DIETARY TREATMENT IN PKU FROM EXPERIENCE TO EVIDENCE

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DIETARY TREATMENT IN PKU FROM EXPERIENCE TO EVIDENCE

Proefschrift

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	Prof. dr. F.A. Wijburg

Denken
Eerst dacht ik: 'niet aan denken',
Dat heb ik toen gedaan
Maar twee seconden later,
Dacht ik er toch weer aan

Nee, zo eenvoudig is dat niet,
Want weet je, wat je doet,
Je denkt er ook aan als je denkt
Dat j' er niet aan denken moet.

Uit Toon Hermans "wijsheid"

Paranimfen:	Ems Carbasius Weber
	Marieke Hoeksma

Contents

List of abbreviations

BH4	tetrabiopterin
HPA	hyperphenylalaninemia
KIVA	L-[1-^{13}C] ketoisovaleric acid
PAH	phenylalanine hydroxylase
Phe	phenylalanine
PKU	phenylketonuria
RDA	Recommended Daily Allowance
Tyr	tyrosine

CHAPTER 1

General introduction and outline of the thesis

Historical highlights I
In 1950 the famous novelist Pearl S. Buck (1892-1973) published the novel "The Child Who Never Grew"[1]. In this autobiographical book (her daughter Carol was born in 1920) Buck wrote that she did not know when Carol's intellectual development stopped. She informs the reader that there was nothing in her family history to suggest that her child would fail to grow mentally. The concern that something was wrong with Carol slowly built as the baby approached her third birthday. By four years of age, Buck could no longer ignore her fears: her daughter suffered from severe mental retardation and had profound learning disabilities. Pearl Buck did not learn the reason for Carol's retardation for some forty years. Carol suffered from the inheritable disease Phenylketonuria (PKU). This condition resulted from an inability to metabolize phenylalanine, an essential amino acid. In addition to causing mental retardation, PKU is associated with blond hair, blue eyes, eczema and a musty odour. It was another mother in Norway who in 1934 found a special doctor, Asbjörn Følling who unlocked the reason for the severe mental retardation of her two children. The report that doctor Følling made in 1934 led to the identification of this disease in children all over the world[5]. Another persistent mother whose child was diagnosed at the age of 17 months could not accept that there was no treatment for the disease. The first attempts of Professor Horst Bickel to try to reduce the child's phenylalanine by using glutamic acid were not successful and he and his colleagues of the Children's Hospital in Birmingham (Evelyn Hickmans and John Gerrard) consulted doctor Louis Woolf at the Great Ormond Street Hospital in London. Woolf had a theory that it might be possible to treat PKU with a diet. A recent report had been published that showed that it was possible to remove phenylalanine from a protein hydrolysate by filtering it through activated charcoal. The work of Bickel and Hickmans in their laboratory resulted in the first phenylalanine free formula[11]. Treatment of the girl with the phenylalanine free formula together with a small amount of whole milk (as phenylalanine is an essential amino acid) improved the condition of the child remarkably[14].

Description of the disorder
Phenylketonuria is an autosomal recessive inborn error of phenylalanine metabolism. It is one of the most common inborn errors of metabolism in Europe (1:5,000-20,000) with an incidence of 1:18,000 in The Netherlands[9;10]. PKU results from deficient or defective activity of the liver based enzyme phenylalanine hydroxylase (PAH). In 1- 2 % of the cases the disorder is due to impaired synthesis of its cofactor, tetrabiopterin (BH_4). PAH is necessary for the hydroxylation of Phenylalanine (Phe) into Tyrosine (Tyr) and the defect leads to an accumulation of Phe and its derivates (phenylpyruvate, phenyllactate and phenylacetate)[12].
Left untreated the disorder causes severe mental retardation, neurological abnormalities (microcephaly, delayed speech, epileptic seizures and movement disorders), eczema, decreased pigmentation, and behaviour abnormalities[15;16].
PAH deficiency is genetically and phenotypically diverse. Normal fasting plasma Phe concentrations range from approximately 35 to 100 µmol/l (0.6 - 1.7 mg ·100 ml)[30;31]. In subjects with little or no hydroxylase activity, blood Phe concentrations rise to more than 30 times normal, but even in the biochemically severe forms of the disorder, there is a wide variation in clinical phenotype. Lesser degrees of biochemical disturbance are associated with a lower risk of mental handicap[12].

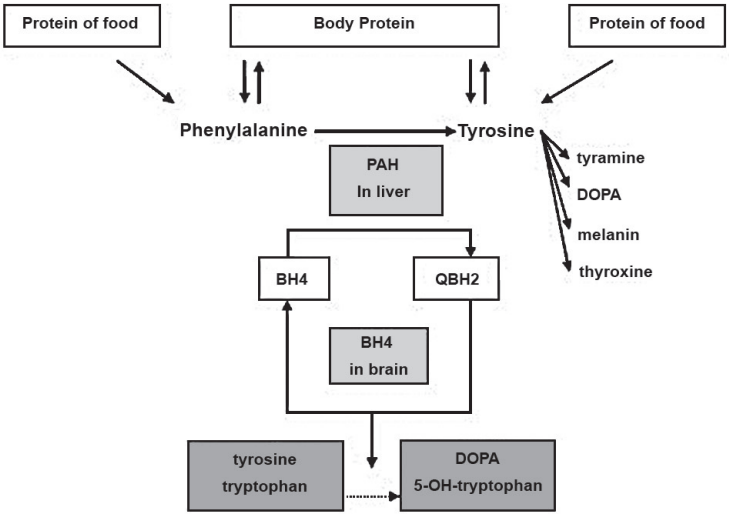

Figure 1. Metabolic pathway of phenylalanine in the body PAH: phenylalaninehydroxylase, BH4: tetrabiopterin, QBH2: quinoïddihydrobiopterin; BH4 metabolism includes 5 enzymatic reactions in synthesis and recycling

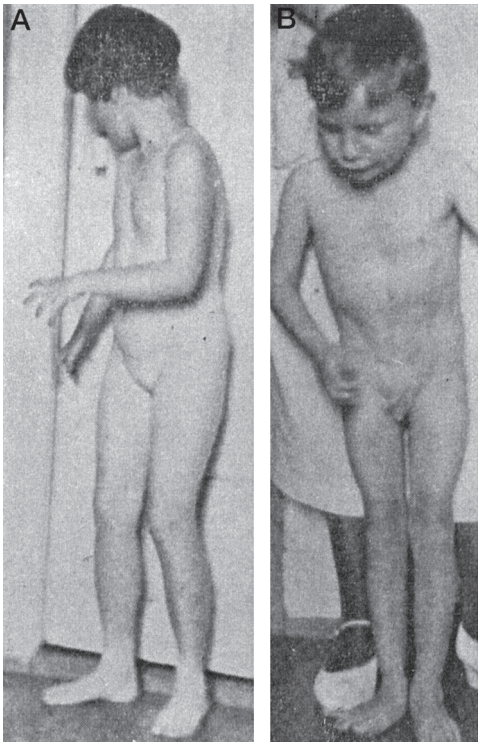

Figure 2. A and B, The first children (Liv, 7 years of age, and Dag, 4 years of age) diagnosed with PKU -from the 1934 publication of Dr Følling on the discovery of PKU.

Historical highlights II

The next breakthrough in PKU came in 1957 when Doctor Willard Centerwall of Los Angeles developed the first diagnostic screening test for the disorder in urine[3].This diaper test was the first step toward mass screening for PKU. Projects were initiated in which all of the residents of institutions for the mentally retarded were tested. It was through such testing that Pearl Buck's daughter was found to have PKU. Family members and new-born sibs of diagnosed patients were advised to be tested. Treatment was most successful when started at an early age before mental retardation was obvious. Of even more importance in the PKU story was the development by Robert Guthrie in 1961of a simple blood test in which an elevated blood phenylalanine concentration could be detected[6].This opened the road to primary screening of newborns on a population wide basis, and the possibility of early dietary treatment before significant brain damage had occurred. In the northern part of The Netherlands neonatal screening for PKU started in 1969 as a pilot, followed in 1974 by national screening.

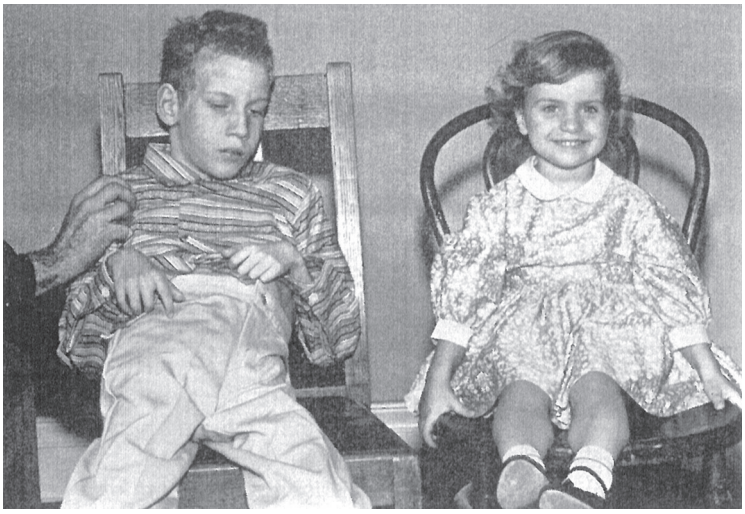

Figure 3. Contrast untreated and treated phenylketonurics. The 11-year-old boy is severely retarded, whereas his 2½ -year-old sister, diagnosed in early infancy and promptly treated with the mind-saving diet, is normal. (from: Diet and recipes. In: Lyman FL, ed. Phenyketonuria. Springfield, IL: Charles C. Thomas; 1963:318)

Screening and diagnosis

Early diagnosis is essential in PKU treatment as the most severe damage due to high Phe concentrations may occur during early infancy.

Neonatal screening is based on the detection of raised blood Phe concentrations. In the Netherlands it is advised to obtain blood samples between 48 -168 hours after birth. Children with blood Phe concentrations > 480 µmol/l are referred to an academic center immediately. If the result is between 240 – 480 µmol/l, the screening is repeated as soon as possible. If the result of the second screening is again > 240

µmol/l referral takes place at the same day. The diagnosis of hyperphenylalaninemia (HPA) is confirmed when quantitative analysis shows a Phe ≥ 240 µmol/l. Based on history (co-morbidity, protein intake), physical examination (signs of co-morbidity, signs of liver disease), liver function parameters and analyses of amino acid concentrations in plasma, causes of HPA other than a deficiency of PAH or BH4 can be excluded.
Different methods and criteria are exploited to determine the severity of the PAH defect. In general, the importance of a classification lies in the implications for prognosis or treatment. The relationship between genotype, enzymatic phenotype (in-vitro or in –vivo severity of PAH deficiency), biochemical phenotype (Phe concentrations) and clinical phenotype is not clear-cut[12]. At present DNA analysis cannot fully predict the metabolic phenotype. It is not currently possible at the time of diagnosis to predict the severity of the defect and the later outcome in Phe tolerance. In clinical practice, two in vivo methods are applied to measure the severity of the PKU: i.e. plasma Phe concentration at time of diagnosis and Phe tolerance i.e. the amount of Phe that a PKU patient can take with plasma Phe concentrations within the target range.

Dietary treatment

Dietary treatment can almost completely prevent cerebral damage in PKU patients, when started in the first weeks of life. This became possible with the introduction of newborn screening. The aim is to start dietary treatment as early as possible, because the greatest damage due to high Phe concentrations occurs during early infancy. The success of the diet is due to the fact that Phe is an essential amino acid and restriction is possible by just reducing the amount of Phe in the diet while providing adequate intake. Plasma Phe concentration can only increase by oral protein intake and by degradation of body protein. The diet is based on a natural protein (i.e. Phe) restriction with supplementation of all other amino acids, vitamins and minerals. Because Phe is an essential amino acid, Phe should not be eliminated entirely from the diet: the Phe intake needs to be restricted to a level preventing too high concentrations but not limiting protein synthesis. The non essential amino acid tyrosine becomes an essential amino acid in PKU, and adequate quantities must be provided to promote growth and repair of body tissue. Adequate intake of Phe, protein and energy must be provided to prevent breakdown of body tissues, which can lead to elevated plasma Phe concentrations. Intake of Phe, sufficient amino acid supplements and energy, as well as growth rate influence Plasma Phe concentrations. Catabolic states e.g. during illness also result in elevated blood Phe concentrations.

Dietary protein in relation to PKU treatment

Essential aspects of protein in the dietary treatment of the PKU patient will be considered in the next paragraphs, including Phe restriction as well as the restoration of the deficiencies that result from this restriction.
The primary function of dietary protein is to provide amino acids for 1) growth, 2) replacing tissue protein that is broken down during normal metabolism and 3) synthesizing several specialized products that contain nitrogen. Growth and replacement of tissue protein requires 23 amino acids. Ten of the amino acids are

essential components of the diet for children and nine for adults. The human body is not capable of synthesizing these essential amino acids out of other products. The non essential amino acids can be synthesized from common intermediates in metabolic pathways or from essential amino acids. If the nitrogen intake is equal to the amount of nitrogen excreted, a state of nitrogen balance exists. The concept of nitrogen balance is important in defining the protein nutritional state of an individual.

Digestion and absorption of proteins

After ingestion, proteins are denatured by acid in the stomach and pass into the small intestine, where the peptides are hydrolyzed by proteolytic enzymes. The resultant mixture of free amino acids and small peptides is then transported into the enteral cells by specific carrier systems and secreted as free amino acids into the bloodstream or further metabolized within the gut itself[36]. Absorbed amino acids are transported via the portal vein to the liver where a proportion of the amino acids is transported into the cell and used. The remainders pass through into the systemic circulation and are transported to all tissues.

Protein intake as free amino acids results in a faster rate of appearance in plasma compared to natural protein. Free amino acids can be immediately absorbed in the first part of the small intestine. Amino acids derived from protein will have to be digested first and are absorbed in the more distal part of the small intestine at a slower rate[37-39].

The amino acid pool

The size of the free amino acid pool in the body is kept rather stable with only small fluctuations. High concentrations of amino acids, especially of essential amino acids, can be toxic and are kept at a rather low constant concentration by the body (40). The amino acid pool is derived primarily from the turnover of tissue protein. In adults about 75% of amino acids derived from tissue protein degradation are used for rebuilding tissue protein. The remainder is used for synthesis of glucose, ketone bodies, and a variety of specialized nitrogen containing products. The essential amino acids that are removed from the pool must be replaced by essential amino acids derived from dietary protein or from body protein. Dietary amino acids can only be stored as body protein. When amino acid appearance rate exceeds protein synthesis capacity, excess amino acids are degraded.

Turnover

Body proteins are in a constant state of degradation and (re)synthesis, with a turnover of about 1-2 % of the total body protein each day. In adults about 30-40 g of nitrogen (0.1 g N_2 · kg · bodyweight ≈ 0,625 g protein · kg · bodyweight) derived from amino acids are excreted daily and not available for resynthesis of protein. This obligatory nitrogen loss results from oxidation of amino acids and excretion of nitrogen containing compounds in sweat, urine, and faeces[41]. Different proteins are degraded at different rates of time. In the adult human body more than 250 g of protein are synthesized and degraded daily, while dietary protein intake is in general far less.

Stable isotopes tracer studies

The possibilities of stable isotopic techniques have improved the understanding of protein and amino acid requirements both in health and disease. In the area of human

studies of protein synthesis and breakdown the use of stable isotopes is often applied, avoiding the hazards of radioactive isotopes as tracer. A schematic representation of whole-body protein and amino acid metabolism, useful for calculating rates of protein turnover from data obtained using a tracer, is shown in figure 4.
Isotopic enrichment is measured using gas chromatography / mass spectrometry (GC/MS).

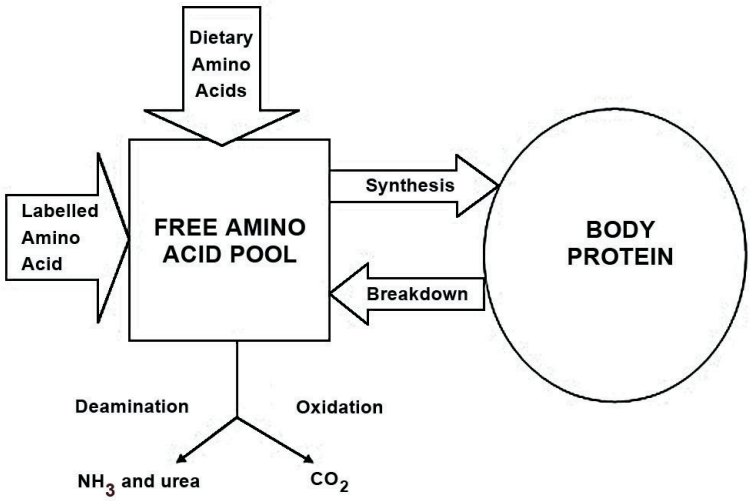

Figure 4: Schematic representation of whole-body protein and amino acid metabolism.

Prerequisites for isotopic dilution techniques are:

a. the tracer is metabolized at the same way and at the same rate as the natural occurring substrate
b. the amount of tracer in the pool being sampled is equal to the pool, or pools of the substrate under study
c. the kinetics of the naturally occurring substrate is not altered by the administration of the tracer
d. steady state is necessary to do reliable calculations of the metabolic processes and it is assumed that the system is in a steady state for both tracer and tracee metabolism.

Protein requirements in relation to PKU treatment

Recommended daily allowance

The recommended daily allowance of protein for adults is based on the amount needed to maintain nitrogen balance. Additional protein is required by women who are pregnant or breastfeeding. The amount of protein required by children is based on the amount needed for optimal growth, together with the amount needed to maintain nitrogen balance. The recommended daily allowances are regularly updated according to the latest evidence. Examples of frequently applied recommendations

Table 1. Protein recommendations in the healthy population

In 2001 the Dutch Health council has revised and lowered the recommendations for protein for the healthy population, which are now more in line with the recommendations of the American RDA and other European countries[2]. The average intake in the Dutch population is much higher, In the Netherlands the protein intake of healthy individuals of all ages is still rising and this tendency is world wide in the developed countries[8]. Figures are shown in Table 1.

Advised and consumed protein intake in healthy individuals

Recommendation	5 year		15 year		40 year	
	m	f	m	f	m	f
United States 1989	24	24	59	44	63	50
Great Britain 1991	20	20	55	45	56	45
European Committee 1992	19	19	54	46	56	47
Dutch Health Council 1992	47	43	70	65	71	55
Scandinavia 1996	53	51	85	68	86	68
Germany,Switserland,Austria 2000	18	17	60	46	59	47
Consumed						
Dutch Food Consumption 1997-1998	56	53	84	70	95	76

are: the American Recommended Dietary Allowances, the European, the British the German and Scandinavian guidelines (Table 1).

There are small differences in the figures (Table 1), but there also are different basic principles concerning the origin of the protein. Recommendations are not completely consistent and sometimes not precise about the quality of the protein advised. The quantification of protein and amino acid requirements is still an incompletely resolved issue.

Protein recommendations in PKU

In recent years there has been a trend in PKU to advise larger amounts of protein (amino acid supplement) both in children and adults with PKU. Recommendations about the optimal amount of supplementation of the amino acids are based on protein recommendations for healthy individuals (Table 1) , on studies on the nutritional value of free amino acids as compared to natural protein[39;49;50], on studies on growth in relation to higher or lower protein intake[57-62], and on the studies supporting that a higher protein intake is related to a better metabolic control[66;67].

Historical highlights III

In the first decades of PKU treatment only a few manufactures produced protein hydrolysed mixtures low in Phe and later Phe free synthetic amino acid mixtures. The same product was used for all age groups in different amounts and in different combinations of fat and carbohydrates. Flavour and smell of all these products are very pronounced and unpleasant. The taste and smell and the high volume that has to be taken (at least there times a day) makes it very hard to comply with, especially for those patients who stopped dietary treatment and those who were not on diet before. In the last decades more manufacturers have developed new products adapted to different patient groups (age, pregnancy), to a better taste (flavoured low volume products) and to a more convenient format (flavoured ready to use drinks, bars, sachets, tablets). The amino acid composition of the products has been optimized according to the amino acid pattern of human milk.

Protein intake in relation to growth in PKU patients

The relation of growth and metabolic control and protein intake in PKU is part of an ongoing debate. Restriction of protein intake carries the risk of negative effects on growth of height and head circumference. In early reports by the collaborative study group of the USA on PKU treatment, restriction of growth of height as well as head circumference occurred, but later this observation was no longer reported[68;69]. In the Netherlands Verkerk and colleagues showed that Dutch patients treated for PKU showed a height growth restriction in the first three years of life (70). This finding prompted a search for factors that might be responsible for this restriction in growth in the Dutch PKU population.

Amino acid supplement and micro nutrients in PKU treatment

Due to the strict limitation in natural protein, the diet is insufficient in most recommendations for vitamins and minerals. Together with the supplementation of amino acids, vitamin- and mineral intake is restored according to European recommendations. Legal guidelines for production of foods formulated for use under medical supervision, such as amino acid supplements, are found in the legislation concerning Foods For Special Medical Purposes (FSMP). The guidelines include complete nutrition formulas for infants and adults, as well as specialised supplements. In the guidelines supplementation of micronutrients is related to energy, while amino acids are prescribed based on protein content. For this reason supplementation is adapted. All micronutrients mentioned in the FSMP have to be added at a minimum level with a defined maximum.

European legislation is based on an average of the different national guidelines of European countries for supplementation and sometimes still far away from evidence based practice.

Optimal supplementation of the amino acid supplement with macro- and micronutrients depends on the amount that is prescribed and the intake of other foods. Phe tolerance is individually quite different and determines other food intake. The differently interpreted total protein requirements result in different amounts of amino acid supplementation and thereby the intake of other essential nutrients.

