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Review article

International best practice for the evaluation of responsiveness to sapropterin dihydrochloride in patients with phenylketonuria

Ania C. Muntau[a], Darius J. Adams[b], Amaya Bélanger-Quintana[c], Tatiana V. Bushueva[d], Roberto Cerone[e], Yin-Hsiu Chien[f], Ana Chiesa[g], Turgay Coşkun[h], Javier de las Heras[i], François Feillet[j], Rachel Katz[k], Florian Lagler[l], Flavia Piazzon[m], Fran Rohr[n], Francjan J. van Spronsen[o], Paula Vargas[p], Gisela Wilcox[q], Kaustuv Bhattacharya[r,*]

[a] University Children's Hospital, University Medical Center Hamburg Eppendorf, Hamburg, Germany
[b] Atlantic Health System, Morristown Medical Center, Morristown, NJ, USA
[c] Hospital Ramón y Cajal, Madrid, Spain
[d] National Medical Research Center of Children's Health of the Ministry of Health of the Russian Federation, Moscow, Russia
[e] G. Gaslini Institute, University of Genova, Genova, Italy
[f] National Taiwan University Hospital, Taipei, Taiwan
[g] Centro de Investigaciones Endocrinologicas "Dr Cesar Bergadá" CEDIE, CONICET, Hospital de Niños Ricardo Gutierrez, Fundacion de Endocrinologia Infantil, Buenos Aires, Argentina
[h] Hacettepe University Faculty of Medicine, Ankara, Turkey
[i] Hospital Universitario de Cruces, Biocruces Health Research Institute and University of the Basque Country UPV/EHU, Vizcaya, Spain
[j] Children's University Hospital, CHU Brabois, Vandoeuvre les Nancy, France
[k] Ann and Robert Lurie Children's Hospital of Chicago, Chicago, IL, USA
[l] Paracelsus Medical University, Salzburg, Austria
[m] Associação de Pais e Amigos dos Excepcionais de São Paulo (APAE DE SÃO PAULO), São Paulo, SP, Brazil
[n] Boston Children's Hospital, Boston, MA, USA
[o] Beatrix Children's Hospital, University Medical Center of Groningen, University of Groningen, Groningen, the Netherlands
[p] Hospital Materno Infantil Presidente Vargas, Porto Alegre, RS, Brazil
[q] University of Manchester & Salford Royal NHS Foundation Trust, Salford, UK
[r] Genetic Metabolic Disorders Service, University of Sydney, Children's Hospital Westmead Clinical School, Sydney, NSW, Australia

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ABSTRACT

Phenylketonuria (PKU) is an inherited metabolic disease caused by phenylalanine hydroxylase (PAH) deficiency. As the resulting high blood phenylalanine (Phe) concentration can have detrimental effects on brain development and function, international guidelines recommend lifelong control of blood Phe concentration with dietary and/or medical therapy. Sapropterin dihydrochloride is a synthetic preparation of tetrahydrobiopterin (6R-BH4), the naturally occurring cofactor of PAH. It acts as a pharmacological chaperone, reducing blood Phe concentration and increasing dietary Phe tolerance in BH4-responsive patients with PAH deficiency. Protocols to establish responsiveness to sapropterin dihydrochloride vary widely.

Two meetings were held with an international panel of clinical experts in PKU management to develop recommendations for sapropterin dihydrochloride response testing. At the first meeting, regional differences and similarities in testing practices were discussed based on guidelines, a literature review, outcomes of a global

Abbreviations: ADHD, attention deficit hyperactivity disorder; APAC, Asia and Pacific; BH4, tetrahydrobiopterin; EMA, European Medicines Agency; EUMEA, Europe and Middle East; FDA, Food and Drug Administration; HPA, hyperphenylalaninemia; IVS, intervening sequence; LATAM, Latin America; MHP, mild hyperphenylalaninemia; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; PKU MOMS, Maternal Phenylketonuria Observational Program; PKUDOS, Phenylketonuria Demographics Outcomes and Safety; Tyr, tyrosine

* Corresponding author at: Genetic Metabolic Disorders Service, Children's Hospital at Westmead, Cnr Hawkesbury Road and Hainsworth Street, Westmead, Locked Bag 4001, Westmead, NSW 2145, Australia.

E-mail addresses: muntau@uke.de (A.C. Muntau), Darius.Adams@atlantichealth.org (D.J. Adams), amaya.belanger@salud.madrid.org (A. Bélanger-Quintana), tbushueva1@yandex.ru (T.V. Bushueva), robertocerone@gaslini.org (R. Cerone), chienyh@ntu.edu.tw (Y.-H. Chien), achiesa@cedie.org.ar (A. Chiesa), tcoskun@hacettepe.edu.trc (T. Coşkun), javieradolfo.delasherasmontero@osakidetza.eus (J. de las Heras), f.feillet@chru-nancy.fr (F. Feillet), RKatz@luriechildrens.org (R. Katz), florian.lagler@pmu.ac.at (F. Lagler), flaviapiazzon@apaesp.org.br (F. Piazzon), frances.rohr@childrens.harvard.edu (F. Rohr), f.j.van.spronsen@umcg.nl (F.J. van Spronsen), paula_triagem_neonatal@yahoo.com.br (P. Vargas), Gisela.Wilcox@srft.nhs.uk (G. Wilcox), kaustuv.bhattacharya@health.nsw.gov.au (K. Bhattacharya).

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physician survey, and case reports. Statements developed based on the discussions were sent to all participants for consensus (> 70% of participants) evaluation using a 7-level rating system, and further discussed during the second meeting.

The experts recommend sapropterin dihydrochloride response testing in patients with untreated blood Phe concentrations of 360–2000 μmol/L, except in those with two null mutations. For neonates, a 24-h sapropterin dihydrochloride loading test is recommended; responsiveness is defined as a decrease in blood Phe ≥30%. For older infants, children, adolescents, and adults, a test duration of ≥48 h or a 4-week trial is recommended. The main endpoint for a 48-h to 7-day trial is a decrease in blood Phe, while improved Phe tolerance is the endpoint to be assessed during a longer trial. Longer trials may not be feasible in some locations due to lack of reimbursement for hospitalization, while a 4-week trial may not be possible due to limited access to sapropterin dihydrochloride or public health regulation. A 48-h response test should be considered in pregnant patients who cannot achieve blood Phe ≤360 μmol/L with a Phe-restricted diet. Durability of response and clinical benefits of sapropterin dihydrochloride should be assessed over the long term. Harmonization of protocols is expected to improve identification of responders and comparability of test results worldwide.

