A Multiplatform Metabolomics Approach to Characterize Plasma Levels of Phenylalanine and Tyrosine in Phenylketonuria

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Abstract *Background:* Different pathophysiological mechanisms have been described in phenylketonuria (PKU) but the indirect metabolic consequences of metabolic disorders caused by elevated Phe or low Tyr concentrations remain partially unknown. We used a multiplatform metabolomics approach to evaluate the metabolic signature associated with Phe and Tyr.

Material and methods: We prospectively included 10 PKU adult patients and matched controls. We analysed the metabolome profile using GC-MS (urine), amino-acid

analyzer (urine and plasma) and nuclear magnetic resonance spectroscopy (urine). We performed a multivariate analysis from the metabolome (after exclusion of Phe, Tyr and directly derived metabolites) to explain plasma Phe and Tyr concentrations, and the clinical status. Finally, we performed a univariate analysis of the most discriminant metabolites and we identified the associated metabolic pathways.

Results: We obtained a metabolic pattern from 118 metabolites and we built excellent multivariate models to explain Phe, Tyr concentrations and PKU diagnosis. Common metabolites of these models were identified: Gln, Arg, succinate and alpha aminobutyric acid. Univariate analysis showed an inverse correlation between Arg, alpha aminobutyric acid and Phe and a positive correlation between Arg, succinate, Gln and Tyr ($p < 0.0003$). Thus, we highlighted the following pathways: Arg and Pro, Ala, Asp and Glu metabolism.

Discussion: We obtain a specific metabolic signature related to Tyr and Phe concentrations. We confirmed the involvement of different pathophysiological mechanisms previously described in PKU such as protein synthesis, energetic metabolism and oxidative stress. The metabolomics approach is relevant to explore PKU pathogenesis.

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Abbreviations

¹ H NMR	Nuclear magnetic resonance
CV ANOVA	ANalysis Of VAriance testing of cross validated predictive residuals, used to evaluate the robustness of multivariate model
GC-MS	Gas chromatography coupled with mass spectrometry
HCA	Hierarchical cluster analysis

KEGG	Pathway database
LC	Liquid chromatography
METPA	A web metabolomics tool to analyse metabolic pathways
OPLS-DA	Orthogonal partial least-squares discriminant analysis
PLS	Partial least square
Q^2	Parameter to estimate of the predictive ability of the model, used to evaluate the robustness of multivariate model
R^2	Parameter defined as a fraction of the variance explained by a component, used to evaluate the robustness of multivariate model
ROC	Receiver-operating characteristics
UV Scaling	UV scaling is defined by a variable that is centred and scaled to “Unit Variance”, i.e. the base weight is computed as $1/SD$, where SD is the standard deviation of variable computed around the mean.
VIP	Variable importance parameters

Introduction

Phenylketonuria (PKU) is a metabolic disorder due to a deficiency in phenylalanine hydroxylase (PAH, RC 1.14.16.1), leading to high concentrations of Phenylalanine (Phe) and low concentrations of Tyrosine (Tyr) in blood and brain. A specific phenylalanine-restricted diet is prescribed following newborn screening but frequently discontinued by adult patients in some countries (Giovannini et al. 2012; Rocha et al. 2013; Jahja et al. 2014). Numerous reports highlighted the neurotoxic role of Phe, thus causing neurological and neuropsychiatric disturbances in PKU patients (van Spronsen et al. 2011; Christ et al. 2012; Bilder et al. 2013). Central mechanisms have been associated with neurotoxicity, such as amino-acid imbalance, reduced glutamatergic transmission, bioenergetics deficit, oxidative stress, impairment of lipid and protein metabolism, disruption of calcium homeostasis (Schumacher et al. 2008; Martynyuk et al. 2010; Schuck et al. 2015), but the interactions between these mechanisms await elucidation. The same questions are raised by the low concentrations of tyrosine responsible for the depletion of catecholamines related to brain dysfunction (McKean 1972; Christ et al. 2010; de Groot et al. 2010; Harding et al. 2014). Thus, a better knowledge of the metabolic pathways indirectly disturbed by both high Phe and low Tyr concentrations may be beneficial to better understand the pathogenesis of the disease, and finally to adjust the nutritional care (van Spronsen et al. 2009; Hennermann and Querfeld 2013; Longo et al. 2015; Schuck et al. 2015;