Supplementation of tyrosine

The available protein substitutes contain 6.8 – 14.7 g tyrosine per 100 g protein equivalent[71]. Based on theoretical reasoning and in-vivo investigations in PKU patients, a tyrosine supplementation of 6 g per 100 g protein equivalent in the amino acid supplement seems to be sufficient[71;72].

Long-chain polyunsaturated fatty acids (LCPUFA's)

In recent literature evidence was shown that breast fed infants have an advantage in visual and cognitive function compared to formula fed infants[73;74]. Both infants and older children with PKU are reported to have low levels of LCPUFA's either due to inadequate intake or a decreased capacity to synthesise these fatty acids[75;76]. Therefore, LCPUFA's enriched amino acid supplements are recommended in PKU patients of various ages.

Distribution of the amino acid supplementation

Metabolic and dietary handbooks advise to divide the daily intake of the amino acid supplements into three equal parts or even more frequently and to combine the intake of natural protein with the amino acid supplement[15;77;78]. A Cochrane review performed in 2000 is much less definite and shows a lack of evidence based studies to confirm these recommendations[79].

Table 2. Various recommendations of countries other than The Netherlands

	Age in years	Phe limits	Frequency of Phe monitoring	Frequency of clinical monitoring
British (MRC 1993)	0 – 5	120 – 360 µmol/l	Weekly	Every 2-3 months
	5 – 10	120 – 480 µmol/l	Fortnightly	Every 3-4 months
	> 10	120 – 700 µmol/l	Monthly	Every 6 months
US (Koch 1996)	Childhood	180 – 480 µmol/l		
	> adolescence	480 – 720 µmol/l		
German (Burgard 1999)	0 – 1	40 – 240 µmol/l	Weekly to fortnightly	Every 3 months
	1 – 10	40 – 240 µmol/l	Fortnightly to monthly	Every 3 months
	10 – 15	40 – 900 µmol/l	Monthly	Every 6 months
	> 15	< 1200 µmol/l	Every 2-3 months	
US (Wappner 1999)*	0 – 12	120 – 360 µmol/l	Approximately monthly	
	> 12	120 – 600 µmol/l		

* These are not recommendations but results of interviews amongst 87 clinical directors

Indications for treatment, target blood phenylalanine concentrations and clinical follow-up

The aim of dietary treatment is to prevent accumulation of Phe in the body and to keep plasma Phe concentrations within target ranges that are thought acceptable to prevent irreversible brain damage.

The Dutch Advisory Committee of PKU holds the view that treatment should start at the upper limit of the target blood Phe concentrations in treatment. Therefore, in the

Historical highlights part IV

When doctors began treating PKU in the early sixties it was thought patients could safely discontinue the diet during early childhood. No one knew for sure when the diet could safely be discontinued, but many professionals felt that once the child's brain had fully developed, probably sometime between the age of six to eight years, the diet was no longer necessary. Besides the risks of maternal PKU, observations of intellectual deterioration, learning difficulties, neuropsychological defects, and psychiatric and psychosocial problems after discontinuation of the diet led to the current recommendations in most treatment centres of "diet for life"[24-29].

Netherlands dietary treatment is started when untreated Phe concentrations > 360 µmol/l[80].

Recommendations concerning target Phe concentrations and frequency of sampling differ widely between different authors[25;27;29;81]. An overview of these different recommendations in the European countries and the US is given in the survey of Schweitzer-Krantz[82]. In Table 2 we show the most important results.

The differences between the recommendations concerning the target Phe concentrations exist partially because of differences in interpreting available data and partially because of lack of sufficient data. The question as to why the blood Phe concentrations in PKU should be higher than in healthy individuals is difficult

Table 3 Target blood Phe concentrations, and the frequency of blood Phe monitoring and clinical follow-up[80].

Age	Target Phe concentrations in µmol/l	Frequency of blood Phe monitoring	Frequency of clinical monitoring
0 – 1 years	120 – 360	Weekly	Every 1 – 3 months
1 – 4 years	120 – 360	Fortnightly	Every 3 months
4 – 12 years	120 – 360	Monthly	Every 4 months
12 – 15 years	120 – 600	Monthly	Every 6 months
> 15 years	120 – 600	Monthly	Every year
(Possible)pregnancy	120 – 240	Weekly	Every 2-4 weeks

to answer. Diurnal variations of plasma Phe concentration in PKU show an inverse pattern throughout the day, compared to healthy individuals. In PKU patients Phe concentrations measured after an overnight fast are the highest, and decrease during the day, which may cause plasma Phe concentrations < 40 µmol/l[29;83-85].

There seems to be enough evidence to suggest an upper limit in blood Phe concentration of 360 µmol/l during at least the first 12 years of life based on IQ outcome and neuropsychological findings. In case of strict dietary control, frequent measurement of the blood Phe concentrations is necessary[29]. Frequent clinical evaluation however is unnecessary. Therefore, it is recommended to have a rather low frequency of clinical evaluation and a rather high frequency of blood Phe concentration measurements, especially at early age when growth velocity varies greatly and the frequency of intercurrent illness and feeding problems can be quite high. Table 3 shows the Dutch guidelines for blood Phe target ranges and frequency of monitoring.

Breastfeeding PKU infants

In PKU, there is no contra-indication for breastfeeding provided that Phe intake does not exceed individual Phe tolerance. Dietary guidelines about breast-feeding the PKU infant can be based on two different principles (exact measurement or feeding to satiety), both with comparable results in metabolic control and growth[86;87]. Breast-feeding PKU infants has long been uncommon in the Netherlands. Recent

data showed that of 97 PKU infants being breast-fed at the time of diagnosis, only 4 continued to be breast-fed after diagnosis (Crone MR, personal communication).

Historical highlights V
Clinicians caring for persons with PKU have been perplexed by the occasional normal individual with the classical biochemical profile consistent with the diagnosis of PKU. Usually untreated subjects with the biochemical profile of blood Phe concentrations >1200 micromol/L are severely mentally retarded and may have neurological findings. Preliminary reports, however suggested that low brain Phe concentrations, despite elevated blood Phe concentrations, account for the occurrence of these occasional unaffected individuals with the biochemical profile consistent with PKU.

Parents of infants with PKU were advised to switch to bottle-feeding after diagnosis. However, recommending breast-feeding is in accordance with the WHO/UNICEF recommendation. Aside from the general advantages of breastfeeding, an extra reason to breastfeed the PKU infant is the low amount of protein (i.e. Phe) in breast milk.

Historical highlights VI
About two decades after screening had started, obstetricians began seeing women with PKU who were not on diet. They found that 95 % of the babies born to these women had serious abnormalities. The phenomenon of maternal PKU was already described by Dent in 1957 in the offspring of untreated, apparently "normal" women who turned out to have higher plasma phe concentrations[4]. The publication of Dent is followed by many others, which Levy summarized in an historical overview in 2003[7]. In 1982 Kirkman predicted that if women with hyperphenylalaninaemia reproduce at a normal rate, the mental retardation prevented by newborn screening could return after only one generation[13]. Fortunately however, the offspring of PKU patients, who maintained a strict diet, turned out to have a normal potential for bearing healthy infants. The offspring of male PKU patients are not considered at a higher risk for abnormalities than the general population[17;18].

Treatment beyond childhood
Dietary treatment of PKU was not considered to be indicated beyond childhood. This policy was based on the assumption that neurological damage in adulthood would not occur. However, some recent reports suggest otherwise, while others do not. Although no data show an obvious decline of Intelligence (measured by IQ) when dietary treatment is stopped after 10 – 12 years of age, individual experiences of parents, adolescent and adult patients and studies using neuropsychological evaluation and Positron Emission Tomography showed that higher Phe concentrations are related to less positive outcome and neurological problems[88-95].
At present it is unclear whether it is safe to stop dietary treatment in adult PKU. Therefore, it is advised to continue dietary treatment throughout life[25-29]. Adolescents and adults with PKU are at risk for nutritional deficiencies if dietary treatment is not carefully

monitored. Particularly the group of previously treated patients who discontinued dietary treatment, but often still avoiding protein rich foods, may show deficiencies in their meal plan.

Maternal PKU

The first report of a successful pregnancy in a patient with PKU under dietary treatment is followed by many studies that emphasize strict metabolic control during pregnancy and at time of conception[96-99]. A clear relationship exists between the (maternal) plasma Phe concentrations and the outcome[100]. Although many questions are still present regarding the mechanism of fetal brain damage, dietary restriction of Phe in the pregnant mother to lower Phe concentration to near normal values is the treatment of choice. Maternal values would need to be kept between 120 – 240 µmol/l prior to conception and during pregnancy.

The PKU treatment team

In the Netherlands, PKU patients are only treated in academic centers. Ideally, the PKU treatment team at the university hospital consists of physicians (both paediatricians and adult physicians), dieticians, nursing staff, clinical biochemists specialised in metabolic diseases, social workers and psychologists. Treatment strategies are primarily focused at normal growth and psycho-motoral development by keeping the blood Phe concentration as low as possible, with adequate supplementation of amino acids, macro- and micro nutrients. However apart from this, treatment aims concern also the quality of life of the patient and the family. Treatment strategies focussing on the patient's and /or parents' own responsibility may be essential to maintain the strict dietary regimen both in the short and the long term. This implies knowledge of the disease itself, the role of nutrition in PKU, and the possibility of regular blood sampling and dietary counselling.

The means for obtaining blood samples at home are offered early after diagnosis. It

Alternative treatments

Large Neutral Amino Acids (LNAA) The origin of the large neutral amino acid (LNAA) hypothesis to reduce brain Phe stems from the work of Andersen et al, Oldendorf et al, Pardridge 1982, Hargraeves and Pardridge 1988 et al on the transport of amino acids across the blood brain barrier, concluding that Phe and other LNAAs (tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, and histidine) share a common active transporter to the brain and therefore compete with one another[19-23]. Clinical trials with one or more LNAAs have been tried. The first study of LNAA supplementation in the treatment of PKU was conducted by Dotremont et al[15;32-35]. This treatment was problematic in that subjects developed a negative nitrogen balance as a result of lysine deficiency secondary to low-protein intake. Many other studies followed, but more randomised long term clinical trials are needed to show whether LNAAs can be safely used as a supplement to the conventional treatment of PKU patients and allow a more liberal diet that can improve compliance[42-48].

Tetrahydrobiopterin (BH4) Many more patients than initially assumed respond to BH4 loading and treatment. About two-thirds of all mild phenylketonuria (PKU) patients are tetrahydrobiopterin (BH4)-responsive and thus can be potentially treated with BH4 to lower Phe concentrations instead of or in combination with a low- Phe diet[51-56]. Tetrahydro-

biopterin (BH4)-responsive phenylalanine hydroxylase (PAH) deficiency is a subgroup of hyperphenylalaninemia (HPA) caused by specific mutations in the PAH gene. Although there has been an increase in the amount of information relating to the diagnosis and treatment, very little is known about the mechanisms of BH4-responsiveness. Besides the mechanisms of the BH4 responsiveness, studies are necessary to fine tune the performance of the loading test and the optimal treatment doses. Also for this treatment there is a lack of reports on the long-term follow-up of HPA/PKU patients on treatment with BH4 with comparable treatment protocols, either as monotherapy or in a combination with the low-Phe or low-protein diet. Several long-term crossover or double-blind studies are currently running in different countries and it is to be expected that BH4 will be available for pharmacological therapy at least of the mild variant of PAH deficiency in the next years.
Phenylalanine Ammonia Lyase (PAL) Another therapeutic resource currently under development for PKU treatment is based on the oral administration of phenylalanine ammonia lyase. This non-mammalian enzyme degrades Phe in the intestinal lumen to a harmless acid and thus prevents Phe absorption[63-65]. In the development of this treatment for PKU two problems occurred, the problem of inactivation by digestive enzymes and the high costs. Further developments are necessary to be able to do clinical trials. Application of PAL treatment is not expected in the near future.
Gene therapy The ideal treatment of genetic diseases would consist of taking a normal copy of the defective gene and transfer it into the patient. PAH gene is expressed mainly in the liver. In mouse models different PAH gene transfer vehicles have been tried and showed that only some in vivo activity of PAH is enough to induce normal Phe metabolism. Still, the development of a safe and successful gene transfer vector is required before clinical trials in humans become possible. So far none of the animal experiments have lead to such a favourable outcome.
Transplantation Full correction of the enzyme defect can be achieved by a liver transplantation. The consequences of this procedure (surgery risks and immune suppressive medication post transplantation) do not make this treatment in its current form a realistic alternative.

is advisable to stimulate the child to actively participate in the diet in an early stage. Self-monitoring might have a positive influence on the compliance of the diet and the ultimate outcome[101]. The information to and education of the patient is a continuous process form early infancy into adulthood. Meetings in age-specific groups create extra possibilities to enhance the patient's knowledge of PKU and its treatment. These meetings may also contribute to improvement of coping mechanisms in all daily life aspects both of the PKU patients and their parents.
Children with PKU may have a higher risk of emotional and behavioural difficulties, probably due to a combination of factors related to the disease itself and the dietary treatment. Therefore, both support and diagnostic evaluation by social workers and psychologists should be easily available[102].

Alternative treatments
Despite the success of dietary treatment, the diet cannot be considered the most desirable management for PKU patients. Research to find alternatives for the dietary management has resulted in the development of various treatment strategies, most of which are still experimental. Treatment can be additional to, or a replacement of, the traditional dietary treatment.

OUTLINE OF THE THESIS

From experience to evidence

Fine tuning a treatment that has been applied successfully for over half a century is challenging. Asking the patient to comply with a life long treatment, however, requires the commitment of caregivers to generate the best practice evidence for the efficacy of such treatment. The aim of this thesis is to provide additional new evidence to the merits of the dietary treatment we prescribe to the patient and to give tools to the patient to obtain better outcomes with less effort and more individual choices.

Protein recommendations in PKU, especially the optimal degree of supplementation with amino acids are still frequently a subject of discussion. The trend to advise larger amounts of amino acid supplementation is based on different principles and assumptions. In **chapter two** protein turnover and growth are addressed. Protein turnover in PKU patients is studied as a first step to see whether PKU patients have an intrinsically different protein metabolism compared to healthy controls. The carbon-labelled amino acid [1-^{13}C]-valine was applied as tracer in the study on protein metabolism (chapter 2a).

The second part of chapter two is focused on the question of a possible relationship between growth and the two fractions of the protein used by PKU patients, i.e. the natural protein and the protein substitute. To our knowledge, no study has paid special attention to the specific amount of protein obtained from the protein substitute or the amount of natural protein in relation to growth of either height or head circumference in PKU infants. A relationship with natural protein rather than total protein would favour further optimizing the composition of protein substitutes.

In the first part of **chapter three** the possibilities of feeding the PKU infant (as normal as possible) are addressed. Positive newborn screening for PKU resulted in almost all cases in termination of breastfeeding. Promoting breastfeeding for healthy infants has been intensified in the Netherlands in the last two decades. To support breastfeeding also in PKU patients, application of different guidelines for breast feeding the PKU infant were studied. The second part of chapter three is aimed at the question whether an unequally divided Phe intake results in larger diurnal fluctuations in plasma Phe concentrations than when Phe intake is equally divided over the feedings of PKU infants. The first part of **chapter four** focuses on the possible prediction of the individual Phe tolerance. The second part describes the first results of a six month's period of a self management procedure of the dietary treatment. We also found that patients and parents apply different methods in "measuring" the diet. The results of a questionnaire about this practice in relation to the individual metabolic control are elucidated in the third part of chapter four. Fluctuations from day to day and week to week in plasma Phe concentrations in adult PKU patients with and without dietary interventions are addressed in the last part of chapter four. Aim of this study is to give more evidence, especially in adults to dietary changes advised to keep plasma Phe concentrations within target ranges. In **chapter five** the significance of the performed studies for the PKU patient is discussed and directions for future studies indicated.

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CHAPTER 2a

Protein metabolism in adult patients with Phenylketonuria

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ABSTRACT

Background: Protein intake recommendations in Phenylketonuria (PKU) are frequently subject of discussion. For healthy adults, the Recommended Daily Allowance (RDA) is 0.8 $g \cdot kg^{-1} \cdot d^{-1}$, which is generally lower than observed in the general western population.
Objective: To study whether whole body protein metabolism in PKU patients is comparable to healthy controls at a RDA rate of protein intake.
Design: Six adult well-controlled PKU patients and 6 healthy individuals of comparable age, height and weight were studied using a primed-continuous infusion of [1-^{13}C]-valine for 8h after an overnight fast before and during frequent meals. Normal protein was given to controls, whereas PKU patients received a combination of an amino acid mixture and natural protein.
Results: No significant differences were observed between PKU patients and controls in preprandial (pp) and prandial (p) rates of valine appearance and oxidation and protein breakdown (B), protein synthesis (S) and net protein balance (NPB). Feeding resulted in a significant (P<0.01) decrease of B (PKU: 94±15 (pp) to 49±10 (p); controls: 97±10 (pp) to 55±10 (p) $\mu mol \cdot kg^{-1} \cdot h^{-1}$), whereas no effects were observed in S (PKU: 77±10 (pp) to 73±7 (p); controls: 76±8 (pp) to 71±5 (p) $\mu mol \cdot kg^{-1} \cdot h^{-1}$). NPB increased from negative (p) to positive (pp) values (PKU: –17±6 (pp) to +23±8 (p); controls: –21±4 (pp) to +16±9 (p) $\mu mol \cdot kg^{-1} \cdot h^{-1}$).
Conclusion: Whole body protein metabolism in adult PKU patients is fully comparable to healthy controls at the RDA level of protein intake.

KEYWORDS:
Phenylketonuria, protein requirement, amino acid oxidation, whole body protein turnover, stable isotopes, [1-^{13}C]-valine, L-[1-^{13}C] ketoisovaleric acid (KIVA).

INTRODUCTION

Patients with phenylketonuria (PKU, McKusick 261600) cannot convert phenylalanine (Phe) into tyrosine (Tyr) due to a deficiency of phenylalanine hydroxylase (EC1.14.16.1) activity in the liver. Left untreated, PKU leads to high Phe concentrations in blood and tissues and low to normal Tyr concentrations, clinically resulting in severe mental retardation, epilepsy and behavioral problems[1]. Treatment consists of restriction of the essential amino acid Phe by reducing the natural protein intake with concomitant supplementation of all amino acids but Phe. Patients, treated by this dietary Phe restriction have a more or less normal outcome although some minor neuropsychological dysfunction remains[1-3].
Recommendations about the optimal amount of supplementation of the amino acids are based on protein recommendations for healthy individuals and factors that may influence optimal protein intake in PKU. Studies have been carried out about nutritional value of free amino acids as compared to natural protein, about growth in young PKU patients and about metabolic control in PKU patients[4-18]. The question arose as to whether the results of all these studies are suitable to determine the amount of amino acid supplementation for adults. The number of adult PKU patients on diet is still growing since the first PKU patients have reached adulthood after the start of newborn screening and the issue of evidence for optimal protein recommendations requires attention. Lifelong dietary treatment with high level supplementation of

amino acids may be unnecessary and has several drawbacks both economically and socially, since amino acid supplements are expensive and adherence to the intake prescription is difficult[19-21].

The aim of the present study was to compare whole body protein metabolism in healthy adults and adult PKU patients preprandial and a subsequent prandial period at a protein intake comparable to the RDA of 0.8 g · kg^{-1} · day^{-1}. Our hypothesis was that protein metabolism in PKU patients is intrinsically not different from healthy controls.

SUBJECTS AND METHODS

Study subjects:

Six patients with PKU and 6 healthy adult individuals were studied. The 6 PKU patients (males and females) had a mean age of 27 ± 7 years with a normal height (mean Z score –0.6 ± 1), and a mean body weight of 70 ± 9 kg and a mean body mass index (BMI) of 23 ± 3 (kg/m^2) (Table 1). All patients had an intellectual development within the normal range. Tolerance of dietary Phe (based on daily intake of natural protein) at 5 years of age was 21 ± 9 mg Phe · kg^{-1} · d^{-1} and 11 ± 4 mg Phe kg^{-1} · day^{-1} at the time of the test. Treatment of patients was considered adequate as the mean Phe concentration was 522 ± 106 µmol/L during the past 2 years and the mean Phe concentration was 502 ± 150 µmol/L during the past 6 months. Blood Phe concentrations were within the target range one week before the study (120 – 600 µmol/L). The 6 healthy individuals (males and females) had a mean age of 32 ± 4 year with a normal height (mean Z score –0.1 ± 1), body weight (mean 67 kg ± 14) and BMI (mean 23 ± 3 kg · m^{-2}) (Table 1).

All participants were in good clinical condition at the time of the study and free of concomitant disease and their body weight was stable within the past 6 months. Participants were asked to keep a record of their entire food and beverage intake for 3 days. From this 3-day period the mean 24 hours energy intake was calculated. Participants were excluded when their normal level of exercise resulted in an energy requirement >25% above mean RDA for energy, as protein requirements under extreme physical activity have not been defined.

The nature, purpose, and potential risks of the study were explained to all subjects before they gave their written informed consent to participate. The Medical Ethical Committee of the University Medical Center Groningen approved the study protocol.

Materials

Isotopes: L-[1-^{13}C]-valine and $NaH^{13}CO_3$, both with enrichment over 99 atom percent excess, were purchased from Cambridge Isotope Laboratories (Andover, MA). Chemical purities were confirmed before use. Pyrogen- and bacteria-free solutions were prepared in sterile saline by the hospital pharmacy the afternoon before the study day and were used within 24 hours after preparation.