1. Introduction

Phenylketonuria (PKU) is a rare autosomal recessive metabolic disorder caused by deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1). PAH deficiency results in a complete or partial inability to convert phenylalanine (Phe) to tyrosine (Tyr) in the liver, leading to high blood Phe (hyperphenylalaninemia, HPA), which can cross the blood-brain barrier [1]. Exposure of the brain to high Phe concentrations can have detrimental effects on brain development and function [2,3]. Without treatment, PKU can result in irreversible intellectual disability, behavioral problems, psychiatric symptoms, motor deficits, seizures, and eczematous rash [1–3]. Patients with PKU are genotypically and phenotypically diverse. As of June 28, 2018, the Phenylalanine Hydroxylase Gene Locus-Specific Database described 1044 known variations in the human *PAH* gene (http://www.biopku.org). Phenotypes can vary from mild HPA to severe phenotypes with untreated blood Phe concentrations exceeding 1200 μmol/L, also referred to as classical PKU [1]. The prevalence of PKU varies widely across the world, from 1 out of every 4000 live births in Turkey [4] to < 1 out of 200,000 live births reported for Thailand [1,5].

With the advent of newborn screening programs for PKU and widespread implementation of early control of blood Phe concentration, the most severe neuropsychological complications of the disease can be prevented [1,6]. In potential cases identified by newborn screening, inherited disorders of BH4 metabolism, liver diseases, and DnaJ heat shock protein family (Hsp40) member C12 (DNAJC12) deficiency that can also result in elevated blood Phe concentrations must be ruled out [6,7]. Patients with DNAJC12 deficiency show HPA, but have a normal PAH gene and normal levels and proportions of pterins in urine and blood. Diagnosis can be confirmed by evaluation of the Phe concentration, Phe:Tyr ratio, and complete amino acid profile in plasma [3]. Deficiencies of BH4 metabolism should be excluded by measuring relative levels of pterins (neopterin, biopterin, and primapterin) in blood or urine and dihydropteridine reductase activity in dried blood spots [2,8]. To prevent brain damage, American and European guidelines for the management of PKU recommend lifelong control of blood Phe concentration, starting as soon as possible after birth [2,3]. Whereas the American guidelines recommend to maintain blood Phe concentration in the range of 120–360 μmol/L in patients of all ages [3], the European guidelines advise to keep blood Phe below 360 μmol/L in children until the age of 12 years and in pregnant women, and below 600 μmol/L in (non-pregnant) patients older than 12 years [2].

Treatment strategies for PKU aim at lowering blood Phe concentration by dietary and/or pharmacologic therapy. Dietary therapy consists of a Phe-restricted diet in combination with Phe-free or Phe-restricted L-amino acid-rich medical foods and/or modified low-protein foods [3,9]. However, a strict Phe-restricted diet is very difficult to maintain in the long term, particularly for adolescents and adults [1]. A synthetic preparation of 6R-BH4, the naturally occurring cofactor of PAH, is available as sapropterin dihydrochloride (Kuvan®, BioMarin Pharmaceutical Inc., Novato, CA, USA). Sapropterin dihydrochloride was approved by the Food and Drug Administration (FDA) in 2007 and the European Medicines Agency (EMA) in 2008 for the treatment of HPA in patients with BH4-responsive PKU or BH4 deficiencies [10,11]. It was subsequently approved by both regulatory agencies for the treatment of all patients, including children < 4 years of age [10,11]. The drug is available as 100 mg tablets and powder (100 and 500 mg) for oral solution. Availability of formulations and reimbursement varies between countries. In patients with BH4-responsive PKU, clinical trials of sapropterin dihydrochloride have demonstrated significant reductions in blood Phe concentration, increased Phe tolerance, and good long-term tolerability [12–17]. The benefits of sapropterin dihydrochloride are bestowed by aiding patients, in concert with a Phe-restricted diet, to achieve lower blood Phe concentrations. In patients with good metabolic control, it allows the inclusion of more natural protein in the diet, while maintaining blood Phe concentration in the target range (i.e. increased Phe tolerance). Improvements in attention deficit hyperactivity disorder (ADHD) inattentive symptoms and executive function have also been associated with sapropterin dihydrochloride treatment [12–17]. To identify those patients who would benefit from treatment, a sapropterin dihydrochloride response test or trial can be performed [18]. Originally, the BH4 response test was only used for differential diagnosis, to discriminate between patients with PAH deficiency and disorders of BH4 metabolism [6]. However, since the approval of sapropterin dihydrochloride for the treatment of PKU, centers across the world have adapted the test to evaluate responsiveness to the drug.

There is a wide variety in protocols used to establish responsiveness between and even within countries, as illustrated by a literature review of studies on this subject (Additional file 1). The literature review reveals that testing protocols differ, particularly with regard to test duration (from 8 h to several weeks), Phe loading strategies before sapropterin dihydrochloride testing, and diet requirements during the test. More similarities were seen for other aspects of the testing protocol. The majority of studies used a sapropterin dihydrochloride dose of 20 mg/kg, taken either one time or daily for several days, which was found to be associated with greater responsiveness rates than a 10 mg/kg dose [19,20]. In addition, most studies defined responsiveness as a ≥ 30% reduction in blood Phe concentration. Some general conclusions could be made with regard to the impact of phenotype, genotype, and test duration on BH4 responsiveness. Although patients with milder phenotypes (lower baseline blood Phe concentration before start of any treatment) appeared to respond better to BH4 than more severe phenotypes, responders were identified across the disease spectrum (Fig. 1). Genotype-phenotype correlation studies have shown that genotype determines residual PAH activity and may help determine BH4 responsiveness in some instances: whereas two null-mutations are highly predictive of non-responsiveness, missense mutations in the oligomerization domain appear to predict responsiveness [21–24]. Studies comparing different response periods suggested that an 8-h test

