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A biochemical, genetic, and nutritional characterization of tetrahydrobiopterin responsiveness in patients with phenylketonuria

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B.S., University of New Mexico, 2007

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Abstract

A biochemical, genetic, and nutritional characterization of tetrahydrobiopterin responsiveness in patients with phenylketonuria

By Meghan Elise Quirk

In a subset of patients with phenylketonuria (PKU), pharmacological doses of tetrahydrobiopterin (BH_4) work in conjunction with the mutated phenylalanine hydroxylase (PAH) enzyme to promote disposal of phenylalanine. Current algorithms for assessing BH_4 responsiveness rely on acute change in blood phenylalanine concentrations as the only criterion for patient classification. This approach inadequately characterizes responses seen clinically.

We explore the use of a novel set of criteria to classify BH_4 responsiveness in 58 patients with PKU. "Definitive responders" experienced ≥15% decrease in plasma phenylalanine concentrations after one month of BH_4 therapy and had substantial improvements in dietary phenylalanine tolerance (n=19). "Provisional responders" also experienced an initial ≥15% decrease in plasma phenylalanine concentrations, but had limited improvements in dietary phenylalanine tolerance (n=9). Patients with <15% decrease in plasma phenylalanine concentrations after one month of BH_4 therapy were classified as "non-responders" (n=25), and patients lost to follow-up remained unclassified (n=5).

Next, we explore the clinical utility of assessing PAH genotype severity to classify BH_4 response using a previously developed tool (assigned value sum). While the majority of definitive responders (17/19 patients) had genotypes with molecular basis for responsiveness, most of the provisional responders (7/9 patients) had severe genotypes indicative of a false-positive response. Furthermore, the heterogeneity in genotype severity within the non-responder group suggests that false-negative classification may have occurred. The simple genotype severity tool which was assessed has the potential to reveal misclassified patients and may have implication for identifying candidates for BH_4 therapy.

The potential response misclassification, however, could not be attributed to overt or divergent trends in dietary total protein, phenylalanine, and medical food intake during the first month of BH_4 therapy. Pediatric definitive responders reported consuming significantly more dietary phenylalanine and less medical food than the provisional responders, further highlighting the phenotypic differences between the two groups.

Thus, dichotomization of patients' acute plasma phenylalanine response to BH_4 therapy is clinically insufficient. As demonstrated by our provisionally responsive group, patients can experience a marked decrease in plasma phenylalanine concentrations, but not have the added benefit of diet liberalization. A comprehensive approach is necessary to sufficiently characterize BH_4 responsiveness in patients with PKU.

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CHAPTER 1

INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is the most common inherited metabolic disorder of amino acid metabolism. In an affected patient, both copies of the gene encoding phenylalanine hydroxylase (PAH; E.C. 1.14.16.1) carry a deleterious mutation, impairing the synthesis and functionality of the PAH enzyme. This derangement inhibits the primary metabolic pathway of phenylalanine, causing a state of hyperphenylalaninemia [1]. Early and prolonged exposure to supraphysiological concentrations of phenylalanine can have severe and potentially irreversible neurocognitive effects [2, 3]. It is imperative that patients with PKU are diagnosed in the newborn period and continuously maintain circulating phenylalanine concentrations in a relatively benign range over the course of their lifespan [4].

There is no cure for PKU. Until recently, the only management option available to patients was lifelong nutrition therapy, consisting of a phenylalanine-restricted diet and an amino-acid, phenylalanine-free medical food [4]. While efficacious, nutrition therapy has proven to be highly burdensome to patients [5-8]. Factors such as access, out-of-pocket expense, and social stigma can lead to non-compliance—consuming too much dietary phenylalanine and/or insufficient medical food. Consequently, circulating phenylalanine concentrations rise and can cause the emergence or reemergence of clinical manifestations. A need exists to develop a PKU management approach that addresses the limitations of conventional nutrition therapy.

Tetrahydrobiopterin (BH_4; sapropterin dihydrochloride) therapy has the potential to help a subset of patients overcome some of the known barriers to adequate management. As the first and only drug approved for the treatment of PKU [9], BH_4 therapy is believed to enhance PAH enzymatic hydroxylation of phenylalanine, leading to a dramatic decrease in circulating phenylalanine concentrations [10, 11]. With improved phenylalanine metabolism, patients have the potential to increase their dietary phenylalanine tolerance and decrease their reliance on medical food while maintaining circulating phenylalanine concentrations in the preferred treatment range [12-18].

BH_4 therapy does not work in all patients with PKU and is only indicated for a subgroup of patients deemed clinically responsive [19]. At the present, responsiveness to BH_4 cannot be predetermined from clinical characteristics, therefore it is recommended that all patients be evaluated [19]. Published protocols assessing responsiveness generally follow a paradigm in which blood phenylalanine concentrations are measured before and after the patient is administered BH_4; percent change between the two time points is used to classify a patient's response. Patients meeting a pre-determined threshold (typically $\geq$30% decrease in blood phenylalanine concentration) are classified as "responders" and continue BH_4 therapy, while those not meeting this criterion are classified as "non-responders" and discontinue BH_4 therapy.

An acute decrease in blood phenylalanine concentrations after being administered BH_4, however, does not always result in sustained clinical benefits with long-term use [20-23]. Current BH_4 response classification protocols may not adequately categorize the

responses seen clinically. By dichotomizing patients based solely on change in a biochemical measurement, current BH_4 response classification overlooks the spectrum which exists within the disorder. PKU embodies a monogenic disorder with complex molecular, biochemical, and clinical characteristics [24]. Alternative and expanded criteria encompassing the totality of the disorder need to be employed when evaluating BH_4 responsiveness.

The goal of this dissertation is to improve the clinical evaluation of BH_4 responsiveness, using a single clinic population as a model. In the first study, we offer an in-depth description of our novel BH_4 response classification algorithm which categorizes patients based on their change in plasma phenylalanine concentrations after one month of BH_4 therapy and subsequent ability to modify their nutrition therapy prescription. In our second evaluation, we use patients' PAH genotype to assess the molecular basis of responsiveness and we explore the utility of using genotype severity to predict response classification. In our third and final analysis, we evaluate trends in reported dietary protein intake, within and between groups, during the first month of BH_4 therapy to determine if protein intake affects initial change in plasma phenylalanine concentrations. This collection of work explores various clinical aspects of PKU in an effort to best characterize BH_4 responsiveness.

CHAPTER 2

THE BIOCHEMICAL, GENETIC, AND CLINICAL PRINCIPLES OF PHENYLKETONURIA

The underlying cause of phenylketonuria (PKU) is well-characterized. The primary metabolic pathway for phenylalanine is inhibited due to defects in a single gene encoding the enzyme phenylalanine hydroxylase (PAH). A firm understanding of the biochemical and genetic basis of the disorder elucidates the reasoning behind the clinical management, therapeutic goals, and emerging treatments for patients with PKU.

The Affected Biochemical Pathway

Normal Phenylalanine Metabolism

Phenylalanine is an essential aromatic amino acid. As such, it cannot be synthesized in the body and must be obtained through exogenous sources [25-27]. Approximately 25% of free phenylalanine is used for protein synthesis [28]. The remaining phenylalanine is metabolized via the pathways outlined in **Figure 2-1**.The transamination and decarboxylation pathways play minor roles under normal physiological conditions [29]. The contribution of the transamination pathway is dependent on circulating phenylalanine concentrations [29] and exhibits considerable interpersonal variation [30]. The decarboxylation pathway, in contrast, is considered a relatively inconsequential contributor to phenylalanine metabolism [31].

Free phenylalanine is primarily metabolized by being converted to the conditionally essential amino acid tyrosine [32]. The reaction is catalyzed by the enzyme phenylalanine hydroxylase (PAH), and results in a hydroxyl group being added to the *para* position of phenylalanine's benzyl group. PAH requires molecular oxygen and a cofactor called tetrahydrobiopterin (BH_4) [33]. BH_4 is oxidized during the reaction, and the resulting q-dihydrobiopterin is recycled back to BH_4 by the enzyme dihydropteridine reductase [34].

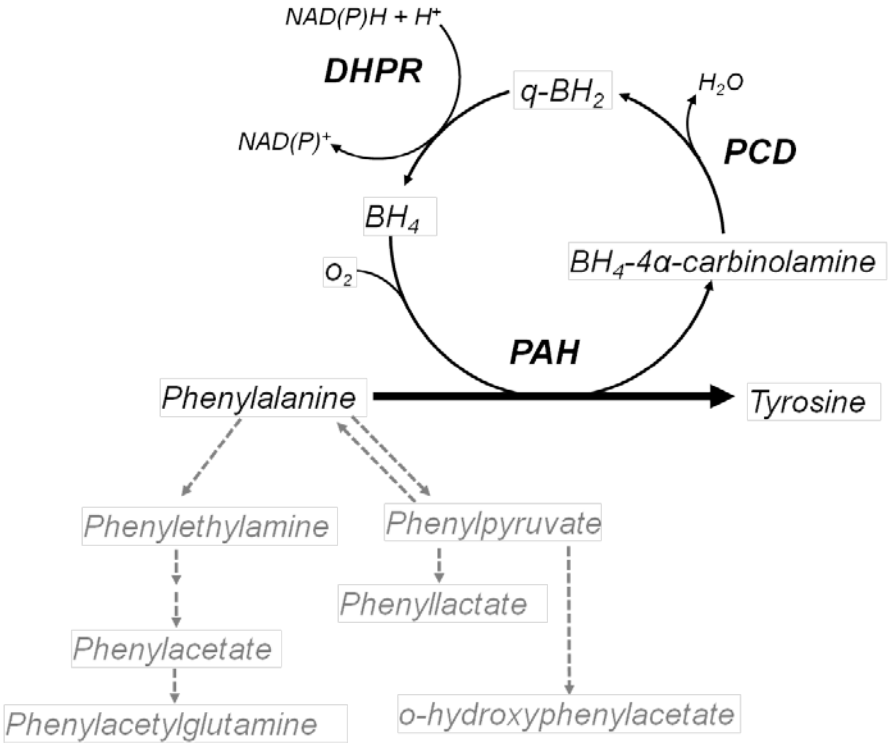

Figure 2-1: Phenylalanine metabolism
Phenylalanine is primarily metabolized by being converted to tyrosine. This process requires PAH, BH_4, and O_2. Enzymes are shown in uppercase, bold acronyms. Minor, alternative pathways (decarboxylation on left, transamination on right) are shown in grey.

Abbreviations: BH_4, tetrahydrobiopterin; DHPR, dihydropterin reductase; PAH, phenylalanine hydroxylase; PCD, pterin-4α-carbinolamine dehydratase; q-BH_2, quinonoid dihydrobiopterin

Phenylalanine Hydroxylase (PAH)

PAH is a multimeric enzyme that is expressed in the liver and in the kidneys [35-37]. It can exist as a tetramer and as a dimer [38-42], with the tetrameric form considered the most active [43]. Each PAH monomer is approximately 50 kDa in size [44, 45] and is composed of three domains: regulatory, catalytic, and tetramerization [41, 46, 47]. The PAH active site is found in a deep pocket of the catalytic domain and requires a non-heme iron for enzymatic activity [48, 49].

PAH exists in activated and in inactivated states [50], with a propensity to exists in its activated state in humans [51]. Phenylalanine is the primary activator of PAH [52-54]. Debate exists as to whether this activation occurs through an allosteric binding site in the regulatory domain [55], or if it is a function of phenylalanine binding to the active site [56, 57]. Phosphorylation has also been shown to play a modest role in PAH activation [44], potentially encouraging interactions between the regulatory and catalytic domains [58, 59]. Phosphorylation lowers the phenylalanine concentration necessary for PAH activation, and likewise elevated phenylalanine concentrations enhance PAH phosphorylation [60, 61]. In contrast, BH_4 promotes PAH inactivation [62] when it interacts with the enzyme without an excess of phenylalanine. The inactivated complex is believed to prevent BH_4 oxidation, degradation, and/or transport out of the cell and is thought to stabilize the PAH enzyme [54, 63]. Rising phenylalanine concentrations can counteract the BH_4 inactivation of PAH [54].

Phenylalanine Metabolism in Patients with PKU

In patients with PKU, there is insufficient PAH enzymatic activity for the hydroxylation reaction to proceed at a biologically relevant rate [1]. As a result, phenylalanine accumulates in the body. The rising phenylalanine concentrations will promote use of the minor phenylalanine metabolism pathways, creating metabolites which can be excreted in the urine [64-66]. The alternative pathways, however, are insufficient to lower blood phenylalanine concentrations in the normal physiological range and hyperphenylalaninemia persists. Fasting blood phenylalanine concentrations of affected patients consuming an unrestricted diet can range anywhere from two to more than fifty times normal (normal concentration: approximately 60-100 μmol/L) [31, 67]. While various factors can influence blood phenylalanine concentrations—anabolic/catabolic state, efficiency of alternate metabolic pathways, dietary intake—one important determinant is the structure and function of available PAH enzymes, which is dictated by the mutations patients harbor in the PAH gene.

Genetic Basis of PKU

The gene encoding PAH is located on the q-arm of chromosome 12 [68, 69]. It is approximately 90 kilobases in size and is composed of 13 exons [70]. PKU is inherited in an autosomal recessive manner; both the maternal and the paternal copy of the gene carry a mutation (**Figure 2-2**). Although rare, de novo mutations have been reported [71, 72]. In the United States the incidence of PKU is approximately 1:11,400 live births [73] and varies with ethnicity [74-76].

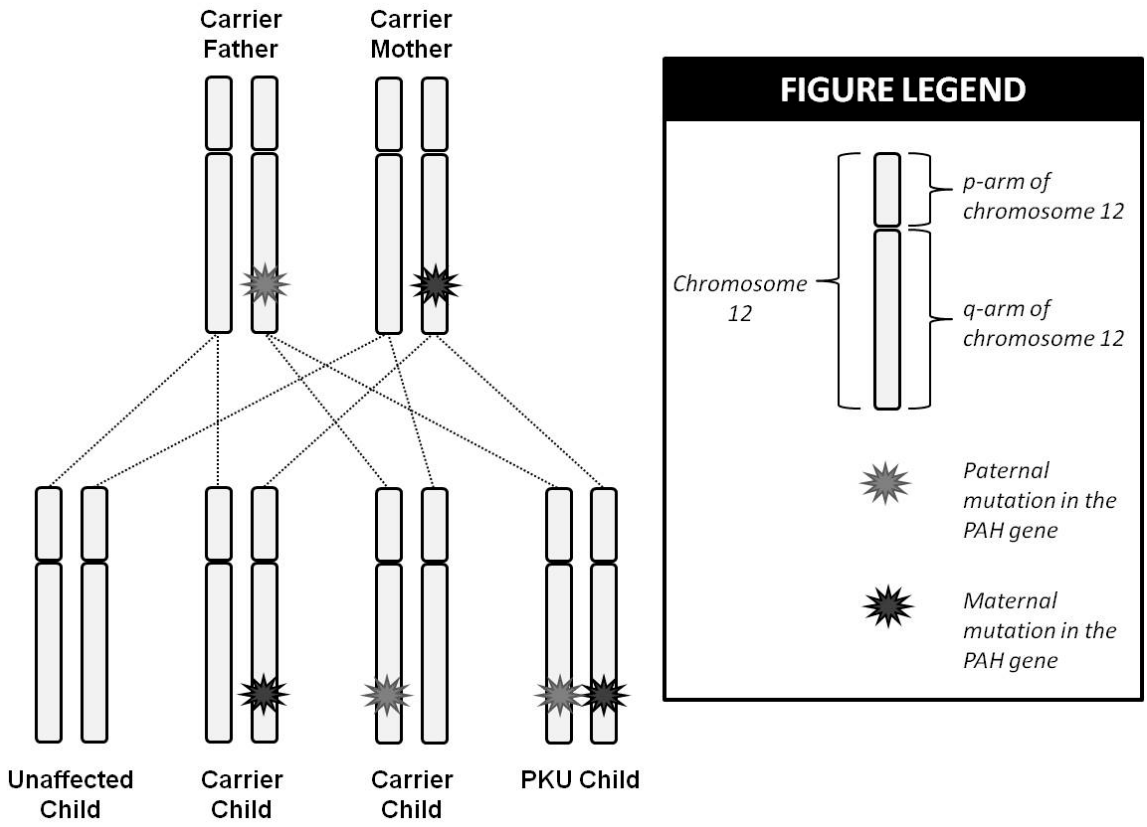

Figure 2-2: Autosomal recessive inheritance of PKU
Patients with PKU inherit two mutated copies of the same gene, one copy from their mother and one copy from their father. Carriers of the PKU gene have one mutated gene and one normal gene copy. With each pregnancy between two carriers, there is a 25% chance of having an unaffected child, a 50% chance of having a carrier child, and a 25% chance of having a child with PKU.

PKU exists as a spectrum rather than a single genotype. Over 560 different mutations have been identified in the PAH gene and cataloged in the open-access, online Phenylalanine Hydroxylase Locus Knowledgebase (www.pahdb.mcgill.ca) [77, 78]. **Table 2-1** summarizes the cataloged mutations by type [78]. It is important to note that the presented distribution does not necessarily reflect that which exists in the PKU

population as a whole, as not all mutation are private and not all mutations have been cataloged. The distribution shows the wide range of mutation types which exist in the PAH gene, with the majority being missense mutations. Missense mutations can range the gamut, from relatively benign to highly deleterious. In contrast, nonsense mutations, most mRNA processing mutations, and deletions in the PAH gene are considered null mutations, as they typically will not produce a viable enzyme.

Table 2-1: Types of cataloged mutations in the Phenylalanine Hydroxylase Locus Knowledgebase

Mutation Type	% of Cataloged Mutations (out of 564 mutations)
Missense Mutations	60.5%
Deletions	13.5%
mRNA Processing Mutations	11.0%
Silent Mutations	5.7%
Nonsense Mutations	5.0%
Insertions	1.8%
Other or Unclassified	2.7%

As a tetramer, PAH can exist as a homotetramer (all four subunits stemming from a single allele) or a heterotetramer (a combination of subunits from both alleles) [79]. Thus, the functionality of the PAH enzyme in patients with PKU will depend on the combination of mutations a patient inherits, the ability of those mutations to be translated into a stable protein, and the monomers' ability to interact with themselves, the other mutated monomers, and the substrates.

Detection and Clinical Manifestations

PKU Screening and Differential Diagnosis

In the United States, incident cases of PKU are identified through state-mandated newborn screening. A blood sample, spotted on a filter paper in the first days of life, is analyzed for abnormal concentrations of phenylalanine. Neonates who screen positive for PKU proceed to diagnostic testing [80]. Elevated blood concentrations of phenylalanine due to PKU must be differentiated from other causes of hyperphenylalaninemia such as defects in BH_4 synthesis or recycling, prematurity, low birth-weight, or false-positive readings [81-83]. If diagnosed with PKU, the newborn must have treatment initiated immediately to prevent irreversible damage.

Clinical Manifestations of PKU

PKU can lead to a wide variety of clinical manifestations depending on the timing and length of exposure to elevated circulating phenylalanine concentration. Patients with chronically elevated blood phenylalanine concentrations during infancy and early childhood generally have poorer outcomes than patients who are exposed only later in life.

Untreated patients have been noted to exude a distinct “musty” odor, have eczema, have light pigmented skin and eyes, and develop learning disabilities or mental retardation often accompanied by reduced head circumference [84-86]. While improvements in cognitive function have been noted in previously untreated patients who lower their elevated phenylalanine concentrations, irreversible damage can occur [2, 3]. In contrast,

patients who began treatment in infancy can achieved normal IQ scores [87] and can have a prognosis similar to their unaffected counterparts, although minor deficits have been noted [88-90]. To optimize the health of patients with PKU, it is advised to initiate early and continuous management over the course of the lifespan.

Nutrition Therapy

Modern PKU management is rooted in a 1954 report in which an affected toddler was successfully treated with a low-phenylalanine diet [91]. The patient had striking reversal of mental and motor aberrations while on the specialty diet and rapidly deteriorated when an excess of phenylalanine was consumed. The clinical solution which emerged was to avoid relying on the affected pathway by simply restricting the influx of phenylalanine from the diet.

While PKU nutrition therapy should ensure overall adequacy, the primary focus has remained on dietary protein intake. Patients are clinically advised to achieve protein adequacy while limiting phenylalanine intake through a specially prescribed diet. The two components of prescribed total protein intake are: intact protein from a low-phenylalanine diet and an amino acid, phenylalanine-free medical food (**Figure 2-3**). Each patient's prescription is tailored to their individual needs and can change over the lifespan.