Turki et al. 2015). Metabolomics is a powerful strategy to ascertain metabolic signatures from a combination of small molecules in biological fluids. One of the main objectives of the metabolomics approach is to improve understanding of pathophysiology, especially based on the non-a priori metabolic signature. Gas chromatography coupled with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) is often used to perform metabolomics studies, but rarely in combination. The aim of our study was then to characterize the metabolic disturbances associated with plasma Phe and Tyr concentrations using a multiplatform targeted metabolomics approach. We used a combination of three analytical methods, namely routine determination of amino-acid profiles using amino-acid analyzer, GC-MS and NMR in order to obtain the most comprehensive metabolic pattern.

Material and Methods

Patients

Ten adult PKU patients (1–10) and ten age- and sex-matched healthy (11–20) controls were prospectively recruited from September 2014 to February 2015. This study has been approved by the ethics committee of Tours Hospital (Comité de Protection des Personnes). All subjects were informed and signed a consent. No subject has Phe-restricted diet at the time of inclusion. Clinical data available for their routine clinical follow-up were collected.

Samples Collection

Blood and urinary samples were collected at the inclusion visit after a night fasted. Blood samples were quickly centrifuged (2,500 g, 10 min). Urine samples were centrifuged in the same conditions. All samples were split into three aliquots and stored at -20°C until analysis. Blood samples were used to analyse amino-acids by aminotac, and urine samples were used to analyse (1) amino-acids by aminotac, (2) organic acids by GC-MS and (3) metabolomics profile by NMR. A brief description of these techniques is detailed below and additional material (document named “Additional Material” in supplemental data) provides complementary information about these analytical methods.

Amino-Acids Chromatography

We determined concentrations of amino-acid by Jeol Aminotac 500 analyzer, based on the ion-exchange chromatography with a post-column derivatization with ninhydrin and colorimetric detection. We used 500 μL of blood

and urine samples to perform the analysis. The determination of concentrations was based on the peak areas of amino-acids compared to that of internal standard.

Urinary Organic Acids Chromatography

Organic acids were extracted from urine by liquid/liquid extraction using ethyl acetate. The organic phase was evaporated (SpeedVac, ThermoFisher). We used two internal standards (4 phenylbutyric acid and heptadecanoic acid in acetonitrile). The derivatization was based on a silylation, by adding *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and incubation for 40 min at 80°C. The samples were then injected in GC-MS (QP2010 PLUS (Shimadzu)). We normalized the peaks of metabolites to the internal standard and the urinary creatinine concentration.

NMR Analysis

Urine samples were thawed at room temperature and centrifuged at 4,000 g for 10 min. 500 µL of supernatant was added with 100 µL of deuterated phosphate buffer 0.2 M (pH 7.4) and 8 µL of external reference (TSP). All the ¹H-NMR spectra were obtained on a Bruker Avance III HD NMR spectrometer (Bruker, SADIS, Wissembourg, France) operating at 700 MHz for ¹H resonance frequency using an inverse detection 5 mm ¹H-¹³C-¹⁵N cryoprobe.

Statistical Analysis

Multivariate Analysis

The plasma and urine signatures explored by the 1 or 3 analytical methods previously described were implemented in statistical software to analyse the merged data. The models were built to explain Phe and Tyr concentrations, and we completed the analysis by a discrimination of PKU and control group. For all the models, we excluded all metabolites directly modified by PKU (Phe, Tyr and derived organic acids).

We performed a multivariate analysis using Simca-P + -13, as done previously (Blasco et al. 2014). Principal component analysis (PCA) (Kemsley et al. 2007) was first performed as unsupervised clustering to identify similarities or differences between sample profiles.

Orthogonal partial least-squares discriminant analysis (OPLS-DA) is a supervised analysis that aims at evaluating the variations in metabolites between groups: the variation in the measured data was partitioned into two blocks by the program; one containing variations that correlated with the class identifier (PKU/controls) and the other containing variations that were orthogonal to the first block and thus did not contribute to discrimination between groups. OPLS-

DA finds the algorithm of metabolites that will enable the best discrimination of the groups (PKU patients and controls) (Madsen et al. 2010).