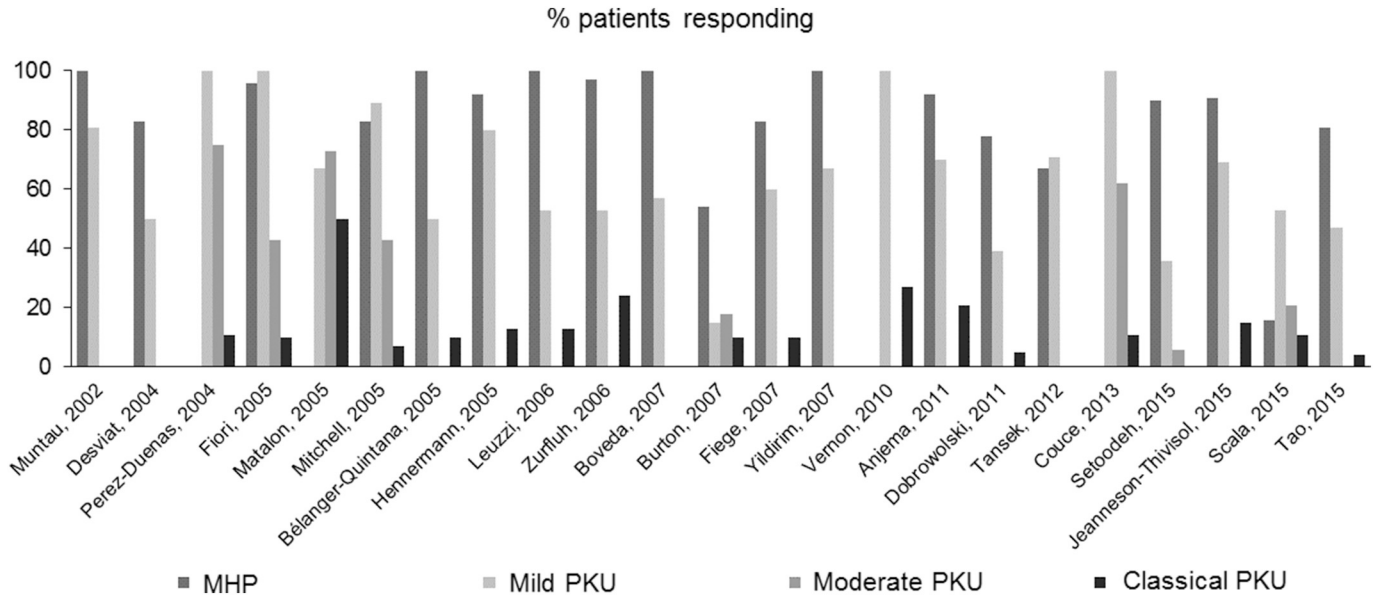


Fig. 1. Responsiveness to tetrahydrobiopterin (BH4) according to phenotype in 23 prospective studies including ≥20 patients [12,19,23,27–46]. See Additional file 1 for more detailed information on the studies. MHP: Mild hyperphenylalaninemia; PKU: phenylketonuria.

is too short to identify all responders and that most, but not all, patients respond to BH4 within 24 h. A study by Anjema et al. suggested that a positive 8-h neonatal response test also predicts responsiveness in the long term, but that a negative 8-h neonatal response test does not rule out responsiveness in a 48-h response test performed at an older age [25]. In addition, a retrospective study by the same investigators in a cohort of 177 (non-neonatal) patients showed that a 48-h test is a good predictor of responsiveness in the long term, although patients who only respond > 24 h after the first BH4 dosing are more likely to be false responders [26].

The wide variety in sapropterin dihydrochloride response testing practices suggested by the literature review is confirmed by the outcomes of the Global BH4 (KUVAN®) Response Survey, an online physician survey that assessed differences and similarities in testing practices between regions worldwide (Additional file 2). The analysis focused on 73 participants who trialed a higher proportion of their PKU patients than their respective regional medians. The outcomes of the survey showed considerable differences between the regions (Europe and Middle East [EUMEA, $n = 40$], Asia and Pacific [APAC, $n = 25$], and Latin America [LATAM, $n = 8$]; no participants from the USA were included in the survey), particularly with regard to test duration, target blood Phe concentration, and definitions used to identify partial responders to sapropterin dihydrochloride. In general, most participants do not exclude patients from sapropterin dihydrochloride response testing, although 40–50% use selection criteria to determine which patients are recommended for a trial. The most frequently used selection criteria were blood Phe concentration and increased disease severity. In addition, the participants tend to utilize short protocol durations (24–72 h), but with a wide variability between regions (Fig. 2). Most participants collect 2–5 Phe measurements prior to testing and 4–5 measurements during testing (independent of protocol duration), and use an initial sapropterin dihydrochloride dose of 20 mg/kg. Sapropterin dihydrochloride response was most commonly defined as a 30% reduction in blood Phe concentration relative to baseline. Whereas increased Phe tolerance was the criterion used to determine durability of response, non-responders were mostly discontinued from treatment. While results for LATAM were often not in line with those of EUMEA and APAC, it should be noted that these results were based on answers from only eight participants. The limited availability of sapropterin dihydrochloride in LATAM greatly limited the number of clinicians experienced with this medication.

The great variety between countries and regions in sapropterin dihydrochloride response testing practices can, at least partly, be explained by the lack of worldwide guidelines for response testing and the lack of comparison studies. Until now, guidelines published in peer-reviewed international journals only exist for Europe and the USA

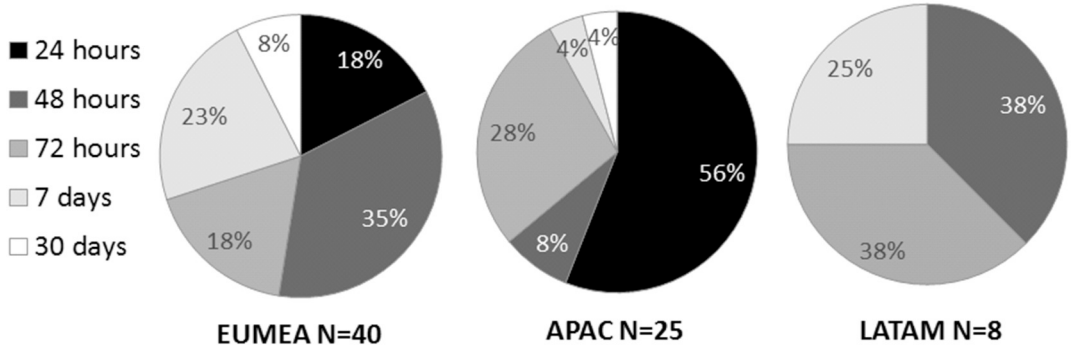


Fig. 2. Most frequently utilized protocol (1 per participant) in the Global BH4 Response Survey (see Additional file 2). Data in the graph are based on answers to the question "*What is the duration of your most frequently utilized protocol for BH4 response testing?*". The survey did not include participants from the US, where the standard procedure is a 4-week (30 day) trial. APAC: Asia and Pacific; EUMEA: Europe and Middle East; LATAM: Latin America.

Table 1
Summary of international guidelines on sapropterin dihydrochloride/BH4 response testing.

	Europe [2,6,47]	USA [3,48]
Patient selection	All PKU patients except those: • without 2 known null mutations (no response) • with 2 known BH4-responsive mutations (systematic response)	All PAH-deficient patients, except those with 2 null mutations in *trans*
Sapropterin dihydrochloride dose	20 mg/kg/day	20 mg/kg/day
Test duration	48 h (24 h in neonates), followed by a trial of ≤6 months	4 weeks[a]
Definition of responsiveness	Increase natural protein ≥100% ***or*** > 75% of Phe levels in target range in previously uncontrolled patients	Decrease blood Phe ≥30% ***or*** improvement in neuropsychiatric symptoms *or* increase in Phe tolerance

[a] If testing is to be done in early infancy, the American College of Medical Genetics and Genomics (ACMG) guidelines recommend to lower blood Phe level first to 480–600 μmol/L. Blood samples for Phe determination are taken at 24 h, 1 week, 2 weeks, and in some cases, 3 or 4 weeks.

(Table 1) [2,3,6,47,48]. The protocols suggested in these guidelines differ considerably, particularly with regard to test duration and definition of responsiveness (Table 1). Differences in test protocols may also be due to differences between countries in reimbursement policies. Whereas some countries do not allow hospitalization of patients for response testing, it is common practice elsewhere. In addition, in some countries it is not possible to obtain reimbursement for a long trial, or it can take months before patients get reimbursement for treatment after a successful response test. Differences also exist with regard to laboratory newborn screening practices for PKU and the laboratories' interface with clinical units, particularly in regard to the timing of when clinicians become aware of the results.

