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Teresa D. Douglas Date

Impact of sapropterin (tetrahydrobiopterin, BH4) treatment, with and without diet liberalization, on monoamine status and quality of life in a phenylketonuria (PKU) cohort

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Masters of Science, University of Hawaii-Manoa, 2005

Advisor: Rani H. Singh, PhD RD

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2012

Abstract

Impact of sapropterin (tetrahydrobiopterin, BH4) treatment, with and without diet liberalization, on monoamine status and quality of life (QOL) in a phenylketonuria (PKU) cohort

By Teresa D. Douglas

Background: Phenylketonuria is an autosomal recessive inborn error of metabolism characterized by impaired phenylalanine hydroxylase activity. To prevent neurological disability caused by high neurotoxic phenylalanine (Phe) concentrations, a strict low Phe, medical food (formula) based diet from infancy is required. Sapropterin is a pharmaceutical for treating PKU that can lower blood Phe and increase dietary Phe tolerance. **Objective**: To evaluate sapropterin's effect on urinary monoamine neurotransmitter concentrations and whether subsequent diet liberalization improves QOL outcomes. **Methods:** 58 PKU subjects were asked to provide an overnight 12 hour urine sample, diet record, and plasma amino acid blood draw for 5 study visits: Baseline, 1 month after initiating sapropterin, then 4, 8, and 12 months. Those above age 10 were asked to complete a self-report QOL questionnaire. Responders (≥ 15% decline in plasma Phe the first month) continued taking sapropterin and were identified after 3 month diet challenge as "definitive" or "provisional" dependent on increases in Phe tolerance. Sapropterin nonresponders identified at one month remained on their standard PKU diet regimen. Urinary monoamines, analyzed by HPLC, are reported in ratio to creatinine. Data was analyzed with linear regression techniques in SPSS 19.0 while controlling for age. **Results:** Provisional responders had significantly lower epinephrine than the other two groups (P=.018). At one month, homovanillic acid (HVA) had significantly increased in the study cohort (P=.015). When controlling for sapropterin response category, HVA increase was significant only for nonresponders (P=.016) but not sustained longterm. 5-hydroxyindole acetic acid (5HIAA) for definitive responders had a modest decline over 1 year (P=.019). Plasma Phe and formula protein were strongly associated with longterm monoamine outcomes (P<.0001). No other significant variations to monoamines occurred. All three sapropterin response groups had significant improvement in longterm subscores measuring impact of PKU on life quality (nonresponder P=.05, provisional P=.01, definitive P<.0001). Definitive responders had longterm improvement in subscores measuring satisfaction with life and health management (P=.001). Both provisional and definitive groups experienced longterm improvement in total QOL scores (P=.001, P=.028). For definitive responders, QOL improvement associated most strongly with increases to dietary Phe tolerance (P=.005). **Conclusions:** Sapropterin has potential to improve dopamine metabolism in PKU, though plasma Phe control and dietary management remain critical to patient health. Lessening dietary restriction when possible improves patient satisfaction and overall QOL, while PKU's impact on life quality improved for all long-term study participants.

Impact of sapropterin (tetrahydrobiopterin, BH4) treatment, with and without diet liberalization, on monoamine status and quality of life in a phenylketonuria (PKU) cohort

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CHAPTER 1: INTRODUCTION

Focus of Investigation

The purpose of this research investigation was to determine whether the levels of neurotransmitter markers and quality of life (QOL) for people living with Phenylketonuria (PKU) change as a result of treatment with sapropterin (tetrahydrobiopterin, BH4).

Phenylketonuria is among the most common of the inborn errors of amino acid metabolism, with an approximate incidence of 1 case per 15,000 live births in the United States [6, 7] and worldwide [8]. Dietary management has historically been the only effective treatment for PKU; untreated patients or those with poor dietary adherence experience the accumulation of harmful levels of phenylalanine (Phe), which can lead ultimately to neurological and psychological dysfunction [9, 10]. Though newborn screening has improved patient outcomes in western society through early diagnosis and early nutrition treatment, neurological deficits are still frequent within the PKU population [6, 11, 12].

Kuvan® (sapropterin dihydrochloride) is an FDA approved prescription pharmaceutical form of tetrahydrobiopterin (BH4). Several studies have demonstrated that the sapropterin form of BH4 effectively decreases plasma Phe concentrations and improves protein tolerance for at least 30% of PKU patients [13-16]. BH4 functions as a cofactor for not only phenylalanine hydroxylase (PAH), the enzyme responsible for converting Phe into tyrosine, but also for other pteridine dependent mono-oxygenase

enzymes involved in neurotransmitter synthesis [17, 18]. Nevertheless, the full range of biological effects of sapropterin in the treatment of PKU, particularly in neurological outcomes, has yet to be explored.

Central Hypothesis

PKU subjects receiving sapropterin will exhibit increased urinary concentrations of catechol and serotonergic analytes due to improved plasma Phe control and direct cofactor activity in the monoamine metabolic pathway. Additionally, those who respond to sapropterin with improved plasma Phe control and liberalized diet will exhibit improved quality of life (QOL) scores.

Objective and Specific Aims

Because sapropterin has the potential to further minimize PKU related neurological impairment, this proposal's objective is to study changes to important markers of neurological health, namely urine monoamine neurotransmitter concentrations and QOL, in a cohort of PKU subjects prescribed Kuvan®.

To accomplish this objective, the following specific aims were addressed:

Specific Aim 1

To investigate monoamine neurotransmitter concentrations in the urine of PKU subjects responsive and nonresponsive to sapropterin during 1 year of follow-up.

- Aim 1 Hypothesis

  Treatment with sapropterin will increase monoamine neurotransmitter markers long-term in PKU subjects responsive to sapropterin due to both lowered plasma

Phe and greater availability of BH4 cofactor for hydroxylase mediated monoamine synthesis.

Specific Aim 2

To evaluate the long-term QOL for both sapropterin responders and nonresponders during 1 year of follow-up.

- Aim 2 Hypothesis

  Sapropterin responders will demonstrate improved QOL long term due to improved Phe control and a decreased psychosocial burden consistent with a more liberal diet.

Background and Significance

Metabolic management of PKU and neurobehavioral effects

Among the multiple disorders detected by newborn screening techniques, PKU is most famous for the dramatic effect of early dietary therapy in preventing severe cognitive and developmental disability [19]. This inherited metabolic disorder is characterized by impaired conversion of the amino acid phenylalanine into tyrosine as a result of absent or reduced phenylalanine-4-hydroxylase (PAH) enzyme activity (**Figure 1**) [20].

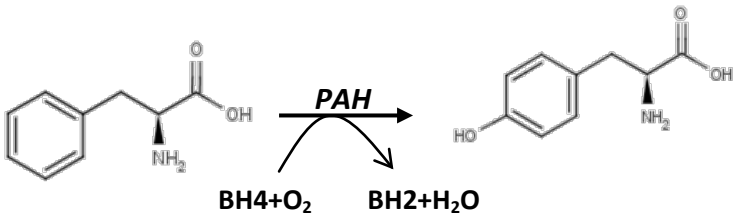


Figure 1: Conversion of Phe to tyrosine with BH4 cofactor addition of hydroxyl group. Not pictured is simultaneous conversion of Fe^{++} cofactor to Fe^{+++}

Treatment for PKU incorporates a phenylalanine-restricted diet wherein most protein-rich foods are replaced with an amino acid-rich medical food (formula) devoid of phenylalanine that serves as the primary source of essential protein for this patient population [20]. The current recommendation states the PKU medical diet should be lifelong [21]. In addition to dietary therapy, blood Phe levels are monitored frequently and ideally kept within 2-6 mg/dL (120-360 μmoles/L) [6, 22].

Despite the overall success of dietary treatment for PKU, adhering to such a strict diet is financially and socially burdensome, time-consuming to manage, and requires a tolerance for the amino acid-rich medical foods, which may be lacking in palatability [23]. Due to these factors, patient compliance is a frequent problem, particularly as PKU children approach adolescence [24, 25].

When PKU is untreated or poorly managed, there are severe consequences. These include mental retardation, seizures [26], poor growth, and psychosis [27]. Research has demonstrated that early and lifelong treatment is associated with improved neurological and overall health outcomes [21, 28-30]. Today in the United States, with the benefits of newborn screening, diagnosis, and improved treatment protocols, most children and adolescents with PKU have IQs and growth approximating normal [31, 32]. Even so, pediatric and adult individuals with PKU are still at a higher risk for certain behavioral and psychological disorders such as mood instability [33], ADHD [34, 35], depression, tics, and anxiety disorder [6, 36]. Mild and classic PKU patients also exhibit impaired learning ability, cognition and motor skills compared to healthy non-PKU controls [37, 38]. Additional problems with autonomy, social functioning, and education

attainment are also recognized [39, 40]. These multiple issues within the PKU population are further magnified when phenylalanine control (determined by blood Phe levels) is substandard [6, 11, 12, 30, 33, 41].

<u>Sapropterin as treatment for PKU</u>

Sapropterin dihydrochloride (Kuvan) is a synthetic formulation of a naturally occurring cofactor BH4 (**Figure 2**), which is required for the enzymatic activity of PAH.

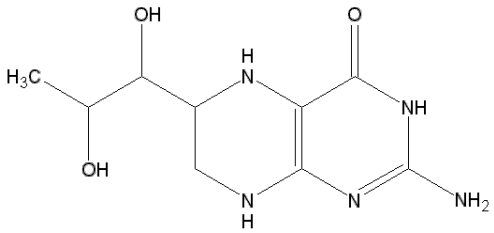


Figure 2: Tetrahydrobiopterin
Molecular Weight: 241.25

BH4 is intrinsically synthesized from GTP (guanosine triphosphate) (**Figure 3**) and cannot be consumed in adequate amounts from external dietary sources [42]. Several studies have shown that even though BH4 synthesis is normal in classical PKU, BH4 supplementation can improve PAH enzyme function and conversion of phenylalanine to tyrosine in over 1/3 of the PKU population [14, 43, 44]. This may be due to impaired binding of the misfolded enzyme to the BH4 cofactor, which is overcome when BH4 levels are artificially increased [45, 46]. BH4 also seems to stabilize mutant PAH proteins, lengthening the PAH turnover rate; this results in more available enzyme for phenylalanine metabolism [47, 48].

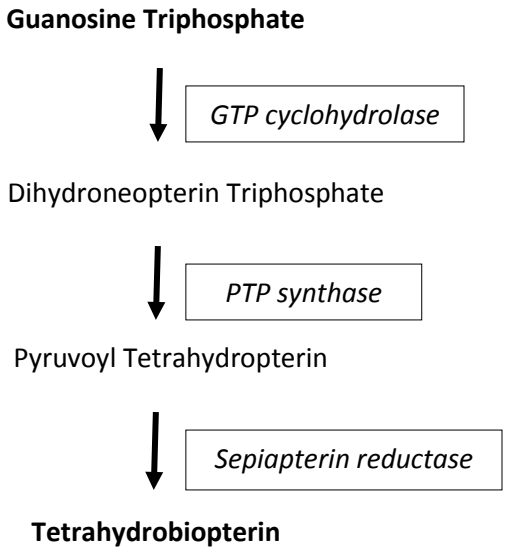


Figure 3: Synthesis pathway of BH4.
Adapted from Richardson [5].

In addition to its function as a PAH cofactor, BH4 is an important cofactor for other biologic enzymes (**Table 1**) [42], which suggests that the benefits of sapropterin therapy in people with PKU may extend beyond improved plasma Phe control, particularly with respect to neurological health.

Table 1: Enzymes dependent on BH4 as a cofactor. NOS enzymes listed in order of greatest affinity for BH4. References for NOS action: [1-4]

ENZYME	FUNCTION
Phenylalanine Hydroxylase (PAH)	**Conversion of Phenylalanine to Tyrosine**
Tyrosine Hydroxylase (TH)	**Conversion of Tyrosine to L-DOPA substrate for dopamine synthesis**
Tryptophan Hydroxylase(TPH)	**Conversion of Tryptophan to 5-OH-Tryptophan substrate for serotonin synthesis**
Nitric Oxide Synthase (NOS)	**Catabolism of Arginine to Citrulline and Nitric Oxide (NO)**
◆ Endothelial NOS (eNOS)	♦ Moderates vascular tone, reduces platelet and white blood cell adhesion, involved in bone formation.
◆ Neuronal NOS (nNOS)	♦ NO initiated signal transduction and neurotransmitter release in neurons. Cardiac contraction. Bone turnover.
◆ Inducible NOS (iNOS)	♦ Induced by macrophages during immune response. Has antipathogenic action. Increases superoxide production.

Several cofactor molecules are vital to the function of enzymes involved in monoamine metabolism, including nutrient cofactors such as B6, niacin, ascorbate, iron, and copper [49, 50]. In fact a deficiency of any one of these cofactors can result in neurological deterioration due to impaired neurotransmitter synthesis [51-54]. The concept of supplementation with a small molecule cofactor in order to improve the metabolic efficiency of a defective enzyme has been demonstrated in other inborn errors of metabolism. Maple syrup urine disease (MSUD) characterized by a deficiency

in branched chain alpha-ketoacid dehydrogenase (BCKDH) activity, and also treated with dietary protein restriction, is one example. This disorder, if left untreated, results in mental retardation and sometimes death [55]. However, a subset of MSUD cases respond to pharmacological doses of thiamin, a cofactor for BCKDH. In this MSUD subgroup, additional thiamin enhances residual BCKDH function and results in decreased buildup of leucine, isoleucine, and valine in the blood, thus improving survival and neurological outcome [55]. Also, a handful of genetically based epilepsies treatable with either pyridoxine (B6) or its bioactive derivative pyridoxal-5-phosphate have received recognition in the literature [56]. As these and other cofactor dependent metabolic disorders have demonstrated, the capacity of small molecule cofactor supplementation to improve neurotransmission, metabolic control, and neurological outcome is not a novel phenomenon and supports the therapeutic potential sapropterin has to improve both PAH function and neurological outcome in a portion of the PKU population.

Phenylketonuria and impaired monoamine metabolism

Ordinarily, once Phe catabolizes tyrosine with the assistance of BH4, cells that synthesize dopamine or serotonin have TH which metabolizes tyrosine into the catecholamine precursor **L**-3,4-dihydroxyphenylalanine (L-DOPA), while TPH converts tryptophan into the serotonin precursor 5-OH-tryptophan. L-DOPA is then metabolized into dopamine, which is subsequently converted to norepinephrine and epinephrine, or catabolized and excreted as HVA. Serotonin can be utilized for melatonin synthesis, or catabolized into 5HIAA [57].

Recent research has implicated abnormally high intrinsic blood Phe concentrations while directly inhibiting other hydroxylase enzymes within the monoamine metabolic pathway [58, 59]. Several research studies also provide evidence that an imbalance in large neutral amino acids (LNAA), as a result of high blood Phe concentrations, is responsible at least in part for PKU related neurological problems [60-63]. Phenylalanine is one of several LNAA that compete for absorption across cellular membranes, including the blood brain barrier (BBB). Eight other LNAA (tryptophan, tyrosine, leucine, isoleucine, valine, threonine, methionine, and histidine) compete with phenylalanine for access to the LAT1 amino acid transporter [64] and to a lesser degree the B^0AT2 transporter (B^0 Amino Acid Transporter 2) [65]. Thus, high concentrations of phe competitively inhibit transport of other LNAAs at the BBB. This results in the transport of high toxic concentrations of Phe into the brain, but not enough of the other LNAA important for neurological function, including tyrosine and tryptophan for monoamine neurotransmitter synthesis. This LNAA competitive inhibition by high phenylalanine explains why serotonin concentrations are subnormal in PKU subjects, particularly in cases of poor Phe control (though also occurring in those adhering to diet), even though BH4 metabolism and TPH function are normal [66].

Also occurring in PKU, since conversion of Phe to tyrosine is limited, excess Phe is shunted (albeit inefficiently) through other metabolic routes forming phenylketones such as phenylpyruvate, phenyl lactate and phenylacetate (**Figure 4**) [67].

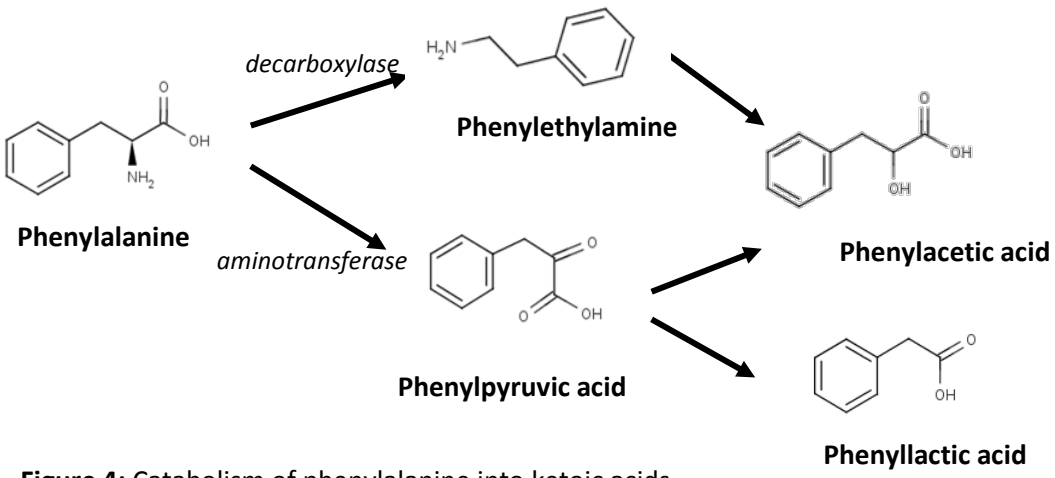


Figure 4: Catabolism of phenylalanine into ketoic acids when PAH conversion to tyrosine is impaired.

Physiologically high concentrations of both Phe and phenylketones can have neurotoxic effects due to disruption of calcium transport, glucose metabolism, glutamate signaling, and ATP metabolism in the brain [68-72], as well as consequential increases in brain oxidative stress [73].

These consequences are important since monoamine neurotransmitters have several critical functions within the CNS (central nervous system) including mood regulation, pain perception, sleep, cognition, hormone activity, gastrointestinal motility, attention, and neuromuscular control [57], all of which can be affected when blood phenylalanine concentrations are too high.

Several decades of clinical and laboratory research have implicated abnormal monoamine neurotransmitter metabolism as a critical factor in the neurological damage suffered by phenylketonuria patients with poorly controlled blood phenylalanine concentrations.

As early as the 1950s, Weil-Malherbe documented subnormal levels of the catecholamines adrenaline and noradrenaline (also known as epinephrine and norepinephrine) in the plasma of individuals with “phenylpyruvic oligophrenia” [74], a now obsolete term descriptive of PKU. Early studies with rodents, where hyperphenylalanemia (high blood Phe concentrations) was induced with high Phe diets and PAH inhibitors, revealed similar deficiencies in monoamine neurotransmitter synthesis [75-77]. The development of true PKU (PAH mutant) experimental mouse models [78] confirmed results from these earlier animal studies, demonstrating that the hyperphenylalaninemic state in PKU correlates with subnormal concentrations of serotonin and catecholamines in the brain [79-81], and to both neurological damage and abnormal behaviors when blood phenylalanine control is poor or nonexistent [82-86].

Clinical research trials investigating monoamine and catecholamine concentrations in the blood, urine, CSF (cerebral spinal fluid) and post-mortem brains of untreated phenylketonurics have further implicated hyperphenylalanemia as contributory to the pathogenesis of neurological dysfunction and in the subnormal synthesis of monoamine neurotransmitters [87, 88]. Further support of this interaction came with the advent of dietary therapy for PKU. Researchers soon discovered that Phe restricted diet therapy for PKU led to marked improvements in behavior [89], intelligence [90], and neurologic symptoms [91]. Eventual acceptance of newborn screening, diagnosis, and diet therapy by the health establishment led to an effective

and dramatic reduction in the severity and incidence of PKU related mental retardation [92].

A culmination of research endeavors has demonstrated that dietary phenylalanine restriction improves monoamine neurotransmitter synthesis in PKU subjects, offering insight into how diet therapy for PKU prevents neurocognitive decline. Butler,et al first published how Phe restriction in 3 PKU subjects increased CSF concentrations of serotonin, epinephrine, and norepinephrine [93]. Several studies since then have confirmed the inverse association between phenylalanine intake, or similarly blood phenylalanine control, and concentrations of serotonin and catecholamine metabolites in CSF [94, 95], urine [96], plasma [97], and blood platelets [98].

Two studies in particular demonstrate the connection that poor blood Phe control has with impaired monoamine synthesis and neurological health in PKU patients, with reversal of declines in neurological and monoamine status possible with adequate dietary control. Krause, et al. showed a correlation between diet induced plasma Phe changes, urinary dopamine excretion, and performance when testing choice reaction time. In 9 of the 10 study patients, urine dopamine levels were lower and reaction times longer when plasma Phe concentrations were high. When plasma Phe concentrations fell, urine dopamine increased and reaction times improved [96]. Another study by Lou et al. demonstrated that visual reaction times improved significantly with either tyrosine supplementation on free diet or decreases to plasma Phe via dietary Phe restriction. Concentrations of CSF monoamine catabolites HVA (homovanillic acid) and 5HIAA (5-hydroxyindoleacetic acid) were inversely correlated

with plasma Phe concentrations and dietary Phe restriction. When tyrosine was supplemented to free diet, CSF HVA increased even with no decrease in plasma Phe concentrations [94].

Brain scan studies corroborate the existence of CNS damage occurring in PKU. Magnetic resonance imaging (MRI) reveal abnormalities in white matter and grey matter distribution [38, 99] as well as decreased functional connectivity between neurons [100], with the degree of abnormality correlating to measures of Phe control. A positron emission tomography (PET) study showed impaired glucose metabolism in the brains of 10 adult PKU subjects who had been receiving diet therapy since two weeks of age [101]. Another PET study, in line with other mentioned research on neurotransmitter metabolism, showed impaired uptake and clearance of dopamine in a group of 7 adult PKU patients [102].

These study results, along with regular presentations of mild psychiatric and neurocognitive problems in PKU patients despite early life exposure to diet therapy [6, 35, 38], suggests that though dietary Phe restriction has been effective at improving health outcomes in people born with PKU, it does not provide for optimal development or maintenance of the CNS.

Quality of Life issues in PKU

Quality of life (QOL) scores have been reported to differ significantly in PKU patients when compared to a non-PKU reference sample [103]. The traditional PKU diet; which requires rigorous limits on phenylalanine intake and a lifelong dependence on synthetic free amino acid formula (Medical Food, MF); can be psychologically, socially, and

financially burdensome to someone with PKU and their family [23]. Children and adults with PKU must eat meals that are dramatically different in composition from that of friends and family members [104]. Grocery shopping can be a discouraging challenge, and eating out is often not possible. In addition, formula and special low protein foods are costly, and the complexities of diet management and blood Phe control can be a hardship for patients and their families [23]. For these reasons, compliance with prescribed Phe intake recommendations, and thus blood Phe control, is a frequent clinical problem, especially as PKU patients grow into adolescence and adulthood [24, 25]. Even though individuals coping with PKU become more nutritionly noncompliant in an effort to decrease the burden of PKU and improve QOL, studies have shown that dietary noncompliance actually has a negative effect on QOL. For example, loss of Phe control from dietary noncompliance results not only in abnormal executive function and psychological problems, but also in poorer QOL scores [105, 106]. When patients are reintroduced to strict dietary therapy, thereby decreasing plasma Phe, QOL scores significantly improve [106, 107]. In spite of patients' own acknowledgement that they feel better while on diet and experiencing good Phe control [108], attrition related to dietary management is common among PKU adults, with 60%-80% demonstrating poor Phe control by the time adulthood is reached [25, 109]. Patients report inability to maintain the recommended diet due to cost, difficulty of dietary management, or psychosocial pressure to eat regular foods [110-112]. Essentially, those with PKU must must sacrifice dietary freedom to improve their well being, or must risk the

consequences of poor Phe control in order to achieve greater dietary freedom, all in pursuit of improved QOL.

For individuals with PKU who respond to sapropterin with a decrease in plasma Phe, there is potential to improve QOL as a result of improved Phe control while also granting greater flexibility in the diet. Published research has clearly shown that treating PKU patients with sapropterin can not only assist with blood phenylalanine control, but can also allow the patient to eat a more relaxed diet with more intact protein while reducing dependence on free amino acid formulas [16, 113]. Reducing the burden associated with the PKU diet could increase patient compliance as well as alleviate the difficulties of cost and psychosocial pressure associated with traditional low-Phe nutrition therapy, including the financial burden of amino acid medical food and specialty phenylalanine free foods. Two small long term studies of infants and children provided sapropterin therapy suggest that the outcome of dietary freedom while achieving healthy Phe control may benefit subject QOL [114, 115], though neither study actually conducted formal QOL analysis in the subject population. Only one other study to date has explicitly evaluated QOL in PKU patients following treatment with sapropterin, though it only evaluated a small pediatric sample over a short period of 90 days [116]. Given the lifestyle burden associated with a low-Phe dietary regimen, and indirect evidence that sapropterin may improve QOL in the PKU population, the proposed study seeks to quantify possible improvements in QOL following treatment with sapropterin which can lower plasma Phe concentrations and liberalize the PKU diet.

Investigative intent and theoretical mechanisms by which sapropterin can improve monoamine dysfunction and QOL in PKU

The intention of this investigation was to evaluate (i) the effect of sapropterin on selected monoamine concentrations, and (ii) treatment-related differences in QOL in people with PKU. Urine monoamine metabolites were selected as the primary biochemical marker for monoamine levels, a common method of analysis reported in the literature for PKU and other disorders [96, 117, 118]. Self reported QOL measured quantitatively with a 5-point Likert scoring system was the psychosocial marker selected to evaluate the personal, social, and financial burdens of following a strict medical diet which can affect QOL but could be alleviated with sapropterin dependent diet liberalization.

<u>Mechanisms through which sapropterin can improve monoamine status in PKU</u>

- Since BH4 functions as a cofactor for hydroxylases that synthesize the monoamine precursors, treatment with additional cofactor could directly increase production of serotonin and the catecholamines which have been shown to be subnormal in PKU [93, 95-98].
- Since high plasma Phe is capable of inhibiting enzyme activity of both tyrosine hydroxylase (TH) [59] and tryptophan hydroxylase (TPH) [58], lower plasma Phe as a result of sapropterin response could augment improved monoamine synthesis due to less Phe inhibition of other monoamine hydroxylases.
- Improvements in plasma Phe control as a result of sapropterin would increase the ratio of large neutral amino acids (LNAA) to Phe, thus allowing for more balanced

competition at the blood brain barrier (BBB) between Phe and the many other LNAA crucial to neurological health [60, 119]. In particular, with more tyrosine and tryptophan entering the brain in place of the excess Phe, concentrations of serotonin and catecholamines would increase due to the availability of their amino acid substrates. This effect can also be seen peripherally as reported by Ormazabal in which sapropterin increased platelet serotonin to normal concentrations in PKU subjects who had subnormal platelet serotonin at baseline [66].

- PKU patients who respond to sapropterin with a decrease in plasma Phe would experience a decrease in the amount of neurotoxic Phe and phenylketones in the brain, which could inhibit these biochemicals' interference with neuron metabolism (i.e.: kinase activity, mitochondrial respiration, and energy metabolism) [68, 120, 121], reducing oxidative stress [73, 122], and subsequently improving neurotransmitter availability.