After these multivariate analyses (PCA, OPLS-DA), we created a score plot to visualize the OPLS-DA model: it represents points corresponding to the different subjects defined by an algorithm of selected metabolites. We show only the models using the more robust algorithm of metabolites to separate the groups. We also characterized the contribution of each metabolite to the separation of the groups using a loading plot. The metabolites with large values of w^*c (loading plot) (positive or negative) are highly correlated with Phe or Tyr values. The position of the metabolites in the loading plot enables to explain the group of subjects represented in score plot. The most discriminant metabolites called variable importance parameters (VIP) ranked the compounds according to their contribution to the model.

The quality of the models was described by different parameters: the CV ANOVA, R^2 and Q^2 values. R^2 is defined as a fraction of the variance explained by a component. Cross validation of R^2 gives Q^2 . Q^2 is an estimation of the predictive ability of the model. The OPLS-DA was cross validated by withholding one-seventh of the samples in seven successive simulations such that each sample was omitted once in order to guard against over fitting. The set of multiple models resulting from the cross validation was used to calculate jack-knife uncertainty measures (Westerhuis et al. 2010).

Supplemental material (see “Additional Material” in supplemental data) provides complementary information.

Based on the same strategies, we also used partial least square (PLS) models to explain the outcome in question (i.e. concentrations of plasma phenylalanine and tyrosine analysed by aminotac, considered as the reference method). We also performed a hierarchical cluster analysis (HCA).

We compared the metabolic pathways identified from PLS analysis (to explain Phe and Tyr concentrations) and OPLS-DA (to explain PKU diagnosis) and we highlighted the common metabolites between all these approaches.

Univariate Analysis

We performed the univariate analyses on the VIPs as a complementary means to improve understanding of mechanistic pathways involved in the disturbance of phenylalanine metabolism. We compared metabolites concentrations between PKU and controls with Wilcoxon tests for categorical variables and we used Spearman tests to evaluate the correlation between quantitative variables (Phe or Tyr versus other metabolites). We applied a correction (Bonferroni) to the p value to correct for multiple statistical tests. We performed statistical analyses with JMP

statistical software version 7.0.2 (SAS Institute, Cary, North Carolina). We used KEGG pathway database (<http://www.genome.jp/kegg/pathway.html>) and METPA web application (<http://metpa.metabolomics.ca/MetPA/faces/Home.jsp>) to discuss the highlighted metabolic pathways. To focus on the most relevant data, we highlighted only the pathways that had significant Holm p and a pathway impact >0.10 . The pathway impact value is the cumulative percentage from the matched metabolite nodes. Even if all the pathways having a significant Holm p are relevant, the threshold of 0.1 was fixed to focus our results and discussion only on the main metabolic pathways.

Results

Patients

The characteristics of PKU patients are provided in Table 1. The concentrations of Phe and Tyr are reported in Fig. S1 and will be used for further metabolomics models. Overall, the mean ($\pm$ standard deviation) concentration of plasma Phe was 679.9 ± 157.5 $\mu\text{mol/L}$ and the mean concentration of Tyr was 56.5 ± 3.0 $\mu\text{mol/L}$ (Fig. S1). As expected we found higher concentrations of Phe ($1,325 \pm 465$ vs 63 ± 15 $\mu\text{mol/L}$) and lower concentrations of Tyr (43 ± 9 vs 65 ± 11 $\mu\text{mol/L}$) in PKU patients, as compared to controls.

Metabolites Analysis

At the end of samples analysis, we detected 131 metabolites: 26 amino-acids measured in plasma and 105 molecules measured in urine: 27 organic acids measured by GC-MS, 26 amino-acids measured by the amino-acid

analyzer and 52 metabolites measured by NMR. After identification of the main metabolites, we found that 92 out of the 105 urinary metabolites correspond to distinct molecules. Indeed, some metabolites may be detected by different analytical methods. Thus, our dataset used to perform statistical analysis was based on 118 different variables: 26 amino-acids measured in plasma by the amino-acid analyzer and 92 metabolites measured in urine (26 amino-acids measured by amino-acids analyzer, 23 metabolites measured by NMR and 27 by GC-MS and 16 unidentified metabolites measured by NMR). We provided on the supplementary data only the list of the identified metabolites, including metabolites measured both, in urine and plasma (Table S1).