To increase uniformity in sapropterin dihydrochloride response testing practices for patients with PKU worldwide, an international panel of experts gathered at two expert meetings to develop global best practice recommendations, which are presented here.

2. Methodology

The expert group (reflected in the authorship) included 18 clinicians with expertise in the management of PKU with sapropterin dihydrochloride from Argentina, Australia, Austria, Brazil, France, Germany, Italy, The Netherlands, Russia, Spain, Taiwan, Turkey, the United Kingdom, and the USA. At the first meeting (Madrid, Spain; June 20, 2017), the experts discussed regional differences and similarities in current response testing practices based on the results of a literature review (Additional file 1e), the outcomes of the Global BH4 Response Survey (Additional file 2), and case reports presented by the experts (Additional file 3). Based on the discussions during the first meeting, statements were developed. These statements were subsequently sent to all participants as an online survey for consensus evaluation, using a 7-level rating system (strongly agree, agree, somewhat agree, neither agree nor disagree, somewhat disagree, disagree, strongly disagree), before the second meeting (Additional file 4). Percent agreement was defined as the percentage of participants selecting "strongly agree" or "agree" in the pre-meeting survey. The outcomes of the pre-meeting survey were presented at the second consensus meeting (London, UK; December 12, 2017). All statements that achieved < 70% agreement in the pre-meeting survey were further discussed and fine-tuned during workshops (in three smaller groups) and subsequently presented and discussed by all participants. The accepted statements (achieving ≥70% agreement) and the revised statements developed during the second consensus meetings were sent to all participants after the meeting for additional comments. The best practice recommendations presented below include the final statements, as well as any associated caveats, and a summary of the rationale behind the statements. Best practice recommendations for sapropterin dihydrochloride response testing were prepared for three patient groups: (1) neonates, (2) non-neonatal infants, children, adolescents, and adults, and (3) pregnant women, and are discussed separately. Recommendations applying to all three patient groups are discussed as "general".

3. Best practice recommendations

3.1. General

Key recommendations: General[a]

- Both early- and late-treated PKU patients can benefit from sapropterin dihydrochloride
- Although PKU patients with certain mutations are more likely to respond to sapropterin dihydrochloride, no patients should be excluded from a response test (either a short response test or a longer treatment trial), except those with two null mutations
- The primary population to receive a sapropterin dihydrochloride response test are those with untreated blood Phe concentrations > 360 and < 2000 μmol/L, although there may be exceptions
- Patients with an untreated blood Phe concentration > 2000 μmol/L should be initiated on a Phe-restricted diet as soon as possible
- If the response test is performed, efforts to collect relevant medical history, nutritional history, and social history information should be made before sapropterin dihydrochloride response testing

[a] Any caveats and further details regarding these recommendations are discussed in the text.

3.1.1. Testing criteria

Taking into account findings from literature and clinical practice, the experts agreed that PKU patients should not be excluded from sapropterin dihydrochloride response testing based on age or blood Phe concentration. An exception was made for patients with untreated blood Phe concentrations < 360 μmol/L or > 2000 μmol/L, although it can be justified to perform a response test in neonates with blood Phe < 360 μmol/L under certain circumstances (see section on neonatal patients).

The lower (untreated) blood Phe limit of 360 μmol/L recommended for sapropterin dihydrochloride responsiveness testing is in line with statements in the European and American PKU management guidelines. According to these guidelines, patients with untreated blood Phe concentrations below 360 μmol/L do not require treatment, as they are already in the target range [2,3]. The upper blood Phe limit for response testing of 2000 μmol/L was set based on the experts' clinical experience that patients with Phe concentrations above this threshold are unlikely to respond to sapropterin dihydrochloride. In these patients, dietary treatment should be started as soon as possible. Response testing might occasionally be considered in patients with baseline blood

Phe > 2000 μmol/L, depending on how Phe levels evolve over time (higher Phe levels in HPA patients or a milder presentation than expected in an initially severe case).

When the patient's genotype is known, it can give clues about the likelihood of sapropterin dihydrochloride response. The PAH activity landscapes website (http://pah-activitylandscapes.org/) shows frequency of genotypes by country, and links to clinical and response data [49]. In a large retrospective study by Wettstein et al., using data from 4181 patients from the BIOPKU database, genotype could predict responsiveness in 71% of cases [24]. Genotyping can also help decide whether a response test is indicated and which test duration is most suitable. A sapropterin dihydrochloride test is not recommended for patients with two null mutations, as these patients are very unlikely to respond [24]. This recommendation is largely in line with the European and American guidelines for the management of PKU (Table 1) [2,3]. In all other patients, a response test remains the best method to determine responsiveness. It should be noted that the presence of two null mutations can generally not be used as a selection criterion for neonatal patients identified with newborn screening, as information on genotype is usually not available at the time of sapropterin dihydrochloride response testing.

3.1.2. Collection of medical, nutritional, and social history data

Before a sapropterin dihydrochloride response test, efforts should be made to collect medical and social history data that could prohibit or influence responsiveness to the drug. Relevant medical history data include physical and neurological exam data, medical issues (including allergies), (birth) anthropometrics, concomitant medications, and relevant laboratory data. Anthropometric data, including growth history, are important because of the negative impact PKU can have on physical growth, body stature, and body composition [50,51]. In non-neonatal infants, children, adolescents, and adults, blood Phe history (average blood Phe concentration and variability), Tyr status (average blood Tyr concentration relative to the normal range and Phe:Tyr ratio), developmental history, and neurocognitive status should be collected as well. Information on a patient's neurocognitive status is important to identify the need for any additional assistance or needs during the test. The ability to adhere to treatment is key, especially for adolescent and adult patients, as they are responsible for taking their medication daily. Folate, B12, and micronutrient status may also be considered as part of the baseline assessment. Medical history data that should be collected in pregnant women with PKU are the same as for non-pregnant patients. However, although a neurocognitive test can be performed in women who are planning pregnancy, it should not be performed in pregnant patients if it delays treatment. In addition, the same medical history data as for pregnant healthy women should be collected, including obstetric/gynecologic history (number of weeks gestation, first child or not, ultrasounds, considered high risk pregnancy), and enteral tolerance.

Important nutritional history data to collect in neonates include the method of feeding (breastfeeding or formula feeding) and frequency of feeding (for total volume calculation). Other nutritional history data such as outcomes of standard pediatric assessments of infant feeding abilities and mother-child feeding dynamics may also be useful, particularly if retesting is required later. In non-neonatal patients, information on dietary history, feeding skills (in children), Phe tolerance, possible food phobias, use of medical foods/amino acid supplements, low protein foods, and supplements (e.g. tyrosine, vitamin/mineral), and treatment adherence should be collected.

Social history information that should be collected includes the distance to the clinic, and insurance coverage of e.g. medical foods and low protein foods. In addition, family dynamics should be documented for children, while for adults their living situation and support system, school history, and employment history/status are relevant. However, treatment should not be delayed for the collection of information that will not affect patient care.

