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Metabolomics in Phenylketonuria disease: A systematic review

Revisió bibliogràfica

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Summary

Systematic review

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Resumen: La fenilcetonuria es una trastorno hereditario que es necesario diagnosticar correctamente durante los primeros años de vida. La metabolómica podría ser una buena herramienta para mejorar las técnicas usadas actualmente para detectar esta enfermedad identificando biomarcadores en muestras biológicas. El objetivo de esta revisión sistemática es proporcionar un resumen de los últimos avances conseguidos mediante la metabolomica en la identificación de biomarcadores de la fenilcetonuria. Se realizó una búsqueda sistemática de artículos publicados en bases de datos como PubMed y Web of Science hasta el mes de marzo del 2021. Los estudios se incluyeron solo si aplicaban técnicas utilizadas con la metabolómica para detectar biomarcadores en muestras humanas. Resultaron incluidas diez publicaciones en esta revisión y se identificaron 34 metabolitos relacionados con la fenilcetonuria. Varios metabolitos fueron identificados por diferentes estudios y se clasificaron como diferenciales com es la fenilalanina, el ácido fenilpirúvico, la arginina, la tirosina, la carnitina, la dimetilarginina asimétrica y el ácido docosahexaenoico. Mediante el Pathway Analysis se observó una mayor implicación del metabolismo de los amino ácidos en la fenilcetonuria. En esta revisión se resumieron los estudios metabolómicos publicados sobre biomarcadores de la fenilcetonuria. Además, se identificaron desregulaciones metabólicas que suceden en pacientes con fenilcetonuria. Se necesitan más estudios metabolómicos para comprender mejor todos los cambios metabólicos de la fenilcetonuria e identificar metabolitos diferenciales más significativos para mejorar el diagnóstico y el tratamiento nutricional.

Abstract: Phenylketonuria is an inherited disorder with an important need to be diagnosed at early ages. Metabolomics could be an excellent tool to improve the actual techniques used to detect this disease by identifying biomarkers. The aim of this review is to provide a summary of the last advances achieved through metabolomics on the identification of phenylketonuria biomarkers. A systematic search was conducted using PubMed and Web of Science through February-March 2021. Studies were included only if metabolomics were applied and metabolomics technics were used to detect the biomarkers on human samples. At the end, ten publications were included in this review and 34 metabolites were identified to be related to phenylketonuria. Several metabolites were identified repeatedly and classified as differential metabolites of phenylketonuria, including phenylalanine, phenylpyruvic acid, arginine, tyrosine, carnitine, asymmetric dimethylarginine and docosahexaenoic acid. The pathway analysis showed a major implication of amino acids metabolism in phenylketonuria. In this systematic review, published metabolic studies on phenylketonuria biomarkers were summarized. Moreover, metabolic disorders in PKU patients were identified. More metabolomic studies are needed to better understand all metabolic changes of phenylketonuria and to identify more significant differential metabolites to improve the diagnosis.

Keywords: phenylketonuria, metabolomics, dietary treatment, biomarkers

Abbreviations: Phenylketonuria (PKU), phenylalanine (Phe), Tyrosine (Tyr), Tryptophan (Trp), Amino Acid (AA), asymmetric dimethylarginine (ADMA), hydroxykynurenic acid (3HK), homovanillic acid (HVA), 3-methoxytyramine (3MT), 5-hydroxyindole acetic acid (5HT), kynurenic acid (KA)

1. Introduction

Phenylketonuria is a metabolic disorder caused by a mutation in the phenylalanine hydroxylase (PAH) gene. It is characterized by a phenylalanine (PHE) accumulation in plasma with decreased

tyrosine biosynthesis. Intellectual disability, motor deficits and psychiatric problems are some the possible effects of phenylketonuria [1]. Approximately, the worldwide prevalence of phenylketonuria (PKU) is 6,002 per 100,000 neonates [2], with Turkey being the country with the highest prevalence. An early detection is essential because newborns with PKU seem to be normal during the first days of life but over the months the disease starts to be more visible [2]. In this case, the diagnosis is based on the criteria established by the European Society for PKU and Allied disorders Treated as PKU [3].

For the management of PKU, the dietary treatment is the basis and it consists of following a diet with a protein restriction and supplemented with phenylalanine-free amino acid formula [4]. The restricted diet consists in avoiding food rich in protein and food and drinks containing aspartame flour or soya. Moreover, recently sapropterin (BH4) has been approved to treat PKU patients, especially the ones with high residual activity of phenylalanine hydroxylase (PAH), the responsible of converting Phe into L-tyrosine [3]. As seen in several publications, a correct supplementation of BH4 improves levels of some of the most important biological markers that suffer modifications in PKU patients [5,6].

