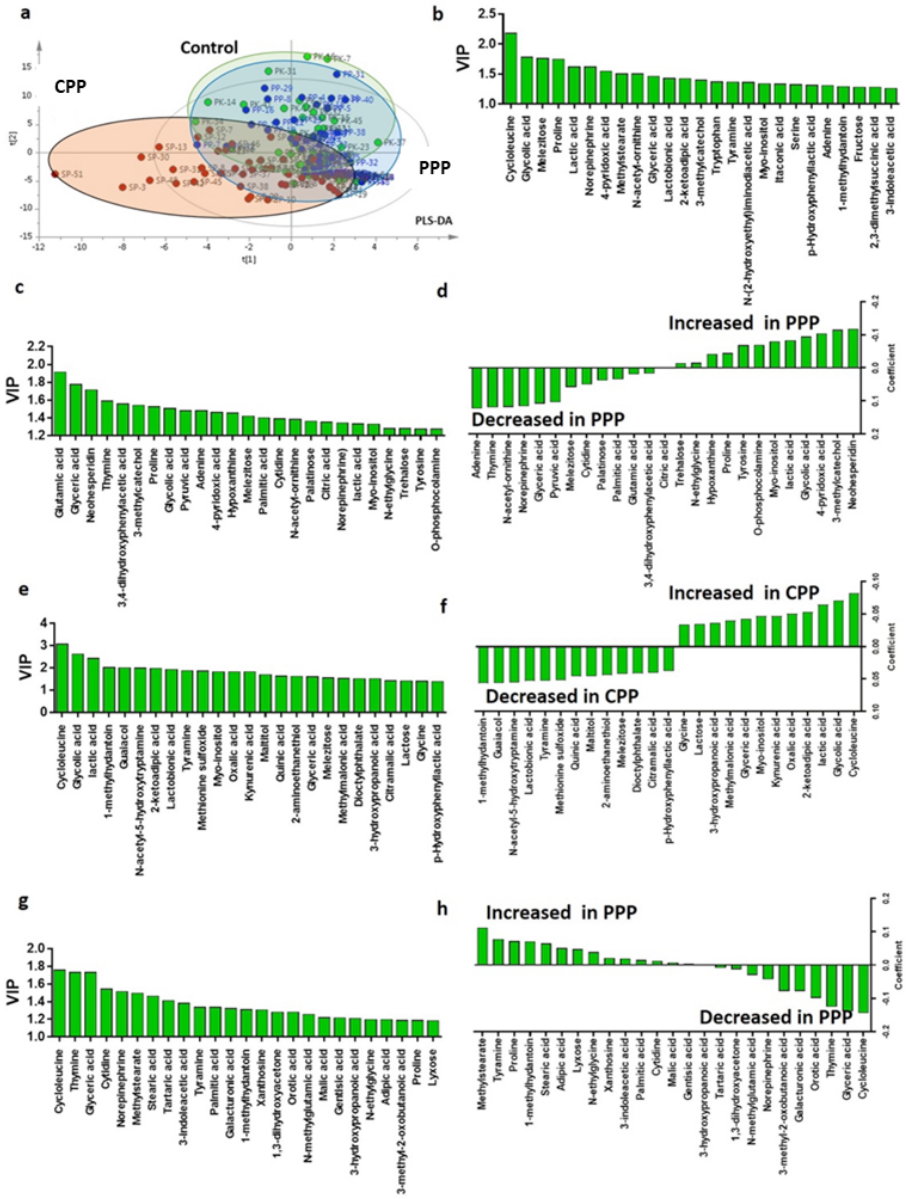

Figure 2. Urine samples.

a) Partial least squares-discriminant analysis (PLS-DA) score plot: Control, PPP, and CPP.
b) Changes in variable importance in projection (VIP) values for 24 metabolites in plasma samples.
c) VIP plot: Control, and PPP.
d) Increase, and decrease in several metabolites: Control, and PPP (*p<0.05).
e) VIP plot: Control, and CPP.
f) Increase, and decrease in several metabolites: Control, and CPP (*p<0.05).
g) VIP plot: PPP, and CPP.
h) Increase, and decrease in several metabolites: PPP, and CPP (*p<0.05).
*In graph a, red circle shows group CPP, while light green circle shows control group, and blue circle shows PPP group.
**The PLS-DA VIP is used to screen out what may be the within-group difference variable, which is easily misleading.
***The comparative analysis can be made only two by two because in the search for differentials it is determined whether the substance is a differential based on the difference in content. The amount of a substance in one group rises/declines relative to the other group while the amount of change relative to the other two groups cannot be calculated simultaneously.