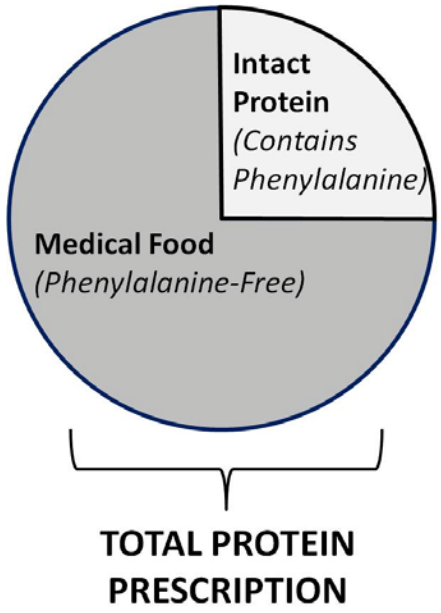

Figure 2-3: Components of total protein prescription in patients with PKU Patients are clinically advised consume a fraction of total protein from phenylalanine-containing sources (intact protein), with the remainder coming from an amino acid, phenylalanine-free medical food. The proportion of total protein coming from each component is individualized to the specific needs and tolerance of each patient.

Low-Phenylalanine Diet

A primary tenet of PKU management is to restrict what the body cannot metabolize. It is therefore recommended that patients with PKU follow a lifelong, low-phenylalanine diet [4]. Phenylalanine cannot be completely eliminated from the diet, since it is an essential amino acid. A delicate balance must be struck between adequacy and excess.

Phenylalanine is pervasive in protein sources typically consumed in the diet (intact protein). One gram of dietary protein provides approximately 50 mg of phenylalanine, although variability exists [92, 93]. High phenylalanine foods include meat, eggs, dairy products, legumes, nuts, and certain grains [94-97]. The sugar substitute aspartame is also

considered a concentrated source of dietary phenylalanine, as it is metabolized to its amino acid monomers: aspartic acid and phenylalanine [98, 99]. Patients with PKU are advised to abstain from both protein-rich foods and aspartame, and monitor intake of foods containing a moderate amount of phenylalanine. Sugar and fat are permissible within the confines of a healthy diet; foods that are encouraged include most fruits and vegetables [92].

Dietary phenylalanine tolerance—the amount that can be consumed while achieving clinical goals—is highly individualized and can fluctuate for various reasons such as growth, changes in body composition, and pregnancy [100-102]. Dietary phenylalanine tolerance is often used as a benchmark for disorder severity. In general, the less dietary phenylalanine tolerated, the more severe the PKU phenotype [103]. Patients with PKU can be prescribed as little as 200 mg phenylalanine/day or approximately 4 grams intact protein/day, in the most severe cases. As a comparison, young children in the United States report typically consuming approximately 56 grams of protein/day (an estimated 2,800 mg of phenylalanine/day), while adults report typically consuming approximately 91 grams of protein/day (an estimated 4,550 mg/day) [104]. Thus, the low-phenylalanine diet a patient with PKU is prescribed can be highly restrictive.

Medical Food

Restricting phenylalanine in the diet concomitantly restricts all other amino acids and micronutrients commonly found in protein-rich foods. It is therefore insufficient to merely limit dietary phenylalanine intake to manage patients with PKU. Adequate protein

for growth and maintenance is supplied to a patient in an amino acid, phenylalanine-free medical food, which can account for upwards of 80% total protein intake [105]. The amount of medical food prescribed to a patient depends on the patient's age, dietary phenylalanine tolerance, and the practices of the prescribing clinic [106, 107]. Medical foods are now available in a wide variety of forms including mixable powders and ready-to-drink packets [108], designed to enhance compliance.

Monitoring Nutrition Therapy – Circulating Phenylalanine Concentrations

A primary goal of nutrition therapy is to ensure patients are not exposed to harmful concentrations of circulating phenylalanine. Patients with PKU are clinically advised to maintain their blood phenylalanine concentrations in a "therapeutic range," which can be achieved if they adhere to their clinically prescribed diet. There is no consensus on what constitutes a benign range of circulating phenylalanine concentrations. Guidelines widely vary between metabolic clinics internationally. In general, it is recommended to maintain blood phenylalanine concentrations below 360 µmol/L for the first 10 years of life [109, 110]. Recommendations for adult patients are more diverse. Some centers recommend maintaining the same strict metabolic control as the patient did in childhood, while others allow relaxation of dietary restrictions, considering blood phenylalanine concentrations as high as 1200 µmol/L clinically acceptable [109].

Issues with Nutrition Therapy

Many barriers exist for patients and their families trying to comply with the PKU diet, including inability to pay for low-protein and medical foods, lack of adequate insurance

coverage, social stigma, and the burden of following a restrictive diet [5-8]. As a result, dietary compliance often wanes, especially in adolescence and adulthood [111]. With nutrition therapy non-compliance—consuming too much dietary phenylalanine and/or insufficient medical food—circulating blood phenylalanine concentrations will rise, and can contribute to the emergence or reemergence of physical, neurological, and cognitive deficits (**Figure 2-4**). These issues significantly limit the effectiveness of current PKU management and indicate nutrition therapy alone may not optimize the health of PKU patients.

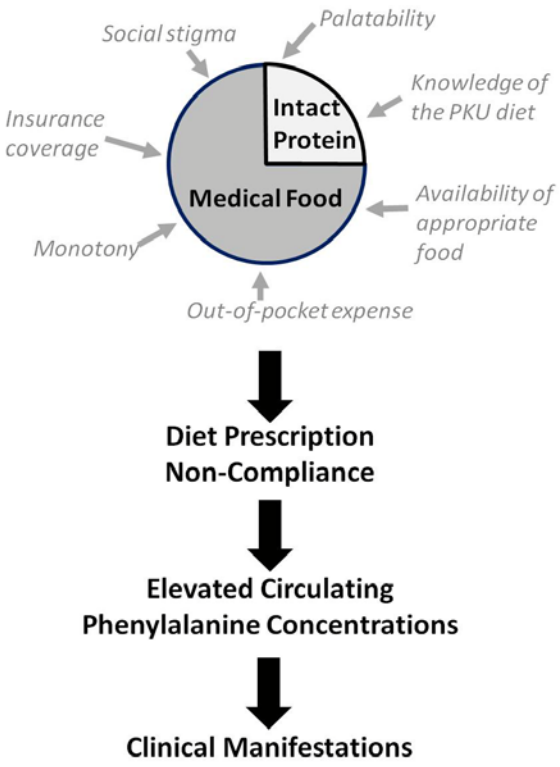

Figure 2-4: Factors affecting diet prescription compliance in patients with PKU and potential consequences
A host of factors can affect a patient's ability to adhere to their clinically advised diet prescription. Diet prescription non-compliance can potentially lead to elevated circulating phenylalanine concentrations and clinical manifestations.

CHAPTER 3

TETRAHYDROBIOPTERIN RESPONSIVENESS IN PATIENTS WITH PHENYLKETONURIA

In 1999, four patients mildly affected with hyperphenylalaninemia experienced dramatic decreases in their serum phenylalanine concentrations after being administered pharmacological doses of BH_4 [112]. It has since been established that certain patients with PKU respond to BH_4 therapy. Approved by the Food and Drug Administration in December 2007, BH_4 (sapropterin dihydrochloride; Kuvan®, BioMarin Pharmaceutical Inc, Novato, CA) is the first and currently the only drug available for the treatment of PAH-deficient hyperphenylalaninemia [9]. While substantial scrutiny led to BH_4's approval [16, 22, 113, 114], considerable clinical ambiguity still exists in this relatively nascent field.

The percent of the PKU population which responds to BH_4 therapy is unknown. Estimates range from 20-56% [16, 113], varying due to selection biases and differences in classification approaches. Patients classified as BH_4 responsive represent a range of clinical phenotypes and genotypes [12, 115, 116], although the distribution is skewed toward the milder end of the spectrum [117]. Correspondingly, patients with genotypes harboring high residual activity are often classified as BH_4 responsive and patients with mutations retaining little to no residual activity are usually classified as BH_4 unresponsive [118], although inconsistencies exist [14, 118, 119]. The reasoning for the milder patients responding is rooted in BH_4's modes of action.

Modes of Action of Pharmacological BH_4

BH_4 therapy is contingent on the mutant PAH enzyme retaining some functionality. In responsive patients, BH_4 is believed to optimize cellular BH_4 concentrations, overcome kinetic defects, and/or acts as a chaperone [10, 120, 121], thereby promoting and prolonging the functionality of the mutant PAH enzyme. These mechanisms are not mutually exclusive. However, BH_4 acting as a chaperone is believed to be the primary mode of action, since few PAH mutations map to the cofactor binding region and not all patients carrying those mutations are responsive to BH_4 [122-125]. BH_4 therapy is not believed to up-regulate PAH gene expression [63], as once hypothesized [123]. BH_4 therapy substantially increases blood biopterin concentrations (upwards of 34-39 times the basal concentration) [126], and responsiveness has not been attributed to differences in drug absorption or distribution [127].

Variability of Protocols Assessing BH_4 Responsiveness

Presently, responsiveness to BH_4 cannot be adequately predicted from clinical characteristics and must be evaluated in all patients. The general paradigm for assessing responsiveness is to calculate percent change in blood phenylalanine concentrations measured immediately prior to and after initiating BH_4, as presented in **Figure 3-1**. While seemingly straightforward, this model has led to a wide range of responsiveness testing protocols, as outlined in **Appendix A**. The most frequently manipulated variables are discussed below.

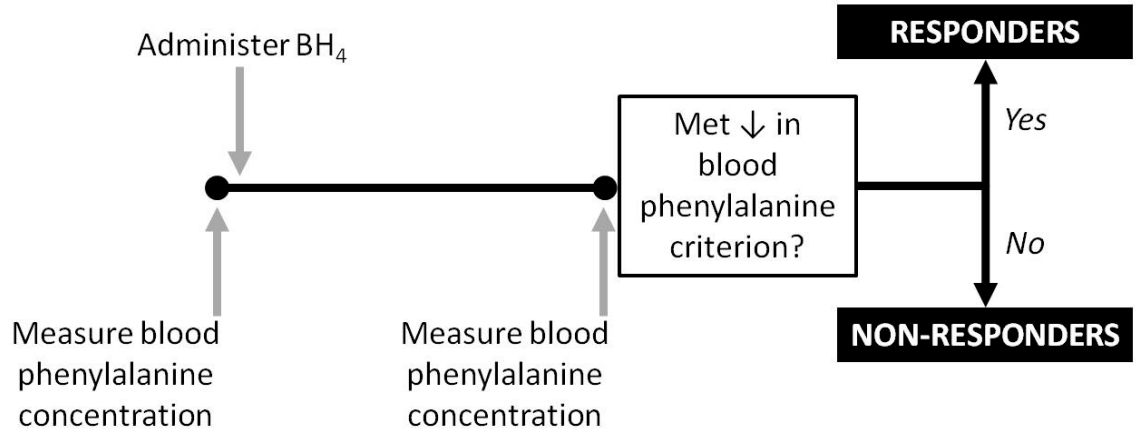

Figure 3-1: General paradigm used to classify responsiveness to BH_4 therapy

BH_4 Doses Used in Responsiveness Protocols

The dose of BH_4 used to assess responsiveness has ranged from 5-40 mg/kg/day. More patients are classified as being BH_4 responsive when given higher doses of BH_4, although the effect appears to plateau by the 40 mg/kg/day dose [128-131]. While protocols have evaluated patients only using the lower doses (5 or 10 mg/kg/day) [112, 113, 131-133], the general consensus is to initiate patients at the 20 mg/kg/day dose [134-136].

Number of BH_4 Doses Administered Prior to Classification

Patients have customarily been assessed after a single dose of BH_4. However, repeated or escalating dose protocols are not atypical and have been used since the first clinical trial [13, 16, 17, 112, 113, 115, 119, 127-131, 137-140]. A small number of protocols report distributing the BH_4 dose over the course of the day [13, 112, 132, 137], although the majority of protocols administer BH_4 once daily. There is currently no consensus as to

how many doses should be consumed when evaluating BH_4 responsiveness, although expert opinion appears to favor multiple days of BH_4 consumption over a single dose protocol [141].

Use of a Phenylalanine Challenge

The use of a phenylalanine challenge during a BH_4 response protocol is not universally accepted and its application varied. Consumption of a 100 mg/kg phenylalanine load one or three hours prior to BH_4 administration has been used in certain protocols, primarily in patients with moderately elevated phenylalanine concentrations (specified as <360 or <400 μmol/L in most protocols) [14, 122-124, 127, 142-147]. In such protocols, the phenylalanine load is used to maximize blood phenylalanine concentrations prior to BH_4 administration. In other protocols, a drug effect has been evaluated by administering a phenylalanine load to a patient on two separate occasions: once without BH_4 and once with BH_4 [140, 143]. A greater reduction in blood phenylalanine concentrations with BH_4 would indicate a drug effect. As protocols using a phenylalanine challenge may exceed the typical dietary phenylalanine intake of the patients, these trials explore the efficacy of BH_4 to reduced maximal circulating phenylalanine concentrations. This may be a slightly different endpoint than protocols not using a phenylalanine load, depending on the diet regimen of patients before and during the trial.

Dietary Regimen Prior to and During the Protocols

Modulation of dietary intake in and around the BH_4 testing protocol has the potential to affect the outcome and the interpretation of test results, since dietary intake can affect

blood phenylalanine concentrations. A description of patients' dietary practices prior to or during testing is often inadequate or overlooked [20, 115, 117, 119, 122, 127, 132, 142, 148-156], and protocols with descriptions significantly vary.

Akin to the pre-BH_4 phenylalanine challenge, some protocols encouraged patients to maximize their blood phenylalanine concentrations by consuming an unrestricted diet in the days before testing [14, 112, 130, 137, 139, 143, 145, 157-159]. Other protocols elected to assess BH_4 response while patients consume their typical diet (regardless of dietary compliance) or while patients adhere to their phenylalanine-restricted diet [15-17, 128, 129, 133, 138, 140, 144, 146, 147, 160, 161]. A distinction in response classification has not been made, despite these approaches measuring different outcomes; all patients have been simply classified as "responsive" or "non-responsive" to BH_4.

Duration of the Protocols

For protocols with a pre-BH_4 phenylalanine challenge, blood is typically drawn before the patient consumes the phenylalanine load and again before consuming BH_4. In protocols not using a phenylalanine challenge, assessment can begin several hours or even days before initiating BH_4 [116, 131, 139, 162]. The majority of protocols typically collect a single baseline phenylalanine concentration immediately prior to BH_4 administration. The length of time spent on BH_4 prior to classification can be as short as eight hours [20, 117, 122, 145, 148-150, 152, 161, 163-165] or as long as four weeks [128], with most protocols being 24 hours in length (see **Appendix A**).

Timing and Frequency of Sample Collections

Both regular and irregular sample collection intervals have been used in BH_4 responsiveness testing protocols. Samples collected at regular intervals have been spaced as close as two hours apart for a total of eight or 24 hours [153, 161]. Many protocols collected blood at four time points: baseline and hours 4, 8, and 24 [12, 14, 117, 123, 129, 133, 137, 138, 143, 166]. Still, other protocols sampled blood at just two time points, once at baseline and again after 24 hours [154, 155, 160] or after several days of BH_4 therapy [16, 113, 119, 132]. The timing and frequency are, in part, dependent on the number of doses of BH_4 administered and the overall length of the test, but considerable heterogeneity exists.

Definition of Responsiveness

Perhaps the most contentious element of the BH_4 protocols is the definition of a "responder." In the early investigations, a patient was considered BH_4 responsive if they experienced a considerable and/or sustained decrease in blood phenylalanine concentrations after BH_4 administration [112, 131, 132, 142, 148, 149, 163]. In 2002, the European Metabolic Group recommended that a threshold of at least a 30% decrease in blood phenylalanine concentrations be used to classify a patient as responsive [167]. This admittedly arbitrary cutoff [130, 168] has given rise to dichotomizing patients as "responders" or "non-responders" strictly based on percent change in blood phenylalanine concentrations over a set period of time.

A subset of protocols has attempted to further categorize responsiveness based on the rate at which patients respond. The concept of a “slow responder” was introduced when it was found that certain patients can achieve the ≥30% decrease in blood phenylalanine concentrations if the test is extended beyond eight hours [13-15, 143, 147]. It has been suggested to further differentiate patients as “rapid responders,” “moderate responders,” or “slow responders” based on percent decline in blood phenylalanine concentrations at hours 8, 24, and 48 of the BH_4 testing protocol [115, 127]. The term “fast responder” has also been described in the literature, defined as a patient who experiences ≥30% decrease in blood phenylalanine concentrations within the first two hours of testing [161]. Additionally, protocols have attempted to classify patients with a lesser response as “adequate responders” (with a 17-30% response) [129, 138] or “partial responders” (with a 10-29% response) [119]. While alternative definitions have been explored [116, 123, 124, 139, 146, 150, 169], dichotomizing patients using the 30% decrease in blood phenylalanine concentration threshold is considered the standard approach to response classification.

Clinical Outcomes

Of utmost importance is the long-term clinical outcome of patients classified as BH_4 responsive. Two outcomes routinely assessed in BH_4 responsive patients are blood phenylalanine concentrations and change in dietary phenylalanine tolerance.

Blood Phenylalanine Concentrations

The hallmark of BH_4 responsiveness is decreased blood phenylalanine concentrations in the hours or days after the administration of the drug. Long-term BH_4 therapy has been shown to help responsive patients maintain blood phenylalanine concentrations in the therapeutic range [152]. Data suggest BH_4 therapy may decrease diurnal and long-term fluctuations in blood phenylalanine concentrations in responders, although the results are preliminary [170-172].

Despite the potential benefits of BH_4 therapy, an initial decrease demonstrated in the short-term does not always lead to improved metabolic control. BH_4 therapy may not compensate for acute peaks in blood phenylalanine concentrations attributed to a catabolic state (e.g. fever or illness) [12, 153]. Furthermore, in the Phase III evaluation, less than half of previously classified "responders" had a sustained ≥30% decrease in blood phenylalanine concentrations after 6 weeks of BH_4 therapy [22]; this trend was also apparent in the 22-week evaluation of patients [172] and has been reported in other protocols [20, 21]. Thus BH_4 therapy, even in patients classified as responsive, does not always result in improved metabolic control.

Dietary Phenylalanine Tolerance and Medical Food Needs

Where diet therapy affects blood phenylalanine concentrations by restricting exogenous intake of the offending amino acid, BH_4 therapy works in conjunction with the mutant enzyme and affects the hydroxylation reaction itself, improving the disposal of phenylalanine [11]. In theory, greater phenylalanine disposal will increase dietary

phenylalanine tolerance, which would, in turn, decrease reliance on medical food. This may help certain patients overcome barriers traditionally experienced by patients with PKU. Thus, BH_4 therapy potentially has added benefits compared to nutrition therapy alone.

Certain responsive patients have been reported to be able to increase their dietary phenylalanine tolerance two to six times their pre-BH_4 tolerance [12-18]. In an evaluation of patients with >45% decrease in blood phenylalanine concentrations during the initial testing period, 11/14 patients were able to subsequently increase their dietary phenylalanine tolerance from 356 ± 172 mg/day to 1546 ± 192 mg/day and discontinue their medical food [23]. This can translate into meeting protein needs through intact protein while maintain blood phenylalanine concentrations in the recommended therapeutic range [146].

BH_4 responsiveness does not always lead to dramatic changes in a patient's diet prescription. Some responsive patients are reported using BH_4 in addition to a moderately restricted diet [152], while other patients need to continue medical food in addition to BH_4 therapy [14, 169]. Still other patients classified as responsive cannot significantly change their dietary tolerance [16-18]. As is the case with metabolic control, liberalization of dietary restrictions does not always results from being classified as a BH_4 responder.

Limitations of the Current BH_4 Response Classification Approaches

The wealth of information available on BH_4 responsiveness in patients with PKU has emerged from the diverse approaches investigators have implemented internationally. The totality of variety which exists in protocols is considerable. This diversity, however, has limited the ability to consolidate data and identify associations across studies, since distinct endpoints are often measured.

The core issue with the current state of BH_4 response classification is inadequate characterization. PKU is a disorder which embodies a complex interplay between exogenous and endogenous factors, and yet BH_4 classification has reduced it to two categories: responsive and non-responsive. The heterogeneity which exists in the disorder and the apparent heterogeneity in BH_4 response have been overlooked in favor of simplicity.

The emergence of a subgroup of patients who do not have sustained clinical benefits from BH_4 therapy suggests that dichotomizing patients may insufficiently capture the outcomes of patients. At the present these patients are only identified as “responders,” but they may in fact represent a false-positive response or be experiencing issues with treatment compliance. The root cause of this subgroup is unknown, as these patients are not systematically identified or thoroughly investigated. Segregating patients who have an initial response but do not experience sustained benefits from other patients in the responder group may illuminate shortcomings of the current BH_4 response protocols or long-term BH_4 therapy.

