Lipids and phenylketonuria: current evidences pointed the need for lipidomics studies

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Abstract

Phenylketonuria (PKU) is the most prevalent inborn error of amino acid metabolism. The disease is due to the deficiency of phenylalanine (Phe) hydroxylase activity, which causes the accumulation of Phe. Early diagnosis through neonatal screening is essential for early treatment implementation, avoiding cognitive impairment and other irreversible sequelae. Treatment is based on Phe restriction in the diet that should be maintained throughout life. High dietary restrictions can lead to imbalances in specific nutrients, notably lipids.

Previous studies in PKU patients revealed changes in levels of plasma/serum lipoprotein lipids, as well as in fatty acid profile of plasma and red blood cells. Most studies showed a decrease in important polyunsaturated fatty acids, namely DHA (22:6n-3), AA (20:4n-6) and EPA (20:5n-6). Increased oxidative stress and subsequent lipid peroxidation have also been observed in PKU.

Despite the evidences that the lipid profile is changed in PKU patients, more studies are needed to understand in detail how lipidome is affected. As highlighted in this review, mass spectrometry-based lipidomics is a promising approach to evaluate the effect of the diet restrictions on lipid metabolism in PKU patients, monitor their outcome, namely concerning the risk for other chronic diseases, and find possible prognosis biomarkers.

Keywords: Inborn errors of metabolism; phenylketonuria; lipid changes; oxidative stress; lipidomics; mass spectrometry.
Abbreviations and acronyms

AA – Arachidonic acid; ALA – Alpha-linolenic acid; BBB – Blood-brain barrier; BH₄ –
Tetrahydrobiopterin; CE – Cholesterol esters; DHA – Docosahexaenoic acid; EPA –
Eicosapentaenoic acid; FA - Fatty acid(s); FAME – fatty acid methyl ester; FID – flame
ionization detector; GC – Gas chromatography; GSHPx – Glutathione peroxidase; HDL-
C – High density lipoprotein cholesterol; HFA – Hyperphenylalaninemia; IEM - Inborn
error(s) of metabolism; LA – Linoleic acid; LDL-C – Low density lipoprotein cholesterol;
LNAA – Large neutral amino acid(s); MDA – Malondialdehyde; MS – Mass spectrometry;
MS/MS – Tandem mass spectrometry; MUFA – Monosaturated fatty acid(s); PAH –
Phenylalanine hydroxylase; Phe – L-Phenylalanine; PKU – Phenylketonuria; PL –
Phospholipid(s); PUFA – Polyunsaturated fatty acid(s); Q₁₀ – Ubiquinone-10; RBC – Red
blood cells; ROS – Reactive oxygen species; SAA – serum amyloid A; Se – Selenium;
SFA – Saturated fatty acid(s); SPE – Solid phase extraction; TAG – Triacylglycerols; TLC
– Thin layer chromatography; TXB₂ – Thromboxane B₂; TXB₃ – Thromboxane B₃; Tyr –
L-Tyrosine; VLDL-C – Very low-density lipoprotein cholesterol;
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1. Inborn errors of metabolism (IEM)

IEM are a phenotypically and genetically heterogeneous group of disorders caused by alteration of a specific chemical reaction in metabolism [1]. IEM are individually rare (some with a birth prevalence less than 1 per 100 000), but collectively they account for a significant proportion of life-threatening and/or chronic illnesses, particularly in children [2].

The pathogenesis of an IEM can be attributed to loss or, more rarely, gain of function of a mutant protein (usually an enzyme or a transporter), and thus, the disease is generally associated with an altered metabolite flux through the involved pathway [1,2]. Pathological consequences can be due to: 1) direct toxicity of accumulating upstream metabolites, 2) deficiency of downstream products beyond the block of the metabolic pathway, 3) activation of alternative metabolic pathways leading to unusual metabolite production, and/or 4) feedback inhibition or activation by the substrate(s) or the product(s) on the same or different pathways (Figure 1) [1,3].

A subgroup of IEM, in which phenylketonuria (PKU) is included [3], is due to malfunction of the catabolic pathways of specific amino acids, with accumulation of the substrates of the defective enzyme and/or production of an alternative product. Those disorders, caused by “endogenous” intoxication, are amenable to dietary intervention with restriction of the accumulated amino acids and supplementation of deficient metabolites.

2. Phenylketonuria (PKU)

PKU is the most prevalent disorder of amino acid metabolism. Its occurrence varies among ethnic groups and geographic regions worldwide. In Europe, the mean birth prevalence is 1 in 10 000 [4]. In Portugal, the estimated frequency was 1 in 10 867, in 2018 [5]. PKU is characterized by elevated levels of L-phenylalanine (Phe) in the blood, a condition known as hyperphenylalaninemia (HFA). In more than 98% of the patients, HFA is due to deficiency of the liver enzyme phenylalanine hydroxylase (PAH) that converts Phe into L-tyrosine (Tyr) [6]. Accumulation of Phe is associated with central nervous system toxic effects, leading to progressive intellectual impairment, autism, microcephaly, seizures, and motor deficits. Some patients are blond and present eczematous rash due to Tyr (and melanin) deficiency. In order to prevent possible irreversible complications, the early diagnosis and implementation of treatment is essential [7,8].
Phe is an essential amino acid, which is mainly metabolized in the liver. When Phe is in excess in the body, and is not used for protein synthesis, it is hydroxylated into Tyr by PAH, with tetrahydrobiopterin (BH₄) as cofactor, as well as iron and molecular oxygen (Figure 2) [9]. PAH deficiency results in a total or partial inability to convert Phe, from the diet or derived from catabolism of proteins in the body, into Tyr, leading to an increase of Phe concentration in the blood [6]. Beyond enzymatic protein deficiency, PKU can also be caused by defects in the enzymatic cofactor (BH₄) synthesis or reduction [10]. These are extremely rare, have specific pathogenic mechanisms and clinical phenotype and will not be further discussed.