Pathway analyses were performed by using metabolites that differ significantly between groups (Figure 3). While creating these graphs, data on plasma, and urine samples were combined. As a result, when the control group, and PPP group biological samples were compared, there were important differences in phenylalanine, tyrosine, tryptophan biosynthesis, arginine biosynthesis, and aminoacyl-tRNA biosynthesis. In addition, there were also significant differences between these two groups concerning alanine, aspartate, and glutamate metabolism (Figure 3a). When we compared control, and the CPP groups, we observed that there were significant differences in phenylalanine, tyrosine, tryptophan biosynthesis, arginine biosynthesis, aminoacyl-tRNA biosynthesis, and in glycine, serine, and threonine metabolism (Figure 3b). Differences were found in phenylalanine, tyrosine, tryptophan biosynthesis, arginine biosynthesis, aminoacyl-tRNA biosynthesis, alanine, aspartate, and glutamate metabolism between PPP, and CPP groups (Figure 3c).

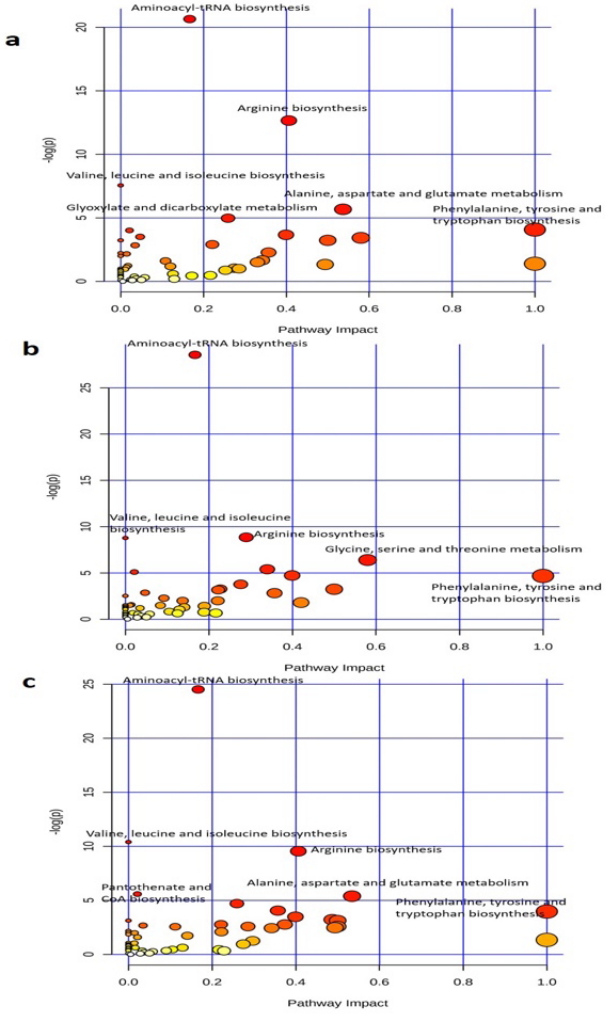

Figure 3. Metabolomics pathway analysis. (Plasma+Urine samples.)

a) Control, and PPP.
b) Control, and CPP.
c) PPP, and CPP.