Multivariate Models to Explain Phenylalanine Concentrations

We used UV scaling and logarithmic transformation to minimize the impact of noise or high variance of the variables.

The best model describing the concentrations of Phe was obtained from 13 metabolites.

Six were from plasma: threonine, glutamine, proline, alpha aminobutyric acid, isoleucine and arginine, and seven from urine: glycerate, 3-OH-3-Me-glutarate, methylmalonate, succinate and three unknown molecules: unknown 3, 8, 13 (U3, U8, U13).

The performances of the models are as follows: R^2X (cum): 0.802, R^2Y (cum): 0.973 and Q^2 (cum): 0.934. CV ANOVA was 1.4×10^{-6} . The score plot is shown in Fig. 1 and the metabolites contributing to the model are shown in Fig. S2. Patients having high concentrations of Phe (with high $t(1)$ and $t(2)$, on the top right in the Fig. 1) are particularly discriminated from the others based on the high levels of U13, U8 and 3-OH 3-Me-3Glu.

Table 1 Clinical and biological characteristics of PKU patients

Patient	Age	Gender	Type of PKU	Phe concentration (μM)	Tyr concentration (μM)	Age of stopping Phe restricted diet (years)
1	34	F	Classical	1,641	37	6
2	22	M	Mild	1,051	61	16
3	30	F	Classical	1,281	51	5
4	34	F	Classical	1,854	46	5
5	27	F	Mild	1,069	30	5
6	36	M	Classical	1,449	56	5
7	30	F	Classical	1,472	39	5
8	39	M	Classical	1,790	52	5
9	30	F	Mild hyperphenylalaninemia	363	52	8
10	18	M	Mild	1,007	51	13

Fig. 1 Score plot based on the partial least square (PLS) models (13 metabolites) to explain Phe concentrations. The intensity of colour is proportional to the concentrations of Phe ($n = 20$, PKU patients from

1 to 10, healthy controls from 11 to 20). To note, the patient 9 is classified within the control group. $R^2X(\text{cum})$: 0.802, $R^2Y(\text{cum})$: 0.973 and $Q^2(\text{cum})$: 0.934, CV ANOVA: 1.4×10^{-6}

The univariate analysis performed on the VIPs and using the p value corrected by Bonferroni (13 metabolites in the model, so cut-off: $p = 0.0004$) showed a significant inverse correlation between glycerate (adjusted R^2 value: 0.61, $p < 0.0001$), alpha aminobutyric acid (adjusted R^2 value: 0.50, $p = 0.0003$), arginine (adjusted R^2 value: 0.56, $p < 0.0001$), glycerate (adjusted R^2 value: 0.61, $p < 0.0001$) and Phe and positive correlation between 3-OH-3-Me-glutarate (adjusted R^2 value: 0.61, $p < 0.0001$) and Phe (Fig. S2).

The dendrogram based on these data showed five different subgroups (Fig. 2a), visualized in the corresponding score plot (Fig. 2b). We noted that one patient was classified within the control group, especially because the concentration of Phe is lower than other patients (373 $\mu\text{mol/L}$).

Multivariate Models to Explain Tyrosine Concentrations

The best model describing the concentrations of Tyr was obtained from 13 metabolites.

Six from plasma: glutamine, alanine, alpha aminobutyric acid, methionine, isoleucine and arginine, seven

from urine: 2-Me succinate, fumarate, isovalerylglutamine, pyruvate, 3-OH isovalerate, succinate and one unknown molecule (U11).

The performances of the models are as follows: $R^2X(\text{cum})$: 0.685, $R^2Y(\text{cum})$: 0.922 and $Q^2(\text{cum})$: 0.898. CV ANOVA was 7.8×10^{-7} . The score plot is shown in Fig. 3 and the metabolites contributing to the model are shown in Fig. S3.

The univariate analysis performed on the VIPs and using the p value corrected by Bonferroni (cut-off: $p = 0.0004$) showed a significant positive correlation between glutamine (adjusted R^2 value: 0.51, $p = 0.0003$), methionine (adjusted R^2 value: 0.48, $p = 0.0004$), isoleucine (adjusted R^2 value: 0.54, $p = 0.0001$), arginine (adjusted R^2 value: 0.53, $p = 0.0002$), pyruvate (adjusted R^2 value: 0.57, $p = 0.0001$), succinate (adjusted R^2 value: 0.56, $p = 0.0001$), U11 (adjusted R^2 value: 0.53, $p = 0.0003$) and Tyr.