In this context, metabolomics is considered the omic approach that can best characterize the phenotypes of human beings. Metabolomics is used to study the metabolic products that can be found in biological samples. The objective of this discipline is to analyse the maximum number of metabolites and select those that truly provide information on the studied situation. Nowadays, it is possible to study unexpected metabolic pathways by using these metabolomic technologies [7]. In inborn metabolic errors, metabolomics has been used to improve diagnostic efficiency given the advances in the technology that is applied in this discipline [8]. Some of the improves that were recently implemented in PKU patients is the identification of profiles to urine samples so the adherence to the treatment could be well documented. Several studies used metabolomics analysis to determine differences in PKU patients metabolism and some alterations that were observed were in cholesterol concentrations, monoamines, vitamins and minerals, etc. [9,10].

The aim of this systematic review was to provide an overview of the published research on metabolomics technics being useful on improving phenylketonuria diagnosis. Namely, by identifying inherent phenylketonuria biomarkers and existing metabolic profiles in humans. Despite of the lack of evidence, having an overview of the current research might be practical for future applications improving the diagnostic for this metabolic disorder. One of the application of interest, related to metabolomics, is to treat different Inborn Errors of Metabolism by measuring secondary metabolites common in these to increase the efficiency of diagnosis [8]. In the future, having additional information, would be positive given that the single analyte for diagnosis could be amplified.

2. Materials and Methods

2.1. Search Strategy and Study Selection

This systematic review was conducted using Pubmed (https://pubmed.ncbi.nlm.nih.gov/) and the Web of Science (https://apps-webofknowledge-com) databases in February 2021 using the syntaxes reported in Supplementary Table S1.
Electronic searches were supplemented with manual searches of references from included studies and reviews on related topics. Studies were included in the present systematic review, if i) they were scientific findings on phenylketonuria using different metabolomics technics, ii) they were done with humans and not animals, iii) they provided information on differential metabolic markers, iv) if metabolomics technics were performed to analyse human samples (blood, feces or urine) v) dietary adherence or type of dietary management was recognized as a variable to study. Exclusion criteria included i) not reported in a European language. No restrictions for the characteristics of study participants (e.g., age, sex, weight, and other health conditions) were applied when selected.

2.2. Quality assessment

The QUADOMICS [11], an adaptation of QUADAS [12], criterion was used to evaluate the methodological quality of the included studies. The criterion consists of 16-item scale which includes: inclusion and exclusion criteria, sample characteristics, differential conditions in preanalytical, clinical and physiological characteristics of research subjects, uninterpretable test results and the presence or avoidance of over-fitting. In this review, only the studies that fulfilled with 70% or more were included.

2.4. Data extraction and pathway analysis

Data were extracted from each identified study and the following information was collected: name of the authors, year of publication, number of participants/controls, dietary management that was controlled and if supplementation formula was used, levels of the metabolites that were studied, outcomes (phenylalanine levels, tyrosine levels, other diseases presented…), main findings. The pathway analysis resulted from including the metabolites from several biological sample implementing the MetaboAnalyst [13] software (version 5.0) on the basis of Kyoto Encyclopedia of Genes and Genomes and Human Metabolome Database.

3. Results

3.1. Study selection

The study selection process that was done in this systematic review is shown in Figure 1 specificizing all the phases and why the excluded articles were not included in our work. A total of 515 records were identified through the database search that was carried out in both PubMed and Web of Science databases. After removing 53 articles that were duplicated, 552 articles were screened and 502 were excluded based on the title or the abstract.
A total of 50 eligible records went under the full text screening process. After this process we excluded 40 articles because they did not meet the inclusion criteria that was specified above.
Ten articles met eligibility criteria, providing data on a total of 7 prospective cohorts and 3 cross-sectional studies on patients with phenylketonuria (Figure 1).

3.2. Quality of the Included Studies

All the studies that were evaluated using the QUADOMICS tool were phase I, all of these used tests to identify patients or cases with one specific disease from healthy controls [11]. All the results are shown in Supplementary Table S2. Only studies that identified differential and remarkable metabolites were included. Seven studies met the criteria of including in detail the inclusion and exclusion criteria, sources of samples and a comparison between the included and excluded patient [14–20]. The item 14 was not applied to either of the included studies because it only applies to studies of phase IV [11]. All the ten included studies described the index test in sufficient detail to permit replication of the test or mentioned the guidelines or studies previous to the publication of the recommendations that were followed.

