

## PHENYLKETONURIA: A SUMMARY OF EXISTING AND PROSPECTIVE TREATMENTS

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### Abstract:

Phenylketonuria (PKU) is an autosomal recessive genetic disorder characterised by the inability to break down the amino acid phenylalanine (Phe), resulting in toxically elevated levels of Phe in the body that may cause serious mental and physical disability. There are two main types of PKU, typical where Phenylalanine Hydroxylase (PAH) is defective and atypical where a defect is found in the biosynthesis or recycling of tetrahydrobiopterin (BH<sub>4</sub>), a cofactor for PAH. In 1954 it was established that the key to treating PKU was a diet low in Phe starting from birth. Since then dietary restriction and medical substitutes for foods have been the most accessible treatment options. However compliance with this treatment is suboptimal, resulting in impairments and lower quality of life. In the last couple of years new therapies have been developed which include supplementation with compounds preventing Phe entry into the brain or allowing its breakdown: (enzyme Phe-ammonia lyase), saproprotein, large neutral chain amino acid supplementation, genetically modified probiotics for the delivery of PAH, glycomacropptide foods and gene therapy. The purpose of this review is to examine prospective PKU treatments and their necessity in improving the quality of life of affected individuals.

**Keywords:** phenylketonuria: a summary of existing and prospective treatments

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### Introduction:

PKU is a genetic disorder due to a loss of function inborn error of amino acid metabolism resulting in a deficiency in PAH, an enzyme that catalyzes the hydroxylation of Phe to tyrosine (Tyr) [1]. Based on the degree of enzyme deficiency the spectrum of clinical and metabolic phenotypes spans from typical PKU, to atypical PKU, to the benign condition non-PKU hyperphenylalaninemia (HPA) [2]. If undiagnosed and untreated, PKU can result in impaired postnatal cognitive development due to the neurotoxic effect of HPA. PKU is inherited in an autosomal recessive pattern, with males and females having the same risk. Therefore for PKU to occur an individual must inherit one abnormal gene from each parent. The locus of the gene encoding for PAH is on chromosome 12 on

the distal part of 12q [1], [9], while genes encoding for BH<sub>4</sub> are found on chromosomes 4, 10 and 14 [1]. Overall more than 300 genetic mutations have been identified to be responsible for PKU. Due to the fact that different mutations result in varying degrees of elevation in blood Phe, each patient is different in regards to their tolerance of the amino acid and may require different management [1].

PKU was first described under the designation oligophreniaphenylpyruvica, in 1934 by Dr. Asbjørn Følling. The metabolic error was then localized as an inability to oxidize Phe to Tyr by Jervis, in 1947. Deficiency of PAH in the liver of a patient was shown by Jervis, in 1953, while newborn screening for PKU was given by Guthrie, in 1996. Before that, newborn screening with the

heel stick began in 1961 and was reported by Guthrie and Susi [1].

Statistically, the occurrence of PKU is rare and varies among different ethnic groups and geographic regions worldwide [5]. People from most ethnic backgrounds can be affected by PKU, although it is less prevalent in ethnic groups descending from sub-Saharan Africa and South Asia, Finland and Jews of Ashkenazi ancestry [1], [5]. Newborn screening programs showed that the incidence of PKU is 1:10,000 in Europe [9].

PKU can be identified by collecting dried blood spots after 24 hours of age and performing tandem mass spectrometry. HPA is manifested as a time-dependent increase in blood Phe concentration, so attempts for earlier detection might yield inaccurate results [4]. Early clinical findings that lead to suspicion of the disease and condition screening a necessity in all developed countries, even required by law in some (eg. in Europe, USA), include vomiting, developmental delay, or eczematoid rash [3], [4]. The test requires a drop of blood taken from the baby's heel. If the screening test is positive, further blood and urine samples are required to confirm the diagnosis. Genetic testing is also provided [4]. Diagnosing PKU prenatally has become feasible with the use of DNA probes, as the related genes have been cloned and the exact locations of numerous mutations in the enzyme have been spotted. Determining whether parents are carriers of the PKU gene or not can be done by an enzyme assay or genetic testing. Chorionic Villus Sampling or Amniocentesis can be done during pregnancy to screen the unborn baby for PKU [3].

As far as symptoms of undiagnosed and unmonitored patients are concerned, high amounts of Phe affect melanin synthesis and result in the fair complexion and light hair pigmentation observed in affected children. Even though phenylketonurics seem normal at birth, they are severely defective by age 1 if left untreated. The rest of the symptoms of PKU, as will be discussed later on, include severe mental retardation, abnormal brain weight, defective nerve fiber myelination which accounts for numerous neurological disorders and hyperactive reflexes. Untreated PKU patients have a drastically reduced life expectancy, as half die by age 20 and three-quarters by age 30 [8].

Management of the disease requires a low-Phe diet supplemented with Tyr, as Tyr is normally synthesized from Phe. The purpose of this treatment is to provide just enough Phe to meet

the growth and replacement needs. Low Phe-content proteins, like milk casein, are hydrolyzed and Phe is removed by adsorption [7]. Plasma phenylalanine must be maintained 120-360  $\mu\text{mol/L}$  (2-6  $\text{mg/dL}$ ) [9]. A carefully monitored diet results to it and prevents intellectual disability and further onset of symptoms. Treatment must start at a very young age (under 3 months), or some degree of intellectual disability may be expected. The average IQ of PKU patients treated soon after birth is 93, in comparison to a control group treated at 1 year of age, which had an average IQ of 53 [8]. It is nearly universally accepted, among clinicians treating PKU that the diet needs to be continued indefinitely [9].