The dendrogram showed five different subgroups (Fig. S4A), visualized in the corresponding score plot (Fig. S4B). We noted that one control was classified within the PKU group, due the low concentration of Tyr (45 $\mu\text{mol/L}$).

Fig. 2 (a) Dendrogram obtained from hierarchical cluster analysis (HCA) based on the 13 relevant metabolites used in the partial least square (PLS) model to explain Phe concentrations, and showing 5 subgroups of subjects; the X axis represents the patients and the Y axis

the distance between the clusters; (b) Score plot characterized by the same colours as identified in the dendrogram. To note, the patient 9 (asterisk) is classified within the control group

Multivariate Models to Explain PKU

The best OPLS-DA model was based on 12 metabolites: six from plasma: threonine, glutamine, proline, alpha amino-butyric acid, methionine and arginine, and six from urine: glycerate, 2-OH isobutyrate, 3-OH isovalerate, 3-Me-2-oxovalerate, succinate and one unknown molecule (U10) (Fig. S5). $R^2X(\text{cum})$: 0.719, $R^2Y(\text{cum})$: 0.837 and $Q^2(\text{cum})$: 0.761. CV ANOVA was 0.0003.

Common Metabolites Explaining Phe and Tyr Concentrations

We found four common molecules with the two PLS models to explain Phe and Tyr concentrations and the OPLS model to explain PKU (Fig. 4). The most relevant pathways involved in the different models are shown in Fig. 4 (defined by a significant p-value and a pathway impact >0.1). The ways found in the three models

Fig. 3 Score plot based on the partial least square (PLS) models (13 metabolites) to explain Tyr concentrations. The intensity of colour is proportional to the concentrations of Tyr ($n = 20$, PKU patients from

1 to 10, healthy controls from 11 to 20). $R^2X(\text{cum})$: 0.685, $R^2Y(\text{cum})$: 0.922, $Q^2(\text{cum})$: 0.898, CV ANOVA: 7.8×10^{-7}

correspond to the metabolism of Arg and Pro, and also of Ala, Asp and Glu metabolism.

Discussion

According to the partially unknown mechanisms associated with neurotoxicity in PKU, we hypothesized that a metabolomics profile of different subjects characterized by a large distribution of Phe and Tyr concentrations may be relevant to identify metabolic pathways disturbances.

Few studies reported metabolomics approach in PKU and they especially focused on new diagnosis biomarkers of this disease (Denes et al. 2012; Janeckova et al. 2012; Xiong et al. 2015) to develop a diagnosis from less invasive samples (for example, from urine collection or dried spot) or to limit false positive cases, for example. A recent metabolomics approach based on GC-MS aimed at developing a new standard to diagnose PKU from urine samples. They reported 19 differentially expressed compounds in PKU patients (for example, phenylacetic acid or phenylpyruvic acid) with a relevant receiver-operating characteristic (ROC) analysis (Xiong et al. 2015), thus validating the metabolomics approach from urine to diagnose PKU. They

observed a high specificity (93.6%) and sensitivity (100%) from the ROC curve based on urinary phenylacetic acid concentrations. They noted a low false positive rate (6.4%) that could be improved from the use of additional specific markers. Interestingly, one other study evaluated the metabolic influence of the long term PKU diet (Mutze et al. 2012). The present study is the first metabolomics study in PKU based on multiplatform analysis and only focused on the pathophysiology of the disease.