<u>Mechanisms through which sapropterin can improve QOL in PKU</u>

- Decreases in plasma Phe and improvement in plasma Phe control as a result of sapropterin response can improve patients' emotional stability [33] and reduce emotional distress [106], thus improving QOL perception [107].
- Decreases to formula dependence along with increases to dietary Phe tolerance as a result of sapropterin response--without sacrificing adequate plasma Phe control--would reduce the psychological, social, and financial burden of the PKU medical diet, therefore enhancing patient QOL.

CHAPTER 2: METHODS

Protocol approval and oversight

Study protocol along with the consent and assent forms were prepared according to Emory IRB standards. These documents were reviewed and approved by Emory University's internal review board (eIRB), BioMarin Pharmaceutical's IRB, Atlanta Clinical and Translational Science Institute clinical interaction network (ACTSI-CIN) reviewers, and the Emory office of clinical research (OCR). In addition any recruitment documents, patient handouts, information materials, and study questionnaires also received approval from eIRB, BioMarin IRB and the ACTSI-CIN. Edits and revisions of the study protocol, consent form, or other documents during both preparation for the study and during the study period itself were submitted to the eIRB, BioMarin IRB and CIN for review and approval prior to incorporation into the study procedures. Emory staff, faculty, and students involved in handling patients or patient data as part of the study received required training in human subject's research ethics. A protocol summary for the study was entered into the clinicaltrials.gov online registry as required by the FDA Modernization Act of 1997.

A research coordinator was involved with management of study patients, documenting and reporting of adverse events, as well as budget and protocol approval measures. Two registered dietitians on the study team were tasked with providing nutrition management and guidance to study subjects.

Recruitment, informed consent, and adverse event management

Recruitment of probands

Adult and pediatric PKU patients were recruited primarily from the patient population at Emory Genetics Clinic (EGC) and from female campers attending the 2009 Emory Annual Metabolic Camp. Flyers and brochures provided information about the study and were placed in the patient waiting room and in consultation rooms of the Emory Genetics Clinic. Letters announcing the study were also mailed to PKU patients at the EGC and information about the study was made available through an online website http://genetics.emory.edu/NUTRITION/BH4andPKU. Patients not meeting screening criteria as defined by the inclusion/exclusion criteria listed below were not included in the cohort trial.

Inclusion criteria

- Age 4 years and older
- Diagnosed with PKU through newborn screening or by clinical evaluation at some other point in time
- Interest in trying sapropterin as a treatment for PKU
- Capable of providing informed consent (self or through legal guardian)

Exclusion criteria

- Nursing, pregnant, or planning to become pregnant
- Currently on sapropterin therapy, or having taken sapropterin in the previous 8 weeks

- Active participation in another clinical trial

Recruitment of controls

Adult and pediatric control subjects were recruited through both the Emory University email system and by printed flyers that were posted at on-campus locations such as the School of Public Health and Whitehead Research Building. Once interested individuals agreed to participate by returning the signed consent form, a date for clinic visit was scheduled. Potential control subjects not meeting screening criteria as defined by the inclusion/exclusion criteria listed below were not included in the study.

Inclusion criteria

- Age 4 years and older
- Generally healthy
- Able to arrive fasting at scheduled visit
- Capable of providing informed consent (self or through legal guardian)

Exclusion criteria

- Nursing or pregnant
- Personal or family diagnosis of an endocrine disorder or inborn error of metabolism
- Personal history or family history of psychiatric, behavioral, or mood disorder
- Chronic illness including neurologic disorder (i.e.: epilepsy, multiple sclerosis)
- Taking neurotropic or psychotropic medication
- Currently on sapropterin therapy, or having taken sapropterin within the previous 8 weeks

Informed consent and authorization

Individuals who expressed interest--in person, by email , or by phone—in being study participants were provided a consent form (in hard copy or pdf) to review prior to scheduling a baseline study visit at the EGC or EUH-CIS, so the prospective participant could be familiar with study details before agreeing to enroll. Sections of the consent form that pertained specifically to control subjects were highlighted in yellow to avoid confusion with the protocol detailed for probands. Research personnel explained to prospective enrollees the study purpose and protocol prior to acquiring any signatures. Potential risks of participating in the study were also covered at this time. For all subjects risks included possibility of bruising or infection at the sight of venous puncture, possible anxiety or embarrassment over certain procedures such as providing urine samples or when completing forms or questionnaires, and a remote risk of loss of confidentiality. Additional risks for probands included the possibility of neither a reduction in medical food prescription nor increase in Phe tolerance, the possibility that there would be no BH4 induced improvement in plasma Phe concentrations, that plasma Phe concentrations may exceed the therapeutic range during the study period, and a risk of medication side effects—digestive upset, sinusitis, headache, and elevated liver enzymes. In addition to the study consent form, probands were requested to sign a consent form granting permission to research staff to retrieve study pertinent data and information from the subject's medical records. For participants under age 11 years, signed consent was received from the legal guardian and verbal consent from the study participant. For subjects between the ages of 11 and 17 years, signed consent was

received from subject's legal guardian at the same time that signed assent was received from the prospective subject. Participants age 18 years and over were required only to provide their own consenting signature. All patients also provided signed authorization for the use and disclosure of their personal study results and information to particular entities and individuals in defined circumstances. Patients were also informed in the authorization document of Emory University's compliance with federal HIPAA regulations. Any questions from would-be participants about the consent form or study protocol were answered in person on sight by research personnel. In one circumstance where low literacy made it difficult for a patient to review the consenting and authorization literature, the literature was read aloud and explained by a study staff member before signed consent was received.

Interested individuals who had passed the screening criteria and agreed to participate were enrolled in the study upon return of the signed consent and authorization forms. All enrolled participants were also provided a form in which they could voluntarily revoke at any time their consent to participate in the study or revoke consent for any further collection or analysis of their information. Research participants had the right at any time to refuse further participation in the study or to decline a test or procedure that was part of the research protocol, as well as refuse to provide information requested on study forms and questionnaires.

Facilities and Resources

Emory University Hospital Clinical Interaction Site (EUH-CIS) (a part of the ACTSI-CIN) in Atlanta GA provided inpatient and outpatient rooms, nursing staff, bionutrition staff—

including onsite registered dietitians--with access to a metabolic kitchen and low phenylalanine food items, phlebotomy service, and freezer storage for specimens for 3 of 5 study timepoints for each patient. Emory Genetics Clinic (EGC) at 2165 North Decatur Road provided outpatient rooms, nursing staff, and phlebotomy service for 3 of 5 study time points for each patient. EGC also provided dietetic counseling and diet record analysis for all study timepoints for each patient. Plasma amino acids analysis for Phe , tyrosine, and tryptophan were performed at Emory Genetics Clinical Laboratory (EGL) using Amino Acid Analyzer (Hitachi Inc.) HPLC detection. EGL also assisted with processing and freezer storage for plasma samples and with urine samples that were collected for later monoamine analysis. Computer access, statistical software access, as well as technology support were provided by the technology office at EGC. Plasma amino acids were analyzed by Emory Genetics Laboratory using HPLC Biochrom 30 HPLC Amino Acid Analyzer and reported in μmoles/L. Standard of care lab analyses were performed by either LabCorp or Quest Diagnostics, dependent upon patient insurance coverage. Urine creatinine analysis was performed by Quest Diagnostics using colorimetric assay. Analysis of urine monoamine biomarkers was conducted with the assistance of Dr. Hyder Jinnah and his laboratory facilities within the Emory University Department of Neurology. Assistance and resources from Dr. Hyder included staff guidance, access to HPLC equipment and corresponding Coularray software, and -80°C freezer storage. Assistance with statistical protocol planning and planned analysis of study results were provided by ACTSI statisticians and George Cotsonis from Emory Department of Biostatistics. Dr. Julie Kable of the Marcus Institute in Atlanta GA and Dr.

Usha Ramakrishnan of the Emory Nutrition Health Sciences Program provided guidance in analysis of QOL score for the PKU study participants. Research staff and personnel included two registered dietitians with access to the University of Minnesota Nutrition Database System for Research (NDSR) for completing diet record analysis, several physicians specializing in treatment of inborn errors of metabolism, an RN credentialed research coordinator, an administrator to assist with data entry and patient management, and graduate research assistants.

Research costs were supported with an independent investigator award provided by BioMarin Pharmaceuticals.

Protection and management of patient information and study data

Each subject upon enrolling in the study was assigned a study number preceded by the subject's initials to reduce incidence of error in data entry and sample handling. Hard copies of study related information were placed in patient charts and in numerically labeled binders. Patient charts were kept in a locked cabinet at the EGC when not in use and study binders were kept locked inside the metabolic nutrition research office at all times. During the course of the study, data was entered into an Excel database that could only be accessed with a pass code. All EGC computers that were used for management or analysis of patient information were both password protected and encrypted with software provided by the technology office at the EGC. In addition all thumbdrives used to store patient data were also encrypted. Prior to statistical analysis or provision of data to statistical personnel, the data was stripped of initials and any

other identifying information. Reported study results do not include any identifying information.

Study Design

The study was designed as a 1 year prospective non-blinded cohort evaluation of 60 male and female PKU subjects from age 4 years through adulthood who intended to try sapropterin (Kuvan®) therapy as a method of blood phenylalanine control. No randomization or research directed dietary intervention or drug intervention were a part of the investigation. Dietary or drug interventions were conducted by clinicians as part of the standard quality of care treatment that any PKU patient beginning sapropterin therapy would expect to receive at the EGC. All patients who enrolled were informed they could complete the full course of the study, regardless of whether the patient was deemed a responder or nonresponder to sapropterin, and regardless of whether the patient's sapropterin prescription was continued or discontinued. All PKU enrollees were subject to therapeutic changes to intact protein allowance or formula prescription dependent upon blood Phe control. BH4 was provided at a dose of 20 mg/kg body weight per day for all study patients. Study patients were instructed to consume the dose at 1 routine time during the day. The number of pills prescribed was adjusted for changes in patients' body weight over the course of the study.

PKU subjects were scheduled for a total of 5 visits: a baseline screening visit and 4 follow-up visits at 1 month, 4 months, 8 months, and 12 months after baseline, with patients exiting the study at the final 12 month visit (**Figure 5**).

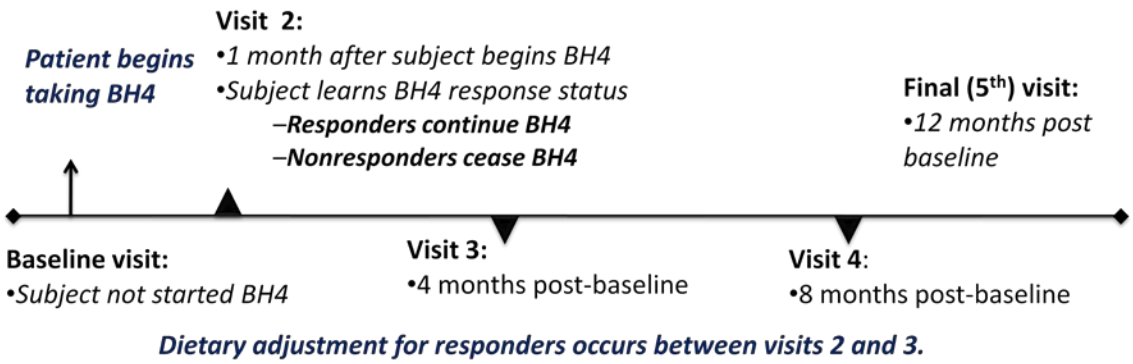


Figure 5: Schematic timeline of study protocol for PKU participants from baseline to 1 year.

Baseline and exit visits were spent overnight as inpatients at Emory University Hospital Clinical interactive site (EUH CIS). Study patients below 18 years of age were accompanied by a parent throughout the inpatient experience. One month outpatient follow-up appointments were scheduled either at the EGC or at the EUH CIS. 4 month and 8 month outpatient visits were entirely at the EGC. Since we anticipated that patients would need to reschedule appointments due to uncontrollable circumstances during the course of a year, flexibility in scheduling allowed for patients to be rescheduled as much as 2 weeks prior or 6 weeks after an original appointment date for all visits, except for the 1 month follow-up for determining initial BH4 response status.

The study protocol allowed for a maximum of 15 male and female healthy non-PKU control subjects, age 4 through adult. Controls came to the EGC for one scheduled visit only and provided urine and fasting plasma samples for the purpose of comparative analysis with PKU probands when evaluating monoamine concentrations. Controls also provided demographic and health history information and a 3 day diet record.

The biomarkers evaluated for research participants and collected during the 5 study visits are listed in **Table 2**. The length of time devoted to study preparation, recruitment, study protocol, data analysis, and publication of results has been summarized within **Table 3**. The study involved a total of 4 years from the time that organization and planning began to final publication of research results.

Table 2: Clinical and investigative biomarkers collected for research participants at study time points

Biomarker	Study group	Time points
• **Demographic information**	Controls and probands	Study visit 1
• **Health history**	Controls and probands	Study visit 1
• **Diet intake record**	Controls and probands	Controls: study visit 1 Probands: study visits 1-5
• **Health status updates**	Probands	Study visits 2-5
• **QOL questionnaire**	Probands (age 10+years)	Study visits 1-5
• **Height and weight**	Probands	Study visits 1-5
• **Blood pressure and vitals**	Probands	Study visits 1-5
• **Plasma amino acids**	Probands	Study visits 1-5
• **Serum creatinine**	Controls and probands	Controls: study visit 1 Probands: study visits 1-5
• **Urine volume**	Controls and probands	Controls: study visit 1 Probands: study visits 1-5
• **Urine creatinine**	Controls and probands	Controls: study visit 1 Probands: study visits 1-5
• **Urine monoamine metabolites**	Controls and probands	Controls: study visit 1 Probands: study visits 1-5

Table 3: Sequential completion of project goals

Research Project Activity	Time required
♦ **Prepare study materials, obtain IRB approval, coordinate use of Emory CIS, train and prepare staff**	♦ January-September 2008
♦ **Recruitment and screening**	♦ October 2008-October 2009
♦ **Baseline evaluation and I year follow up of all subjects**	♦ October 2008-October 2010
♦ **Study close out procedures, final reports and paperwork**	♦ November 2010-February 2011
♦ **Data analysis, publications, disseminate study results**	♦ May 2012

EGC protocol for following patients on BH4 and determining response status

Chapter 5's Table 1 lists occasions where standard of care procedure of a study patient deviated from that described below due to extenuating patient circumstances during the cohort period.

General management of all patients initiating sapropterin

Patients met with both a physician and registered dietitian to discuss sapropterin as a treatment option. All patients were informed that less than ½ of those who try sapropterin therapy respond with lower plasma Phe concentrations and that diet liberalization is not guaranteed. Clinical methods for monitoring plasma Phe, importance of maintaining plasma Phe concentrations within the therapeutic range (< 360 µmoles/L), and a dietary monitoring plan were described in detail to the patients. A genetics physician specialist wrote a prescription for one month sapropterin supply for all subjects. The pharmaceutical company BioMarin provided sapropterin to the patients free during this period. Patients were requested to provide 3 day diet records, but when

these were not provided by the patient 24 hour recalls were collected and analyzed instead. NDSR diet analysis software was used by an RD to determine dietary phenylalanine intake as well as adequacy of other nutrients in the patients' diets. Any adjustments to sapropterin intake, formula prescription, and prescribed Phe tolerance were at the discretion of the genetics physician and RD. Patients were provided with an ample supply of filter papers, finger lancets, diet record forms, stamped envelopes addressed to the clinic, as well as instructions for carrying out the tasks of recording dietary intake and submitting blood spot filter papers. Patients were provided frequent written, electronic, and phone reminders of upcoming appointments as well as in regards to submitting diet records and filter papers.

Sapropterin has the FDA classification of pregnancy category C. Breast feeding and pregnancy were exclusionary criteria for women participating in the research study.

Evaluating BH4 response based on change in plasma Phe concentration

Prior to being prescribed sapropterin, the patient provided a fasting baseline blood draw for analyzing plasma Phe and tyrosine as well as a 3 day diet record. The patient was prescribed 20 mg/kg/day of sapropterin once daily for 1 month. This is the maximum FDA approved dose and studies have demonstrated that it results in the highest yield of response among patients when compared to smaller doses [15, 123]. Sapropterin was provided in the form of 100 mg pills. Patients were instructed to swallow pills whole or to crush pills and mix with apple juice or water. Patients were instructed not to alter dietary Phe or medical food intake during the 1 month trial period and encouraged to submit a 3 day diet record and blood spot filter paper to the EGC once every week

between the first and second clinic visits. At the end of the 1 month trial period when all subjects were provided sapropterin patients returned for the second visit for another fasting plasma blood draw. Five days scheduling flexibility was allowed for the second visit and a prescription bridge for sapropterin was provided until the patient was informed of their sapropterin response classification. A minimum 20% decrease in plasma Phe concentration was the criteria for classification as a "responder" and the patient was permitted to continue taking sapropterin. Those without this decline in Phe were classified as "nonresponders" and discontinued sapropterin while continuing with their standard PKU diet. Within 3-5 days of the 2nd clinic visit, plasma Phe concentration was determined and communicated to the patient along with the patient's initial sapropterin response status. Both responders and nonresponders continued to be followed for the remainder of the 1 year follow-up. For responders, diet challenge over the next 12 weeks for evaluating changes to Phe tolerance and formula dependence, was conducted systematically with close attention to responders' plasma Phe concentrations. Diet challenge followed previously published protocol [124]. At the end of 12 weeks, initial responders with higher Phe tolerance and reduced formula dependence were classified as "definitive responders". Those that demonstrated an initial drop in plasma Phe but were unable to liberalize their PKU diet while maintaining plasma Phe control were classified as "provisional responders" per published recommendations [125]. Patients were notified of their provisional status once known and were permitted, along with definitive responders, to continue taking sapropterin since they had met the initial response criteria.

Response specific detailed patient management

Initial responders

These standard of care management practices for PKU patients on sapropterin are in agreement with published guidelines based on the experiences of metabolic clinics which allowed diet liberalization for patients responsive to sapropterin [124, 126].

Once a PKU subject was designated a sapropterin responder, the patient iwas given the option to continue on sapropterin at which point the drug cost was picked up by the patient or their insurance. Responders were also free to decline further sapropterin treatment and continue with their current low-Phe diet and medical food intake.

Responders continuing sapropterin underwent Phe-challenge over the next 4-6 weeks to determine their new sapropterin dependent dietary Phe tolerance under close clinical supervision. Patients with plasma Phe concentrations below the 360 µmoles/L therapeutic threshold were instructed to add 20mg of nonfat milk powder or 6.7g of egg white powder (equaling to 340mg additional dietary Phe) to their current medical food formula each week while submitting weekly blood spot filter papers and 3 day diet records to the EGC. Sapropterin dependent Phe tolerance was determined based on the additional dietary Phe the patient could safely consume without exceeding the therapeutic threshold for plasma Phe. If plasma Phe ever exceeded 360 µmoles/L, the patient was advised to reduce dietary Phe intake by 340mg weekly until plasma Phe returned to within the therapeutic limit. For patients entering the study with high plasma Phe and poor dietary adherence, instead of milk powder challenge, patients

were encouraged to reduce dietary phenylalanine intake by 340mg per week until plasma Phe concentrations dropped below 360 µmoles/L. Once plasma Phe was in the therapeutic range, Phe tolerance was calculated and compared to the patient's prior (before sapropterin) Phe tolerance on record.

Once the patient's BH4 dependent Phe tolerance was determined, the patient was allowed 4 transition weeks in which ordinary foods containing Phe (in place of milk or egg powder) were added to the diet. The patient continued to monitor Phe intake with diet intake records and submit weekly blood spot filter papers for monitoring blood Phe concentrations.

The final step in the patient's dietary adjustment involved systematically decreasing the amount of prescribed free amino acid medical food over the next four weeks to determine the essential amount of medical food required for proper macronutrient balance while maintaining plasma Phe control. The patient's baseline formula prescription was decreased by 25% once a week over 4 weeks. Diet records and blood spot filter papers were submitted weekly by the patient for analysis to ensure that the patient's blood Phe levels remained within the therapeutic limit while meeting dietary protein and caloric requirements. Medical food reduction was ceased in one of the following circumstances 1) The patient's blood Phe levels exceeded the therapeutic threshold at any time during the 4 week medical food adjustments 2) optimal protein and caloric intake could not be achieved with further reduction to medical food and 3) Medical food has been reduced to 0 intake for males and no less than 25% of original prescription for females of reproductive potential. Females of child bearing age or

younger were strongly encouraged to consume at least 25% of their original formula prescription in order to accommodate nutrition needs in case of future pregnancy. Patients not consuming medical food regularly were encouraged to do so at the start of this final dietary adjustment phase. The dietitians and clinical staff worked diligently with patients to remove financial or social barriers to consuming prescribed formula. The patient was encouraged during medical food adjustment phase to continue consuming regular foods within the limit of his/her sapropterin-dependent Phe tolerance. Patients who were able to completely eliminate medical food from their PKU diet plan, or who consumed a micronutrient free medical food, were strongly encouraged to take a multivitamin/mineral supplement to prevent micronutrient deficiencies which could develop when not consuming a micronutrient fortified amino acid formula. Standard of care clinic visits occurred after each 4 week interval in the dietary adjustment period to monitor plasma Phe concentrations and to discuss with an RD the next step in the dietary adjustment process or other nutrition and dietary issues that may have arisen.

Once the patient's diet had been adjusted the patient was asked to submit monthly diet records and blood spot filter papers henceforth. The patient was also encouraged to return to the EGC for biannual visits with the doctor and dietitian for continued monitoring. Prescribed Phe allowance and medical food intake could have been altered later after the initial dietary adjustment period, depending on Phe control and nutrition needs such as calorie and protein intake. Sapropterin dose could also have

been adjusted at the physician's discretion for reasons pertaining to growth, weight change, or effectiveness in maintaining plasma Phe control.

Characteristics and management of provisional and definitive responder classes

Provisional responders, though demonstrating an apparent initial decrease in plasma Phe during the first month on sapropterin, were unable to increase dietary Phe intake or decrease medical food intake. Also, shortly after the first month, plasma Phe concentrations in this group rebounded to baseline values and continued to climb during the study despite continue sapropterin intake. Definitive responders were capable of both significant increases to Phe tolerance along with decreasing dependence on medical formula after initiating sapropterin, and maintained generally good Phe control during the study period.

For provisional responders, original formula prescription and dietary Phe tolerance were maintained at baseline levels except for needed adjustments to accommodate growth, physical activity, or weight change. At the 1 year study exit visit, the provisional status of the patient was reviewed and discussed again, as well as the option to continue or discontinue sapropterin.

Nonresponders

After being identified as a nonresponder to sapropterin after the second clinic visit, sapropterin treatment was withdrawn and the patient was encouraged to follow current recommendations for dietary Phe intake and medical food prescription in order to maintain optimal blood Phe control. Diligent efforts were made to ensure that all study

patients had access to formula, and to reduce any financial or social barriers inhibiting adherence to prescribed formula. Nonresponders were also encouraged to return for the remaining study visits and to submit monthly blood spot filter papers and diet records. Prescribed Phe tolerance and medical formula intake could still have been adjusted at later times by the Emory Genetics' clinical care team, depending on factors such as nutrition needs, plasma Phe control, changes in physical activity, or growth and weight changes.

All subjects

At the end of the study, all PKU patients regardless of response category, were encouraged to return to the EGC for biannual visits with the doctor and dietitian and to submit monthly blood spot filter papers and diet records. Prescribed Phe allowance and medical food intake could be adjusted at later time by the patient's clinical care team, depending on factors such dietary nutrition needs, blood Phe control, and growth and weight changes. Sapropterin dose could also be subject to change at the physician's discretion, or dose decreased at the patient's request, for responders opting to continue sapropterin.

Study visit procedures

Study visit 1

PKU patients

The baseline visit was an overnight inpatient visit at the EUH CIS. A medical doctor completed a physical exam on day 1 of the visit. A 3 day diet record, vitals, and

anthropometric data were also collected. Patients filled out demographic and health history forms as well as a QOL questionnaire. A registered dietitian counseled patients on how to accurately complete diet records and how to submit blood spot filter papers during follow ups. Patients were provided with finger lancets, measuring cups and spoons, a 12 inch English/metric ruler, stamped envelopes, and multiple blood spot filter papers and diet record forms to make these tasks more convenient. Patients were also provided with an information booklet that explained the methods and purpose of the study and contained resources for contacting study personnel, keeping track of appointments, recording medications and illnesses, and instructions for completing diet records and filter papers. At the EUH-CIS, patients were provided a low-Phe dinner and breakfast prepared by dietetic staff. Low-Phe drinks and snacks were also available. At the CIS, patients were given a 1 liter urine collection container and toilet hat for collection of the 12 hour overnight urine sample. Prior to breakfast, fasting blood samples were drawn for plasma amino acids determination, a complete metabolic panel that included liver enzymes and serum creatinine, and for monoamine analyte determination.

Upon checkout, patients were given a prescription for sapropterin, written confirmation of their next scheduled follow up visit, and a urine collection kit (consisting of a 1 liter urine collection container, cooler bag, cold pack, toilet hat, and printed instructions) to assist with collectin a 12 hour overnight urine sample to bring to the next study visit.

Controls

Control subjects were scheduled for a single outpatient study appointment at the EGC. Prior to the appointment, controls were provided with a "study kit" which consisted of a 1 liter overnight urine collection container (along with a toilet hat for female participants) inside of a cooler bag, a demographic questionnaire and a health history questionnaire which could be filled out before arrival, and instructions on completing a 3 day diet record.

Controls arrived at the study visit at least 8 hours fasting, with a completed 3 day diet record. 12 hour overnight urine specimens were brought in the 1 liter overnight container inside the cooler bag with ice or cold packs. Completed demographic and health history forms were turned in. At the study visit, a 2 ml gold top serum separator (SST) vacuum tube of blood was drawn for serum creatinine evaluation along with a 7 mL lithium heparin monovette for plasma monoamine analysis. Snack and drink were available for control subjects after blood draw.

Study visits 2-4 for PKU patients

Visits 2-4 were single day outpatient visits. By the second time point, PKU patients had been on sapropterin for 1 month. For visits 2-4, the patient was asked to arrive at the appointments at least 8 hours fasting and with a 12 hour overnight urine sample along with a 3-day diet record. Anthropometric and vital signs were collected and blood drawn for plasma amino acid analysis, complete metabolic panel, and monoamine analyte determination. A low-Phe drink and snack were available to patients after blood draw. Patients were asked about any changes to their health status or medications between visits, which included a verbal query as well as completing a health update form.

Subjects had opportunity to speak with a registered dietitian during study visits and were asked to complete the QOL questionnaire.

Prior to departure, the patient was provided with another urine collection kit along with additional blood spot filter papers, stamped envelopes, diet record forms, and written confirmation of their next standard of care or study visit. Clinician adjustments to sapropterin intake, formula prescription, or recommended Phe tolerance as well as clarification of sapropterin response status were conducted as described in earlier paragraphs.