3.2. Neonatal patients

Key recommendations for sapropterin dihydrochloride response testing in neonates[a]

- Neonates with an untreated blood Phe concentration of 360–2000 μmol/L should receive a sapropterin dihydrochloride response test for testing BH4 responsiveness; testing of patients with blood Phe < 360 μmol/L could be considered to rule out most disorders of BH4 metabolism; patients with blood Phe > 2000 μmol/L should not typically be tested, but should be initiated on a Phe-restricted diet immediately
- Although a test should be administered as soon as possible after birth, efforts should be made to collect baseline information before sapropterin dihydrochloride response testing in neonates, including blood Phe concentration and body weight (for determining dose); in addition, it may be useful to estimate Phe intake
- Parent education before sapropterin dihydrochloride testing in neonates should include: setting of expectations of the trial, establishment of requirements for an accurate trial, and review of treatment goals
- A response test in neonates should be performed with 20 mg/kg sapropterin dihydrochloride (crushed tablets or powder mixed in a small amount of formula or breast milk)
- During sapropterin dihydrochloride testing in neonates, blood Phe concentration should (initially) be monitored over a 24-h period. During this period, it is best practice to collect samples for determining blood Phe concentration just prior, and at 4 h, 8 h, 12 h, 16 h, and 24 h after administering sapropterin dihydrochloride
- A neonate with a decrease in blood Phe ≥30% during 24-h response testing can be considered a sapropterin dihydrochloride responder
- In neonates identified as responsive, sapropterin dihydrochloride dose should be adjusted between 5 and 20 mg/kg/day
- Critical evaluation of clinical benefit of sapropterin dihydrochloride in neonates should include assessment of blood Phe control, dietary Phe tolerance, need for/amount of Phe-free amino acid supplements/medical food, and tolerability of the drug

[a] Any caveats and further details regarding these recommendations are discussed in the text.

3.2.1. Timing of the neonatal test

Sapropterin dihydrochloride testing practices in neonatal patients vary across the world: e.g., while neonatal tests are common practice in Europe, this is not always the case in the USA [3]. If neonatal testing is not performed, guidance for non-neonatal infants, children, adolescents, and adults should be followed (see next section).

In neonates with elevated blood Phe concentrations, treatment (with diet and/or sapropterin dihydrochloride) before day 10 is critical, as the intelligence quotient of PKU patients has been shown to decrease by about 4 points for every 4 weeks delay in starting treatment [2,52]. Therefore, if a sapropterin dihydrochloride response test is considered, it should be performed as soon as possible after birth using a test with a maximum of 24 h.

3.2.2. Indications for testing

As previously discussed, sapropterin dihydrochloride response testing is recommended in patients with baseline blood Phe concentrations between 360 and 2000 μmol/L. Only untreated blood Phe concentrations should be used to determine the need for a test. Ideally, the decision to test for sapropterin dihydrochloride responsiveness is based on diagnostic blood Phe concentrations, as these may deviate from neonatal screening concentrations. However, in countries where diagnostic testing for PKU takes a long time due to local circumstances, it can be justified to start sapropterin dihydrochloride based on screening blood Phe concentrations before diagnostic concentrations are available, to avoid any treatment delay. If diagnostic Phe concentrations turn out to be below 360 μmol/L, sapropterin dihydrochloride can be discontinued at this time. Diagnostic tests may also reveal defects in BH4 metabolism, liver diseases, or DNAJC12 deficiency which require another treatment approach [6,7]. While defects in BH4 metabolism can in most cases be ruled out quickly by measuring blood spot or urine pterin concentrations [53,54], diagnostic pterin concentrations cannot be obtained quickly in some areas. In this case, sapropterin dihydrochloride response testing may be considered also in patients with blood Phe below 360 μmol/L to rule out disorders of BH4 metabolism. In addition, the *in vivo* ^{13}C-Phenylalanine Oxidation Test,

which quantifies PAH enzyme activity by measuring $^{13}CO_2$ in exhaled air, as described by Muntau et al. [27], can be useful to discriminate between PKU and disorders of BH4 metabolism if diagnosis is delayed. Although this test is relatively cheap and can be performed quickly, it is not yet commercially available.

3.2.3. Before testing

Important baseline information to collect before response testing in neonates includes blood Phe concentration and body weight (for determining sapropterin dihydrochloride dose). Baseline blood Phe concentration should be collected prior to sapropterin dihydrochloride administration and, if there is a delay between diagnosis and response testing, on the day the drug is administered. As the exact composition of the mother's breastmilk is unknown, it is difficult to calculate dietary Phe intake in breastfed babies. Nevertheless, it may be useful to estimate Phe intake, as it can be used as a baseline to follow the evolution in Phe intake over the long term. Phe intake in neonates can be estimated by tracking estimated fluid intake and baby's weight, considering a mean Phe content of 40 mg per 100 mL mature breastmilk. If the test is performed in the first 2 weeks of life, Phe content may vary between 40 and 80 mg/100 mL breastmilk (with higher concentrations earlier after birth) [55].

It is important to discuss the protocol of the sapropterin dihydrochloride loading test with the patient's parents in advance, including the (frequent) collection of blood samples, diet records, and safety issues, and requirements for an accurate trial. For example, the patient should not be ill or in a stressed state at the time of the loading test, as this could influence outcomes [8,56], while the test can be influenced by the fact that the neonate is in a catabolic or anabolic state. In regions where access to sapropterin dihydrochloride treatment cannot be guaranteed, parents need to be informed that the neonatal test can also benefit their child in terms of excluding other HPA diagnoses and help in the selection of future treatments. If the loading test is performed in an outpatient setting, parents can be taught to collect filter paper blood samples at home. However, this could be difficult for new parents who are still adjusting to the PKU diagnosis for their baby. In this case, hospitalization of the infant during the test should be considered, if there are no legal or reimbursement constraints. In addition to the test protocol, treatment goals should be reviewed with the parents. Parents need to be aware that responsive patients may still require a Phe-restricted diet and PKU dietary supplements in conjunction with sapropterin dihydrochloride, although diet will be less restrictive than without treatment.

3.2.4. Testing protocol and outcome measures

The 24-h neonatal sapropterin dihydrochloride loading test is recommended to be performed with a 20 mg/kg initial dose, which has been shown to result in a higher response rate than a 10 mg/kg dose in clinical studies (Additional file 1) [19,20]. It is recommended to collect blood samples for determining Phe concentrations at 4 h, 8 h, 12 h, 16 h, and 24 h after administering sapropterin dihydrochloride. The experts are aware that 12 and 16 h samples for determination of blood Phe during the 24-h test period may be difficult to collect in an outpatient setting, and that practices vary widely between and within countries. Nevertheless, they strongly recommended obtaining these samples whenever possible, since late responders may show a decrease in blood Phe concentration between 8 h and 24 h after dosing, and the lowest Phe concentration can be missed if 12-h and/or 16-h samples are not collected.