Unfortunately, there are two major drawbacks in treating PKU. Firstly, Phe occurs in practically all natural nutrition sources, thus it is impossible to adequately restrict the diet using natural foods alone without compromising health. For this reason, special Phe-free food preparations are helpful [23]. Secondly, severe limitation of Phe intake results in vitamin B12 and other micronutrient deficiencies and symptoms of Phe deficiency may develop [15]. The U.S. Food and Drug Administration (FDA) approved Kuvan (Sapropterin hydrochloride) in 2007 for the treatment of PKU. Blood Phe levels are reduced by Kuvan in patients with HPA due to  $\text{BH}_4$ -responsive PKU [25].

PAH genotyping is recommended for improved therapy planning. Any combination of therapies, medical foods, Kuvan, Palyngiq etc., that improve a patient's Phe levels is appropriate and should be individualized. Individuals with PKU and their families should consider genetic counselling. Blood Phe levels should be controlled at a consistent time during the day, preferably 2-3 hours after eating. Regular mental health monitoring is warranted due to an increased number for neurocognitive psychological issues. A number of screening tests is recommended to identify those in need of further assessment [9]. Conclusively, PKU patients should be closely monitored by a metabolic team of experts.

In 2018, Palyngiq (pevgaliase-pqpz) was approved by the FDA for adults with PKU. Palyngiq is an enzyme therapy for patients who have uncontrolled blood Phe concentration on current treatment. It is understandable that the need for new therapies is a necessity due the tedious existing ones [6]. This review aims to investigate the new PKU treatment options, either already implemented or still experimental and assess the extent to which recent research

has managed to overcome the drawbacks of the 60 year old common practice.

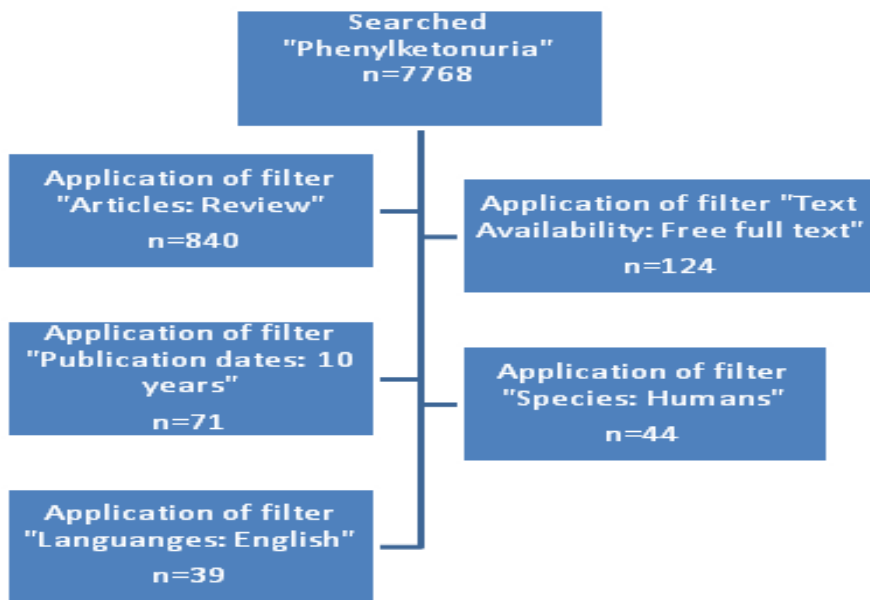


Figure 1: Flow chart of article filtering and exclusion process.

**Methods:**

The current review followed established methods for knowledge synthesis, including a comprehensive search strategy, screening citations against inclusion/exclusion criteria and extraction of important data elements from eligible studies. The following primary research question was addressed: “What are the prospective treatments in PKU?” A secondary research question was “What is the pathophysiology of PKU?” All research used for this review was published in PubMed. The inclusion and exclusion criteria were formed as such: due to resource constraints all results were limited to reviews written in the English language and to the publications of the last 10 years. Animal-only records were removed from the results, while due to reduced accessibility, the review was performed on publications with free full text availability. The research yielded 39 results.

The inclusion criteria used to select the articles included in the survey were the following:

- (1) articles were five or less than 10 years old,
- (2) articles have been published on credible journal databases,
- (3) articles were directly related to the topic of this part of the systematic review; “Current and future treatments of PKU”,
- (4) articles were written in the English language,
- (5) reviews were included (studies included in qualitative synthesis).

The exclusion criteria were as follows: (1) articles not published in English, (2) articles published more than 10 years ago, (3) articles not published in a peer reviewed journal database, (4) articles

published in non-credible journal databases or databases not approved by national health and scientific organizations.

Overall the filters used in Pubmed were: (1) Article types: Reviews, (2) Text availability:Free Full Text, (3) Publication dates:10 years, (4) Species:Human, (5) languages:English Language. Additionally titles were excluded due to mismatch to the research questions, for example reviews studying the prevalence of the disease only in certain populations, focusing only on certain aspects of diagnosis of the disease, like infant screening or examining only one type of the disease, like mild HPA.

Additional research keywords were used in order to yield results more specific to treatments, symptoms and pathophysiology. Our research using the keywords “Phenylketonuria treatment” yielded 33 results out of which we included 5. The search using

“Phenylketonuria new treatment” yielded 6 results, out of which 4 were included. The search “Phenylketonuria symptoms”: yielded 21 results, out of whichwe included 6. Searching “Phenylketonuria pathophysiology” yielded 6

results, out of which we included 5. Overall 20 articles were selected to be reviewed.

Other key publications of interest focus on historical facts, inheritance patterns, diagnosis and screening, psychological aspects of living with the disease and monitoring guidelines. These publications were partially identified by conducting an additional search and were mainly used for the introduction and discussion parts, along with certified textbooks and the papers reviewed. The search strategy for this additional search has been developed for Governmental Organizations such as OMIN, the National Library of Medicine (NLM) or the U.S. Food and Drug Administration (FDA).