Table 1 Clinical characteristics of PKU patients and healthy controls studied for whole body protein metabolism*

Characteristics	PKU patients	Healthy volunteers
Age (y)	27 (± 7)	32 (±) 4
Sex (M/F)	3/3	2/4
Weight (kg)	70 (± 9)	67 (± 14)
BMI (kg/m^2)	23 (± 3)	23 (± 3)
Height (cm)	172 (± 3)	172 (± 9)
Height (Z score)	-0,6 (± 1)	-0,1 (± 1)
Energy intake (kcal · kg^{-1} · day^{-1})	40 (± 14)	38 (± 7)
Protein intake (g · kg^{-1} · day^{-1})	1.1 (± 0.1)	1.2 (± 0.1)
Phe tolerance at 5 y (mg Phe · kg^{-1} · day^{-1})	21 (± 9)	
Phe tolerance at test (mg Phe · kg^{-1} · day^{-1})	11 (± 4)	
Phe concentration last 2 years (µmol/L)	522 (± 106)	
At start of test:		
Phe concentration in plasma (µmol/L)	443 (± 100)	49 (± 10)
Tyr concentration in plasma (µmol/L)	38 (± 8)	53 (± 14)
Val concentration in plasma (µmol/L)	222 (± 42)	193 (± 52)
Albumine g/L	43 (± 2)	45 (± 2)
Total protein g/L	70 (± 3)	71 (± 3)
Urea mmol/L	4 (± 1)	5 (± 1)
Creatinine (µmol/L)	81 (± 12)	85 (± 5)
ASAT U/L	21 (± 5)	23 (± 8)
ALAT U/L	18 (± 7)	19 (± 11)

* All values mean ± SD

Diet:

For the PKU patients the protein intake conform the RDA advise (0.8 g protein · kg^{-1} · d^{-1}) was composed out of amino acid mixture (PKU 3® Milupa, Friedrichsdorf, Germany) and the individual Phe tolerance as natural protein (0.1- 0.2 g protein · kg^{-1} · d^{-1}). Patients used to another amino acid supplement were changed at least two weeks before the study to PKU 3®.

The protein intake for the healthy individuals conform the RDA advise (0.8 g protein · kg^{-1} · d^{-1}) was composed out of 67% milk protein (high quality) and 33% vegetable protein (low quality).

On the test day meals for both groups consisted of liquid meal portions, fruit and biscuits. For PKU patients the liquid meal portions were composed of water, amino-acid mixture PKU 3® (Milupa, Friedrichsdorf, Germany), fat and malto-dextrin modules (Solagen® and Fantomalt®,- Nutricia- Zoetermeer, The Netherlands) and an artificial flavor (®Flavour Sachet -SHS, Liverpool, United Kingdom). For healthy individuals the liquid meal portions were composed of milk, a milk protein module (Protifar plus®- Nutricia, Zoetermeer, The Netherlands), fat and malto-dextrin preparations (Solagen® and Fantomalt®). The valine content of milk protein was 6.3%, of wheat protein 4.5% and of protein of fruit 3.6%[22]. The valine content of the amino acid supplement PKU

3® was 7.9 %[23]. This resulted in an average valine intake of 78 ± 5 µmol·kg $^{-1}$ · h $^{-1}$ in PKU patients and 56 ± 1 µmol·kg $^{-1}$ · h $^{-1}$ in healthy controls for the 4 h prandial period.

Experimental Design

The adaptation period of the test diet with 0.8 g protein ·kg^{-1} · d^{-1} (described as above) was 1 day, in which the intake was divided over 3 main meals and 3 snacks. The energy intake during the adaptation day was equal in both groups to the computed individual intake of the 3 days records, 40 (± 14) kcal ·kg^{-1} ·day^{-1} for the PKU patients and 38 (± 7) kcal ·kg^{-1} ·day^{-1} for the control group. The last snack was taken between 8 and 10 PM. After 10.00 PM no food or beverages aside from water were allowed. The study started at 08.00 AM after overnight fasting in the preprandial state. The first meal on the study day at 12.00 AM contained one third of the daily individually calculated energy and protein intake. After that, hourly meals provided one twelfth of the energy and protein intake. The energy intake for both groups was based on the individual's 3 day food record. Calculation of the records was done by a dietician (MvR) with the ZIS -food calculation computer program based on NEVO[24]. A schematic diagram of the study day is shown in Figure 1.

Individuals were admitted to the Hospital Research Unit at 7:45 AM. An intravenous catheter was inserted into a vein for blood sampling, and another was placed in the opposite arm for the infusion of the labeled materials. Subsequently, baseline breath and blood samples were taken. The $NaH^{13}CO_3$ infusion started at 8:00 AM. During the 1st hour, whole body $NaH^{13}CO_3$ production was measured using a primed constant infusion of $NaH^{13}CO_3$ (5 µmol·kg^{-1} bolus followed by a continuous infusion of 5 µmol·kg $^{-1}$ · h $^{-1}$). Four breath samples were taken from 15 to 60 min after the start of the $NaH^{13}CO_3$ infusion at 15-min intervals. The $NaH^{13}CO_3$ infusion was discontinued immediately after

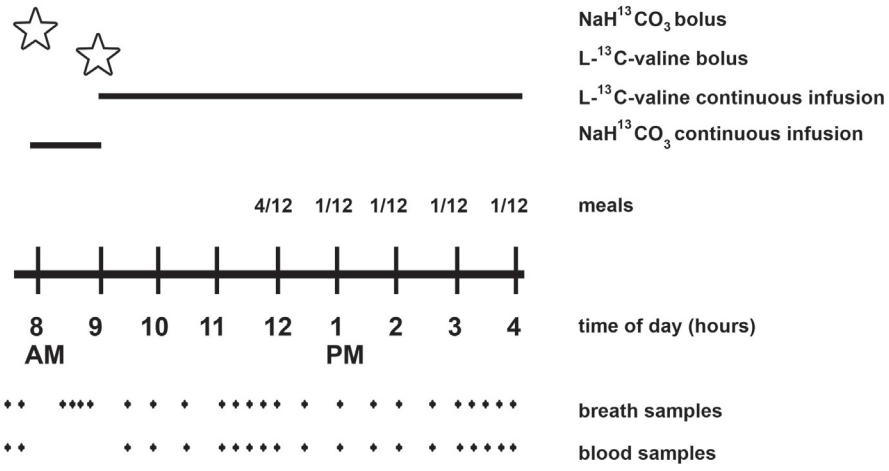

Figure 1. Study protocol for whole-body L[1-^{13}C]valine kinetics in PKU patients and controls pre-prandial and during hourly meals.

the last breath sample was taken, and replaced by L-[1-^{13}C]-valine infusion bolus of 7.5 μmol·kg^{-1} followed by a continuous infusion of 7.5 μmol · kg^{-1} · h^{-1} for the next 7 h. Blood and breath samples were taken simultaneously every half hour for the first 2 h after the start of the L-[1-^{13}C]-valine infusion. During the third hour, blood and breath samples were taken every 15 min. At 12:00 AM, the meal period was started by consumption of the first meal and continued for 4 h by consumption of a meal every 60 min. After the start of the meal period blood and breath samples were taken every 30 min for 3 hr and during the last hour samples were taken every 15 minutes. Amino acids, insulin and glucose were determined at start, at the end of the fasting period and at the end of the meal period. Total protein, albumin concentrations, platelet counts, liver enzymes, urea and creatinine were determined at the start of the test day by standard clinical chemistry methods. Urine was collected during the 24 hour adaptation and during the study in 2 periods of 4 hours (preprandial and prandial). Urine samples of these three periods were taken to determine urea and creatine by standard clinical chemistry methods.

Analytical procedures

Blood (4 ml) was drawn for each sample in liquid-heparinized vacuum tubes and centrifuged at 3,000 rpm. Plasma was extracted and stored at -20°C until analysis. Breath samples were collected in gas collection tubes with a straw, as described earlier[25]. Subjects exhaled normally through a straw in the glass container. After exhalation was completed, tubes were closed immediately and stored at room temperature until analysis. Phenylalanine concentrations in bloodspot (1 week before the testing day) were measured by the AccQ Tag method using high performance liquid chromatography according to the manufacturer's protocols (Waters, Breda, The Netherlands). Analysis of all amino acid concentrations in plasma at the testing day were measured on a Biochrom 20 amino acid analyzer with the ninhydrine- method, according to manufacturer's protocols (Biochrom, Cambridge, United Kingdom). Measurement of $^{13}CO_2$ isotopic enrichment was performed by sampling directly the glass container with a Heliview (Medichems, Seoul, Korea) continuous-flow isotope ratio-mass spectrometer as described by Vonk et al.[25]. L-[1-^{13}C]-ketoisovaleric acid (KIVA) isotopic enrichment was determined according to Kulik et al[26]. In short, standards with a tracer mole ratio for L-[1-^{13}C]-KIVA ranging from 0 to 22% were prepared by enzymatic conversion with L-amino acid oxidase type 1 of standard mixtures of L-[1-^{13}C]-valine with natural valine, as described earlier[27]. Standards of L-[1-^{13}C]-KIVA and patient plasma samples were processed in the same series. KIVA was converted to its quinoxalinol-*O*-*t*-butyldimethylsilyl derivative. Isotopic enrichment of the derivatized samples was performed by gas chromatography coupled with mass spectrometry (GC-MS). The mass detector was a quadropole mass spectrometer (Finnigan Trace-MS Plus; Thermoquest-Interscience, Breda, Netherlands) used in electron impact mode. The gas chromatograph was fitted with a capillary column (J&W Scientific DB-1701, length 20 m, internal diameter 0.18 mm, film thickness 0.40 μm; Alttech, Netherlands). The mass spectrometer was operated in the selected ion-monitoring mode recording fragments at *m*/z 245 and 246 of unlabeled KIVA and L-[1-^{13}C]-KIVA, respectively. All

isotopic enrichments were calculated against standard calibration curves.

Evaluation of Primary Data

The whole body rate of appearance of valine (R_a) was calculated at isotopic steady state using the inverted pool model described by Matthews et al for leucine kinetics[28]. When this isotopic model is applied to L-[1-^{13}C]-valine, enrichment of plasma L-[1-^{13}C]-KIVA is assumed to provide an adequate estimate of intracellular enrichment of valine[26]. The R_a (µmol valine·kg^{-1}·h^{-1}) was calculated according to the following equation:

$$R_a = [MPEi(V)/MPE(KIVA) - 1] \times i(V)$$

where MPEi(V) is the isotopic enrichment of the valine in the infusate in mole percent excess, MPE(KIVA) is the isotopic enrichment of KIVA in plasma in mole percent excess, and i(V) is the infusion rate of L-[1-^{13}C]-valine (µmol valine·kg^{-1}·h^{-1}).

The rate of oxidation of valine was calculated following the approach used by Van Goudoever et al. and described by Veeneman et al[29;30]. We did not use indirect calorimetry in our study to determine CO_2 production as a measure of whole body bicarbonate production. In the approach of Van Goudoever et al., whole body bicarbonate flux is estimated before the L-[1-^{13}C]-valine infusion using a primed continuous infusion of NaH$^{13}CO_3$ of short duration[30].

The NaH$^{13}CO_3$ production (I_{bic}[V]) from L-[1-^{13}C]-valine during valine infusion (i_{bic}(V) was calculated according to:

$$I_{bic}(V) = [IECO_2(V)/IECO_2(B)] \times i(b)$$

Where $IECO_2$(B) is the isotopic enrichment in atom percent enrichment (APE) of $^{13}CO_2$ in expired air at isotopic steady state during the NaH$^{13}CO_3$ infusion, $IECO_2$(V) is the isotopic enrichment in APE of $^{13}CO_2$ in expired air at isotopic steady state during the L-[1-^{13}C]-valine infusion, and i(b) is the NaH$^{13}CO_3$ infusion rate in micromoles per kilogram per hour. Valine oxidation (Ox) was calculated according to:

$$Ox = I_{bic}(V) \times [100/MPE(KIVA)] \text{ (µmol valine·kg}^{-1}\text{·h}^{-1}\text{)}$$

In this way, the oxidation rate of L-[1-^{13}C]-valine could be calculated without measuring VCo_2.

In the preprandial period:

$$Ox = O \text{ (preprandial)}$$

where O (preprandial) is the oxidation rate in the preprandial period.

During the meal period, recovery of labeled CO_2 will be increased in comparison with preprandial. Estimates from the literature have been used, i.e. 0.74 ± 7 to 0.84 ± 8 prepandial and during and meal intake, respectively[31]. This represents an average increase of ~13%. Correction of the rate of oxidation of valine during the meal period is necessary because the two-point calibration was done while the patient was fasting.

O (prandial) was thus calculated according to:

O (prandial) = Ox/1.13

Calculation of Whole Body Protein Metabolism

In Figure 2 the isotopic model for whole body protein metabolism is shown in a schematic diagram. In this model, influx of valine comes from whole body protein breakdown (B) and, when appropriate, from dietary intake (I) as described by Veeneman et al[29](Veeneman 2003). Valine leaves the plasma amino acid pool by whole body protein synthesis (S) and oxidation (O). The input fluxes in this model result in label dilution of infused L-[1-^{13}C]-valine in plasma. These fluxes have to be differentiated from those that result in changes in size of the plasma amino acid pool. This is of particular importance for the calculation of the R_a of valine in plasma in the experiments in which the influence of protein intake has been studied. During protein intake, plasma amino acid concentrations increased gradually.

The R_a of dietary valine participating in whole body protein metabolism was calculated starting from the dietary intake of valine (I). This rate was multiplied by 0.8 to correct for first pass metabolism[32;33]. Subsequently, this rate was corrected for the enlargement of the plasma valine pool resulting in the flux of dietary valine participating in whole body protein metabolism (J_I). The amount of dietary valine entrapped in the enlarged pool size of valine (ΔQ) was calculated by multiplying the increase in valine concentration

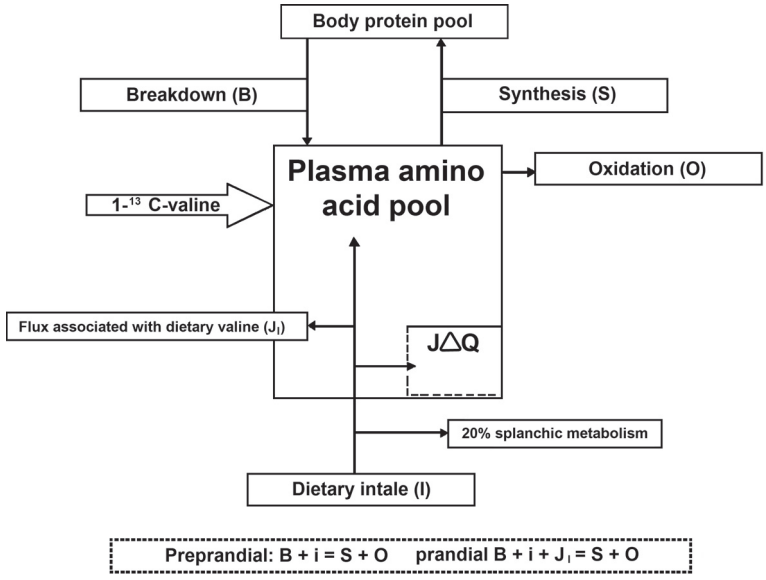

Figure 2. The isotopic model of whole-body protein metabolism during preprandial and prandial period

The fluxes considered in this model shown are whole-body protein breakdown (B), synthesis (S) and oxidation (O) after correction for splanchic metabolism (0.8 I) and the flux associated with the plasma amino acid pool enlargement (J(ΔQ), infusion of ^{13}C-valine (i), the flux (J_I) associated with dietary intake of valine (I).

in plasma by total body water, defined as 60% of body weight in these patients[34]. The difference of plasma valine concentration before and at the end of the meal period was used to calculate the increase in the whole body valine pool. In a previous study, a continuous increase of plasma valine concentration during dietary protein intake was observed during the study period[29]. This increase was assumed to be linear in time and the flux associated with the enlargement of the pool size of valine ($J\Delta Q$) to be constant. Accordingly, the total R_a of valine comprises the R_a of valine released from whole body protein breakdown, infusion of L-[1-^{13}C]-valine (i), and during the meal period the flux associated with dietary valine (J_l). At steady state, the R_a of valine equals the rate of disappearance (R_d) of valine. The total rate of disappearance of valine comprises whole body protein synthesis and oxidation. At steady state:

$R_a = B + J_l + i = S + O = R_d$ (μmol valine·kg^{-1}·h^{-1})

This results in the following calculations.
During fasting:
$B = R_a\ i$
and
$S = R_a - O$ (preprandial)
After meal:
$B = R_a\ i - J_l$
and:
$S = R_a - O$ (prandial)
Protein balance was calculated by subtracting protein breakdown from protein synthesis.

Statistics

All values are given as means ± SD. Statistical analysis was done using Excell 2000 Microsoft computer program. The changes in protein metabolism in the preprandial and prandial states were compared using a paired 2-tailed Student's *t*-test. Differences between the two groups (PKU patients and controls) in protein metabolism parameters were tested using the 2-tailed unpaired Student's *t*-test. Statistical significance was assumed at $P < 0.05$.

RESULTS

The demographic and clinical details of the studied PKU patients and the healthy controls are given in Table 1. Both groups were comparable in demographics and base line laboratory values (albumine, total protein, platelet counts, transaminases, urea, creatinine). Excretion of urea and creatinine in urine was also within the normal range.

Primary data on concentrations and isotopic enrichments

Preprandial and prandial concentrations of glucose, insulin and amino acids for both healthy adults and adult PKU patients are shown in Table 2. Analysis of glucose and insulin

Table 2.Amino acids, glucose and insulin concentrations at the start and end of the test day*

	Controls		PKU	
	At start	**At the end**	**At start**	**At the end**
Asp	16 ± 2	15 ± 2	15 ± 2	14 ± 3
Thr	120 ± 29	111 ± 16	132 ± 18	141 ± 36
Ser	112 ± 19	110 ± 15	95 ± 12	102 ± 25
Asn	47 ± 11	46 ± 6	38 ± 7	25 ± 6†§
Glu	54 ± 19	67 ± 23	55 ± 18	58 ± 20
Gln	432 ± 76	418 ± 53	422 ± 60	427 ± 44
Pro	163 ± 66	248 ± 57†	165 ± 62	258 ± 59†
Gly	205 ± 34	188 ± 20	229 ± 57	201 ± 41
Ala	254 ± 53	374 ± 47†	356 ± 99	418 ± 52
Citr	27 ± 7	22 ± 4	33 ± 8	18 ± 6†
Val	193 ± 52	233 ± 43†	222 ± 42	337 ± 42†§
Cys	3 ± 1	5 ± 2	7 ± 2‡	5 ± 2
Met	22 ± 6	20 ± 3	21 ± 7	24 ± 4
Ile	58 ± 15	52 ± 9	56 ± 15	80 ± 15†§
Leu	116 ± 32	106 ± 21	112 ± 26	152 ± 32†§
Tyr	53 ± 14	50 ± 11	38 ± 8	48 ± 15
Phe	49 ± 10	52 ± 7†	443 ± 100‡	409 ± 105‡
Orn	75 ± 23	55 ± 16†	70 ± 23	58 ± 10
Lys	157 ± 34	165 ± 22	168 ± 37	203 ± 16§
His	78 ± 11	83 ± 5	73 ± 5	85 ± 9
Arg	39 ± 5	38 ± 8	28 ± 17	49 ± 12†§
Ess AA‖	744 ± 61	770 ± 70	783 ± 69	1021 ± 99
Non ess AA¶	1425 ± 126	1585 ± 142	1512 ± 140	1631 ± 151
glucose	4 ± 1	4 ± 1	4 ± 1	5 ± 1†§
insuline	7 ± 2	76 ± 29†	9 ± 3	118 ± 49†

* All values mean ± SD, amino acid concentrations in µmol/L, glucose in mmol/L, insuline mE/L

† *P* <0,05 2-tailed paired Student's *t*-test between preprandial and prandial

‡ *P* <0,05 2-tailed unpaired Student's *t*-test between PKU and control at start (preprandial)

§ *P* <0,05 2-tailed unpaired Student's *t*-test between PKU and control at the end (prandial)

‖ Ess AA: total essential amino acids minus Phe: Thr, Val, Met, Ile, Leu, Lys, His

¶ NEs AA: total non essential amino acids minus Tyr: Asp, Ser, Asn, Glu, Gln, Pro, Gly, Ala, Citr, Cys, Arg, Orn

showed the expected insulin response in the meal period, which tended to be higher in the PKU group without reaching statistical significance. The response to the meals of the concentrations of amino acids varies depending on the amino acid considered. In the control group the rise in proline, alanine and valine concentration and the fall in ornithine concentration in the meal period were significant. In the PKU group the rise in proline, valine, isoleucine and leucine concentration and the fall in asparagine and citrulline concentration were significant in the meal period. Differences in plasma amino acid concentrations between the two groups in the preprandial period were statistically significant for phenylalanine and cystine. Plasma concentrations at the end of the meal period of valine, isoleucine, leucine, phenylalanine and lysine were

significantly higher in PKU patients than in controls.
In Figure 3 and Figure 4 the time courses are shown of $^{13}CO_2$ enrichment in expired air and ^{13}C KIVA enrichment respectively, in plasma for both groups. As is clear from Figure 3, isotopic steady state of $^{13}CO_2$ in expired air during the $NaH^{13}CO_3$ infusion was reached between 30 and 60 min after the start of the infusion. The majority of the individuals reached isotopic steady state for $^{13}CO_2$ and ^{13}C-KIVA between 180 and 240 min during the preprandial period and between 390 and 480 min during the prandial period. However some individuals showed a tendency to a small decrease at 180 minutes. The values of isotopic enrichment obtained during these time periods were used to calculate the steady-state valine fluxes reflecting whole body protein metabolism.

Parameters of whole body protein metabolism
In Table 3 the primary data are given of the valine fluxes in control subjects and PKU patients during both the preprandial and the prandial period. Rate of appearance of valine is not different between both groups, both before and after meals. The higher oxidation rate during the prandial period compared to the preprandial period reached significance only in the PKU group (P <0.01 for the PKU group and 0.07 for the controls). During the prandial period, the resulting R_a of dietary valine into the peripheral circulation was 42 ± 4 in the control group and 51 ± 8 µmol valine $\cdot kg^{-1} \cdot h^{-1}$ in the PKU group (P <0.02).