Furthermore, the basis for dichotomizing patients—a change in blood phenylalanine concentrations—may inadequately characterize the effect of BH_4. Blood phenylalanine concentrations can be affected by the metabolic state (i.e. anabolic or catabolic) of the patient or normal diurnal variation [173-175]. These factors can potentially affect the interpretability of percent change in plasma phenylalanine observed. But beyond the possibility of patient misclassification due to normal fluctuations in blood phenylalanine concentrations, the definition of responsiveness is limited in its scope. An acute change in blood phenylalanine is clinically relevant, but other aspects comprise PKU management. Response classification needs to acknowledge the gradation in effects BH_4 therapy has not only on blood phenylalanine concentrations, but also nutrition therapy.

CHAPTER 4

USING CHANGE IN PLASMA PHENYLALANINE CONCENTRATIONS AND ABILITY TO LIBERALIZE DIET TO CLASSIFY RESPONSIVENESS TO TETRAHYDROBIOPTERIN THERAPY IN PATIENTS WITH PHENYLKETONURIA

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Abstract

Tetrahydrobiopterin (BH_4) responsiveness is currently defined as a decrease in plasma phenylalanine concentrations in patients with phenylketonuria (PKU). This definition does not offer insight beyond the initial assessment of patients, which may lead to treatment ambiguity in patients who only experience an initial decrease in plasma phenylalanine concentrations. We present our experience with a novel classification approach using sequentially-applied criteria. Plasma phenylalanine concentrations were measured at baseline and after one month of BH_4 therapy (20 mg/kg/day) in 58 PKU patients (34M, 24F; age 17.3 ± 11.0 years). Thirty-two patients (55.2%) were classified as "preliminary responders" at one month, experiencing at least a 15% decrease in plasma phenylalanine concentrations. Preliminary responders' ability to liberalize their dietary restrictions was then systematically assessed. "Definitive responders" were defined as preliminary responders who could increase their dietary phenylalanine tolerance by at least 300 mg/day and lower prescribed medical food needs by at least 25% while maintaining metabolic control (plasma phenylalanine $\leq$360 μmol/L) and consuming adequate dietary protein. Preliminary responders who could not liberalize their diets according to these criteria were classified as "provisional responders." Nineteen patients (32.8% of patients initiating BH_4 therapy) met the definitive responder criteria, increasing dietary phenylalanine tolerance from 704 ± 518 mg/day to 1922 ± 612 mg/day and reducing medical food to 16.7 ±19.5% of their baseline prescription. Nine patients (15.5% of patients initiating BH_4 therapy) were classified as provisional responders, all remaining on 100% of their baseline medical food prescription. From this classification approach, a subgroup of provisionally responsive patients emerged who experienced an

initial decrease in plasma phenylalanine concentrations but who could not substantially increase their dietary phenylalanine tolerance or decrease medical food needs. Diet liberalization is an essential component of BH_4-responsiveness classification.

Keywords: phenylketonuria; tetrahydrobiopterin; sapropterin dihydrochloride; Kuvan®

Abbreviations: PKU – phenylketonuria; BH_4 – tetrahydrobiopterin; RDA – Recommended Dietary Allowance

4.1. Introduction

Patients with phenylketonuria (PKU; OMIM 261600) are instructed to follow a lifelong diet restricted in phenylalanine [4]. Dietary protein adequacy is achieved by adding a phenylalanine-free amino acid medical food, which supplies the majority of protein in the diets of treated patients [176, 177]. The burden of such a limited and often unpalatable diet can lead to treatment non-compliance and prolonged periods of elevated blood phenylalanine concentrations, which can negatively impact a patient's development and health [84, 85, 178].

Effective PKU management must strike a balance between diet liberalization and maintenance of blood phenylalanine concentrations in the therapeutic range (preferably 120-360 μmol/L). Tetrahydrobiopterin (BH_4) is the first drug therapy that may help certain PKU patients strike such a balance. With its potential first clinically identified in four mild hyperphenylalaninemic patients in 1999 [112], BH_4 has since been the subject of numerous investigations and clinical protocols internationally [13, 14, 17, 113, 137, 179]. It is believed that pharmacological doses of BH_4 can correct kinetic defects and/or can act as a chemical chaperone [10, 120], thereby increasing and/or prolonging the functionality of mutant phenylalanine hydroxylases harboring some residual activity.

Where conventional diet therapy maintains blood phenylalanine concentrations in the therapeutic range by simply limiting the amount of the offending amino acid ingested, BH_4 therapy enhances the catabolism of phenylalanine and therefore has the potential to improve responsive patients' dietary phenylalanine tolerance [15, 16, 23]. The current

definition of BH_4-responsiveness typically found in the literature – a clinically significant decrease in blood phenylalanine concentrations, with a threshold usually set at $\geq$30% decrease [167] – fails to capture the added benefit of improved intact protein tolerance. Clinical ambiguity can arise when patients experience an initial marked decrease in blood phenylalanine concentrations, but cannot subsequently increase their dietary phenylalanine tolerance.

Assessing dietary phenylalanine tolerance to classify BH_4-responsiveness has been previously suggested [136, 180], but in-depth descriptions of the implementation of such protocols in the clinical setting are still lacking. We present our clinic's approach to and experience with a novel and expanded BH_4-responsiveness classification protocol which uses both change in plasma phenylalanine concentrations and ability to liberalize diet restrictions in patients prescribed the BH_4 analog sapropterin dihydrochloride (Kuvan®; BioMarin Pharmaceutical Inc., Novato, California, USA).

4.2. Material and Methods

4.2.1. Patient Eligibility

Patients seen at the Emory University Genetics Clinic were recruited from October 2008 through October 2009 to participate in a yearlong clinical trial evaluating BH_4-responsiveness. Inclusion criteria were: being diagnosed with hyperphenylalaninemia or PKU and being at least 4 years of age. Patients were excluded if they were pregnant or breastfeeding, were previously determined to be BH_4-responsive, or had taken biopterin in the previous 8 weeks. Informed consent, and when necessary assent, was obtained

from all study participants and from pediatric patients' legal guardians. This study was approved by the Emory University Institutional Review Board.

4.2.2. Responsiveness Classification Algorithm

Patients were classified using the algorithm presented in **Figure 4-1**. The approaches are detailed as follows:

4.2.2.1. Preliminary Responsiveness Criterion: Change in Plasma Phenylalanine Concentrations

Patients' plasma amino acid concentrations were assessed immediately prior to and after one month of BH_4 therapy (20 mg/kg/day). A patient was considered a "*preliminarily responder*" if their month one plasma phenylalanine concentration was at least 15% lower than their baseline plasma phenylalanine concentration. Patients meeting this threshold continued using BH_4 and proceeded to diet liberalization. Patients not meeting the 15% threshold were classified as "*non-responders*" and discontinued BH_4 therapy.

4.2.2.2. Definitive Responsiveness Criteria: Ability to Liberalize Diet

Preliminary responders' diets were liberalized using an adapted version of a previously published protocol [136]. The approach taken was dependent on the patient's reported dietary prescription compliance and plasma phenylalanine concentrations at the month one assessment (detailed in Sections 4.2.2.2.1 and 4.2.2.2.2).

Regardless of diet liberalization approach, two criteria were ultimately used to classify patients. A "*definitive responder*" was defined as a preliminary responder who could increase dietary phenylalanine tolerance by at least 300 mg/day (approximately 6 grams of intact protein) and decrease medical food need by at least 25% while maintaining their blood phenylalanine concentrations in the therapeutic range ($\leq$360 μmol/L) and meeting their age- and sex-specific Recommended Dietary Allowance (RDA) for protein. Preliminary responders who could not increase their dietary phenylalanine tolerance and decrease their medical food needs while maintaining metabolic control were classified as "*provisional responders*."

Patients who electively ate diets rich in phenylalanine (meeting RDA protein needs through intact protein) and had plasma phenylalanine concentrations $\leq$360 μmol/L after one month of BH_4 therapy had no need to have their diets liberalized and were considered definitive responders. If medical food was being consumed, its necessity was evaluated.

4.2.2.2.1. Diet Liberalization of Patients with Plasma Phenylalanine Concentrations in the Therapeutic Range: Milk Powder Challenge

Patients who reported restricting intact protein and had plasma phenylalanine concentrations $\leq$360 μmol/L after one month of BH_4 therapy were instructed to add 20 grams of non-fat dry milk powder (approximately 350 mg phenylalanine or 6.8 grams protein) to their diet each week. A patient's new dietary tolerance was established as the quantity of dietary phenylalanine consumed prior to blood phenylalanine concentration

exceeding 360 μmol/L. After the new dietary phenylalanine tolerance was established, medical food intake was progressively decreased by 25% of baseline prescription each week. The patient's new medical food prescription was established as the intake associated with the last blood filter paper phenylalanine concentration in the therapeutic range, ensuring dietary protein adequacy. Once dietary phenylalanine and medical food tolerance were established, intact protein sources displaced milk powder in the diet. Female definitive responders of childbearing potential were encouraged to maintain the taste for medical food by consuming a fraction of their baseline prescription al food, typically 25%, even if intact protein tolerance could meet the patient's RDA. This routine is intended to ease the transition back to diet therapy alone if a woman chooses to discontinue BH_4 therapy during pregnancy.

4.2.2.2.2. Diet Liberalization of Patients with Plasma Phenylalanine Concentrations Exceeding the Therapeutic Range

Patients who reported consuming medical food and whose plasma phenylalanine concentration exceeded the therapeutic range after one month of BH_4 therapy were instructed to decrease dietary phenylalanine intake by approximately 350 mg (6.5-7 grams of intact protein) per week. A patient's dietary phenylalanine intake was decreased until blood phenylalanine concentration was in the therapeutic range. Medical food intake was then progressively decreased using the method described in Section 2.2.2.1.

Patients consuming completely liberalized diets without medical food and whose blood phenylalanine concentration exceeded 360 μmol/L were instructed to decrease dietary

phenylalanine intake until metabolic control was achieved, with medical food progressively added back into the diet 25% at a time as needed to ensure dietary protein adequacy.

4.2.3. Plasma and Blood Amino Acid Analysis

Plasma amino acids were measured at baseline and after one month of BH_4 therapy. A fasting blood sample was drawn from each patient in a heparinized tube and assessed using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd, Cambridge, UK). During diet liberalization, patients were instructed to spot a filter paper weekly with finger-stick blood drops after an overnight fast. Filter paper amino acid concentrations were analyzed using liquid chromatography/tandem mass spectrometry (Waters 2795 HPLC system/Micromass Quattro micro; Waters Corporation, Milford, Massachusetts, USA), as previously described [181].

4.2.4. Dietary Intake and Diet Prescription

Patients were instructed to record dietary intake for the three days prior to both baseline and month one assessments and before each filter paper submission. If no 3-day diet record was received, a metabolic dietitian conducted a 24-hour recall to approximate energy, macronutrient, and medical food intake. Diets were analyzed using the Nutrition Data System for Research (University of Minnesota, Minneapolis, MN, USA) diet analysis program. Baseline diet prescriptions were the last prescription recorded prior to the initiation of the protocol. Subsequent changes to the diet prescriptions were established and adjusted by the research metabolic dietitian.

4.2.5. Statistical Analysis

Data were analyzed using SAS 9.2 (SAS Institute Inc, Cary, NC, USA). Descriptive statistics are presented as count (%) and mean ± standard deviation. Differences between the classification groups were assessed using Student's *t*-tests for continuous variables and χ^2-test for categorical variables. A p-value<0.05 was considered statistically significant.

To determine if changes in dietary intake during the first month of BH_4 therapy were associated with preliminary classification, percent change in plasma phenylalanine concentrations after one month of BH_4 therapy was modeled against percent of change in reported dietary intake (energy, protein, phenylalanine, and medical food). Exclusion of diet records containing less than three days did not affect the associations, so all diet records and recalls have been included in the analysis. Due to the diversity of the patient population, the effects of age, sex, baseline diet phenylalanine prescription (a proxy for disorder severity), and bodyweight were evaluated in the models.

4.3. Results

4.3.1. Baseline Characteristics and Preliminary Responsiveness Classification

Of the 83 patients approached for study enrollment, 58 participated at baseline, 57 of which returned at month one for preliminary responsiveness classification. Thirty-two patients (55.2% of patients evaluated at baseline) were classified as preliminary responders. The remaining 25 patients (43.1% of patients evaluated at baseline) were

classified as BH_4 non-responders. Characteristics of patients at baseline and after one month BH_4 therapy are displayed in **Table 4-1**.

Mean plasma phenylalanine to tyrosine ratios were significantly different between the two groups at baseline (preliminary responders: 12.9 ± 9.8, non-responders: 20.4 ± 14.7; p=0.033) and after one month of BH_4 therapy (preliminary responders: 6.4 ± 6.7, non-responders: 24.1 ± 15.9; p<0.0001). These differences were driven by plasma phenylalanine concentrations since plasma tyrosine concentrations did not differ between the groups at baseline (preliminary responders: 50.5 ± 15.5 μmol/L, non-responders: 52.1 ± 22.4; p=0.755) or month one (preliminary responders: 46.1 ± 21.4, non-responders: 48.4 ± 26.3; p=0.720).

4.3.1.1. The Effect of Dietary Intake during Preliminary Responsiveness Assessment

Table 4-2 displays a summary of the reported intake of 53 patients with complete diet records at both baseline and the month one evaluations. Percent change in reported baseline intake of energy, total protein, phenylalanine, and medical food protein equivalents did not have a relationship with percent change in plasma phenylalanine concentrations between baseline and month one. Models were not improved with the addition of clinical characteristics (all predictor *p-values* >0.05).

4.3.2. BH_4 Responsiveness: Diet Liberalization

Thirty-two preliminary responders were eligible for diet liberalization assessment. Prior to diet adjustments, one preliminary responder was electively removed from BH_4

treatment while another preliminary responder discontinued BH_4 therapy due to protocol non-compliance. A third preliminary responder was lost to follow-up prior to the establishment of a new diet prescription. One additional patient, who only experienced a 10.8% decrease in plasma phenylalanine concentration after one month of BH_4 therapy, was further evaluated through the diet liberalization process because of a reported 12.9 gram increase in intact protein intake over the first month of BH_4 therapy. A total of 30 patients were assessed using diet liberalization approach. A flow diagram of patient classification is displayed in **Figure 4-2**.

4.3.2.1. Diet Liberalization of Patients with Plasma Phenylalanine Concentrations in the Therapeutic Range

After one month of BH_4 therapy, three patients eating completely liberalized diets had plasma phenylalanine concentrations below 360 μmol/L and were classified as definitive responders. All three patients no longer needed medical food to meet their age- and sex-specific RDA for protein or to maintain plasma phenylalanine concentrations in the therapeutic range.

Twenty-three patients initiated the milk powder challenge. Of note are two patients who were assessed despite having month one plasma phenylalanine concentrations exceeding 360 μmol/L (439 and 441 μmol/L). Both patients were eating diets high in phenylalanine. To establish their new dietary tolerance they displaced a portion of their intact protein intake with milk powder and proceeded through the challenge.

Of the 23 patients who initiated the milk powder challenge, 15 were classified as definitive BH_4 responders while the other 8 were classified as provisional responders.

4.3.2.2. Diet Liberalization of Patients with Plasma Phenylalanine Concentrations Beyond the Therapeutic Range

Four patients following liberalized diets had plasma phenylalanine concentrations exceeding 360 μmol/L after one month of BH_4-therapy (range: 496-1504 μmol/L). Patient 1, who reported consuming his full medical food prescription while eating approximately 500 mg phenylalanine/day, failed to reduce his dietary phenylalanine intake enough to lower his blood phenylalanine concentrations into the therapeutic range (as per the protocol outlined in section 4.2.2.2.2*).* Since his reported phenylalanine intake was less than 300 mg above his prescription, this patient did not meet the responsiveness criteria and was classified as a provisional responder.

Patient 2 was not consuming medical food at baseline but progressively decreased his phenylalanine intake in his second month of BH_4 therapy. After adding 25% of his original medical food prescription to his diet, Patient 2's blood phenylalanine concentrations fell within the therapeutic range. His dietary tolerance was increased by 1,100 mg phenylalanine/day as compared to his baseline prescription and he was classified as a definitive BH_4-responder.

Patients 3 and 4 electively followed completely liberalized diets prior to baseline and neither consumed medical food. Both patients attempted to decrease their dietary

phenylalanine intake and incorporate 25% of pre-BH_4 medical food prescription into their diet. Due to noncompliance issues, these patients' blood phenylalanine concentrations never fell in the therapeutic range. Since patient 3 was the participant with a 10.8% decrease in plasma phenylalanine concentration after one month of BH_4 therapy, he was ultimately classified as a non-responder. Patient 4 could not be accurately classified.

4.3.2.3. Differentiating Provisional Responders from Definitive Responders

The changes in dietary phenylalanine tolerances and medical food prescriptions after diet liberalization are detailed in **Table 4-3**. All definitive responders who underwent diet liberalization could tolerate at least twice the dietary phenylalanine they could at baseline. In comparison, none of the provisional responders could double their prescription or meet the 300 mg phenylalanine/day criterion. Medical food was discontinued in 10 of the 19 definitive responders. An additional four female definitive responders could meet their dietary protein needs through their phenylalanine tolerance, but remained on a reduced medical food prescription. Thus, only five of the 19 definitive responders had nutritional needs for their medical food prescription. In comparison, all nine provisional responders continued on 100% of their medical food prescription, with one participant needing a slight increase due to growth.

Provisional responders were similar to definitive responders in terms of baseline plasma phenylalanine concentrations, month one plasma phenylalanine concentrations, and reported change in dietary intake between baseline and month one assessment.

Provisional responders were comprised entirely of children and adolescents (range of baseline age: 4.6-17.8 years) while definitive responders encompassed a wider age range (6.1-36.8 years), leading to a significant difference in age between the groups (p=0.038). All other demographic characteristics were similar between the groups.

4.3.3. Summary of Responsiveness Classification

Preliminary responders comprised 55.2% (32/58) of participants who were evaluated at baseline. This group was further differentiated into definitive BH_4-responders and provisional responders, 32.8% (19/58) and 15.5% (9/58) of patients who initiated the protocol, respectively. This protocol resulted in 8.6% (5/58) of patients being unclassified due to protocol non-compliance and loss to follow-up.

4.4. Discussion

While the current definition of BH_4-responsiveness in the literature appears simple, in clinical practice responsiveness determination is less straightforward and many factors must be considered. Our approach to BH_4-responsiveness classification differs from other previously reported protocols. First, the minimum change in plasma phenylalanine concentrations during the responsiveness testing period was lowered to 15% from the typical 30% cutoff. This criterion allowed us to identify one additional definitive responder who only experienced a 25.4% decrease in plasma phenylalanine concentrations, but could subsequently increase his dietary phenylalanine intake by 1,100 mg/day. This lower cutoff appears appropriate for protocols of longer duration. The length of time used to assess preliminary responsiveness in our approach was longer than

in other protocols, which generally span from 8 to 48 hours [182]. The extension of the testing period beyond a single BH_4 dose has been suggested to identify "slow responders" [115]. One month of therapy was selected for the current protocol to maximize the number of potential responders identified and to minimize patient burden of repeated clinic visits. It should be noted that the longer the testing period, the more likely changes in blood phenylalanine concentrations are to be affected other factors such as dietary intake or illness. While our data suggest that percent change in baseline dietary intake of energy, protein, phenylalanine, and medical food did not have an association with percent change in plasma phenylalanine concentrations, these results are preliminary and subject to diet record reporting biases by the study participants. Additionally, medical food consumption was fairly consistent in the majority of patients between baseline and month one in our clinic population, which may lead to the false assumption that changes in medical food consumption have no effect on plasma phenylalanine concentrations. To prevent the effects of potential confounders, it would be of value to determine how our responsiveness classification varies between the shorter and longer protocols to expedite the determination process while maximizing accuracy.

The diet liberalization phase of BH_4-responsiveness was a critical element of our protocol. While it has been reported that BH_4-responsive patients can have an increase in dietary phenylalanine tolerance and a decreased need for medical food [12, 14-16, 179, 183], it is known that this is not the case for all patients who experience the threshold change in blood phenylalanine concentrations. A substantial subset of patients that we describe as "provisional responders" was identified by the diet liberalization criteria.

The provisional responder group highlights critical aspects of BH_4 responsiveness determination that must be considered when implementing clinical protocols. Two unrelated provisional responders each had a biological sibling who did not experience a decrease in plasma phenylalanine concentrations during the month-long trial of BH_4. While it has been documented that responsiveness cannot necessarily be predicted from a patient's genotype [119, 184], discordant responsiveness classification between biological siblings begs for further evaluation. Additionally, two of the nine provisional responders had acute illness at baseline, believed to inflate their blood phenylalanine concentrations and cause misclassification. Thus, illness or other catabolic states at baseline and/or follow-up must be taken into consideration, as they can lead to false-positive or false-negative classification. The remaining provisional responders, however, had no remarkable changes in reported health status or dietary intake over the course of the first month of BH_4 therapy.