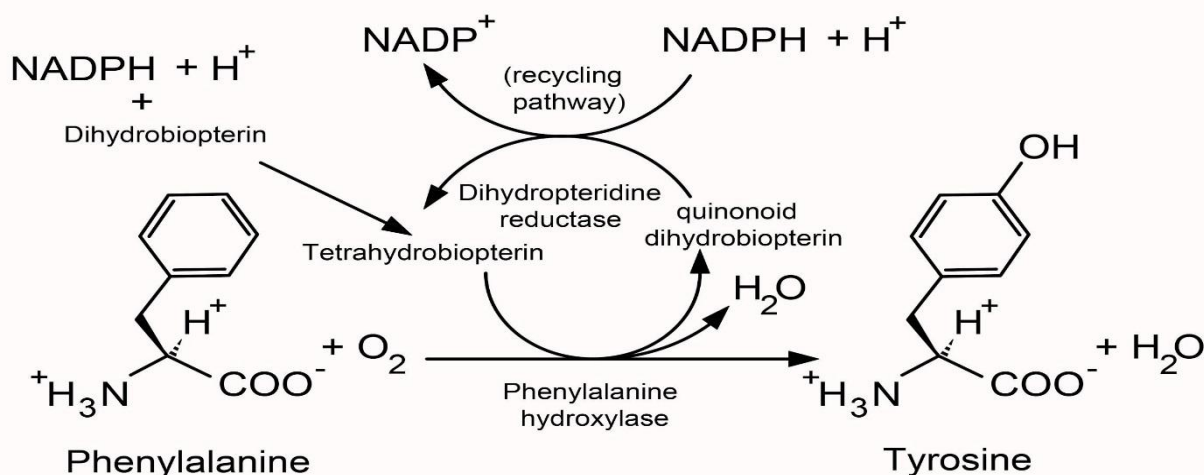
### Normal Phenylalanine (Phe) Degradation

In the normal Phe degradation, 75% of Phe molecules are converted into tyrosine (Tyr), and the other 25% become incorporated into proteins. [7], [10]

Analyzing the first and rate limiting step of the normal Phe catabolism is critical for the comprehension of the pathologic mechanism and symptoms of PKU, as well as the treatments implemented.

1st step (shown in the figure): The degradation of Phe begins with its hydroxylation to Tyr, a

reaction catalyzed by the phenylalanine hydroxylase enzyme (PAH), which is a mixed function oxidase. This means that it uses a reacting diatomic oxygen molecule ( $O_2$ ), of which one atom is placed on Phe (substrate hydroxylation) and then appears on the product (Tyr), as its hydroxyl group. The other atom is reduced to water (released  $H_2O$ ) [8], [10]. PAH is mainly found intrahepatically, removing excess Phe and thus preventing the neurotoxic damage of hyperphenylalaninemia (HPA). [10], [12] the reductant in this reaction is tetrahydrobiopterin ( $BH_4$ ), a cofactor acting as an electron carrier molecule from NADPH to  $O_2$  [8].  $BH_4$  is synthesized by dihydrobiopterin, in the presence of NADPH and  $H^+$  ion, by the enzyme dihydrofolatereductase, which transfers the reducing power of NADPH to dihydrobiopterin. PAH uses the reducing power of  $BH_4$  to form Tyr. The reducing power of  $BH_4$  is also used to produce quinonoid dihydrobiopterin, which regenerates  $BH_4$  back after it has been used in the hydroxylation of Phe. This is the recycling pathway of  $BH_4$ , catalyzed by the enzyme dihydropteridinereductase. Therefore, this enzyme is considered a part of the PAH system, which conditions the normal Phe breakdown pathway sensitive to defects in numerous genes [10], [12].



**Figure 2: First step of normal Phe degradation pathway**

### Phenylketonuria/ Pathologic Phe breakdown pathway

PKU can be classified into two types, "Typical PKU", where the PAH enzyme is defective and "Atypical PKU" ( $BH_4$ -deficient HPA), which occurs when the PAH enzyme is normal, and a defect is found in the biosynthesis or recycling of the cofactor  $BH_4$  [12], [15]. Atypical PKU accounts for approximately 2% of the cases demonstrating elevated Phe levels [8], [15]. In

both cases, the problem lies within the first step of the normal pathway [12].

Minor fates of Phe in normal people, such as the formation of phenylketones (phenylpyruvate, phenyllactate and phenylacetate) become major fates in phenylketonurics [7]. In this otherwise little used pathway, Phe is transaminated with pyruvate to form phenylpyruvate, and both are excreted in the urine, after building up in the



blood and tissues [8], [10] - hence the name given to the disease - while Tyr is deficient. Phenylpyruvate molecules are decarboxylated to phenylacetate or reduced to phenyllactate to

be excreted. The characteristic musty body and urine odor is attributed to Phenylacetate, and it was traditionally used by nurses to detect infantile PKU [8].