PAH deficient activity leads to an increase of Phe plasma levels and high Phe/ Tyr ratio. Tyr concentration can be normal or low. Due to the high concentrations of Phe, an alternative pathway for Phe degradation, less active in healthy individuals, becomes prominent in PKU. In this pathway, Phe undergoes a transamination, leading to the formation of phenylpyruvate. This metabolite, together with Phe, is accumulated in the blood and excreted in the urine [11,12]. Phenylpyruvate molecules that are not excreted may be decarboxylated to phenylacetate, or reduced to phenyl-lactate [10] (Figure 2). The excretion of excessive Phe and its metabolites can create a characteristic musty body odor [6,13]. The decrease or absence of PAH activity may also lead to a deficiency of Tyr and its downstream products, which includes the decrease of melanin production (Figure 2). Consequently, PKU patients may have hair, skin and eye hypopigmentation [14].

The mechanisms by which high Phe concentrations disturb cerebral metabolism and cognitive function are not yet fully understood. However, several factors have been proposed as contributing to the neurotoxicity in PKU, including:

i) The effect of elevated Phe concentrations on the transport of other metabolites across the blood-brain barrier (BBB): Phe concentrations in the brain occur from its passage across the BBB. There is a competition between Phe and other large neutral amino acids (LNAA), such as Tyr, leucine, tryptophan, threonine, isoleucine, valine, methionine and histidine, for the transport at the BBB, which is mediated by the LNAA transporter 1 [15–18]. The increased Phe concentrations in blood lead to high levels of Phe and a decrease of the other LNAA in the brain. This unbalance negatively influences the protein synthesis rate, causing the impairment of the dendritic projections and myelination (increase of myelination turnover and decrease of myelin production) [17];

ii) Tyr deficiency: In PKU, Tyr becomes a semi-essential amino acid. The reduced levels of Tyr in brain lead to impaired synthesis of catecholamines such as dopamine,
norepinephrine and epinephrine (Figure 2), which are important neurotransmitters [15,19,20];

iii) The effect of elevated Phe concentrations on the production of free radicals:

The accumulation of Phe and its metabolites induces, directly or indirectly, the production of free radicals and/or depletion of the central nervous system antioxidant capacity, contributing to increased oxidative stress and neurological impairment [21];

All those factors contribute to the pathophysiology of the neurological impairment observed in PKU, especially in non-treated or late-treated patients.

2.1 Diagnostic

In Europe, PKU diagnosis is based on the criteria established by the European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria [4]. Newborn screening is done in most European countries [7]. Blood is collected at a Guthrie card by pricking the baby’s heel [4], as it has high density of blood capillaries and few nerves, causing less pain to the newborn. Also, the heel is easily accessible. Usual time collection is between the third and sixth day after birth to ensure that the newborn had, at least, 48 hours of feeding. Earlier blood collection can lead to false negative results, as there has not yet been time for diet Phe to reach diagnosable levels [22].

In the past, for PKU screening, two laboratory methods were used: 1) the Guthrie card bacterial inhibition assay, a time-tested, inexpensive, simple and reliable test, and 2) the fluorometric analysis, a reliable quantitative and automated test, which produces fewer false-positives test results than the bacterial inhibition assay [23]. Nowadays, the analysis of dried blood spots is based on tandem mass spectrometry (MS/MS). This is a very sensitive technique, and in a single analysis, it can measure Phe and Tyr concentrations [4,13,23], as well as of other metabolites related to other IEM [24,25]. MS/MS is now used in newborn screening programs in all Europe. In case of a positive result, Phe is higher than 2.0 mg/dL [12], Tyr values may be normal or low, and Phe/Tyr ratio is three or above (normal Phe/Tyr ~1) [26]. If this happens, it is crucial to track down defects on biopterin metabolism [27], especially when normal plasma Tyr values are found, through the study of urinary biopterin and neopterin profiles and blood dihydrobiopterin reductase activity determination.

PKU phenotype determination is not always straightforward because Phe concentrations are measured in newborn babies when blood Phe might not have time to reach its highest value. Also, Phe tolerance levels change with age. Nevertheless, three forms of the
disease, according to the Phe values found at screening can be considered: hyperphenylalaninemia (3-6 mg/dL), moderate or atypical PKU (6-20 mg/dL), and classic PKU (higher than 20 mg/dL) [12]. Once diagnosis is established, dietary treatment by Phe restriction is implemented as soon as possible, in order to prevent possible irreversible neurological sequelae.

2.2 Therapeutic approach in PKU

Phe levels in the body are a result of a balance between diet, catabolism of endogenous proteins and conversion of Phe into other metabolites [18]. Therapeutic approach for PKU aims to normalize Phe and Tyr concentrations in the blood, preventing the development of neurological manifestations. PKU patients follow a Phe-restricted diet, which is achieved by lowering natural protein intake and supplementing with amino acid mixture free of Phe, as needed [28]. Blood Phe levels must be regularly evaluated and kept below 6 mg/dL until 12 years of age, as well as before and during pregnancy. In late adolescence and in adulthood, Phe levels under 8 mg/dL are recommended [12,28]. As Phe levels fall during the day and rise during the night until reaching a maximum value in the early morning, blood samples should be collected in the morning after fasting overnight [4].

A diet restricted in Phe should be implemented after PKU diagnosis and maintained for life [28]. The normal daily variation of blood Phe levels is lower than 50% in healthy individuals, but it can be much higher in PKU. Such variation in PKU patients may be influenced by the adherence to diet, but also changes in growth rate, illness and genotype [29].