3.3. Characteristics of the Included Studies

The main characteristics of the included studies are reported in Table 1. Out of the 10 included articles, 9 of them characterized or determinated levels of specific metabolites in PKU patients following a restricted diet, with or without supplementation. The remaining 2 publications [18,20] investigated specific relations like docosahexaenoic acid status in PKU patients following a restricted diet and cognitive performance and the other one studied the possible modifications in tryptophan pathways when a phe-restricted diet is followed. Most of the articles were performed in Germany (n=4), followed by Spain and France (n=3), the United States of America (n=2) and Canada (n=1).
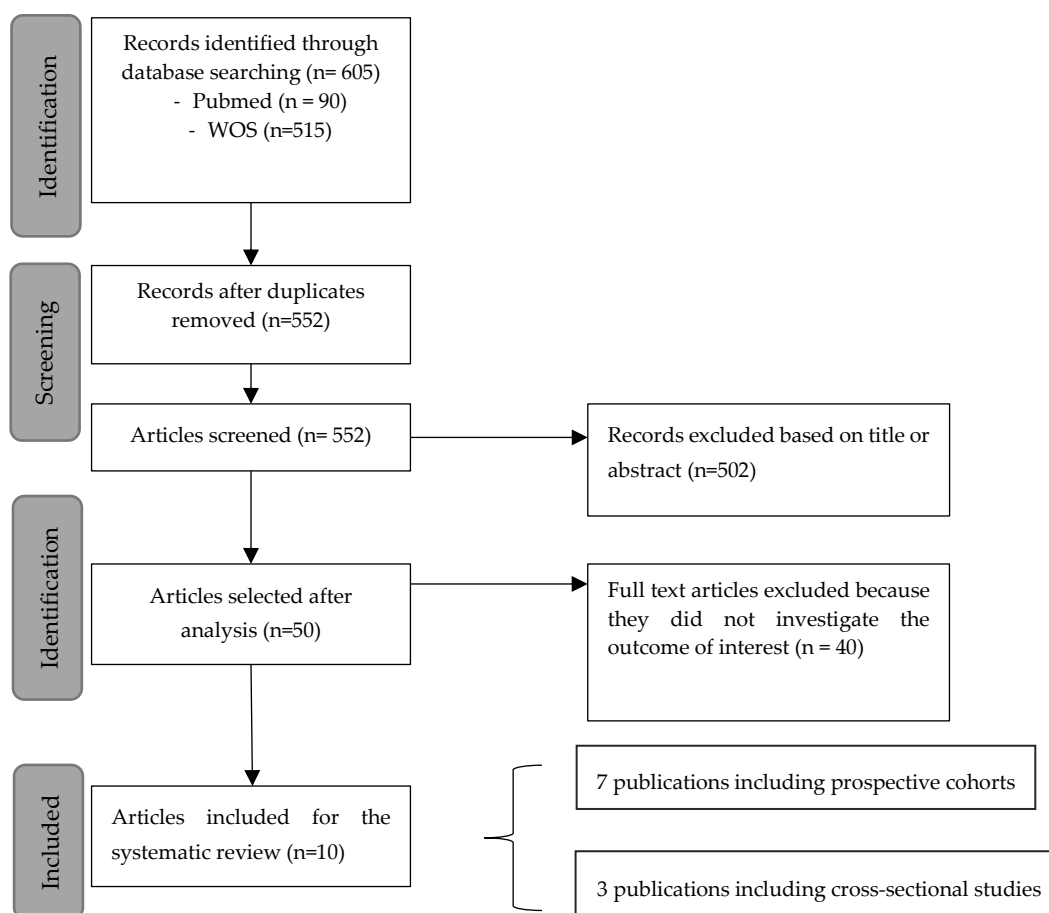
Figure 1. Flow diagram of literature search and study selection for metabolomics and phenylketonuria. Legend: WOS: Web of Science

Patients with Phenylketonuria must follow a phenylalanine restricted diet or a protein restricted diet. From the selected articles, 8 clearly mentioned that a certain percentage of the patients participating in the study were following a special restricted diet [14–19,21,22]. One publication just mentioned that a classical and strict dietary treatment was followed. About the remaining study, it does not mention what type of treatment the patients were receiving [23]. The number of study participants ranged from n= 12 [22] to n=151 [19], while mean age ranged from 1 [18] to 54 years [14].

A Phe-free amino acid formula is also part of the dietary treatment for PKU patients. In the included studies, 5 articles [14,16,19,20,22] had PKU patients with amino acid supplementation devoid of Phe but enriched in tyrosine and other essential aa. Two studies [15,18] had PKU patients with a restricted diet but also with sapropterin (BH4) as supplementation. Vilaseca et al. [18] included the phe-free amino acid formula in the treatment too. And the remaining two studies [21,23] did not mention if a supplementation was prescribed as part of the dietary treatment of the PKU participants.

In relation with the type of biological sample that was used to identify metabolic markers, 7 studies [14,17–22] applied metabolomic technics in plasma samples of PKU patients, 2 studies [15,23] used urine samples and the remaining 2 publication [15,16] used both urine and plasma samples to analyze different metabolites.

Table 1. Characteristics of the included studies investigating on metabolomics and the disease of phenylketonuria.