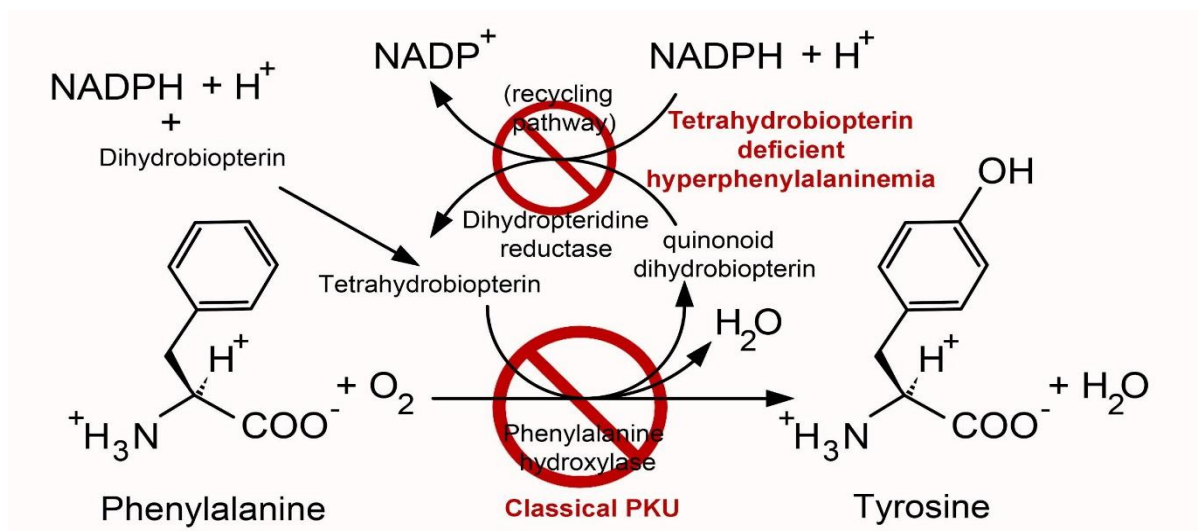


Figure 3: Sites of defects in the normal reaction

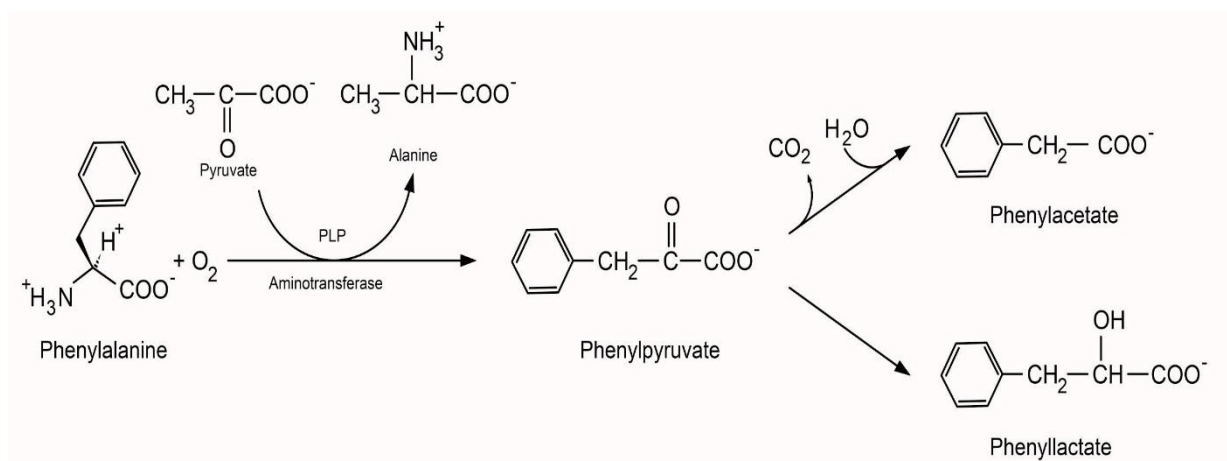


Figure 4: Pathologic Phe breakdown pathway

### Determining the basis of the pathophysiology of PKU

The observed symptoms in untreated PKU are epilepsy, brain damage, progressive mental retardation, microcephaly, motor deficits, eczematous rash, autism, seizures, neurological and behavioral problems, purposeless movements, all of which result from the neurotoxic action of HPA. Depression and psychiatric symptoms are also included among the symptoms [9], [10], [12], [14], [15], [19], [20]. Some of the neurological symptoms cited above can appear later in adulthood of early treated patients, if they relax their diet [15].

Though not completely understood, the molecular basis for the neurological symptoms of the disease is associated with the saturation of the LAT-1 transporter of the blood-brain barrier by Phe [7], [10], with defective myelination [15], and lack of Tyr. LAT-1, which has a high affinity for Phe, is at the same time the only passage of eight large neutral amino acids (LNAAs) to the brain. LNAAs are essential for the synthesis of the proteins of the brain [10], while Tyr and Tryptophan also function as precursors for neurotransmitters [8], [10], [20]. Elevated Phe levels saturate LAT-1, resulting in a disturbance in neurotransmitter- and protein synthesis, including hypomyelination. Thus, providing LNAAs supplementation is one

treatment under investigation [10], since Tyr specifically is a precursor of dopamine (DA). Higher order cognitive functions performed by the prefrontal brain cortex (executive functions like working memory, cognitive updating, inhibitory control) rely on prefrontal pyramidal neurons, for which DA is necessary [11], [15], [19], [20].

In addition, white matter damage has been demonstrated by histopathological and neuro-imaging studies both in treated and untreated PKU patients [9], [11], [15].

Oxidative stress is also seen in phenylketonurics and is associated with the pathophysiology of many neurodegenerative diseases, including Parkinson's and Alzheimer's disease, epilepsy, and demyelination [9], [13], [15]. Oxidative Stress is defined as the disturbance of the equilibrium between the production of reactive oxygen and nitrogen species (ROS & RNS respectively) and the antioxidant system. The brain tissues are especially sensitive to oxidative stress due to increased consumption of oxygen, high concentrations of iron, diminished level of antioxidant defenses, the presence of excitatory amino acids and dopamine metabolism, which generate hydrogen peroxide [13]. There is evidence relating oxidative stress to poor metabolic control and deficiency of numerous micronutrients (selenium, zinc, co-enzyme Q<sub>10</sub> and perhaps L-Carnitine) [9], which can be attributed to the strict diet followed by the PKU patients [15]. However biochemical monitoring is not proposed, because of inadequate clinical data about the antioxidant status of phenylketonurics. Instead monitoring and maintaining low blood Phe levels appears to help reduce oxidative stress [9].