Study visit 5 for PKU patients

For the final visit, subjects were encouraged to spend the night at the EUH CIS as inpatients. A physical exam by a medical doctor was conducted on day 1 of the visit. A 3 day diet record, vitals, and anthropometric data were also collected. Patients were asked about any changes to their health status or medications since the previous visit, which included a verbal query as well as completing a health update form. Subjects had opportunity to speak with a registered dietitian about their PKU diet and any other nutrition concerns. Subjects were also asked to complete the QOL questionnaire. Patients were provided a low-Phe dinner and breakfast prepared by dietetic staff. Low-Phe drinks and snacks were also available. At the CIS, patients were given a 1 liter urine collection container and toilet hat for collection of the 12 hour overnight urine sample. Prior to breakfast, fasting blood samples were drawn for plasma amino acid analysis, a complete metabolic panel, and for monoamine analyte determination.

Prior to checkout, the patient was provided with additional blood spot filter papers, stamped envelopes, and diet record forms. At this time, the RD and research coordinator explained post-study routine standard care and follow up to the patient. Standard monitoring of patient metabolic control, dietary adherence, and therapeutic effectiveness was continued post-study via biannual clinical appointments at EGC and via continued submission of filter papers and diet records.

Data collection and biological sample handling

Patients were provided time at visits to fill out requested forms and questionnaires. Hard copies of these, in addition to hard copy print outs of laboratory results, diet records, and anthropometric data were maintained in binders specified by study ID number and ordered according to date and clinic visit. All data was also entered into an Excel database maintained within a password protected research folder. Stored biological samples have labels with the printed subject ID number, date acquired, and study visit number.

Demographic and health information

A form was developed for requesting information on demographic variables as they relate to age, gender, marital status, educational status, and socioeconomic variables for the purpose of controlling for demographic factors that may be associated with study outcomes. The form was modeled after previous demographic questionnaires used for clinical and study purposes by the EGC with modifications best suited for the purpose of the study.

A general health history questionnaire applicable for both control and proband study subjects was created. The questionnaire was modeled after health history questionnaires that had been used for other research protocols or in the clinical setting. Questions were included based on potential relevance and associations with study outcomes. An additional health history form requesting information specifically in regards to a proband subject's PKU medical management was also utilized. Questions were related to dietary monitoring as well as laboratory and clinical management for PKU. A health update questionnaire was also created and provided to study probands which could be filled out if any change in the patient's medical status occurred during the cohort investigation (such as changes in regular medicines, medical diagnoses, surgeries, or physical activity level).

Quality of Life

QOL was determined with a self-report Quality of Life Questionnaire (QOLQ) created to specifically address psychosocial QOL issues as they relate to PKU. The PKU-QOLQ was developed by Dr. Rani Singh and adapted from a validated QOL instrument constructed for youths with diabetes [127]. The PKU-QOLQ has been validated for female adolescents and used in prior investigations [128]. Validation for adults and males is in process. In order for the questionnaire to be applicable to both adults and adolescents in this study, some minor modifications were made to age sensitive questions that pertained to school, work, and home circumstances. The questionnaire was provided only to PKU study patients who were at least 10 years of age. Patients could fill out the questions themselves or have the questions read aloud to them in a private setting with

the proxy completing the answers on the questionnaire. The questionnaire consisted of 53 questions evaluating subject attitudes, sense of satisfaction, perception of emotional support, and worries in relation to his/her PKU condition. Two questions related to work or school activities were skipped by enough participants to warrant exclusion from final scoring. Qualitative questions were designed on a 5 point Likert scale. The most positive responses would be a "1" with the most negative responses being a "5". This design is to limit negative bias when answering the questionnaire. Calculation of scores was then reversed so that higher scores could reflect better QOL while lower scores would reflect poorer QOL when reporting results. The highest QOL score could equal 255 while a score of 51 would indicate the lowest measurable QOL. In addition, 5 qualitative questions asking about patients' thoughts and feelings towards PKU and medical management thereof were included on the last page of the questionnaire.

Collection, storage, and analysis of biological samples

Urine collection and storage

Patients were requested to bring overnight 12 hour urine samples in the 1L collection containers provided and kept chilled inside a cooler bag given to the patient. On the collection container was a label by which patients could record start and stop times of the 12 hour collection period as well as date and study ID number. Urine volume was recorded at the time of visit and the specimen was taken to the EGL. Prior to aliquoting, the urine was mixed at least twice with tipping or inversion. A serological pipette was used to remove a 10mL aliquot into a yellow capped solution free plastic vial to be sent on ice to Quest Diagnostics for determination of urine creatinine concentration. For

monoamine analysis, four 5 mL aliquots were frozen and stored at -80°C. Smaller volumes however were needed for monoamine analysis of the urine samples. Therefore, one 5mL aliquot was thawed on ice at a later date when 300 uL ice cold urine was aliquot into 6 labeled cryogenic vials, quickly frozen on dry ice and stored at -80°C.

Plasma collection and storage

Blood for plasma amino acid analysis was collected into a green top sodium heparin tube, centrifuged to separate out the plasma and taken to EGL for analysis. Plasma amino acid results were reported within 3-5 days.

Blood for serum creatinine analysis as part of a complete metabolic panel was collected into a green top sodium heparin tube, centrifuged down to separate out the plasma, frozen on dry ice, then shipped frozen to Quest Diagnostics. Results were reported within one week.

Blood stored for future analysis of plasma monoamine analytes was collected using a 7.5ml green labeled lithium heparin monovette. The sample was immediately wrapped in foil and placed on ice to reduce the oxidation of analytes. The blood sample was centrifuged and plasma aliquot into 3-4 2.0 ml cryogenic vials pretreated with 1.25 µM of EDTA and SMBS (sodium metabisulfate) to inhibit oxidation. Samples were immediately frozen and stored at -80°C.

Chromatography analysis of urine monoamine analytes

Subject ID number was utilized for random ordering of urine samples for HPLC preparation and analysis, with all available study timepoints for each subject batched together. Frozen urine samples were thawed on ice and microcentrifuge spun to

separate out precipitate. 200 µL supernatant was treated with 20 µL 1.0M perchloric acid (PCA) to yield 0.1M PCA equilibrated urine specimen. Equilibrated specimens were diluted tenfold in 0.1M PCA prior to centrifugation through 0.45 µm PVDF Micro-Spin columns (Grace Davison, Deerfield, IL). Filtrate was transferred to HPLC vials for chromatography analysis. Samples were kept chilled throughout preparation process.

Tenth dilution of urine samples for HPLC analysis was selected after experiment evaluating the chromatograms of a single control urine sample at 1/2, 1/10, 1/20, and 1/100 dilutions. Dilutions of 1/10 provided the greatest visibility of the most monoamines with the least interference from co-eluting substances present in the urine. Tenth dilution also was less prone to eluting in the next sample's chromatogram and better tolerated by the HPLC equipment than 1/2 or 1/5 dilutions. All 13 control samples were HPLC analyzed at 4 different times and compared to evaluate variation between batched runs. Use of monoamine standard (described below) also controlled for between run and within run variation.

HPLC analytical method was adapted from previously described technique [129]. Mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium, 75 mM NaH_2PO_4, 0.25% triethylamine, and 8% acetonitrile at pH 2.9. An ESA 5600A Coularray detection system incorporating an MD-150 X 3.2 mm C.18 column and 5020 guard cell with 6210 detector cell (ESA, Bedford, MA), eluted samples at 0.4 mL/min flow rate. Guard cell potential was set at 450 mV while analytic cell potentials were -175, 100, 350 and 425 mV. HPLC would run for 2-3 hours on blank 0.1M PCA samples prior to beginning sampling of unknowns.

Monoamines in prepared urine samples were identified by comparing electrochemical profiles and retention times with those of 100 ng/mL known standards (Sigma Chemical Co., St. Louis MO) in 0.1M PCA: epinephrine (E), norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3MT), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA). Monoamine peaks in urine were further verified by co-elution of test samples supplemented with standards. MHPG and NE could not be identified with certainty in urine due to prevalent co-eluting substances and are excluded from this report. 3MT and 5HT concentrations were effectively considered to be 0 when falling below the lower limit of detection (0.2 ng/mL in 1/10 diluted sample). Efforts to rerun samples at 1/2 or 1/5 urine dilution for better 3MT and 5HT resolution were unsuccessful due to higher incidence of co-eluting substances and stress of the less diluted urine samples on the sensitive HPLC equipment.

Approximately 20 urine samples were batched at a time, so that all available timepoints for 4-5 randomly selected ID numbers were completed with each batched run. Monoamine standard was inserted at the beginning and end of each batched run, and inserted between each ID specific set of timepoint samples. Blank PCA was inserted at the beginning and end of each run to ensure no contaminants were being eluted, and also between ID sets of timepoint samples before each monoamine standard. Fourteen batched runs were required to analyze all available urine monoamine samples.

Data reporting and management

<u>Urine Monoamines</u>

Monoamines were recorded as ng/mL concentrations determined by HPLC. To control for urine dilution effects on monoamine concentrations, monoamines were reported in ratio to urine creatinine (Cr)[130]: mg/g Cr for HVA and 5HIAA, μg/g Cr for all other monoamines. Urine creatinine, reported by Quest Diagnostics in mg/dL, and monoamines were converted to the appropriate units for ratio calculation. Log10 transformation of monoamine ratios was performed prior to statistical analysis to control for the skewness of the outcome variables. Means and confidence intervals of monoamine ratios reported in tables are back-transformed values.

Quality of life

Scoring the quantitative portion of the PKU specific QOL questionnaire required recording the reverse score for each question answer (for example, questions with a 1 on the likert scale were reverse-scored as 5) then summing up the answers for both a total score and a sub-score for each of the 5 sections in the quantitative portion of the questionnaire. The 5 sections each focused on a different aspect of life quality in relationship to PKU: impact of PKU in the person's life, worries relevant to life with PKU, sense of satisfaction, sense of support (from family, friends, and the medical community), and perspective on general well-being. If ≤25% of questions within a section were missing for any subject, the missing answers were imputed by averaging the answers to the other section questions. If >25% of questions were skipped within a section, the data from that section was not included in the analysis. Likewise, if >25% of questions total were skipped in the questionnaire, the total QOL score for that

questionnaire was not included in the data analysis, as was the case also if an entire section was eliminated from scoring due to skipped questions.

Dietary variables

Dietary Phe (prescribed or actual) is reported in mg/day, whereas formula protein (prescribed or actual intake) is reported in g/day. Energy (kcalories) and total protein intake are reported per kg per day. Adherence to prescribed Phe intake, representative of Phe tolerance, was calculated by subtracting the prescribed dietary Phe from actual intake, providing the value of excess Phe the patient consumed above prescribed amount. Adherence to formula protein prescription was calculated by subtracting actual intake from the prescribed value, providing a value of how much less formula protein the patient is consuming in relation to their prescribed amount. For these two calculations of adherence, data had a skewed distribution and was thus transformed by Log10 for covariate use in statistical analysis.

Statistical analysis

General statistical approach

Statistical technique for analyzing data

SPSS 19.0 was used for the descriptive and statistical analyses. Graphs representations of results were also prepared in SPSS 19.0. All tests were two tailed with α=0.05. Normality and skewness were evaluated for all variables in the analysis. When necessary, variables were transformed to improve normality, thus controlling for intrinsic skewness in the data. Exploratory analysis of covariate associations with

outcomes at baseline was conducted with multivariate analysis and Pearson correlation. Confirmation of associations with covariates in model was conducted with partial Pearson correlation and linear regression. Comparison of outcomes variables with covariates between baseline and 1 month, or between responder categories, was evaluated with generalized linear ANOVA or ANCOVA, with Bonferroni post-hoc adjustment for multiple comparisons when indicated. For long-term analysis of change across 1 year of study visits for each sapropterin response group, mixed linear regression analysis was utilized. Mixed linear regression analysis was selected since the method is robust for analyzing data from unbalanced study designs with frequent missing values and for handling significant variation in both slopes and intercepts [131-133]. Mixed linear regression was chosen over mixed generalized linear ANCOVA due to greater accuracy in reported p-values for the latter method, and the ability to report results as change across time. In the mixed regression models, changes and trends within each response group were evaluated by including the "group*time" interaction variable. Association of a covariate with the outcome variable within sapropterin response groups was evaluated with three way interaction terms (for example: formula protein*group*time). Variables associated with outcome regardless of group (such as age) were included as added covariates in the linear regression model. For example, a linear regression model controlling for age while evaluating change in total QOL score across time for all three response groups would be represented as:

$$\text{Total QOL} = \text{intercept} + b_1(\text{age}) + b_2(\text{time*group})$$

Main effects on outcome for time and group would have been previously evaluated. This is typical of mixed longitudinal regression incorporating multiple linear regression modeling [133, 134]. All outcome variables were evaluated for presence of significant variation in intercepts and slopes (change over time). In most circumstances, intercepts varied significantly among study subjects, but slopes did not. In these circumstances a mixed- intercept with fixed-slope mixed regression model was used in longitudinal analysis. On the rare occasion when slope did vary significantly among research subjects for an outcome variable, mixed-intercept with mixed-slope and "unstructured" covariance designation was utilized.

Sample size and power analysis

Sample size requirements for the study were determined using SPSS SamplePower 2.0 software. SamplePower's sample size and power analysis technique is based on Cohen's calculations for determining effect size [$f^2=R^2/(1-R^2)$] and study power [135].
For the aims listed, a minimum sample size of 51 subjects was calculated as essential for a power of 0.8 with $\alpha=0.05$, an effect size of 0.33, and a maximum of 7 covariates in the linear model. Effect size was determined with the conservative r^2 value of 0.25, based on the increase in platelet serotonin seen in PKU subjects on dietary control at one month and at 6 months as reported by Ormazabal [66]. A paper by Schulpis demonstrating significant correlation between plasma catecholamines and plasma Phe control in PKU subjects [97], and another by Bik-Multanowski demonstrating association between diet related plasma Phe improvements and quality of life in PKU subjects [110], had even larger r^2 values (0.55 and 0.55).

Our final sample size at baseline was 58 PKU subjects, with a subcohort of 37 subjects for the QOL analysis. At end of study, total sample size was decreased to 46 PKU subjects, with 29 remaining in the QOL subcohort (attrition includes both lost to follow-up and exclusions). One monoamine outcome model (HVA) had 6 independent variables included in the final analysis (age, energy intake, formula protein intake, plasma Phe, time, response status), though time and response were part of an interaction term. All other monoamine outcomes had fewer covariates in the regression model. Lowest observed r^2 within the level of significance was 0.236. This yields a total study power of 0.87 at baseline that was 0.75 at end of study. For the QOL subcohort analysis which incorporated at most 5 covariates in the regression model and had a lowest observed r^2 of 0.35 within level of significance, statistical power was 0.90 at baseline and 0.78 at end of study. If using the 0.236 r^2 value for QOL subcohort power analysis, power is 0.67 at baseline and 0.51 at QOL study end. Ability of multiple measurements for each subject across time to increase power was not incorporated into the analysis due to limitations of the SPSS software.

Therefore, in regards to the outcomes reported in this study, sample size and sample power were adequate for detection of significant results. An even larger sample size would certainly have been ideal; however, recruiting large numbers of individuals affected by a rare genetic disorder can be extraordinarily difficult. Our study sample is certainly much larger than what is seen and reported in other published research investigations for PKU, and proven adequate for the longitudinal regression techniques utilized for this investigative analysis.

CHAPTER 3: THE EFFECT OF SAPROPTERIN ON URINARY MONOAMINE METABOLITES IN PHENYLKETONURIA

Introduction

For individuals with the autosomal recessive inborn error of metabolism known as phenylketonuria (PKU), disruption of phenylalanine hydroxylase (PAH) function leads to abnormally high concentrations of phenylalanine (Phe) and phenylketones. If left untreated, severe neurological damage and cognitive impairment occur as early as infancy [9, 10]. Standard treatment for PKU consists of a specialized low Phe diet which includes consumption of phenylalanine-free amino acid rich medical food (formula) as a primary source of protein [20]. This specialized diet, which involves regular monitoring of medical food and Phe intake, enables patients with PKU to maintain plasma Phe concentrations within the safe range of 100-360 umoles/L [22]. Though newborn screening has enabled prompt diagnosis and diet intervention for PKU affected infants, thereby avoiding irreparable lifelong neurologic damage, patients with PKU are still reported to have a higher frequency of psychiatric and behavioral diagnostic disorders [39, 40] as well as other neurological complications [38, 136, 137] that are most pronounced when Phe levels are above therapeutic threshold and dietary compliance wanes [30, 41, 99].

Subclinical monoamine deficiency in PKU patients has been documented in blood, CSF, and brain tissue samples [88, 138]. Observation of lower monoamines neurotransmitters in PKU urine has also been observed [87, 96, 138]. The cognitive and

neurologic ramifications could be attributed to the abnormal monoamine metabolism demonstrated in PKU as a consequence of high Phe [81, 88, 95, 139, 140]. However even when PKU patients are early treated, monoamine deficiency exists and has been observed in PKU mouse model as well [66, 81, 97].

Sapropterin dihydrochloride (Kuvan) is a pharmaceutical form of tetrahydrobiopterin (BH4), an essential cofactor in the metabolism of monoamines due to its chaperone activity for PAH, tyrosine hydroxylase, and tryptophan hydroxylase (Figure 1), though it is also a cofactor for nitric oxide synthase [42]. Though BH4 levels are normal for patients with PKU, several studies have demonstrated that supplemental BH4 in the form of sapropterin can enhance impaired hydroxylase activity [141], particularly PAH [46, 47], thus improving the efficiency of phenylalanine turnover and lowering plasma Phe concentrations [44, 48]. 30-50% of patients with PKU respond to sapropterin treatment (dose: 20mg/kg/day) with a plasma Phe decrease of at least 20% [125, 142].

As just summarized, monoamine neurotransmitter profiles in PKU patients typically exhibit lower concentrations in multiple physiological substrates including lower urinary monoamine concentrations. We hypothesized that sapropterin cofactor action within the monoamine metabolic pathway as well as improved plasma Phe control would improve urinary monoamine profiles for PKU patients responsive to sapropterin therapy.

Methods

Study design and patient enrollment

Patients 4 years of age and older with PKU, who were planning on trying sapropterin dihydrochloride (Kuvan, BioMarin Inc.) to improve plasma Phe control, were asked to volunteer for a 1 year prospective cohort study to evaluate monoamine status at 5 time points: baseline prior to sapropterin treatment, after 1 month of sapropterin when initial response was determined; then 4, 8, and 12 months after the baseline visit for all response categories. Enrollment lasted from October 2008 through October 2009. Participants were asked to provide a 12 hour overnight urine sample at each study visit. Fasting blood samples were collected at each time point for evaluation of plasma amino acids (Biochrom 30 HPLC Amino Acid Analyzer) by the Emory Genetics Lab, while either 3 day diet records or 24 hour diet recalls were collected to estimate dietary Phe and formula intake.

Study subjects provided written informed consent, or the legal guardian's written informed consent with age appropriate patient verbal or written assent, prior to study participation. Study protocol and informed consent procedures were approved by Emory University institutional review board (IRB). Inclusion and exclusion criteria for PKU patients and controls are listed in Table 1.

All study patients were provided sapropterin for 1 month to determine plasma Phe response. Patients were instructed by a registered dietitian not to alter formula and dietary Phe consumption habits during that month to ensure that observed changes in plasma Phe concentration were attributed to sapropterin response rather than diet

changes. If plasma Phe decreased by at least 15% after 1 month on 20mg/kg/day of sapropterin, the patient was classified as a "responder" and permitted to remain on sapropterin thereafter. Patients without this plasma Phe response at 1 month were classified as "nonresponders" and, with one exception, sapropterin was discontinued. Sapropterin nonresponders were requested to return for follow-up study visits under their regular clinical diet treatment plan. Over the next 3 months initial responders were provided with diet challenge and determined to be either "definitive" or "provisional" responders in accordance with previously published criteria [124, 125]. Both definitive and provisional responders remained on sapropterin throughout the study, with two exceptions where parents discontinued sapropterin for an initial responder after 3 months and for a provisional responder after 9 months.

Baseline and exit visits for PKU subjects were collected during an overnight stay at ACTSI-CIS (Clinical Interaction Site) at Emory University Hospital (EUH). Study visits for 1, 4, and 8 months as well as control subject visits were conducted as outpatient at the Emory Genetics Clinic (EGC).

Sample collection, storage, and analysis

12 hour overnight urine samples were collected for each study visit and were elected over 24 hour samples for patient convenience, since overnight urine sampling is a valid technique for evaluating monoamine status [143-145] . Patients were provided light protected collection jugs with gel cold packs to reduce degradation of urine analytes from collection time to appointment time. At time of appointment urine volume was

recorded. Samples were aliquotted into light protected labeled cryogenic tubes and stored at -80°C. A 5mL aliquot for each collected urine sample was submitted to Quest Diagnostics for creatinine evaluation.

Monoamines in urine samples were analyzed by high performance liquid chromatography with electrochemical detection (HPLC-ED). Subject ID number was utilized for random ordering of urine samples for HPLC preparation and analysis, with all available study timepoints for each subject batched together. Frozen urine samples were thawed on ice and centrifuged to sediment any precipitate. A 200 µL supernatant was treated with 20 µL 1.0M perchloric acid (PCA) to yield 0.1M PCA then diluted tenfold with additional 0.1M PCA prior to centrifugation through 0.45 µm PVDF Micro-Spin columns (Grace Davison, Deerfield, IL). Filtrate was transferred to HPLC vials for chromatography analysis. Samples were kept chilled throughout the preparation process.

HPLC analytical method was adapted from previously described technique [129]. Mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium, 75 mM NaH2PO4, 0.25% triethylamine, and 8% acetonitrile at pH 2.9. An ESA 5600A Coularray detection system incorporating an MD-150 X 3.2 mm C.18 column and 5020 guard cell with 6210 detector cell (ESA, Bedford, MA) was used and samples eluted at 0.4 mL/min. Guard cell potential was set at 450 mV while analytic cell potentials were -175, 100, 350 and 425 mV.

Monoamines were identified by comparing electrochemical profiles and retention times with those of known standards (Sigma Chemical Co., St. Louis MO) in

0.1M PCA: epinephrine (E), norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3MT), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA) (Figure 2). Monoamine peaks were further verified by co-elution of test samples supplemented with standards. MHPG and NE could not be identified with certainty in urine due to prevalent co-eluting substances and were not examined for this report. 3MT and 5HT concentrations were effectively considered to be 0 when falling below the lower limit of detection (0.2ng/mL in 1/10 diluted sample).

Data management and statistical analysis

Data from one sibling pair included for baseline and 1 month were excluded from one year follow-up analysis due to deviation from medical protocol. Data from one subject who was 12 weeks gestation by the 5th study visit, and from another who arrived with alcohol intoxication at the 4th study visit, was excluded for those timepoints due to the effect of these variables on laboratory markers. Seven additional PKU subjects who completed the study failed to provide a urine specimen for one or more time points.

Monoamine values from urine were multiplied by 10 to adjust for dilution during sample preparation and were analyzed as ratios to creatinine (Cr) to control for effects of urine volume on concentration (HVA and 5HIAA: mg/g Cr per mL urine. E, DA, DOPAC, 3MT and 5HT: μg/g Cr) [130]. Due to intrinsic differences in urine creatinine excretion between pediatric control and PKU subjects, control monoamine:Cr ratios were adjusted by using the median pediatric PKU creatinine concentration (59.5mg/dL), a method found elsewhere in the literature to control for cohort specific creatinine

differences when calculating ratios [146]. Monoamine:Cr ratios as well as values for urine creatinine (mg/dL), excess Phe intake (mg/day) and formula protein intake below prescribed (g/day), were log10 transformed to adjust for skewness and improve normality of the data.

Statistical analyses utilized SPSS 19.0, and were two-tailed with α=0.05. Baseline analysis of variable associations with monoamine outcomes was conducted with Pearson correlation and multivariate linear regression. Cox regression survival analysis was used to investigate the impact of baseline variables on study attrition. ANCOVA by generalized linear method was used for comparing baseline and 1 month differences in outcome. For associations and trends across the full year of follow-up, mixed linear regression analysis was utilized since the method is robust for analyzing data from unbalanced study designs [131, 133]. For 5HIAA long-term analysis, random-intercept/random-slope mixed modeling was used due to significant patient variation in both slopes and intercepts. For all other long-term outcomes, random-intercept/fixed-slopes mixed models were used due to significant variation in intercepts but not slopes among subjects. The impact of age, gender, socioeconomic variables, plasma amino acid concentrations, dietary factors, and sapropterin response on monoamine outcomes were among the variables examined. Means and 95% confidence intervals were back-transformed into non-logarithmic values for reporting.

Results

Demographics, response, and attrition

Fifty-eight PKU patients, 4-49 years of age at baseline (mean ± SD: 17 ± 11 yrs), enrolled in the study. Thirteen healthy non-PKU controls between the ages of 7-37 years (20 ± 11 yrs) enrolled for one study visit only. Baseline demographic information for patients and controls is described in Table 2. Fifty-three percent (n=31) of PKU subjects exhibited a preliminary response to sapropterin, though only 34% (n=20) were definitive responders. One patient lost to follow up after baseline was not classified. One initial responder did not complete the diet challenge, though he remained in the study, thus his final responder class was not determined. Thus final sample size for each sapropterin response group was 26 nonresponders, 20 definitive responders, and 10 provisional responders, not accounting for attrition by end of study.

Ten subjects (7 nonresponders, 1 provisional responder, 1 definitive responder, and 1 undetermined) withdrew or were lost to follow up prior to study completion. Two other nonresponders deviated from study protocol, resulting in exclusion of their follow up data. Baseline urine 3MT:Cr ratio had the strongest association with attrition, in that patients with higher 3MT at baseline spent longer time in the study (P=.01) and were more likely to complete the study (P=.037) than patients with lower baseline 3MT. Survival analysis revealed patients diagnosed with ADHD at baseline were more inclined to exit the study after the first month (P=.047) (Figure 3). The majority of attrition occurred within nonresponders, although the relationship was significant only after

controlling for 3MT and ADHD covariates (*P*=.045). Presence of anxiety, bipolar, or depression at baseline was not associated with study attrition.

Urine creatinine

Due to urine creatinine concentrations being significantly lower in pediatric (<19 years) PKU subjects compared to pediatric controls (*P*<.0001), control pediatric monoamine:Cr ratios were adjusted as described in the methods section. No urinary creatinine difference was observed between adult subjects with and without PKU. This difference with the PKU cohort remained highly significant even after controlling for urine volume (mL), age, protein intake (g/kg/day), and self reported physical activity level. Gender was not associated with urine creatinine in this study sample.

Serum creatinine also differed significantly between controls and PKU (*P*=.02), even after controlling for age, gender, and per kg protein intake (*P*=.028). Physical activity was unrelated to serum creatinine.

Neither urine nor plasma creatinine concentrations differed significantly between sapropterin response groups or across study visits.

Monoamine: Cr ratios at baseline

Table 3 provides the statistical relationship between baseline monoamine:Cr ratios and covariates. Control subjects had significantly greater mean concentrations of all monoamines (*P*<.0001) except for E and DOPAC. An example of this can be seen in Figure 4, with means and confidence intervals listed in Table 4. Age was inversely associated with DA, DOPAC, HVA, 5HIAA ratios. When evaluating the PKU sample only

without controls, association between age and DOPAC became insignificant (*P*=.074), while 5HT was marginally significant (*P*=.052). Males had higher E compared to females, though when controls were excluded gender had no association with monoamine outcomes in the PKU study sample.

Only E:Cr ratio differed significantly between prospective sapropterin response groups at baseline (Figure 5) with provisional responders having lower E (*P*=.007) than other response groups, irrespective of plasma Phe control.