In PKU diet, rich protein foods, such as eggs, milk, cheese, meat, fish and dried beans, are excluded [30]. Drinks containing aspartame are also avoided because, when metabolized, it releases Phe, L-aspartic acid and methanol [6]. The low-Phe diet provides a low amount of natural protein, which may not be enough for growth requirements. Thus, in order to provide a nutritionally adequate diet, in most PKU patients, it is necessary a semi-synthetic diet based on commercial formulas with Phe-free essential amino acids, which also contain minerals, vitamins and other nutrients [4,28]. In particular, the diet restrictions lead to a low dietary intake of polyunsaturated fatty acids (PUFA). To ensure that the dietary needs for essential PUFA are met, amino acid mixtures containing PUFA and PUFA supplements are prescribed to PKU patients [4]. However, some imbalance in the level of PUFA can occur, as described below (section 2.3.2).
The dietary treatment is complex, demanding, and lifelong. It requires the patient's full understanding of the disease and cooperation. Therefore, any methods that relieve the dietary requirement are welcomed by the patients [31]. In this context, there has been a great interest in alternative and complementary therapeutic approaches for PKU.

BH₄ has recently been approved to treat PKU. Patients with high residual activity of PAH, but also a minority of patients with classical PKU, can benefit from this treatment. Some mutations are associated with BH₄-sensitive phenotype of PKU [32], in which giving pharmacological doses of exogenous BH₄ results in an increase in the activity of PAH that is enough to reduce circulating Phe to a therapeutically relevant extent [33,34].

Efficacy and safety of BH₄ has been demonstrated in children below the age of 4, which led to the European approval for BH₄ in this age category [35,36]. Nevertheless, few clinical studies are available to demonstrate long-term therapeutic efficacy, as well as the long-term neurocognitive consequences and the impact on behavior and quality of life of PKU patients [37,38].

Because Phe competes with other LNAA for transport across the BBB, supplementation with LNAA (Phe-free) is another alternative therapeutic approach. As supported by quantitative magnetic resonance spectroscopy analysis, supplementation with LNAA reduces the influx of Phe into the brain, consistent with competitive inhibition of Phe transport due to increased plasma levels of other LNAA [18]. LNAA supplementation is most effective in lowering plasma Phe concentrations in individuals who have difficulties in complying with the low-Phe diet [6,28]. However, this approach has some side effects at the gastrointestinal level, since a similar transporter for LNAA exists in the gut [28].

An alternative therapeutic approach for PKU, approved for adult patients, involves the oral administration of phenylalanine ammonia lyase (PAL), a bacteria-derived enzyme that catalyzes the conversion of Phe to transcinnamic acid and ammonia in the intestinal lumen, preventing its absorption [39].

Despite the alternative approaches that have been developed, low-Phe diet remains the cornerstone for lifelong treatment of PKU. This type of diet favors a dietary intake rich in carbohydrates. The high carbohydrate intake has been pointed out, for example, as the cause for PKU patients are at risk of carbohydrate intolerance and insulin resistance [40]. However, there is currently no clear evidence that PKU patients may be at higher risk of developing diabetes mellitus and most studies only include children or young adults [41]. On other hand, dietary restrictions may lead to deficiencies, namely of PUFA, contributing for changes in the lipidome of PKU patients. Such changes reported to date are detailed below.
2.3 Changes to the lipidome in PKU

Lipids include a heterogeneous group, comprising a high number of structurally and functionally distinct molecules. They play a central role in almost aspects of biological life, either as structural components of cell membranes or regulatory and signaling molecules in many metabolic and hormonal pathways [42].

The uptake of lipids from diet is important for the maintenance of the lipidome stability [43]. The human lipid profile is regulated by a plethora of metabolic pathways and enzymes. Its disturbance can be associated with a disease state [44]. As detailed below, changes in lipid profile of PKU individuals have been reported from analysis of plasma and serum samples, as well as red blood cells (RBC).

In the plasma/serum, the most abundant lipid classes include phospholipids (PL), sterol lipids (including free cholesterol and respective esters), triacylglycerols (TAG) and minor non-esterified free fatty acids [45,46]. PL and sterol lipids are also the most abundant lipids in RBC. Cholesterol in RBC is mainly free, whereas in plasma/serum it is mainly esterified with fatty acids (FA), being a component of cholesterol esters (CE) [47]. As they are insoluble in water, lipids in the plasma/serum are mostly associated with proteins, forming lipoproteins [45,46]. Studies carried out to date have reported changes in lipoprotein components, as well as in the FA profile of plasma/serum and RBC of PKU individuals.

2.3.1 Changes in lipoprotein components

Disturbances in lipoproteins components have been reported from the analysis of serum and plasma of patients with PKU, even with good metabolic control (Table 1). In some studies, PKU patients (age: 6 months to 50 years) on dietary therapy exhibited lower total cholesterol (TC) levels compared with healthy controls [48–52]. The low TC levels in treated PKU patients could be attributed to their diet, where one of the main sources of lipids is olive oil [48–51]. Moreover, these low TC levels may also be explained by the impairment of cholesterol synthesis due to down-regulated expression of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase. The reduced expression of this enzyme might derive from the increased levels of Phe and its metabolites in plasma [49–51]. However, one study showed that TC levels did not differ between PKU patients (9 months to 7 years) and healthy individuals [53], and another study revealed that TC levels were higher in PKU patients (18-47 years) [54].
Lower low-density lipoprotein cholesterol (LDL-C) levels were also observed in PKU patients on dietary treatment when compared with healthy controls [49–51]. However, this difference was not found in other studies [48,53]. Schulpis et al [50] reported an increase in LDL-C/apolipoprotein B (Apo B) ratio in phenylketonurics (5-8 years). Regarding the very low-density lipoproteins cholesterol (VLDL-C), both an increase [49,53] and a decrease [48,50] have been observed. In several studies, it was reported low levels of plasmatic high-density lipoprotein cholesterol (HDL-C) in PKU patients (6 months to 50 years) compared with controls [48,49,51,52,54]. However, one study showed no significant differences in plasma HDL-C between PKU patients and controls [53].