In the end, all nine provisionally responsive patients were prescribed duplicative PKU treatments: a maximum BH_4 prescription along with their entire pre-BH_4 medical food prescription. These patients emphasize the need for establishing guidelines for what constitutes BH_4-responsiveness. It is interesting to note that the provisionally responsive patients were comprised entirely of pediatric patients. While there is a possibility that provisional responsiveness is a function of age, it should be noted that we had pediatric patients as young as 6 years of age who met this dietary tolerance threshold. Significant increases of dietary phenylalanine tolerance—far beyond 300 mg phenylalanine per

day—have also been previously observed in pediatric patients [15]. While we used absolute cutoffs for diet liberalization in this protocol, alternative dietary criteria could be considered (such as a doubling of dietary phenylalanine prescription, creating age- or weight-adjusted dietary criteria, etc). The purpose of the diet tolerance criteria is to prevent the over-management of patients, considering the expense of either treatment approach. Continued BH_4 therapy in a patient who cannot substantially increase their dietary tolerance can only be justified if it improves long-term metabolic control or improves a clinically significant secondary outcome (such as quality of life, ADHD symptomaology, etc) as compared to diet therapy alone. These benefits have yet to be demonstrated specifically in the provisionally responsive patients. Until they are, the added benefit of BH_4 therapy as opposed to diet therapy alone must be evaluated on a case-by-case basis.

We are not the first clinic to identify a group of patients who cannot increase their dietary tolerance despite an initial marked decrease in plasma phenylalanine concentrations. In 2005, Lambruschini et al [23] reported three patients who experienced 45.7-74.5% decrease in blood phenylalanine concentrations 21 hours after BH_4 loading, but could not improve their diet prescription and subsequently stopped BH_4 therapy. Additionally, Trefz et al report two “pseudo-responders” with initial responses of 60.8% and 33.7% decreased, respectively, who could not increase their dietary phenylalanine tolerance [21]. While it is possible that our provisionally responsive group is an artifact of our month-long protocol – that is all nine patients are false-positive responders – the emergence of a similar subgroup of patients in alternative and shorter protocols [21, 23]

suggests that they represent a legitimate subgroup of patients. A need exists to systematically identify and closely follow these patients to form a uniform guideline for proper management.

Two caveats to our proposed diet titration guidelines are exemplified by Patient 4, who remains unclassified due to protocol noncompliance. First, the patient was not actively managed prior to the initiation of BH_4 therapy. The lack of an established diet prescription hampered our ability to definitively classify him. Secondly, after consuming an unrestricted diet for the majority of his adult life, this patient would not reduce his intake to sufficiently lower his blood phenylalanine concentrations into the therapeutic range even with BH_4 therapy. For diet liberalization criteria to be successfully applied, patients initiating BH_4 must be closely monitored by their metabolic clinic, must have a current diet prescription, and must be willing to comply with the diet liberalization process.

In conclusion, using both changes in plasma phenylalanine concentrations and ability to liberalize dietary restrictions as criteria to determine BH_4-responsiveness in patient with PKU led to the identification of a sub-group of provisional responders. This classification approach aids in the identification of patients who can use BH_4 to both liberalize dietary restrictions while achieving blood phenylalanine concentrations in the therapeutic range.

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Table 4-1: Baseline and month one characteristics of 58 PKU patients who initiated BH_4 therapy
Data are presented collectively and separated into preliminary responsiveness groups.

Characteristic	All Patients (N=58)	Preliminary BH_4 Responders[a] (n=32)	BH_4 Non-Responders[b] (n=25)	Difference Between Groups *(p-value)*
Age (years)	17.3 ± 11.0	15.2 ± 10.3	19.7 ± 11.7	0.128
Gender (male)	34 (58.6%)	21 (65.6%)	13 (52.0%)	0.298
Height (cm)	149.0 ± 23.0	145.0 ± 24.8	153.4 ± 20.1	0.175
Weight (kg)	56.3 ± 30.7	48.4 ± 27.2	63.3 ± 29.7	0.053
Baseline plasma phenylalanine concentration (μmol/L)	693 ± 412	564 ± 307	843 ± 479	0.016
Month 1 plasma phenylalanine concentration (μmol/L)	555 ± 478[c]	250 ± 213	947 ± 437	<0.0001
Change in plasma phenylalanine concentrations (% change from baseline)	-17.4 ± 58.0[c]	-55.3 ± 19.8	+31.2 ± 54.6	<0.0001

Values are presented as mean ± standard deviation or n (%); p-values are calculated using Student's t-test or χ^2 test, as appropriate

[a] Preliminary BH_4-responder experience $\geq$15% decrease in plasma phenylalanine concentrations after one month of BH_4 therapy

[b] BH_4 non-responder is a patient experiencing <15% decrease in plasma phenylalanine concentrations after one month of BH_4 therapy

[c] n=57; one patient did not return for month one assessment

Table 4-2: Reported dietary intake of 53 patients with diet records at baseline and after one month of BH_4 therapy

	Preliminary BH_4 Responders (n=31)[a]		**BH_4 Non-Responders (n=22)[b]**		**Association with % Change in Plasma Phenylalanine Concentration[c]**
	Baseline	***Month 1***	***Baseline***	***Month 1***	***P-value***
Energy Intake (kcal/day)	1803 ± 579	1793 ± 460	1687 ± 481	1846 ± 828	0.516
Total Protein Intake (g/day)	60.9 ± 22.1	57.1 ± 18.6	61.8 ± 14.1	59.5 ± 20.7	0.619
Total Protein Intake (g/kg/day)	1.5 ± 0.6	1.4 ± 0.7	1.2 ± 0.6	1.1 ± 0.5	0.583
Dietary Phenylalanine Intake (mg/day)	1,034 ± 968	876 ± 634	822 ± 802	849 ± 910	0.479
Dietary Phenylalanine Intake (mg/kg/day)	20.3 ± 12.8	17.7 ± 8.2	14.4 ± 12.1	14.1 ± 12.3	0.481
Protein Equivalents from Medical Food Consumption (g/day)	37.6 ± 21.0	36.6 ± 20.7	42.9 ± 17.5	39.2 ± 19.2	0.145
Medical Food Consumption (% of prescription)	87.6 ± 28.9[d]	85.6 ± 29.6 [d]	82.1 ± 30.2	73.1 ± 35.0	---

[a] One patient excluded for incomplete diet record at baseline

[b] Three patients excluded for incomplete diet records at baseline and/or month one

[c] Linear regression of % change plasma phenylalanine concentration (between baseline and month one) modeled against % change in reported dietary intake (between baseline and month one) for all participants with a complete diet record at both time points

[d] n=29; two patients did not have an established medical food prescription at baseline; excluded from analysis

Table 4-3: Change in dietary phenylalanine and medical food prescription in 19 definitive BH_4 responders and 9 provisional responders

	Definitive Responders		Provisional Responders
	All Definitive Responders (n=19)[a]	***Subset of patients who underwent diet liberalization (n=16)[b]***	***All Provisional Responders (n=9)***
Baseline dietary phenylalanine prescription (mg/day)	704 ± 518[c]	512 ± 177[d]	313 ± 119[c,d]
Liberalized dietary phenylalanine prescription (mg/day)	1922 ± 612[e]	1958 ± 632[f]	457 ± 177[e,f]
Phenylalanine tolerance (% of baseline)	356.0 ± 157.4[e]	403.9 ± 119.0[f]	149.3 ± 28.3[e,f]
Baseline medical food prescription (grams protein equivalents/day)	43.3 ± 20.3	50.1 ± 13.6	48.8 ± 17.1
Liberalized medical food prescription (grams protein equivalents/day)	7.8 ± 10.5[e]	9.3 ± 10.9[f]	49.5 ± 16.6[e,f]
Medical food prescription (% of baseline)	16.7 ± 19.5[e, g]	18.8 ± 19.7[f]	101.8 ± 5.1[e,f]

[a] Includes three patients who did not undergo diet liberalization process due to month one intact protein intake at their RDA

[b] Excludes the three patients who did not undergo diet liberalization

[c] $p = 0.005$; comparison of all definitive responders and all provisional responders

[d] p =0.007; comparison of definitive responders who underwent diet liberalization and all provisional responders

[e] p<0.0001;comparison of all definitive responders and all provisional responders

[f] p<0.0001; comparison of definitive responders who underwent diet liberalization and all provisional responders

[g] n=18; one patient did not have medical food prescribed at baseline

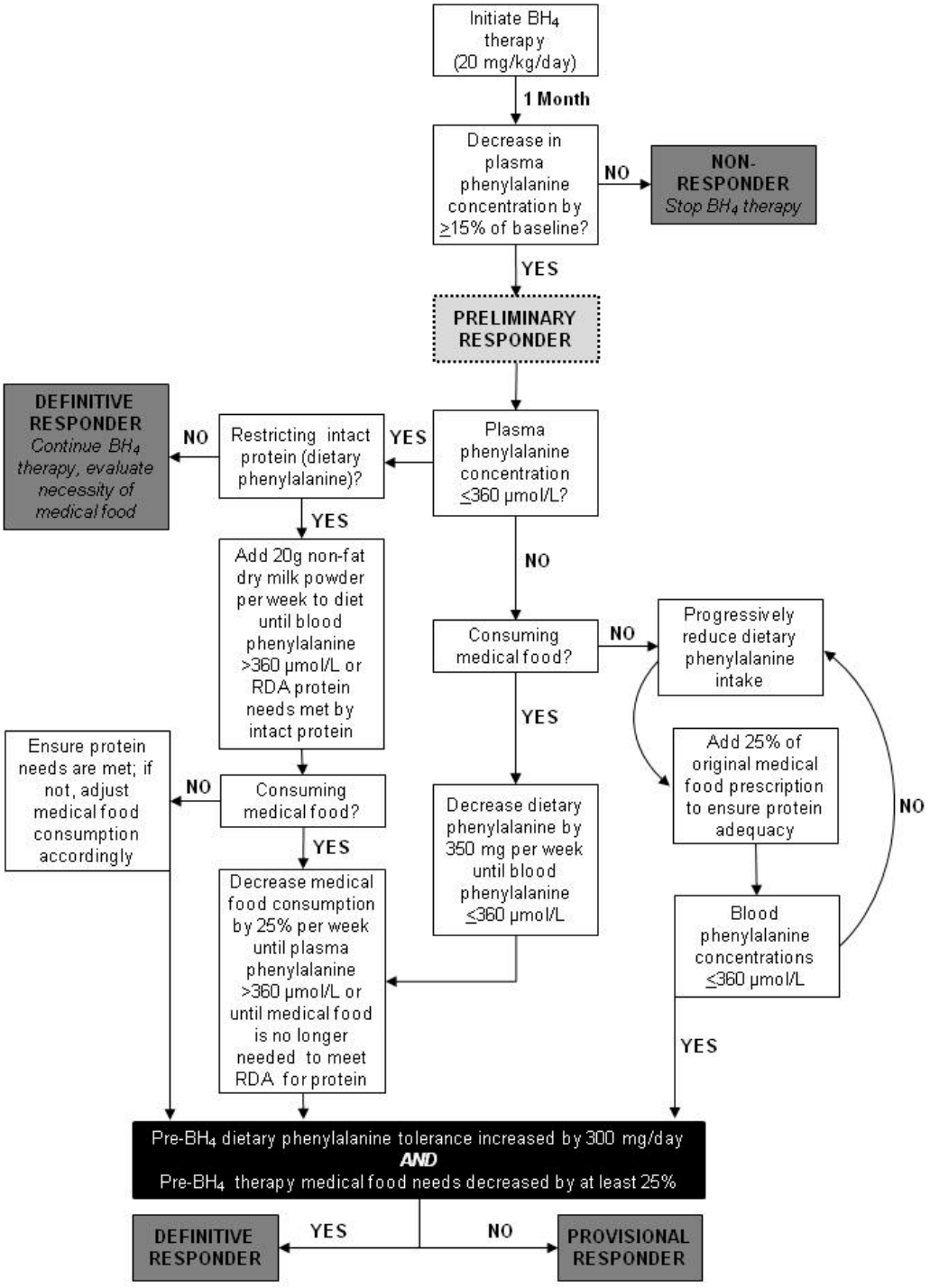

Figure 4-1: Practical algorithm used to classify BH_4 responsiveness in patients with PKU

Criteria include both change in plasma phenylalanine concentrations and ability to liberalize diet restrictions.

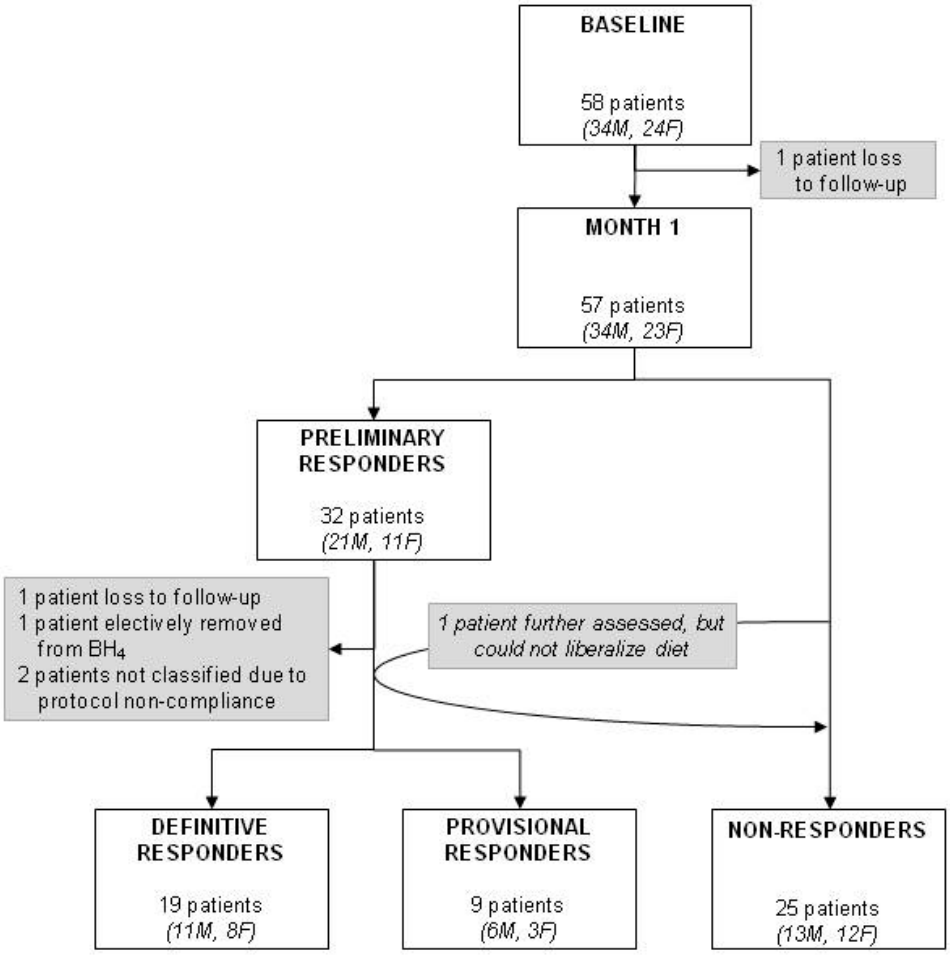

Figure 4-2: Flow diagram of BH_4 response classification in 58 PKU participants

CHAPTER 5

UTILITY OF PHENYLALANINE HYDROXYLASE GENOTYPE FOR TETRAHYDROBIOPTERIN RESPONSIVENESS CLASSIFICATION IN PATIENTS WITH PHENYLKETONURIA

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Abstract

Background: A need exists to expand the characterization of tetrahydrobiopterin (BH_4) responsiveness in patients with phenylketonuria (PKU), beyond simply evaluating change in blood phenylalanine concentrations. The clinical interpretation of BH_4 responsiveness should be evaluated within the context of phenylalanine hydroxylase (PAH) genotype.

Aim: This investigation seeks to use a previously developed PAH genotype severity tool, the assigned value (AV) sum, to assess the molecular basis of responsiveness in a clinical cohort and to explore the tool's ability to differentiate BH_4 responsive groups.

Methods: BH_4 response was previously clinically classified in 58 patients with PKU, with three response groups emerging: definitive responders, provisional responders, and non-responders. Provisional responders represented a clinically ambiguous group, with an initial decrease in plasma phenylalanine concentrations, but limited ability to improve dietary phenylalanine tolerance. In this retrospective analysis, mutations in the PAH gene were identified in each patient. PAH genotype was characterized through the AV sum approach, in which each mutation is given an AV of 1, 2, 4, or 8; the sum of both mutations' AV corresponds to genotype severity, with a lower number representing a more severe phenotype. An AV sum cutoff of 2 (indicative of the most severe genotypes) was used to dichotomize patients and predict BH_4 responsiveness. Provisional responders were classified with the definitive responders then the non-responders to see with which group they best aligned.

Results: In 17/19 definitive responders, at least one mutation was mild or moderate in severity (AV sum >2). In contrast, 7/9 provisional responders carried two severe or null mutations (AV sum=2), suggesting little molecular basis for responsiveness. Non-responders represent a heterogeneous group with 15/25 patients carrying two severe mutations (AV sum=2), 5/25 patients carrying one moderate or mild mutation in combination with a severe or null mutation (AV sum >2), and the remaining five patients carrying an uncharacterized mutation in combination with a severe mutation. Predictive sensitivity of the AV sum was maximized (89.5% vs. 67.9%) with limited detriment to specificity (79.4% vs. 80.0%), by classifying provisional responders with the non-responders rather than with the definitive responders.

Conclusions: In our clinical cohort, the AV sum tool was able to identify definitive responders with a high degree of sensitivity. As demonstrated by both the provisional responder group and the substantial number of non-responders with AV sums >2, a potential exists for misclassification when BH_4 response is determined by relying solely on change in plasma phenylalanine concentrations. PAH genotype should be incorporated in the clinical evaluation of BH_4 responsiveness.

Abbreviations: AV – assigned value; BH_4 – tetrahydrobiopterin; PAH – phenylalanine hydroxylase; PKU – phenylketonuria

Key Words: phenylketonuria; tetrahydrobiopterin; sapropterin dihydrochloride; phenylalanine hydroxylase; genotype

5.1. Introduction

Phenylalanine hydroxylase (PAH; EC 1.14.16.1) genotype is playing an increasingly important role in the management of patients with phenylketonuria (PKU; OMIM 261600), especially with the emergence of tetrahydrobiopterin (BH_4) therapy. Found to lower blood phenylalanine concentrations in a subset of patients with PKU [112], BH_4 is believed to improve the activity of certain dysfunctional PAH enzymes by optimizing cellular BH_4 concentrations, acting as a chemical chaperone, and/or overcoming kinetic variants [10, 120, 121]. BH_4's modes of action are contingent on the enzymes produced from the mutated gene. As such, PAH genotype should play a pivotal role in defining BH_4 responsiveness.

PAH genotype is currently not a standard criterion for BH_4 response classification. Patients are typically categorized as either "responders" or "non-responders" based only on percent change in blood phenylalanine concentrations after being administered BH_4 [118, 150]. Protocols assessing responsiveness are highly divergent with respect to variables that can affect circulating phenylalanine concentrations, such as diet prescription compliance, length of evaluation, dose of BH_4, and use of a pre-BH_4 phenylalanine load [118, 135]. Not surprisingly, inconsistencies in the relationship between PAH genotype and response classification have emerged [14, 118, 119]. Discordant categorization is rarely attributed to response misclassification, despite some "responsive" patients having severe PAH genotypes [116, 128] or limited to no long-term clinical benefits with continued use [16-18, 21-23]. Thus, a need exists to expand the scope of BH_4 response classification.

We recently described a novel clinical algorithm for assessing BH_4 responsiveness which includes both change in plasma phenylalanine concentrations and ability to modify dietary restrictions as criteria [185]. This approach allowed us to identify a subgroup of patients which experienced an initial marked decrease in plasma phenylalanine concentrations, but had only marginal improvements in dietary phenylalanine tolerance. Similar patients have been reported in protocols different from ours [21, 23]. It is unclear if these patients represent a truly responsive group or are merely artifacts of the protocols assessing responsiveness. PAH genotype may help to shed light on the nature of this subgroup.

From a clinical perspective, PAH genotypes are often difficult to interpret. The severity of a mutation or genotype can be explored through open-access databases like the Phenylalanine Hydroxylase Locus Knowledgebase (www.pahdb.mcgill.ca) and BIOPKU (www.biopku.org). However, a simple BH_4 response-specific clinical tool has yet to be created. Prior to the emergence of BH_4 therapy, a large multi-center study developed a relatively straightforward approach to assign phenotypic severity to a patient's PAH genotype [186]. While not intended for BH_4 response classification, this tool may serve as a starting point for incorporating PAH genotype into the clinical definition of BH_4 responsiveness. The goals of this investigation are to use this tool to assess the molecular basis of responsiveness in our clinical cohort and to explore the utility of using a genotype severity tool to differentiate BH_4 responsive groups.