The combination of several analytical methods adds complexity but might be the currently most expeditious approach in a disease with perturbations focused on metabolism. The present approach is based on the combination of two methods usually performed in routine practice to help in the diagnosis of metabolic disease or to adapt patients' diet or therapeutics. We added the exploration of urine samples by NMR, using a 700 MHz NMR spectrometer which is very sensitive and reproducible and actually rarely used for metabolomics approaches. We applied a targeted analysis to obtain a limited number of metabolites, mostly identified and with sufficient signal to be representative of physiological and pathological variations. A combination of different "omics" approaches has never been performed for a PKU condition, but has been tried in

Fig. 4 Venn diagram showing metabolites identified in the partial least square (PLS) models to explain Phe concentration (the *left circle*), Tyr concentration (the *right circle*) and Orthogonal partial least-squares discriminant analysis (OPLS-DA) to explain clinical status (the *bottom circle*). This diagram highlights the common and specific metabolites according to the objectives of the models. The

metabolic pathways identified by METPA web application from the metabolites noted in the Venn diagram are shown for each model: PLS to explain Phe concentration (graph on the *left*), Tyr concentration (graph on the *right*) and OPLS-DA to explain clinical status (graph at the *bottom*). We noted on the figures only the most relevant pathways defined as significant Holm p and pathway impact >0.1 .

other fields (Weckwerth 2008). One of the limitations of this strategy is the inadequate number of metabolites compared to a limited samples size. In our study, we used merged data, and we optimized the different multivariate models to obtain the better performances with a limited number of metabolites (always <13), thus avoiding misinterpretation. Moreover, we validated our PLS modeling based on concentrations values of Phe and Tyr by the OPLS-DA model.

The different multivariate models enable a correct classification of subjects, except the subjects 9 and 11. The multivariate models performed to explain Phe concentrations revealed a clear discrimination of patients (except the subject 9), even after exclusion of metabolites directly altered by PKU. Some of discriminant metabolites included in the models were also significantly associated with Phe concentrations after univariate analysis. HCA enabled us to

identify some subgroups of subjects, especially the patient 5, who seems different and the subject 9 who is classified with controls. Importantly, the statistical analysis of outsiders performed using SIMCA analysis did not reveal any outsider in the included subjects (data not shown). After a deeper analysis of the clinical characteristics of the patients, we noted that the patient 5 has also type 1 diabetes, thus explaining the different metabolic profile despite the high concentrations of Phe. To note, the OPLS-DA discriminating PKU patients from controls also found the patient 5 as an outlier. The patient 9 has a low concentration of Phe ($363 \mu\text{M}$) compared to other PKU patients, so associated metabolism modification is similar to that of controls.

Inhibition of tyrosine transport across the presynaptic membrane by phenylalanine (McKean 1972; Knudsen et al. 1995), and decreased urinary excretion of dopamine and serotonin or their metabolites (Curtius et al. 1972) have

been largely reported in the literature since many years but the metabolic perturbation associated with Tyr in PKU context remains partially unknown. To explain Tyr concentrations, the best fit was obtained from 13 metabolites, with excellent performances. However, we observed a bigger dispersion of subjects in the score plot. We noted that the patient 5, but also the 7 are quite different (Fig. 3). The control 11 was misclassified, that could be explained by its particularly low concentration of Tyr compared to other controls. The score plots of both PLS models showed that the dispersion of subjects is higher within the group of PKU patients, thus highlighting the heterogeneity of these patients for the discriminant metabolites. According to the misclassified subjects, the potential impact of diet has to be taken into account. In this study, subjects were fasted and the analysis of the amino-acids profile suggests that subjects respected the conditions of samples collection. However, the major role of diet on metabolic explorations has to be considered to avoid misinterpretation. Moreover, fasting, as other physiological challenges increased inter-individual variations, even in similar subjects (Krug et al. 2012). These findings may explain some of our unexpected results.

The analysis of the common metabolites found in both PLS models and the OPLS-DA model revealed an important role of glutamate, succinate, arginine and alpha aminobutyric acid. METPA analysis highlighted the involvement of Arg, Pro, and Ala, Asp, Glu metabolism.

One of the most described pathophysiological ways in PKU is the defect in central nervous system protein synthesis (de Groot et al. 2015). Numerous authors highlighted a significant negative correlation between plasma Phe levels and the cerebral protein synthesis rate, based on positron emission tomography in PKU patients, and experiments in animal or in vitro models (Schuck et al. 2015). We observed a relation between different amino-acids largely involved in protein synthesis and Phe or Tyr (such as alanine, isoleucine). Alpha aminobutyric acid is an amino-acid not taking part in protein synthesis but is considered as a non-specific biomarker of liver, malnutrition or protein catabolism (Chiarla et al. 2011). An old report showed that alpha aminobutyric acid concentration was significantly higher (Thalhammer et al. 1980) in PKU that is consistent with our findings. This observation needs complementary data to understand the role of this metabolite in PKU pathophysiology.