Concerning TAG plasma levels, most of the studies demonstrated that they were higher in PKU patients (9 months to 36 years) with a good metabolic control compared with the healthy group. This trend has been related to the high consumption of carbohydrates in PKU diet [48,49,52,53]. A decrease in plasma TAG levels was reported in one study with PKU patients with a diet that included an additional supplementation with long-chain PUFA (fish oil) [55], but two other studies, with supplementation as well, reported no significant changes neither in serum TAG levels, nor in other lipoprotein components [56,57].

The alteration of lipoprotein profile is a key risk factor in the etiology of atherosclerosis and, thus, cardiovascular diseases. Some of the studies above mentioned reported increased levels of TC and TAG, and decreased HDL-C levels in PKU patients (9 months to 47 years). These changes are known to contribute to a higher risk of atherosclerosis and cardiovascular diseases [53,54]. A positive correlation between increased levels of HDL-C and serum amyloid A (SAA) was found in PKU patients, suggesting that HDL-C is enriched with SAA. This change may indicate an alteration in function of this lipoprotein, acquiring a pro-inflammatory phenotype [54]. However, the above mentioned increase of LDL-C/Apo B ratio suggests that PKU patients (6 months to 50 years) are not at risk of developing atherosclerosis [50,51]. High LDL-C/Apo B ratio is associated with the presence of larger and less atherogenic particles, which are less susceptible to oxidative damage than small LDL-C particles [50]. As pointed out by the literature cited, there is currently no consistent evidence that PKU patients exhibited an atherogenic lipid profile and, consequently, higher risk of developing atherosclerosis. More studies, with adequate data statistical analysis, are required to clarify the potential risk of PKU patients develop atherosclerosis and cardiovascular diseases. Due to the low prevalence of PKU, most of the reported studies analyze the lipid profile of children and adults without the separation by age range. The lipid profile changes with age [58], therefore children will
not have a similar lipid profile to adult patients. The conjugation of children and adult
patients with PKU in the same study, as if they had a similar lipid profile, may lead to
non-significant or even odd results. Thus, in the future, it is important to perform studies
with different age ranges and genders, separating children from adults, in order to obtain
significant and representative results of PKU population.

2.3.2 Changes in fatty acid profile

FA play multiple biological roles in human body, and they are structural components of
different lipids [59,60]. If the diet is changed, as it happens with PKU patients, and
consequently the FA uptake is dissimilar, it will lead to changes in lipid profile in
membranes and in lipid signaling cascades that will affect lipid metabolism and their
role and functionality at cell and organ level [60]. In fact, changes in FA profile of plasma
and RBC, where FA occur mainly esterified with PL, TAG and CE [59], have been
reported both in PKU patients maintained on a Phe-restricted diet with and without an
additional long-chain PUFA supplementation (Table 2).

The analysis of FA performed by gas chromatography (GC), in most of the studies using
GC with flame ionization detector (GC-FID), requires the derivatization of FA. With the
exception of one out of the fourteen studies analyzed in this review (Table 2), which
converted FA into pentafluorobenzyl ester (PFBE), FA were converted to FA methyl ester
(FAME) derivatives, prepared by either acid-catalyzed methods (which allow the analysis
of free and esterified FA) or basic-catalyzed methods (which limits the analysis to
esterified FA).

The FA composition (free and esterified FA) of total plasma/plasma PL fraction of PKU
patients (age: 2 months to 42 years), who have been maintained on a Phe-restricted diet,
was evaluated in different studies [61–66]. In three of the six cited studies, a significant
reduction of both PUFA docosahexaenoic (DHA) (22:6n-3) and arachidonic acid (AA)
(20:4n-6) levels in total plasma and plasma PL fraction was reported [61,62,64]. Different
authors proposed diverse explanations for the plasmatic reduction of DHA and AA.
Sanjurjo et al [64] suggested that the lower levels of plasma DHA were probably a
consequence of the prohibited intake of fish (rich in n-3 PUFA). In fact, the low dietary
intake of DHA should be the main cause for the decrease in DHA levels, as most of DHA
comes from the diet and its endogenous synthesis is very low [67]. In the study of
Sanjurjo et al [64], PKU patients also showed higher levels of linoleic acid (LA) (18:2n-6)
in plasma than those observed in healthy controls, which was associated to the
relatively high LA intake by PKU individuals [64]. In other two studies [61,62], it was
suggested that the reduction of plasmatic DHA and AA could be associated with an impairment in the endogenous synthesis of these FA in patients on a Phe-restricted diet. This impaired synthesis could be the result of the inhibition of enzymatic processes by Phe derived metabolites, within which are phenyl-lactate and phenylpyruvate. Other three studies revealed no significant differences levels of AA in total plasma/plasma PL fraction [63,65,66]. Some authors also showed a significant decrease on plasma levels of eicosapentaenoic acid (EPA) (20:5n-3) [61–63,66], but only Gramer et al [68] demonstrated no significant differences in the plasma levels of DHA and EPA. Only Giovannini et al [65] revealed that PKU patients had a lower level of DHA in plasma, while other PUFA, such as AA and EPA, did not show significant differences between PKU patients and healthy controls.

The inconsistent results obtained in the different studies, relatively to the plasma FA profile, may probably be due to the use of either plasma PL fraction or total plasma. When the FA profile is analyzed in total plasma, FA are esterified with various lipid classes (TAG, CE and PL) which are components of lipoproteins or free, whereas when in plasma PL fraction, FA are only esterified with PL [59].

The FA composition (free and esterified FA) of total RBC/RBC PL fraction of phenylketonurics with a low-Phe diet was evaluated in five studies [61–64,69]. In four of these studies, a significant decrease of DHA (22:6n-3) and EPA (20:5n-6) levels were reported [62–64,69]. In the study of Stroup et al [69], PKU patients also showed, in total RBC, higher levels of alpha linolenic acid (ALA) (18:3n-3), the precursor of DHA and EPA. This suggests that the significant decrease of DHA and EPA levels was due to the diminished efficiency in the conversion of ALA in these PUFA. However, Galli et al [61] found no significant differences for those PUFA (EPA and DHA) between PKU and healthy individuals. Moreover, Van Gool et al [63] also found a decrease in AA levels RBC PL fraction, besides that observed in DHA and EPA. Galli et al [61] found no significative differences in the RBC AA levels, in contrast to the decrease that was reported in plasma lipids of PKU patients. Thus, it was suggested that RBC can efficiently control their AA levels, even in the presence of AA deficits in plasma. The different results obtained from total RBC and respective PL fraction can be due to the lower amount of FA in PL fraction, where only esterified FA in PL are analyzed [47].