We observed significant changes in amino acid metabolism of PP patients, along with alterations in biosynthesis of aminoacyl t-RNA. Our results are discussed under five sub-titles:

Aminoacyl tRNA biosynthesis

Aminoacyl tRNAs are carrier tRNAs to which their cognate amino acids are chemically bonded. These tRNAs, with particular elongation factors, deliver the amino acid to ribosomes for incorporation into the polypeptide chain that is produced during translation. During binding, aminoacyl t-RNA synthetases are involved. Final aim is to ensure that protein synthesis occurs smoothly by binding amino acids to carrier RNAs. Problems in biosynthesis of aminoacyl t-RNAs cause significant problems in protein synthesis (Moutiez et al., 2017; Ibba & Soll 2000). Herein, aminoacyl t-RNA biosynthesis was altered in girls with PP, and this can significantly affect protein synthesis patterns in girls with PPP, and CPP.

Phenylalanine, tyrosine, and tryptophan metabolism

Accumulation of phenylalanine, and its metabolites disrupts brain development in early life, causing phenylketonuria, and mental retardation (Qi et al., 2012). Phenylalanine is converted to tyrosine by phenylalanine hydroxylase. Tyrosine has roles in thyroid hormone production, melanin production of melanocytes, and is essential for production of dopamine, noradrenaline, and adrenaline (Fernstrom & Fernstrom 2007, Ploder et al., 2008). Its insufficient production can cause deficiencies in production of hormones, and neurotransmitters (Hase et al., 2015). Thyroid hormone deficiencies can lead to mental retardation, growth disorders, obesity, and metabolic problems in early life (Braun & Schweizer 2018). Disruptions in synthesis of neurotransmitters may also have significant consequences (Ruhé et al., 2007).

Tryptophan is involved in production of serotonin, melatonin, and nicotinic acid (Dejong et al., 2007, Comai et al., 2020). Serotonin deficiency can cause anxiety/depression/insomnia, eating, and memory problems (Dell'Osso et al., 2016). Under- or over-secretion of melatonin can also lead to many pathologies (Tordjman et al., 2017). Decreases in serotonin can lead to reduction in melatonin levels. Serotonin levels were decreased in plasma samples of PPP group vs. control. Several studies showed that use of melatonin in children with PPP had positive effects (Boafo et al., 2019). Plasma tryptophan levels of PPP group were lower than CPP. When plasma, and urine analyses were combined, phenylalanine, and tryptophan levels were found to be lower in the PPP group vs. control. When all data are evaluated, it may be interpreted that measuring phenylalanine, and tryptophan levels in adolescence can be considered as an early diagnosis criterion for PP.

Alanine, aspartate, and glutamate metabolism

Glutamate is synthesized by two main pathways: from α-ketoglutarate, and ammonia (with glutamate dehydrogenase), and from glutamate family amino acids (glutamine, arginine, proline, and histidine) (Tapiero et al., 2002, Brosnan & Brosnan 2013). Glutamate serves as a precursor in synthesis of alanine, glutamine, arginine, aspartate, and purine, and pyrimidine bases. It serves as a major excitatory neurotransmitter, and precursor in synthesis of gamma-aminobutyric acid (GABA). Moreover, it has roles in production of insulin, and in synthesis of atypical tripeptide glutathione. Glutamate is also responsible for taste associated with food addiction, and obesity. Besides, glutamate has important roles in regulation of growth, development, and reproduction (Tapiero et al., 2002, Brosnan & Brosnan 2013, Petroff 2002, Plaitakis & Shashidharan 2000, Sookoian & Pirola 2012). Glutamate, and particularly its N-methyl-D-aspartic acid (NMDA) subtype receptor regulates sexual maturation. Multiple daily injections of immature rats, and monkeys with NMDA could induce PP (Smyth &Wilkinson 1994). Intrinsic central nervous system (CNS) mechanisms responsible for LHRH pulse generator inhibition during childhood are provided by inhibitory