Reference	Study participants	Study design	Restricted diet (yes/no)	Formula (yes/no)	Technique	Type of sample	Metabolites in ↑ [ ]	Metabolites in ↓ [ ]	Final findings
Cannet, Claire et al. 2020 [14]	22 (16F-6M); 30-54yr	Cohort	Yes	Yes	NMR	Plasma	Tyrosine, glutamine, creatinine	-	The level of adherence with diet did correlate with blood phe levels
Douglas, Teresa D. 2013 [15]	58 (34M-24F); 4-49yr	Cohort	Yes	Yes	HPLC, aa analyzer	Plasma and urine	Dopamine, homovanillinic acid , methoxytyramine, 5-hydroxyindole acetic acid, serotonin	-	Sapropterin had a significant correlation with plasma phe and monoamine outcomes, specially increasing HVA
Kanzelmeyer, Nele et al. 2012 [16]	52	cohort	Yes	Yes	GC-MS/MS	Plasma and urine	Asymmetric dimethylarginine, arginine	-	Thee cardiovascular riks could originate from disminished NO bioavailability and/or from other pathways that do not implicate ADMA..
Yi, Sarah H.L, et al. 2011 [17]	41F; 12-17 and > 18yr	Cross-sectional study	Yes	No	GC/MC	Plasma	Docosahexaenoic acid	Phe	Lower DHA levels in PKU patients are suspected to be due to the restrictive diet
Vilaseca, Maria A, et al 2010 [18]	13; 1-16yr	Cross sectional	Yes	Yes	HPLC +UV detection, GC + flame ionisation	Plasma, serum and dried blood spots	Docosahexaenoic acid, carnitine	Phe	More advantages for PKU patients were found from BH4 therapy, specially to those who respond correctly to this cofactor
Boulet L. et al, 2020 [19]	151	Cohort	Yes	Yes	HPLC coupled to tandem MS	Plasma	Kynurenine, hydroxykynurenic acid	Kynurenic acid	Plasma 3HK appears to be a better reflection of the metabolic status of dietary amino acid intake in PKU patients
Wil J, et al 2019 [20]	22 (11M+11F); 2m-50yr	Cohort	Yes	Yes	UPLC-UV, MS/MS, aa analyzer, LC/MS	Plasma	Arginine, tyrosine, 2-aminobutyric acid, propionylcarnitine, carnitine, 2-aminoadipic acid, aminohippuric acid, urocanic acid, trymethylamine N-oxide, butyrylcarnitine	Phe, phenylpyruvic acid, phenylactecylglutamine, hydroxyphenylacetic acid, imidazole lactic acid	Nontargeted metabolite profiling revealed that excessive plasma phe on untreated patients correlated with several circulatory metabolites.

Reference	Study participants	Study design	Restricted diet (yes/no)	Formula (yes/no)	Technique	Type of sample	Metabolites in ↑ [ ]	Metabolites in ↓ [ ]	Final findings
Andrade F, et al. 2017 [21]	42 (23M + 19F);	Cross-sectional	Yes	No	HPLC coupled to a triple quadrupole MS	Plasma	Asymmetric dymethylarginine, homocysteine, arginine	-	Due to low Hcys levels, there is an impairment of methylation cycle, which is related to the lack of protein quality.
Mütze U, et al. 2012 [22]	12 (6M + 6F); 5-14yr	Cohort	Yes	Yes	LC-MS/MS	Plasma	Carnitine, acylcarnitine	γ-linolenic acid ,dihomo-γ-linolenic acid	Both, acylcarnitine concentrations and LC-PUFA satuts are influenced by the strict diet of PKU patients
Xiong X, et al. 2015 [23]	47	Cohort	-	-	GC/MS	Urine	-	Phenylpyruvic acid, phenylacetic acid, phenyllactic acid, 2-hydroxyphenylacetic acid	Phenylacetic acid might be a good diagnostic value for discriminating PKU from non-PKU patients

[ ]: concentrations, PKU: phenylketonuria, PHE: phenylalanine, HVA: homovanillic acid, 3HK: hydroxykynurenic acid, LC-PUFA: Long-chain polyunsaturated fatty acid, NMR: Nuclear magnetic resonance , HPLC: high-performance liquid chromatography, GC/MS: Gas Chromatography coupled to Mass Spectrometry, MS: Mass Spectrometry

3.4. Metabolites Identified for Phenylketonuria

Metabolomics technics are used to identify and characterize phenylketonuria metabolic biomarkers. Several publications study and compare the sensitivity and specificity of some of the conventional technics [24–27]. A multiplatform approach is preferred to analyse the metabolome profile [28]. Thus, the involvement of different pathophysiological processes could be confirmed as a system that happens in every PKU patient's metabolism. Nevertheless, most of the included studies used one technic like high-performance liquid chromatography (HPLC) that is used with several modifications. Douglas, et al. used HPLC with electrochemical detection to study plasma amino acids and other metabolites. Vilaseca et al. [18] applied HPLC with UV detection in their study and Andrade et al. and Boulet et al. [19,21] used it coupled to mass spectrometry. Only one of the included articles used Nuclear magnetic resonance (NRM) technic [14].