The experts recommend defining “responsiveness” in the neonatal loading test as a reduction in blood Phe concentration from baseline of at least 30%. Although arbitrary, a 30% reduction in blood Phe concentration from baseline is the most widely used criterion in studies and clinical practice (see Additional files 1 and 2). After the 24 h test, sapropterin dihydrochloride treatment should be stopped and dietary treatment should be started immediately until the results of the test are known. It should be noted that a reduction in blood Phe during the test may also occur in patients who have a deficiency in BH4 metabolism or a DNAJC12, underlining the importance of a correct diagnosis.

In neonatal patients shown to respond, sapropterin dihydrochloride dose should be adjusted between 5 and 20 mg/kg/day over time, taking into account changes in blood Phe concentration while the child is growing. Dose adjustment should either lead to increased Phe tolerance, or to stable tolerance under a lower dose. It should be noted that patients may have a relatively high Phe tolerance without sapropterin dihydrochloride during the first weeks of life, which could bias long-term results. Responsiveness to sapropterin dihydrochloride should therefore be critically evaluated by assessing long-term control of blood Phe, taking into account initial blood Phe concentration and genotype (if available). Changes in dietary Phe tolerance may be monitored in relation to the Phe intake estimated at baseline. In addition, the need for and/or the amount of Phe-free amino acid supplements/medical foods, as well as the tolerability of the drug, should be assessed. Dietary Phe intake should be adapted in accordance with published recommendations [9].

3.3. Non-neonatal infants, children, adolescents, and adults

Key recommendations for sapropterin dihydrochloride response testing in non-neonatal infants, children, adolescents, and adults[a]

- A previous BH4/sapropterin dihydrochloride response test, or an untreated blood Phe concentration < 360 or > 2000 μmol/L in the neonatal period, should not preclude a response test at a later age
- If a patient previously received a BH4/sapropterin dihydrochloride test, the following information should be collected about that trial: length of the test, results of the test, timing of the test (neonatal period or not), reason why treatment was not started
- For non-neonatal infants, children, adolescents, and adults, test duration should be at least 48 h and up to 4 weeks
- Potential responsiveness in a 48-h to 7-day test can often be evaluated by measuring the reduction in blood Phe concentration, with a ≥ 30% reduction in blood Phe indicating response; a longer trial is needed to identify an increase in Phe tolerance (see Fig. 3 and text for protocol details and caveats)
- Baseline data that should be collected before starting sapropterin dihydrochloride include blood Phe concentration (≥3 measurements), body weight (for determining dose), dietary Phe intake and tolerance, amino acid supplements/medical food intake; a neurocognitive test should be collected whenever possible
- Before starting a sapropterin dihydrochloride response trial, PKU patients and/or parents should be educated to establish expectations of the trial and requirements for an accurate trial, review treatment goals, and discuss the possibility to connect with other parents/patients who have experience with sapropterin dihydrochloride response testing and/or treatment
- A response test or trial must be performed with 20 mg/kg sapropterin dihydrochloride (tablets, crushed tablets, or powder in liquid or soft foods[b]), administered daily at the same time of the day with a meal
- After final Phe tolerance has been determined, the sapropterin dihydrochloride dose should be adjusted between 2 and 20 mg/kg/day
- In sapropterin dihydrochloride responsive patients who have been following a Phe-restricted diet, dietary Phe intake should be progressively increased, per published guidelines [9]
- Critical evaluation to determine clinical benefit of sapropterin dihydrochloride should include assessment of improvement in blood Phe control, dietary Phe tolerance, need for special low protein foods, need for/amount of amino acid supplements/medical food, drug tolerability, and improvements in neurocognitive status, behavior, and/or quality of life

[a] Any caveats and further details regarding these recommendations are discussed in the text.

[b] Not all sapropterin dihydrochloride formulations may be available in different countries.

3.3.1. Indications for testing

Sapropterin dihydrochloride testing in non-neonatal infants, children, adolescents, and adults with PKU should be considered when untreated blood Phe concentrations are above the target range, whether they are on dietary therapy or not. However, unknown untreated blood Phe concentrations should not preclude a sapropterin dihydrochloride

test. As previously mentioned, blood Phe target ranges for non-neonatal PKU patients differ between European and American guidelines. The European guidelines recommend keeping blood Phe below 360 µmol/L in children until the age of 12 years, and below 600 µmol/L in older (non-pregnant) patients [2]. American guidelines recommend lifelong maintenance of blood Phe concentration in the range of 120–360 µmol/L [3]. Even if patients already have Phe levels within the recommended range with diet, sapropterin dihydrochloride can help to reduce the burden caused by a Phe-restricted diet. Patients who were not tested in the neonatal period should be offered a response test. A previous response test in the neonatal period should not preclude sapropterin dihydrochloride testing later in life, particularly if the patient's genotype suggests a BH4-responsive phenotype. Slow responders can be missed in the shorter neonatal test [25].

3.3.2. Before testing

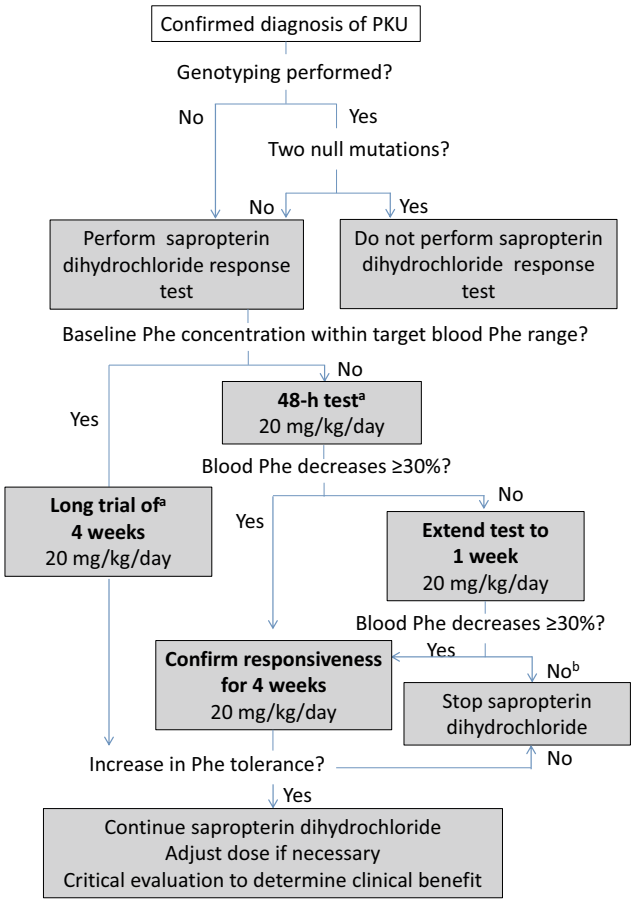
Baseline information that should be collected before starting sapropterin dihydrochloride in non-neonatal PKU patients includes blood Phe concentration, body weight (for determining dose), dietary Phe intake, and amino acid supplements/medical food intake. For patients under good metabolic control, it is essential for the interpretation of the test results to assess maximum Phe tolerance by progressively increasing Phe intake prior to response testing. Assessment of maximum Phe tolerance at baseline assures that an observed increase in Phe tolerance after the test is due to sapropterin dihydrochloride. In addition, it may be valuable to perform a baseline neurocognitive test to allow assessment of changes in neurocognitive function in the long term. However, as no short-term effect of sapropterin dihydrochloride treatment on neurocognitive function is expected, the test can also be performed shortly after starting treatment. Formal psychometric evaluation of neurocognitive function is best, but if not available, neurocognitive evaluation may consist of questions on e.g. patient performance, concentration, attention, and hyperactivity.