An example of a nutrient deficiency associated with adult pathophysiology of the disease imposed by chronic dietary restriction is that of Vitamin B<sub>12</sub>. This vitamin is naturally found in animal protein which is usually avoided by PKU patients. Vitamin B<sub>12</sub> deficiency accounts for numerous clinical symptoms such as neuropathy, subacute combined degeneration of the cord, glossitis, anaemia, dementia and psychiatric states such as depression and psychoses [15].

Additionally decreased skin and hair pigmentation described in PKU affected individuals is attributed to diminished melanin, since Tyr is a precursor of this molecule as well [8].

There is a suggested link between Phe levels and low bone mineral density [15], but two more

recent studies insist that further research should be conducted, due to unreliable data [16], [17].

Finally, among the complications related with PKU is the maternal PKU syndrome, concerning the pregnancy of a phenylketonuric woman. In untreated pregnancies of affected women with uncontrolled Phe levels, the offspring are in risk of developing microcephaly, mental retardation, intrauterine growth retardation, developmental delay and congenital heart defects, even if the baby hasn't inherited the defective gene [5], [9], [15], as the toxic metabolites accumulating in the mother may cross the placenta and reach the fetus [18].

### **New perspectives in treatment for PKU**

The basic and common treatment of PKU includes a specialised low-protein and low-Phe dietary plan, patients should follow for a lifetime under the supervision of a metabolic team consisting of a physician, a dietician specialised in metabolic disorders and a (neuropsychologist). For the initiation of treatment of PKU there are certain criteria the patient must meet. No treatment is recommended if the patient's plasma Phe levels are <360µmol/L. However, during the first year of life the blood Phe levels should be closely monitored. According to the review "The complete European guidelines on phenylketonuria: diagnosis and treatment" [9] the monitoring of the plasma Phe levels until 1 year of age is recommended as a minimum to determine whether levels are going to rise above 360µmol/L. Moreover, all the patients with HPA (blood Phe levels >360µmol/L) should be treated immediately, while patients with untreated Phe concentrations of 360-600µmol/l should be treated until the age of 12. The European guidelines point out the following: "all adults with PKU should have life-long, systematic follow up in specialized metabolic centers due to specific risks which may occur during adulthood" [9].

The majority of PKU patients still rely on a specialised diet as the basic treatment of this disease, although this approach holds some significant drawbacks. PKU patients have a significantly low phenylalanine tolerance thus the PKU diet demands low protein food intake such as different types of meat, dairy products and plants or grains rich in protein. Moreover, Aspartate based artificial sweeteners must be avoided because their metabolism causes the release of Phe in the blood and thus increases blood Phe concentrations.[european guidelines] Adhering to the PKU diet is very restrictive and thus difficulty in compliance and control of Phe blood levels is commonly observed especially in

adolescent and adult patients. Additional to poor compliance is the nutrient deficiency observed as a result of the PKU diet. New treatment strategies for PKU have developed the past few years which in combination with the already existing dietary treatment have shown promising results in controlling and stabilizing the Phe blood levels. New perspectives in treatment of PKU include:

1. Enzyme substitution therapy Phenylalanine ammonia-lyase (Phe-lyase) (Pegvalian) drug
2. Sapropterin dihydrochloride (Kuvan/phenoptin) - BH<sub>4</sub> supplementation (cofactor of PAH)
3. Large neutral amino acids (LNAAs)
4. Gene therapy
5. Glycomacropetides (GMP)
6. Production and delivery of PAL enzyme by genetically modified (GM) probiotics

### Existing treatments for PKU

#### 1. Enzyme substitution therapy-Phenylalanine ammonia-lyase

A prospective approach for treating PKU is enzyme therapy. This can be done by replacing either the PAH enzyme or the phenylalanine ammonia-lyase enzyme. Thus, Phe ammonia lyase (PAL) or PAH substitution therapy are the two new treatment strategies of enzyme therapy which were first studied under animal models [2], [5]. In the first case, studies have demonstrated that PAH-fusion proteins tested in mice appear to have beneficial effects. However, enzyme therapy with PAL enzyme appears to be more promising. PAL catalyses the conversion of Phe to Trans-cinnamic acid and to small amounts of ammonia. A major difference between the PAH and PAL enzyme is that the former is a mammalian enzyme which requires the presence of cofactors, while the latter has a monomeric structure and no cofactors are required for it to function. PAL is found in higher plant organisms, yeast and some mammalian species [2]. Its main function in micro-organisms is the utilisation of L-phenylalanine (L-Phe) which is the only source of carbon and nitrogen for these microorganisms. Moreover, in higher plants PAL takes part in their defence mechanism. PAL may also be found in mammalian species, where it has a half-life of almost 21 hours after intravenous injection to the host organism (mouse models).

The physiological and pharmacological principles of the PAL enzyme therapy have been demonstrated by examining the effect of PAL

enzyme given orally or by injection in PKU mice. The injection of PAL enzyme has some serious limitations and side effects, such as triggering an immune response to the host organism (mice models in this case) and causing serious reactions. The oral administration on the other hand, reduces the effectiveness of this therapy because it is more likely to lead to the proteolytic degradation of the enzyme [5]. The reduction of these side effects was achieved by the conjugation of the PAL enzyme with polyethylene glycol (PEG) which lead to the decrease of the immune responses and appeared to lessen the enzyme degradation. Two clinical trials in PKU patients were conducted -Phase I and Phase II. PKU patients were injected with the PAL enzyme which was conjugated with the PEG analogue. The subcutaneous administration of this enzyme showed promising results, was safe and efficient, while patients could tolerate the procedure [26]. Reduction of blood Phe levels was observed in the patients who received the highest doses for approximately six days after both injection and drug PEG-PAL therapy with was interchanged during these days. Recently, significant changes on the oral PEG-PAL have been made in order to prevent the degradation of the enzyme and improve the effectiveness of the therapy. Finally, PEGylated PAL was developed as a novel enzyme substitution therapy for PKU and it was approved by the U.S Food and Drug Administration (FDA) in 2018 under the name "Pegvalian" (pegvalianase-pqqz).