Mütze et al. applied liquid chromatographic tandem mass spectrometry (LC-MS/MS) to analyse the influence of moderate long-term fatty acids restriction in *PKU* patients and gas chromatography to determine the fatty acid composition of plasma glycerophospholipids [22]. Wild, et al. [20] carried on a urine metabolite profiling by MSI-CE-MS which resulted to offer a valuable analytical tool for dietitians to better monitor phenylalanine intoxication due to poor dietary adherence in *PKU* patients. About the others included studies [16–18,23] used Gas Chromatography coupled to Mass Spectrometry (GC/MS) to obtain metabolomic information. Xiong and colleagues used an improved oximation-silylation method combined with GC/MS and based on the results they concluded that this improved technique might be reliable for the diagnosis and differential diagnosis of *PKU* [23].

In total, 34 differential metabolites were extracted from the 10 included publications in this review. Based on the number of times the metabolites were reported, phenylalanine, arginine and carnitine are the most significant metabolites because they were reported up to three times [16–18,20–22] followed by phenylpyruvic acid, asymmetric dimethylarginine (ADMA) and docosahexaenoic acid, which were reported two times [16–18,20,21,23]. Seven high frequency (reported twice or more) differential metabolites were identified which are displayed in Table 2.

Table 2. High Frequency Differential Metabolites Related to Phenylketonuria.

Metabolite Name	HMDB ID	Hits	Biological samples
Phenylalanine	HMDB0000159	3	Plasma
Arginine	HMDB0000517	3	Plasma
Carnitine	HMDB0000062	3	Plasma
Phenylpyruvic acid	HMDB0000205	2	Urine
Tyrosine	HMDB0000158	2	Plasma
ADMA	HMDB0001539	2	Urine
DHA	HMDB0002183	2	Plasma

ADMA: asymmetric dimethylarginine; DHA: Docosahexaenoic acid, HMDB ID: The Human Metabolome Database

Phenylpyruvic acid was determined as the most significant urinary biomarker by two studies [20,23]. Additionally, Wild et al. detected low levels in plasma and urine samples of arginine, tyrosine, 2-aminobutyric acid, propionylcarnitine, 2-aminoadipic acid, aminohippuric acid, urocanic acid and carnitine. Contrary, elevated levels of phenylacetylglutamine, hydroxyphenylacetic, imidazole lactic acid and phenylpyruvic acid were found in plasma of PKU patients [20]. Xiong, et al. [23] identified phenylacetic acid as a possible reliable discriminator for the diagnosis of PKU. This was determined with the largest area under the curve (AUC) of 0,987, and a high specificity and sensitivity of 0,936 and 1.000, respectively. Furthermore, 19 differentially compounds were reported in PKU patients such as phenylpyruvic acid, 2-hydroxyphenylacetic acid and N-phenylacetylglutamine [23].

The decreasing of DHA, asymmetric dimethylarginine (ADMA), Hcys, carnitine, acylcarnitine, Tyr, glutamine, hydroxykynurenic acid (3HK) and the increasing of homovanillic acid (HVA), 3-methoxytyramine (3MT), 5-hydroxyindole acetic acid (5HT), serotonin, kynurenic acid (KA) and KYN were observed in several publications included in this review.

3.5. Pathway analysis

The metabolomic information of 10 studies on phenylketonuria [14–23] were integrated to study enriched pathways and impact pathways at the significance level of 0.05 on both p value and FDR (False Discovery Race). In Table 3 and 4, those pathways are shown with also the impact and the match status of each one.

Table 3. Pathway analysis of metabolic biomarkers in plasma on PKU

Pathway Name	Match Status	P value	FDR	Impact
Phenylalanine metabolism	**5/10**	**7,48E-7**	**6,28E-5**	**0,619**
Phenylalanine, tyrosine and tryptophan biosynthesis	**3/4**	**3.472E-5**	**0.0015**	**1.0**
Tyrosine metabolism	4/42	0.0108	0.3018	0.287
Aminoacyl-tRNA biosynthesis	4/48	0.0171	0.34	0.0
Cysteine and methionine metabolism	3/33	0.0310	0.467	0.233
Arginine biosynthesis	2/14	0.0341	0.467	0.076
Biosynthesis of unsaturated fatty acids	3/36	0.039	0.467	0.0
Tryptophan metabolism	3/41	0.0541	0.568	0.213

Thirty-four differential metabolites, which were reported in plasma and urine, were included in the software MetaboAnalyst for pathway analysis and enrichment analysis. A visual graphic was created with metabolic signatures related to the phenylketonuria metabolites that were integrated in this analysis (Figure 2).