Independent of later response classification, baseline plasma Phe had a strong negative effect on monoamine:Cr ratios for DA (*P*<.0001), HVA (*P*=.008), 5HIAA (*P*<.0001), and 5HT (*P*=.006) but not E, DOPAC, or 3MT. Baseline plasma tyrosine and tryptophan were not associated with monoamine outcomes after controlling for age and plasma Phe covariates.

Phe intake, either total or above designated tolerance, was not associated with any monoamine outcomes. However, greater noncompliance with formula prescription (calculated by subtracting reported formula protein intake from prescribed formula protein) was associated with lower DA (*P*=.015), HVA (*P*=.045), 5HIAA (*P*=.045), and 5HT (*P*=.018) ratios. Energy intake (kcal/kg), regardless of formula or Phe intake, had a positive association with HVA concentrations (*P*=.014), but no other monoamines.

Neither ADHD nor psychiatric diagnosis of depression, anxiety, or bipolar were associated with any monoamine outcomes at baseline. Reported physical activity level, household marital status, income, and education level were not associated with monoamine outcomes, nor was gender.

Dietary Phe, formula protein, and plasma amino acids post-baseline

Even though patients were provided instructions not to alter dietary Phe or formula intake during the first month on sapropterin, definitive responders still exhibited a significant decrease in Phe intake (*P*=.019) (figure 6a), potentially contributing--in concert with sapropterin--to the observed 1 month decline in plasma Phe. There were no significant long-term changes to mean dietary Phe for provisionals and nonresponders. In addition, nonresponders had a decrease in mean reported formula protein intake over the length of the study (*P*=.021) (Figure 6b), though no significant change during their month on sapropterin. Only definitive responders experienced significant increases to Phe intake and tolerance (*P*<.0001) along with significant decreases to both formula protein Rx and intake (*P*<.0001) across the length of the study.

Plasma Phe concentrations decreased significantly for both definitive (*P*=.001) and provisional responders (*P*=.033) after 1 month on sapropterin while there was no significant change for nonresponders. Controlling for formula intake in the model strengthened the significance for provisionals (*P*=.02) and definitive (*P*<.0001) responders. Over the course of year, trends for each response group demonstrate no statistically significant change in plasma Phe for definitive responders (*P*=.139), a marginal increase at best for nonresponders (*P*=.052) and a significant increase for provisional responders (*P*=.021) (figure 7a). However, after controlling for effect of medical food intake (or lack thereof) on plasma Phe levels, longterm plasma Phe change

for definitive responders became strongly significant (*P*<.0001), although nonsignificant for nonresponders and provisional responders.

Plasma tryptophan did not change for any of the response groups during the first study month, regardless of formula intake in the model. However plasma tryptophan continued to significantly decrease for nonresponders over the course of year (*P*=.002) (Figure 7b) in direct association with declining formula protein intake (*P*=.02) and inversely related to plasma Phe (*P*=.001). These associations were not significant in provisional and definitive responders.

Plasma tyrosine concentrations did not significantly change for any response groups from baseline to 1 month or during the course of a year.

Monoamine:Cr ratios during follow-up

HVA increased significantly for the entire PKU cohort during the first study month (*P*=.015) (Figure 8a). Surprisingly, when evaluating change within each response group, only nonresponders had an increase to HVA that was significant (*P*=.016), albeit not without either formula intake or plasma Phe covariates in the regression model (Figure 8b). The increase to HVA was not sustained long-term for nonresponders once sapropterin was discontinued, nor for the two responder categories.

No significant changes in E, DA, DOPAC, 3MT or 5HT ratios occurred at any time during the one year follow-up, regardless of sapropterin response status or other covariates. 5HIAA did decline slightly across time in definitive responders, but this was only significant after controlling for effects of plasma Phe (*P*=.019)(Figure 9), and was unrelated to other laboratory or dietary variables.

Baseline variables associated with monoamine outcomes remained so for the length of the study with the exception of formula protein and 5HIAA association which became nonsignificant long-term. DOPAC and plasma Phe, though not associated in the baseline analysis, were associated in the long-term analysis (*P*=.035).

Discussion

This study is the first to evaluate short term and long term monoamine changes in PKU urine samples after exposure to sapropterin and, in the case of responders, diet liberalization. Other publications provide evidence that significant improvements to monoamine status in PKU generally follow improvements to plasma Phe control [94, 96, 147] as well as following initiation of sapropterin [66, 148]. However in this investigation, though several monoamines had a significant inverse association with plasma Phe concentrations at baseline and over 1 year, the influence of plasma Phe was not enough to yield significant changes in monoamine status, even in the case of sapropterin responders where plasma Phe concentrations declined dramatically over the course of a month. Although our study evaluated urine monoamines rather than cerebral spinal fluid or platelet concentrations, our results differed also from Krause's 1985 analysis of urine dopamine and serotonin (μg/g Cr) in PKU patients who were crossover treated with high and low Phe diets. This incongruity in results may be due to method of analysis or statistical approach; Krause used radioimmunoassay for undiluted urine and reported only individual results without statistical interpretation. In all likelihood though, our findings simply reflect the high natural variability in monoamine status that occurs in a diverse patient cohort of 58 subjects. In addition, one could

argue that lack of significant decreases to most monoamines in relation to sapropterin use or diet liberalization in PKU patients indicates these therapies are not deleterious in regards to monoamine status, and thus a beneficial outcome in that respect.

An interesting observation in this study was the significant increase in HVA:Cr ratios during the 1 month trial of sapropterin for the whole PKU cohort, and more specifically, sapropterin nonresponders. Since nonresponders had no significant change to plasma Phe during the first study month, this indicates a direct effect of sapropterin on catecholamine metabolism in this group. Though increases in mean HVA were also seen for definitive and provisional stratified responder groups, it did not reach statistical significance, even with covariates in the model. Since no other dopaminergic analyte concentrations were affected by sapropterin therapy in this analysis, it is reasonable to conclude that urinary HVA, a catabolite end product of monoamine synthesis with no known neurotransmitter properties, may be reflecting increases in all three areas of catecholenergic metabolism: synthesis, utilization, and degradation. If only reflective of enhanced degradation, as some may assume, then lower amounts of other catechols would have been detected, which was not the case. An explanation for the significant HVA increase seen specifically in nonresponders is the greater availability of BH4 for TH utilization. Since little to none of the sapropterin was being used by the misfolded PAH enzyme of nonresponders, that left more available for affinity binding to TH, thus stimulating tyrosine conversion to the catecholamine precursor L-DOPA. As to why a similar increase in 5HIAA concentrations as a result of enhanced TPH activity wasn't seen may be due to reduced substrate availability of tryptophan resulting from LNAA

competition by high Phe or high Phe levels directly inhibiting TPH activity, as some studies have indicated [58, 79, 81]. Interpretation of this outcome with HVA must be cautious however, since studies have shown that a placebo effect related to expectation of benefit is also capable of stimulating dopamine metabolism [149, 150].

Of some concern in our long-term analysis is the slight but meaningful decreasing trend in 5HIAA concentrations across 1 year for definitive responders, independent of plasma Phe and dietary factors evaluated. No obvious changes were seen in serotonin levels to corroborate this finding in 5HIAA, however a large portion of PKU patients had serotonin levels that fell below the limits of detection for our HPLC technique (38% of responders, 46% of all PKU subjects). Therefore, what changes may have occurred with serotonin may have gone undetected. At this point it would be premature to conclude serotonin metabolism may be affected by long term exposure to either sapropterin or a liberalized diet given that the long term 5HIAA slope in responders was not independently significant, and since dietary factors were not significantly associated with long-term 5HIAA trends in the definitive responder stratified analysis.

Another important finding within the study was the association observed between formula intake and monoamine concentrations, notably DA, HVA, 5HIAA, and 5HT. In our analysis and others, greater formula protein intake has been shown to be effective at maintaining lower plasma phenylalanine concentrations [151, 152], not surprising since it is a primary source of protein nutrition in the form of free essential amino acids for patients with PKU. The benefit to monoamine status in our investigation

was often an effect of the inverse association between formula intake and plasma Phe control, as the presence of plasma Phe in the model would negate the impact of formula protein. Though in the case of HVA levels, both formula and plasma Phe were significant covariates, indicating some other aspect of formula, such as large neutral amino acid competition [153], was also influential. Our results indicated that degree of adherence or non-adherence to dietary Phe restriction had no significant correlation with monoamine outcomes, implicating formula intake as the critical dietary modulator of not only plasma Phe but also monoamine status. This is true even of patients on sapropterin and with increases to Phe tolerance.

The relationship between ADHD diagnosis and attrition is relevant to future trials and has been reported elsewhere [154, 155]. The prevalence of ADHD in our study supports other literature reporting higher prevalence of ADHD in children with PKU, as in our study sample there were 7 pediatric PKU patients diagnosed with ADHD and 2 over the age of 18. This equates to 18.4% prevalence of ADHD within our PKU pediatric group, nearly double the 9.5% estimate for U.S. children ages 4-17 [156]. Within this study however, psychiatric diagnoses such as depression, anxiety, and bipolar disorder only affected 4 of the adult PKU patients, thus a prevalence of mental illness in this adult sampling (20%) that was similar to the 25% reported for the background U.S. population [157].

The influence of 3MT on attrition is worthy of future investigation and indicates that although 3MT has traditionally been considered a non neuro-active intermediary dopaminergic metabolite, it may well have some nueromodulatory capability. One

animal study in particular supports this possibility [158]; though exactly how lower amounts of 3MT influence attrition, particularly in a cohort in which the baseline mean is already low, remains to be determined.

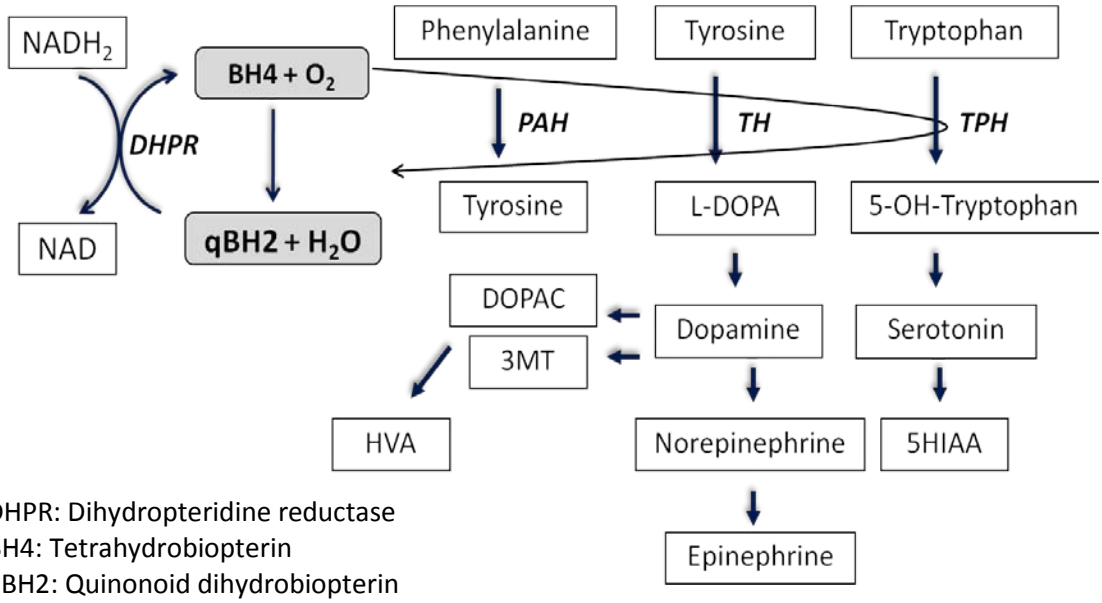
The clinical implications of the biochemical outcomes observed and discussed within this manuscript is beyond the scope of this investigation, but future study designs may discover effects in areas of cognitive, social, neurologic, and psychological outcomes.

In agreement with other published studies which evaluated monoamines between healthy controls and PKU [88, 95, 159], our study also demonstrated reduced monoamine profiles for PKU patients compared to controls, with urinary concentrations being our method of investigation. Baseline catecholamines for both PKU and controls were within population reference ranges reported by Pussard [130].

Conclusions

In summary, this analysis supports the conclusion that sapropterin may be capable of moderately stimulating the dopaminergic pathway in phenylketonuria, particularly for patients who do not respond to sapropterin with decreases in plasma phenylalanine. It also highlights the importance of both formula intake and plasma phenylalanine control to monoamine health, regardless of sapropterin response status. In addition, we found no significant deleterious effects of sapropterin treatment or diet liberalization to current monoamine indicators in a PKU cohort followed over multiple time points in a year.

Figure 1: BH4 within the MA metabolic pathway, adapted from prior source [160].



DHPR: Dihydropteridine reductase
BH4: Tetrahydrobiopterin
qBH2: Quinonoid dihydrobiopterin
PAH: Phenylalanine hydroxylase
TH: Tyrosine hydroxylase
TPH: Tryptophan hydroxylase
L-DOPA: L-3,4-dihydroxyphenylalanine
DOPAC: 3,4-dihydroxyphenylacetic acid
3MT: 3-methoxytyramine
HVA: Homovanillic acid
5HIAA: 5-hydroxyindole acetic acid

Table 1: Inclusion and exclusion criteria for study enrollment

	All	*PKU*	*Controls*
Inclusion		• Minimum age of 4 years • Plan on beginning sapropterin treatment	• Within age range of PKU cohort • Good physical, mental, and cognitive health
Exclusion	• Inability to provide informed consent • Taken sapropterin or BH4 within past 8 weeks • Pregnancy/planning on pregnancy		• Prescribed psychotropic or neurotropic medication • Diagnosed with psychiatric, cognitive, neurologic, or behavioral disorder • First degree family member diagnosed with psychiatric, cognitive, neurologic, or behavioral disorder

Figure 2: Monoamines at 100ng/mL concentration in standard solution. Numerical values are retention times.

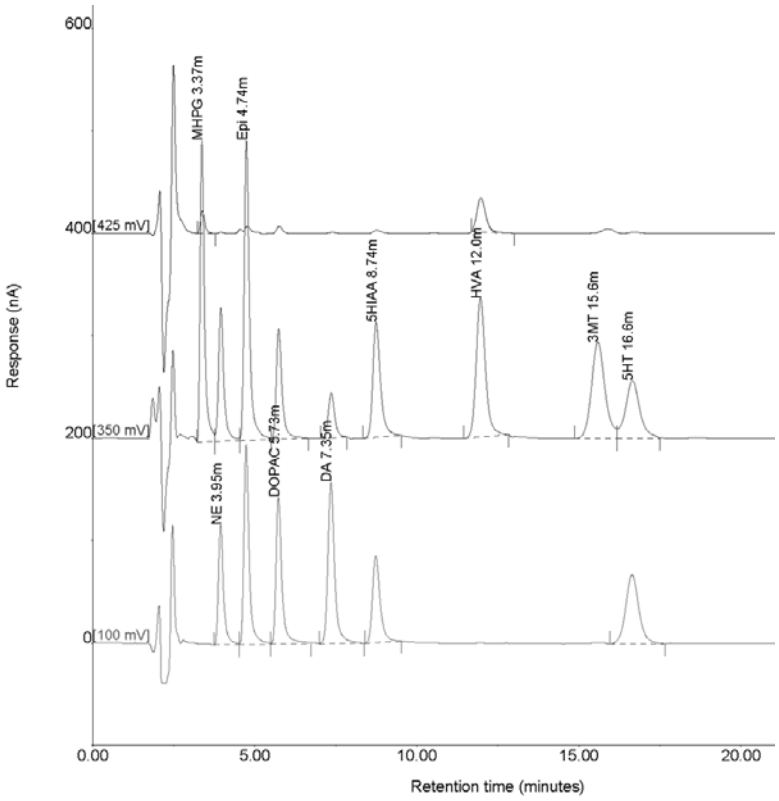


Table 2: Demographics of controls and PKU study participants at baseline

Demographic	N=58 PKU (%)	N=13 controls (%)
Gender		
• Male, Female	34, 24 (59%, 41%)	7, 6 (54%, 46%)
Age (years)		
• Child (4-11.9)	24 (41%)	3 (23%)
• Adolescent (12-18.9)	14 (24%)	5 (38.5%)
• Adult (19+)	20 (34%)	5 (38.5%)
Pairs of biological siblings	5 pairs (17%)	2 pairs (31%)
Neuropsychiatric and behavioral diagnoses		
• No diagnosis	46 (79%)	13 (100%)
• ADHD	7‡ (12%)	0
• Developmental delay (unspecified)	1 (2%)	0
• Other (Depression, Bipolar, Anxiety)	4 (7%)	0

‡2 additional pediatric patients diagnosed with ADHD mid-study and provided medication by last study visit (ADHD final n=9)

Figure 3: Survival diagram demonstrating relationship between baseline psychiatric or ADHD diagnosis and attrition.

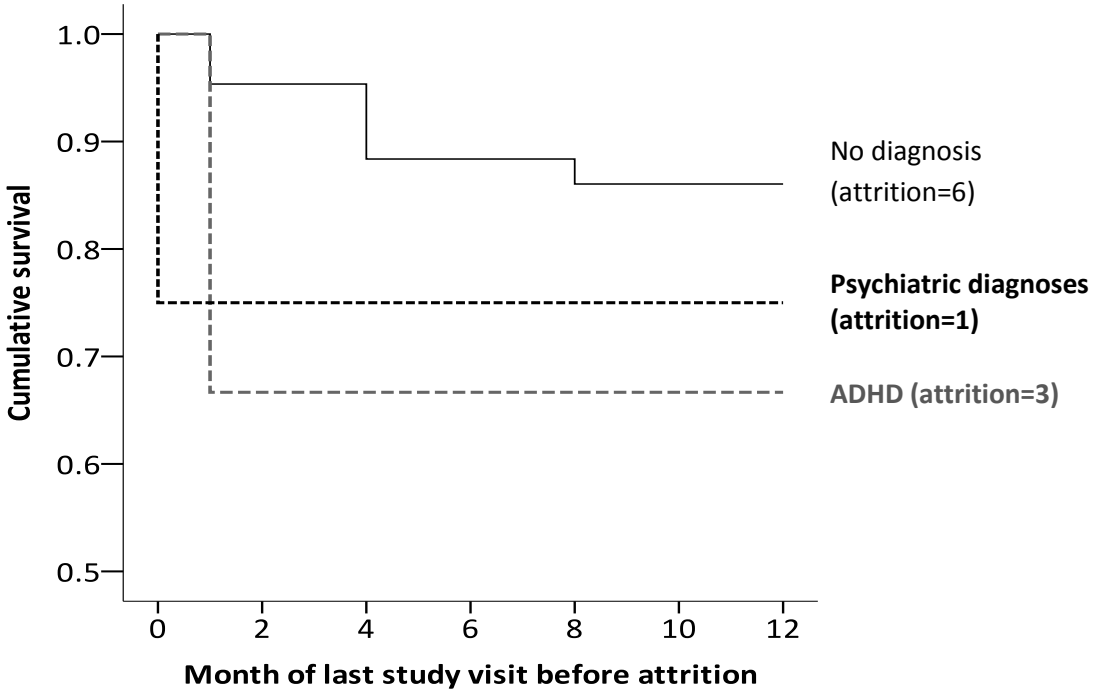


Table 3: Baseline associations of study variables with the adjusted log10(monoamine:Cr) ratios. Only variables with significant associations are included in the table.

	E	DA	DOPAC	HVA	3MT	5HIAA	5HT
Variables	**Pearson coefficient**						
Age	.09	-.523***	-.278*‡	-.628***	.011	-.471***	-.149
Energy intake	-.104	.224	.006	.268*	.160	.217	.171
Plasma Phe §	.043	-.534***	-.138	-.442**	-.079	-.636***	-.386**
Formula compliance§	.140	-.402**	-.172	-.342*	-.132	-.329*	-.325*

*P<.05, **P<.01, ***P<.001

Controlled for age when evaluating variable correlations with DA, DOPAC, HVA, 5HIAA, and 5HT

‡Not significant in PKU cohort with controls excluded.

§PKU cohort only

Figure 4: Baseline monoamine chromatograms for control subject (a) and PKU subject (b) matched by age and gender. Numerical values are concentrations.

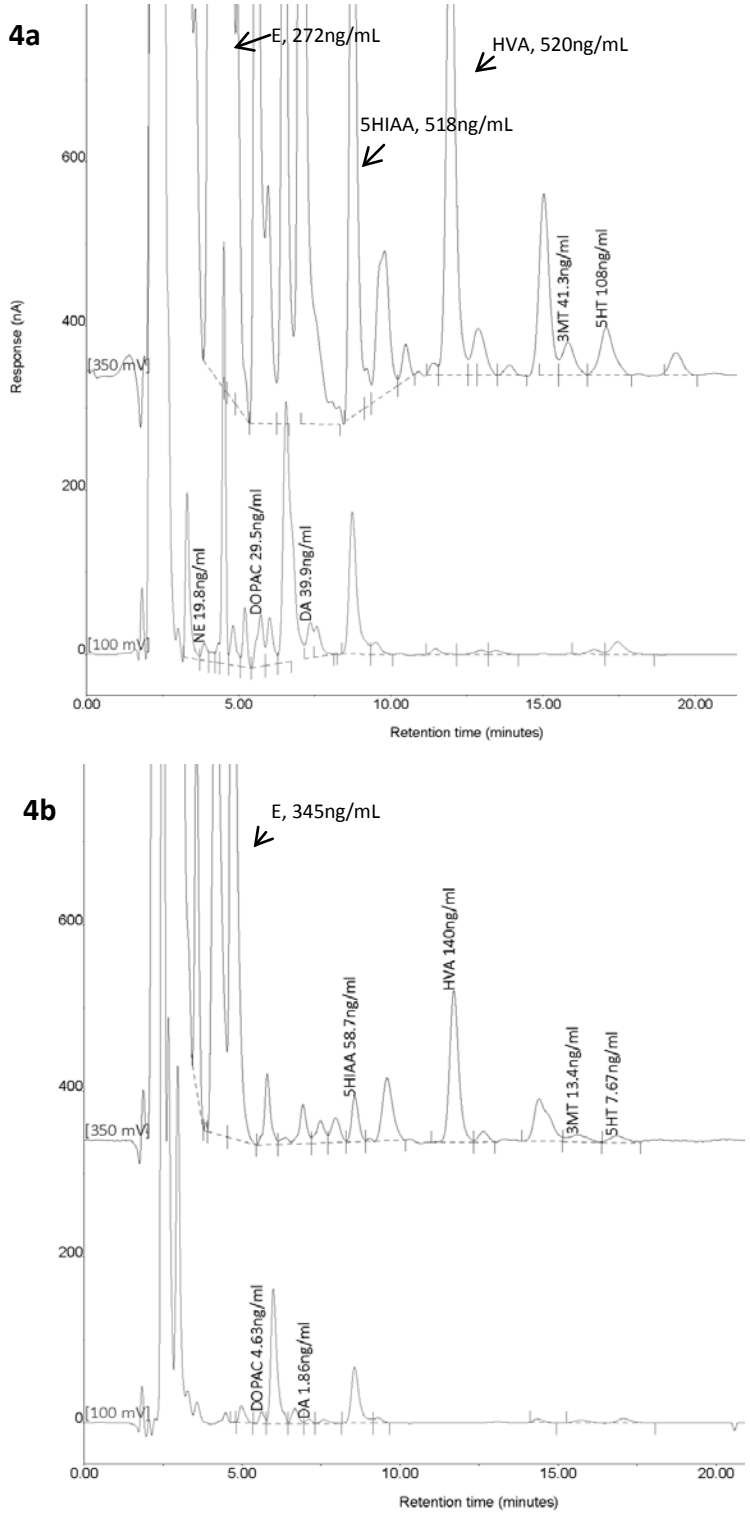


Table 4: Means (with 95% CI) of baseline adjusted urine monoamine:Cr ratios, back transformed from log10 values.

Monoamines (unit per g Cr)	PKU	Controls
E (µg)	2703 (2098-3482)	2715 (1358-5445)
DA (µg)	101 (83-123)	266 (158-450)***
DOPAC (µg)	233 (165-329)	389 (230-661)
HVA (mg)	3.43 (2.94-3.98)	6.80 (5.06-11.99)***
3MT (µg)	21 (11-40)	387 (255-590)***
5HIAA (mg)	2.25 (1.86-2.69)	7.78 (5.56-13.84)***
5HT (µg)	17 (9-32)	1021 (649-1609)***

Significant differences between PKU and controls marked with *** (*P*<.0001)

Figure 5: Mean (± 95% CI) baseline log10(E:Cr) ratios differentiated by sapropterin response classification (*P* values are Bonferroni adjusted for multiple comparisons)

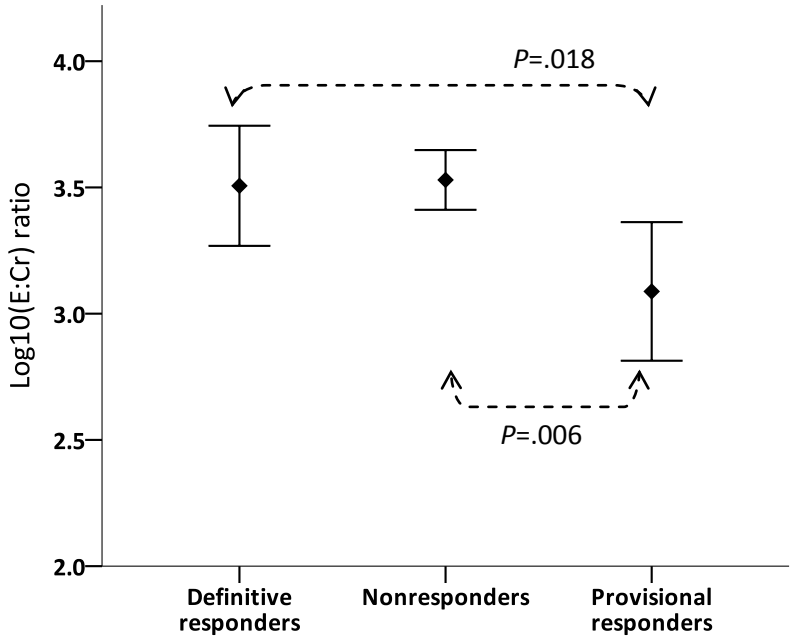
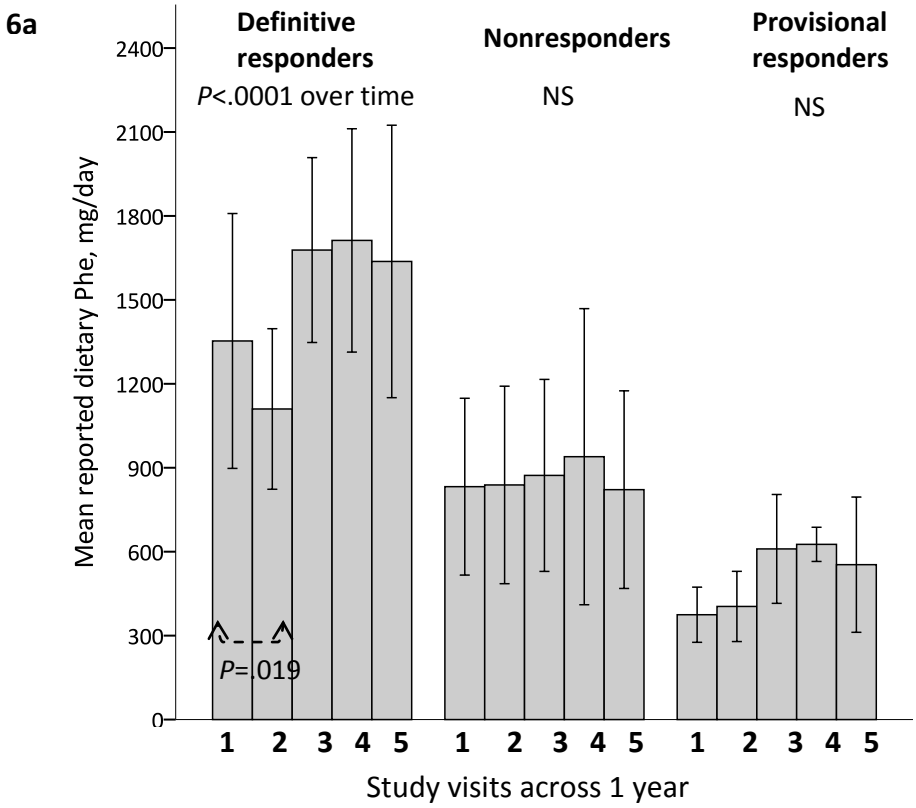


Figure 6: Changes to reported mean (± 95% CI) dietary Phe (a) and formula intake (b) for each response group across study visits. NS=not significant.