5.2. Patients and Methods

5.2.1. Patients and Clinical BH_4 Response Classification

Patients at least 4 years of age, diagnosed PAH-deficient hyperphenylalaninemia were enrolled in a single-center, clinical trial assessing BH_4 responsiveness. Response was classified using a multi-criteria approach outlined in **Table 5-1** and detailed elsewhere [185]. Briefly, patients were first categorized based on change in plasma phenylalanine concentrations after one month of 20 mg/kg/day BH_4 therapy (sapropterin dihydrochloride; Kuvan®, BioMarin Pharmaceutical Inc, Novato, CA). Patients with ≥15% decrease in plasma phenylalanine concentrations continued BH_4 therapy and were further segregated based on subsequent ability to increase dietary phenylalanine tolerance and decrease medical food needs while maintaining plasma phenylalanine concentrations ≤360 μmol/L. Three BH_4 response groups emerged: definitive responders, provisional responders, and non-responders. Provisional responders represent a clinically ambiguous group, experiencing an initial decrease in plasma phenylalanine concentrations but being unable to substantially change their dietary phenylalanine tolerance or medical food needs. Noncompliant patients or those lost to follow-up remain unclassified. Informed consent was received for all patients. This study was approved by the Emory University Institutional Review Board.

5.2.2. PAH Mutation Identification

PAH genotypes were assessed retrospectively, and were not evaluated as part of the clinical BH_4 response classification. When available, PAH genotypes were taken from participants' medical records. These PAH mutations were identified using polymerase

chain reaction and DNA sequencing of the 13 coding exons and flanking regions. If only one mutation was identified, a second sample was analyzed using a PAH gene-specific comparative genomic hybridization array [187]. For patient who had not been clinically genotyped, a filter paper blood spot was collected which provided DNA that was analyzed using high-resolution melt profiling, as previously described [188]. Mutations were characterized by location (i.e. exon, intron, untranslated region) and by type (missense, mRNA processing, nonsense, or deletion).

5.2.3. Assessing PAH Genotype Severity Using Assigned Value (AV) Sum

PAH genotype severity was assessed using the assigned valued (AV) sum approach developed by Guldberg et al [186]. The method was created by evaluating nearly 300 functionally hemizygous patients with PKU and using the patients' phenotypic severity to classifying a total of 105 different mutations. Each mutation was given an AV of 1, 2, 4, or 8. A lower mutation AV corresponds to a more severe phenotype. Mutations with an AV of 1 are considered particularly severe in nature, with many classified as putative null mutations. Mutations with AV >1 are associated with moderate or mild phenotypes, suggesting that the mutation retains some functionality. To assess the severity of a patient's genotype, both mutations' AVs are added together (the "AV sum"). AV sums range from 2 to 16, again with a lower number indicating a more severe phenotype.

Some minor modifications to the AV sum approach were necessary for our analysis. First, there were certain mutations which had been assigned to multiple AVs due to a wide range of clinical phenotypes observed in the original analysis. In those instances, we

only used the mutation AV most frequently designated by Guldberg et al (see Appendix in ref [186]). To expand our ability to give a patient an AV sum, decidedly severe mutations not previously evaluated in the AV sum analysis—such as large deletions, frame shift mutations, and disruptions of canonical splice site motifs—were given a mutation AV of 1. Finally, since not all mutations identified in our clinic population had a designated mutation AV, some patients were given an "indefinite AV sum" (e.g. ≥2, ≥3, ≥4, etc). The indefinite AV sum is, at minimum, one greater than the AV for the characterized mutation.

5.2.4. Assessment of Classification Approaches and Statistics

The ability of the AV sum to differentiate the clinically designated BH_4 responses and the genetic basis of responsiveness were simultaneously assessed, as outlined in **Figure 5-1**. Patients were first dichotomized into "true responder" and "true non-responder" groups based on the clinical response classification described in Section 5.2.1 and Table 5-1. Due to the clinical ambiguity of the provisional responder group, two iterations were evaluated: (1) provisional responders were classified with the definitive responders in a single "true responder" group and (2) provisional responders were classified with the non-responders in a single "true non-responder" group. Patients were then classified by their AV sum. Patients with an AV sum >2 were classified "AV sum responders"; those with an AV sum=2 were classified as "AV sum non-responders." This threshold was selected, as an AV sum of 2 represents a severe genotype with limited to no molecular basis for responsiveness. Patients with the indefinite AV sum of ≥2 were obligate "AV sum non-responders," since an AV sum above 2 could not definitively be assigned. Since

obligate AV sum non-responders have the potential to bias the analysis, results are presented both with and without these patients. To quantify the ability of AV sum to classify BH_4 response, sensitivity, specificity, positive predictive value, and negative predictive value were calculated. Patients with an unclassified BH_4 response, while presented in the descriptive and summary statistics, were excluded from this portion of the analysis.

5.3. Results

5.3.1. Summary of Identified Mutations

A total of 58 patients were genotyped: 19 definitive responders, 9 provisional responders, 25 non-responders, and 5 unclassified patients. Of the expected 116 alleles, 114 mutations were identified (98.3% detection rate). In two patients, only one mutation could be identified, although their clinical and biochemical profiles indicated PAH-deficient hyperphenylalaninemia. There were 47 different mutations identified within our clinical cohort. Mutations affected all 13 exons, 7 introns (intron 1, 4, 5, 6, 8, 10 and 12), and the 3' untranslated region. As **Table 5-2** shows, missense mutations comprise the majority of the 47 distinct mutations and the majority of 116 alleles.

5.3.2. PAH Genotype AV Sum by BH_4-Response Classification

Table 5-3 presents the PAH genotypes and AV sums of all patients, separated into their respective BH_4 response groups. The majority of definitive responders (17/19 patients) had an AV sum >2, indicating that at least one mutation is moderate or mild in severity. The remaining two definitive responders carried a severe mutation (AV=1) in

combination with an uncharacterized mutation, and were given an indefinite AV sum of ≥2. In contrast, 7/9 provisional responders had a severe PAH genotype (AV sum=2). The two remaining provisional responders had AV sums of 5.

Non-responders represented a particularly heterogeneous group. The majority of non-responders (15/25 patients) had an AV sum of 2, indicating a severe PAH genotype. However, 5/25 non-responders had an AV sum >2, carrying a mild or moderate mutation in combination with a severe mutation. The remaining 5 non-responders had a severe mutation (AV=1) in combination with an uncharacterized mutation, and were assigned an indefinite AV sum ≥2. The unclassified patients' AV sums indicate their genotypes are primarily severe. One unclassified patient, who was lost to follow-up, has an AV sum of 6.

5.3.3. Discordant BH_4 Response Classification of Matching PAH Genotypes

Several patients had a PAH genotype matching one or more enrolled patient, including five pairs of siblings, four pairs of unrelated patients, and one unrelated patient matching a sibling pair. Of these, two sibling sets and two unrelated sets had discordant clinical BH_4 response classification. In these four instances, one patient was classified as a non-responder and the other patient was classified as a provisional responder. The PAH genotype AV sum in each instance was 2, indicating that both mutations were severe in nature. Interestingly, none of these discordant classifications included a patient being classified as a definitive responder.

5.3.4. Ability of AV Sum to Predict BH_4 Response

Table 5-4 shows the ability of the AV sum to predict clinical BH_4 response classification. Categorizing provisional responders with the non-responder group improved sensitivity and negative predictive value with little detriment to specificity and positive predictive value. As expected, excluding patients with an indefinite AV sum of ≥2 improved the sensitivity of using AV sum to classify BH_4 responsiveness.

5.4. Discussion

PAH genotype severity has important implications for clinical classification of BH_4 responsiveness. With seven of nine of our provisional responders carrying two severe or null mutations, there is strong evidence to suggest they do not represent a truly responsive group. The discordant classification of four sets of patients with matching PAH genotypes—with one patient being classified as a non-responder and one patient being classified as a provisional responder—further suggests that the initial change in plasma phenylalanine concentrations in the provisional responders cannot necessarily be attributed to a drug effect. These findings highlight the potential for patient misclassification in extended protocols relying solely on change in plasma phenylalanine concentrations. As BH_4 response classification continues to evolve, it is essential that the definition becomes more comprehensive to encompass change in plasma phenylalanine concentrations, change in dietary phenylalanine tolerance, and PAH genotype. Identification of misclassified patients must also become a crucial element of BH_4 response assessment.

In our clinical cohort, one mild or moderate mutation was necessary but not sufficient for BH_4-responsiveness. In two instances, a definitive responder carried an uncharacterized mutation in combination with a severe mutation. The literature, while sparse, indicates that the uncharacterized mutation in each of these patients—p.P275S [23, 189] and p.P366H [145, 190], respectively—does not produce a severe phenotype, even when coupled with a severe or null mutation. Thus, it appears that all of our definitive responders have an AV sum >2, including these two patients. Surprisingly, the ability of AV sum to differentiate our non-responder group is less straightforward. Assuming our clinical classification of BH_4 responsiveness is accurate, relying solely on AV sum to predict response classification led to a substantial number of false-positive cases. These genotypic inconsistencies, however, may potentially expose inherent limitations of current BH_4 response protocols, especially those spanning days or weeks. The lack of demonstrated decrease in plasma phenylalanine concentrate may have been affected by numerous factors, including: overall metabolic state of the patient, change in health status, non-compliance with BH_4, or alteration of dietary intake [30, 121, 125, 191]. Extensive evaluation of these potentially misclassified patients may elucidate limitations of the AV sum approach or clinical BH_4 response protocols.

The concept of evaluating PAH genotype for BH_4 responsiveness is not a novel one. Efforts have been made to identify “responsive” alleles from the clinical results of various BH_4 response protocols [123, 125]. This approach, however, is limited in that it is reliant on divergent protocols which do not assess patient misclassification, and ambiguity has arisen. A simple, BH_4-specific clinical tool has yet to be developed. In

contrast, PAH genotype AV sum is an easy tool, developed independent of BH_4 response classification. While our data may be preliminary in nature, the AV sum approach appears to provide a high degree of sensitivity for identifying patients who have both biochemical and dietary benefits from BH_4 therapy. AV sum, in its current state, may serve as a tool for screening patients who should be evaluated for responsiveness. In retrospective analyses, the AV sum may help identify potentially misclassified patients.

While our data are promising, some limitations of our study should be noted. Although a group of 58 patients with PKU assessed at a single clinic is substantial, the external validity of our findings needs to be assessed. Moreover, we could not confirm that the two mutations are in trans in each patient due to incomplete parental studies. There is a potential that some patients' mutations are in cis and that these patients may harbor an additional unidentified mutation; however, these cases are relatively atypical [156]. Furthermore, some adjustments to the AV sum approach should be considered before widespread implementation. For example, the mutation c.1066-3C>T is classified as a severe mutation (AV=1), but is known to maintain some normal splicing properties and can result in a mild phenotype [143, 192]. An expansion of the number of mutations with an AV score would also be necessary. The AV sum tool, should be considered a starting point for the clinical utilization of PAH genotype for response classification.

In conclusion, AV sum appears to be a useful clinical tool for identifying potential candidates for BH_4 therapy and retrospectively evaluating BH_4 response misclassification. As our provisional responder group exemplifies, a change in

phenylalanine concentrations does not always indicate BH_4 responsiveness. Our findings underscore the importance of factors such as genotype and dietary phenylalanine tolerance when assessing a patient's response to BH_4.

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***Conflict of Interest*:** This investigator-initiated protocol was supported in part by BioMarin Pharmaceutical Inc. Rani H. Singh and Meghan E. Quirk currently have an investigator-initiated protocol with a material supply agreement with BioMarin Pharmaceutical Inc. Additionally, Rani H. Singh is involved in four sponsor-initiated protocols in collaboration with BioMarin Pharmaceutical Inc.

Table 5-1: Clinical BH_4 response classification of patients with PKU using a novel, multi-criteria algorithm

Response Classification	Classification Criteria
Definitive Responder	• ≥15% ↓ in plasma phenylalanine concentrations[a] • ↑ baseline dietary phenylalanine tolerance by ≥300 mg/day or could consume a fully liberalized diet[b] • ↓ baseline medical food need by ≥25% or completely discontinue medical food[b]
Provisional Responder	• ≥15% ↓in plasma phenylalanine concentrations[a] • Could not significantly ↑ baseline dietary phenylalanine tolerance (<300 mg/day) and ↓ baseline medical food needs[b]
Non-Responder	• <15% ↓ in plasma phenylalanine concentrations[a]
Unclassified	• Lost to follow-up or noncompliant with protocol

[a] Change in plasma phenylalanine concentrations assessed after one month of BH_4 therapy (20 mg/kg/day)

[b] Dietary criteria contingent on maintaining plasma phenylalanine concentrations ≤360 μmol/L

Figure 5-1: Classification of clinical BH_4 response and assigned value (AV) sum to evaluate the utility of a PAH genotype severity tool

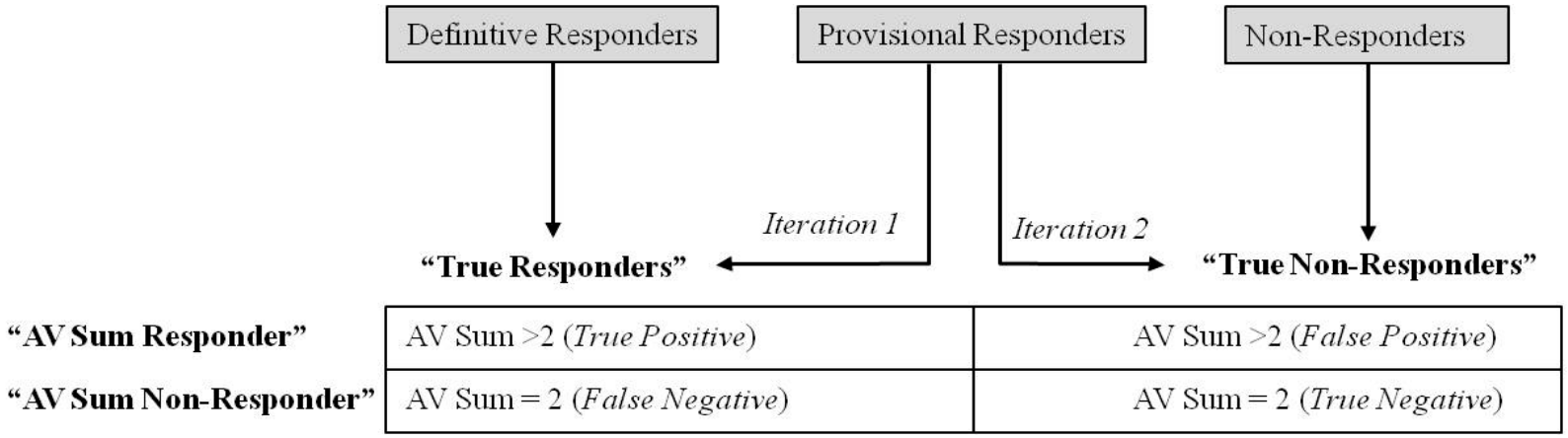

Table 5-2: Frequency of PAH mutation types in patients with PKU evaluated for BH_4 responsiveness (N=58)

Mutation Type	Of the 47 distinct mutations, n (%)	Of the 116 alleles, n (%)
Missense Mutations	30 (63.8%)	78 (67.2%)
mRNA Processing Mutations	8 (17.0%)	24 (20.7%)
Nonsense Mutations	4 (8.5%)	7 (6.0%)
Deletions	5 (10.6%)	5 (4.3%)
Mutation Not Identified	---	2 (1.7%)

Table 5-3: PAH genotypes and AV sums of 58 patients evaluated for BH_4 responsiveness

Pt ID	Mutation 1	Mutation 2	Mutation 1 AV	Mutation 2 AV	AV Sum
Definitive Responders (n=19)					
148	p.I65T	p.A403V	2	8	10
128	p.V190A	p.X453_453+2del	8	-	≥9
155	c.441+1G>A	p.V190A	1	8	9
135	p.V245A	p.F299C	8	1	9
104	p.A104D	p.Y414C	4	4	8
102	p.L48S	p.I65T	4	2	6
110	p.V245L	p.Y414C	-	4	≥5
158	p.R68S	c.1065+3A>G	4	1	5
111	p.R68S	c.509+1G>A	4	1	5
122	p.N133_Q134>Rfs	p.Y414C	1	4	5
113	p.L348V	p.L348V	2	2	4
114	p.L348V	p.L348V	2	2	4
132	p.I65T	p.E205D[a]	2	-	≥3
107	p.I65T	-	2	-	≥3
134	p.F39L	p.G272X	2	1	3
131	p.I65T	c.1066-3C>T	2	1	3
105	p.I65T	p.F299C	2	1	3
106	c.1066-11G>A	p.P366H	1	-	≥2
136	p.P275S	EX9_EX13del	-	1	≥2
Provisional Responders (n=9)					
144	p.A104D	p.R408W	4	1	5
100	p.L48S	c.1315+1G>A	4	1	5
112	p.R408W	EX6_IVS6del	1	1	2
153	c.509+1G>A	p.R408W	1	1	2
117	p.Q20X	p.R408W	1	1	2
109	p.R408W	c.1315+1G>A	1	1	2
138	p.P281L	c.1315+1G>A	1	1	2
115[b]	p.R408W	p.R408W	1	1	2
126[c]	c.912+1G>A	p.R408W	1	1	2

Table 5-3, continued

Non-Responders (n=25)					
129	p.R241C	c.912+1G>A	8	1	9
154	p.A403V	p.R408W	8	1	9
141	p.R68S	p.R408W	4	1	5
121	p.L348V	p.R408W	2	1	3
123	p.I65T	p.R111X	2	1	3
101	c.1315+1G>A	-	1	-	≥2
108	p.L41P	p.E280K	-	1	≥2
120	p.R252W	p.Q304R	1	-	≥2
124	p.I283F	p.R408W	-	1	≥2
145	p.L311P	p.Y386C	1	-	≥2
103	c.1315+1G>A	c.1315+1G>A	1	1	2
116[b]	p.R408W	p.R408W	1	1	2
119[d]	p.R261X	c.1066-11G>A	1	1	2
125	p.R252Q	c.1315+1G>A	1	1	2
151	p.R252Q	c.1315+1G>A	1	1	2
127	p.R158Q	c.1315+1G>A	1	1	2
133	p.R408W	c.1315+1G>A	1	1	2
137	p.R252W	p.R408W	1	1	2
139	p.P281L	c.1315+1G>A	1	1	2
140	p.A395P	p.R408W	1	1	2
142[e]	p.E280K	p.F299C	1	1	2
147[e]	p.E280K	p.F299C	1	1	2
149[f]	c.60+5G>T	p.G272X	1	1	2
150[f]	c.60+5G>T	p.G272X	1	1	2
152[c]	c.912+1G>A	p.R408W	1	1	2
Unclassified Patients (n=5)					
130	p.I65T	p.Y414C	2	4	6
146	p.G218V	p.S349P	-	1	≥2
143	p.E280K	EX6_IVS6del	1	1	2
118[d]	p.R261X	c.1066-11G>A	1	1	2
157[b]	p.R408W	p.R408W	1	1	2

[a] Variant of unknown pathogenesis (c.615G>C)

[b] Patient 115 and patient 116 are siblings; patient 157 is unrelated

[c] Patient 126 and patient 152 are siblings

[d] Patient 118 and patient 119 are siblings

[e] Patient 142 and patient 147 are siblings

[f] Patient 149 and patient 150 are siblings

Table 5-4: Sensitivity, specificity, positive predictive value, and negative predictive value using genotype AV sum cutoff of >2 to predict clinical BH_4 response classification

"True Responder" Criteria	"True Non-Responder" Criteria	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Iteration1: Analysis of All Classified Patients (n=53)					
Definitive Responders, Provisional Responders	Non-Responders	67.9%	80.0%	79.2%	69.0%
Definitive Responders	Non-Responders, Provisional Responders	89.5%	79.4%	70.8%	93.1%
Iteration 2: Analysis Excluding Patients with Indefinite AV Sums of ≥2 (n=46)					
Definitive Responders, Provisional Responders	Non-Responders	73.1%	75.0%	79.2%	68.2%
Definitive Responders	Non-Responders, Provisional Responders	100%	75.9%	70.8%	100%

CHAPTER 6

DIETARY PROTEIN INTAKE OF PATIENTS WITH PHENYLKETONURIA DURING AN EXTENDED TETRAHYDROBIOPTERIN RESPONSE PROTOCOL

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Abstract

Background: Patients with phenylketonuria (PKU) who experience a decrease in plasma phenylalanine concentrations during a tetrahydrobiopterin (BH_4) response protocol do not always have long-term clinical benefits with continued use. In protocols spanning multiple days or weeks, factors other than BH_4 may lead to an apparent initial response. We recently identified a subgroup of "provisional responders" who experienced marked decreases in plasma phenylalanine concentrations after one month of BH_4 therapy but could not subsequently improve their dietary phenylalanine tolerance.