AA, EPA and DHA, also classified as highly unsaturated FA, have important functions in the living systems. EPA and AA can be released from PL by the action of phospholipase A. Most of the biological functions of AA are mediated by the so-called eicosanoids [70,71]. Eicosanoids are the active end-products of arachidonate metabolism involving...
cyclooxygenase and lipoxygenase, and the mediators are implicated in inflammatory, coagulative and vasoactive responses, and are effectors of the homeostatic processes [72]. Platelets are the main producers of thromboxane from AA. Thus, Mütze et al [72] and Agostoni et al [73] hypothesized that, if plasma level of AA is lower in PKU patients, it could result in some changes in platelets arachidonate and it will cause alteration in the production of platelet-derived eicosanoids. To explore this hypothesis, both studies measured the levels of PUFA and eicosanoids metabolites in a group of PKU patients (23 to 37 years and 2 to 17 years). In the Mütze et al [72] study, the levels of AA and thromboxane B$_2$ (TXB$_2$) did not differ between PKU patients and controls. In contrast, Agostoni et al [73] found reduced levels of TXB$_2$ and AA. On the other hand, the levels of thromboxane B$_3$ (TXB$_3$), a metabolite of EPA, were significantly lower in the PKU group when compared with the controls [72]. This may indicate a reduction on n-3 PUFA metabolism in patients with PKU, although the amount of EPA and DHA was adequate [72]. Also, Mütze and coworkers [72] hypothesized that the dietary restriction of long-chain PUFA affect the platelet function, but no differences between the PKU patients and controls were found concerning the aggregation and platelet eicosanoid release.

As shown in the studies above mentioned, the long-chain PUFA status in PKU patients is often compromised. Considering that long-chain PUFA are important structural and functional constituents of all cell membranes and essential for the normal cognitive and visual development, it has been hypothesized that lipid metabolism in PKU can be improved by dietary long-chain PUFA supplementation. In seven studies performed with PKU patients submitted to long-chain PUFA supplementation, either through fish oil or other supplements, it was noticed a significant increase of DHA in total plasma/plasma PL fraction and in the incorporation into PL in the RBC membranes [55–57,74–77]. Two of these studies showed an increased level of EPA in plasma [55,76]. In most of the studies, no modifications were observed in AA levels [55–57,74,75]. However, Beblo et al [76] observed a decrease in AA concentrations and Koletzko et al [77] observed an increase in AA levels, after the supplementation (Table 2).

Despite the differences in results between studies, the great majority of the studies on the effect of the PUFA supplementation in FA profile of PKU patients reported an increase in the n-3 levels and a decrease in the n-6 levels, as well as a higher n-3/n-6 ratio. These changes may have positive impacts in the health of patients, as n-3 PUFA have been associated with anti-inflammatory properties and reduction of the risk of development cardiovascular disease, while n-6 PUFA are associated with the promotion of inflammatory processes [78,79]. The changes observed in PUFA levels after supplementation may be due to the high percentage of n-3 PUFA in the supplements.
However, the detailed composition of the supplements was not reported in all studies, and that is important because their composition may influence the results.

The conflicting results reported on the changes in FA profile in PKU, as well as in lipoprotein components (discussed in section 2.3.1), when comparing the same type of sample, may be driven by small numbers of patients involved in the studies, as well as by other variables, such as age, sex, body mass index and disease severity, that are not taken into account. Data from adults are particularly limited and the oldest PKU patients are in the age of the 50s. Further studies in older populations of PKU patients are required to confirm the risk for the development of dyslipidemia, as well as of other comorbidities associated to PKU. Also, methodological aspects, such as the procedure used for lipid extraction from biological samples, the approach used for sample analysis and the way how results are expressed, may contribute to the conflicting results. The results obtained also depend on the type of sample analyzed. For example, plasma FA profile is an indicator of recent fat intake, while the RBC FA profile reflects longer-term intake [80]. Regarding the FA analysis, the different conditions, such as reagents, temperature and time, used to prepare derivatives from FA for GC, also influence the FA profile obtained and lead to different results. In future studies, standardization is needed to make results comparable.

2.4 Oxidative stress in PKU

Over the last years, the role of oxidative stress in PKU pathogenesis has been investigated in PKU animal model and biological samples from PKU patients under treatment [81,82]. The results indicate that oxidative stress may represent an important element in the pathophysiology of PKU. Although the cause of increased oxidative stress in this disease is poorly understood, it is assumed to result from the accumulation of toxic metabolites which induce the production of free radicals and/or from the reduction of antioxidant defenses, possibly due to the dietary treatment that lead to a deficient intake of micro or macronutrients with antioxidant properties [83]. The altered redox status in PKU patients with a low-Phe diet has been associated, in particular, with selenium (Se), ubiquinone-10 (Q10) and L-carnitine deficiencies (Figure 3).

Se deficiency has been observed in plasma of PKU patients with Phe-restricted diet [81,82,84,85]. The reduced levels of Se may impair normal plasma/RBC glutathione peroxidase (GSHPx). GSHPx is a Se-containing enzyme that removes hydrogen peroxide by coupling its reduction to water with oxidation of reduced glutathione [81]. Consequently, Se deficiency reduces the ability to cope with the usual production of
reactive species, which may result in increased ROS levels and oxidative stress. In fact, it was demonstrated that RBC GSHPx activity in PKU patients is significantly lower than in healthy controls [84,86] due to a poor Se intake. Furthermore, Se supplementation restored the activity of GSHPx [85], and the concentration of plasma Se was strongly correlated with the GSHPx activity in RCB [84,85].