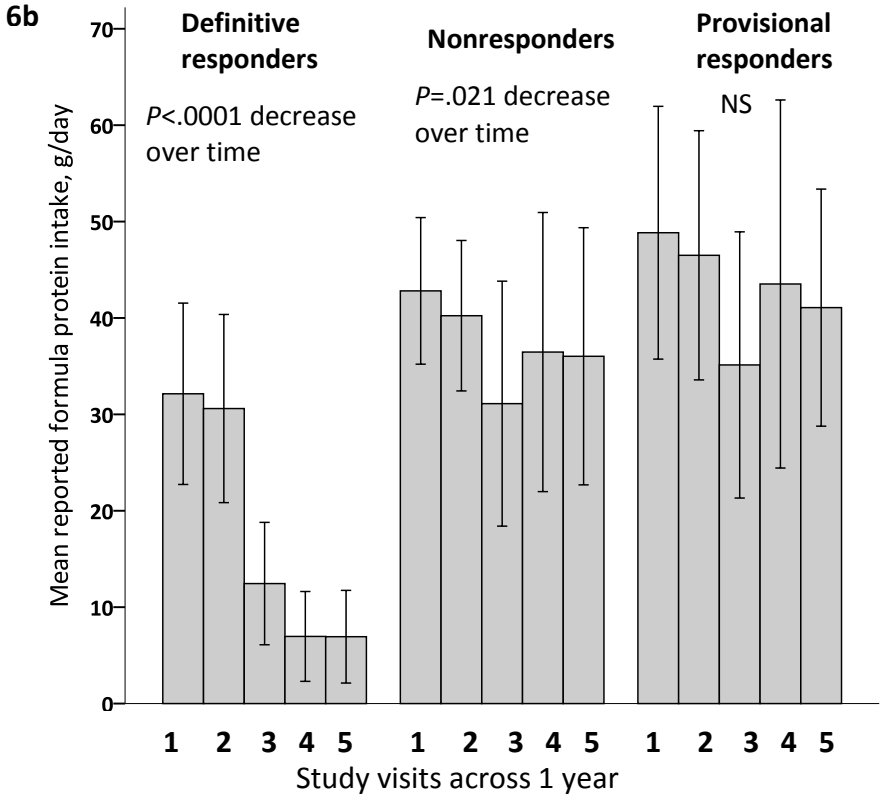


Figure 7: Plasma Phe (a) and plasma tryptophan (b) (mean with 95% CI) across timepoints for all sapropterin response groups. Normal plasma Phe < 150 µmoles/L. Therapeutic threshold for PKU is 360 µmoles/L.

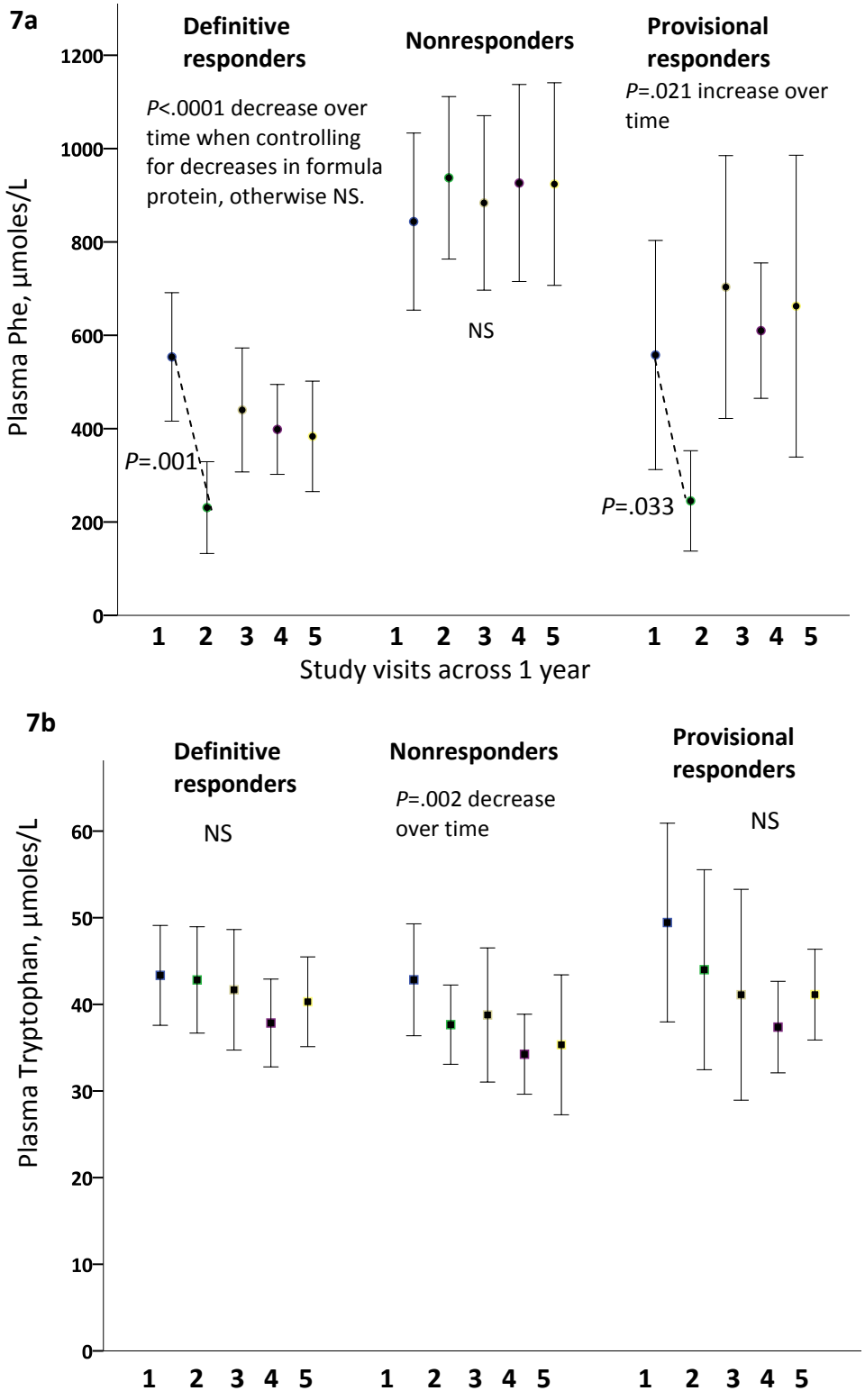
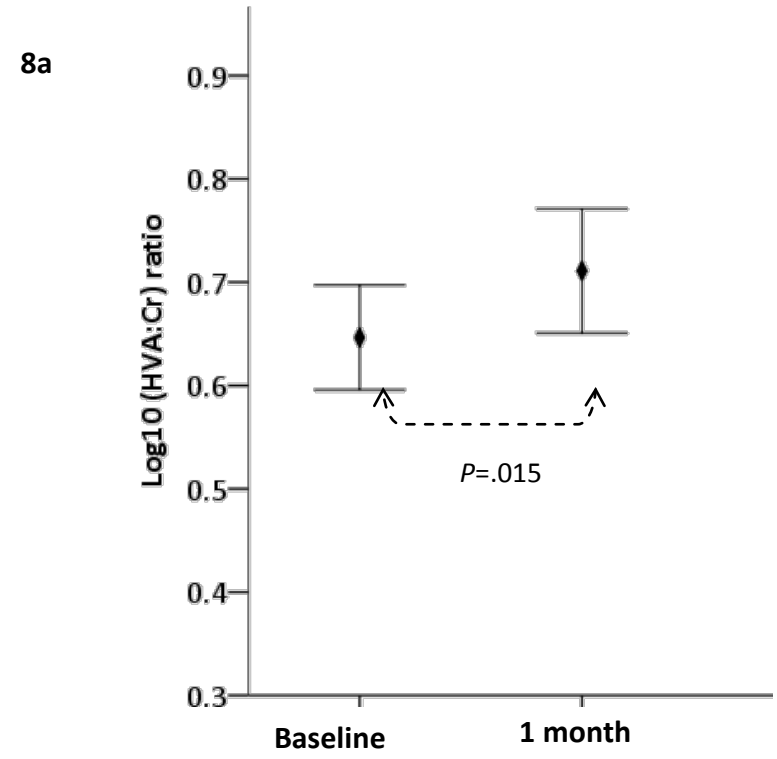


Figure 8: HVA (mean ± 95% CI) (a) for entire PKU cohort at baseline and 1 month (b) stratified by response category across 1 year follow-up.



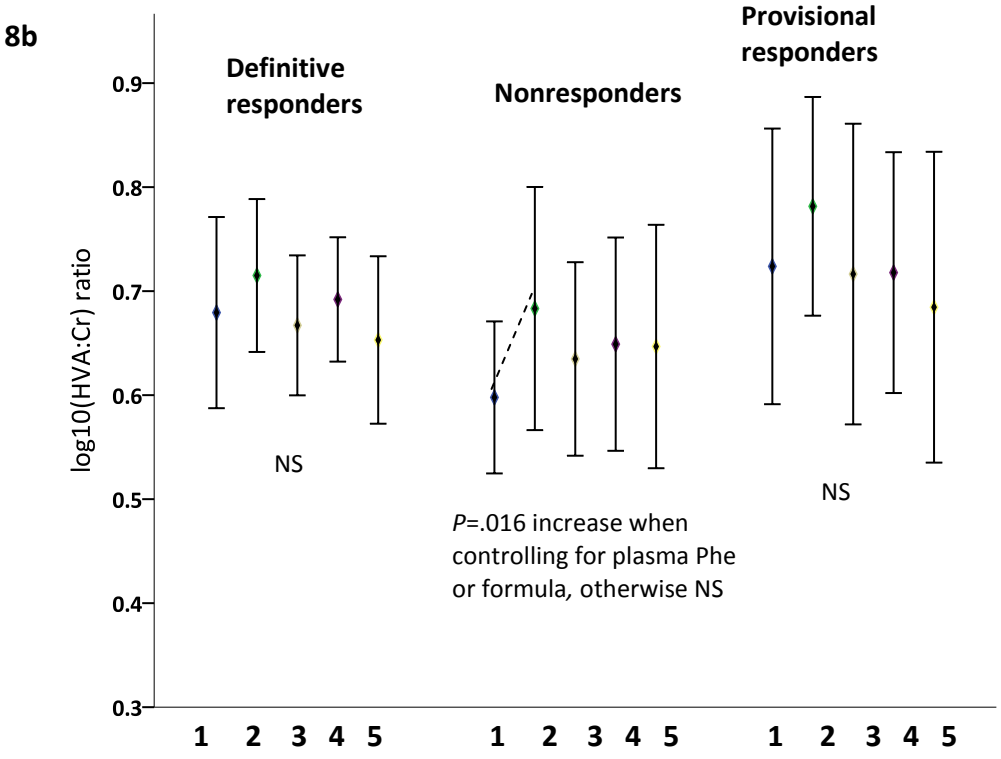
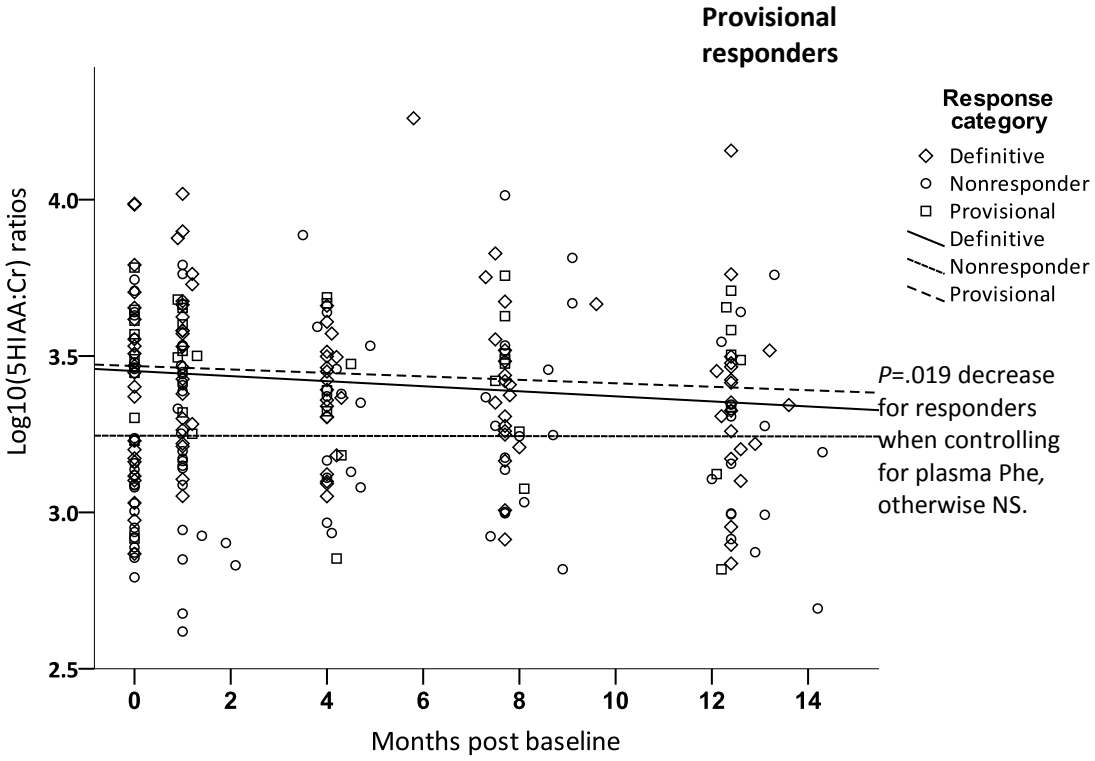


Figure 9: 5HIAA (mean ± 95% CI) mean long-term trends stratified by response status across 1 year follow-up



CHAPTER 4: QUALITY OF LIFE OUTCOMES FOR PHENYLKETONURIA PATIENTS PROVIDED SAPROPTERIN DIHYDROCHLORIDE (TETRAHYDROBIOPTERIN, BH4) WITH AND WITHOUT INCREASES TO DIETARY PHENYLALANINE TOLERANCE

Introduction

In individuals with phenylketonuria (PKU), an autosomal recessive inherited inborn error of metabolism, phenylalanine hydroxylase (PAH) cannot efficiently metabolize the essential amino acid phenylalanine into tyrosine. This leads to high physiological concentrations of phenylalanine (Phe) and phenylketones that when left untreated result in severe neurological damage to patients with PKU.

Standard treatment for PKU consists of a specialized diet centered on lifelong consumption of phenylalanine-free amino acid rich medical food (formula) as a primary source of protein nutrition along with foods low in Phe content. Dietary treatment, which involves the monitoring of medical food and Phe intake, allows patients with PKU to maintain plasma Phe concentrations within the safe therapeutic threshold of 100-360 µmoles/L. Newborn screening has enabled early diagnosis of PKU within industrialized countries, thus allowing infants to begin dietary treatment for PKU within the first few days of life, thereby avoiding neurological damage that could otherwise lead to severe lifelong developmental and cognitive disability.

The dietary regimen for PKU however can be rigorous, expensive, and socially burdensome to both families and patients [23, 112]. As patients move from childhood into adolescence and adulthood, dietary compliance decreases, and plasma phenylalanine concentrations increase beyond the therapeutic range [109, 161]. Both

high plasma Phe concentrations, as well as the strict medical diet those with PKU are encouraged to follow lifelong, have been implicated in reduced quality of life (QOL) for this patient group [106, 107].

Sapropterin dihydrochloride is a pharmaceutical form of the chaperone PAH cofactor molecule tetrahydrobiopterin (BH4). An estimated 1/2 of patients with PKU are reported to respond to sapropterin treatment (20mg/kg/day) with plasma Phe decreases of at least 20% [142] and approximately 1/3 with a minimum 300mg/day increase in dietary Phe tolerance, resulting in a more liberalized diet [125].

We hypothesized that improved plasma Phe control along with increased dietary Phe tolerance would increase QOL outcome measures in an adolescent and adult cohort of PKU patients taking sapropterin.

Methods

Study design and patient enrollment

Patients 10 years of age and older with PKU, who were planning on trying sapropterin dihydrochloride (Kuvan, BioMarin Inc.) to improve plasma Phe control, were asked to volunteer for a 1 year prospective cohort study in which QOL would be evaluated in the form of a self-report questionnaire. Enrollment lasted from October 2008 through October 2009. Participants were asked to complete the questionnaire prior to sapropterin initiation as well as at 1 month, 4 months, and 8 months, and 12 months post-baseline. Blood was collected during corresponding clinic visits for evaluation of plasma amino acids (Biochrom 30 HPLC Amino Acid Analyzer), while 3 day diet records or 24 hour diet recalls were collected to estimate dietary Phe and formula intake. Diet

and laboratory data from the corresponding clinic visits was obtained from patient medical charts after receiving written legal guardian or patient authorization to access medical record information.

Patient volunteers provided written informed consent, or the legal guardian's written informed consent with age appropriate patient verbal or written assent, prior to study participation. Study protocol and informed consent procedures were approved by the Emory University institutional review board (IRB). Inclusion criteria were a diagnosis of PKU, minimum age of 10 years, and interest in sapropterin as a treatment for PKU. Exclusion criteria were having been on a BH4 therapy within 8 weeks prior to start of study, pregnancy or planning to become pregnant, noncompliance with study protocol, and literacy or comprehension difficulties that would limit patient ability to provide informed consent or complete the QOL questionnaire.

All patients were recruited through the Emory University Genetics Clinic in Atlanta Georgia. Patients were determined by their medical practitioner to be a responder to sapropterin if plasma Phe decreased by at least 20% after 1 month of sapropterin therapy (20mg/kg/day) and were permitted to remain on sapropterin thereafter. Patients not demonstrating this degree of response at 1 month were classified as nonresponders (Figure 1). With one exception, sapropterin was discontinued per the discretion of the medical practitioner for nonresponders. All patients were instructed by their registered dietitian not to alter formula and dietary Phe consumption habits during the 1 month trial period on Kuvan to ensure that observed changes in plasma Phe concentration were attributed to sapropterin response

rather than dietary manipulation. Sapropterin nonresponders were asked to continue with follow-up study visits under their regular clinical diet treatment plan. Classified responders over the next 3 months were provided with diet challenge and determined to be either definitive or provisional responders according to previously published criteria [125] (Figure 1). Both definitive and provisional responders remained on sapropterin throughout the study period, with exception of one provisional responder whose parent discontinued sapropterin prior to the final visit.

QOLQ data collection and management

The QOL questionnaire selected for this investigation, originally developed by Rani H. Singh, is structured similar to the validated juvenile diabetes QOLQ [127]. The PKU QOL questionnaire has been shown to predict positive adaptive outcome in an adolescent female cohort of PKU patients [128] and successfully correlated to child behavior checklist scores in a preliminary validation performed with adolescent PKU females.

The QOLQ consists of 5 quantitative subsections, each with a subset of numbered questions that can be answered on a five-point Likert scale, with 1 being the most positive response and 5 being the most negative response (subsections and scoring ranges described in Table 1). For the purpose of this investigation, the QOL questionnaire was adapted for use in a combined adult and adolescent PKU cohort by altering questions which ask about school to refer to “work or school”. However two “work or school” questions had to be eliminated from final scoring due to over 20% of patients leaving the questions blank. Adapting the QOL questionnaire to a male inclusive

cohort was not necessary since no questions were female specific in nature and no gender associated differences were found in the results.

Subsections evaluated 5 areas in which PKU could influence quality of life: impact of PKU on life quality, PKU related life worries, satisfaction with life and medical management, sense of support from social network and clinical community, and perspective on general well-being. Subscores for the quantitative subsections were combined into a total QOL score for each study subject. To avoid confusion when reporting improvements to QOL in association with study variables, all QOL subscores were reversed (such that 1 indicates lower QOL and 5 indicates better QOL) and then summed to equal a total reverse-score. All QOL results being reported here are represented by reverse-scores.

Statistical analysis

Statistical analyses utilized SPSS 19.0 and were two-tailed with $\alpha=0.05$. Baseline analysis of variable associations with QOL scores was conducted with Pearson correlation and multivariate linear regression. Cox regression survival analysis was used to investigate the impact of baseline variables on study attrition. Baseline and 1 month analyses conducted with Pearson correlation and ANCOVA methods. For associations and trends across the full year of follow-up timepoints, mixed linear regression analysis was utilized since the method is robust in circumstances when study data includes unevenly spaced time points, unequal n values between study groups, and missing data points [131]. For all long-term QOL outcomes other than Worries, random-intercept/fixed-slopes mixed

model was used due to significant variation in intercepts but not slopes among subjects. For long-term analysis of Worries sub-score, random-intercept/random-slopes model was used due to significant variation in both intercepts and slopes. The impact of age, gender, socioeconomic variables, formula and dietary Phe intake, plasma amino acid concentrations, and sapropterin response on QOL outcomes were among the variables evaluated.

Results

Demographics and sapropterin response

Thirty-seven male and female patients, age 10-49 years old at baseline (mean ± SD: 22.1 ± 9.4), provided consent for participation in the QOL analysis. Demographic data from the baseline study sample (N=37) is portrayed in Table 2. Figure 1 provides the number of patients classified as nonresponders, provisional responders, and definitive responders during the study period.

Attrition

N values for reporting total QOL scores and outcomes for each subsection differ from the baseline N of 37 (Table 3) due to the following circumstances: one patient with undetermined response status ceased participation after baseline while seven subjects classified as nonresponders were lost to follow-up before the final visit. Of the 29 patients that completed the long-term QOL study, 8 failed to return a QOL questionnaire for one or more timepoints during the study period. In addition, 2 study participants did not complete all subsections of the QOLQ for at least one timepoint.

Study attrition was significantly related to both nonresponder status (*P*=.043) and single marital status (*P*=.037). In addition, baseline Support sub-scores had a positive association with length of time in the study (r=0.45), even after controlling for response and marital status (*P*=.005). Associations remained significant after controlling for same-household siblings. No other variables, including other QOL sub-scores or total QOL score, were associated with attrition.

QOL at baseline

Table 3 describes the average QOL scores, and scoring ranges, for the PKU cohort at baseline. Total and sub-scores averaged high, with at least 74% of participants having baseline sub-scores above the midscore and 94% having a total QOL score above midscore.

Adjusting for one low scoring outlier (> -2 SD below mean) who discontinued the study after baseline, QOL total and sub-scores—except for Support sub-score—were inversely associated with age; associations with QOL scores other than Support sub-score are therefore age adjusted (Table 4). Plasma tyrosine was inversely associated with Worries sub-score (*P*=.025) but no other QOL scores. Physical activity (reported on a 5-point Likert scale) had a positive association with General sub-score (*P*=.018), while patients diagnosed with a psychiatric disorder reported lower Satisfaction scores (*P*=.028) (Figure 2). Plasma Phe, prescribed diet, marital status, income and education level were not associated with baseline QOL scores. There were also no differences in baseline QOL scores among prospective sapropterin response groups. Inclusion of single low scoring outlier in analyses yielded null associations for all variables except for age's

associations with Total score (*P*=.042), Satisfaction sub-score (*P*=.021) and Generally sub-score (*P*=.005) and physical activity's association with Generally sub-score (*P*=.012). Controlling for same-household siblings did not affect results.

QOL over 1 year

Age continued to have a significant association during long-term follow-up with all QOL outcomes except for Support sub-score, and was thus controlled for in relevant mixed regression models. Likewise physical activity was associated long-term with General well-being subscores, even though these scores did not change during one year. In spite of a baseline association, plasma tyrosine was not associated long-term with Worries sub-scores for any of the sapropterin responder groups. Due to post-baseline missings for subjects diagnosed with represented psychiatric and behavioral conditions, conclusions regarding the effect of psychiatric status on long-term QOL outcomes could not be determined.

Even though plasma Phe significantly dropped for sapropterin Responders (P<.0001) (Figure 3) during the 30 day Kuvan trial, no statistically significant change in QOL scores occurred within or between response groups from baseline to the 1 month visit.

Longterm analysis indicates that even though there were no statistically significant differences in QOL scores between sapropterin response groups during the 1 year follow-up, significant within group changes did occur as detailed below and in Figures 4A-4C.

Sub-scores

All response categories demonstrated an improvement in Impact sub-scores during the 1 year follow-up, with Definitive Responders experiencing the greatest improvement (*P*<.0001) compared to Provisional Responders (*P*=.01) and Nonresponders (*P*=.05).

Satisfaction sub-scores improved significantly for Definitive Responders only (P=.001), with no significant changes for the other two response groups.

Worries, Support, and General well-being subscores did not change significantly over 1 year, regardless of response category.

Total QOL scores

Sub-scores summed to evaluate total QOL benefit yielded statistically significant improvement for both Definitive (*P*=.001) and Provisional responders (*P*=.028). In spite of modest improvement in Impact sub-scores for Nonresponders as well as for the other sapropterin groups, total QOL score for Nonresponders did not change significantly over one year.

Associations with Phe tolerance, formula protein, and plasma Phe

During one year of follow-up, plasma Phe concentrations decreased (*P*=.002) for Definitive Responders (Figure 5) while dietary Phe tolerance increased (*P*<.0001) and formula dependence decreased (*P*<.0001) (Figure 6A, 6B), though not significantly for Provisional Responders and Nonresponders. These biomarkers also differed between groups during the long-term follow-up in that Nonresponders had higher plasma phenylalanine in general (P=.018) while Definitive Responders had higher Phe tolerance (*P*<.0001) and lower formula protein needs (*P*=.022) when compared to other response

groups. Plasma tyrosine levels did not differ for any response groups during the study or change significantly across time.

Table 5 provides *t* and *F* statistics for regressed Phe and formula associations with long-term QOL outcomes.

Prescribed increases to dietary Phe (mg/day) were associated strongly with the improved total (*P*=.005), Impact (*P*<.0001), and Satisfaction (*P*=.022) QOL scores seen in Definitive Responders across time. Interestingly, long-term Phe tolerance associated significantly with Impact subscores for Nonresponders (*P*=.011) as well, though not for Provisional Responders (*P*=.062).