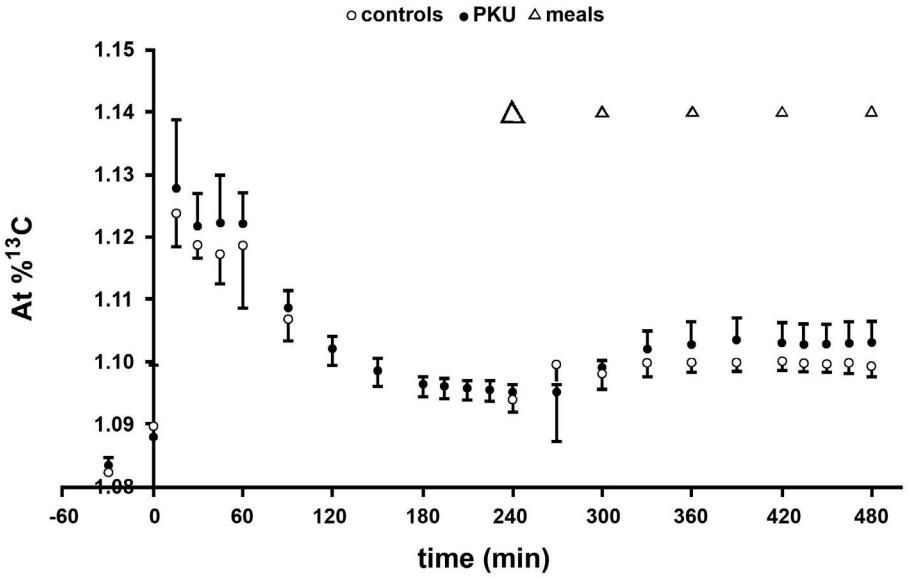

Figure 3. Breath 13CO2 enrichment for the control group and the PKU group

○ open circles for the control group
● closed circles for the PKU group
△: meal with 2/3 energy and protein
△ : meal with 1/12 energy and protein

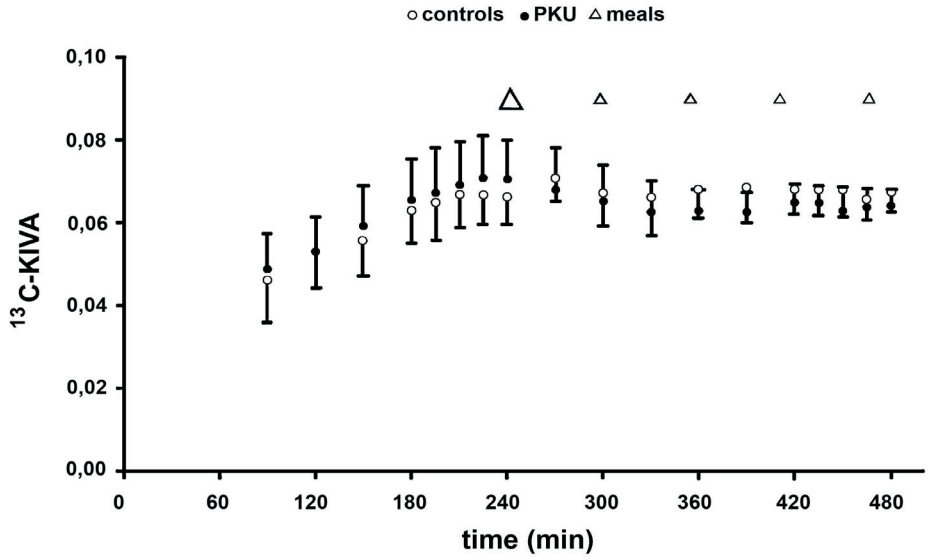

Figure 4. Plasma 13C-KIVA enrichment during the test
○ open circles for the control group
● closed circles for the PKU group
△: meal with 2/3 energy and protein
Δ: meal with 1/12 energy and protein

Table 3. Primary results of valine metabolism in control subjects and in PKU patients, obtained during the preprandial and prandial period*

	Controls		PKU	
	preprandial	prandial	preprandial	prandial
†I	-	56 ± 1	-	78 ± 5¶
‡J(ΔQ)	-	3 ± 4	-	12 ± 7
§R_a	104 ± 9	104 ± 8	102 ± 15	108 ± 7
‖Ox	28 ± 4	33 ± 7	24 ± 6¶	35 ± 2

*Values expressed as mean ± SD, micromoles valine per kilogram per hour
†I dietary intake of valine, ‡J(ΔQ): flux of valine associated with the enlargement of the plasma valine pool, §R_a: Rate of Appearance, ‖Ox: Oxidation, ¶$P < 0,01$ between controls and PKU

In Figure 5 whole body protein breakdown, synthesis and net protein balance during the preprandial and the prandial period are shown. As can be seen from this figure, the meal diminished whole body protein breakdown in both groups to a similar extent. Whole body protein synthesis was not influenced by the meal. Collectively, this changed the net protein balance from protein loss during the preprandial period (-21

± 4 *vs.* –17 ± 6 µmol valine · kg^{-1} · h^{-1} control *vs.* PKU, NS) into protein accretion during the prandial period. (16 ± 9 *vs.* 23 ± 8 µmol valine · kg^{-1} · h^{-1} control *vs.* PKU, NS). In both groups the effect of the meal on net protein balance was significant (*P* <0.01).

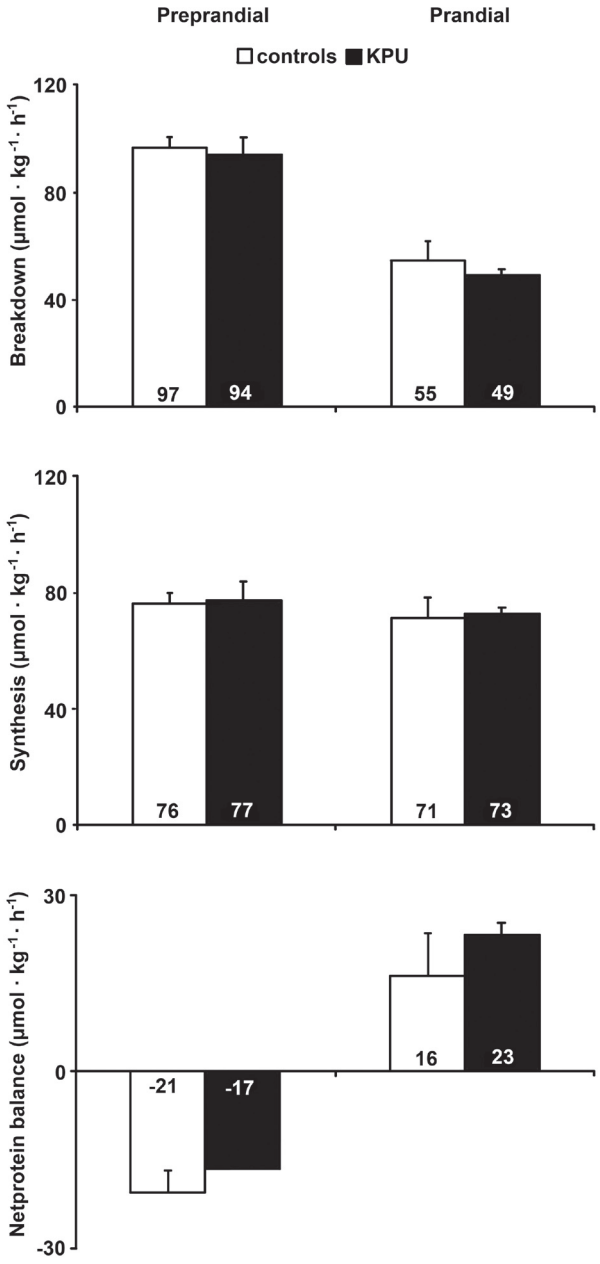

Figure 5. Protein Breakdown, Synthesis and Balance, for control subjects and PKU patients during both preprandial and prandial.

DISCUSSION

The most important findings of this study were that turnover, oxidation, and net balance of L-[1-^{13}C]-valine in adult PKU patients were comparable to the healthy adult individuals, while both groups received a protein intake at RDA level in the prandial period with frequent meals. Before discussing the results, some methodological issues should be addressed.

First, L-[1-^{13}C]-leucine has been applied in many studies to trace whole body protein metabolism, however the use of L- [1-^{13}C]-valine as tracer has advantages over [1-^{13}C]-leucine metabolically. It has been reported that leucine compared to [1-^{13}C]-valine shows a larger insulinomimetic effect on protein metabolism[35;36]. In our laboratory L- [1-^{13}C]-leucine has been used previously in the study of whole body protein metabolism[29;37-39]. At doses normally used in the study of whole body protein metabolism, valine and leucine give similar values of the fluxes of protein breakdown, synthesis, and oxidation[40].

Second, in general protein can be given as natural protein, as hydrolysate, and as free amino acids. In the studied healthy individuals the intake was given as natural protein conform the RDA advise (0.8 g protein $\cdot kg^{-1} \cdot d^{-1}$) and was composed out of 67% milk protein (high quality) and 33% vegetable protein (low quality). In the PKU patients, the protein intake was given almost exclusively as amino acids. Free amino acids harbor differences in biological value and in absorption rates when compared to natural protein[18;41-44]. In RDA an adjustment is proposed of approximately 20% to compensate both for losses due to digestibility and protein quality for mainly vegetarian diets. In line with this we used an incremental factor of 1.2 to compensate for using amino acids instead of natural protein.

Third, the differences in Phe tolerance (range 6-15 mg Phe $\cdot kg^{-1} \cdot d^{-1}$) in the studied PKU group resulted in small differences in the intake of Phe in the PKU group (range 4-10 mg Phe $\cdot kg^{-1} \cdot d^{-1}$) in the meal period (t 240- t 480). However in all patients only marginally lower concentrations of Phe were found in the meal period compared to the preprandial period. This is in line with previous studies and shows that we measured in a rather stable situation of Phe metabolism[45;46].

Further discussing the result we see that in response to the meals the concentrations of especially the branched chain amino acids in blood showed a larger increase in the PKU patients compared to the controls (Table 1). This effect is most likely due to the effect of the free amino acids consumed by the PKU patients and the higher concentrations of essential amino acids in the mixture compared to the natural protein taken consumed by the healthy controls (Table 3). Although a tendency of larger increases of insulin in the PKU group during the meals was observed, this tendency did not result in statistically significant differences of insulin, protein turnover and synthesis in the prandial period between healthy controls and PKU patients (Figure 5).

The enrichment of both $^{13}CO_2$ in expired air and ^{13}C KIVA in plasma, reached at the end of both studied periods enabled us to calculate the steady-state valine fluxes reflecting whole body protein metabolism. These calculations showed that the increase in oxidation rate during the meal period was the only parameter that reached statistical significance in the PKU group, where the differences between the

two groups preprandial and prandial were not significantly different (Table 3). The results of this study showed that turnover, oxidation, and net balance of L-[1-^{13}C]-valine in PKU patients were comparable to the control group of healthy individuals both during the preprandial and prandial period.

Very limited data are available on whole body protein metabolism in PKU patients. Thompson et al studied whole body protein metabolism in PKU patients at high plasma Phe concentrations under preprandial conditions using a primed continuous infusion of L-[1-^{13}C]-leucine, and showed that the protein turnover was comparable to healthy individuals[47]. Notwithstanding that we studied patients at lower plasma Phe concentration, the results of the preprandial period in our study were comparable with the results of Thompson et al[47]. Together with the findings of the present study during meals the studies strongly suggest that protein turnover in PKU patients is comparable with healthy controls under these study conditions. This conclusion, however, necessitates a restriction. The results are achieved under the strict conditions of continuous nutrition to achieve steady state as in the studies of Veeneman et al[29;38]. However, in PKU it has been shown that unequal distribution of amino acids and natural protein might be hypothesised to have specific influences on the use of phenylalanine and other amino acids[16;46;48-50]. Further studies under less ideal nutritional conditions are warranted to test the hypothesis for day to day care.

CONCLUSION

The results of the present study show that there is no difference in valine turnover, oxidation and net protein balance between healthy adults and PKU patients. Therefore, these data do not support the recommendation of a protein intake in PKU patients higher than RDA as suggested in the guidelines of the Medical Research Council and the Ross Metabolic Nutrition Support System and in studies in PKU children[51;52]. Knowing that protein metabolism is normal in PKU patients under the present ideal study conditions, further studies are necessary to investigate whole protein turnover under less ideal nutritional conditions as practiced in daily life.

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CHAPTER 2b

The intake of total protein, natural protein and protein substitute and growth of height and head circumference in Dutch infants with phenylketonuria.

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SUMMARY

In a previous study, Dutch children with phenylketonuria (PKU) were found to be slightly shorter than their healthy counterparts. In the literature, it has been hypothesised that a higher protein intake is necessary to optimize growth in PKU patients. The study aimed to investigate whether protein intake (total, natural and protein substitute) in this group might be an explanatory factor for the observed growth. Growth of height and head circumference and dietary data on protein intake (total, natural and protein substitute) from 174 Dutch PKU patients born between 1974 and 1996 were analysed retrospectively for the patients' first three years of life. Analyses were corrected for energy intake during the first year of life and for the clinical severity of the deficiency of phenylalanine hydroxylase by means of plasma phenylalanine concentration at birth.
Neither protein nor energy intake correlated with height growth. A positive, statistically significant relation between head circumference growth and natural protein and total protein intake was found, but not with the intake of the protein substitute or energy. Therefore, this study suggests that improvement of the protein substitute rather than an increase of total protein intake may be important to optimise head circumference growth in PKU patients.

INTRODUCTION

Phenylketonuria (PKU) is caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). Treatment of PKU aims at lowering plasma phenylalanine (Phe) concentrations by dietary Phe restriction [1]. Since Phe is part of natural protein, Phe restriction implies restriction of natural protein. Consequently, PKU patients consume individually tailored limited amounts of natural protein so that Phe requirements are just met. The requirement for the remaining essential amino acids and nitrogen is fulfilled in the form of a protein substitute containing all amino acids but Phe, and minerals, vitamins and other micronutrients, that constitute natural protein sources. This treatment has proven its effectiveness in preventing mental retardation, although defects in neuropsychological functioning remain[1;2].

Restriction of protein intake during infancy carries the risk of negative effects on growth of height and head circumference, as well as negative effects on neurodevelopment and immunity, growth being probably the latest characteristic affected [3]. In early reports of the collaborative study group of the USA on PKU treatment, restriction of height as well as head circumference occurred, but in later reports of that group this observation was not reported[4;5]. Verkerk and colleagues (1994) showed that Dutch patients treated for PKU have a lower mean height and head circumference at the time of diagnosis. In the first three years of life, further height growth restriction occurred while head circumference normalised in most children [6]. This finding prompted a search for factors that might be responsible for the restriction in growth in the Dutch PKU population. Studies that investigated causes for the retarded height growth in PKU patients focused on low blood Phe concentrations, a deficiency of Tyr, or a deficient intake of total protein. The first two suggestions could not be shown to cause growth restriction in length[5;7;8].

The recommended protein intake of PKU infants is the subject of ongoing debate[9-

[11]. Most studies investigating the effects of the dietary treatment on growth in PKU have focused predominantly on the restriction in height growth [5;8;12]. A smaller number of studies focused on the relation of head circumference and protein intake in PKU infants, but the results of these studies were not conclusive[13-15].

Theoretically, the conclusion that the total protein intake is not related to growth does not exclude the possibility of a relation with one of the two fractions of the protein PKU patients consume, i.e. the natural protein and the protein substitute [11;16]. The finding of Acosta and collegues, that growth is related to total protein may also be explained by a relation with either one of these components rather than the total intake. Specification of the possible relation between growth and protein intake would be of biological, social and economic interest. An increase in total protein intake will especially increase the intake of the chemically manufactured protein substitute. Protein substitutes have unpleasant taste and are expensive. A relation with natural protein rather than total protein would favour research to further optimize the biological composition of protein substitutes. To our knowledge, no study has paid special attention to the specific amount of protein obtained from the protein substitute or the amount of natural protein in relation to growth of either height or head circumference in PKU infants.

The aim of this study was to determine the relation between the intake of the total amount of protein, the amount of protein substitute, and the amount of natural protein on the one hand, and growth of height and head circumference on the other hand in a large group of early diagnosed and continuously treated Dutch PKU patients during the first three years of life. The hypothesis tested was that a lower intake of natural protein rather than a lower level of total protein intake causes a retarded growth of height and head circumference in PKU children during the first 3 years of life.

METHODS

The population studied consisted of 174 early (<21 days) diagnosed and continuously treated Caucasian Dutch PKU patients as reported in previous studies on growth in Dutch PKU patients [6;8].They were treated in the eight Dutch University child clinics. The study period ranged from 1 September 1974 to 31 December 1995. None of the 174 patients needed to be excluded because of major congenital anomalies such as severe heart, lung renal or liver disease.

We studied growth of height and head circumference in relation to the intake of protein and energy during the first three years of life. The period of 3 years was chosen because previous studies showed that this was the period in which the growth restriction was most evident [6]. For the analyses of growth of head circumference, there were 13 dropouts because of insufficient data, leaving 161 subjects to be analysed. Because of insufficient dietary data, there were 41, 39 and 57 dropouts in the data on total protein, protein substitute and the natural protein intake, respectively, leaving 133, 135 and 117 subjects to be analysed.

Height (cm) and head circumference (cm) were measured at time of diagnosis (in 74% before the age of 2 weeks), at the age of 6 months and yearly as close as

possible to the child's birthday. Height and head circumference were converted into standard deviation scores (SDS) by subtracting the expected mean measurement for that age and sex and dividing by its standard deviation. As reference population we used the results of the National Dutch growth study 1955-1997[17].

To assess the individual protein intake, prescribed amounts of protein in the diet were registered at the same ages as for the anthropometric measurements. Data of the prescribed intake of total protein, natural protein and the protein equivalent from the protein substitute (calculated from the amount of prescribed protein substitute and the protein equivalent of the specific protein substitute) were recorded. The relative contribution of natural protein in proportion to the protein equivalent of the protein substitute in the diet was calculated by dividing the natural protein intake by the intake of protein from the protein substitute. Mean individual intake of total and natural protein and protein substitute during the studied period was calculated by multiplying the daily intake (in g per kg per day) by the period (in days) during which the specific amount was prescribed and dividing the sum of these products by the age (in days) at which the final anthropometric measurements were taken.

$$\text{Mean intake} = \frac{[(\text{intake1} * \text{daysI}) + (\text{intake2} * \text{daysII}) + (\text{intake3} * \text{daysIII}) + (\text{intake4} * \text{daysIV})]}{\text{final age}}$$

where intake is daily intake and 1 = 1 month, 2 = 6 months, 3 = 12 months and 4 = 24 months of age and I = 1-6 months, II = 6-12 months, III = 12-24 months and IV = 24-36 months

Energy intake is an important factor in protein turnover and growth. Therefore, the individual mean energy intake was taken into account as a confounding factor. Mean energy intake was calculated in the same way as the mean of total protein, natural protein and protein substitute intake. After the first year of life energy intake is less precisely recorded and consequently prescribed energy intake cannot be assumed to match actual intake. Therefore, energy intake was only based on the prescriptions of the first year. Total energy intake during the first year was divided by weight at age 1 year. To investigate probable effects of the clinical severity of the PAH deficiency on growth, Phe concentration - as measured at diagnosis - was regarded as an indicator of clinical severity of the PAH deficiency.

Table 1 shows total protein intake, natural protein intake and protein intake from the protein substitute, the relation between natural protein and protein substitute at the different time intervals, and the means of the different protein intakes and of the energy intake (for year 1). The ratio of natural protein and protein substitute during the first 6 months of life is significantly different from the other time intervals. The protein substitutes mainly used were: "PKU-1®" Milupa, Friedrichsdorf, Germany; "Fenyldon®", Nutricia Nederland, Zoetermeer The Netherlands; "Phenyldon AM®" Nutricia Nederland, Zoetermeer The Netherlands; and "Phenystrict B®", Friesche Vlag, Leeuwarden, The Netherlands.

Table 1. Protein intakes (mean and SD) in the observation periods during the first 3 years of life and mean (SD) energy intake during the first year

Source of intake	Age (months)	Mean intake (g/kg per day)	SD (g/kg per day)
Total protein	0-6	2,70	0,50
	6-12	2,36	0,51
	12-24	2,25	0,56
	24-36	2,20	0,65
Mean	0-36	2,33	0,42
Natural protein	0-6	1,22	0,53
	6-12	1,03	0,41
	12-24	0,97	0,46
	24-36	0,93	0,51
Mean	0-36	0,99	0,34
Protein substitute	0-6	1,46	0,58
	6-12	1,28	0,38
	12-24	1,26	0,34
	24-36	1,22	0,42
Mean	0-36	1,29	0,28
Ratio natural protein:	0-6	1,11*	0,91
protein substitute	6-12	0,87	0.45
	12-24	0,84	0,53
	24-36	0,84	0,62
Mean energy intake in first year of life	0-12	27 KJ per kg per day	2,6 KJ per kg per day

* differs significantly from values at other time intervals.

Statistical analysis

Growth of height and head circumference was defined as the change in SDS of height and the change in SDS of head circumference over the period investigated. These slopes were obtained by applying least-square regression analysis to the data of each patient separately, and expressed in SDS/year. The regression coefficients of the individual patients were then used in a linear regression analyses to investigate the possible relation between total protein intake, protein substitute and natural protein intake on the one hand, and growth of height and head circumference on the other hand. As potential confounding factors we considered the clinical severity of PAH deficiency and the energy intake during the first year of life. Since we considered that only a lower intake and not higher intake of protein mightlead to growth restriction, we considered a one tailed *p*-value of < 0.05 as statistically significant.

RESULTS

Only 90 patients used the same protein substitute during the complete study period ("PKU-1®" N=13, "Fenyldon®" N=42, "Fenyldon AM®" N=30, "Phenystrict B®" N=5).