#### 2. Sapropterin dihydrochloride-BH<sub>4</sub> supplementation

In 1970s studies indicated that a group of patients diagnosed with HPA developed neurological problems despite following the specialised PKU diet [25]. After further studies were conducted it became apparent that there were differences in the response to dietary treatment. This was due to the fact that there were two types of PKU: a) Typical PKU which is caused due to the mutation of the PAH enzyme gene and thus it leads to the misfolding of the PAH enzyme needed in the pathway which converts phenylalanine to tyrosine, b) Atypical PKU which is caused by defects in the BH<sub>4</sub> synthesis or recycling and thus by a mutation responsible for the encoding of the BH<sub>4</sub> gene. The group of patients who didn't respond to the basic dietary treatment were diagnosed as atypical PKU patients. In 1999 a study conducted by Kura [25] demonstrated that some PKU patients-mainly atypical PKU patients and patients with mild or moderate typical PKU-responded to pharmacological doses of BH<sub>4</sub> and this resulted in the reduction of the Phe levels. The distinction between typical and

atypical PKU is clarified by the BH<sub>4</sub> loading test and by identifying the quantity of the neurotransmitters such as dopamine, noradrenaline and adrenaline and their metabolites and pterins in urine [21], [25]. According to the European guidelines for PKU [9], patients with Phe levels >360 μmol/L should have the BH<sub>4</sub> loading test. This test includes measuring the initial plasma Phe concentrations and comparing them to the Phe concentrations after short-term (24-48 hours) or long-term (several weeks) administration with BH<sub>4</sub> (20 mg BH<sub>4</sub>/kg/day).

If the baseline plasma Phe concentrations decrease by 30% after 8 hours or by 50% after 24 hours then the BH<sub>4</sub> loading test is considered positive and the patient is classified as atypical PKU patient. Based on the above protocol it has been found that 60-70% of patients with mild PKU resulted in a greater decrease of the plasma Phe levels and thus responded to the oral loading test significantly [25]. However, there have been cases in which the patient's response to the oral loading test is delayed. In order to identify the severity of the atypical PKU and classify it as mild or moderate PKU an extended protocol is used which demands the repeated administration of 20 mg BH<sub>4</sub>/kg/day for 24 hours. Moderate PKU patients are considered to be slow responders to the 24 hour administration [25].

Despite the use of BH<sub>4</sub> loading test to determine whether elevated Phe levels of PKU patients are due to PAH or BH<sub>4</sub> deficiency, an additional synthetic analogue to BH<sub>4</sub> known as sapropterin was developed. Sapropterin is considered to be a pharmacological chaperone which has beneficial effects on the PAH activity by stabilizing it and correcting its misfolded form. It was developed mostly for the treatment of the atypical PKU patients, although studies have demonstrated that a subset of PAH-deficient PKU patients responded to the BH<sub>4</sub> treatment as well [25]. In atypical PKU patients this treatment is successful because results indicate an increased tolerance in Phenylalanine which allows the patients to be less strict with their specialized PKU diet and in some cases the patients were able to give up the Phe free diet entirely. It must be pointed out that only PAH-deficient PKU patients with mild or moderate forms of PKU appeared to be responsive to this treatment. However, about 90% of patients with the typical PKU, comprising 50-80% of the PKU patients, detected in newborn screening show no response to the BH<sub>4</sub> therapy [25], [26]. These patients can turn to other alternative therapy strategies such as the enzyme therapy as aforementioned. Conclusively, BH<sub>4</sub> therapy resulted in an at least

30% reduction of blood Phe concentrations in atypical PKU patients and in typical PKU patients with mild phenotype and it also leads to the increase of Phe dietary tolerance in patients that respond to it

### 3. Large neutral amino acids (LNAAs)

Large neutral amino acids (LNAAs) supplementation treatment aims to reduce cerebral Phe concentrations, plasma Phe concentrations and to increase neurotransmitter and cerebral essential amino acid concentrations [27].

LNAAs intake results in the prevention of phenylalanine entry into the brain and thus the reduction of cerebral Phe concentrations. The mechanism responsible for this is due to the fact that LNAAs and Phenylalanine have the same transport system across the blood-brain barrier. Therefore, when Phe blood levels rise, Phe and LNAAs are competing on their passage through the blood-brain barrier. LNAA supplementation has been found to achieve the reduction of brain Phe concentrations in PKU patients, even at mean plasma Phe concentrations >1,000 μmol/L [24], [27]. It was also observed that in some cases a decrease in cerebral Phe levels was paired with an increase in plasma Phe concentrations. Although, there were also cases of PKU patients in which both the cerebral and blood Phe levels had been reduced with LNAAs supplementation indicating that LNAAs apart from competing with Phe for their transport across the blood-brain barrier, also compete with Phe for the transport across the intestinal mucosa. Thus, the therapeutic effect of LNAAs is based on the fact that LNAA transport occurs across both the blood-brain barrier but also at the gut-blood barrier.

An open and double-blinded trial in PKU patients was performed (Matalon et al. 2006-2007 in which the patients received LNAAs three times daily with their meal, while their regular diet was unchanged [27]. A decrease in blood Phe levels up to 50% of the initial values was observed on patients on LNAA supplementation of 0.5-1.0 g kg<sup>-1</sup> day<sup>-1</sup> [27]. This indicated that LNAA supplementation, while following a regular diet, may induce a reduction of Phe absorption by the gastrointestinal tract. Therefore, the reduction in blood Phe concentrations may result from a decreased gastrointestinal tract absorption. This decrease in blood Phe levels may also be a result of increased synthesis of protein as a result of the increased availability of essential amino acids in case the patients had essential amino acid deficiencies, although no evidence of that is present. The reduction of Phe levels might also



be due to just the timing that the blood sample was taken. Conclusively, LNAAs supplementation may reduce blood Phe levels, however not all reviews report this effect [21], [24], [27].