To a lesser extent, but still significant, changes to prescribed formula protein (g/day) were associated with better Satisfaction sub-scores (*P*=.037) for Definitive Responders, and improved Impact scores in Provisional responders (*P*=.039), but not with any other QOL outcomes.

Plasma Phe control was associated with better Satisfaction (*P*=.007) and total QOL scores (*P*=.028) for Definitive Responders, and improved Impact scores for Nonresponders (*P*=.042) after controlling for other relevant variables such as age, dietary Phe, and formula protein intake. Plasma Phe was not associated with any QOL outcomes in Provisional Responders.

Discussion and conclusion

The high baseline scores of our study patients however could have resulted in a ceiling effect when evaluating long-term changes. In essence, improvements to quality of life may not be as measurable when quality of life is already near the upper scale limits. This

phenomenon may be a reflection of patient adaptation, in an emotional and psychological sense, to their disorder [162, 163] or it may indicate a need for improved sensitivity in the questionnaire's QOL scale. In addition, 83% of the cohort reported Phe intake above Phe tolerance at baseline (Figure 7), thus improvements to QOL extending from sapropterin induced increases to Phe tolerance could be attenuated in patients already consuming liberal amounts of Phe.

An interesting observation in this analysis was that even though differences in Phe tolerance were not associated with QOL outcomes at baseline, increases to Phe tolerance over the course of one year were significantly associated with improved QOL outcomes. The lack of baseline association may well be due to the psycho-emotional adaptation of these patients to the constraints of PKU diagnosis, as discussed earlier, particularly since patients have lived with the condition since birth. However, a fast dramatic increase in Phe tolerance that is sustained as a result of sapropterin treatment could certainly boost QOL self-perception when measured over time. Whether that improvement in QOL can be sustained, or whether patients will habituate to their higher Phe tolerance with QOL gradually returning to baseline, remains to be seen in future analysis where follow-up extends beyond one year.

Also of interest was that both nonresponders and provisional responders, although without significant change to Phe tolerance or formula dependence nevertheless had significant improvement in regards to the impact of PKU on life quality. Though the trend for provisionals and nonresponders was modest and not to the extent seen for definitive responders who had significant diet liberalization, the

increase was still notable. This may be the equivalent of a Hawthorne effect in these groups in which patient's answers to QOL questions change over time as a result of monitoring within the study parameters. A placebo effect in the case of provisional responders in also possible, as patients within this group anecdotally reported the benefit they felt sapropterin had on their lives even though their diets were not significantly liberalized.

Study results indicate baseline Support scores, in addition to other factors such as marital status and sapropterin response classification, could effectively predict long-term attrition within our PKU study group. In fact, sense of emotional support had the strongest relationship with study attrition, such that the amount of weeks spent in the study could be predicted by how high the support scores were at baseline, especially within the Nonresponder group where most of the attrition occurred. Interestingly the support question represents the smallest section of the QOLQ with only 4 queries asking about support received from the person's family, friends, medical team, and nutrition team. An improvement in just one or two of these support aspects could dramatically reduce attrition risk in future studies, thus it may be worthwhile to ensure patients are provided an emotionally supportive clinical environment from their very first appointment when longterm follow-up is indicated.

Due to several adult PKU patients in this study being neither employed nor in school, two work/school questions were frequently skipped. Modifications to the PKU-QOLQ may be necessary to accommodate this circumstance in adults, however for this

study we resolved the issue by eliminating those two specific questions from final scoring and analyses.

Our results support other QOL studies which demonstrate an inverse association between age and QOL scores [164-166], thereby stressing the need to control for age when evaluating QOL differences in clinical groups. It was a pleasant surprise to observe that the majority of PKU study patients had QOL scores exceeding the midscore, with high means at both baseline and throughout the study (Table 3, Figures 4A-4C). However, a 2011 study evaluating QOL in PKU subjects discovered low QOL scores in pediatric PKU subjects compared to a non-PKU reference group [103] while no differences have been found with adults when compared to a non-PKU reference sample [103, 167], or with a combined adult adolescent cohort such as ours [168]. Our results do not necessarily conflict with these studies since the focus of our study was to investigate the effects of sapropterin intake and corresponding changes in Phe tolerance on QOL outcomes within the context of the specific medical disorder PKU, rather than to compare QOL with that of non-PKU populations. Hence our tool for evaluating QOL is specific and relevant only to individuals diagnosed with PKU. The questionnaire would have not been applicable to a control group unaffected by PKU. In addition, non-PKU controls would not have had access to the sapropterin, which is an important variable in this investigation. Therefore, the PKU cohort served as their own control group by comparing pre and post sapropterin and pre and post diet liberalization depending on specific Phe response to sapropterin. Also, the QOLQ utilized in this study was designed for self-report by adolescents and adults, whereas in Cotungo's study, pre-adolescent

patients were included in which parents completed the QOL forms on their child's behalf, which could certainly lead to a difference in results.

To emphasize, though this study focused on patient self reported QOL only, there is also potential for improved QOL outcomes in parents, caregivers, spouses, or other patient family members when the individual with PKU is able to increase dietary freedom due to a new treatment such as sapropterin or PEG-PAL. Though our clinicians have received many positive anecdotal reports from family members and caregivers of sapropterin Responders (both Definitive and Provisional), a formal analysis is needed to determine measurable QOL benefits to family members and caregivers as well. However such an analysis was outside the scope of this investigation.

Conclusion

The results of this study provide opportunity to better assist PKU patients in areas where they report feeling most challenged or discouraged from a social-medical perspective. The knowledge can also assist with the technicalities of adjusting therapeutic protocols as patients gain access to emerging treatment options, including treatments which may offer more dietary freedom, as well as with planning of long-term clinical studies. In this way, patients can receive the emotional, medical, and dietary support they need for effective lifelong management of their disorder.

Though self reported quality of life is high in PKU patients, particularly for adolescents and young adults, marked improvements still occurred for QOL areas that measure impact on various aspects of life and self-sense of satisfaction. Improvements were most dramatic for patients who experienced increased Phe tolerance while taking

sapropterin. These improvements were accomplished while maintaining good Phe control in the majority of Definitive Responders. Patients who did not experience greater Phe tolerance, whether on or off sapropterin long-term, still demonstrated modest improvements to QOL during the study, though to a lesser extent than those with increased Phe tolerance. Aspects of general well being, sense of social support, and worries in relation to PKU and life were not affected by sapropterin response classification, Phe tolerance, formula prescription, or plasma amino acids during the long-term study. However baseline Support scores were highly indicative of later study attrition in Nonresponders. Also, psychiatric diagnosis was associated with lower baseline Satisfaction scores while higher physical activity levels correlated with greater general wellbeing.

Figure 1: Response classification of study group, per criteria of Singh and Quirk [125]

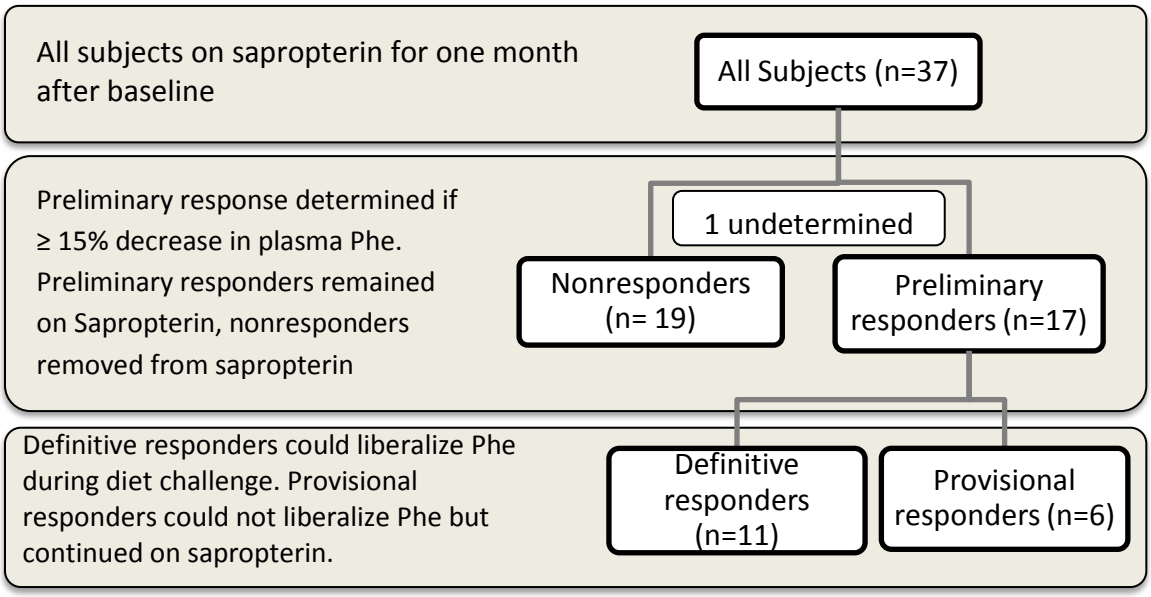


Table 1: Attrition within the study group for each timepoint

Timepoint	Available n	Withdrawals or loss to follow up (cumulative)	Unreturned forms
Baseline	35	0	2
Study visit 2	34	2	1
Study visit 3	28	5	4
Study visit 4	23	7	7
Final study visit	28	8	1

Table 2: Demographics of study participants (N=37) at baseline

Variable	N (%)
Gender	--
• Male	20 (54 %)
• Female	17 (46 %)
Age (years)	--
• Adolescent (10-19)	19 (51 %)
• Adult (20+)	18 (49 %)
3 pairs of biological siblings	6 (16 %)
Neuropsychiatric and Behavioral diagnoses	--
• No diagnosis	29 (78 %)
• ADHD	4 (11 %)
• Other (Depression, Bipolar, Anxiety)	4 (11 %)
Marital status‡	--
• Single	7 (19 %)
• Married	26 (70 %)
• Divorced	3 (8 %)
• Widowed	1 (3 %)

‡Marital status represents participants themselves age 19+, and of the legal guardians for subjects <19 years.

Table 3: Summary of PKU cohort baseline QOL scores (Total and section subscores). Scoring limits are *minimum score*: lowest score possible if all questions answered as 1 on Likert scale, *maximum score*: highest score possible if all questions answered as 5. *Midscore* is center score between minimum and maximum.

				QOL Scoring	
	Mean ± SD	Range	n with scores > midscore	*Min-Max*	*Midscore*
Scoring total (n=34)	**206 ± 29**	**109 to 246**	**32 (94%)**	***51-255***	***153***
Impact (n=35)	71 ± 11	31 to 85	32 (91%)	*18-90*	*54*
Worries (n=35)	44 ± 8	13 to 50	33 (94%)	*10-50*	*30*
Satisfaction (n=35)	30 ± 7	15 to 40	26 (74%)	*8-40*	*24*
Support (n=34)	18 ± 3	10 to 20	30 (88%)	*4-20*	*12*
Generally (n=34)	44 ± 8	30 to 54	29 (85%)	*11-55*	*33*

Table 4: Baseline associations of independent variables to QOL outcomes (Rx=Prescribed, MF=Medical food/formula), reported as the r coefficient with 95% confidence interval (CI) for strength and direction of association.

	Impact	Worries	Satisfaction	Support	Generally	**Total score**
Variables	r (CI)	r (CI)	r (CI)	r (CI)	r (CI)	r (CI)
Age (years)	-.37* (-.63,-.04)	-.35* (-.61,-.02)	-.48** (-.70,-.17)	-.25 (-.54, .10)	-.55** (-.75,-.26)	-.58*** (-.77, -.30)
Plasma Phe (µmoles/L)	.03 (-.31, .36)	.24 (-.10, .53)	.10 (-.24, .42)	-.22 (-.52, .13)	-.13 (-.45, .22)	.03 (-.31, .36)
Plasma Tyrosine (µmoles/L)	.09 (-.25, .41)	-.39* (.07, .64)	.02 (-.31, .35)	.12 (-.23, .44)	.12 (-.23, .44)	.00 (-.34, .34)
Phe tolerance (mg/day)	-.05 (-.38, .29)	.09 (-.25, .41)	.04 (-.30, .37)	-.02 (-.35, .32)	.10 (-.25, .42)	.07 (-.27, .40)
Formula protein Rx (g/day)	.01 (-.32, .34)	.13 (-.21, .44)	.18 (-.16, .48)	-.23 (-.53, .12)	-.13 (-.45, .22)	.02 (-.32, .35)
Physical activity	-.04 (-.37, .30)	-.24 (-.53, .10)	.29 (-.05, .57)	.30 (-.04, .58)	.41* (.08, .66)	.17 (-.18, .48)

*P<.05, **P<.01, ***P<.001

Controlled for age when evaluating covariate associations with all QOL outcomes except Support

Figure 2: Mean differences (± 95% CI) in baseline Satisfaction sub-scores for PKU subjects with documented ADHD or psychiatric condition (Depression, Anxiety, Bipolar). Midscore noted by grey shading on y-axis.

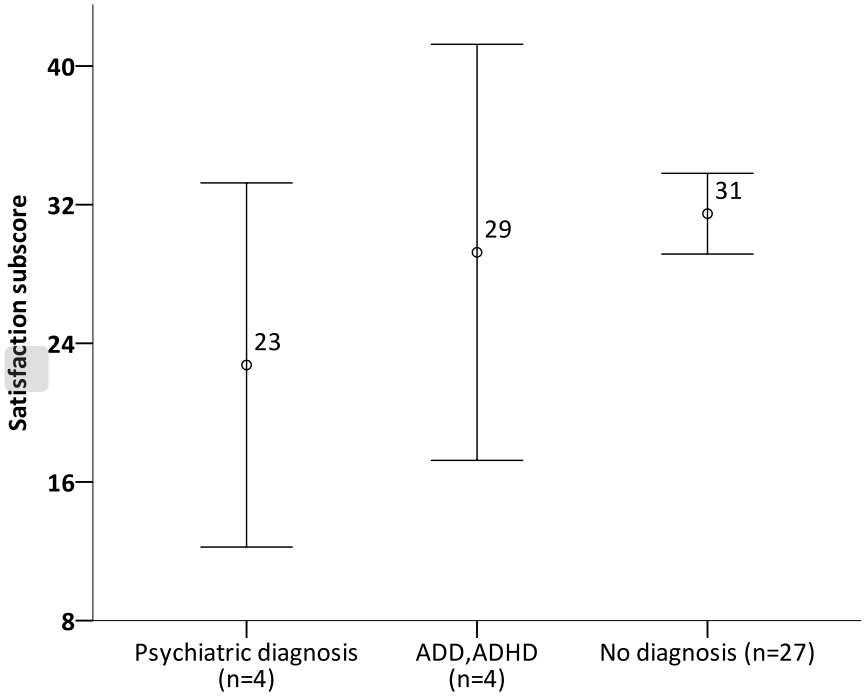
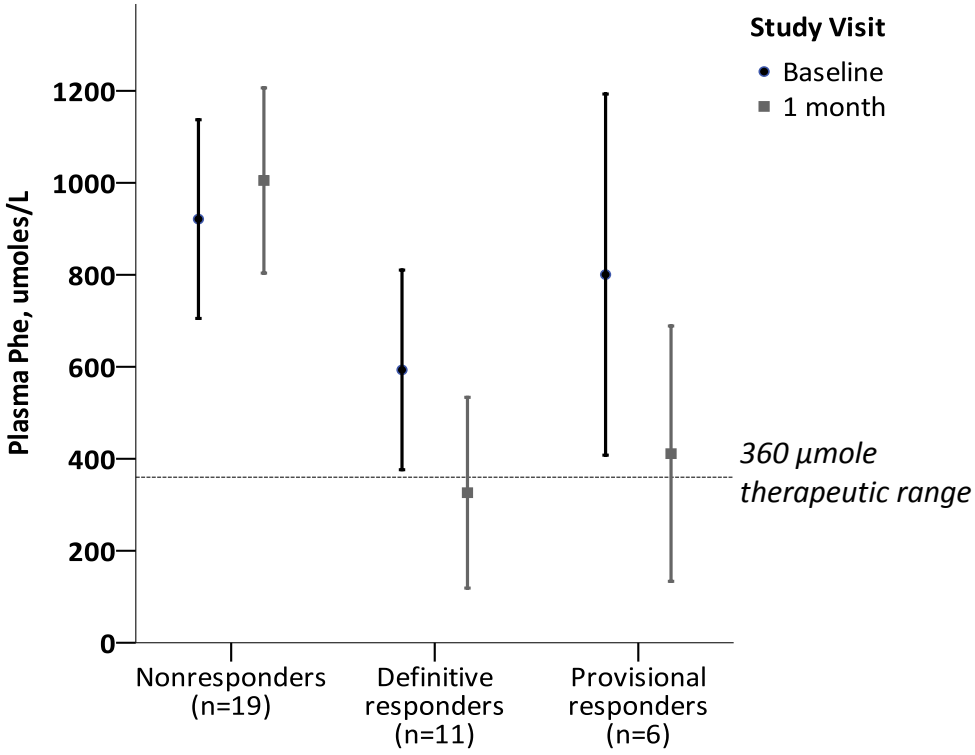
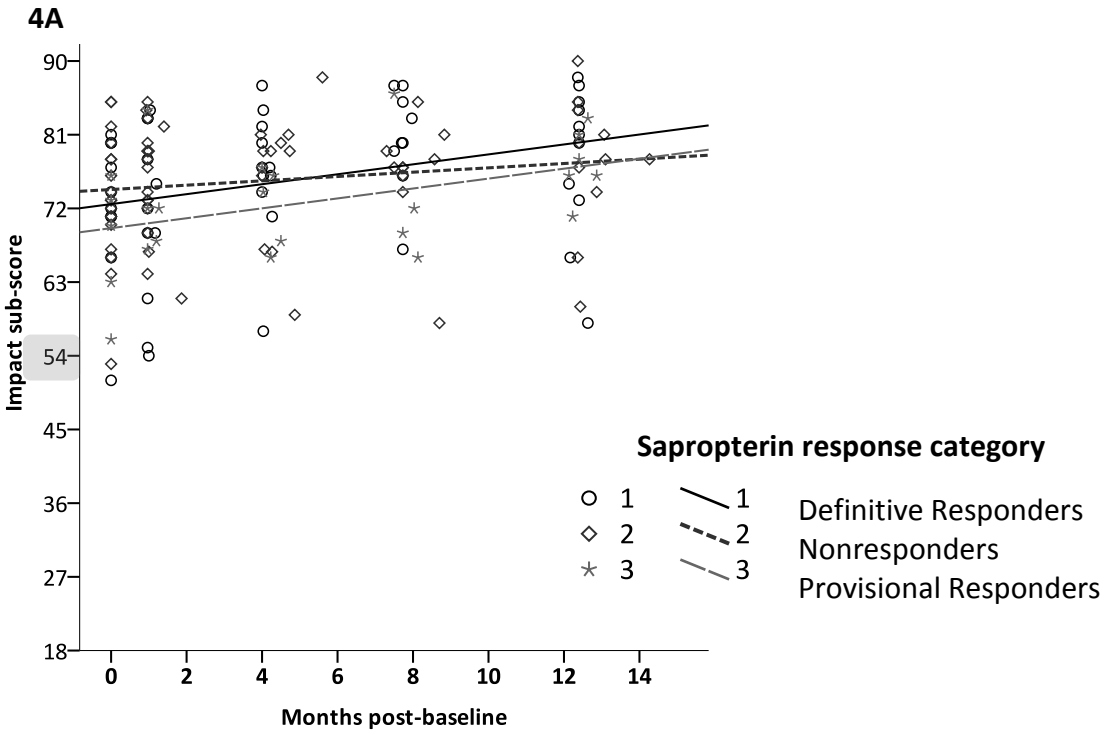
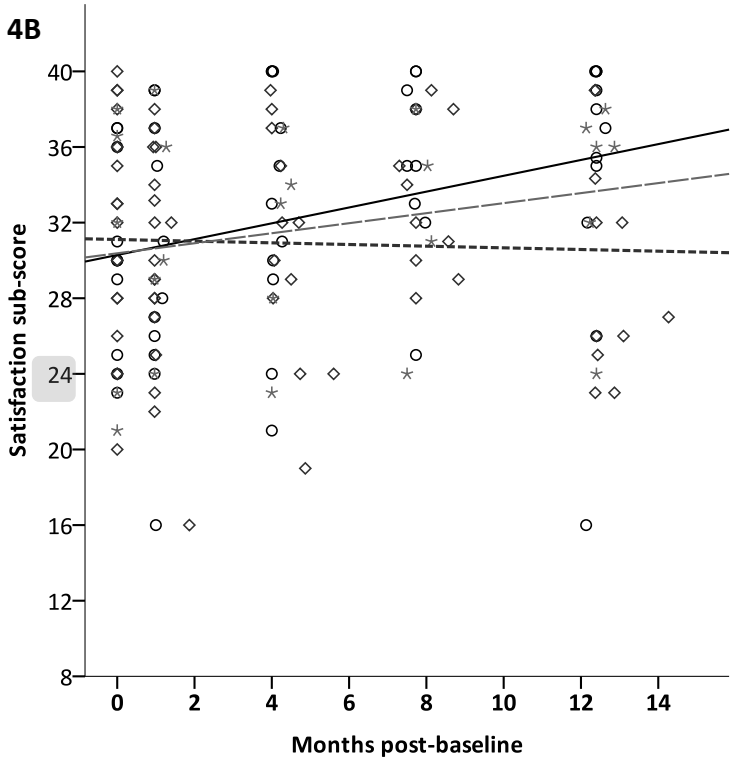


Figure 3: Plasma Phe (mean ± 95% CI) for prospective response groups (age 10+ years) comparing baseline to 1 month after sapropterin initiation.



Figures 4A-4C: 1 year trends for Impact and Satisfaction sub-scores (A and B) and total QOL score (C). Midscore noted by grey shading on y-axis. Axis begins at minimum score. Figures A-C not adjusted for covariates.





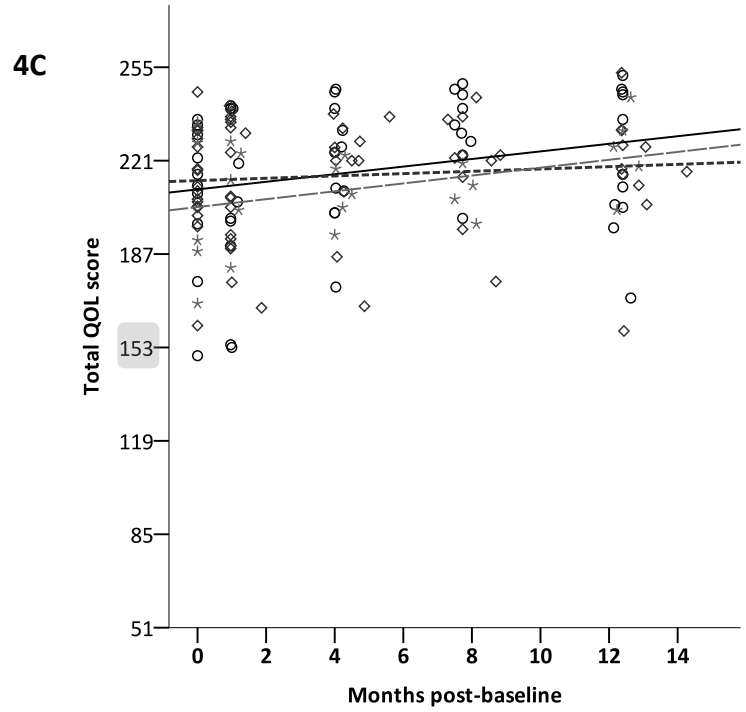
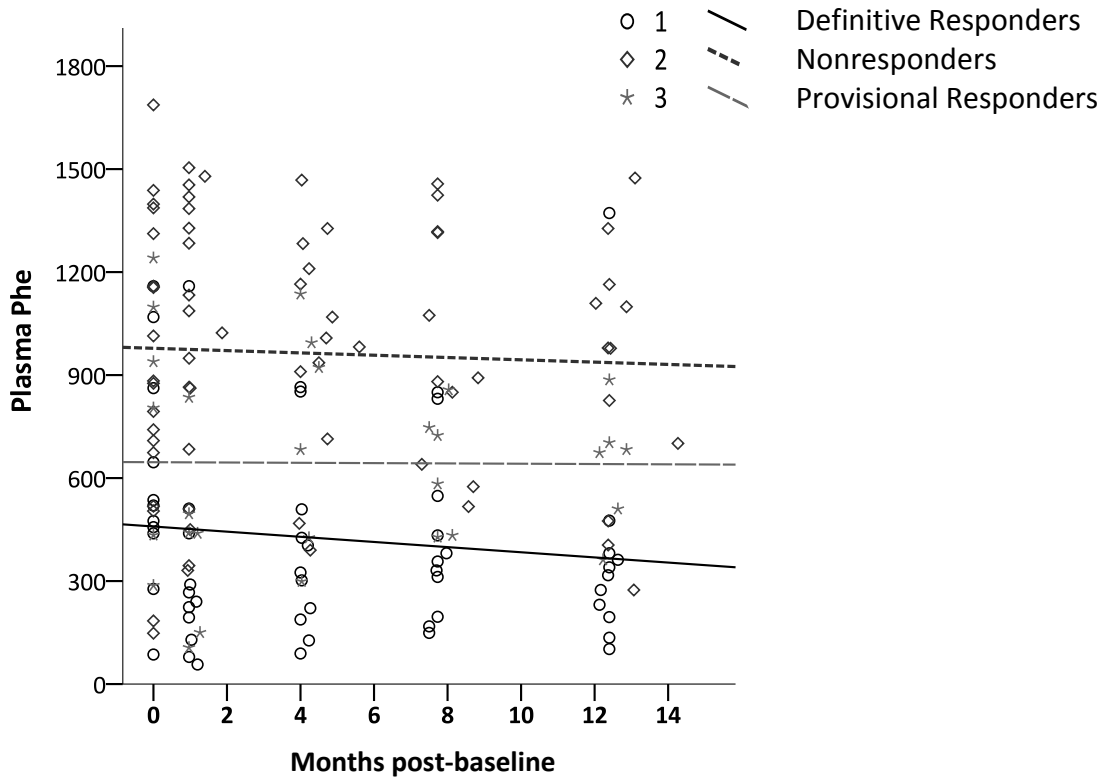


Figure 5: 1 year plasma Phe trends (µmoles/L) for sapropterin response groups. Trend lines not adjusted for covariates.



Figures 6A, 6B: Mean (± 1 SD) Phe tolerance (mg/day) and prescribed medical formula protein (g/day) for each sapropterin response group for all five study visits.