Figure 1 shows SDS of height and SDS of head circumference from diagnosis until 3 years. For height growth, the mean slope was –0.057 SDS/year. For head circumference

growth the mean slope was 0.29 SDS/year.

Table 2 shows the relationships between intake and growth. After adjustment for the confounding factors, (i.e. energy intake during first year of life, severity of the deficiency of PAH) height growth was not significantly related to the total protein intake, natural protein intake, or protein substitute. After adjustment, head circumference growth was significantly related to total protein intake (p = 0.04) and natural protein intake (p = 0.035), but not to the amount of protein substitute. There was no significant relation between head circumference and height growth and diagnostic Phe concentration.

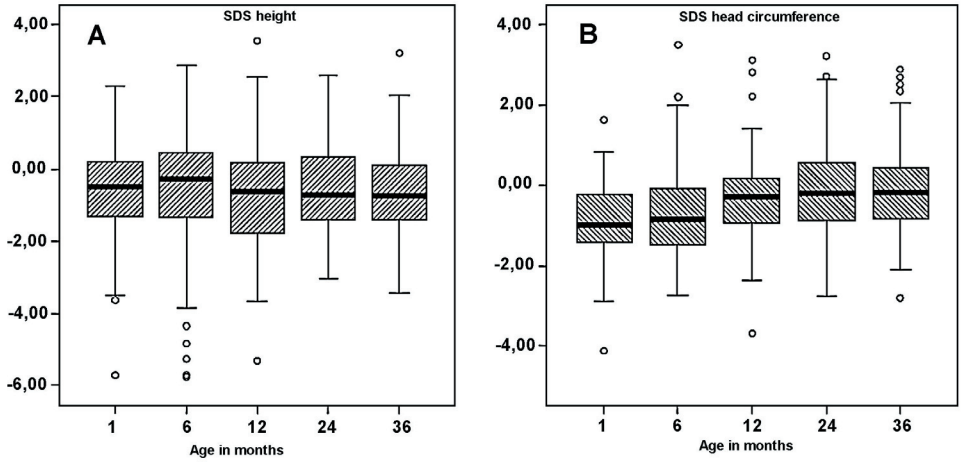

Figure 1 Boxplots of SDS of (A) height and (B) head circumference from 1 to 36 months.

Table 2. Regression coefficients and 90% confidence interval (CI) of regression analyses of protein intake and growth.

Independent variable	Dependent variable	Unadjusted Regression coefficient	90% CI	Adjusted[a] Regression coefficient	90% CI
Height growth	Total protein	0.15*	0.00 to 0.30	0.09	-0.07 to 0.25
	Protein substitute	0.05	-0.20 to 0.30	0.13	-0.12 to 0.38
	Natural protein	0.04	-0.16 to 0.24	-0.02	-0.23 to 0.19
Head circumference growth	Total protein	0.30*	0.10 to 0.50	0.22*	0.02 to 0.42
	Protein substitute	-0.07	-0.35 to 0.21	0.07	-0.21 to 0.35
	Natural protein	0.32*	0.09 to 0.55	0.28*	0.03 to 0.53

[a]Adjusted for Phe and kcal/ weight
*significant (one sided p-value <0.05)

DISCUSSION

The present study addressed the question whether there is a relation between protein intake and growth in PKU patients. The most important findings were that the total protein intake and natural protein intake showed a significant relation with head circumference growth, but not with height growth during the first three years of age. In contrast, growth of height and head circumference did not correlate with the intake of energy during the first year, suggesting that nutritional demands were adequately met during this period. The third, and in our opinion most interesting finding was that head circumference growth was affected by the intake of natural protein rather than total protein, and not by the intake of protein from the protein substitute.

Two methodological issues need to be addressed before the results are discussed. First, when discussing the dietary data of the present study, it should be taken into account that the study is based on diet history data of the prescribed diet. This study, therefore, lacks information about the precise amount of protein consumed, which is not necessarily the same as the amount prescribed in the diet. However, even in the prospective study of Acosta et al, the prescribed intake was not the consumed intake[15]. Both our study and those of Acosta assume that there is a strong correlation between the prescribed and the consumed intake, especially during the first years of life. This seems a realistic assumption for various reasons. The frequency of dietary counselling is rather high during this period. During these consultations the various amounts of intake (natural protein, protein substitute) and energy intake are checked and the difficulties encountered by the parents are discussed. Especially during the first years of life, parents can fairly easily control the nutritional intake of their child. During the first 3 years of age, parents control more or less easily nutritional intake of their child.

Secondly, the severity of the PAH deficiency was controlled for by the use of plasma Phe concentration at diagnosis. It should be taken into account that there is no gold standard for the severity of the PAH deficiency.

The findings of this study are in line with observations in non-PKU low-birth-weight infants, in which head circumference rather than height growth was positively affected by natural protein intake[18]. In non-PKU term infants, no clear relations were found[19]. It is not clear why head circumference growth rather than height growth is affected by natural protein intake in PKU patients.

The clinical importance of a small degree of height growth restriction can be questioned. However, head circumference could, especially during the first years of life, be regarded as an indicator of brain growth[20]. Moreover, a correlation was found between microcephaly in infants and mental retardation in later life[21-23]. Therefore, knowing that mental development in PKU patients still is not equal to that in non-PKU individuals, head circumference growth in PKU infants should be monitored closely and needs to be optimised in order to try to achieve maximum patient IQ in later life.

We hypothesised that in PKU patients growth rate is related to the intake of natural protein rather than the total protein intake. Although there is a significant relation

between head circumference growth and total protein intake, of the two components that compose total protein only the relation with natural protein intake was significant and not the relation with protein substitute.

It could be hypothesised that the clinical severity of PAH deficiency affects both head circumference growth and the intake of natural protein (by its aims of treatment), and that, therefore, a relation was found between head circumference and (natural) protein intake. The clinical severity of PAH deficiency seemed to reduce the strength of the relations found between head circumference and the protein intake to a small degree. At the same time, however, no significant relation was found between the clinical severity of the PAH deficiency and head circumference ($p = 0,20$), suggesting that the retarded head circumference growth did not stem from the clinical severity of the PAH deficiency. Since natural protein only accounts for only a minor part of the total protein, the present data strongly suggest that an increase in total protein does not affect growth in PKU children as it increases the intake of natural protein only to a small degree.

The lack of a relation with the protein substitutes can be due to various reasons. For example, the composition of the protein substitutes varies between the companies that manufactured the substitutes during these years. This study comprised a large time window in which the composition of the protein substitutes changed more than once in terms of both the amino acids and the other substances that constitute the protein substitute. For example, some substitutes contained carnitine, others did not; some manufacturers included long chain poly unsaturated fatty acids, others introduced these at a later date. The numbers of patients using a specific protein substitute during the entire study period where too small to allow a detailed statistical analysis on differences in growth parameters of patients using different substitutes. The relationship between natural protein and protein from substitute differs between intervals studied. Especially during the first six months of life, natural protein intake is higher, whereas in the years thereafter the relationship between natural protein and protein from the protein substitute no longer changes significantly. However, this does not seem to explain the significant relation found between natural protein intake and growth over the first 3 years of life, as during the first 6 months of life neither natural protein intake nor the protein substitutes correlated significantly with growth.

The data of this study suggest that the composition of the protein substitutes used may less optimal in terms of the composition of natural protein. One reason may be that the metabolic use is less effective for the amino acids supplied by the protein substitute than for the protein derived from milk or egg[9;24]. Especially when the daily intake of protein substitute is not adequately distributed over the day, plasma amino acid concentrations rise more rapidly, and amino acids are used for oxidation rather than protein synthesis[25;26].

Factors other than amino acids, however, may be important as well. Recent studies showed that deficiencies of long chain polyunsaturated fatty acids may also cause a lower growth rate of head circumference. For example, the ratio of arachidonic acid and docosahexaenoic acid seems to be important for the increase in head circumference in young infants [27]. Other studies pointed to a clear deficit of the long

chain polyunsaturated fatty acids in the protein substitute given to PKU children in the past, offering a good alternative explanation[10;28].

Apart from this, with the knowledge that excessive protein intake in early life is related to a number of negative effects in later life (obesity, renal dysfunction and even reduced intellectual outcome), caution against too high a protein intake in (PKU) children is warranted[3].

In conclusion, our study showed that height growth is not clearly related to protein intake and that the amount of natural protein intake, rather than the total protein intake seems an important factor in head circumference growth. Therefore, in investigating the association between growth and protein intake in PKU patients, not only the amount of total protein, but also the amount of natural protein and protein substitute should be taken into account. We did not find a relation between growth and the intake of protein substitute. The relation with natural protein intake suggests that further improvement of the protein substitute, to make it more comparable to natural protein, rather than an increase in intake of total protein is important to achieve optimal (head circumference) growth in PKU children.

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CHAPTER 3a

A different approach to breast-feeding of the infant with Phenylketonuria

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ABSTRACT:

We studied the possibility and safety of a new approach to breast-feeding infants with Phenylketonuria (PKU). We compared a group of PKU infants being breast-fed according to our new protocol to a group of PKU infants receiving formula only. The breast-fed group consisted of 9 infants born between 1994 and 1999 being breast-fed at the moment of diagnosis. The formula fed group consisted of 9 PKU infants, born between 1988 and 1997. In the breast-fed group feedings alternated between breast-feeding and Phenylalanine (Phe) free bottle-feeding. The numbers of breast-feedings were adapted to the plasma Phe concentrations. At each feeding, bottle- or breast-feeding, the child was allowed to drink till satiety. Data about metabolic control and growth during the first 6 months showed no statistically different results. The mean Phe concentration in the breast fed group was 170 μmol /l (range 137-243) and in the formula fed group 181 μmol/l (range 114-257). Compared to a routine where both bottle and breast are offered at each feeding, this new approach is more convenient for the parents and the child will be able to empty the breast, therefore drinking not only foremilk but also hindmilk. *Conclusion:* The results suggest that this feeding protocol is safe in the strict treatment of otherwise healthy infants with phenylketonuria.

INTRODUCTION

Breast-feeding infants with Phenylketonuria (PKU) has long been uncommon in the Netherlands. Recent data showed that of 97 PKU infants being breast-fed at the moment of diagnosis, only 4 continued to be breast-fed after diagnosis (Crone M.R personal communication). Parents of infants with PKU were advised to switch to bottle-feeding after diagnosis. However promotion of breast-feeding is in accordance to the WHO/UNICEF recommendation[15]. General advantages such as the content of long chain polyunsaturated fatty acids[18], immunoglobulins, better absorbtion of iron[9], non protein nitrogen combinations such as lactoferrin, polyamines and nucleotides [3], as well as advantages in emotional attachment and satisfaction are also applicable to the PKU infant.

Mothers, who have to stop breast-feeding abruptly at the time of PKU diagnosis, may experience extra feelings of guilt, making acceptance even more difficult[9]. Another advantage of breast-feeding in PKU is the low amount of Phenylalanine (Phe) in human milk in comparison to standard infant formulas, which makes it possible to give more human milk than standard formula.

Until now, two different guidelines have been described for breast-feeding PKU infants. One is based on the exact measurement of the intake of breast-milk by measuring expressed breast-milk or performing weight checks of the children before and after feeding[1]. The other guideline advises to start each feed with a measured amount of Phe free formula followed by breastfeeding till satiety[4,6,10,11]. Both guidelines have emotional and practical disadvantages in stimulating breast-milk production.

The aim of this study was to investigate the possibility of breast-feeding PKU infants with a set number of breast-feedings per day in a fixed schedule alternated with Phe-free formula. The amount of breast-milk was not controlled by weight checks. We retrospectively compared a group of PKU infants entirely formula-fed with a group of

PKU infants having been breast-fed according to our protocol.

SUBJECTS AND METHODS

Subjects:

All patients were diagnosed with PKU by neonatal screening and treated in the University Hospital of Groningen. From 1994 till 1999 the continuation of breast-feeding was offered to nine new-born babies breast-fed until diagnosis. The fully formula-fed group was born between 1988 and 1997. This group included infants born before breast-feeding was recommended and infants who were bottle-fed at the time of diagnosis. The patient characteristics of both groups are presented in table 1 and are not significantly different with one exception. Median age at diagnosis in the control group was significantly higher without a difference in Phe concentration at time of diagnosis. The difference in median age at time of diagnosis was due in part to the fact that the fully bottle-fed patients were diagnosed between 1988 and 1994. In this

Table 1. Baseline patient characteristics. *

	Breast fed (n=9)	Formula fed (n=9)	Significance†
Age at diagnosis (days)	7(6/8)	12(9/16)	<0.01**
Phe at diagnosis ‡ (µmol/l)	1600(390/2200)	780(200/4150)	0.44
Length (z-score) §	-0.38(-0.45/0.24)	0.07(-1.26/1.79)	0.09
Weight (z-score) §	-0.52(-1.37/0.65)	-.33(-1.79/3.11)	1.00
Headcircumference (z-score) §	-0.20(-0.99/0.96)	0.57(-1.36/2.70)	0.34
Sexe	2♂:7♀	5♂:4♀	-
Ethnicity			
Dutch	5	8	-
Turkish	3	1	-
Georgian	1	0	-
Duration of breastfeeding (weeks)	10(7-33)	-	-
Maternal Phe ‡	114(96-172)	143(91-153)	0.6

* numbers represent median values, ranges min/max are given in parentheses

† p-value resulting from Mann-Whitney test for difference in medians

‡ Phe = plasma phenylalanine concentration in µmol/l

§ 0-1 month of age

period the heel puncture was performed between days 7 and 9 after birth. In 1994 this has changed to days 5 and 7, and referral to our centre occurred a few days earlier[16]. In the bottle-fed group we also found two patients with a dubious first screening test and a delayed diagnosis. In the other group we found only one such patient.

We measured the Phe concentrations of the mothers in both groups.

Feeding method:

The feeding schedule for the breast-fed group was based on alternating breast-feeding and Phe free bottle- feeding, resulting in individual schedules for each child depending on tolerance and age. The first few days after diagnosis the mother usually breast-fed the infant once daily, the numbers of breast-feedings increased over the following days monitoring the plasma Phe concentrations daily. The mother expressed breast-milk a few times a day as long as the infant was allowed to drink breast-feeding only once or twice during the first few days, to stimulate milk production. Bottle- and breast-feeding were divided equally throughout the day and advice was given to keep the same sequence. At all feedings, either bottle or breast, the child was allowed to drink until satiated.

The completely bottle-fed group was fed as usual with a Phe free formula mixed together with a standard formula. The amount of both formulas was adjusted to obtain plasma Phe concentrations within the therapeutic range.

Metabolic control and growth:

The frequency of measuring plasma Phe concentrations was gradually reduced from daily in the first week after diagnosis, to twice a week, weekly and biweekly when levels were stable within the therapeutically aimed range. We studied the length of time needed for the plasma Phe concentrations to reach the therapeutically aimed range (120 to 360 μmol/l) after diagnosis. The Phe concentrations of the first 6 months were plotted in order to track the Phe concentrations were within the aimed range (<360 and >120 μmol/l), above the aimed range (>360 μmol/l), or below the aimed range (<120μmol/l). Phe concentrations were determined in plasma using an amino acid analyser with a coefficient of variation of 2%.

Growth (weight, length and head circumference) was followed from the time of diagnosis until 6 months of age. The results were expressed as individual z-scores according to the latest Dutch Growth diagrams[14]. Statistical analysis was performed using the non- parametric Mann-Whitney-test.

RESULTS

Table 2 presents the data concerning the metabolic control showing that the time to stabilise Phe values and further metabolic control was not different between the two groups.

Table 3 shows that z-scores for growth were satisfactory and statistically not different in both groups. The data of the headcircumference of both groups are further presented in Figure 1. At the start the headcircumference in the bottle-fed group was

Table 2. Metabolic control in breast fed and formula fed PKU patients during the first 6 months of life. *

	Breast fed (n=9)	Formula fed (n=9)	Significance†
Mean Phe ‡ (µmol/l)	170(137-243)	181(114-257)	0.86
Variation coefficient of mean Phe (%)	69(34-139)	66(31-119)	0.93
% Phe <120 µmol/l §	31(5-65)	26(2-65)	0.61
% Phe 120-360 µmol/l ‖	56(20-90)	59(22-98)	0.67
% Phe > 360 µmol/l ¶	6(1-25)	4(0-37)	0.49
Days until Phe 120-360 µmol/l	6(2-15)	6(0-26)	0.80

* numbers represent median values, ranges min/max are given in parentheses
† resulting from Mann-Whitney test for difference in medians
‡ Phe = plasma phenylalanine concentration
§ percentage of time in which plasma phenylalanine concentration was below the specified range
‖ percentage of time in which plasma phenylalanine concentrations were within the specified range
¶ percentage of time in which plasma phenylalanine concentrations were above the specified range

Table 3. Growth in breast-fed and formula-fed PKU patients during the first 6 months of life. *

	Breast fed (n=9)	Formula fed (n=9)	Significance †
Length			
0-1 month	-0.38(-1.45/0.24)	0.07(-1.26/1.79)	0.09
3-4 months	0.48(-0.66/-1.48)	0.17(-0.66/1.62)	0.80
6-7 months	0.33(-0.98/2.42)	0.68(-0.11/2.43)	0.34
Weight			
0-1 month	-0.52(-1.37/0.65)	-0.33(-1.79/3.11)	1.00
3-4 months	0.35(-1.72/1.34)	0.57(-0.56/1.51)	0.34
6-7 months	0.30(-1.46/2.16)	0.51(-0.12/2.44)	0.34
Headcircumference			
0-1 month	-0.20(-0.99/0.96)	0.57(-1.36/2.70)	0.34
3-4 months	-0.01(-1.32/0.57)	0.78(-1.66/2.03)	0.09
6-7 months	-0.15(-1.54/0.36)	0.87(-1.70/2.19)	0.11

* numbers represent median Z-scores, ranges min/max are given in parentheses
† resulting from Mann-Whitney test for difference in medians

larger than in the breast-feeding group. The difference remained but never reached statistical difference. The results of the Phe concentrations of the mothers show that none of the mothers had hyperphenylalaninaemia.

All nine mothers who breast-fed their child at the moment of diagnosis wanted to continue breast-feeding and were very positive afterwards. In three patients it was necessary to implement one "mixed" feeding (combination of Phe- free bottle-feeding and breast-feeding), when the switch to one total breast- or bottle-feeding resulted in too high or too low plasma Phe concentrations. At 4 weeks of age the number of breast-feedings in all the children was 50 % or more of their total amount of feedings. We did not see any problems of nipple- bottle confusion in the babies.

DISCUSSION

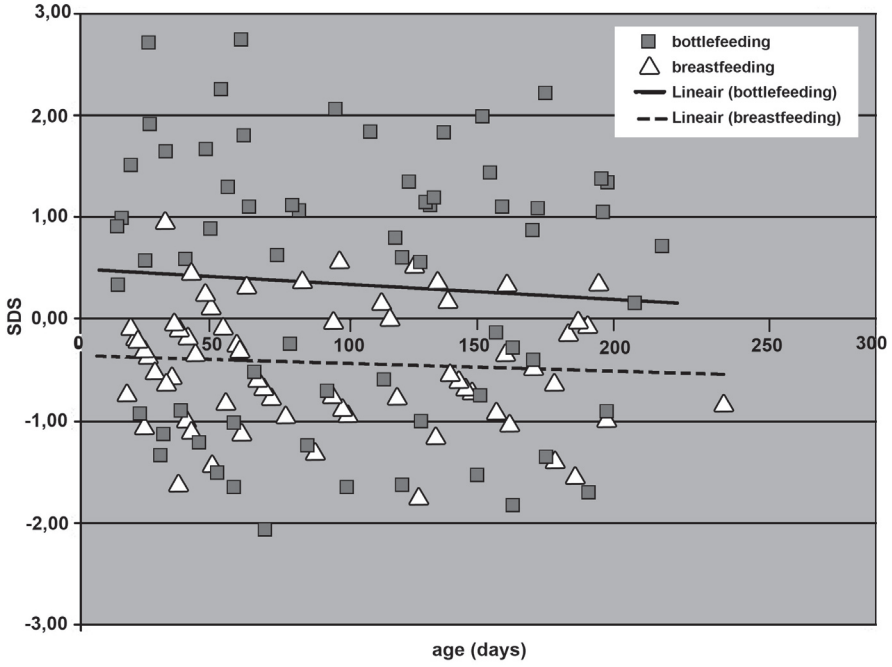

Figure 1. Headcircumference of both groups.

For a long time we have assumed that it is not safe to breast-feed the newly diagnosed PKU infant without monitoring the amount of human milk by weighing the expressed milk or by weighing the baby before and after drinking. Giving a certain amount of Phe free bottle-feed followed by "breast-feeding ad lib" in each feeding was an improvement[4,6,10,11]. This way the satiety controls the amount of breast-feeding.

In our study we investigated further the possibility and safety of alternating Phe free bottle-feedings and breast-feedings. In 1986 MacCabe described this feeding method

in order to improve iron-absorption[9]. In our feeding protocol the breast-feeding was fixed in the total number of breast-feeds per day and in the alternating sequence throughout the day. The child was allowed to drink till satiety both by bottle- and breast-feeding. The purpose of the study was to determine whether good metabolic control could be achieved and maintained in this feeding-schedule. Although the number of patients is relatively small, data showed that applying our breast-feeding protocol to otherwise healthy PKU patients, the Phe concentrations could be kept within the therapeutic range.

The breast-fed group showed a growth comparable to the bottle-fed group and better when compared to the average Dutch PKU population[16]. The differences in head circumference between both groups could not be explained by differences in the Phe concentration of the mothers.

The difference in age at diagnosis might be expected to induce higher Phe concentrations in the bottle-fed group. In contrast there was some tendency of higher plasma Phe concentrations in the breast-fed group indicating that the breast-fed group certainly did not have a less severe phenylalanine hydroxylase deficiency.