When significance is determined by both p value and FDR for the pathways, phenylalanine metabolism and phenylalanine, tyrosine and tryptophan biosynthesis were significantly enriched from the resulted pathways reported in plasma (Table 3).
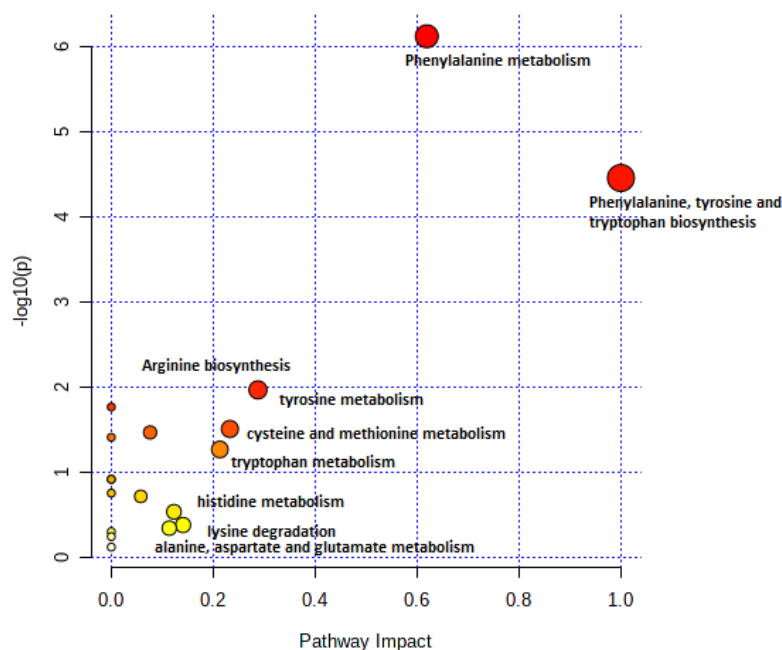
Figure 2. Pathway analysis for significant metabolites of PKU

4. Discussion

In this systematic review, we analyzed 10 studies and determined significant metabolic pathways of phenylketonuria by conducting a Pathway Analysis with MetaboAnalyst software. As a result of this analysis, seven differential metabolites were identified in two studies or more and were determined as high frequency metabolites in PKU. Phenylalanine is the most remarkable, reliable and promising metabolic marker with the highest intervention in most identified metabolic pathways. Due to the significant enriched metabolism pathways found in the conducted analysis, metabolic dysregulations could be more studied in phenylketonuria patients for a better understanding of the disorder.

4.1. Dietary Treatment on PKU and Metabolomics application

Metabolomics technics were used in the included studies to determine differential metabolites in PKU patients based on the concentrations found in urine and plasma. The concentration of monoamines is an important deficiency that appear in patients with a restricted diet as mentioned by several studies [9,29,30]. Douglas et al. [15] studied the impact of sapropterin on monoamine neurotransmissor status in patients with PKU between 4 to 49 years old with a restricted diet and supplementation of sapropterin (20mg/kg/day). They concluded that the degree of adherence to dietary Phe restriction had no significant correlation with monoamine outcomes but with the medical food that was facilitated to patients [15].

Several publications [14,18,22] studied the fatty acid profile in PKU patients following a restricted diet with medical food treatment. Cannet et al. [14]. found that a good adherence was correlated with Phe levels. Two publications [17,18] observed lower concentrations of docosahexaenoic acid (DHA) in PKU patients. However, lower concentrations of DHA are shown due to the restriction of LCPUFA sources in Phenylketonuria restricted diet. Some of these principles sources are fish, meat, eggs, nuts, liver, or milk products [31]. Tough, no improvement was seen when some animal fat and a few portions of yogurt and quail egg yolk were included [18], which means DHA concentrations cannot be improved by only dietary treatment. Another study evaluated metabolic pathways of fatty acids in PKU patients and reduced concentrations of acylcarnitine and others intermediates were found [22].

Furthermore, Boulet, et al. [19] studied the tryptophan metabolism in a cohort of 151 PKU patients and how it is modified when a controlled low Phe diet was followed. Tryptophan is an essential AA involved in protein synthesis, a precursor of serotonin and others active substances [32]. They found that concentrations of hydroxykynurenic acid (3HK), a tryptophan metabolite, was lower in only patients that underwent controlled low Phe-diet, but this could be restored when a Phe-free amino acid substitute was taken[19].

Given that low asymmetric dimethylarginine (ADMA) levels have been observed in adolescents and adults with PKU, most of the evidence refers to a possible cardiovascular risk in PKU patients [33]. Kanzelmeyer, et al. [16] found that the concentrations of the ADMA, an endogenous inhibitor of nitric acid and a novel marker of cardiovascular risk, is unaltered in PKU patients. Despite of ADMA concentrations were unaltered they came to be lower in PKU patients than in the control group [21]. In this present study, homocysteine (Hycs) levels were lower than normal range and it was due to the low-quality protein intake that PKU patients follow because of the disease.