Moreover energy metabolism disturbance was reported in PKU, including pyruvate kinase, hexokinase, other enzymes dependent or not from metal ions, and also ketonic bodies metabolism (Schuck et al. 2015). The inhibition of glucose-6-phosphate dehydrogenase activity by phenylpyruvic acid has also been reported, thus leading to an impairment of NADPH production (Rosa et al. 2012).

Importantly, some author observed a significant decrease of succinate dehydrogenase and mitochondrial respiratory chain in cerebral cortex of rats with hyperphenylalaninemia (Rech et al. 2002). These data are consistent with the involvement of succinate in our different models. To note the alteration of mitochondrial respiratory chain by phenylalanine is still debated (Hargreaves et al. 2002; Rech et al. 2002; Kyprianou et al. 2009). Even if succinate is a non-specific metabolite of energetic disturbance, this finding merits more explorations. As all these patients were not under rigorous Phe-restrict diet therapy, we suggest that these observations are probably not due to a poor nutritional status.

We showed that arginine was a relevant metabolite to explain Phe and Tyr concentration. Arginine has numerous roles in inflammation response (Rath et al. 2014), growth hormone regulation (Merimee et al. 1965), wound repair (Williams et al. 2002), glucose metabolism (Piatti et al. 2001) and so on. Among the different effect of arginine, its link with oxidative stress seems one of the most relevant mechanisms in PKU. Indeed, NO produced from L-arginine by NO synthases is largely involved in oxidative stress described in PKU patients (Ribas et al. 2011; Sanayama et al. 2011).

Interestingly, some metabolic pathways were highlighted only in models to explain Tyr concentrations (Fig. 4, citrate cycle, butanoate metabolism) but they were closely linked with that associated with Phe.

The main perspectives of this pilot study are to pursue on a larger cohort composed of patients from childhood to adult age. We may assess the evolution of metabolome that may be related to the Phe and Tyr equilibrium as well as the effect of age. As metabolism is largely age-associated and as metabolomics provide a global metabolism view without a priori, we have to plan some longitudinal studies to explore the evolution of the altered metabolic pathways during several years. As metabolomics profile may be influenced by gender and age (Thevenot et al. 2015), the robustness of this present study and the followings is based on the matching between PKU patients and controls. Importantly, such kinds of study will not be able to interpret the metabolism disturbance directly influenced by Phe, but it would establish a specific metabolic profile using high throughput techniques of PKU patients, deeply linked to the disequilibrium of Phe and Tyr (Kaufman 1976).

Conclusion

Importantly, we observed metabolism alterations in plasma and urine, although most studies are focused on brain metabolism to explain PKU pathogenesis. This pilot study

based on a new strategy of pathophysiology exploration based on multiplatform metabolomics to analyse biological fluids is promising for further experiments. The approach based on easily available samples enabled us to obtain a large metabolic pattern without a priori and we identified the involvement of relevant pathways, thus opening new perspective of pathogenesis understanding.

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Synopsis

The metabolomics approach based on a multiplatform strategy is promising to improve the knowledge of PKU pathogenesis.

Compliance with Ethics Guidelines

Conflict of Interest Statements

Blasco H, Veyrat-Durebex C, Bertrand M, Patin F, Labarthe F, Henique H, Emond P, Andres CR, Antar C, Landon C, Nadal-Desbarats L and Maillot F declare no conflict of interest

All the authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsor

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Contribution of Authors

Hélène Blasco performed the statistical analysis, interpreted the data and wrote a part of the manuscript

Charlotte Veyrat-Durebex: acquired data

Franck Patin: performed the statistical analysis, interpreted the data and wrote a part of manuscript

Labarthe F: critically revised the manuscript for important intellectual content

Bertrand M: acquired NMR data

Hélène Hénique: acquired clinical data

Patrick Emond: acquired GC-MS data

Christian R Andres: critically revised the manuscript for important intellectual content

Catherine Antar: pre-treated and integrated NMR data

Céline Landon: acquired NMR data

Lydie Nadal-Desbarats: pre-treated, integrated NMR data and identified metabolites

François Maillot: recruited patients, interpreted data and critically revised the manuscript for important intellectual content

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