Median duration of breast-feeding was 10 weeks (range 7 to 33 weeks), which is comparable to the normal Dutch population[2]. The reasons to stop breast-feeding, such as returning to work, stress in family circumstances and lack of milk- production did not seem different from the average in the Dutch population.

Our data further suggests that continuing breast-feeding may have a positive influence on the emotional acceptance by the parents. The emotional advantages were already mentioned by McCabe[9]. Especially the fact that the mother did not have to stop her breast-feeding abruptly is a positive factor in acceptance of the PKU diagnosis.

This study did not investigate the 24- hour fluctuation in plasma Phe concentration. At present, little is known about this daily variation of Phe concentrations in PKU infants younger than one year of age. Both the studies of Macdonald et al and van Spronsen et al concern PKU patients older than one year[7,8,12,13]. The study of Gerdes et al supplies us only with two Phe concentrations during a short time span of the day[5]. It might be hypothesised that supplying such young PKU infants with a rather unbalanced daily distribution of the natural protein and the Phe free formula may result in large daily fluctuations of plasma Phe concentrations. The high feedings-frequency in young infants however will reduce this effect. Future studies should also address the diurnal Phe variations both in case of breast-feeding and formula feeding.

Results of this study suggest that breast-feeding in PKU using our protocol is technically possible. With respect to the growth and metabolic control the protocol appears safe. With respect to the metabolic control in PKU infants, future studies are necessary on the use of breast-milk and formula. We hope that our more convenient breast-feeding method may result into a higher percentage of breast-fed PKU infants, and thus will contribute to the emotional acceptance by the parents and an optimal development of the PKU infant.

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CHAPTER 3b

PKU infants too often too low Phenylalanine concentrations? Diurnal variations in blood phenylalanine under different feeding regimes.

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Submitted

ABSTRACT

Summary: In Phenylketonuria (PKU) patients diurnal patterns of blood phenylalanine (Phe) are different from healthy individuals. Until now this pattern has been studied in PKU patients over one year of age. The aim of this study was to investigate diurnal patterns in PKU infants under one year of age receiving both the natural protein and Phe-free formula at the same time or in an alternating feeding scheme.

Method: In 7 PKU infants (aged 3-8 months), diagnosed by neonatal screening, diurnal variations in blood Phe concentrations were recorded twice: on day A they received natural protein and Phe-free formula combined in each feeding; on day B they received the natural protein and Phe-free formula in an alternating feeding scheme. Number of feedings, total protein, and energy intake were similar on both study days. Blood samples were taken before each feeding.

Results: The medians (±SD) of the difference between the individual minimum and maximum blood Phe concentrations were 81(±50) μmol/L and 104(±26) μmol/L on day A and B, respectively (n.s.). Fifty and 30% of the samples were below target range for age (120 μmol/L), while only 3% and 6% were above target range (360 μmol/L) on day A and B respectively (n.s.).

Conclusion: Both feeding regimes, i.e. the natural protein and Phe-free formula combined in each feeding or alternating, resulted in comparable diurnal fluctuations of blood Phe concentrations in PKU infants. Blood Phe concentrations beyond range within 24 hours were more related to the early morning blood Phe concentration than to large fluctuations.

Take home message: In infants, low blood Phe concentration in the morning after an overnight fast is a good predictor of the chance that blood Phe concentrations will be beyond the target range during the day, irrespective the Phe distribution over the day.

INTRODUCTION

Patients with phenylketonuria (PKU, McKusick 261600) can not convert phenylalanine (Phe) into tyrosine (Tyr) due to a deficiency of phenylalanine hydroxylase (EC1.14.16.1) activity in the liver. Left untreated, PKU leads to high Phe concentrations in blood and tissues and to low to normal Tyr concentrations, clinically resulting in severe mental retardation, epilepsy and behavioural problems[1;2]. Treatment consists of restriction of the essential amino acid Phe by reducing the natural protein intake with concomitant supplementation of all amino acids but Phe. Patients, treated this way, have a more or less normal outcome although some minor neuropsychological dysfunction remains[2-4].

Studies of the diurnal variation of blood Phe concentrations have shown that diurnal variations may depend on variable factors and may be associated with age[1;5-9]. All these studies were performed in patients above one year of age. In PKU infants under one year of age no data have been published. In PKU infants under one year of age, it can be assumed that the variation of blood Phe concentrations is greater and occurs more often, probably due to changes in growth velocity, intercurrent illness, and changes in intake. Especially for young children, the target range of 120-360

μmol/L for the blood Phe concentration is quite small, resulting in a relatively high risk for blood Phe concentrations of being outside the target range.

Infants with PKU consume a more or less fixed amount of Phe (natural protein) and Phe free formula. Phe intake in infants is adjusted based on results of measurements of blood Phe concentrations. When natural protein is given as breast feeding, natural protein and Phe-free formula can be given in the same feeding or in an alternating feeding scheme[10-12].These different feeding regimes might result in different diurnal variations of blood Phe concentration.

The aim of the present study was to study 1) the diurnal variations in blood Phe concentrations in PKU infants and 2) the effect on this diurnal fluctuations of two different feeding regimes i.e. natural protein and Phe free formula given either combined in each feeding or in an alternating feeding scheme.

METHODS AND MATERIAL

Patients:

The study was carried out in seven PKU infants aged three to eight months detected by neonatal screening and treated from time of diagnosis in the Beatrix Children's Hospital of the University Medical Centre of Groningen. Patients had blood Phe concentrations of > 400 μmol/L at time of diagnosis. Patients were free from comorbitity and feeding problems.

After diagnosis parents of PKU infants were instructed how to sample blood on filter paper from heel punctures at home and to send the bloodspots to the laboratory for measurement of blood Phe concentration. Based on the Phe concentration, intake of Phe was adjusted. Frequency of sampling was adapted to age and blood Phe concentrations and varied from daily in the first week after diagnosis, to once a week when the Phe concentration had stabilized, usually in 1-2 months. To be informed about the frequency of blood Phe concentrations out of target range due to the diurnal variation, parents performed one or two blood Phe day profiles when the infant had a stable feeding pattern, mostly when infants were between three and eight months of age.

Design of the study

All patients that will be presented were studied twice. On day A they received the natural protein (breast milk, normal formula or fruit and vegetables) and the Phe-free formula combined in all feedings. Infants receiving breast-milk got Phe-free formula just before being breastfed; the infants receiving normal formula got a mixture of normal formula and Phe-free formula. On day B they received the natural protein and Phe-free formula at different feeding times, i.e. Phe-free formula and natural protein were given in an alternating scheme. Feeding frequency depended on the feeding habits of the infant at the time of the studied day. At 3-7 months of age the prescribed diet contained 2.0 g protein $\cdot kg^{-1} \cdot day^{-1}$ (as natural and Phe-free formula combined) and 100 kcal $\cdot kg^{-1} \cdot day^{-1}$ on both study days. When tested at > 7 month of age energy intake was reduced to 90 kcal $\cdot kg^{-1} \cdot day^{-1}$. Phe content of each

feeding and each day was calculated in mg Phe per kg bodyweight using the ZIS -food calculation computer program based on NEVO with supplementary data of manufacturers (ref NEVO and Milupa manual[13]. Bloodspots on filter paper (type 2992, Scheicher and Schuell 's Hertogenbosch, the Netherlands) were taken before each feeding. Samples were sent by mail to the laboratory for further analysis. Approval of the Medical Ethical Committee was not required as the study used retrospective data of blood Phe concentrations regularly collected for adaptations in dietary treatment.

Analyses of blood Phe concentrations

Blood Phe concentrations were determined in eluates of ⅛ ' discs punched from dried blood spots. Discs were eluted at room temperature for 30 minutes in 150 µl TCA solution (6.6 % $^{w}/_{v}$ TCA in H_2O) containing norvaline as internal standard, after which the debris was precipitated by centrifugation for 5 minutes at 14,000 rpm. Ten µl of the supernatant was transferred to a clean reaction vial and prepared for HPLC analysis by the AccQ-Tag® method according to the manufacturer's protocol (Waters, Breda, the Netherlands).

Statistics

Mean, SD and the difference between the minimum and maximum of the measured blood Phe concentrations on days A and B of all individuals were calculated. Differences in these parameters between both days A and B were tested for significance using the paired Student *t* test. Differences in the number of blood Phe concentration out of the target range of 120 – 360 µmol/L on days A and B were also tested for significance using the paired Student *t* test. The relationship between the severity of the disease defined as the blood Phe concentration at the time of diagnosis or the Phe tolerance, and the resulting mean and ranges of blood Phe concentrations on days A and B, was tested for significance with univariate regression analysis (ANOVA). This test was also applied to test for significant relationship between the first sample of the day and the number of blood Phe concentration beyond the target range. Statistical analysis was done using SPSS version 12 (Chicago, IL, USA). Statistical significance was assumed at $P < 0.05$.

RESULTS

Data of all patients but one in whom PKU was diagnosed in our centre between 2004 and 2006, could be compared. Table 1 shows the clinical data of all seven patients, with blood Phe concentration at the time of diagnosis varying from 847 µmol/L to 2329 µmol/L at day 6-9. Patient 1was diagnosed with a blood Phe concentration of 433 µmol/L, 24 hours after birth as a sib of a PKU patient. Six patients were born after uncomplicated pregnancies of 37- 42 weeks; patient 6 was born at 36 $^{+5}$ weeks. Growth parameters of all patients for height, weight and head circumference are given in Z scores at time of day A.

In table 2 Phe intake per day and for each feeding, and the difference between

Table 1 Patient characteristics

				Growth parameters* at time of study		
	sex	**age at diagnosis (days)**	**Phe at diagnosis µmol/L**	**weight**	**length**	**head-circumference**
patient 1	f	1	433	1.23	0.48	0.8
patient 2	m	6	2329	1.30	1.03	0.84
patient 3	f	8	983	0.35	-0.71	-0.39
patient 4	m	9	1698	-2.04	-2.26	-2.08
patient 5	f	7	910	-1.41	-1.44	-0.81
patient 6	f	6	847	0.33	1.17	-0.15
patient 7	m	7	1774	-0.81	-0.12	-0.91

*all values are expressed as Z-score, based on the growth curves given the growth analyzer version 3.5 (Dutch Growth Foundation)

minimum and maximum blood Phe concentrations observed on both days A and B are shown together with age at time of testing. Age varied from 12 to 33 weeks at study time, while the time period between days A and B varied from one to five weeks. Individual Phe tolerance varied between 22 and 44 mg Phe $\cdot kg^{-1} \cdot day^{-1}$ on day A and between 16 and 42 mg Phe $\cdot kg^{-1} \cdot day^{-1}$ on day B.

Figure 1 shows the individual blood Phe concentrations at feeding times on day A and B. On day A the median of the difference between minimum and maximum blood Phe concentration was 79 µmol/L and on day B 104 µmol/L. Mean blood Phe concentrations and differences between minimum and maximum were not significant different between both days A and B ($p = 0.140$ and $p = 0.184$ respectively).

Figure 1 also shows that on day A, 16 samples were below target and one sample was above the target range. On day B, 10 and 2 samples were below and above the target range respectively. No significant differences were observed in the numbers of blood Phe concentrations out of target range on days A and B ($p = 0.253$).

Univariate regression analysis (ANOVA) showed no significant relationships between the blood Phe concentration at time of diagnosis, the Phe tolerance and the resulting mean and ranges of blood Phe concentrations. The blood Phe concentration measured in the first sample of the day was negatively related to the number of blood Phe concentrations below 120 µmol/L on the same day (data not shown).

Table 2. Age, number of feedings, Phe intake and blood Phe ranges on both study days A and B, in infants receiving either the natural protein and Phe-free formula combined in each feeding (A) or in an alternating feeding scheme (B).

patient	1	2	3	4	5	6	7
study A							
Age (weeks)	21	27	32	19	18	13	14
Weight (kg)	7.5	8.9	8.5	5.3	5.5	5.7	5.7
Phe intake (mg ·kg^{-1} · day^{-1})	33	22	32	31	42	44	29
Number of feedings	4	6	4	5	4	5	5
Phe intake (mg ·kg^{-1} · feeding)	8.4	3.3-5.3	6.9-9.3	6.2	10-12	8.8	5.8
Difference in blood Phe *) (µmol/L)	28	103	135	128	89	19	67
study B							
Age (weeks)	18	24	33	24	21	12	16
Weight (kg)	7.2	8.5	8.6	6.1	6.3	5.5	6
Phe intake (mg ·kg^{-1} · day^{-1})	37	16	29	32	37	42	33
Number of feedings	4	6	5	5	4	5	5
Phe intake (mg ·kg^{-1}) :							
feeding 1	15	0	0	0	17.6	14	11
feeding 2	0	7.8	17.7	10.7	2	14	0
feeding 3	15	0	0	0	17.6	0	11
feeding 4	7.2	0	11.3	7.4	0	14	0
feeding 5		7.8		14.4		0	11
feeding 6		0					
Difference in blood Phe *) (µmol/L)	70	104	91	156	104	113	97

*) difference between minimum and maximum blood Phe concentration within one day

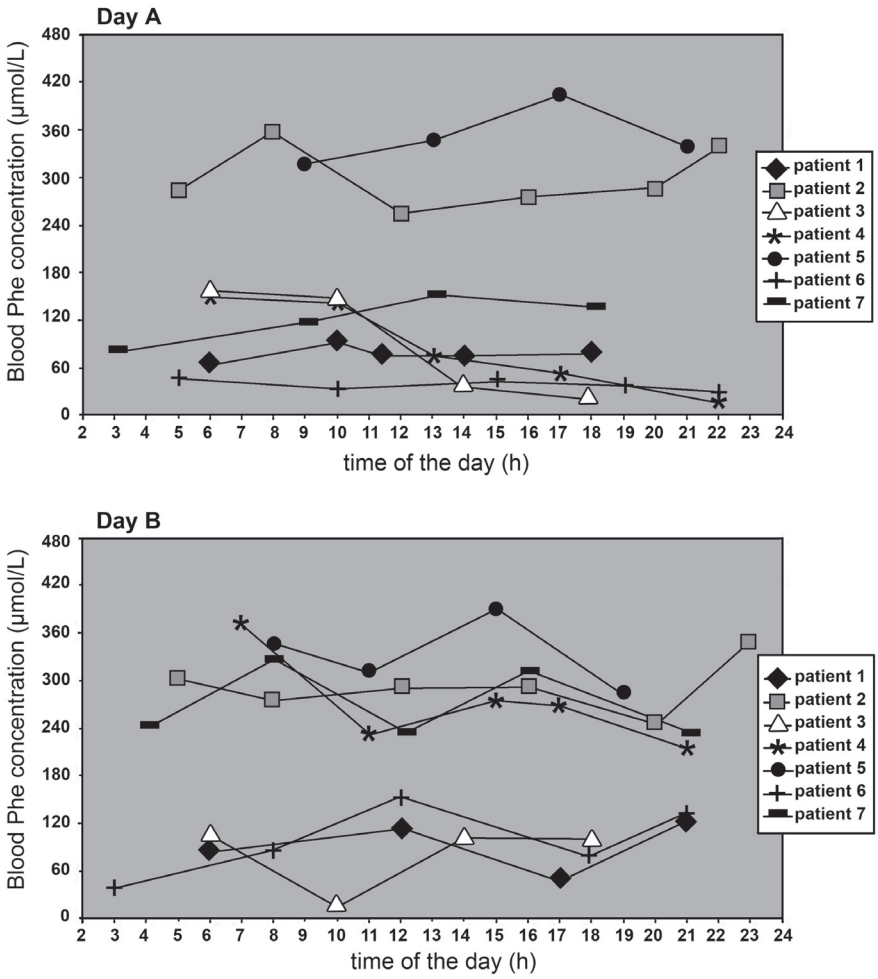

Figure 1. Course of the individual blood Phe concentrations on day A and B. The dashed lines represent the upper and lower limits of the age-adjusted target range of blood Phe concentration.

DISCUSSION

Our results showed that in PKU infants under one year of age different feeding regimes did not result in significantly different diurnal variations of blood Phe concentrations and number of samples out of the target range.

Before discussing the results, first a few methodological issues need to be addressed. To study the diurnal variation of blood Phe concentration in PKU infants, we used the results of samples that parents took routinely at home. The intake might have been less strictly controlled than in a clinical situation. The advantage of performing the sampling at home, however is that the schedule reflects the normal daily situation and enables minimal disturbance of feeding habits of the infant.

We observed that the range between the minimum and maximum blood Phe concentrations on both days A and B was not significantly different and alternating intake of Phe did not result in an increase in blood Phe concentrations. A remarkable number of samples was found to be below the minimum target Phe concentration of 120 μmol/L, irrespective the distribution of Phe intake over the day. In figure 1 we see that especially those infants with a blood Phe concentration in the morning below or just above 120 μmol/L showed blood Phe concentrations below the target range during the rest of the day. Regression analysis showed significant relationship between the first sample of the day and the number of measurements below 120 μmol/L on the same day. Thus, low absolute Phe concentrations at the start of the day rather than large fluctuations were responsible for the blood Phe concentrations below the target range. Various factors, including unbalanced intake of natural protein, energy, age, and morning blood Phe concentration determine the diurnal course of the blood Phe concentration[1;5-8].

This present study was the first to report on the diurnal variations in infants below one year of age. In line with study of MacDonald 1996, that showed larger fluctuations in children compared to adolescents and adults, one might have expected considerable large fluctuations in our population. In contrast, the most important finding was the large number of too low blood Phe concentrations depending on the morning blood Phe concentration rather than the variation.

In PKU treatment most attention is focussed on blood Phe concentrations above the target. On contrast, only few studies addressed possible consequences of too low (≤120 μmol/L) blood Phe concentrations. When dietary Phe restriction was started as treatment of PKU patients in 1956 severe growth retardation was observed[14]. In that time frame treatment aimed at normal blood Phe concentrations of 35 - 100 μmol/l[15;16]. Furthermore the composition of the supplements, which were less than optimal when compared to current formulations, might have caused the severe growth retardation[14;17]. In later years no significant relation was found between blood Phe concentrations ≤ 120 μmol/L at young age and growth retardation[18]. Intellectual development, however, has been more clearly related to low blood Phe concentrations. The study of Smith et al showed that blood Phe concentration below 120 μmol/L during five months in the first two years of age was related to a slightly lower IQ at four years of age[19]. Therefore, possible consequences of too low blood Phe concentrations should be taken into consideration when treatment strategies are concerned.

CONCLUSION

In conclusion, diurnal patterns of blood Phe concentrations were comparable under different feeding regimes i.e. the natural protein and Phe-free formula combined in each feeding or in an alternating feeding scheme, in PKU infants under one year of age. Our results also showed that very often low blood Phe concentrations were observed at this young age as an unwanted side effect of PKU treatment primarily focused on prevention of too high blood Phe concentrations. In infants, blood Phe

concentration on the morning after an overnight fast appeared to be a good predictor of the chance that blood Phe concentration will be below the target range, irrespective the feeding scheme.

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CHAPTER 4a

Phenylalanine tolerance can already reliably be assessed at the age of 2 years in patients with PKU

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Submitted

ABSTRACT

The clinical severity of phenylalanine hydroxylase deficiency is usually defined by pre-treatment phenylalanine (Phe) concentration and Phe tolerance at 5 years of age. Little is known about the ability of these parameters to predict the tolerance at later age. The objective of this study was to assess the predictive value of Phe tolerance at 1 and 6 months, at 1, 2, 3 and 5 years of age as well as pre-treatment Phe concentration for Phe tolerance at 10 years of age. Pearson's r correlation was used to calculate the effect size. Data of 236 early and continuously treated Dutch PKU patients up to 10 years of age were used. Phe tolerance decreased logarithmically with age. Pearson's r correlation with tolerance at 10 years was highest at the ages of 2 ($r=0.61$, $p<0.0005$), 3 ($r=0.73$, $p<0.0005$) and 5 years ($r=0.66$, $p<0.0005$). Pearson's correlation of Phe tolerances before the age of 2 years and Phe tolerance at 10 years of age varied between 0.11 (1 month) and 0.39 (1 year). There was no significant correlation of the pre-treatment Phe concentration and Phe tolerance at 10 years of age ($p= 0.1$). We conclude that already at an age of 2 years Phe tolerance can be reliably assessed.

INTRODUCTION

Phenylketonuria (PKU; McKusick/OMIM 261600) is an inborn error of metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) due to mutations in the human PAH gene. The deficient activity of PAH results in a decreased conversion of the essential amino acid phenylalanine (Phe) into tyrosine. Consequently, blood concentrations of Phe are elevated. Left untreated this condition usually results in severe mental retardation. Neonates are screened for PKU by measuring Phe concentrations in blood a few days after birth. Treatment aims to reduce Phe concentrations by means of a life-long diet, low in natural protein, and supplemented with a Phe-free protein substitute. Early diagnosed and continuously treated individuals with PKU are of normal intelligence although some minor neuropsychological dysfunction remains[1,2].

There is a large scale of severity of the PAH deficiency. The strongest severity (almost no residual PAH activity) results in lower amounts of Phe in the prescribed diet. This is necessary to meet treatment goals, expressed as target ranges for the blood Phe concentrations. One of the difficulties of PKU is how to define the clinical severity of PAH deficiency and the resulting Phe restriction in the diet. So far, various methods have been proposed including measurement of enzyme activity in a liver specimen, expression analysis of mutations within the PAH gene, in-vivo protein or Phe loading-tests with measurement of the Phe concentration after 72 hours, studies with radioactively labeled Phe, and continuous tracer or bolus tracer techniques with stable isotopes[3-8]. None of these methods is satisfactory because they are rather invasive, do not have a clear relation with the clinical severity of the PAH deficiency, might be hazardous because of causing very high Phe concentrations for a period of time during and after the test, or because of radioactivity.