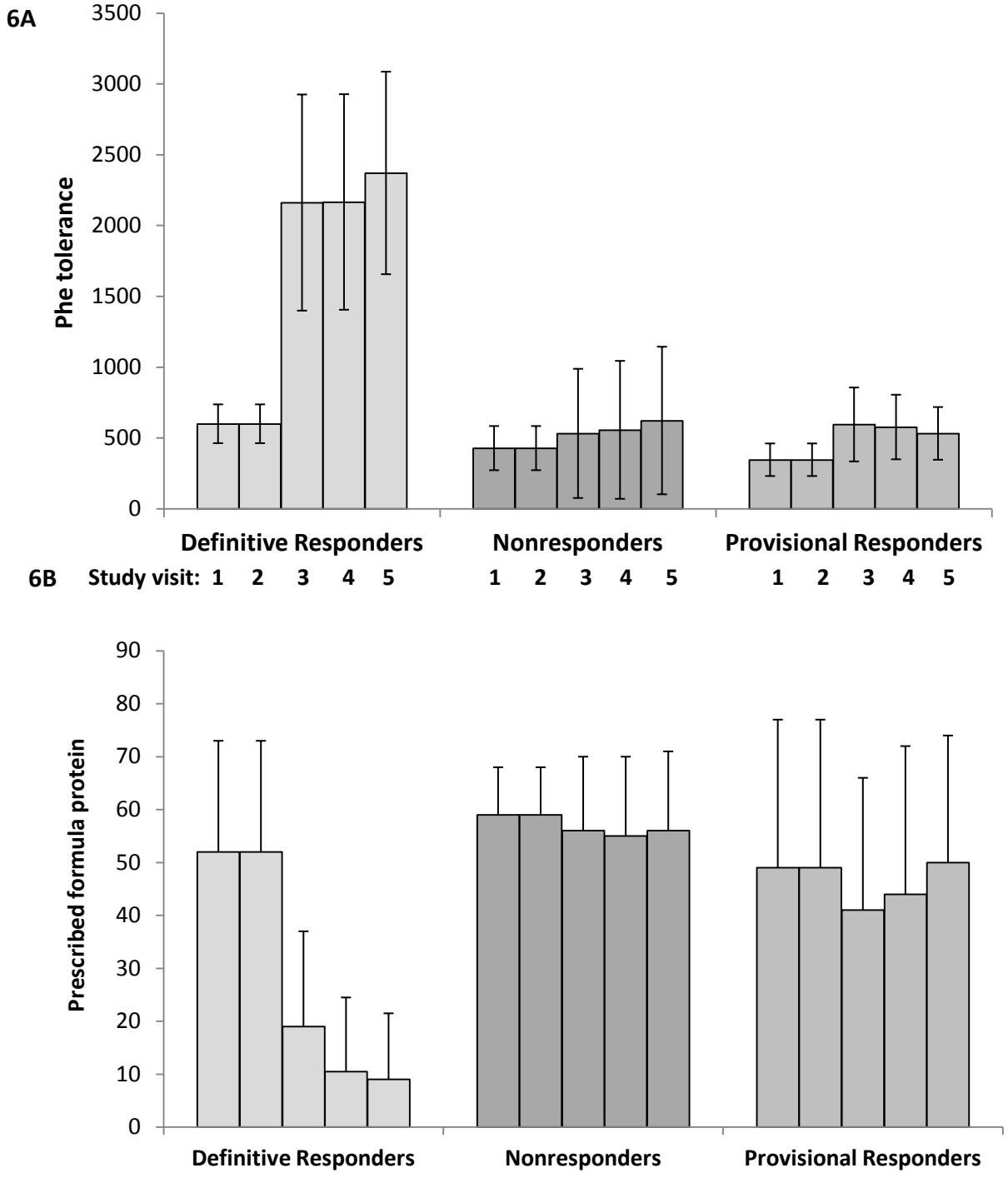


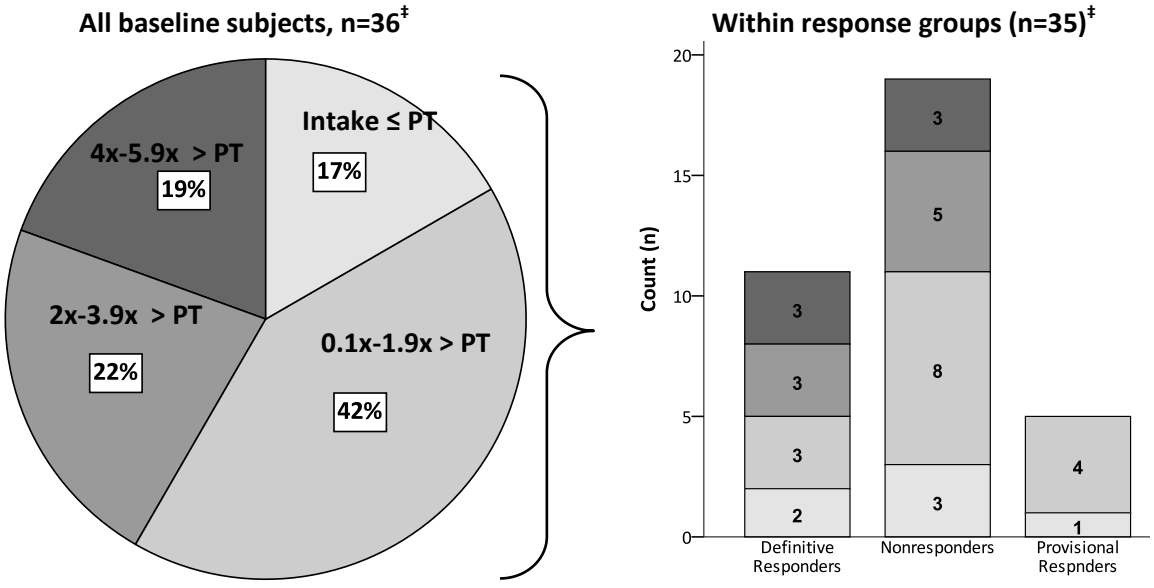
Table 5: *t*-statistic and significance of interaction variables for each response group across time, and *F* statistic with significance for interaction term within the whole age controlled mixed regression model. Statistics for Worries, Support, and General well being sub-scores not included since no significant changes occurred across time for those outcomes.

	Impact				Satisfaction				**Total score**			
	t			***F***	***t***			***F***	***t***			***F***
‡ Interaction variables	D	N	P	IT	D	N	P	IT	D	N	P	IT
• Phe tolerance (mg/day)	4.5 ***	2.6 *	1.9	9.6 ***	2.3 *	-1.8	0.1	3.0 *	2.9 **	0.1	0.5	2.8 *
• Formula protein Rx (g/day)	0.5	1.7	2.1 *	2.4	2.1 *	-1.2	-0.4	2.1	1.6	-0.2	0.7	1.0
• Plasma Phe (µmoles/L)	1.2	2.0 *	1.9	2.8 *	2.7 **	-1.6	-0.2	3.6 *	2.2 *	-0.1	0.4	1.7

*P<.05, **P<.01, ***P<.001

‡*(variable**time*response category)*
D: Definitive Responders, N: Nonresponders, P: Provisional Responders, IT: Interaction term

Figure 7: Dietary Phe compliance at baseline [mg Phe consumed in excess of Phe tolerance (PT)]. Pie chart depicts whole study group percents, stacked bar chart depicts n-values within prospective response groups.



‡One subject with undetermined Phe tolerance not included in figure. One other subject with undetermined response status not included in bar chart.

CHAPTER 5: INTENT-TO-TREAT DIVISION OF PHENYLKETONURIA PATIENTS PROVIDED SAPROPTERIN INTO THREE DISTINCT CLINICAL MANAGEMENT GROUPS

Introduction

Sapropterin is a pharmaceutical that lowers plasma Phe in a subset of phenylketonuria (PKU) patients with residual phenylalanine hydroxylase (PAH) activity. This subset of the PKU patient population can utilize sapropterin's tetrahydrobiopterin (BH4) cofactor function which allows responsive PKU patients to more efficiently metabolize phenylalanine (Phe), thus decreasing the concentration of neurotoxic Phe in the bloodstream. In addition to improved plasma Phe control, responders may also experience a decrease in medical food (formula) dependence while being able to increase dietary Phe [124, 169].

Since the introduction of sapropterin as a pharmaceutical treatment capable of managing blood Phe levels in PKU [170], many efforts have been made to accurately identify responders from nonresponders by evaluating BH4 dose, isomer, and length of exposure on plasma Phe response, while also considering genotypic influence [142, 171]. Response analysis has often been in situations where patients undergo Phe challenge prior to sapropterin loading, then followed by several hours or days of monitoring plasma Phe levels [171, 172]. These early tests for response classification set the arbitrary standard of two primary PAH response types, responder or nonresponder, with responders exhibiting a minimum 30% decrease in plasma Phe several hours or days after sapropterin exposure, and nonresponders experiencing less of a decrease in plasma Phe or none at all. A recent publication by Singh and Quirk [125] described the

necessity of also evaluating changes in Phe tolerance and formula dependence when determining response status, the efficacy of a lower plasma Phe response criteria (15% instead of 30%) at increasing response sensitivity while not sacrificing specificity, and the presence of a third response group that demonstrated an obvious decrease in plasma Phe after one month on 20 mg/kg/day sapropterin, but without tolerable changes to prescribed formula or Phe intake. Ultimately, this demonstrates the existence of 3 comparative groups: definitive responders able to liberalize diet and maintain lower plasma Phe levels long-term, provisional responders with rebounding plasma Phe levels and unable to liberalize diet, and the nonresponder group without a minimum 15% sapropterin dependent plasma Phe decrease.

However, even within the classification system outlined by Singh and Quirk, some patients evaluated at baseline, 1 month, and after dietary Phe challenge remained undetermined. Though it is essential to recognize both the capabilities and limitations of this newer method at accurately determining sapropterin response, and thus also accept how there will occasionally be subjects whose response classification remains ambiguous due to various circumstances, all PKU patients on sapropterin arriving at clinic require a treatment plan regardless.

Therefore the purpose of this article is to describe an intent-to-treat (ITT) classification system for the same PKU cohort described in the Singh and Quirk paper. This ITT system, though acknowledging the difficulty of classifying some PKU patients due to extraneous circumstances, none-the-less provides a response specific treatment plan to all patients completing one month of sapropterin response evaluation followed

by dietary Phe challenge for responders. In this way clinicians can evaluate sapropterin response according to the method described by Singh and Quirk, yet be able to refer to this ITT classification system to ensure that all patients, even those with more ambiguous response status, are provided an appropriate best-fit classification and treatment plan.

Methods

Recruitment of PKU patients, study design, sapropterin response evaluation, and therapeutic patient management were accomplished as described by Singh and Quirk and according to methods detailed on pages 19, 24-37 of this dissertation manuscript.

ITT classification was initially based on plasma Phe response to sapropterin, followed by effectiveness of sapropterin at increasing Phe tolerance and decreasing formula dependence. However at every step, clinical judgment was incorporated, taking into account other factors affecting outcomes and ensuring patients received appropriate knowledge and instructions regarding sapropterin response and dietary treatment plan. PAH genotype was not a contributing factor for ITT classification since genotypes were unavailable for the majority of patients at the time when sapropterin response was being determined.

Classification outcomes

<u>Classification schema</u>

All subjects except four were placed into a sapropterin response group and followed the general treatment plan of that group longterm: nonresponders remained on standard diet therapy without sapropterin, provisional responders were provided sapropterin

longterm but were unable to liberalize diet by increasing dietary Phe or decreasing prescribed formula, definitive responders were provided sapropterin longterm and capable of liberalized diet (Figure 1).

Of the four exceptions mentioned above, 2 were siblings determined clinically to be nonresponders at 1 month, but upon retrying sapropterin several months later were reclassified as provisional responders. For the integrity of research data analysis, post one month data from this sibling pair was excluded, however that does not negate their final clinical classification. The other two had no conclusive determination of response status (see Figure 1) and remained on their pre-existing PKU diet plan but also were not included in the research data after the first month.

These extenuating circumstances along with other situations that initially resulted in difficulty classifying sapropterin response are described in Table 1 along with final resolution of an assigned patient treatment plan.

Biomarkers

Baseline plasma Phe, tyrosine, and dietary Phe and formula intake are described in Table 2 for the entire PKU cohort and stratified by initial ITT response classes (stratified sample excludes the two who were undetermined).

At one month, all subjects classified as responders had plasma Phe decreases exceeding 20%, despite the minimum 15% criteria. No dietary changes indicative of final responder status were initiated until preliminary response status had first been determined.

Longterm data demonstrates the difference between provisional and definitive responders as provisional responders could not tolerate significant changes to dietary Phe (Figure 2) or prescribed formula (Figure 3) and did not demonstrate improved longterm plasma Phe control (Figure 4).

Discussion: biomarker utility and importance of clinical discretion

Short term changes in plasma Phe, particularly 20% or greater declines, in response to sapropterin combined with success of diet challenge are appropriate methods for evaluating response, even when genotypic PAH mutation analysis is not available. However, even with this more accurate system for determining sapropterin response in PKU patients, occasions arise where confounding circumstances make final response classification difficult. Genotyping can further assist with clarifying true sapropterin status of more ambiguous cases, however even mutation analysis does not always provide a concrete answer, nor is expensive genotype analysis always an option in clinical practice.

A helpful and important contributor to final determination of both sapropterin response and effective longterm treatment when cases are ambiguous is clinician knowledge of the situation. The attending clinician, whether a medical doctor or metabolic dietitian, may be aware of circumstances involving the patient's health, home environment, compliance with dietary or prescription instructions, and other behaviors or unique situations which could easily skew biomarker criteria for response status. For example, if a patient is ill with fever at baseline and thus catabolic, but has recovered by 1 month, this could be misinterpreted as a sapropterin Phe response. Likewise,

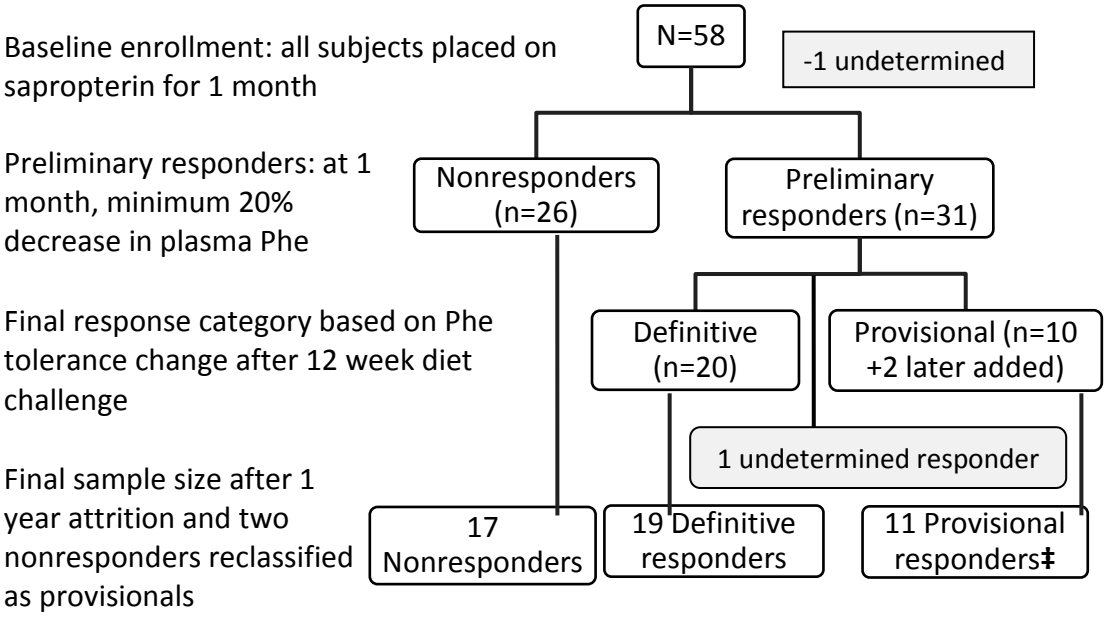
increases in physical activity during the 1 month trial or during diet challenge can have a similar confounding effect. Unknown pregnancy and changes in drug prescriptions can also add to the difficulty of determining sapropterin response status. One must also consider that when patients come in with poor dietary compliance and plasma Phe concentrations at near-saturation, the high Phe levels have no direction to go other than down, a phenomenon called “regression towards the mean”. In addition, some patients may attempt manipulation of dietary Phe or formula intake in an effort to influence plasma Phe levels towards a lower concentration while trying out sapropterin.

When taking special circumstances like these, or others, into account a clinician may ultimately classify a patient differently from what the biomarker criteria would indicate, or evaluate the situation further to ensure the initial classification is indeed the correct one. In this way, patients who complete a one month trial on sapropterin, and diet challenge when applicable, yet remain ambiguous are still provided a clinical grouping and longterm therapeutic plan relevant to both dietary needs and plasma Phe control. In the case of provisional responders, clinicians must work together with patients to discuss cost and benefit. Even though provisional responders may desire to remain on sapropterin longterm, discussing the cost to insurance and lack of longterm benefit to blood Phe control and diet restriction will enable both clinicians and patients to determine if continued sapropterin use is warranted. For occasions when a patient stops taking sapropterin before a response can be determined, continuing with the PKU diet plan the patient had in place at baseline is appropriate.

Conclusion

Even in circumstances where PKU patients may not exhibit a clear sapropterin response status after BH4 trial and diet challenge, ITT classification and clinical discretion can still provide these patients with a response class to identify with and longterm treatment plan appropriate for optimal Phe control while meeting dietary needs.

Figure 1: Response classification and attrition of PKU study group.



‡Two reclassified provisionals included in chart were excluded from longterm research data analysis due to deviation from study protocol. 1 provisional attrition.

Table 1: Extraneous circumstances and resolution when determining sapropterin response status.

n=9	Circumstance
n=2	One sibling had 18% decrease in plasma Phe at 1 month while other was a clear nonresponder. Extraneous factors, including intentional diet manipulation, led clinicians to classify both siblings as nonresponders. Family lost to followup until returning at 8 months with request for a second sapropterin trial. Both siblings exhibited a >30% plasma Phe response with second trial and remained on sapropterin, though unable to liberalize diet and with poor Phe control. For research purposes, these two were excluded from data after one month. However ITT classification was "nonresponder" at 1 month and revised to "provisional responder" after the second trial.
n=1	Adult patient had been declared nonresponder in previous sapropterin trial from a few months prior. Patient had 33% decrease in plasma Phe after 1 month in this trial, though also demonstrated 38% decline in dietary Phe during that period and was not consuming formula. Plasma Phe control remained poor longterm, patient was self-liberalized and would not adhere to instructions to reduce Phe intake or begin drinking formula. Patient final classification as provisional responder.
n=1	Adult patient had 10.8% decrease in plasma Phe which indicates nonresponder status, but had increased dietary Phe intake by 12.5%. Patient received physician and insurance approval to remain on sapropterin. Patient was unable to decrease formula intake or increase Phe tolerance. Due to insurance difficulties and no change in clinical status, patient ceased sapropterin after 11 months. Final classification is nonresponder, albeit permitted an extended time on sapropterin.
n=1	Pediatric patient had 45% decrease in plasma Phe indicative of sapropterin response. Patient was unable to improve Phe tolerance or decrease medical food and was thus classified as a provisional responder. Parents elected to remove child from sapropterin after 9 months due to perceived lack of benefit and insurance costs and child continued with standard PKU diet.
n=1	Child responded to sapropterin with 62% decrease in plasma Phe. However the parent had little trouble maintaining good Phe control in the child and saw little benefit from the medicine at that time, so after 2 months the parent elected to remove the child from sapropterin. Child did not complete diet challenge and remained on standard PKU diet.
n=1	Adult patient disliked swallowing pills and did not return for 1 month followup. Response status not determined and patient encouraged to follow pre-exisitng PKU diet plan.
n=1	Adult patient was planning a vacation directly after baseline appointment stating she would not be following any diet restrictions while on vacation. As a result, patient did not begin taking sapropterin until 34 days after baseline. 1 month followup was scheduled for 30 days after patient began taking sapropterin. Lack of plasma Phe response indicated patient was nonresponder.
n=1	Physician approval for adult patient to take sapropterin had to be cleared since patient was on many psychotropic medications and there was uncertainty regarding potential drug interaction. Approval was received and patient began sapropterin 23 days after baseline. 1 month folllowup was scheduled for 30 days after patient began taking sapropterin. Lack of plasma Phe response indicated patient was nonresponder.

Table 2: Plasma phe, plasma tyrosine, Phe intake, and formula intake of PKU patients at baseline (mean ± SD). Values listed for entire cohort and stratified by response status. Not age adjusted.

	PKU cohort	Definitive responders	Provisional responders	Nonresponders‡
Plasma Phe (µmoles/L)	693 ± 412	533 ± 288	626 ± 370	844 ± 470
Plasma tyrosine (µmoles/L)	52 ± 19	53 ± 16	49 ± 14	51 ± 23
Phe tolerance (mg/day)	452 ± 266	600 ± 352	313 ± 119	395 ± 178
Reported Phe intake (mg/day)	1002 ± 920	1292 ± 928	692 ± 1009	848 ± 794
Prescribed formula (g protein/day)	49 ± 16	43 ± 20	49 ± 17	54 ± 12
Reported formula intake (g protein/day)	38 ± 20	34 ± 21	44 ± 22	41 ± 18

‡Sibling pair initially classified as nonresponders but reclassified as provisionals after sapropterin retrial were included with nonresponders in this table since that was their original ITT classification at one month.

Figure 2: Prescribed Phe intake (mean ± 95% CI), representative of biological Phe tolerance, stratified by sapropterin response status across one year (Baseline, 1 month, 4 months, 8 months, 12 months respectively)

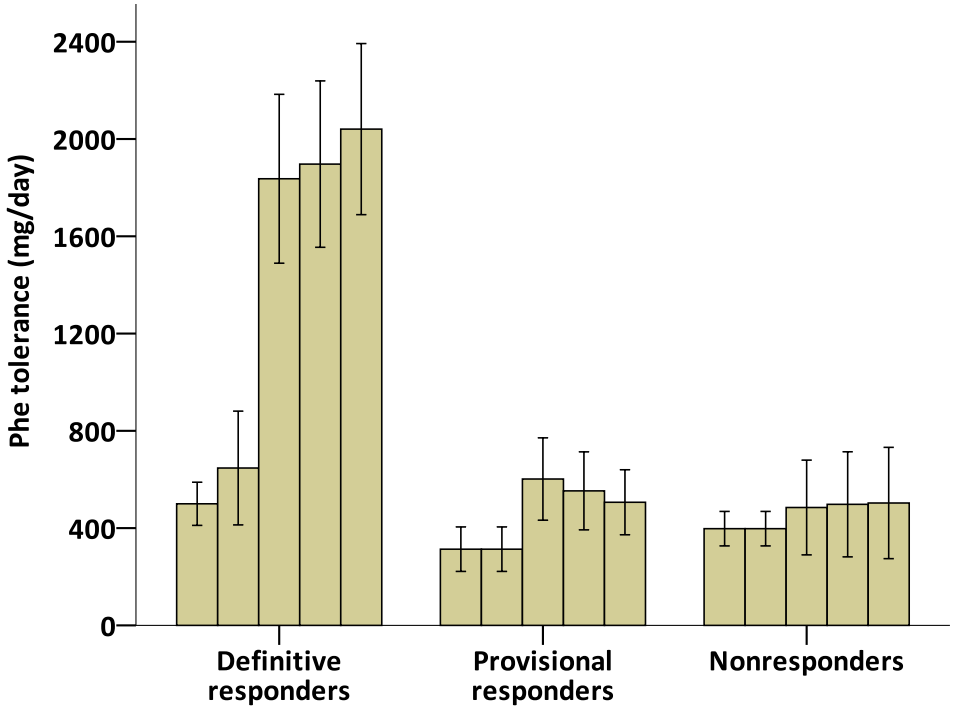


Figure 3: Formula protein prescription (mean ± 95% CI) stratified by sapropterin response status across one year (Baseline, 1 month, 4 months, 8 months, 12 months respectively)

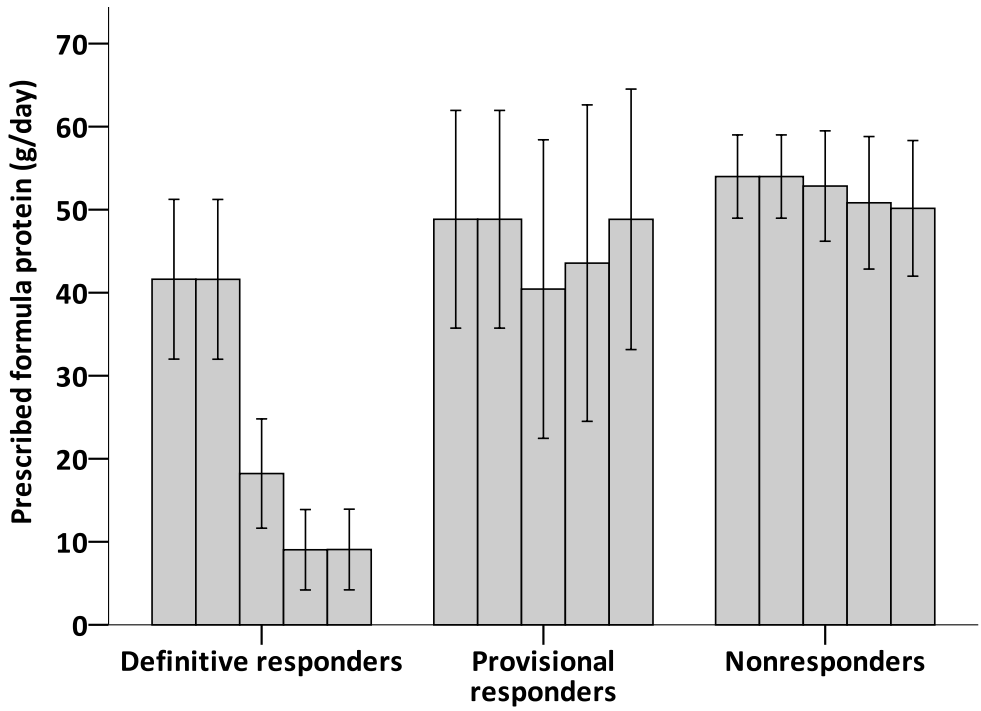
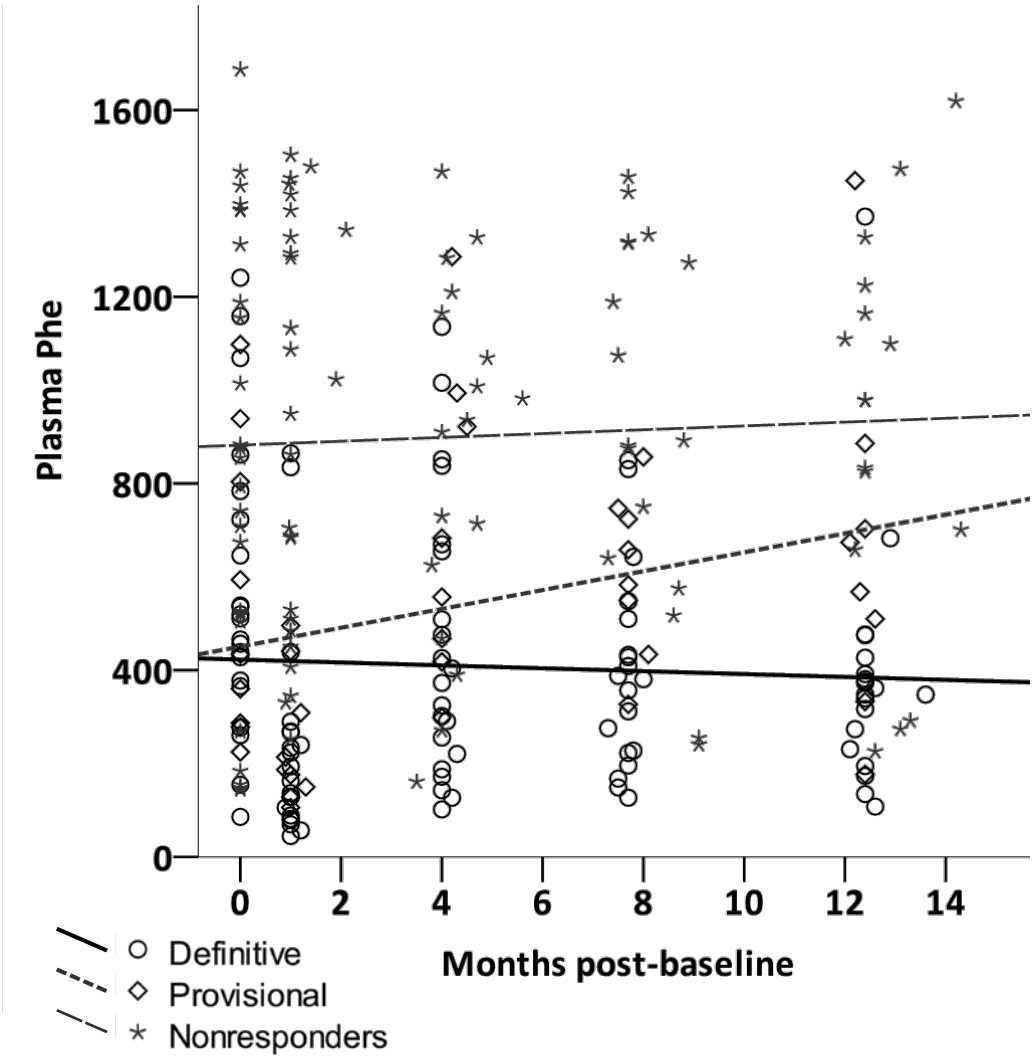


Figure 4: Plasma Phe trends (µmoles/L) across one year stratified by sapropterin response status



CHAPTER 6: CONCLUSIONS AND DISCUSSION

The primary objectives of this research investigation were to determine if sapropterin treatment, along with corresponding changes to PKU dietary needs, was capable of improving both monoamine status and QOL, either short term or long-term, for an early diagnosed early treated PKU cohort. PKU subjects had variable responses to sapropterin treatment and were separated into three response and clinical treatment categories defined by plasma Phe change in addition to whether increased dietary Phe tolerance and reduced formula dependence occurred. The study design provided opportunity to compare response groups at baseline, 1 month, and after diverging into treatment groups based first on initial plasma Phe response then followed by dietary determinants of definitive response. The aims and objectives described earlier were achieved for this investigation as modest yet statistically significant changes to both neurotransmitter and quality of life outcomes were observed in association with sapropterin response and more liberalized diet. The implications of these observations are discussed below.