Metabolomics could be used to improve the diagnosis of phenylketonuria by detecting more differential metabolites in biological samples.

4.2. Metabolic Dysregulations Implicated in Phenylketonuria Pathogenic Mechanisms

By the obtained results of the different pathways, amino acid dysregulation was the most notable characteristic of phenylketonuria metabolic pathways. Phenylalanine resulted to be an important biomarker in the pathways of phenylalanine, tyrosine and tryptophan. Moreover, a relation was indicated between these metabolites because phenylalanine dietary intake can influence in both phenylalanine hydroxylation and tyrosine oxidation as well as at the rates of plasma phenylalanine and tyrosine concentrations [34].

High Phe concentrations are due to a less strict compliments of the diet and this can result in a range of neuropsychiatric problems like depression and anxiety [35]. Furthermore, different type of cognitive dysfunction in PKU patients were hypothesized to be related to elevated blood Phe concentrations or reduced blood Tyr concentrations [36]. Some reports have already characterized the role of Phe in the deficiencies of several precursors of monoamine neurotransmitters, as Tyr and Trp. This happens due to the competitive inhibition from Phe for the large neutral amino acid (LNAA) transporter 1 (LAT1) [36,37]. Likewise, tyrosine was also identified as a high frequency biomarker in phenylketonuria. Tyrosine is a non-essential amino acid that is synthesized from phenylalanine. But, since PKU patients cannot synthesize Tyr from Phe on account of the sever deficiency of PAH enzyme, Tyr is considered as an essential amino acid in these patients [34].

Tryptophan metabolism was also shown to be crucial for the development of phenylketonuria as an essential amino acid involved in protein synthesis and a substrate to serotonin synthesis [32]. Tryptophan also has a role as the precursor of an active substance called kynurenine (KYN), this metabolite was also identified in PKU patients biological samples in lower levels than normal [19]. Furthermore, tryptophan metabolism is linked with neurological perturbation, which several PKU patients suffer when there is not a good adherence to dietary treatment and supplementation [1]. Tryptophan metabolism is considered important in phenylketonuria because several similarities are presented between both metabolic pathways of phenylalanine and tryptophan. For example, both amino acid use the same L-large neutral amino acid type 1 transporter to cerebrospinal fluid, and this has an affinity stronger to Phe than to Trp [38,39].

4.3. The role of metabolomics in clinical implications of Phenylketonuria

Following a restricted diet on animal protein a low cholesterol concentration might be found in blood. This, being known to be a protector effect on cardiovascular diseases such as atherosclerosis.

Although, it doesn't apply to several PKU patients because of having elevated levels of ADMA, a metabolic by-product from protein metabolism found in blood [44].

One of the main metabolites that could be a cardiovascular risk if was found in high levels is ADMA. This tends to happen with homocystinuria, which is presented in several PKU patients. Kanzelmeyer et al. implemented a MS technique to find that the levels of ADMA were lower in PKU patients than in controls. Consequently, it should not be considered a risk and it may be due to the decreased nitric oxide (NO) synthesis, an endogenous vasodilator, or other pathways [16].

Furthermore, when a restricted diet is followed a nutrient deficiency can occur and then an oral supplementation might be necessary. In PKU patients a Phe free amino acids supplementation is prescribed. Though, it should be considered that these formulas may suffer from oxidation, and consequently these amino acids would not be efficient enough [21]. Thus, it is important to evaluate first if patients are good responders to such supplementation and if not, other options should be considered.

Tetrahydrobiopterin (BH4), also known as sapropterin, is used along with the restricted diet to control blood Phe levels in adults and children with 1 month of age and older with PKU [45]. Vilaseca, et al. found that DHA levels were normal in PKU patients treated with BH4[18]. Douglas, et al. studied the effects of sapropterin on urinary monoamines using metabolomics techniques to identify modifications in differential metabolites. Homovanillic acid (HVA), a marker of dopaminergic activity, was increased in PKU patients after 1 month of sapropterin (20mg/kg) [15].

5. Conclusions

The findings in this review on 34 high-frequency metabolites have been indicated to be in relation with Phenylketonuria. The noted metabolic pathways may provide a better understanding of all the implicated biological mechanisms of the disease. Metabolomics approach could be used to determine more differential metabolites to improve the diagnosis and treatment.

Due to the vast majority of studies being conducted in small cohorts with limited number of participants, generalizability and applicability are limited. Furthermore, considering the wide variability in terms of study design, technics, methods of outcome assessment there is limitations of the comparability of these studies. From all the studies selected studies, we could see that is not difficult to study PKU metabolites in patients that present nutritional deficiencies and that need to be guided by a prepared dietitian.

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Supplementary Table S1.   Used syntaxy in both Data Bases for the systematic literature.