neurotransmitter GABA, and GABAergic neurons. Disinhibition, and reactivation of LHRH pulse generator is associated with a fall in GABAergic neurotransmission, and concomitant increase in the input of excitatory neurotransmitters like glutamate, and possibly astroglial-derived growth factors are observed with the onset of puberty (Grumbach 2002; Richter &Terasawa, 2001, Kasuya, 1999). Pulse frequency of hypothalamic GnRH secretion increases at puberty onset. In rodents, and primates, this process involves facilitatory, and inhibitory effects mediated through NMDA, and GABA receptors, respectively. In a case study, PP was observed in an 11-month-old girl with nonketotic hyperglycemia. Researchers suggested that PP resulted from high concentrations of glycine acting on NMDA receptors as a co-agonist of glutamate. Pubertal development regression during anticonvulsive treatment with GABA agonists like loreclezole, and vigabatrin suggested that stimulatory effects of glycine could be overcome by GABA-receptor-mediated inhibition. An *in vitro* experiment was conducted on the explanted hypothalamus of infantile male rats. Glycine (1-10 μmol/L) increased pulse frequency of GnRH secretion. This increase was prevented by glycine antagonist 7-chlorokynurenic acid, and loreclezole, and they suppressed developmental increase in the frequency of pulsatile GnRH secretion. These findings showed that stimulatory inputs mediated through NMDA receptors, and inhibitory inputs through GABA receptors could be involved in the initiation of puberty (Bourguignon et al., 1997).

Brain irradiation in prepubertal children with malignomas can lead to PP. Roth et al., (2001) developed a selective cranial cobalt (Co^{60})-irradiation technique in rats. Juvenile (13-15 days old) female rats received a single dose of 5 Gy or sham irradiation. At pubertal age (postnatal days 33-34), irradiated rats had higher serum estradiol, and LH levels. Irradiated rats had higher GnRH mRNA levels in the preoptic area vs. controls ($p<0.05$). Release rates of GABA from preoptic mediobasal hypothalamic areas of irradiated rats were significantly reduced after stimulation with GABA(A) receptor agonist muscimol. CPP induced by radiation might be caused by damage to inhibitory GABAergic neurons leading to premature activation of GnRH-pulse generator (Roth et al., 2001).

Aspartate takes part in synthesis of pyrimidine bases, has regulatory functions in CNS, developmental processes, and endocrine system (Sookoian & Pirola 2012; Hashimoto & Oka 1997; Nadler 2011). Alanine has roles in synthesis of almost all proteins, and peptides, gluconeogenesis, and various reactions used as nitrogen sources (Sookoian & Pirola 2012; Nadler 2011, Kohlmeier 2003).

When plasma, and urine metabolomics data of the PPP group were evaluated, metabolism of alanine, aspartate, and glutamate was accelerated vs. control, and CPP groups. Therefore, we can suggest that these amino acids were not present in the body sufficiently probably due to their higher excretion compared to other groups, and their physiological functions may not be fully achieved. On the other h, and, the data show the opposite in the CPP group. The metabolism of these amino acids was slower compared to the control, and PPP. This may cause bioaccumulation of these amino acids, the importance of which should be evaluated. Yang et al., (2012) also observed changes in alanine, aspartate, and glutamate metabolism in girls with CPP (Yang et al., 2012).

Arginine biosynthesis

Arginine, synthesized from glutamate, plays a role in synthesizing creatine together with glycine (Morris 2016, Joncquel-Chevalier Curt 2015, Wu 2009). Creatine is an important energy source, and is stored in the muscles as creatine phosphate. In cases where creatine cannot be synthesized due to disruptions in arginine biosynthesis, significant problems in energy balance may be occur. Lower creatine synthesis may lead to a lack of movement, fatigue, and metabolic diseases (Joncquel-Chevalier Curt 2015). Arginine also has antioxidant effects, roles in hormone secretion, and immune system. It

is transformed into nitric oxide, and is effective in nutrient metabolism, vascular tonus, hemodynamics, and angiogenesis (Morris 2016; Joncquel-Chevalier Curt, 2015; Albaugh, 2017).

Arginine is converted to another amino acid called "agmatine" by arginine decarboxylase (Wu 2009). Agmatine functions in nitric oxide synthesis, in operation of ion channels, and in membrane transporters. Agmatine also has vasodilator effects, is involved in the release of neurotransmitters, and may be associated with depression, anxiety, neurodegenerative diseases, and epilepsy (Piletz 2013, Neis 2017).