Similar to parents of neonatal patients, older PKU patients and/or their parents should receive proper education before sapropterin dihydrochloride testing to establish expectations of the trial and requirements for an accurate trial, and to review treatment goals (Table 2).

3.3.3. Testing protocol and outcome measures

A response test in non-neonatal individuals with PKU is preferably performed with sapropterin dihydrochloride at 20 mg/kg/day. As BH4 is water soluble, and thus not expected to be absorbed in fat tissue, the experts suggest using ideal body weight rather than actual body weight to calculate the dose for obese individuals.

Test duration should be at least 48 h and up to 4 weeks. Several factors can determine the choice for a short or longer test (Fig. 3). For patients with baseline blood Phe concentrations in the target range due to dietary therapy, and particularly those with concentrations of 180 µmol/L or lower, a longer-term trial of 4 weeks with an increase in maximum Phe tolerance may be most suitable (Fig. 3). These patients do not always show a significant decline in blood Phe in a shorter response test, even if they are responsive to sapropterin dihydrochloride (Additional file 3) [3,26]. An increase in maximum Phe tolerance can be determined by progressively increasing Phe intake, while keeping the patient in the target blood Phe range. According to the European guidelines for the diagnosis and treatment of PKU "establishing an increase in natural protein tolerance of ≥100% from pre-treatment with blood Phe concentrations remaining consistently within the target range" after 6 months provides evidence for responsiveness [8]. It



[a]A short test can also be performed if a longer trial is not possible due to local circumstances
[b]Continuing sapropterin dihydrochloride response testing for 4 weeks can be considered if the patient is suspected to be a responder

Fig. 3. Recommended algorithm for sapropterin dihydrochloride response testing in non-neonatal infants, children, adolescents, and adults with phenylketonuria (PKU).

Table 2
Recommendations for parent/patient education before sapropterin dihydrochloride testing.

Establish expectations of the trial:	• Collection of a sufficient number of blood samples • Diet record • Documentation of potential adverse events
Establish requirements for an accurate trial:	• No fever or illness • Decreased stress • No travel plans • Standardization of blood sampling • Consistent dietary protein intake (intact and medical food/amino acid supplements) during the trial
Review treatment goals:	• Set expectations of the effects of sapropterin dihydrochloride o Some patients still need dietary protein restriction and medical food/amino acid supplements in combination with sapropterin dihydrochloride o A more relaxed diet can have a positive impact on the patient's life • Parents or patients may be connected with other parents/patients who have experience with sapropterin dihydrochloride treatment and/or a response trial

remains to be established what increase in Phe tolerance should be considered as a response after 4 weeks.

During a long-term sapropterin dihydrochloride response test, blood samples for determining Phe concentrations should be collected at least once weekly, and daily if possible. Although conditions may vary between centers, blood samples should always be collected under the same condition, by the same method (i.e. venous vs. finger prick) and analyzed the same way (i.e. HPLC, tandem MS) in each patient (at the same time of the day, preferably fasted), as Phe concentrations can vary considerably based on these factors [56]. Any illness during the trial should be documented as it may temporarily increase Phe concentration, and thus affect trial outcomes [8,56]. An intercurrent illness during a test can make the results uninterpretable.

Patients who have blood Phe concentrations above target range at baseline can have a short response test (Fig. 3). A short response test can also be considered if a long trial in a patient with blood Phe concentrations in the target range cannot be done due to local circumstances (e.g. limited access to sapropterin dihydrochloride, no reimbursement). Potential responsiveness in a shorter test should be evaluated by measuring the reduction in blood Phe concentration. To increase the likelihood of a positive response to sapropterin dihydrochloride in the shorter test in patients with blood Phe concentrations in the target range, blood Phe may have to be increased before the test by diet relaxation or Phe loading [26]. It should be noted that raising blood Phe concentration for a response test in children with PKU under dietary control can be problematic as parents may be reluctant to temporarily increase their child's protein intake. Moreover, blood Phe concentration does not always rise quickly in response to a higher Phe intake [57]. Together, these issues can impede stabilization of blood Phe at a sufficiently high concentration, which is necessary for correct interpretation of the response test. Adding a source of Phe (e.g. powdered milk) into the amino acid supplement/medical food to provide a hidden source of extra Phe can be considered for the trial period.

In a shorter test, blood Phe concentration should initially be monitored over a 48-h period. Samples for determining blood Phe should be collected just prior to administering sapropterin dihydrochloride, followed by 3–5 samples during the test period at the same time of the day and measured by the same method. A patient who shows a decrease in blood Phe concentration of ≥30% within 48 h can be considered a responder. Otherwise, it can be considered to prolong testing for 1 week. During this period, blood samples for determining Phe concentration should attempt to be collected daily. In potential responders (Phe reduction ≥30%), durability of response should be monitored over an extended period of time of around 4 weeks by assessing changes in overall blood Phe control (variability in blood Phe concentration, Phe:Tyr ratio, long-term blood Phe reduction), and dietary Phe tolerance. Continuing response testing for 4 weeks can also be considered if the patient shows insufficient decrease in blood Phe in the short test, but is suspected to be a responder based on genotype.

3.3.4. After testing

After the response test, and once final maximum Phe tolerance has been determined, the sapropterin dihydrochloride dose should be adjusted between 5 and 20 mg/kg/day. Ideally, sapropterin dihydrochloride should be administered with a meal to increase absorption, and to minimize possible side effects due to the acidity of the drug. It should be noted that in patients with certain mutations, higher doses might result in a lower reduction in blood Phe than lower doses (http://pah-activitylandscapes.org/) [49].

To critically evaluate clinical benefit of sapropterin dihydrochloride in the long term, it is necessary to continue measuring blood Phe control (blood Phe concentration, variability in blood Phe, Phe:Tyr ratio), and dietary Phe tolerance. In order to determine the impact of sapropterin dihydrochloride on Phe tolerance in the long term, it is necessary to continue increasing Phe intake in line with published guidelines [9]. Changes in the need for or the amount of special low protein foods, amino acid supplements/medical foods, as well as any tolerability issues should also be documented. Finally, changes in neurocognitive status, behavior, and/or quality of life might be evaluated in the long term.