DATABASE	SYNTAX
PubMed	(human* OR subject* OR adult* OR child* OR men OR "male" OR women OR "female" OR patient OR patients OR volunteer* OR participant* OR adolescent* OR overweight OR infant* OR newborn* OR neonate*) AND (trial OR experiment OR study OR studies OR intervention OR "Clinical Trial" OR "Controlled Clinical Trial" OR "Randomized Controlled Trial" OR "observational study" OR observational OR cohort OR "cohort study") AND (metabolomics OR metabonomics OR metabolome OR "metabolic profile" OR "metabolomic profile" OR biomarker* OR metabolite OR metabolic* OR fingerprint*) AND (PKU OR phenylketonuria* OR Hyperphenylalaninemia* OR Hyperphenylalaninemia, Non-Phenylketonuric OR "BH4 Deficiency" OR "Atypical PKU" OR BH4 OR "Tetrahydrobiopterin Deficiency" OR "phenylketonuria II" OR "DHPR Deficiency" OR "Dihydropteridine Reductase Deficiency") AND (serum OR urine* OR plasma OR feces OR faeces OR blood) AND (LC-MS OR LC/MS OR NMR OR chromatography OR " Nuclear magnetic resonance" OR "gas chromatography" OR "mass spectrometry")AND (diet* OR nutritional OR "nutritional intervention" OR dietary OR phytochemical* OR "medical foods" OR "low-phenylalanine restricted diet" OR "low-phenylalanine diet" OR "fatty acids" OR protein* OR lipid* OR "dietary adherence" OR "diet records" OR "nutritional status" OR "nutrient status")
Web of Science	(human* OR subject* OR adult* OR child* OR men OR "male" OR women OR "female" OR patient OR patients OR volunteer* OR participant* OR adolescent* OR overweight OR infant* OR newborn* OR neonate*) AND (trial OR experiment OR study OR studies OR intervention OR Clinical Trial OR Controlled Clinical Trial OR Randomized Controlled Trial OR observational study OR observational OR cohort OR cohort study) AND (metabolomics OR metabonomics OR metabolome OR metabolic profile OR metabolomic profile OR biomarker* OR metabolite OR metabolic* OR fingerprint*) AND (PKU OR phenylketonuria* OR Hyperphenylalaninemia* OR hyperphenylalaninemia OR non-phenylketonuric OR BH4 deficiency OR atypical PKU OR BH4 OR tetrahydrobiopterin Deficiency OR phenylketonuria II OR DHPR Deficiency OR dihydropteridine reductase deficiency) AND (serum OR urine* OR plasma OR feces OR faeces OR blood) AND (LC-MS OR LC/MS OR NMR OR chromatography OR nuclear magnetic resonance OR gas chromatography OR mass spectrometry) (diet* OR nutritional OR "nutritional intervention" OR dietary OR phytochemical* OR "medical foods" OR "low-phenylalanine restricted diet" OR "low-phenylalanine diet" OR "fatty acids" OR protein* OR lipid* OR "dietary adherence" OR "diet records" OR "nutritional status" OR "nutrient status")

Supplementary Table S2 Methodological quality assessment using the QUADOMICS Tool.

Ref.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	score
Cannet (2020)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	N	11/13
Douglas (2013)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	Y	12/13
Kanzelmeyer (2012)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	N	11/13
Yi (2011)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	Y	12/13
Vilaseca (2010)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	N	11/13
Boulet (2020)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	N	11/13
Wild (2019)	Y	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	Y	12/13
Andrade (2017)	N	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	Y	11/13
Mütze (2012)	N	N/A	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N/A	N	Y	11/13
Xiong (2015)	N	N/A	Y	Y	Y	Y	Y	N/A	N/A	Y	Y	N	Y	N/A	N	Y	9/12

Table 1. Used items are described in the next list.

1. Were selection criteria clearly described?

2. Was the spectrum of patients representative of patients who will receive the test in practice?

3. Was the type of sample fully described?

4. Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail? 4a. Clinical and physiological factors 4b. Diagnostic and treatment procedures

5. Were handling and pre-analytical procedures reported in sufficient detail and similar for the whole sample? And, if differences in procedures were reported, was their effect on the results assessed?

6. Is the time period, between the reference standard and the index test short enough to guarantee that the target condition did not change between the two tests?

7. Is the reference standard likely to correctly classify the target condition?

8. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?

9. Did patients receive the same reference standard regardless of the result of the index test?

10. Was the execution of the index test described in sufficient detail to permit replication of the test?

11. Was the execution of the reference standard described in sufficient detail to permit its replication?

12. Were the index test results interpreted without knowledge of the results of the reference standard?

13. Were the reference standard results interpreted without knowledge of the results of the index test?

14. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?

15. Were uninterpretable / intermediate test results reported?

16. Is it likely that the presence of over-fitting was avoided; Y=criteria achieved, N=criteria not achieved, N/A=not applicable.

    Y=criteria achieved, N=criteria not achieved, N/A=not applicable.