Aim: To better understand what led to the initial decrease in plasma phenylalanine concentrations in our provisional responders, we explore reported dietary protein intake during the response protocol, prior to assessment of change in plasma phenylalanine concentrations.

Methods: In this retrospective analysis, three groups of patients were evaluated: definitive responders, provisional responders, and non-responders. All patients had initiated BH_4 therapy (20 mg/kg/day) for one month, during which they were instructed to maintain a consistent dietary intake. Starting with the baseline visit, patients submitted self-reported, 3-day diet records on a weekly basis until the month one evaluation. Six dietary measures were evaluated: energy, total protein, phenylalanine, percent of phenylalanine prescription, medical food protein equivalent, and percent of medical food prescription. Linear mixed modeling analysis was used to evaluate differences in baseline intake and trends intake over time, within and between the three groups. Least squares

means was used to evaluate differences in typical intake between groups during the first month of BH_4 therapy. Analyses were first run with all patients, and then restricted to only pediatric patients.

Results: A total of 705 days worth of intake were evaluated from 53 patients (19 definitive responders, 9 provisional responders, 25 non-responders). When all patients were evaluated, trends in intake over time did not differ between the three response groups for any of the dietary measures. Definitive responders reported consuming more dietary phenylalanine than the provisional responders, but did not differ from the non-responder group. All other intakes of interest were comparable. In contrast, when the analysis was restricted to the 33 pediatric patients, the non-responders reported a slight decrease in total protein intake (approximately 6 grams) and appeared to become slightly more compliant with their dietary phenylalanine prescription over the course of the first month, compared to the negligible change in intake in the provisional responder group. Pediatric definitive responders reported typically consuming more dietary phenylalanine and less medical food than both the pediatric provisional responders and pediatric non-responders.

Conclusions: We cannot attribute the initial decrease in plasma phenylalanine concentrations experienced by the provisional responders to any unique or overt trends in dietary protein intake. Definitive responders appear to collectively represent a less severe phenotype compared to the provisional responder, with significantly greater dietary phenylalanine intake.

Abbreviations: BH_4 – tetrahydrobiopterin; PAH – phenylalanine hydroxylase; PKU – phenylketonuria

Key Words: phenylketonuria; tetrahydrobiopterin; sapropterin dihydrochloride; phenylalanine hydroxylase; protein; dietary intake

6.1. Introduction

Phenylketonuria (PKU; OMIM 261600) is a rare autosomal recessive disorder in which deleterious mutations in the gene encoding phenylalanine hydroxylase (PAH; EC 1.14.16.1) hinder the enzyme's ability to metabolize phenylalanine. In untreated patients, phenylalanine accumulates and can negatively impact their development and neurocognitive status [84, 85, 178]. Until recently, nutrition therapy—consisting of a phenylalanine-restricted diet and a phenylalanine-free, amino acid medical food—was the only management approach available to help patients maintain blood phenylalanine concentrations in a relatively benign range. The treatment paradigm shifted when a subset of patients with PKU were found to experience a significant decrease in blood phenylalanine concentrations after being administered pharmacological doses of PAH's cofactor, tetrahydrobiopterin (BH_4) [22, 112, 113]. BH_4 therapy is believed to enhance the hydroxylation reaction by working in conjunction with certain mutant PAH enzymes [10, 120]. With improved phenylalanine metabolism, patients who respond to BH_4 are often able to liberalize their dietary restrictions and decrease their reliance on medical food [12-18].

Responsive patients are identified by measuring blood phenylalanine concentrations before and after initiating BH_4. Published protocols, despite being highly divergent in approach, inevitably dichotomize patients as "responders" or "non-responders" based on a threshold percent change in blood phenylalanine concentrations (typically $\geq$30% decrease). Yet an acute response does not always confer long-term benefits [16-18, 21-23]. In both a 21-hour protocol [23] and an 8-day protocol [21], a subgroup of patients

experienced clinically significant reductions in blood phenylalanine concentrations (33.7-74.5% decreases), but had limited ability to subsequently improve their dietary phenylalanine tolerance. These patients do not exemplify the expected phenotype of a BH_4 responder, suggesting current approaches to classifying patients may not adequately characterize the gradation of responses to BH_4 seen clinically. It is vital to understand the emergence of this subgroup, as they may elucidate limitations in current BH_4 response classification protocols or identify factors that reduce the effectiveness of long-term BH_4 therapy.

We recently identified a group of these "provisional responders" through our clinical algorithm [185]. Upon evaluating their PAH genotypes, we found that the majority of these patients had limited molecular basis for responsiveness [193]. As such, alternate explanations for the apparent initial change in plasma phenylalanine concentrations to BH_4 must be explored. In longer protocols such as ours, decreases in plasma phenylalanine concentrations may be attributed to factors other than BH_4, such as modulation of dietary intake. To better understand what led to the initial response in our provisional responder group, we explore reported dietary protein intake of patients during the first month of BH_4 therapy, between the two time points used to assess change in plasma phenylalanine concentrations.

6.2. Patients and Methods

6.2.1. Patient Selection

Patients at least 4 years of age with PAH-deficient hyperphenylalaninemia were recruited from a single center to evaluate their BH_4 responsiveness. Patients were excluded if they were pregnant or breastfeeding, were previously determined to be BH_4 responsive, or had taken biopterin in the previous 8 weeks. Informed consent was obtained for all participants. This protocol was approved by the Emory University Institutional Review Board.

6.2.2. Classification of BH_4 Response

Fasting plasma phenylalanine concentrations were measured immediately before and after one month of 20 mg/kg/day BH_4 therapy (sapropterin dihydrochloride; Kuvan®; BioMarin Pharmaceutical Inc., Novato, California, USA). BH_4 response was classified using sequentially applied criteria, detailed elsewhere [185]. Briefly, patients were initially classified based on the percent change in plasma phenylalanine concentrations after one month of BH_4 therapy. Non-responders experienced less than a 15% decrease in plasma phenylalanine concentrations, discontinued BH_4 therapy, and continued their diet therapy regimen. Patients with at least a 15% decrease in plasma phenylalanine concentrations at month one were further evaluated and differentiated. Definitive responders were able to improve their dietary phenylalanine tolerance by at least 300 mg/day (or consume an unrestricted diet) and could decrease their medical food needs by at least 25% compared to baseline (or no longer needed medical food) while maintaining blood phenylalanine concentrations below 360 µmol/L. Provisional responders, despite

an initial decrease in plasma phenylalanine concentrations, could not meet both of the diet liberalization criteria while maintaining blood phenylalanine concentrations below 360 μmol/L.

6.2.3. Assessment of Dietary Intake

During the first month of BH_4 therapy, patients were instructed to consume a diet consistent with their baseline intake, regardless of adherence to their diet prescription (the amount of dietary phenylalanine and medical food clinically advised to consume in a day). Patients were asked to submit 3-day diet records on a weekly basis, starting with their baseline study visit. If a 3-day diet record was not received, a metabolic dietitian attempted to capture a 24-hour recall. Diet records and recalls were analyzed using the Nutrition Data System for Research diet analysis program (2010 version, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, USA).

There were six dietary measures of interest captured from the diet records and recalls: energy, total protein, phenylalanine, phenylalanine prescription compliance, medical food protein equivalent, and medical food prescription compliance. Energy intake was evaluated to assess whether any identified trends were a function of change in overall consumption. Total protein represented the sum of intact protein and medical food protein equivalents, while phenylalanine served as a proxy for intact protein intake, as all medical foods were phenylalanine-free. Baseline dietary phenylalanine and medical food prescriptions were drawn from patients' medical charts and compared to reported intake

to evaluate diet prescription compliance. Compliance was expressed as a percent of the patient's baseline diet prescription.

6.2.4. Statistical Analysis

Due to the age distribution among the groups, data were assessed in two ways. First, all participants with a classified BH_4 response were evaluated and groups compared. Analysis was then restricted to patients <18 years of age, since the provisional responder group consisted entirely of pediatric patients. Differences in demographic characteristics between the definitive, provisional, and non-responder groups were assessed by one-way ANOVA, with a Tukey-Kramer adjustment for post-hoc pairwise comparisons.

Linear mixed modeling of repeated measures was used to evaluate the effect of response group, time, and the interaction of the two (response group*time) on the six dietary intake measures of interest (described in *Section 6.2.3*). A significant group effect in the model would indicate that the dietary intake of interest differed between at least two of the groups at baseline, while a significant time effect would suggest that patients collectively reported a change in intake over the course of the first month of BH_4 therapy, regardless of response group. A significant interaction term would indicate that trends in intake over time differed between at least two of the response groups. The interaction term was removed from models when the Type 3 test for fixed effects indicated it was not a significant term in the model.

In all models, time was a continuous variable expressed as the number of days since initiating BH_4 therapy. Each diet record day or recall was counted as its own data point (i.e. 3-day diet records were not averaged). Estimated values of change in intake between the baseline and the month one visit (either a collective or a group-specific change) was calculated using with the time equal to day 24 of BH_4 therapy, as the majority of patients' last diet record was submitted on or after this day. Exclusion of diet records with exceptionally high reported energy intake (>3500 kcal/day for females, >4200 kcals/day for males) did not alter the significance of the findings, so all diet records and recalls were used in the final analysis.

In addition to trends over time, models provided an estimate of the "typical" intake of the groups during the first month of BH_4 therapy (essentially the mean intake, accounting for an unbalanced dataset). Differences between groups' typical intake were evaluated using least squares means approach with a Tukey-Kramer adjustment. Statistical analyses were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). A p-value of less than 0.05 was considered statistically significant. The findings of the mixed model analysis were alternatively assessed through generalized logit models in which the outcome was response group and the predictors included estimated intake at baseline and change over time (results presented in Appendix B).

6.3. Results

6.3.1. Analysis of All Patients

The demographic characteristics of the 53 patients evaluated are presented in **Table 6-1**. At baseline, the definitive responders were prescribed more dietary phenylalanine, but similar amounts of medical food when compared to both the provisional and non-responder groups. Baseline plasma phenylalanine concentrations were lower in the definitive responders compared to the non-responders, and month one plasma phenylalanine concentrations segregated the non-responders from both the definitive and the provisional responder groups. A total of 705 days of diet records were collected, with a mean of 13.3 ± 2.8 days of diet records (range: 4-18 days) received from each participant. The mean number of diet record days collected during the first month did not differ between the BH_4 response groups [$F(2,49)=1.96$, $p=0.151$].

Table 6-2 summarizes dietary intake and trends of all patients during the first month of BH_4 therapy. The interaction term did not reached statistical significance in any of the models, thus identified changes in intake were collective rather than group-specific. There was an estimated 2.2 gram decrease in total protein intake from baseline to the month one study visit. This change appears to be primarily driven by a decrease in medical food intake, with a slight decrease in medical food prescription compliance emerging over time. Changes in reported intake of energy, phenylalanine, and phenylalanine prescription compliance over the course of the first month of BH_4 therapy were not statistically significant.

Typical intake of energy, total protein, and medical food along with medical food compliance during the first month of BH_4 therapy did not differ between the three response groups. Typical phenylalanine intake, on the other hand, was significantly higher in the definitive responders compared to the provisional responders; the non-responders did not differ from either group. While the difference in typical dietary phenylalanine intake would be expected due to the greater baseline dietary phenylalanine prescription, it appears to be slightly exaggerated by greater prescription non-compliance in the definitive responder group. However, group differences in percent of phenylalanine prescription typically consumed did not reach statistical significance.

6.3.2. Pediatric Analysis

Table 6-3 presents the characteristics of the 33 pediatric patients. All demographic characteristics were comparable between the three response groups, except month one plasma phenylalanine concentrations, which again segregated the non-responder group from the definitive responder and the provisional responder groups. A total of 433 days of diet records were received from the pediatric patients. Patients submitted a mean of 13.1 ± 3.0 days of diet records, and the mean number of diet record days collected did not differ between response groups [$F(2,30)=1.88$, $p=0.170$].

Results of the pediatric analysis are presented in **Table 6-4**. In contrast to the full analysis, group-specific trends in intake over the course of the first month of BH_4 therapy emerged. The differences in trends in intake that emerged were between the non-responder group and the provisional group; the trends in intake in the definitive responder

group did not differ from either the non-responder or provisional responder groups. The pediatric non-responder group decreased total protein intake by an estimated 6.0 grams between baseline and the month one study visit, while the provisional responders had a negligible change during this time (**Figure 6-1A**). The different trend in total protein intake in the non-responder group was partially due to a decrease in phenylalanine intake over time. While the group*time interaction effect did not reach statistical significance (Type 3 test p-value=0.074), the model estimate suggested dietary phenylalanine intake trended differently in the non-responder compared to the provisional responder group (non-responder*time β coefficient p-value=0.023; provisional responders serving as referent group). The non-responder group decreased phenylalanine intake by an estimated 117 mg between baseline and month one visit, while the provisional responder group had a slight, non-significant 50 mg increase in phenylalanine intake over time. Definitive responders did not differ from either group, with an estimated decrease of 23 mg phenylalanine between study visits, which did not reach statistical significance. The difference between the pediatric non-responders and provisional responders was amplified when evaluating dietary phenylalanine prescription compliance. The non-responder group appears to become more compliant with their dietary phenylalanine prescription over the course of the first month of BH_4 while provisional responders consumed a relatively consistent diet (**Figure 6-1B**). No group-specific trends emerged for medical food consumption, although a collective decrease in medical food consumption over the course of the month did appear (an estimated decrease of 1.9 grams of protein equivalents between the baseline and the month one evaluation). Energy consumption did not differ between the groups or change over time.

Typical intake of energy and total protein in pediatric patients during the first month of BH_4 therapy were not statistically different between groups. However, the definitive responder group reported consuming more phenylalanine and less medical food compared with both the provisional responder and non-responder groups. Differences may be a function of greater diet prescription non-compliance in the definitive responders, but group differences in phenylalanine and medical food prescription compliance did not reach statistical significance.

6.4. Discussion

From our analysis, we cannot attribute the emergence of our provisional responder group to overt or divergent trends in self-reported dietary protein intake prior to BH_4 response classification. The provisional responders reported consuming less phenylalanine than definitive responders in both the full and pediatric-restricted analyses. Additionally, the provisional responders reported consuming a relatively consistent diet while the pediatric non-responder group reported a decrease in total protein consumption over the course of the first month of BH_4 therapy. From a clinical perspective, neither of these differences explains the appearance of our provisional responder group.

The relationship of plasma phenylalanine concentrations and dietary protein intake is complex. Significant variation in blood phenylalanine concentrations is seen both with and without modulation of dietary phenylalanine intake [194], and in an acute evaluation, excess intact protein intake was not correlated with blood phenylalanine concentrations measured the next morning [195]. On the other hand, improved diet prescription

compliance over the course of a week has been associated with decreased plasma phenylalanine concentrations [196]. Furthermore, the distribution of medical food intake over the course of a day appears to affect diurnal changes in blood phenylalanine concentrations [195]. Thus, dietary protein intake has the potential to influence plasma phenylalanine concentrations, but the relationship is not straightforward. A need exists to further explore how the composition and timing of meals consumed prior to and during the BH_4 responsiveness protocols affects patient classification.

Assuming our diet records and recalls are accurate, the decrease in plasma phenylalanine concentrations experienced by the provisional responders could be attributed to a non-dietary source, including but not limited to: a true effect of BH_4, resolution of a catabolic state which was present at baseline, or usual fluctuations in plasma phenylalanine concentrations. The subsequent inability to liberalize dietary phenylalanine restrictions could then be attributed to a loss of BH_4 efficacy, a decreased compliance with the therapy, and/or a false-positive response. However, it is important to recognize that self-reported dietary intakes are not always accurate. Under- and over-reporting occurs in non-PKU populations [197-201] and most likely occurs in PKU patients as well [202]. So while self-reported diet records are the best possible tool for capturing acute change in dietary intake, their ability to capture actual intake is an inherent limitation.

Some general findings can be drawn from our analysis. First, the definitive responders appear to collectively represent a less severely affected group than the provisional responders. They reported consuming significantly more dietary phenylalanine than the

provisional responders, yet showed no differences in plasma phenylalanine concentrations. This finding is best demonstrated in the pediatric analysis, where definitive responders also reported consuming significantly less medical food. While variability exists at the individual patient level, the differences in reported intakes corroborate the group differences found in diet prescriptions and genotype severity [193]. Furthermore, our analysis highlights the level of diet prescription non-compliance in a PKU cohort. Overall, our patients reported consuming more dietary phenylalanine and less medical food than clinically prescribed. As a result, all response groups had mean baseline plasma phenylalanine concentrations exceeded our preferred clinical threshold of 360 μmol/L. This is not surprising, as diet prescription non-compliance is well-documented in the PKU population [8, 111, 203]. Unexpectedly, the definitive responder group appears to be the least compliant group, although group differences did not reach statistical significance. While the definitive responders may have been more non-compliant, the clinical data also suggest that patients may have had an inappropriate baseline diet prescription. We did not systematically evaluate diet prescriptions prior to initiating patients on BH_4. Some patients may have been able to consume more dietary phenylalanine and less medical food than prescribed without detriment to their plasma phenylalanine concentrations. We recommend systematic evaluation of phenylalanine tolerance and medical food need prior to evaluating BH_4 responsiveness.

Surprisingly, the pediatric non-responders reported a small but clinically significant decreased their total protein and phenylalanine intake during the first month of BH_4 therapy compared to the relatively consistent intake of the provisional responder group.

Despite becoming more compliant with their diet phenylalanine prescription over time, the pediatric non-responders' plasma phenylalanine concentrations did not decrease. This finding potentially indicates that dietary intake during an extended BH_4 response protocol may not affect response classification. However, this finding must be viewed with caution, given our sample size and statistical approach. As there were no group-specific trends when evaluating all patients, restricting the analysis to pediatric patients may have biased the non-responder group or allowed an influential patient to significantly affect the overall trend. Further evaluation of protocol compliance, especially of patients classified as non-responders, is warranted.

In conclusion, change in self-reported dietary protein (total protein, phenylalanine, or medical food) intake over the course of the first month of BH_4 therapy does not appear to be a hallmark of the provisionally responsive group. With greater dietary phenylalanine intake, the definitive responder group collectively appears to have a less severe clinical phenotype, although individual phenotypes within the group are variable.

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***Conflict of Interest*:** This investigator-initiated protocol was supported in part by BioMarin Pharmaceutical Inc. Rani H. Singh and Meghan E. Quirk currently have an investigator-initiated protocol with a material supply agreement with BioMarin Pharmaceutical Inc. Additionally, Rani H. Singh is involved in four sponsor-initiated protocols in collaboration with BioMarin Pharmaceutical Inc.