3.4. Pregnant women

Both the European and American guidelines for PKU management recommend maintaining blood Phe concentration within the range of 120–360 μmol/L throughout pregnancy [2,3]. Exposure of unborn children to high Phe concentrations can lead to intrauterine growth retardation, intellectual disability, behavioral problems, microcephaly, and congenital heart disease, depending on the timing and duration of the exposure [58–60]. Given the serious risks for the offspring of PKU patients, is it essential to bring blood Phe concentration ≤ 360 μmol/L as quickly as possible. Information regarding the use of sapropterin dihydrochloride during pregnancy is limited. Available data from the Maternal Phenylketonuria Observational Program (PKU MOMS), a subregistry of the Phenylketonuria Demographics Outcomes and Safety (PKUDOS) registry, and a European cohort study showed that sapropterin dihydrochloride can reduce blood Phe concentration during pregnancy and is generally well-tolerated [61,62]. According to the experts, the considerable benefit of metabolic control for the unborn child, together with the limited risk of teratogenicity of sapropterin dihydrochloride use during pregnancy, justify response testing during pregnancy when metabolic control cannot be achieved with diet alone. However, it is strongly recommended to perform the test prior to pregnancy, if possible.

Key recommendations for pregnant women

- Sapropterin dihydrochloride treatment should be considered in pregnant women with PKU if
  - o They are unable to achieve a blood Phe concentration ≤ 360 μmol/L with dietary management *or*
  - o They are already on sapropterin dihydrochloride, which had been started prior to becoming pregnant, and request to continue taking this medication during pregnancy
- The following baseline information should be collected before sapropterin dihydrochloride testing: blood Phe concentration (≥3 measurements, if it does not delay treatment), pre-pregnancy body weight (for determining dose), and dietary Phe intake and Phe tolerance
- During the sapropterin dihydrochloride test, blood Phe concentration should (initially) be monitored over a 48-h period (see text for protocol details and caveats)
  - o A pregnant woman with PKU who achieves blood Phe ≤360 μmol/L within 48 h of beginning sapropterin dihydrochloride should maintain the same diet while continuing on sapropterin dihydrochloride
  - o If the blood Phe concentration ≤ 360 μmol/L is not achieved within 48 h, but there is a decrease towards this target, dietary protein intake should be adjusted to help lower blood Phe to target while maintaining sapropterin dihydrochloride treatment
  - o If the patient does not respond to sapropterin dihydrochloride within 48 h, pharmacologic treatment should be discontinued and focus should shift to optimizing dietary management to lowering blood Phe to the target range
- Sapropterin dihydrochloride dose should be assessed over the course of pregnancy. The dose may decrease with increasing Phe tolerance due to fetal growth during the third trimester of pregnancy; diet and medical food/amino acid supplements should likewise be adjusted
- Durability of sapropterin dihydrochloride response should be monitored over an extended period of time by monitoring blood Phe control within the target range of 120–360 μmol/L, assessing tolerability, and determining dietary intake and nutritional adequacy
- If continuation of sapropterin dihydrochloride after pregnancy is considered, clinical benefits should be reassessed in terms of blood Phe control, change in dietary Phe tolerance, neuropsychiatric benefits, and quality of life changes

If a pregnant patient is unlikely to respond to sapropterin dihydrochloride (i.e. when she has a severe form of PKU, with a high diagnostic blood Phe concentration, or a genotype suggesting a non-responsive phenotype), it is not recommended to perform a response test. In this case, dietary control should be started as soon as possible. If the

target blood Phe concentration cannot be achieved with either therapy, the patient should be hospitalized. Close monitoring of blood Phe concentrations as well as careful antenatal care throughout pregnancy, particularly ultrasound examinations to follow-up head circumference and body weight of the fetus, are crucial [8].

As time is essential during pregnancy, pregnant PKU patients should have a shorter 48-h test. This shorter test should be performed using sapropterin dihydrochloride 20 mg/kg/day, and following the same protocol as the 48-h test described for non-pregnant patients (see recommendations non-neonatal infants, children, adolescents, and adults).

Patient education recommended for pregnant women with PKU regarding the response test is similar to that for non-pregnant patients (Table 2), but should also include discussion of the importance of blood Phe control throughout the pregnancy and changing Phe tolerance during pregnancy.

As in the neonatal period, it is essential to achieve the target blood Phe concentration in pregnant women as quickly as possible. If the target concentration is achieved within 48 h, the patient can continue on sapropterin dihydrochloride with the same diet. When blood Phe decreases, but remains above 360 μmol/L, the patient should continue on sapropterin dihydrochloride, while her diet should be revised in line with published guidelines to help lower blood Phe concentration to the target range [9]. When blood Phe shows no reduction within 48 h, pharmacologic treatment should be discontinued. A longer trial may be performed after delivery if the patient is suspected to be a responder. During pregnancy, the time window to test responsiveness is limited to the first trimester, as this period is most critical for the development of the child.

Little is currently known about the risk of taking sapropterin dihydrochloride during lactation for the child. More information is to be expected from the PKU MOMS sub-registry, which collects data on lactation in sapropterin dihydrochloride-treated women who are following the standard of care for pregnant women with PKU [61]. If breastfeeding is planned, the following actions should be considered: adjustment of dietary Phe tolerance, adjustment of the sapropterin dihydrochloride dose, evaluation of tolerability, and reimbursement of the drug during breastfeeding.

4. Conclusions and future directions

Since the introduction of sapropterin dihydrochloride for the treatment of HPA in patients with BH4-responsive PKU, a wide variety of protocols have been proposed to assess responsiveness to the drug. The global recommendations for the evaluation of responsiveness to sapropterin dihydrochloride in PKU patients presented here are the result of 10 years of research and clinical experience with the drug in daily practice, and are expected to improve the validity and comparability of response testing results worldwide. It should be noted that while these recommendations represent “best practices” for the performance of a response test, the authors are aware that some centers will not be able to align with all recommendations due to cultural differences, financial hurdles, and local circumstances or legislation. However, in the experts' opinion, it is in the patients' interest that all centers endeavor to implement these best practice recommendations, although this may require considerable efforts and time from clinic staffing and patients.

Mechanisms determining response to sapropterin dihydrochloride are multifactorial and complex, and further optimization of these protocols may be required based on the outcomes of ongoing and future research. Although genotype may be predictive of response to some degree, other factors such as interallelic complementation, post-transcriptional regulation of PAH, Phe transport into hepatocytes, and absence or presence of other enzymes involved in sapropterin dihydrochloride oxidation and cellular uptake such as dihydrofolate reductase, also play an important role [63–65]. Better insight into the interactions between these factors may allow for better prediction of responsiveness. As long as these mechanisms are insufficiently understood, a response test or trial remains the best method to identify potential responders.

Continued follow-up of patients is expected to provide better insight into the efficacy and safety of sapropterin dihydrochloride across the disease spectrum, at different ages, during pregnancy, and in late-treated patients, including long-term effects on neurocognitive tests, neuropsychiatric symptoms, and growth [61,64,66]. More research is warranted to further explore the genetic, dietary, and environmental factors which may underlie the observed variation in responsiveness between populations.

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