The parameters used to observe the clinical severity in day to day life include pre-treatment Phe concentration and Phe tolerance (i.e. the amount of Phe per kg body weight per day a patient can tolerate without blood concentrations above

the highest target concentration). Güttler proposed a differentiation between 3 subgroups using the Phe tolerance at 5 years of age[9]. However, although both the pre-treatment Phe concentration and the Phe tolerance at 5 years of age have been related to the genotype, intellectual outcome and long-term Phe concentrations, the clinical significance of the pre-treatment Phe concentration and Phe tolerance at early age to predict Phe tolerance at a later age has not been studied[10-14]. Only one report studied the course of the dietary Phe tolerance till 8 years of age and only in a very small group of patients[15].

In the present study, the predictive value of pre-treatment Phe concentration and Phe tolerance up to 5 years of age for Phe tolerance at 10 years of age was investigated.

METHODS

From the start of the national screening program for PKU in the Netherlands in 1974, children with positive screening results have been referred to one of the eight Dutch pediatric university clinics. Data have been collected nationwide and registered at the central PKU-registry. The Dutch Steering Committee considered a patient to have PKU when pre-treatment blood Phe concentrations are (a) >500 µmol/L in the untreated newborn and (b) when the Phe tolerance is <50 mg/kg/day at 12 months of age[16].

Between 1974 and 1996, PKU was detected in 236 children by neonatal screening and treated ever since. These 236 patients were included in this study.

Actual weight, dietary Phe intake at 1 and 6 months and at 1, 2, 3, 5, and 10 years of age (all with a margin of ±1 month) were obtained as well as all blood Phe concentrations of the patients, including the pre-treatment Phe concentration. The Phe tolerance (mg/kg/day) was calculated from the prescribed intake for each individual patient at the seven time points described above.

The target range of blood Phe concentrations was 200 - 500 µmol/L for all ages according to Dutch recommendations at that time[17]. The mean of Phe concentrations of blood samples collected at the seven time points were subsequently used to decide on metabolic control and to include or exclude data on tolerance of the individual patient at that moment.

To establish the mean course of the Phe tolerance with age for the total population, we calculated the mean Phe tolerance at the seven times points, making use of the blood Phe concentrations that fell within the target range of 200-500 µmol/L .

Statistics

Correlations between the individual pre-treatment Phe concentration and the individual Phe tolerance at 1 and 6 months, and at 1, 2, 3, and 5 years of age on the one hand, and the tolerance at 10 years of age on the other hand were computed using Pearson's r correlation coefficient (in version 10 of the statistical program SPSS, Inc., Chicago, IL). To predict the Phe tolerance at 10 years of age from the tolerance at an earlier age, linear regression analysis was performed to construct a least square

regression equation of ages with a correlation >0.5. In all analyses a p-value <0.05 was considered to be statistically significant.

RESULTS

Data of 74 patients on the pre-treatment concentration could be included to compare with the Phe tolerance at 10 years. Data of the remaining 162 patients could not be used because of lack of tolerance figures at 10 years of age (n=141), or blood Phe concentrations not within the target range at 10 years of age (n=21). A total of 992 values of Phe tolerance could be included. These 992 values of Phe tolerance concerned 213 patients. Data of the remaining 23 patients could not be used due to incomplete data or values out of target range. The mean number of values of Phe tolerance per patient that could be used for the study was 5 (range 1-7) for the 7 time points.

The pre-treatment Phe concentration varied between 240-6000 µmol/L. The correlations between pre-treatment blood concentrations and Phe tolerance at 10 years of age was -0.192 (p=0.11) (Table 1).

The course of the mean Phe tolerance is shown in Figure 1. At 1 month of age

Table 1. Pearson r correlations between pre-treatment Phenylalanine concentration and Phe tolerance values at six different ages with Phe tolerance at 10 years of age

		Phe tolerance at 10 years of age
Pre-treatment Phe concentration	r	-.181
	p	.125
	N	73
Phe tolerance at 1 month of age	r	.112
	p	.390
	N	61
Phe tolerance at 6 months of age	r	.276
	p	.036
	N	58
Phe tolerance at 1 year of age	r	.387
	p	.002
	N	59
Phe tolerance at 2 years of age	r	.608
	p	.000
	N	57
Phe tolerance at 3 years of age	r	.725
	p	.000
	N	56
Phe tolerance at 5 years of age	r	.661
	p	.000
	N	59

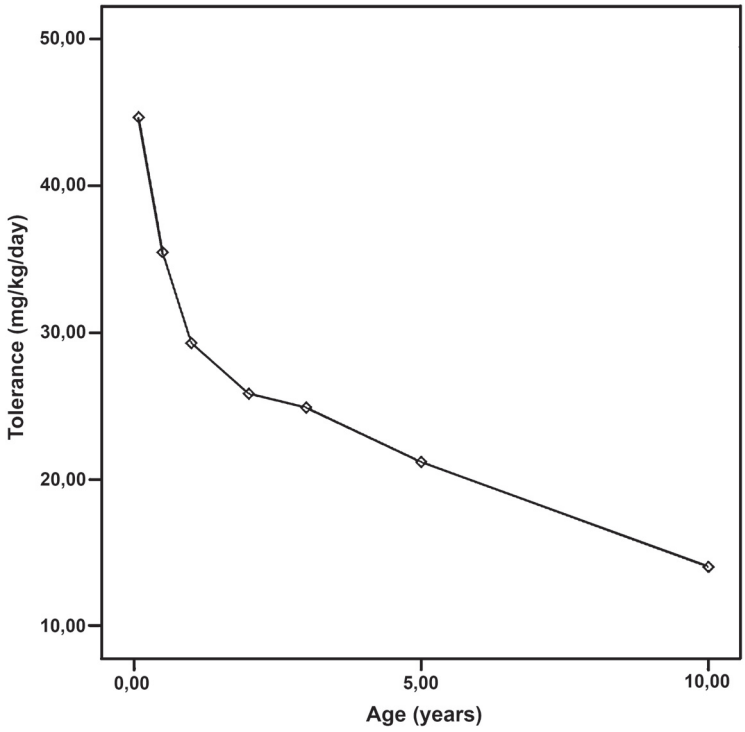

Figure 1. The relationship between mean phenylalanine tolerance and age.

the mean Phe tolerance was 45 mg/kg/day, decreasing to 26 mg/kg/day at 2 years of age. The mean Phe tolerance further decreased to 21 mg/kg/day at 5 years of age and 14 mg/kg/day at 10 years of age. Correlations between the individual Phe tolerance at 1 and 6 months, and at 1, 2, 3, and 5 years of age on the one hand, and the tolerance at 10 years of age on the other hand were highest after the age of 2 years (Table 1).

Linear regression analysis between the tolerance up to 5 years and 10 years of age was performed with the correlations >0.5, i.e. at 2, 3, and 5 years of age, to calculate the least square regression equation. By this, the Phe tolerance at 10 years of age could be predicted as follows:

Phe tolerance [10 years] = 2.2 + (0.5 x tolerance [3 years])
Phe tolerance [10 years] = 5.4 + (0.3 x tolerance [2 years])
Phe tolerance [10 years] = 4.9 + (0.4 x tolerance [5 years])

DISCUSSION

Most important finding of our study is that from the age of two years on the Phe tolerance can be reliably assessed. In contrast, the pre-treatment Phe concentration as well as Phe tolerance before the age of two years are not or only weakly associated with Phe tolerance at 10 years.

Before discussing the results some methodological issues need to be addressed. The period of time between 1974 and 1996 was taken because during that time period no change was made in the target Phe range in plasma, and the target concentration was 200-500 µmol/L in all age groups in the Dutch PKU population. The original classification of Güttler was based on the upper limit of Phe of 600 µmol/L at 5 years of age[9]. As recommended Phe concentrations in young patients were lowered, the upper concentration of Phe used by Güttler was first decreased to 420 and later to 300 µmol/L[18-20]. This however, was done without changing the ranges of the Phe tolerances within the classification, probably because the effect of lowering the target ranges on the tolerance is not well known.

For a large number of patients, data on dietary Phe intake and weight were not available within the chosen small margin around the seven time points (1 month before and after) and patients therefore were excluded at that time point for further analysis. In addition, when the mean Phe concentration was out of range around a certain time moment, data on tolerance were not included at that specific time moment. This especially applied to 5 and 10 years of age were data of 130 and 95 patients were available, respectively. Part of this loss of patient numbers can be explained by the smaller number of patients that had reached that age at the time of the study, part of this loss of patient numbers is explained by the fact that Phe concentrations were out of the target range at that time point. While the mean Phe concentration was within the target range of 178 patients at 1 month of age, the mean Phe concentration of only 111 and 74 patients were within the target range at 5 and 10 years of age, respectively.

The figures of Phe intake in our retrospective study were based on the prescribed intake, rather than the actually consumed intake, because the actually consumed intake figures (e.g. calculation of recorded 3 days food intake, dietary history) could not be obtained in all centres. It should be taken into account that, also reported consumed intake is not 100% reliable[21]. The influence of this is unclear. Probably the actual Phe intake and consequently the tolerance at an older age may be higher than the prescribed amount. However, this does not necessarily need to change the correlations. When children grow older the influence of parents on dietary intake of their child will diminish. Therefore, we expect that at older ages (after the age of two years) this bias towards the null value will be even higher than at younger ages. It is therefore unlikely that this bias has any influence on our conclusions. It should further be taken into account that both in the study of Güttler in 1980, and the present study in the Netherlands, Phe tolerance is based on total Phe intake, whereas in some other countries Phe of low protein food is not included in the calculation of the Phe tolerance[9,22,23].

We found that the Phe tolerance at 10 years can be predicted adequately from 2 years of age rather than at 5 years of age. This shows that nothwithstanding the impact of intercurrent illness and growth, there is a clear relationship between the Phe tolerance at early age and 10 years of age. This study aimed to predict the Phe tolerance at 10 years. We do not suggest that Phe tolerance does not change after 10 years of age. Apart from the activity of PAH various factors may influence

Phe tolerance during childhood. Where growth and intercurrent illnesses are the most important factors in childhood, after childhood, these will probably be mainly illnesses, adolescent growth spurt, and changes in body composition, as well as differences in the target Phe concentrations. None of these factors, however, has been studied in PKU so that they remain hypothetical.

Correlations between pre-treatment Phe concentrations and data on tolerance values throughout the studied period were weak (Table 1). This is in line with the finding that the tolerance before 2 years of age showed weak correlations with tolerance at 10 years of age. This implies that to construct a predictive parameter before 3 years of age, non-clinical in-vivo parameters are necessary. For this reason the early use of a Phe loading-test with ^{13}C-Phe measuring $^{13}CO_2$ production, as performed by Treacy et al, may be useful[24].

In conclusion, the Phe tolerance at 10 years can be adequately predicted with the Phe tolerance as early as 2 years of age. This might help parents and treatment teams to estimate the severity of natural protein restriction for a larger period of time during childhood. Further studies are necessary, especially with patients above 10 years of age.

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CHAPTER 4b

Plasma phenylalanine in patients with phenylketonuria self managing their diet

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Dietary adherence in phenylketonuria (PKU) –measured as plasma phenylalanine concentrations (Phe) - is a major issue.[1 2] In the so-called professionally steered situation, it was our practice to take all blood samples for measurement of Phe during patients' hospital visits. Phe concentrations were interpreted by the paediatrician and the dietician phoned patients/parents with dietary advice. Self-management has been suggested to improve dietary adherence.[1] We report Phe concentrations during our first 6 months experiences with self-management in 48 PKU patients above 1 year of age.

During this period, patients decided frequency of blood sampling, and sent samples (filter paper) to the laboratory by post. A nurse without knowledge of PKU phoned the results to patients/parents without interpretation/advice. Patient/parents decided independently regarding adjustment, but could phone the dietician for advice one day later. The clinical team received all blood test results weekly. When Phe concentrations were frequently deemed unsatisfactory, the dietician called the parent/patient. During hospital visits (Table 1), Phe concentrations, patients' adjustments, and the protein substitute were evaluated.

Self-management was introduced following 1–2 individual meetings of patients with staff, two group sessions, and provision of written information, including advice regarding frequency of blood sampling, appropriate Phe concentrations (adapted from the British recommendations[3]), and dietary adjustments (Table 1). As this was a change in management policy rather than a research study, there was no control group. Therefore, results of six months self-management (456 samples in 48 patients) (Wilcoxon test) were compared with those obtained over three years beforehand (1152 samples in 48 patients).

Table 1. Advised ranges for plasma Phe concentration and frequency for follow-up.

Age (years)	Plasma Phe concentration (µmol/l)		Blood sampling		Clinical follow-up	
	Old	New	Old	New	Old	New
First year	120-240	120-240	Fortnightly	Weekly	Fortnightly	Every 1-2 months
1-4	200-500	120-360	Monthly	Weekly	Monthly	Every 2-3 months
5-10	200-500	120-480	Every 6-8 weeks	Fortnightly	Every 6-8 weeks	Every 3-4 months
11-15	200-500	120-480	Every 6-8 weeks	Monthly	Every 6-8 weeks	Every half year
> 15	200-500	120-600	Every 3 months	Monthly	Every 3 months	Every year

Old refers to the situation steered by professionals. New refers to the situation of self-management

During self-management, most patients took blood samples according to the recommended frequency. The Phe intake changed in 10 patients (mean change 2,3%, SD 5,7%). The largest differences in intake were 32% and 16% of the total Phe daily given in mg/day, observed in 2 patients with changes in growth velocity during puberty.

Parents and professionals adjusted the diet in a comparable way. The dietician was phoned by families once or twice a week, while she phoned them twice a month. In patients aged 1-4 year and 11-15 year the Phe concentrations rose (Table 2). The median proportion of samples within the advised range remained comparable, largely because of the decrease frequency of Phe concentrations below the target range.

Various explanations can be given for the increase in Phe concentrations during self-management, including the normal rise with age,[2] increased frequency of blood sampling, a tendency for patients to attempt a dietary intake compatible with Phe concentrations just below the advised upper limit, and sampling after an overnight fast rather than later in the day during the professionally steered situation.

The question whether this rise in Phe concentrations is important is hard to answer. The mean of the medians within 1-4 years aged patients rose of 214 to 327 μmol/l but remained lower than most of the reported experiences.[2] Younger the patients are more vulnerable to higher Phe concentrations, but the importance of variable

Table 2. Plasma phenylalanine concentration and proportion of samples with plasma phenylalanine above the recommended range in the situation steered by professionals and self-management.

	Mean plasma phenylalanine concentration (μmol/l) during situation			Percentage samples with Phe above recommended range during situation		
	Median (ranges)			Median (ranges)		
Age in years	Steered by Professionals	Self-management	P value*	Steered by Professionals	Self-management	P value*
1-4 (n=11)	214 (183-995)	327 (189-1007)	0.010	75 (8-100)	75 (14-95)	0.929
5-10 (n=12)	309 (186-1414)	326 (208-1282)	0.480	44 (0-100)	68 (8-100)	0.919
11-15 (n=14)	315 (155-433)	392 (277-582)	0.011	100 (50-100)	100 (34-100)	0.483
> 15 (n=11)	587 (290-960)	649 (337-1266)	0.068	67 (0-100)	50 (0-100)	0.075
All ages (n=48)	320 (337-1266)	382 (189-1282)	<0.001	78 (0-100)	75 (0-100)	0.248

Results expressed as median (range)
*(Wilcoxon)

time periods of Phe concentrations far below 360 µmol/l below 10 years of age is uncertain.[4-6]

In conclusion, this is the first study on the effect of self-management on plasma Phe in PKU has shown that self-management is a viable option, but further investigation of the effects and safety is warranted.

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CHAPTER 4c

Natural protein intake in PKU patients: to estimate or to measure?

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Provisionally accepted Jada

ABSTRACT

Objective: This study investigated which methods patients and parents used to realize the Phe intake and the relationship between the methods applied, age and blood Phe concentration.

Patients and methods: A questionnaire was sent to 327 Dutch Phenylketonuria patients (age 0-29 years) to investigate the method used to realize phenylalanine intake (either by estimation, exact measurement or a combination of both). The mean blood phenylalanine concentration of each individual patient was related to the method reported to be used. Three different age groups (<10, ≥10-15, ≥16 years) were distinguished.

Results: The response of the questionnaires was 73%. In these 188 patients, data of both phenylalanine concentrations and questionnaires could be used. Out of these 188, 75 used exact measurement, 75 estimation and 38 used both methods. The number of patients that estimated phenylalanine intake clearly increased with age. Whatever method was used, an increase in phenylalanine concentrations was seen with age. During childhood exact measurement was used more frequently, while from adolescence on estimation was used more frequently. The method (exact measurement and/or estimation) did not result in statistically different phenylalanine concentrations in any of the three age groups, although blood Phe concentration tended to be lower in adolescence using exact measurement.

Conclusion: The data suggest that estimation and exact measurement of the phenylalanine intake are both reliable methods. Therefore, in addition to exact measurement, patients should be instructed in both methods at an early age, so that both methods can be used adequately at each age.

INTRODUCTION

The treatment of Phenylketonuria (PKU) is based on a Phenylalanine (Phe) restricted diet. This diet consists of two inseparable elements: extensive restriction of natural protein to lower the Phe intake in PKU patients, and supplementation of amino acids other than Phe to achieve a normal intake of protein except Phe.

The communication in the treatment of PKU comprises explaining diagnosis, disorder, treatment and care to the parents and training in practical aspects of dietary management. To translate the amount of Phe (i.e. natural protein) into a meal plan, parents are instructed about the Phe content of food and about the use of scales and measuring cups. Dietary protocols and handbooks recommend weighing all protein containing food products. Exact measurement is stated to be the most reliable method to achieve optimal metabolic control, although studies are not available[1,2]. In the Netherlands parents are initially instructed to weigh all protein containing food on a scale that reads in grams and learn to use the exchange food lists based on the Dutch Food Composition Table NEVO to vary in protein containing products[3,4]. In daily practice however, parents and patients may realize Phe intake without scales, i.e. by estimation or a combination of both techniques. Crone et al showed that keeping strictly to the PKU diet without being too rigid is associated with a better metabolic control[5]. Using estimation (or a combination of both estimation and exact measurement) may differentiate between strict and rigid dietary treatment.

Therefore this study aimed to investigate the method applied to realize Phe intake at different ages. We compared blood Phe concentrations of the different age groups using exact measurement, estimating or a combination of both.

PATIENTS AND METHODS

All PKU patients detected by neonatal screening, referred to one of the eight university affiliated hospitals, born between September 1974 and 2004 in the Netherlands, were included. In the study the patients were divided into 3 groups based on age: < 10 years, ≥10-15 years and ≥16-29 years of age.

A questionnaire was developed to investigate the daily practice used to realize the Phe intake of the PKU patient. The questionnaire was based on the same theoretical behavioral model used by Crone[5]. The theory of planned behavior described by Ajzen, is often applied in health related studies[6,7]. This model takes into account the following factors as an explanation of behavior: attitude (positive or negative beliefs and experiences with a certain behavior e.g. estimating or measuring protein containing food), subjective norm (normative beliefs and motivation of the social surroundings, social pressure from significant others to perform- or not to perform- a particular behavior) and perceived behavioral control (the person's belief as to how easy or difficult performance of the behavior is likely to be)[6]. In this study behavior was defined as the methods to realize the Phe intake (i.e. exact measurement, estimation or a combination of both). Exact measurement was defined as the method of realizing Phe intake in daily practice with the use of scales and measuring cups and calculation of the amount of Phe. Estimating was defined as "eyeballing" the amount of protein containing food that can be eaten and estimating the Phe content of the portion that is taken. Less precise pre-measured food such as a glass, a plate or a table spoon was defined equal to estimating. Pre-measured food containing a precise defined amount of food in grams was defined equal to exact measurement. Examples of the questions are given in Table 1. The questionnaire was pre-tested by 5 parents and 5 patients ≥ 16 years of age. The mean time to complete the questionnaire was 15 minutes. The questionnaires were sent out by post with additional information about the purpose of the study. A reminder was sent after 3 weeks. The questionnaires used were sent back within 5 weeks after the first posting. Information was given anonymously with a code to be able to combine with blood Phe concentrations.

The questionnaire was tested for internal consistence (Cronbach's alpha). In the questionnaire answers could be given or could be recoded to a 5-points scale from 0- 4. Measuring = 0, probably measuring = 1, both = 2, probably estimating = 3 and estimating = 4. The questionnaire contained 20 questions about the social economic background and 30 questions concerning the practice of realizing Phe intake by measuring, estimating or both. Of each individual we calculated the mean score. We classified individuals as follows: a score of <1,5 was equal to measuring, 1,5 – 2.49 to both and > 2,5 to estimating.

To define metabolic control we used the mean of the individual blood Phe concentrations from 2001 - 2003. Thus we avoided a large effect of *ad hoc* fluctuations

Table 1 Examples of questions concerning behavior at meal time. The questionnaire was based on the theoretical behavioral model of Ajzen (the theory of planned behavior).

Notice:
Mark in these questions what is most usual in your situation, when certain conditions are not usual you can answer what you or your child would do in such a situation.

I count the amount of phenylalanine daily:
o Yes
o No
o Sometimes

When I am at home I use scales to determine the portions up dinner:
o Never
o Sometimes
o Always
o not applicable

When we have dinner outside home (e.g. in a restaurant or with friends) I use scales to determine the portions for dinner.
o Never
o Sometimes
o Always
o We never go out for dinner
o not applicable

French fries are used at dinner and as a snack. How do you measure the amount of french fries?
o I use the amount in the supplied fixed portion
o I estimate the amount by using a tablespoon
o I use scales
o Not, (depends on my appetite)
o Otherwise:……
o Not applicable

in blood Phe concentrations, due to illness etc. When less than 10 Phe concentrations were available during this period, patients were excluded. All Phe concentrations were measured quantitatively in blood using ion-exchange liquid amino acid analysers.

Metabolic control was not related to the target ranges as the different participating centers did not apply the same target ranges at time of the study. The Dutch National PKU Steering Committee approved the study and the medical ethical committee of the university affiliated hospitals concluded that their approval was unnecessary because the study consisted only of administration of a questionnaire and the retrospective use of blood Phe concentrations. All eight Dutch university affiliated hospitals agreed to participate.