Table 6-1: Demographic characteristics of patients with PKU with a classified response to BH_4 therapy (n=53)

Characteristic	Definitive Responders (n=19)	Provisional Responders (n=9)	Non-Responders (n=25)
Age (years)	14.3 [6.1-36.8]	10.1 [4.6-17.8]	16.8 [4.9-50.4]
Sex (male)	11 (57.9%)	6 (66.7%)	13 (52.0%)
Weight (kg)	55.8 ± 27.9	39.8 ± 21.6	63.3 ± 29.7
Height (cm)	151.5 ± 22.9	140.0 ± 20.9	153.4 ± 20.1
Dietary Phenylalanine Prescription (mg/day)	704 ± 519[a,b,c]	313 ± 118[b]	389 ± 165[c,d]
Medical Food Prescription (grams protein equivalents/day)	45.8 ± 18.0[e]	48.9 ± 17.0	53.9 ± 12.6
Baseline Plasma Phenylalanine (μmol/L)	523 ± 281[f]	558 ± 319	843 ± 479[f]
Month 1 Plasma Phenylalanine (μmol/L)	210 ± 188[g]	245 ± 139[h]	947 ± 437[g,h]
Diet Record Received from Each Patient (days)	13.6 ± 2.7	14.7 ± 0.7	12.6 ± 3.2

Expressed as median [range], n (%), or mean ± standard deviation

[a] Includes three patients with prescriptions meeting their protein needs through intact protein sources

[b] p= 0.017; comparison of definitive responders and provisional responders

[c] p= 0.011; comparison of definitive responders and non-responders

[d] n=24; one patient did not have an established diet prescription at baseline

[e] n=18; excludes an adult patients who discontinued medical food at age six

[f] p=0.027; comparison of definitive responders and non-responders

[g] p<0.001; comparison of definitive responders and non-responders

[h] p<0.001; comparison of provisional responders and non-responders

Table 6-2: Mixed modeling analysis of dietary protein and energy intake during the first month of BH_4 therapy in all evaluated patients (n=53)

	Typical Intake During Month One of BH_4 Therapy[a]			Mixed Modeling Analysis	
Intake	**Definitive Responders (n=19)**	**Provisional Responders (n=9)**	**Non-Responders (n=25)**	**Effect[b]**	**p-value[c]**
Energy (kcal/day)	1,886 (123)	1,678 (178)	1,778 (108)	*Group*	0.606
				Time	0.872
Total Protein (g/day)	59.5 (4.5)	57.8 (6.5)	57.6 (3.9)	*Group*	0.948
				Time	0.030[d]
Phenylalanine (mg/day)	1,260 (150)[e]	426 (217)[e]	812 (131)	*Group*	0.004
				Time	0.678
Phenylalanine (% prescription)	200.5 (21.5)	135.5 (31.0)	180.8 (19.2)	*Group*	0.226
				Time	0.775
Medical Food (grams protein equivalents/day)	31.0 (4.7)	47.9 (6.8)	39.4 (4.1)	*Group*	0.106
				Time	0.002[f]
Medical Food (% prescription)	73.9 (7.6)	98.0 (10.7)	75.4 (6.5)	*Group*	0.144
				Time	0.014[g]

[a] Least squares means estimate from mixed models; presented at estimate (standard error)

[b] Group*time interaction excluded from all models as Type III test for fixed effects did not indicate significance ($p>0.05$)

[c] Type III test for fixed effects p-value

[d] Model β coefficient: -0.090 grams total protein/day since initiating BH_4 therapy

[e] $p=0.005$; comparison of definitive and provisional responder groups

[f] Model β coefficient: -0.071 grams protein equivalents/day since initiating BH_4 therapy

[g] Model β coefficient: -0.187 percent medical food prescription/day since initiating BH_4 therapy

Table 6-3: Demographic characteristics of evaluated pediatric patients (n=33)

Characteristic	Definitive Responders (n=11)	Provisional Responders (n=9)	Non-Responders (n=13)
Age (years)	9.7 ± 3.4	11.0 ± 5.1	11.1 ± 4.0
Sex (male)	8 (72.7%)	6 (66.7%)	7 (53.9%)
Weight (kg)	39.0 ± 17.5	39.8 ± 21.6	42.6 ± 20.0
Height (cm)	138.7 ± 21.2	140.0 ± 20.9	141.3 ± 20.5
Dietary Phenylalanine Prescription (mg/day)	618 ± 451	313 ± 118	360 ± 188
Medical Food Prescription (grams protein equivalents/day)	36.8 ± 15.7	48.9 ± 17.0	47.1 ± 10.5
Baseline Plasma Phenylalanine (μmol/L)	511 ± 275	558 ± 319	591 ± 460
Month 1 Plasma Phenylalanine (μmol/L)	215 ± 231[a]	245 ± 139[b]	700 ± 428[a,b]
Diet Record Received from Each Patient (days)	12.8 ± 3.2	14.7 ± 0.7	12.3 ± 3.5

Expressed as mean ± standard deviation or n (%)

[a] p=0.0018; pairwise comparison of definitive responders and non-responders

[b] p=0.0056; pairwise comparison of provisional responders and non-responders

Table 6-4: Mixed modeling analysis of dietary protein and energy intake during the first month of BH_4 therapy in pediatric patients with PKU (n=33)

	Typical Intake During Month One of BH_4 Therapy[a]			Mixed Modeling Analysis	
Intake	**Definitive Responders (n=11)**	**Provisional Responders (n=9)**	**Non-Responders (n=13)**	**Effect[b]**	**p-value[c]**
Energy (kcal/day)	1,663 (100)	1,678 (109)	1,626 (92)	*Group*	0.930
				Time	0.881
Total Protein (g/day)	44.8 (4.5)	57.8 (4.9)	52.9 (4.1)	*Group*Time*	0.030[d]
Phenylalanine (mg/day)	1007 (133)[e,f]	428 (122)[e]	525 (122)[f]	*Group*	0.005
				Time	0.305
				*Group*Time*	0.074[g]
Phenylalanine (% prescription)	170.2 (17.4)	136.2 (19.0)	149.9 (16.1)	*Group*Time*	0.046[h]
Medical Food (grams protein equivalents/day)	22.5 (5.0)[i,j]	47.9 (5.5)[i]	40.6 (4.6)[j]	*Group*	0.002
				Time	0.011[k]
Medical Food (% prescription)	69.0 (8.8)	98.8 (9.7)	84.9 (8.1)	*Group*	0.085
				Time	0.071

[a] Least squares means estimate from mixed models; presented at estimate (standard error)

[b] Group*time interaction excluded from the model when Type III test for fixed effects indicated it was not statistically significant ($p>0.05$)

[c] Type III test for fixed effects p-value

[d] Provisional and non-responder groups trended differently over time; group-specific time β coefficients -0.099, 0.015, and -0.250 grams total protein/day since initiating BH_4 therapy for definitive, provisional, and non-responder groups, respectively

[e] p=0.010; comparison of definitive and provisional responder group

[f] p=0.022; comparison of definitive and non-responder group

[g] Group-specific time β coefficient for definitive, provisional, and non-responder: -0.956, 2.093, and -4.871 mg dietary phenylalanine/day since initiating BH_4 therapy, respectively

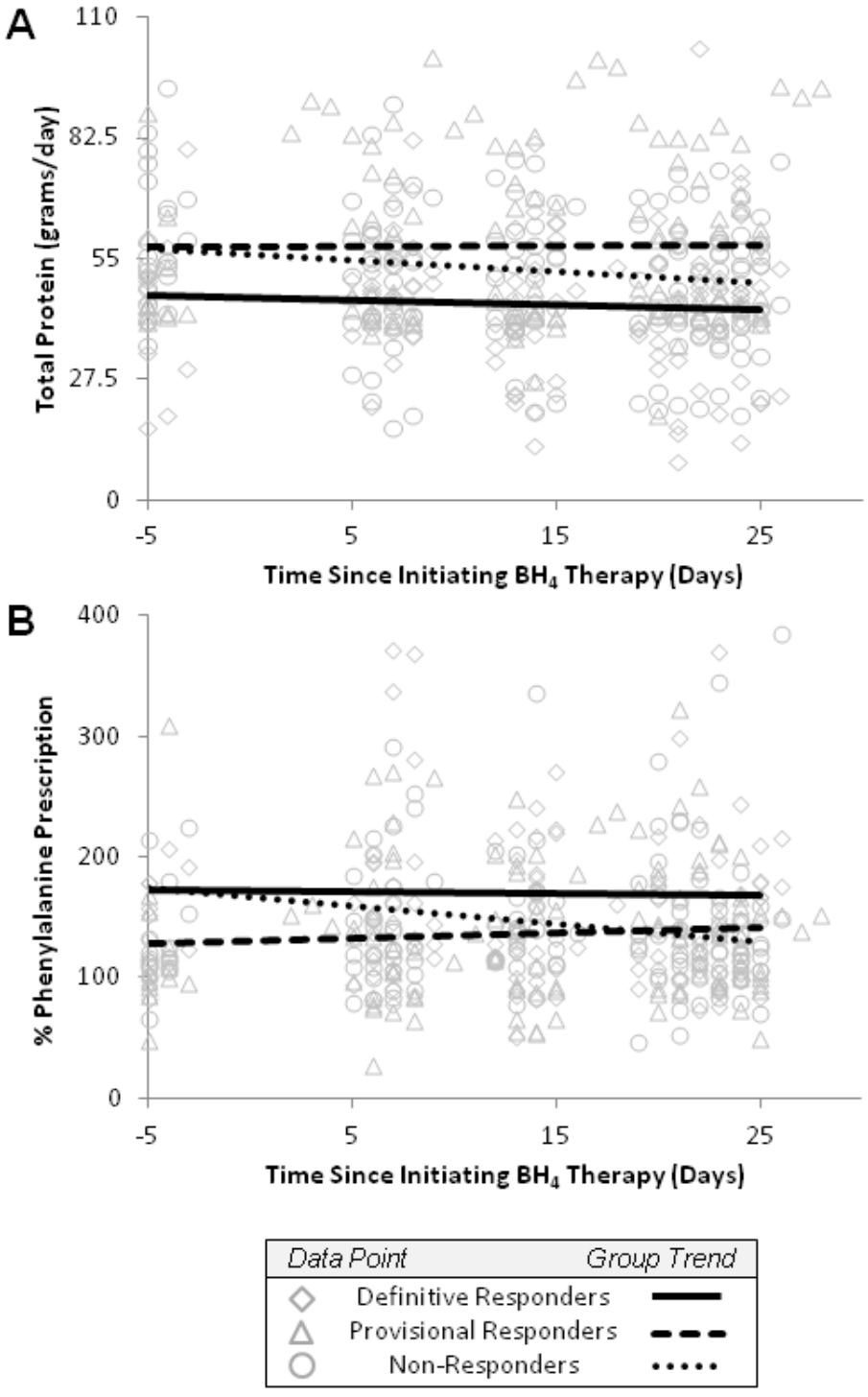
[h] Provisional and non-responder groups trended differently over time; group-specific time β coefficients -0.188, 0.445, and -1.487 percent of dietary phenylalanine prescription/day since initiating BH_4 therapy for definitive, provisional, and non-responder groups, respectively

[i] p=0.002; comparison of definitive and provisional responder groups

[j] p=0.021; comparison of definitive and non-responder groups

[k] Time β coefficients -0.079 grams protein equivalents/day since initiating BH_4 therapy

Figure 6-1: Modeled trends in (A) total protein and (B) phenylalanine prescription compliance in pediatric patients with PKU with a classified BH_4 response (n=33)



CHAPTER 7

SUMMARY OF RESULTS AND CONCLUSIONS

Defining BH_4 responsiveness using a novel set of sequentially applied criteria afforded us the opportunity to identify a subgroup of provisionally responsive patients who could not substantially improve their dietary phenylalanine tolerance despite an initial decrease in plasma phenylalanine concentrations. The separation of the provisional responders from the definitive responders is an important one, as their genotypic and phenotypic information suggests that these are two distinct groups.

PAH genotyping revealed that the majority of provisional responders carry highly deleterious mutations on both alleles, providing little molecular basis for responsiveness. Provisional responsiveness, therefore, may be a transient or false-positive response which occurs when assessing change in plasma phenylalanine concentrations after several weeks. Furthermore, the large number of patients in the non-responder group carrying particularly mild mutations suggests that patient misclassification likely occurred. While preliminary, our results indicate that PAH genotype is a valuable eligibility criterion for BH_4 response evaluation and may be used retrospectively to identify patients whose responses to BH_4 were potentially misclassified. We demonstrated that the AV sum tool may be of clinical utility in this endeavor.

We explored the possibility that changes in reported protein intake over the course of the first month may have contributed to the initial decrease in plasma phenylalanine

concentrations in the provisional responder group. Such a trend was not identified. However, the definitive responder group did report consuming significantly more dietary phenylalanine during the first month of BH_4 therapy when compared to the provisional responders. When restricted to a pediatric analysis, the definitive responders not only reported consuming more dietary phenylalanine but also less medical food than the provisional responders. Given that plasma phenylalanine concentrations at baseline did not differ between the definitive and provisional group, the greater dietary phenylalanine intake suggests that the definitive responder group has less severe phenotype, supporting our PAH genotype data.

Caveats to and Limitations of Implemented BH_4 Classification Protocol

The clinical algorithm presented in Chapter 4 should serve as a starting point for amending BH_4 response classification rather than be regarded as the ideal protocol. The relatively high number of patients that remained unclassified (5/58 patients), the fairly large group of provisional responders, and the identification of patients with mild genotypes in the non-responder group suggest components of our algorithm should be fine-tuned. We recommend systematically assessing dietary phenylalanine and medical food prescriptions prior to initiating patients on a BH_4 response protocol. A more thorough evaluation and characterization of patients in the days or weeks prior to initiating BH_4 therapy may have clarified some clinical ambiguity which arose. Furthermore, one month is most likely too long of a time period to assess percent change in plasma phenylalanine concentrations attributed to BH_4 therapy. Our research group has previously demonstrated that a week-long, educational intervention can lead to a

significant decrease in plasma phenylalanine concentration [196]. This indicates that the longer the protocol, the greater potential the results are to be confounded by factors other than BH_4. Future protocols must be long enough to capture all potential responders, must be flexible enough to accommodate patients' schedules, and must be short enough to prevent misclassification. Finally, the diet liberalization criteria used to classify patients in our algorithm needs to be further developed. The threshold for dietary phenylalanine tolerance used to allocate patients into either the definitive or provisional responder group is admittedly arbitrary. The clinical rationale was that a 300 mg/day increase in dietary phenylalanine tolerance would approximately double a severely affected patients' prescription. Alternative criteria—such as doubling of baseline dietary phenylalanine prescription, percent of total protein need, and/or perceived added benefit to the patient—should be considered. Ultimately, the classification criteria should be an evidence-based guideline maximizing the long-term benefits of BH_4 therapy.

Need for External Validation

PKU is a rare disorder and as such, rigorous trials and unbiased sampling strategies are often difficult in a single-center study. Despite our clinic population being large, our classification approach partitioned patients into three uneven groups. This naturally affected the statistical analyses we performed and limited our ability to draw certain conclusions. While our results are promising, their external validity needs to be verified, especially with respect to the genotype and diet liberalization findings.

External validation may be possible with the expansion of www.biopku.org (curator: N. Blau). This public-access database captures the results of BH_4 responsiveness protocols internationally. Baseline and liberalized diet prescription are currently not included in the database. Our results suggest dietary phenylalanine tolerance may be valuable addition to help clarify future assessments of genotype-phenotype associations. With more than 700 patients' information cataloged, the approach to capturing dietary data should consider retrospective analyses as well as prospective implementation.

Clinical Implications for the PKU Population

Despite the noted limitations, our work underscores the need to reassess the clinical approach to BH_4 classification. The field is primed for a change, as evidenced by expert opinions beginning to recommend a multi-phase approach to BH_4 classification [118]. Our data provide important clinical insight into factors that need to be considered when testing BH_4 responsiveness in patients with PKU, both from a clinical standpoint and from a methodological standpoint. Biochemical, genetic, and nutritional characterization of patients are essential to adequately characterize BH_4 responsiveness.

Of interest is the relationship between ability to liberalize dietary phenylalanine restrictions and PAH genotype. In patients who experienced ≥15% decrease in plasma phenylalanine concentrations, our criteria for change in diet prescription were able to segregate patients with a severe PAH genotype from those with a milder genotype. This has important implications in the clinical setting. In a survey of 19 European countries, less than half of clinics reported routinely genotyping their patients with PKU [182].

Thus, the ability to liberalize dietary phenylalanine restrictions may serve as a proxy for genotype severity when PAH genotype is not clinically available. Alternatively, PAH genotype may elucidate which patients may maximally benefit from BH_4 therapy with an ability to liberalize dietary restrictions. Our preliminary results suggest that the simple tool, the AV sum, may have clinical utility in predicting BH_4 responsiveness or at the very least determining which patients do not have a molecular basis for responsiveness.

Furthermore, our assessment of dietary intake in patients during the month prior to classification offers critical insight for metabolic dietitians and clinicians. Patients were instructed to maintain a consistent dietary intake during the first month of BH_4; from the diet records received, our analysis suggests our patients generally followed those instructions, although the pediatric non-responder group did appear to become slightly more compliant with dietary phenylalanine intake over time. The lack of overt or group-specific trends in dietary protein intake must be taken in context of reporting bias, as recorded intake is often not the same as actual intake [200, 201]. A difference in protein intake over time may still be an underlying contributor to the emergence of the provisionally responsive group, but our method of capturing the data may not have been sensitive enough to identify the change in dietary intake. At a clinical level our data suggest the need for metabolic dietitians to continually reinforce accurate diet record techniques with their patients. Additionally, the excess in reported dietary phenylalanine consumption and less than optimal medical food adherence coupled with the elevated plasma phenylalanine concentrations at baseline emphasize the fact that non-compliance

is typical within this population. It is the role of emerging therapies like BH_4 to help counteract the barriers to compliance and promote metabolic control.

Clinical Implications for BH_4 Beyond the PAH Pathway

As clinically explored herein, BH_4 therapy can dramatically impact the PAH pathway. Yet BH_4 therapy has implications far beyond merely improving the metabolism of phenylalanine. BH_4 plays a critical role in the synthesis of dopamine, serotonin, norepinephrine, and epinephrine as cofactor for the three aromatic amino acid hydroxylase systems (phenylalanine, tyrosine, and tryptophan hydroxylases). A state of excess cofactor, which is created with BH_4 therapy, may affect the metabolism of the aromatic amino acids, and thereby affect neurotransmitter and hormone synthesis. Furthermore, BH_4 is also an essential cofactor for the endothelial nitric oxide synthase (eNOS) system, responsible for regulating vasodilation and vascular tone. In a biological state of altered BH_4, dihydrobiopterin, and eNOS stoichiometry, the eNOS system becomes uncoupled [204, 205], causing a pro-oxidant state [206-209] associated with cardiovascular damage [210-212]. Thus, BH_4 therapy has the potential to serve as a therapeutic for disorders related to neurotransmitters and/or eNOS derangement, such as cardiovascular disease, Parkinson's disease, and Alzheimer's disease [213-219].

From a broader perspective, the emergence of BH_4 therapy for the treatment of PKU may pave the way for future cofactor therapies. PKU, as a monogenic, autosomal recessive disorder, represents a straightforward model of the dysfunction of one specific enzyme. Enzymatic functionality was enhanced by supraphysiological concentrations of its

cofactor. This may serve as a model for other autosomal recessive disorders, such as those which can be identified through newborn screening [220]. But beyond severe enzymatic derangement, the characterization and understanding of the functionality of BH_4 therapy may have implications for the general population. It is believed that BH_4 therapy can create an optimal cofactor concentrations for PAH to function [121]. This finding may be explored and applied other enzyme systems to find the optimal working range of cofactor concentrations to achieve a particular outcome of interest.

Future Directions

BH_4 therapy has dramatically shifted the clinical approach to and management of PKU. In the immediate future, the definition of BH_4 responsiveness needs to be expanded and standardized at an international level. This will promote the prospective collection of data that are able to be compared and consolidated. To address previously collected data, efforts must be made to retrospectively assess patient misclassification. Our data suggest both genotype severity and ability to liberalize dietary phenylalanine restrictions may be valuable in this endeavor. As the first pharmacological agent for the treatment of PKU, BH_4 has set the stage for the new drug therapies on the horizon [221, 222]. The clinical and methodological principles used in the implementation of BH_4 response protocols can be applied to the evaluation of emerging therapeutics.

Conclusions

Our evaluation of BH_4 responsiveness in a clinical cohort of 58 patients with PKU has revealed limitations to the common approach for classifying patients. Dichotomizing

responses based only on change in plasma phenylalanine concentrations is insufficient and has the potential to lead to patient misclassification. It is of utmost importance to comprehensively evaluate BH_4 responsiveness from biochemical, genetic, and nutritional perspectives.

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APPENDIX A: HETEROGENEITY OF PROTOCOLS CLASSIFYING BH_4 RESPONSIVENESS IN PATIENTS WITH PKU

The table below outlines the various protocols that have classified patients as responsive to BH_4 therapy. Excluded are expert opinions. Also excluded are protocols evaluating the long-term effectiveness of BH_4 therapy.