Q\textsubscript{10} is a lipophilic antioxidant, important for the prevention of peroxidation of lipids in blood and tissues [81,86]. Low Q\textsubscript{10} plasma/serum levels have been found in PKU patients when compared with healthy controls [82,86]. The low Q\textsubscript{10} levels in PKU patients were mainly associated with high plasma Phe concentrations and, to a lesser extent, to the natural protein restriction, Tyr deficiency and a down regulation of the mevalonate pathway. Moreover, high levels of Phe seem to inhibit the activity of key enzymes, 3-hydroxy-3-methylglutaryl-CoA reductase (cholesterol synthesis) and mevalonate-5-phosphosphate decarboxylase (mevalonate pathway), leading to decreased Q\textsubscript{10} biosynthesis [81,82,86]. It has been reported that the low Q\textsubscript{10} values in PKU patients are associated with higher levels of plasma malondialdehyde (MDA), a product derived from lipid peroxidation. This suggest an important role of Q\textsubscript{10} in the prevention of lipid peroxidation [81]. However, the high levels of MDA may also report an increase in oxidative stress [87].

L-carnitine protects the cells from the effect of ROS. This molecule can reduce MDA levels by facilitating FA transport and thereby lowering its availability for lipid peroxidation. Decreased plasma total L-carnitine levels were found in PKU patients who strictly adhered to the diet. Also, a significant negative correlation between thiobarbituric acid-reactive substances, a parameter of lipid peroxidation, and L-carnitine plasma levels was observed [81,82,85]. Furthermore, it was demonstrated a significant inverse correlation between blood levels of L-carnitine and MDA, indicating that lipid peroxidation in PKU patients occurs mainly due to shortage of L-carnitine [85].

Oxidative stress and inflammation have been reported in PKU and seem to be related. In fact, increased levels of plasma cytokines, namely interleukin-6 and interleukin-1β, were found in treated PKU patients (age: 10-22 years), indicating a pro-inflammatory state in PKU. Also, it was found an increase in the anti-inflammatory cytokine interleukin-10. Besides that, there is a negative correlation between interleukin-6 and interleukin-10, suggesting an attempt to repair the response to inflammation processes [88]. On the other side, the increase of interleukin-1β was positively correlated with the increase of isoprostanes (lipid peroxidation biomarkers formed by non-enzymatic peroxidation of
AA), excreted in the urine of PKU patients. These results suggest that the inflammatory process is enhanced in PKU patients and is associated with lipid oxidative damage [88].

To the best of our knowledge, the few studies relating oxidative stress and lipids in PKU reported higher levels of oxidative stress and lipid peroxidation markers (MDA), but no studies identified oxidized lipid species. It is well known that oxidized lipids, not only enzymatically produced eicosanoids, but also lipid oxidation products formed by radical induced oxidation, have key roles in the onset of inflammation [89,90], namely in chronic diseases, such as cardiovascular [91] and neurodegenerative disorders [92]. Lipidomics studies at molecular level (i.e. with identification of individual lipid molecular species, including those oxidatively modified) are needed in order to identify oxidized lipid species, understand their role in PKU pathogenesis and establish the possible correlation with the risk of developing other chronic complications.

3. The need for lipidomics in PKU

Lipidomics is the systematic and large-scale study of structure, function and interactions of lipids with other lipids, proteins and other molecules in biological samples (blood, tissues, cells, among others), as well as the study of lipid changes that occur during pathophysiological disturbances [93,94].

Lipidomics analysis uses mass spectrometry (MS) approaches, most often combined with liquid chromatography [95]. These approaches allow the identification and quantification of a large range of molecular species from distinct lipid classes [96]. The main steps of a typical lipidomics workflow (Figure 4) are: extraction of lipids from biological samples, data acquisition by MS methodologies (either by direct infusion or coupled to liquid chromatography) and data analysis. The extraction is commonly performed by Bligh and Dyer [97] or Folch [98], both methods based on the use of chloroform and methanol, as well as using solid-phase extraction (SPE). In what concern to the data acquisition, two different strategies can be considered: untargeted MS, which aims the identification and quantification of as many lipid species as possible, or targeted MS, which usually aims at the detection and quantification of a panel of specific lipids. Untargeted lipidomics is usually used to prospect disease biomarkers. In validation studies, targeted methods are further designed for identified biomarkers, envisioning their implementation in clinical laboratories [99–101]. This type of methodology is also applied in the analysis of oxidatively modified lipids, which is called oxidative lipidomics [102]. The big amount of data obtained by MS are analyzed using bioinformatic tools.
Lipidomics is particularly useful to study changes in lipids at molecular level that can occur as consequence of the metabolic adaptation in a disease environment [103–110]. Thus, lipidomics has been applied in the study of several diseases [103–110], and, in particular, of some IEM, such as FA oxidation defects [111–113] and peroxisomal disorders [114]. To date, there are no lipidomics studies at molecular level, using MS-based strategies, in PKU. Despite the important current knowledge, further studies are needed to understand in detail the changes in the lipid profile of PKU patients. As highlighted in this review, previous studies reported that lipoprotein lipids and FA composition of plasma/serum and RBC can be changed in PKU patients. Such changes can affect molecular species of different lipid classes, and, in particular, changes in PL can be expected.

PL, the major building blocks of biological membranes, incorporate about 50% of the total amount of FA in the plasma. PL are important players in the regulation and control of cellular functions in health and in disease [115]. It is widely recognized that disturbances in PL homeostasis are associated with several diseases and their study can give new insights in the knowledge of disease pathophysiology, new prognosis biomarkers, or risk of other comorbidities [115,116]. However, changes in the PL profile of PKU patients have never been explored.