#### **Tyr and/or tryptophan supplementation for neurotransmitter synthesis increase.**

Recent clinical trials also indicate that another result of LNAAs supplementation at  $0.5 \text{ g kg}^{-1} \text{ day}^{-1}$  may be beneficial in biochemical and neuropsychological levels, correcting abnormalities of this nature [24], [27]. High blood Phe concentrations result in low brain neurotransmitter concentrations such as dopamine, serotonin and other metabolites found in cerebrospinal fluid and in brain tissue [27]. In PKU patients with low compliance and adhering on the specialised low-protein PKU diet, tyrosine and tryptophan supplementation have been shown to appear beneficial for the neurotransmitter metabolism, reaction time, and vigilance.

#### **4. Glycomacropeptides (GMP)**

A Glycomacropeptide is a by-product of the production of cheese that is rich in amino acids other than phenylalanine, such as valine, isoleucine and threonine and is produced by the cleavage of bovine k-casein by chymosin. It is a glycosylated peptide consisting of 64 amino acids and is naturally found in cheese and bovine milk [24]. Moreover, GMP is being released in the newborn and adult gastrointestinal mucosa which occurs after the digestion of milk a process mediated by pepsinogen proteolysis.

When GMP is supplemented paired with the essential amino acids tyrosine, tryptophan, arginine, cysteine, and histidine it functions as a beneficial addition to the PKU diet. Studies have demonstrated that foods containing GMP appear more appealing and palatable to PKU patients than their usual amino acid dietary program [23]. There are several benefits that have been observed in the GMP diet including low levels of ureagenesis, Phe utilisation and protein impermeability/retention. Nutritionally suitable, safe and accepted for PKU patients can be foods and beverages made with GMP as the main source of protein, because GMP is low in phenylalanine but at the same time provides a great variety of proteins. Studies have demonstrated that GMP contains much greater amounts of LNAAs (isoleucine, threonine and valine), almost 2-3 times more, than the other dietary proteins and thus supplementation of other AAs is need during a PKU diet.

The acceptability of GMP medical foods has been tested through blind sensory studies taking place in three PKU camps [23]. The GMP products were rated from a scale of 1 to 5 representing five sensory categories (odor, taste, appearance and overall acceptability). The GMP products were overall scored for  $>3$ , which indicates a positive response. Moreover, another metabolic study established the safety of GMP products as well as their preference in terms of taste and the greater variety of options that they provide compared with the usual amino acid formula supplementation. Therefore, after the findings of these studies were released the production of medical foods based on GMP additional food and beverages were developed, such as snack bars, salad dressings, puffed cereal, crackers and beverages were developed.

#### **Prospective treatments for PKU (Animal models only)**

##### **5. Gene Therapy**

The cloning of the gene that encodes for PAH made gene therapy for PKU a possible option treatment. In gene therapy the functional PAH-gene is targeted to liver cells since PAH activity mainly occurs in the liver. There have been numerous types of possible viral vectors identified such as adenoviral and associated-adenoviral vectors which have shown promising results in delivering the PAH-gene and potentially correcting PKU in mouse models or isolated hepatocytes derived from mice. Although this treatment option is still experimental, several experiments have been conducted both ex vivo and in vivo with the use of different PAH transgene delivery strategies to the cells. More specifically, over the past years there have been a lot of gene therapy methods including [22], [26]:

- a. Ex Vivo Gene Therapy in which patients cells are being transduced in vitro with the correct-therapeutic gene encoding the PAH enzyme and afterwards the cells are re-implanted. In this case, T-lymphocytes taken from patients were transduced with the PAH cDNA gene using a retroviral vector system [22]. This study showed that functional PAH can be targeted mainly in liver cells but also in cells different than the liver, if there are sufficient amounts of the cofactor  $\text{BH}_4$ .
- b. In Vivo Gene Therapy [22]:
  - i) Adenovirus mediated Transgene Delivery, in which the gene encoding PAH enzyme was delivered to the cells via an adenovirus (Adenovirus mediated Transgene delivery).

A major drawback of this method was that a strong immune response of the host organism was activated. However, when a recombinant adeno-associated virus (rAAV) was used to deliver the transgene to the cells, it proved to be nonpathogenic with low immunogenicity and thus efficient for gene therapy.

ii) Recombinant Adeno-Associated Virus, in which no host pathogenic response was observed while it was considered a safer vector for viral gene transfer. They were also able to establish long term expression of the inserted gene in different tissues, not only liver tissue as aforementioned. Additionally, these vectors do not carry viral genes and thus they minimize the possibility of eliciting an immune response.

#### Future Clinical Development of PKU Gene Therapeutics

Several studies have demonstrated that the PAH cDNA delivered to the liver by an injection in the portal vein results in the reduction of blood Phe levels in mice [22]. However, this reduction was observed only in male mice, while the female mice in order to achieve the same blood Phe levels needed three times more vectors [22]. This indicates that there might be a gender dependent mechanism in the delivery of PAH-gene. Researchers suggest that the gender dependent response to the treatment is due to the presence of androgen-dependent pathways in male mice, although this mechanism is not fully understood. This suggests that a possible gender dependent approach on the gene therapy treatment could be introduced. Despite the aforementioned findings in mice, the clinical development of PKU Gene therapeutics and the translation of these results in a clinical setting with humans has not yet been accomplished. According to the review "Gene Therapy for the Treatment of Neurological Disorder: Metabolic Disorders" (published July 2016 on PubMed) [22] no clinical trials for gene therapy of PKU have been conducted. Further research is required to create more stable and safe vectors before implementing Adenovirus (AAV) or adeno-associated virus (rAAV) mediate gene therapies.