The problems observed in glutamate metabolism may also negatively affect arginine biosynthesis. Our results also point out that there can be problems in glutamate metabolism, which may also affect metabolism of arginine in girls with PPP. Yang et al., (2012) also observed significant changes in arginine, and pyrroline pathway in girls with CPP (Yang et al., 2012).

Metabolism of glycine, serine, and threonine

Threonine takes part in protein phosphorylation, and glycine synthesis. It also has roles in the synthesis of mucin, which is necessary to maintain intestinal integrity, and function. Moreover, threonine is crucial in maintaining immune functions (Wu et al., 2009).

Serine is synthesized from three phosphoglycerates. It is converted to glycine by serine hydroxymethyl transferase. Serine is also involved in synthesis of phospholipids, cysteine, purine, and pyrimidine bases. It acts on CNS through NMDA receptors (Hashimoto & Oka 1997, Häusler et al., 2014).

Glycine plays a role glutathione production, serine biosynthesis, production of purine nucleotides, and collagen (Parker &Metallo 2016,Wang et al., 2013, Adeva-, andany et al., 2018, Lee & Kim 2019). It has a mutual role with arginine in producing creatine (Wang et al., 2013, Adeva-, andany et al., 2018, Lee & Kim 2019). Glycine enables absorption/metabolism of lipids, and liposoluble vitamins. It also affects insulin production by pancreas. It has role in proper functioning of chloride channels of leukocytes, and macrophages. Glycine regulates intracellular calcium levels, and has functions in production of cytokines, and superoxide. It acts as a neurotransmitter in CNS affecting behavior, food intake, and whole-body homeostasis (Wang et al., 2013, Adeva-, andany et al., 2018, Lee & Kim 2019). Therefore, glycine plays significant roles in metabolism, growth, and development, and immune system. Disruption in glycine metabolism may reduce glutathione production, leading to important problems in antioxidant defense. Moreover, creatine phosphate production may be disrupted in glycine deficiency, causing decreases in energy production. Several studies associated glycine deficiency with insulin resistance, obesity, and type 2 diabetes (Adeva-, andany et al., 2018; Alves et al., 2019; Magnusson et al., 2015; Choe et al., 2013; Anuradha, 2009).

Plasma glycine, serine, and threonine levels were significantly lower in PPP group vs. control, and CPP. We can suggest that there is a problem in this metabolic pathway in the PPP group. Due to acceleration of metabolism, these amino acids may be insufficiently present, and not fulfill their physiological functions. In the CPP group, our results show the opposite, and metabolism of these amino acids was different vs. control, and PPP groups.

CONCLUSION

Our results showed that plasma is a more suitable material for metabolomic analysis. We can suggest that as urine is a complex biological matrix that contains mainly metabolic breakdown products from foods, drinks, drugs, environmental contaminants, endogenous waste metabolites, and bacterial by-products (Benini et al., 2011; Bouatra et al., 2013), it was not possible to obtain a good metabolomics profile in the current work. Although we applied a mild centrifugation step to remove bacteria, cells, and other materials in suspension before storage, there can still be contamination from different sources.

In two other studies conducted on metabolic profiles of PP, urine samples were used, and metabolomic evaluations were made. Our results, and the results of Yang et al., (2012), and Qi et al., (2012) indicate that mainly "the phenylalanine-tyrosine-tryptophan synthesis pathway" can be used as a biomarker for early diagnosis of CPP, and PPP. Besides, alterations in alanine-aspartate-glutamate pathway can also be evaluated as a biomarker; however, more comprehensive clinical studies are needed to determine reliable early diagnosis criteria that can be used routinely for the early diagnosis of CPP, and PPP. This is the first study conducted on the plasma samples obtained from PPP, and CPP patients. Our study contributes to limited number of studies conducted in this area, and its results may guide further studies as preliminary data.

ACKNOWLEDGEMENTS

The authors would like to thank Hacettepe University Research Fund. This work was supported by Hacettepe University (Grant Number [2017-14278]).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

PE, BKG and SK conceived this research project and were integral in the design of the study. ABO, TR, EN and PE conducted the majority of the study. TR and EN performed all statistical analysis. ABO, PE, TR and EN wrote the manuscript. All authors read and approved the final version of the manuscript.

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