Lipidomics studies are needed to face the lack of knowledge regarding changes in molecular species of PL, but also of other lipid classes, in PKU. Such knowledge would contribute to understand the lipid metabolism adaptation in PKU patients, monitor their outcome, namely concerning the risk for other chronic diseases, and find possible prognosis biomarkers.

4. Concluding remarks
The high dietary restrictions of PKU treatment may lead to nutritional imbalances, namely at the level of important lipids and antioxidants. Also, the accumulation of toxic metabolites could affect enzymes of lipid metabolism. Alterations in lipoprotein component levels and at the FA profile have been disclosed in PKU, as well as increased oxidative stress, lipid peroxidation and inflammation.

Although most of the studies made in PKU patients to date have been done in children, it is important to perform studies in adults in order to understand if there is an association with the development of chronic complications, such as diabetes mellitus and
cardiovascular diseases. Future studies should also consider gender and age stratification.

Further studies using MS-based lipidomics are needed to understand in detail the changes in the lipid profile of PKU patients, particularly at the level of PL, which have important signaling and regulatory functions. The identification of the alterations of the phospholipidome could help to understand not only the role of PL in biological and pathological conditions, but also an important part of molecular mechanisms in PKU. The use of lipidomics approach may contribute to unravel the pathophysiology of PKU, as well as to identify possible biomarkers for disease monitoring and treatment response.

In a first stage, lipidomics studies using untargeted approach are needed in order to identify possible biomarkers. Such biomarkers could be further considered for the development of target methods, envisioning their implementation in clinical laboratories.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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References


[59] P. Risé, S. Eligini, S. Ghezzi, S. Colli, C. Galli, Fatty acid composition of plasma, blood cells and whole blood: relevance for the assessment of the fatty acid status in


M.A. Cleary, F. Feillet, F.J. White, M. Vidailhet, A. MacDonald, A. Grimsley, N. Ma


Ú. Catalán, M. Rodríguez, M.-R. Ras, A. Macia, R. Mallol, M. Vinaixa, S. Fernández-Castillejo, R.-M. Valls, A. Pedret, J. Griffin, R. Salek, X. Correig, M. Motilva, R. Solà, Biomarkers of food intake and metabolite differences between plasma and red blood


Figure 1. Pathogenic mechanism in inborn errors of metabolism (IEM). The enzyme deficiency leads to accumulation of substrate (S) and intermediary metabolites proximal to the blockage and the formation of alternative products (C) and may also lead to deficiencies of products downstream of the blockage (P). Pathological consequences can be due to: 1) direct toxicity of accumulating upstream metabolites, 2) deficiency of downstream products beyond the blockage, 3) activation of alternative metabolic pathways leading to alternative metabolite production, and 4) feedback inhibition or activation by the substrate on the same or different pathway. Adapted from Lanpher et al [1] and Dixon et al [117].
Figure 2. Phenylalanine metabolism. BH$_4$: tetrahydrobiopterin; PAH: phenylalanine hydroxylase; TYRH: tyrosine hydroxylase; Adapted from Rocha and Martel [10] and van Wegberg et al [4].
Figure 3. Systematic representation of oxidative stress in PKU.
Figure 4. Basic lipidomics workflow. Adapted from Domingues et al [101] and from Lydic and Goo [44].
### Tables

**Table 1.** Changes in lipoprotein components observed in serum and plasma of PKU patients, subjected to a Phe-restricted diet and after an additional long-chain PUFA supplementation, reported in published studies with statistical analysis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of sample (results expressed as)</th>
<th>Number of PKU patients</th>
<th>Age range</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schulpi and Scarpelezou [48]</td>
<td>Serum (mg/dL)</td>
<td>20 (10F+10M)</td>
<td>1-10 yr</td>
<td>TC, HDL-C, VLDL-C</td>
</tr>
<tr>
<td>Colomé et al [49]</td>
<td>Serum (mmol/L)</td>
<td>61</td>
<td>1-36 yr</td>
<td>TC, HDL-C, LDL-C</td>
</tr>
<tr>
<td>Schulpis et al [50]</td>
<td>Serum (mmol/L)</td>
<td>44</td>
<td>5-8 yr</td>
<td>TC, LDL-C, VLDL-C, Apo B</td>
</tr>
<tr>
<td>Azabdaftari et al [54]</td>
<td>Serum (mmol/L)</td>
<td>23</td>
<td>18-47 yr</td>
<td>HDL-C</td>
</tr>
<tr>
<td>Couce et al [51]</td>
<td>Plasma (mg/dL)</td>
<td>100 (53F+47M)</td>
<td>6 m-50 yr</td>
<td>TC, HDL-C, LDL-C, Apo B</td>
</tr>
<tr>
<td>Rocha et al [52]</td>
<td>Plasma (mg/dL)</td>
<td>89</td>
<td>7.8-21 yr</td>
<td>TC, HDL-C</td>
</tr>
<tr>
<td>LaVoie et al [53]</td>
<td>Plasma (μmol/L)</td>
<td>21</td>
<td>9 m-7 yr</td>
<td>TAG, VLDL-C</td>
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<tr>
<td>Agostoni et al [55]</td>
<td>Plasma (mmol/L)</td>
<td>21</td>
<td>5-10 yr</td>
<td>TAG</td>
</tr>
</tbody>
</table>

This data represents the result of the comparison of PKU individuals with healthy controls, except in the study of Couce et al [51] (PKU vs Hyperphenylalaninemia) and Agostoni et al [55] (PKU before vs after supplementation).