Monoamine neurotransmitter results

Conclusions from baseline and 1 month analysis

The evaluation of monoamine neurotransmitter status during the first study month provided opportunity to investigate effects of sapropterin itself on monoamine status when all subjects were taking sapropterin without exposure to dietary changes. The only true potential confounder was the observed decrease in plasma Phe that expectedly occured in sapropterin responders, since plasma Phe has been shown in the literature and our own analysis to be inversely associated with monoamine

concentrations. However, inclusion of sapropterin nonresponders into the analysis allowed for comparison of monoamine outcomes in a group that did not experience a mean decrease in plasma Phe, thus providing opportunity to identify monoamine changes independent from the effects of plasma Phe and dietary variables.

Baseline concentrations of monoamines were similar across groups, regardless of prospective response status, with the exception of epinephrine. Provisional responders were found to have less epinephrine excretion in the urine than either nonresponders or definitive responders at baseline and throughout the study. Since neither sapropterin nor diet challenge had been initiated at this point, these factors were ruled out as causal to this noted difference. Since the majority of provisional responders were pediatric (9 out of 10), age was investigated as a covariate, but was found to not be associated with epinephrine levels, a finding consistent with other literature reports in which pediatric patients after the age of 5 years have epinephrine:Cr ratios equivalent to adults [130]. Dietary Phe and formula protein, measured as compliance or as total intake, were also not associated with provisional responders' lower epinephrine concentrations. This leads us to conclude that lower epinephrine seen in provisional responders may be an intrinsic biological difference. Or, rather than a true significant finding, the observed difference could simply be a chance finding reflecting bias within the provisional group's smaller sample size. Future investigations of monoamines that would accommodate provisional responders in the analysis are needed to further verify the association between provisional response status and low epinephrine.

The one significant change in monoamine status that occurred after 1 month on sapropterin was an increase in HVA. This was observed for the entire PKU cohort, though when stratified by sapropterin response status, only nonresponders exhibited a statistically significant increase in HVA (Figure 8, Chapter 3). Since no declines were observed in other catechols, this indicates that the HVA increase is due to the stimulation of dopamine metabolism rather than simply increased catecholamine degradation. One possible mechanism by which this dopaminergic stimulation could occur is sapropterin's cofactor activity, particularly in regards to tyrosine hydroxylase function. The additional BH4 in the form of sapropterin could have induced TH synthesis of the dopamine precursor L-DOPA which would be synthesized into downstream dopamine metabolites at the rate that it becomes available, finally resulting in higher amounts of excreted HVA. This effect would be most enhanced for nonreponsders since little BH4 is being utilized by the abnormal PAH leaving more available for TH activity. Why a similar effect wasn't seen with 5HIAA, since tryptophan hydroxylase also requires BH4, may be due to greater sensitivity of the tryptophan hydroxylase enzyme to inhibition by high Phe levels or high phenylalanine interfering with tryptophan membrane transport and thus substrate availability for serotonin metabolism. One other explanation for the increase in HVA could well be a placebo effect. Studies with Parkinson's disease patients, in which increases to brain dopamine were observed when patients were unknowingly provided placebo, highlight the biochemical effect of expectation; in other words, a patient's own belief that they can or have improved with a provided treatment (regardless of the true activity of the

treatment) can lead to that very improvement, at least in regards to catecholamine metabolism [149, 150, 173]. Whether this is the case for the nonresponders in this study would require a future clinical investigation, a double blind crossover study design ideally, in which predetermined nonresponders are provided either placebo or active sapropterin with catecholamine levels being measured.

The lack of significant change in monoamines at one month within the stratified definitive and provisional responder classes was contrary to our expectation that the sharp declines in plasma Phe which occurred in these groups would be accompanied by associated increases to monoamine concentrations, particularly since the literature has been consistent in showing inverse association between plasma Phe and monoamine status. In fact own baseline results demonstrated significant inverse association between plasma Phe status and several monoamine neurotransmitter analytes. What this outcome indicates is that short term changes in blood Phe levels are much less influential on monoamine outcomes than prior longterm plasma Phe control. The plasma Phe decrease that responders to sapropterin experienced was for a brief 30 day period, whereas the plasma Phe concentrations most patients had at baseline were consistent with their level of Phe control for several months, or an even longer amount of time, prior to the study. This demonstrates that monoamine status in PKU is most reflective of longterm metabolic control rather than of acute blood Phe changes. Therefore, for significant improvements in monoamine status to occur, Phe levels must decrease and remain at those lower concentrations for well beyond one month.

Conclusions from longterm monoamine outcomes

After the second study visit patients diverged in their treatment plans. Both definitive responders and provisional responders remained on sapropterin and had diet challenge, yet provisional responders experienced no significant change in Phe tolerance and formula dependence in the way definitive responders did. Thus the lack of dietary change seen in provisionals on sapropterin was to that of nonresponders who had been removed from sapropterin and kept on standard diet therapy. Evaluating the longterm monoamine trends of these three groups provided opportunity to identify the longterm effects of sapropterin, independently and in concert with changes to formula protein and dietary Phe.

Interestingly, there was no longterm improvement in monoamine neurotransmitter concentrations regardless of sapropterin response, dietary factors, or initial increase in HVA observed during the first study month. The lack of longterm monoamine changes in nonresponders, in spite of significant improvement in HVA status early in the study, can be easily explained by the removal of sapropterin treatment once nonresponder status had been identified at the second study visit. This event, along with no significant improvements to plasma Phe control during the full year, led longterm monoamine concentrations that did not vary from those observed for nonresponders at baseline. For the provisional and definitive responder groups, the explanation is more complex. Perhaps if plasma Phe had remained as low as it had been at study visit two longterm improvement in several monoamines would have occurred. The reasoning is that even though definitive responders exhibited longterm Phe control

that was generally better than baseline values, the incorporation of a more liberalized diet still elevated plasma Phe levels significantly above the much lower concentrations observed at the second study visit, and this elevation in plasma Phe sustained longterm prevented any longterm improvement to monoamine status regardless of sapropterin treatment. Likewise for provisional responders, longterm elevation in plasma Phe after the second study visit also prevented improvements to monoamine levels. What this demonstrates is that when it comes to monoamine status, sapropterin is beneficial in lowering plasma Phe as well as stimulating dopamine metabolism, however maintaining lowered plasma Phe concentrations longterm while on sapropterin is critical for optimal longterm improvement of monoamine neurotransmitter status.

However, even with modest improvement in Phe control for definitive responders, this group experienced a decline in mean 5HIAA across one year of follow-up. The decline, although only mildly significant in trend, is worthy of scrutiny. One could speculate this decline to be a longterm side effect of sapropterin intake, however provisional responders, also on sapropterin longterm, did not experience a significant 5HIAA decline. This may simply be due to the small size of the provisional responder group, or it could indicate that some nutrition component of the more liberalized PKU diet privileged to definitive responders is negatively affecting 5HIAA in the longterm. Since a positive association between formula protein intake and 5HIAA is reported in our results, first thought was that reduced formula protein intake in definitive responders was most likely contributory to the decline in 5HIAA, as this could lead to less LNAA competition against higher Phe levels and thus less provision of tryptophan

substrate for serotonin metabolism. However in the statistical model, decreases to prescribed formula protein intake were not associated with the decline in 5HIAA. Changes to dietary Phe intake were also not associated, and plasma tryptophan remained stable longterm for definitive responders. One possibility is that physiological decreases in micronutrient cofactors essential for serotonin synthesis, such as B6, iron, and zinc, led to gradual reduction of serotonin metabolism and thus lower 5HIAA over time. This could feasibly have resulted from reductions to fortified formula intake and insufficient micronutrient provision from the more liberalized, albeit still restrictive, PKU diet. Though patients with decreased formula dependence were instructed to begin taking a multivitamin to compensate, patient adherence to this instruction was infrequent. Ultimately, these factors could have contributed to the longterm 5HIAA decline observed in definitive responders. However, since patient micronutrient status was not incorporated into this analysis, micronutrient cofactor insufficiency as a contributor to lower 5HIAA in definitive responders on liberalized diet is an unconfirmed theory.

Nevertheless one could argue that the 5HIAA decrease is not of clinical significance, particularly given lower overall plasma Phe in the definitive responders which works to increase 5HIAA. There is less protection though for patients on sapropterin who over-liberalize and develop uncontrolled high plasma Phe concentrations, thus losing that buffer and leading to 5HIAA levels that drop even lower than the already decreased concentrations prevalent at baseline. It is necessary to emphasize to patients on sapropterin the importance of adhering to their prescribed Phe tolerance and ensuring

adequate micronutrient intake. Also worthy of consideration is retaining a portion of medical food protein in the diet of all definitive responders to assist with maintaining lower plasma Phe levels, appropriate micronutrient provision, and optimal monoamine status.

In fact emphasis on Phe control and formula intake is crucial independent of sapropterin treatment or response status due to both the strong inverse association that exists between plasma Phe and monoamine concentrations (excepting epinephrine) and the positive association formula protein intake has with several monoamines. Thus poor adherence to these factors of metabolic control could easily negate any potential benefits of sapropterin to monoamines for those taking the pharmaceutical and have longterm detrimental influence to monoamine and neurocognitive status even for those not on sapropterin. One could argue that if this were the case, how come monoamines did not rise significantly at one month when plasma Phe decreased in responders or decrease sharply in definitive responders consuming less formula. This could be lack of sensitivity within our measurement technique, or it could indicate that chronic exposure to both formula protein intake and plasma Phe concentration have greater impact on current monoamine levels than acute dietary or plasma Phe changes.

To conclude, though sapropterin has capability to improve monoamine status in PKU patients, particularly dopamine metabolism, its potential for beneficial effect on monoamine neurotransmitter concentrations is modest and secondary to the stronger effect of maintaining longterm plasma Phe control and adequate formula protein intake.

Quality of life outcomes

Conclusions from baseline and 1 month analysis

At baseline mean QOL scores were high, averaging well above the midscore, and representative of the QOL subcohort majority. This was seen for total QOL score as well as for all 5 subsections. Variables thought to be relevant to QOL such as plasma Phe control, dietary Phe tolerance, and formula prescription were not associated with PKU-QOL scores at baseline. These findings were surprising given literature evidence, as well as patient anecdotal reports, that dietary restriction and poor Phe control lower life quality in some respects. What our study results indicate however is that most individuals with PKU have excellent quality of life perspective even if there are aspects of living with their metabolic disorder which they dislike, but even those aspects seem to have little bearing ultimately on self perceived life quality, at least prior to sapropterin initiation. These results are unlikely to be questionnaire error as a recently published study also evaluating QOL in 19 pediatric PKU patients before and after sapropterin treatment also demonstrated high baseline QOL scores regardless of PKU phenotypic severity [116].

To explain this outcome, consider that PKU subjects in this study were diagnosed and treated from infancy. Hence, having lived with this disorder their whole lives, it is feasible most patients would have adjusted emotionally and psychologically. Studies have shown this to be a frequent occurrence in those living with chronic conditions as already discussed in chapter 4. Also, this subcohort was comprised of adolescents and adults. Most studies demonstrating lower QOL at baseline incorporated

pediatric subjects, with evaluations based on parent report. Parent report of child QOL may well be different from the self report on QOL completed by adolescents and adults.

The percentage of patients in the baseline analysis who had diagnosis of psychiatric depressive disorders was not outside that of the general population, per reported in the chapter 4 discussion. To provide details, two patients were diagnosed with comorbid depression and anxiety disorder, 1 patient with comorbid depression and bipolar disorder, and 1 patient diagnosed with all three conditions. What this indicates is that even though early treated PKU has been associated with mood instability, particularly when Phe levels are high, it may not increase risk for depressive symptoms beyond general population risk. This result corroborates another study report [174], albeit not with all literature findings [27, 175]. Even so, baseline psychiatric status was evaluated for relevance to QOL outcomes and found to be associated with lower Satisfaction scores when a depressive disorder was present. This was not entirely unexpected since an association between psychiatric diagnosis and lower health-related QOL satisfaction has been reported in other investigations and is apparently a frequent occurrence in populations suffering from chronic disorders [176-178]. This result in our study confirms that depressive disorders within the PKU population have a negative impact on patient satisfaction with life and medical management similar to what has been observed in other chronic disease/disorder populations. Navigating patients towards treatments for diagnosed depressive disorders will assist with improving health related life satisfaction.

Interestingly, many patients on sapropterin during the first study month provided anecdotal reports of feeling better on the medication regardless of eventual response classification. Yet there was a complete lack of change in mean QOL scores between baseline and 1 month for any of the response groups. This indicates that neither sapropterin as an independent factor, nor in conjunction with short term decreases in plasma Phe, has the capability of altering QOL as measured quantitatively, at least within that short span of time. This also coincides with the baseline observation that anecdotal reports are not reflected in quantitative QOL anlaysis, within this study as well as other research [116]. The absence of change to QOL scores in the first month however does provide insight into which variables are responsible for QOL improvements seen longterm within this study, discussed below.

Conclusions from longterm QOL outcomes

During the 1 year of followup, definitive responders demonstrated significant improvement in QOL subscores which measured satisfaction with life and medical management. For both nonresponders and provisional responders however, no longterm significant change in satisfaction subscores occurred. These observations indicate that the advent of diet liberalization as a result of definitive sapropterin response was key to the improved satisfaction scores seen in this circumstance. Statistical regression analysis likewise confirmed the significant association of longterm changes in dietary Phe tolerance, prescribed formula protein, and plasma Phe control with improved satisfaction subscores in definitive responders.

That isn't to say plasma Phe control and diet liberalization were the only contributing factors to QOL improvement. Neither provisional responders nor nonresponders to sapropterin experienced significant changes to Phe tolerance, prescription formula, or blood Phe control over the longterm, yet these two groups along with definitive responders experienced a statistically significant improvement in QOL subscores measuring impact of PKU on life quality. Improvement over time had the highest significance for definitive responders and lowest significance for nonresponders, but all three groups had statistically verified improvement regardless. Though liberalized diet was associated with the improved Impact scores in definitive responders, the improvement also observed for provisionals and nonresponders indicates that some other factor influencing the two latter groups, and possibly the former one as well, contributed some extent to improved perception of PKU's impact on life quality. An explanatory factor is that the increased medical attention and dietary counseling subjects received over one year was responsible, at least in part, for the improvements to Impact QOL subscores seen in all sapropterin response groups.

The sum improvement in both satisfaction and impact subscores for definitive responders resulted in a highly significant improvement in total QOL scores that associates most significantly with increases to Phe tolerance. Interestingly, provisional responders on sapropterin, though not able to increase Phe tolerance or reduce formula intake, also exhibited a significant improvement in total QOL scores. This is most likely the result of the summed increase of both Satisfaction and Impact subscores as well. Though admittedly the increase in Satisfaction for provisionals was not enough to be

significant, when combined with improvements to Impact scores across time, the increase in total QOL score for this group, though modest, was still statistically significant. Since provisional responders were on sapropterin without diet liberalization, and since statistical analysis revealed no association between dietary variables and longterm total QOL score outcome in this group, the observed total QOL score improvement may therefore be indicative of a placebo effect. Essentially, provisional responders may believe their dietary freedom has improved and that they are benefitting from sapropterin, even with evidence to the contrary. Or, because provisionals received some additional medical attention during the diet challenge period, the better QOL scores could simply reflect patient emotional benefit to increases in medical surveillance and monitoring.

To summarize, QOL improvements seen longterm in definitive responders are most certainly the effect of sapropterin response related diet liberalization, where as improvements seen for provisional responders and nonresponders may be the consequence of other medical management influences.

That said, its important to realize that no longterm change occurred in subscores evaluating Worries, Support, and General well-being, regardless of sapropterin response status, dietary variables, or blood Phe control. This absence of longterm improvement for these QOL components could simply be due to lack of change or association, though there is also the possibility of type II error resulting from less study power, given the subcohort's smaller sample size further divided by response status. One final possibility for lack of longterm improvement may be the high QOL scores observed in PKU patients

at baseline; with QOL scores already so high, a ceiling effect would inhibit further improvement in most circumstances.

However if the longterm stability of Worries, Support, and General well-being subscores truly reflects lack of influence by sapropterin, diet, or plasma Phe on these outcomes, then an opportunity arises for development of an abbreviated PKU-QOLQ. The abbreviated questionnaire would be based primarily on the Satisfaction and Impact sections of the current questionnaire, while other sections or the full questionnaire could still be utilized selectively in future QOL investigations, dependent upon study objectives and design. Of note, a common complaint patients had in our study was the time required to complete the questionnaire. Therefore if significant findings can be achieved with an abbreviated PKU-QOL questionnaire that eliminates sections which are unrelated to plasma Phe control, diet, or sapropterin use, this would be beneficial to patients and researchers alike.

On final point in regards to potential effects of baseline psychiatric status on trajectory of QOL, particularly given the finding of lower baseline satisfaction scores in 4 subjects with depressive disorders: our intent was to control for psychiatric condition when evaluating longterm QOL outcomes, though ultimately we could not due to 1 attrition and the division of the other 3 affected subjects into separate responder classes. In essence, the numbers were too small for accurate longterm evaluation of psychiatric status influence on QOL within responder groups. However in studies where sample size is large enough, psychiatric and behavioral diagnoses should be evaluated

for association with the outcome variables being investigated, and thus controlled for in the analysis when relevant.

Implications of monoamine and QOL outcomes in relation to clinical patient management

Attrition

Since patient attrition is a common problem in medical management situations, including our own study cohort, we evaluated associations of analyzed variables with risk of loss to follow-up.

Baseline factors associated with study attrition across the one year period were classification as a nonresponder, ADHD diagnosis, lower QOL Support subscores, and lower urinary 3MT concentrations compared to others in the PKU cohort. The first three influences, though not surprising, are worthy of discussion.

Even though all patients received encouragement to continue participation in the study, and provided explanation at baseline of at least 50% chance of nonresponse, those designated as sapropterin nonresponders felt little incentive to return for follow up once withdrawn from the medicine. Whether or not this issue can be ameliorated with moderate incentives or additional supportive methods will have to be determined through future trials.

Regarding ADHD, of 7 subjects with diagnosis at baseline (all on stimulant medication), three (2 nonresponders and 1 provisional responder) were lost to follow-up after the second study visit. These three included one adult, one adolescent, and one child. After the first month however, there was no further attrition of ADHD diagnosed

study participants, regardless of response category. Other studies also report higher attrition in ADHD diagnosed subjects [155], and though staff persistence is effective in reducing attrition, it is not an eliminating influence [154].

Lower QOL support subscores also had a significant association with attrition. This is not the first time sense of support has been implicated as important to investigative outcomes with the PKU population [108, 179]. Since support scoring involved 4 questions only, 1 asking about family support, 1 asking about support from friends, and 2 asking about support from the patient's healthcare team (physician and dietetic) at least half the weight of the support scores falls into the lap of the medical profession. Admittedly, influencing family dynamics or friendships in a patient's life may not always be feasible, though counseling for these things is, it is critical that patients feel emotionally supported by their medical management team in order to reduce incidence of loss to follow up. Particularly since longterm medical follow up of patients with inborn errors of metabolism is essential to patient well-being and should be encouraged.

The association between lower baseline urinary 3-methoxytyramine and attrition is more difficult to explain but still worthy of consideration and further investigation. 3MT was the only monoamine outcome associated with attrition. This is peculiar given that 3MT is accepted as a non-neuromodulatory intermediary in dopamine catabolism, and since 3MT concentrations were low for the entire PKU cohort although lowest in those with attrition. Hence, our finding may be reflective of some other factor affecting both 3MT concentrations and attriton risk, or it may indicate that 3MT does indeed have

neuromodulatory capability. Controlling for the other factors associated with attrition further magnified the P value significance associating 3MT with attrition risk and makes the possibility of true 3MT neuromodulatory effect more viable. However, with the exception of one mouse study [158], literature is lacking regarding 3MT neuromodulatory capability. Hence 3MT influence on attrition, though intriguing, is speculative at this time .

To conclude, consideration of these 4 variables found to predict attrition risk in PKU patients initiating sapropterin, along with intervention when possible, could potentially reduce the number of patients in clinical practice who are lost to follow-up.

Diet liberalization and plasma Phe control

Dietary change in definitive responders had unique effects on monoamine and QOL outcomes. QOL improved longterm as a result of diet liberalization in the definitive responder group, however monoamine status did not. The absence of longterm benefit to monoamine status in definitive responders may well be due to rebounding plasma Phe concentrations after the first month on sapopterin, consistent with increased dietary Phe and reduced formula intake. Formula intake especially was shown to be strongly correlated to both plasma Phe control and monoamine outcomes at baseline and longterm.

Thus, liberalizing the diet of PKU patients, though effective to QOL outcomes, may be contradictory to improvement of monoamine status. Maintaining full formula prescription in the diet however while on sapropterin may not be feasible since this could lead to excess calorie consumption given higher Phe intake. And though not

liberalizing the diet is also an option, patients with lowered plasma Phe who can consume more intact protein while maintaining plasma Phe levels in therapeutic range will self liberalize regardless. This apparent conflict in outcomes can however be resolved. Since formula protein intake had the greater impact on monoamine status, but less influence on QOL compared to dietary Phe, an appropriate compromise for definitive responders may be to increase Phe intake recommendations while maintaining at least a fraction of their original prescribed formula in the diet.

Additionally, management of plasma Phe control in provisional responders is important to ensure these patients are not self-liberalizing when there has been no biological increase to their Phe tolerance.

Conclusions on sapropterin's null effects and therapeutic potential for PKU patients with and without plasma Phe response

An optimistic conclusion from the null findings in this study is that neither sapropterin treatment nor diet liberalization have a deleterious impact on monoamine concentrations (except possibly long-term 5HIAA in definitive responders) and QOL scores in an age and gender diverse cohort of PKU patients.

Also the increase in HVA found in the PKU cohort at one month, and in particular nonresponders, not only indicates dopamine stimulation but also reveals that sapropterin has the potential to benefit PKU patients both with and without resulting declines in plasma Phe. Neurocognitive and brain imaging studies as part of future clinical investigations are needed to determine the phenotypic benefits of sapropterin induced dopaminergic stimulation in both responders and nonresponders.

Relevance of research to PKU and other medical diagnoses

Diet therapy and sapropterin have both been effective at helping PKU patients maintain plasma Phe control, thus improving longterm patient outcomes. Even so, better outcomes in PKU patients are not equivalent to optimal outcome. Therefore other investigators are developing treatments for PKU with potential for optimal outcomes in relation to blood Phe control, neurologic health, and dietary freedom [180]. Still in the pipeline these include pegylated phenylalanine ammonia lyase (PEGPAL) enzyme substitution therapy [181, 182], PAH chaperones [183], and even potentially curative gene therapy [139]. Provided any one of these therapies become available to PKU patients in the next few years, earlier research evaluating monoamine and QOL effects from pharmacologic intervention and PKU diet liberalization will be relevant as patients with access to these new therapies could have similar outcomes. Additionally, sapropterin will continue to be available longterm for PKU management and such outcomes are certainly important to the longterm health and wellbeing of patients taking this medication, as well as to those (or parents of those) considering initiating sapropterin treatment. The results are also relevant to PKU patients who are classified as nonresponders, as it highlights discrepancies in QOL outcomes, and indicates potential for sapropterin benefit even within this group.

Additionally, phenylketonuria is just one of at least 2 dozen inborn errors of metabolism characterized by neurological disability when diet restriction is not enforced [184, 185]. First diagnosed by Dr. Folling in 1934, PKU has been a forerunner in the recognition, treatment, and efforts towards curing an inborn error of metabolism [19]. It

is frequently a template in the medical management of other inborn errors responsive to diet restriction [186, 187], such as maple syrup urine disease (MSUD), methylmalonic academia (MMA) and citrullinemia to name a few. As treatment options become available for these disorders from research efforts that follow phenylketonuria advances, individuals and families affected by these disorders will likewise face circumstances of potential neurologic benefit, monoamine normalization, and diet liberalization. With over 250 known inborn errors of metabolism and only 81 treatable at this time (though not always with optimal outcomes), inborn errors thereby affecting 3-4/1000 live births worldwide [184, 185], the potential for applicability of this research to other diagnostic groups and populations is considerable.

To note, potential sapropterin benefit to monoamine status, particularly catecholamines as this study indicates, could be equally relevant to medical populations affected by catecholamine dysfunction such as Parkinson's disease, psychiatric disorders [160], and even vascular disorders [188].

Final statement

Sapropterin has the potential to improve dopamine metabolism in PKU subjects, though longterm plasma Phe control and dietary management remain critical to optimizing monoamine concentrations in PKU. QOL scores evaluating patient satisfaction with life and health management improved significantly as a result of diet liberalization within the group of definitive responders, while all sapropterin response groups demonstrated improved scores measuring impact of PKU on their lives.

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