#### 6. Production and Delivery of metabolic enzymes by Genetically Modified (GM) probiotics

A new treatment approach in metabolic deficiencies is the use of GM probiotics to express certain metabolic enzymes that are deficient or completely absent from a certain

metabolic pathway. In the case of PKU, the PAL enzyme has to be delivered to the intestinal wall. The expression of the PAL enzyme in GM organisms and the delivery of this enzyme in animal models are the basic mechanisms of this prospective treatment. In 1999, the first probiotic used to express the PAL enzyme was E.coli which was genetically engineered and delivered to mice [26]. The results of this study indicated a decrease in blood Phe concentrations, although genetically engineered E.coli containing the PAL was resistant to proteolytic activation by intestinal enzymes in vitro. Moreover, some concerns were raised regarding the use of E.coli for delivering the enzymes to the GI tract. The fact that a Proteobacteria, which is a non-pathogenic E.coli component, is part of the normal gut flora and comprises 8% of it. Therefore, there is a danger of the interaction of the normal E.coli in the gut with the engineered one and thus result in the alteration of the normal gut flora and have pathological consequences for the human PKU patients. Moreover, E.coli mostly colonises the large intestine, although the dietary amino acids are absorbed in the small intestine (distal portion) [26].

Another study of genetically modified probiotics has been conducted in which PAL Cdna is expressed in L.lactis which colonizes mainly the small intestine [26]. In this study, rats with HPA were orally administered with the PAL expressing L.lactis and the results showed that the Phe levels were decreased. Although a reduction of the phe levels in both animal studies was achieved the development of orthologous models of a human PKU treatment approach with GM probiotics is not yet completely formed. The findings of the aforementioned studies are a strong indicator that GM probiotics could be a potential alternative therapy for PKU. However, further studies need to be conducted for the evaluation of the safety and efficacy of this prospective treatment method.

#### Discussion:

Provided that PKU is diagnosed within the first few days after birth and treatment is started immediately the prognosis for people with the disorder is positive, without treatment it will result in intellectual disability [26].

The long-term outlook for patients with PKU is excellent if they adhere to therapy and if blood Phe levels remain within the therapeutic range [30], [31]. Life expectancy should be normal, and most patients function independently in adult life with a properly managed diet.

The height and weight are within the normal range compared to healthy children at the age of two. But later, affected persons are more prone to be overweight [28].

Puberty and bone age do not differ from the healthy children, although atypical PKU affected individuals might have an abnormal outcome [16].

Studies have shown that the IQ of individuals with PKU is within the normal range [9], [26]. Although intellectual disability is prevented by the initiation of treatment shortly after birth, treated patients with PKU have an increased incidence of ADHD, deficits in executive functioning, including reduced information processing speed, and psychiatric disorders [30]. Most of these adverse outcomes can be related to mean blood phe levels and how strict the treatment is followed during the first six years of life.

The management of PKU is complex and it is common that the patient's blood Phe levels are not in the normal range, usually due to inadequate compliance. Low Phe blood levels should be strictly maintained even during adulthood to protect from neuropsychological deterioration. Inadequate adherence to the diet usually happens because both the families and PKU individuals find it difficult to comply with [29]. This may be because of the stressful and time consuming food preparation, record-keeping and due to the exclusion of most high protein natural foods which can be socially restrictive. Reestablishing the diet is also difficult because individuals with PKU might have a decreased motivation to maintain the therapy [32]. Other factors hindering compliance include the intake of special medical foods, amino acid formula supplementation and constant hospital visits, both of which are financially challenging for many PKU patients [26], [29].

As already mentioned before there are additional therapies beside the low phe diet which have an impact on the patients' health outcome. These treatments aim to liberalize the strict diet that PKU patients must comply with. There are many options that fit different situations, lifestyles and increase the overall quality of life. For instance new drugs such as enzyme replacement therapy which use PAL, improve mental health and provide a better life quality. The effectiveness other therapies discussed here such as gene therapy have not fully been shown in humans yet.

## Conclusion:

Accessible treatment options (diet and medical foods) for PKU have been mostly the same for over 60 years and are difficult to adhere to due to personal, socio-economic and other factors. Additionally traditional PKU treatment may result in nutritional deficiencies and leaves patients vulnerable to oxidative stress and neurocognitive decline. New therapies for PKU could improve the quality of life of PKU patients and work by preventing Phe entry into the brain or allowing Phe breakdown. The therapies we reviewed were: 1. Enzyme therapy with Phe ammonia-lyase conjugated with polyethylene glycol, which can be injected or ingested and for which two clinical trials showed reduction of Phe levels in typical/atypical patients. 2. Production and delivery of metabolic enzymes by genetically modified probiotics (*E.coli*&*L.lactis*) in the intestinal wall, which reduced Phe levels in animal studies of typical PKU. 3. Sapropterin dihydrochloride supplementation, which resulted in 30% reduction of Phe levels in patients with atypical PKU. It may be used in mild cases of typical PKU patients as well. 4. LNAA's supplementation, which reduced cerebral and plasma Phe levels & increased neurotransmitter concentrations in patients with PKU. 5. Glycomacropeptide medical foods which are medical foods superior in taste and quality than older versions.

6. Gene Therapy: Studies have been done only in animal models or isolated hepatocytes. Ex Vivo therapy & in vivo therapy with adenovirus mediated transgene delivery or recombinant adeno-associated virus delivery of the PAH gene showed reduction of Phe levels. Safety and stability of vectors is in need of further research.

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