Apo B, apolipoprotein B; F, female; HDL-C, high-density lipoprotein cholesterol; M, male; m, months; TAG, triacylglycerol; TC, total cholesterol; VLDL-C, very low-density lipoproteins cholesterol; yr, years;
Table 2. Changes in FA observed in plasma and RBC of PKU patients, subjected to a Phe-restricted diet and after an additional long-chain PUFA supplementation, reported in published studies with statistical analysis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analytical method</th>
<th>Lipid extraction method</th>
<th>Derivatives for GC</th>
<th>Type of sample (results expressed as)</th>
<th>Number of PKU patients</th>
<th>Age range</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Galli et al [61]</td>
<td>GC-MS</td>
<td>Bligh and Dyer</td>
<td>FAME or PFBE</td>
<td>Plasm (%weight total FA)</td>
<td>15</td>
<td>3-12 yr</td>
<td>20:4n-6, 20:5n-3, 22:6n-3 18:1n-9</td>
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<td></td>
<td></td>
<td>Folch et al</td>
<td>(methanolic</td>
<td>Red blood cells (%weight total FA)</td>
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<td>(conditions not</td>
<td>Red blood cells (% total FA)</td>
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<td>clarified)</td>
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<tr>
<td>Van Gool et al [63]</td>
<td>GC-FID</td>
<td>Bligh and Dyer</td>
<td>FAME</td>
<td>Plasma PL fraction (% total FA by</td>
<td>9</td>
<td>6m-25 yr</td>
<td>18:3n-3, 20:4n-3, 20:5n-3, total n-3 20:3n-6, 22:4n-6, 22:5n-6 total n-6</td>
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<td>(14% boron</td>
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Table 2: Changes in FA observed in plasma and RBC of PKU patients, subjected to a Phe-restricted diet and after an additional long-chain PUFA supplementation, reported in published studies with statistical analysis.
<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Extraction</th>
<th>Description</th>
<th>Plasma (% total FA)</th>
<th>Red blood cells PL fraction (% total FA)</th>
<th>Study Age</th>
<th>FA Range</th>
<th>PL fraction (% weight)</th>
<th>RBC FA Range</th>
<th>Effect of additional long-chain PUFA supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanjurjo et al [64]</td>
<td>GC-FID</td>
<td>No extraction step</td>
<td>FAME (methanol-benzene 4:1 (v/v) and acetyl chloride, 100°C, 1h)</td>
<td>40</td>
<td>40 (15F+25M)</td>
<td>2m-20 yr</td>
<td>16:0, 16:1, 20:4n-6, 22:6n-3</td>
<td></td>
<td>18:2n-6, 20:3n-6</td>
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<tr>
<td>Giovannini et al [65]</td>
<td>GC-FID</td>
<td>Folch et al</td>
<td>FAME (methanol/hydrochloric acid, 90 °C, 1h)</td>
<td>45</td>
<td>2m-20 yr</td>
<td>9-14 yr</td>
<td>14:0, 16:1, 18:1n-9, 18:3n-3, 20:5n-3, 22:6n-3</td>
<td>SFA, 22:6n-3, total n-3</td>
<td>MUFA, 18:3n-3</td>
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<tr>
<td>Aldámiz-Echevarría et al [66]</td>
<td>GC-FID</td>
<td>No extraction step</td>
<td>FAME (methanol-benzene 4:1 (v/v) and acetyl chloride, 100°C, 1h)</td>
<td>47</td>
<td>20:5 n-3, 22:6 n-3, SFA, total n-3</td>
<td>INF</td>
<td>20:5 n-3, 22:6 n-3, total n-3</td>
<td></td>
<td>18:1n-9, MUFA, n-6/n-3 ratio</td>
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<tr>
<td>Stroup et al [69]</td>
<td>GC-MS</td>
<td>No extraction step</td>
<td>PFBE (triethylamine and 10% pentafluorobenzyl bromide in acetonitrile, 15 min, room temperature)</td>
<td>25</td>
<td>20:5 n-3, 22:6 n-3, total n-3</td>
<td>18-49 yr</td>
<td>18:0, 18:1n-9, 20:5n-6, 22:6n-6, total n-3, n-3/n-6 ratio, SFA</td>
<td>18:3n-3, 18:3n-6, 20:3n-6, total n-6, n-6/n-3 ratio</td>
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<tr>
<td>Agostoni et al [56]</td>
<td>GC-FID</td>
<td>Folch et al</td>
<td>FAME (methanolic hydrochloric acid)</td>
<td>20</td>
<td>3-17 yr</td>
<td>-</td>
<td>22:6 n-3, total n-3</td>
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<tr>
<td>Study</td>
<td>Extraction Method</td>
<td>Lipid Extraction Method</td>
<td>lipid to be recovered</td>
<td>Component Information</td>
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<tr>
<td>Demmelmaier et al [57]</td>
<td>GC-FID</td>
<td>Folch et al</td>
<td>FAME</td>
<td>Plasma PL fraction (mg/L) 109, 5-13 yr - 22:6 n-3</td>
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<tr>
<td>Koletzko et al [77]</td>
<td>GC-FID</td>
<td>n-hexane/isopropanol (3:2 vol/vol)</td>
<td>FAME</td>
<td>Plasma PL fraction (%weight) 10, (3F+7M), 1-3 w - 20:4n-6, 22:6n-3</td>
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<tr>
<td>Agostoni et al [75]</td>
<td>GC-FID</td>
<td>Folch et al</td>
<td>FAME</td>
<td>Red blood cells PL fraction (%) 42, (22F+20M), 1-5w - 22:6n-3, total n-3</td>
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<tr>
<td>Cleary et al [74]</td>
<td>GC-MS</td>
<td>No extraction step</td>
<td>FAME</td>
<td>Red blood cells PL fraction (%) 53, 1-10 yr - 22:6 n-3</td>
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</table>

Methods to recover phospholipid fraction: aSPE, solid phase extraction; bTLC, thin layer chromatography; FA, fatty acid(s); FAME, fatty acid methyl ester; F, female; GC-FID, gas chromatography-flame ionization detector; INF, information not found; GC-MS, gas chromatography-mass spectrometry; M, male; m, months; MUFA, monounsaturated fatty acid(s); PFBE, pentafluorobenzyl ester; PL, phospholipid(s); PUFA, polyunsaturated fatty acid(s); SFA, saturated fatty acid(s); w: weeks. yr: years.