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
10	Unrestricted 2d prior	0, 2, 4, 24	-	-	-	• ↓ serum Phe concentrations	[112]
10 (T_0, T_{24}); 5 (T_{36}, T_{48})	Unrestricted 2d prior	0, 4, 24, 52	-	-	-		
20	Unknown	0, 8	-	-	-	• ↓ blood Phe concentrations	[148, 149]
20	Unknown	0, 8, 33	-	-	-	• ↓ plasma Phe concentrations	[142]
20	Unknown	-3, 0, 4, 8, 21	Moderately elevated plasma Phe concentrations	100 mg/kg	-3		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Infants breastfed without restriction	0, 4, 8	-	-	-	• ↓ blood Phe concentrations	[163]
10[b]	Unknown	0, 5d	-	-	-	• ↓ plasma Phe concentrations	[132]
0 (T_{0-48}); 10 (T_{48-96}); 5 (T_{96-152}); 0 (T_{152+});	100-150 mg Phe/d	Approx. every 4h over course of 5d	-	-	-	• ↓ plasma Phe concentrations	[131]
0 (T_{0-4}); 10 (T_{4-52}); 5 (T_{52-100}); 0 (T_{100+});	100-150 mg Phe/d	Approx. every 4h over course of 5d	-	-	-	• Sustained ↓ plasma Phe concentrations (<600 μmol/L)	[131]
20	Unknown	0, 8	-	-	-	• ≥50% ↓ in plasma Phe concentrations	[20]
20	Unknown	0, 4, 8	-	-	-	• ↓ plasma Phe concentrations	[122]
20	Unknown	-3, 0, 4, 8, 21	Unknown	100 mg/kg	-3		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, 4, 8	-	-	-	• ≥5% ↓ in plasma Phe concentrations between 0h and 4h and between 4h and 8h; hydroxylation slope >3.75[c]	[150]
20	100 mg/kg Phe meal -1h; infants breastfeed throughout; children received 10 mg/kg Phe between 6h-8h	0, 4, 8, 15	All patients evaluated	100 mg/kg	-1	• ≥30% ↓ in plasma Phe concentrations by 15h	[124]
10	Infants fasted 6h, children fasted overnight	0-3[d]	All evaluated patients	6 mg/kg labeled Phe	0	• ≥15% increase in Phe oxidation	[124]
20	Infants fasted 4h, no dietary restrictions (breastfed infants)	0, 4, 8, (24)[e]	-	-	-	• ≥30% ↓ in plasma Phe concentrations	[164]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, 4, 8, 16, 24	-	-	-	• ↓ plasma Phe concentrations (assessed at 8h)	[151]
20	100-150 mg Phe meal 0.5h after BH_4	0, 4, 8	All evaluated patients	100-150mg Phe meal 0.5h after BH_4	0.5	• ↓ plasma Phe concentrations	[165]
10	Unrestricted diet throughout	0, 4, 8, 24	-	-	-	• ≥20% ↓ in plasma Phe concentrations	[137]
10 (T_0, T_{24}); 5 (T_{36}, T_{48})	Unrestricted diet throughout	0, 4, 8, 24, 52	-	-	-		
20[f]	Unrestricted diet throughout	0, 4d, 7d	-	-	-		
10	Consistent with baseline dietary intake	0, 4, 8, 24	-	-	-	• ↓ plasma Phe concentrations	[133]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, 4, 8	-	-	-	• ↓ plasma Phe concentrations	[152]
20	Unrestricted diet 2d prior	0, 4, 8, 24	-	-	-	• "Responder" if ≥30% ↓ in plasma Phe concentrations at 8h • "Slow responder" if ≥30% ↓ in plasma Phe concentrations at 12-16h	[14, 143]
20	Unrestricted diet 2d prior	-3, 0, 4, 8	Patients with Phe concentrations <360 μmol/L	100 mg/kg	-3		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unrestricted diet 2d prior	-27 through -3; -3, 0, 4, 8	Mild HPA patients	100 mg/kg	-27, -3	• "Responder" if ≥30% ↓ in plasma Phe concentrations at 8h • "Slow responder" if ≥30% ↓ in plasma Phe concentrations at 12-16h	[143]
20 (>36 months old); 7.5 (<36 months old)	Unrestricted diet	0, 1, 2, 4, 6, 8, 12, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations at 12h	[157]
20	Phe restricted diet throughout	-3, 0, 3, 7, 11, 21	All tested patients	100 mg/kg	-3	• ≥30% ↓ in plasma Phe concentrations at 21h	[144]
20	Fasting 4h at T_0; infants breastfed or bottle fed throughout	0, 4, 8, 24	-	-	-	• ≥30% ↓ in serum Phe concentrations at 8-24h	[12]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20[b]	Consumed prescribed diet along with an additional 25 mg/kg/d Phe for 2d prior to starting BH_4 and throughout the study	0, 8, 36, 7d	All tested patients	25 mg/kg/d	T_{-2d}, $T_{0\text{-}7d}$	• "Responder" if ≥30% ↓ in plasma Phe concentrations after 8h • "Slow responder" " if ≥30% ↓ in plasma Phe concentrations after 36h	[13]
10	Consistent with baseline dietary intake	0, 4, 8, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations • "Adequate response" if plasma Phe concentrations ↓ 17-30% in 24h[g]	[129, 138]
10 (T_0); 20 (T_{7d}); 40 (T_{14d})[h]	Consistent with baseline dietary intake	0, 24 (for each of the 3 doses)	-	-	-		
10 ($T_{0\text{-}7d}$); 20 ($T_{14\text{-}21d}$)	Consistent with baseline dietary intake	0, 1d, 3d, 7d (for both doses)	-	-	-		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unrestricted during protocol	0, 4, 8, (24)[e]	-	-	-	• ≥30% ↓ in plasma Phe concentrations at 8h	[145]
20	Unrestricted during protocol	-3, 0, 4, 8, (24) [e]	Patients with Phe concentrations <360 μmol/L	100 mg/kg	-3		
20 (T_0, T_{24})	Unknown	0, 4, 8, 12, 24, 36	-	-	-	• "Rapid responder" if 8h, 24h, and 48h plasma Phe concentrations ↓ by ≥30%, ≥50%, and ≥50%, respectively • "Moderate responder" if 8h, 24h, and 48h plasma Phe concentrations ↓ by ≥20%, ≥30%, and ≥50%, respectively • "Slow responder" if 8h, 24h, and 48h plasma Phe concentrations ↓ by <20% ≥20%, and ≥30%, respectively	[115]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Overnight fasted at T_0; Phe-restricted diet throughout the protocol	-3, 0, 4, 8, 24	All tested patients	100 mg/kg	-3	• ≥30% ↓ in plasma Phe concentrations at 8h *OR* • ≥50% ↓ in plasma Phe concentrations at 24h	[146]
20	Phe intake not modified during the protocol	-24, -20, -16, -12, 0, 4, 8, 12, 24	-	-	-	• At least one plasma Phe concentration (T_{4-24}) was ≥30% lower than T_0 *OR* • Patient exceeded the lower limits of their personal 95% confidence interval for plasma Phe concentration (constructed T_{-24-0}) with at least one measurement (T_{4-24})	[116]
20	Infants breastfed throughout	0, 2, 4, 8, 24	-	-	-	• ≥35% ↓ in plasma Phe concentrations	[169]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, (2), 4, 8, (12), 24[e]	-	-	-	• "Rapid responder" if 8h and 24h plasma Phe concentrations ↓ by ≥30,% and ≥50%, respectively • "Moderate responder" if 8h and 24h plasma Phe concentrations ↓ by ≥20% and 30-50% respectively • "Slow responder" if 8h and 24h plasma Phe concentrations ↓ by <20% and ≥20%, respectively • "Non-responder" if plasma Phe concentrations experience <20% ↓ at all time points • "Not determined" if 8h and 24h plasma Phe concentrations ↓ by ≥20% and <30%, respectively	[127]
20 (T_0, T_{24})	Unknown	0, 4, 8, 24, 32, 48	-	-	-		
20	Unknown	-3, 0, 4, 8, 24	A subset of patients evaluated (no criteria given)	100 mg/kg	-3		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 8h	[153]
20	3h fasted at T_0; meal given 0.5h after BH_4; dietary intake consistent with baseline intake	0, 4, 8, 12, 24	-	-	-	• "Responder" if ≥30% ↓ in plasma Phe concentrations at 8h • "Slow or partial responder" if ≥30% ↓ in plasma Phe concentrations at 12-16h	[15, 147]
20	Dietary intake consistent with baseline intake	-3, 0, 4, 8, 12, 24	Patients with Phe concentrations <360 μmol/L	100 mg/kg	-3	• "Responder" if ≥30% ↓ in plasma Phe concentrations at 8h • "Slow responder" if ≥30% ↓ in plasma Phe concentrations at 12-16h	[147]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Unknown	0, 4, 8	-	-	-	• Different cutoffs for ↓ in plasma Phe concentrations at 8h explored, including : ≥20, ≥30, ≥40, and ≥50%	[117]
20	Unknown	0, 4, 8, 24	-	-	-		
20	Unknown	0, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 24h	[154, 155]
20	Dietary intake consistent with baseline intake	0, 24[i]	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 24h	[160]
10 ($T_{0, 1d, 2d, 3d, 4d, 5d, 6d, 7d, 8d}$)	3h fasted at T_0; Doses T_{1d-7d} taken 10-15 minutes before breakfast; dietary intake consistent with baseline intake	0, 8d	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 8d	[113]
20	Phe-restricted diet throughout	0, 8d	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 8d	[16]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	Dietary intake consistent with baseline intake	0, 2, 4, 6, 8	-	-	-	• "Responder" if ≥30% ↓ in plasma Phe concentrations after 8h • "Fast responder" if ≥30% ↓ in plasma Phe concentrations after 2h	[161]
20	Phe-unrestricted diet during protocol	0, 2, 4, 6, 8, 12, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations after 24h	[158]
20	Unknown	0, 8-24[k]	-	-	-	• "Partial responder" if 10-29% ↓ in plasma Phe concentrations • "Full responder" if ≥30% ↓ in plasma Phe concentrations	[119]
10	Unknown	0, 8d	-	-	-		
20 (T_0, T_{24})	2 weeks before and during testing, Phe intake distributed equally throughout day	0, 4, 8, 12, 24, 32, 48	-	-	-	• ≥30% ↓ in plasma Phe concentrations during testing	[223]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20	3-4d before testing, encouraged to consume unrestricted diet	0, 8, 24, 48	-	-	-	• ‘‘Responder” if blood Phe concentrations ↓ by ≥30% within 24h • ‘‘Slow responder” if blood Phe concentrations ↓ by <20% at 8h and ≥20% but <30% at 24h • ‘‘Not clear” if blood Phe ↓ s by ≥30% at 8h, and <20% at 24h	[159]
20	Trial conducted in infants before initiating low-Phe diet	0, 4, 8, 24	-	-	-	• ≥30% ↓ in plasma Phe concentrations at any time point during the trial	[166]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
10, 20, and 30 (T_0, T_{7d}, and T_{14d})[l]	6d before BH_4 and throughout trial, encouraged to eat ~50 mg Phe/kg/d	*Morning and evening:* -3d, -2d, -1d, *Dose 1*: 0, 4, 8, 12, 16, 24, 36, 48, 60, 72, 84 *Dose 2*: 0, 4, 8, 12, 16, 24, 36, 48, 60, 72, 84 *Dose 3*: 0, 4, 8, 12, 16, 24,	-	-	-	• ≥30% ↓ in plasma Phe concentrations T_{4-24} during any of the dose trials • Exceeded the lower limits of personal fluctuation (constructed using the 7 measurements prior to the Dose 1 trial ±3standard deviations)	[139]
0	Phe and tyrosine restricted diet throughout protocol	-3, 0, 1.5, 3, 6, 9, 12, 24	All tested patients	100 mg/kg	-3	• No criterion used; compared the intrapersonal variation between the three testing scenarios	[140]
20		-3, 0, 1.5, 3, 6, 9, 12, 24	All tested patients	100 mg/kg	-3		
20		0, 3, 4.5, 6, 9, 12, 15, 27	All tested patients	100 mg/kg	3		

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
20 ($T_{Week\ 1}$); 10 (T_{Week2}); 5 (T_{Week3})	Unrestricted diet initiated 3d prior to and throughout protocol	*Week 1:* 0, 1d, 2d, 5d *Week 2*: 7d, 8d, 9d, 12d *Week 3*: 14d, 15d, 16d, 19d, 21d	-	-	-	• ≥30% ↓ in plasma Phe concentrations in one or more of the following scenarios: o T_0 vs. average of $T_{5\&7}$ o T_0 vs. average of $T_{12\&14}$ o T_0 vs. average of $T_{19\&21}$	[130]
10 ($T_{0\text{-}7d}$) 20 ($T_{7d\text{-}30d}$)[m]	Dietary intake consistent with baseline intake	0, 8d, 16d, 30d	-	-	-	• ≥30% ↓ in plasma Phe concentrations during trial *OR* • Lowering of plasma Phe concentrations <360 µmol/L	[17]
20[n]	Unknown	0, 4, 8, (24)[e]	-	-	-	• ≥30% ↓ in blood Phe concentrations during trial • If estimated PAH activity was <1% patient was automatically classified as a non-responder, regardless of challenge results	[156]

APPENDIX A, CONTINUED

BH_4 Dose (mg/kg)	Diet Regimen Prior to and During Test	Time(s) Sample Collected[a]	Phenylalanine Load			Responsiveness Criteria	Ref
			Patient Criteria	Amt of Phe	Time (hrs)		
10, 20	Dietary intake consistent with baseline intake	*10 mg/kg dose:* 0, 24 *20 mg/kg dose:* 0, 24	-	-	-	• "Acute responder" if ≥30% ↓ in blood Phe concentrations or Phe:tyrosine ratio in between T_0 and T_{24} • "Chronic responder" if ≥30% ↓ in blood Phe concentrations or Phe:tyrosine ratio in between T_0 and T_{28d}	[128]
20 ($T_{1\ Month}$)	Dietary intake consistent with baseline intake	0, 1d, 7d, 14d, 28d	-	-	-		
20	Patients had plasma Phe concentrations >360 μmol/L at T_0	-24, -20, -16, 0, 4, 8, 24	-	-	-	• ≥30% ↓ in blood Phe concentrations at 8h • ≥30% ↓ in blood Phe concentrations at 24h	[162]

APPENDIX A, CONTINUED

Abbreviations: Amt, amount; HPA, hyperphenylalaninemia; Phe, phenylalanine; Ref, reference

[a] Times are in hours and 0h corresponds to BH_4 dose unless otherwise noted

[b] Dose divided into two doses

[c] Slope of hydroxylation = $(\sum y_i - \bar{y}) * x_i)/(\sum x_i - \bar{x})$, where yi = % of phenylalanine elimination at time 0, 4, and 8h (x_i) [150]

[d] Sample was ^{13}C-labeled breath samples

[e] Parenthetical times not preformed in all subjects

[f] 20 mg/kg/d dose of BH_4 split into 3 doses over the course of the day

[g] Response criterion not included in [129]

[h] Single doses given with a one-week washout period between each dose

[i] Indicated blood phenylalanine analyzed three times prior to enrollment, but do not use the data to classify responsiveness

[j] Dose in Weeks 11-22 were dependent on

[k] Time points to assess plasma phenylalanine not definite, only listed as a range

[l] Dose order randomized; single dose given on each of the days (T_0, T_{7d}, T_{14d})

[m] Dose was increased to 20 mg/kg/d only if there was no response seen with the first week of the 10 mg/kg/d dose

APPENDIX A, CONTINUED

[n] Before 1999, BH_4 used consisted of a mixture of 6R-BH4 (active) and 6S-BH4 (inactive); a 20 mg/kg/d dose corresponded to 13.3 mg/kg/d dose of the active form

APPENDIX B: SENSITIVITY ANALYSIS USING GENERAL LOGIT MODELS TO EVALUATE PROTEIN INTAKE PRIOR TO BH_4 RESPONSE CLASSIFICATION

For each patient, an individual liner model of intake over time was constructed for each of the six dietary intakes of interest. The resulting model-based estimates of intake at baseline (individual model intercept) and change over time (modeled slope) for each patients were used as predictors in generalized logit models to alternatively assess the findings of mixed modeling analysis. For each logit model, BH_4 response group was the outcome of interest and the provisional responders served as the referent group. Models were evaluated for all patients (n=53) and the pediatric-restricted subgroup (n=33). Statistical analyses were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

Table B.1 displays the results of the full analysis. Similar to what was found in the mixed modeling analysis, the logit model suggested that the definitive responder group was more likely to consume greater amounts of phenylalanine when compared to the provision responders (OR: 1.005, CI: [1.001, 1.009]). No other difference emerged as being statistically significant.

Table B.2 summarizes the pediatric-restricted analysis. Again, the logit model revealed that the definitive responders were at greater odds to consume more dietary phenylalanine compared to the provisional responders (OR: 1.005, CI: [1.000, 1.010]). Pediatric

definitive responders were a lower odds to consume greater amounts of medical food compared to the provisional responders (OR: 0.9, CI: [0.830,0.975]), similar to what was seen it the mixed model analysis. No group-specific trends over time could be identified.

The general logit analysis had findings similar to the mixed modeling analysis. Definitive responders appear to be at greater odds of consuming more dietary phenylalanine when compared to the provisional responders, both in the full and pediatric analyses. The pediatric definitive responders were also at lower odds of consuming greater amounts of medical food compared to the provisional responder group. These findings strengthen our ability to conclude that the definitive responders are collectively a milder group when compared to the provisional responders. We did not see the different trends in total protein and percent of phenylalanine prescription intake over time seen through linear mixed modeling analysis, when comparing pediatric non-responders and provisional responders. There are several possible explanations for this. First, the sample size for this analysis is quite small, especially for the pediatric-restricted analysis. Furthermore, the asymptotic statistic estimates may not be valid. The odds ratios for the trends in intake of total protein and medical food over time appeared to suggest dramatic differences when compared to the provisional responder. However, the confidence intervals were wide, leading to non-significance. This analysis, therefore, should serve as an indicator of patterns.

Table B-1: Logistic regression analysis with all evaluated patients (n=53)

Intake of Interest	Model Effect		Odds Ratio	CI	Estimate p-value
	Parameter	**Response Group**			
Energy (kcal/day)	Baseline Intake	Definitive Responder	1.001	[0.999, 1.003]	0.308
		Non-Responders	1.000	[0.999, 1.002]	0.594
	Intake Over Time	Definitive Responder	0.990	[0.935, 1.049]	0.744
		Non-Responders	0.998	[0.944, 1.055]	0.938
Total Protein (g/day)	Baseline Intake	Definitive Responder	1.013	[0.968,1.061]	0.568
		Non-Responders	1.004	[0.960, 1.050]	0.863
	Intake Over Time	Definitive Responder	0.277	[0.028, 2.753]	0.273
		Non-Responders	0.300	[0.031, 2.868]	0.296
Phenylalanine (mg/day)	Baseline Intake	Definitive Responder	1.005	[1.001, 1.009]	0.026
		Non-Responders	1.004	[1.000, 1.008]	0.072
	Intake Over Time	Definitive Responder	0.957	[0.813, 1.127]	0.602
		Non-Responders	0.962	[0.819, 1.131]	0.643
Phenylalanine (% prescription)	Baseline Intake	Definitive Responder	1.015	[0.995, 1.035]	0.132
		Non-Responders	1.012	[0.993, 1.032]	0.212
	Intake Over Time	Definitive Responder	0.939	[0.557, 1.583]	0.813
		Non-Responders	0.908	[0.542, 1.523]	0.715
Medical Food (grams protein equivalents/day)	Baseline Intake	Definitive Responder	0.957	[0.914, 1.003]	0.066
		Non-Responders	0.980	[0.938, 1.025]	0.379
	Intake Over Time	Definitive Responder	0.373	[0.002, 86.319]	0.723
		Non-Responders	0.089	[<0.001, 14.783]	0.354
Medical Food (% prescription)	Baseline Intake	Definitive Responder	0.939	[0.840, 1.050]	0.271
		Non-Responders	0.940	[0.840, 1.051]	0.274
	Intake Over Time	Definitive Responder	0.406	[0.028, 5.824]	0.507
		Non-Responders	0.437	[0.031, 6.124]	0.539

Table B-2: Logistic regression analysis with all pediatric patients (n=33)

Intake of Interest	Model Effect		Odds Ratio	CI	Estimate p-value
	Parameter	**Response Group**			
Energy (kcal/day)	Baseline Intake	Definitive Responder	1.000	[0.997, 1.003]	0.973
		Non-Responders	1.000	[0.997, 1.002]	0.730
	Intake Over Time	Definitive Responder	1.000	[0.932, 1.072]	0.989
		Non-Responders	0.980	[0.914, 1.051]	0.579
Total Protein (g/day)	Baseline Intake	Definitive Responder	0.944	[0.878, 1.014]	0.114
		Non-Responders	0.990	[0.931, 1.053]	0.748
	Intake Over Time	Definitive Responder	0.077	[<0.001, 6.903]	0.263
		Non-Responders	0.034	[<0.001, 2.749]	0.132
Phenylalanine (mg/day)	Baseline Intake	Definitive Responder	1.005	[1.000, 1.010]	0.035
		Non-Responders	1.002	[0.997, 1.007]	0.403
	Intake Over Time	Definitive Responder	0.928	[0.738, 1.166]	0.520
		Non-Responders	0.827	[0.655, 1.044]	0.110
Phenylalanine (% prescription)	Baseline Intake	Definitive Responder	1.013	[0.987, 1.039]	0.345
		Non-Responders	0.995	[0.968, 1.024]	0.748
	Intake Over Time	Definitive Responder	0.877	[0.400, 1.923]	0.744
		Non-Responders	0.483	[0.207, 1.128]	0.093
Medical Food (grams protein equivalents/day)	Baseline Intake	Definitive Responder	0.900	[0.830, 0.975]	0.010
		Non-Responders	0.967	[0.906, 1.032]	0.309
	Intake Over Time	Definitive Responder	0.052	[<0.001, 117.289]	0.453
		Non-Responders	0.021	[<0.001, 22.142]	0.275
Medical Food (% prescription)	Baseline Intake	Definitive Responder	0.947	[0.867, 1.035]	0.228
		Non-Responders	0.961	[0.879, 1.050]	0.374
	Intake Over Time	Definitive Responder	0.181	[0.006, 5.476]	0.326
		Non-Responders	0.206	[0.007, 6.076]	0.360