Statistics

Kruskal-Wallis tests (in version 10, 2002 of the statistical program SPSS, Inc., Chicago, IL) were performed to compare the Phe concentrations between groups of patients with the same age who determined their Phe intake either by measurement, estimation or using both methods, and to compare the distribution of the methods in the age groups. An $a < 0,05$ was considered statistically significant.

RESULTS

A total of 327 questionnaires were sent out, six questionnaires did not reach the patient. Of the remaining 321, 73% (n233) was returned with information given by the (parents of) patients. Of these, 24 questionnaires had significant missing data and were excluded from analyses. Of the remaining 209 questionnaires, data of 21 could not be related to the metabolic control because less than 10 blood Phe concentrations were available.

Mean (SD) blood Phe concentrations in the different groups and the number of patients that used exact measurement, estimation or both methods to realize the Phe intake in patients of different ages are shown in Figure 1. The group consisted of 88 patients <10 years of age, 44 patients of ≥10-15 years of age and 56 patients ≥16 years of age. In the group below 10 years of age 55% reported that they used exact measurement, in the older groups ≥ 10-15 years and over 16 years this was 35% and 20% respectively. In the group under 10 years of age 24% reported that they used estimation, in the older groups ≥10-15 years and ≥ 16 years of age this was 36% and 63% respectively. The distribution of the methods in the age groups (Kruskall-Wallis test) was significantly different ($p < 0.001$).

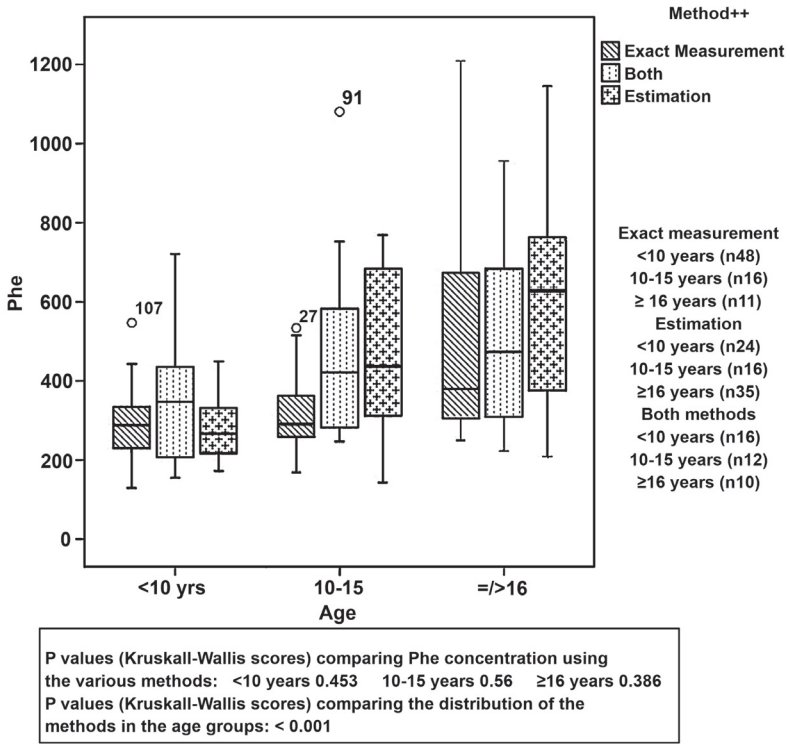

Figure 1. Blood Phe concentrations in the different groups and the number of patients that used exact measurement, estimation of both methods to realize the phenylalanine intake in patients of different ages.

Phe concentration rose with age irrespectively the method used. In all age groups patients used exact measurement or estimation rather than a combination of both methods. The method used did not result in significant differences in the mean Phe concentrations in any age group, although in the group ≥10-15 years, exact measurement tended to result in lower blood Phe concentrations.

DISCUSSION

The most important findings of the present study were that in all age groups the methods did not result in statistically differences in mean Phe concentrations, although in the group ≥10-15 years, exact measurement tended to result in lower blood Phe concentrations.

From the results it appears that there was a gradual change from exact measurement at young age to estimation at later age. At the first confrontation of the parents with PKU they have to learn how to manage the treatment and feel comfortable with clear instructions. When confidence in the treatment and their own capabilities grow, they are likely to develop other methods. Initially measuring with scales and calculating the amount of Phe, parents and patients may develop the skills necessary to measure without scales and calculator. However, at the same time 20% of the adult patients answered that they continue to use the exact measurement.

Differences in target ranges may have influenced differences in resulting blood Phe concentrations. However at the time of the studied period national guidelines were developed. The nationally agreed target ranges were close to the different targets applied in the eight institutes, and not likely expected to influence the study results largely. Severity of the disease might have influenced the choice for a certain method to realize Phe intake. In the study differences in Phe tolerance (as measure of severity of disease) was not taken into account as it was impossible to match individual figures about the Phe intake as these data were not available anonymously. In day to day practice we find patients realizing Phe intake with different methods, irrespective their severity of the disease.

To assess whether findings were subject to selection bias, we compared mean blood Phe concentration in the participating patients with the mean blood Phe concentrations in comparable age groups of previous national Dutch studies, showing comparable mean blood Phe concentrations in the same age groups[5,8].

Phe concentrations were not related to the method of realizing Phe intake within any age group, but in the age group 10-15 years there was a tendency to lower blood Phe concentrations in the group that measured (Figure 1). This could suggest that in this age group the start of experimenting with estimating results in a tendency to higher blood Phe concentrations, rather than a consequence of estimating itself. In contrast, this difference in blood Phe concentration is clearly smaller in adults, in this age group the combination of both methods resulted in a tendency to lower blood Phe concentrations. This would- as a consequence- suggest the importance of being capable to use both methods. In general, we see that beyond childhood a significant percentage of the patients show higher mean blood Phe concentrations[9-11]. This

study tried to contribute to the puzzle how to influence the phenomenon of rising blood Phe concentrations of adolescent and adult patients. The clinical relevance of treatment at the time of diagnosis and throughout childhood is unquestionable and most guidelines also agree that dietary treatment of PKU should be continued throughout adult life[12,13]. Weglage and Burgard described that psychosocial problems seem to arise in adolescent and adult patients and speculated that this may be associated with the burdensome diet[14,15]. Life of adolescents changes completely with regard to school, sports, societies, and the importance of peer groups both in healthy adolescents and adolescents with chronic diseases. In healthy adolescents, responsibilities are transferred from the parents to the adolescents themselves. In chronic diseases, not only normal responsibilities are transferred, but also responsibilities and activities specific for the disease, e.g. medicine, dietary instructions and diet[16,17]. As a consequence adolescents need time to get experienced with their responsibilities in general and with regard to their disease. Measuring without scales can make life with PKU less different from normal. Simplifications in dietary treatment can contribute to a better compliance.

CONCLUSIONS

This study showed that exact measurement of Phe intake compared to estimating Phe intake, is not clearly related to a lower mean blood Phe concentration. It can be more attractive for patients/ parents to be able to realize Phe intake by "eyeballing" rather than by exact measurement, being compliant, but not too rigid. In the dietary treatment it is of importance that after initial instruction in exact measurement, both methods (i.e. to estimate and to measure) are taught to realize Phe intake early in life, so that both can be used adequately and patient and parents can choose the method with which they are most comfortable with in different situations.

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CHAPTER 4d

Well-controlled Adult PKU Patients Tolerate Incidental Additional Intake of Phenylalanine.

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ABSTRACT

Background In patients with Phenylketonuria (PKU) target ranges of blood phenylalanine (Phe) concentrations have become narrow in order to improve long term outcome. Strict dietary treatment necessitates more knowledge about variation of blood Phe concentrations and the effects of changes in Phe intake.

Objective We investigated day-to-day and week-to-week variation in blood Phe concentration and the effect of an additional Phe load on blood Phe concentration.

Design We performed a longitudinal study in six adult PKU patients. The study was divided in five periods of seven days: one period without any intervention (period I) and four periods with a Phe load on day three equivalent to 100% (period II-III) and 200% (period IV-V) of their individual daily Phe intake. Phe loading was given as encapsulated L- Phe. In all periods the diet was similar in energy, natural protein and amino acid supplementation. Bloodspots were taken each morning before breakfast in all periods, to measure blood Phe concentration.

Results Blood Phe concentrations varied considerably from day-to-day and week-to-week with and without intervention in Phe intake. Equal loads of Phe did not result in comparable effects in blood Phe concentrations in all patients. In periods II-IV mean blood Phe concentrations of days 1-3 (pre-load) were not significantly different from days 4-7 (post-load). The 200% load of period V resulted in a significantly larger variation.

Conclusion The results showed that well-controlled PKU patients incidentally tolerate 100% and in some cases 200% of their daily Phe intake additional to their normal Phe intake.

Introduction:

In Phenylketonuria (PKU), caused by a deficiency of the enzyme phenylalanine hydroxylase, the amino acid phenylalanine (Phe) cannot sufficiently be converted into tyrosine. Left untreated, phenylalanine hydroxylase deficiency will result in high concentrations of Phe in blood and tissues, severe mental retardation and behavioral problems[1]. The precise mechanism of the brain damage is still unclear, but is clearly related to high blood Phe concentrations[2]. Therefore, treatment aims at restriction of Phe intake to prevent increases in blood Phe concentrations. In The Netherlands guidelines advise blood Phe concentration between 120 – 360 µmol/L until 12 years of age and between 120 – 600 µmol/L thereafter. Dietary treatment is based on Phe restriction with supplementation of a Phe-free synthetic amino acid mixture enriched with tyrosine, vitamins and minerals to meet protein and micro-nutrient requirements[3,4].

Metabolic control is achieved by regular measurement of blood Phe concentration and subsequent adjustment of Phe intake when blood Phe concentration is not within the target range. Other factors are also taken into account which influence metabolic control eg intercurrent infections, growth, energy intake and amino acid supplementation.

Studies on the variation of blood Phe concentrations have examined changes occurring within 24 hours[5-9]. Advised frequency of blood sampling decreases with age[10,11]. Little is known about individual variations of blood Phe concentrations from day-to-day and week-to-week and the effects of changes in the Phe intake in adolescence and adulthood. Knowledge of normal variation of blood Phe

concentrations and the effect of incidental extra Phe intake can contribute to better understanding of metabolic control. Therefore, the aim of the present study was to investigate 1) day-to-day and 2) week-to-week variation of the blood Phe concentrations in well-controlled adult patients and 3) to investigate the effect of an incidental extra Phe intake on blood Phe concentrations.

PATIENTS AND METHODS

The study was carried out in six adult PKU patients who were continuously treated from diagnosis onwards in the Beatrix Children's Hospital of the University Medical Centre Groningen. They were all well controlled with regard to the blood Phe concentration, i.e. in the year prior to the study over 65% of the monthly blood Phe concentration measurements were within the target range of 120-600 µmol/l, as advised by the Dutch PKU Advisory Committee. The individual Phe tolerance expressed as the total amount of Phe per day tolerated while maintaining blood Phe concentrations within the target range, varied between 360 and 2000 mg per day at the time of the study. Apart from PKU, patients were healthy during the study period. Patients were not pregnant or planning for a pregnancy. Their body weight was stable over the last 6 months prior to the study. Patients were fully informed about the procedure and entered the study with written informed consent. The Medical Ethical Committee of the University Medical Centre Groningen approved the study.

Study design

The study was performed at home. Patients were instructed about the study and the goals by the research dietician (MR). The study consisted of five study periods of 7 days each (Figure 1). All patients who enrolled subsequently finished the study. They consumed their individually tailored diet unchanged in all these five study periods. Study period I did not include any intervention. In each subsequent study period (II – V) one single Phe load was given on day three during dinner. The Phe load was based on the individual daily Phe intake and was given as L-Phenylalanine powder in cellulose capsules, prepared by the hospital pharmacy. In the first two intervention periods (II – III) the additional Phe load was equivalent to the individual daily Phe intake and was designated as "100% load" and in the last two periods (IV – V) the additional load was equivalent to twice the individual daily Phe intake and was designated as "200% load". Participants decided themselves when to perform the consecutive study periods. Blood from finger punctures was collected on filter paper (type 2992, Schleicher and Schuell, 's Hertogenbosch, the Netherlands) each day before breakfast in all study periods. Bloodspots were sent by mail to the laboratory for further analysis. During each study period patients kept a 2-day record of their intake of all food, drinks and amino acid supplementation. Participants decided themselves on which 2 days of the 7 days periods to record intake. The intake of energy, Phe and amino acid supplementation of these 2 days was calculated according the exchange list and the ZIS computer program based on food declaration table NEVO 2001[12]. Besides intake registration, body weight was measured on day one and seven

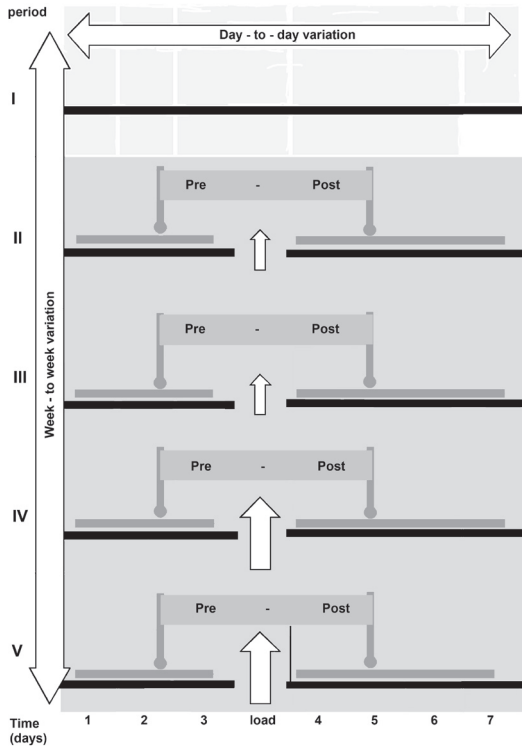

Figure 1. Schematic picture of the study protocol

at the same time of the day in all testing periods.

Analyses of blood Phe concentrations

Blood Phe concentrations were determined in eluates of ⅛ ' discs punched from dried blood spots. Discs were eluted at room temperature for 30 minutes in 150 µl TCA solution (6.6 % $^{w}/_{v}$ TCA in H_2O) containing norvaline as internal standard, after which the debris was precipitated by centrifugation for 5 minutes at 14,000 rpm. Ten µl of the supernatant was transferred to a clean reaction vial and prepared for HPLC analysis by the AccQ-Tag® method according to the manufacturer's protocol (Waters, Breda, the Netherlands).

Analysis of resulting data and statistics

The variations of the blood Phe concentrations, the mean blood Phe concentrations, and the number of blood Phe concentrations above the target range were calculated. Results of days 1-3 and days 4-7 were calculated separately. The individual day-to-day variation without intervention was calculated as the difference between the highest and the lowest blood Phe concentration of each individual during period I. For the individual week-to-week variation without intervention the means and SD of the blood Phe concentrations over days 1-3 of periods II-V were compared with days 1-3 of period I. To evaluate the effect of the different Phe loads, we divided study periods II-V into 2 blocks, i.e. day 1-3 (pre - load block) and day 4-7 (post -load block). Subsequently the means and SD over the daily blood Phe concentrations of each PKU patient measured in the pre- and in the post-load blocks of each period

II-V were calculated. Furthermore, we evaluated the effect of the Phe load on the number of blood Phe concentrations above target range of 600 µmol/L by comparing the number of samples out of the target range of days 1-3 to days 4-7. Tests for statistical significance of differences between days 1-3 and days 4-7, was done by paired Student's *t*-tests. Relationship between the severity of disease defined as the individual Phe tolerance, and the resulting mean and SD of blood Phe concentrations of each period was tested with univariate linear regression analysis (ANOVA). Linear regression analysis (ANOVA) was applied to test the relationship between the blood Phe concentration on day one of each period and the number of days beyond range and the mean an SD of the blood Phe concentrations in the same period. Statistical analysis was done using the Statistical Package for Social Sciences (version 12 SPSS Inc., Chicago IL USA). Statistical significance was assumed at $P \leq 0.05$.

RESULTS

Table 1 shows patient characteristics, protein and energy intake as means ± SD during the study. Intake of energy, natural protein and amino acid supplementation was within 5% of the individual prescribed diet. Amino acid supplementation was taken daily in three equal portions by all individuals and, together with the natural protein, resulted in a total protein intake of 1.2 gram per kg body weight per day in five patients and in 1.1 gram per kg body weight per day in one patient. Bodyweight did not vary during the study periods.

Day-to–day and week-to-week variation

Table 2 shows all individual blood Phe concentrations of the five study periods and the time period between the study periods. The individual course of all resulting blood Phe concentrations is depicted in Figure 2. The individual day-to-day variation in blood Phe concentration in period I varied between 55 µmol/L in patient three and 257 µmol/L in patient six.

Table 1 Patient characteristics, protein and energy intake during the study.

Patient	Age (years)	Sex (M/F)	Phe intake a)	bodyweight b)	Energy c)	Protein d)
1	27	M	2000	98.8 (0.6)	2460 (130)	1,2
2	27	F	550	51.0 (0.2)	1840 (60)	1,2
3	19	F	450	62.8 (0.3)	2380 (185)	1,1
4	18	F	360	54.5 (0.5)	2140 (125)	1,2
5	19	F	1150	64.6 (0.3)	2030 (150)	1,2
6	18	F	800	53.7 (0.4)	1990 (175)	1,2

a) individual Phe intake in mg / day
b) body weight in kg (mean and SD) measured in all study periods on day 1 and 7 before breakfast
c) energy intake in kcal / day (mean and SD), registered on 2 days each period
d) grams per kg body weight (natural protein and amino acid supplementation in total)

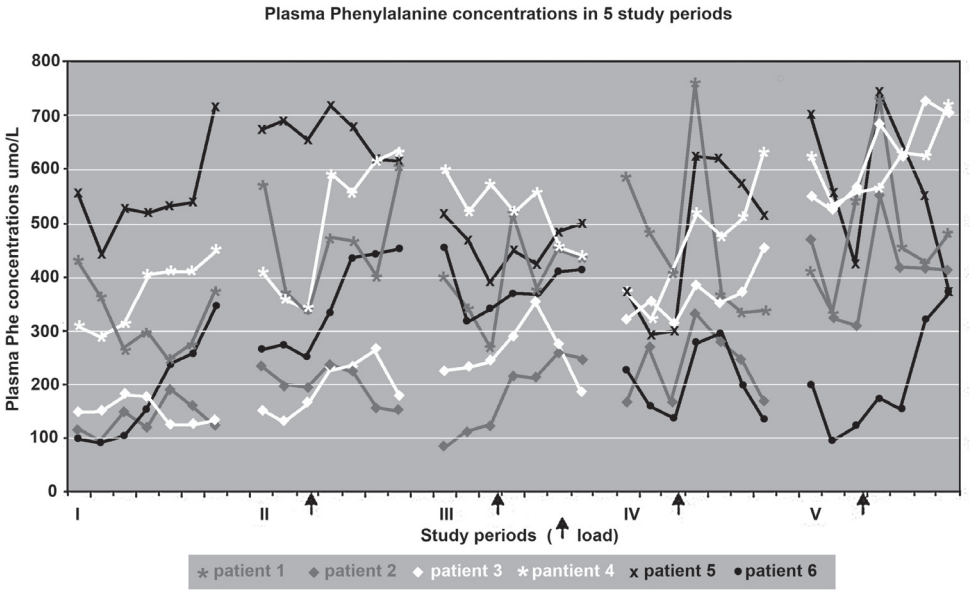

Figure 2. The individual course of all resulting blood Phe concentrations

Table 3 shows the means and SD's of blood Phe concentrations calculated for the study periods and for the days 1-3 and the days 4-7 for each PKU patient. The week-to-week variation was not significantly different in days 1-3 of period III and IV as compared to day 1-3 of period I, but was significantly different in days 1-3 of period II and V when compared to day 1-3 of period I.

Effect of Phe load

Results of the paired 2-tailed Student's *t*-test of the mean blood Phe concentrations of the pre-load blocks compared to the post-load blocks in periods II-V was only significant in period V (p 0.004). In study period I only 1 measurement out of 42 blood Phe concentration was above the target range (600 µmol /L). On days 1-3 of periods II-V, 6 samples out of 72 were above the target range. On days 4-7 in period II-III, 6 samples out of 48 were above the target range. On days 4-7 in period IV-V, 14 samples out of 48 were above the target range. The observed individual maximum blood Phe concentration after a Phe load varied greatly.

Testing for the possible relation between mean blood Phe concentrations of all samples obtained during each study period and the individual Phe tolerance showed no significant results. Blood Phe concentration on day one of each study period was significantly predictive for all other blood Phe concentrations in the same period and for the number of blood Phe concentrations above and below the target range of 120 – 600 µmol /L (data not shown).

Table 2 Blood Phe concentrations per individual per study period in µmol/L

	Patient					
Period/day	1	2	3	4	5	6
I 1	430	114	151	311	556	101
I 2	363	104	153	287	442	91
I 3	264	147	183	310	528	102
I 4	297	118	180	403	518	151
I 5	243	190	128	411	532	237
I 6	273	159	129	413	541	258
I 7	374	124	134	454	716	348
Days in between	7	17	0	0	0	0
II 1	570	233	153	409	673	265
II 2	368	198	135	358	690	273
II 3	337	191	171	341	654	251
II load 100%						
II 4	472	235	237	594	718	333
II 5	466	226	229	557	678	434
II 6	402	159	268	614	616	442
II 7	600	154	179	633	614	453
Days in between	0	37	0	0	0	0
III 1	398	86	226	601	518	455
III 2	341	109	236	522	469	320
III 3	266	123	249	574	388	341
III load 100%						
III 4	522	217	291	519	453	368
III 5	374	213	357	557	420	366
III 6	453	260	279	432	484	407
III 7	433	246	187	402	499	412
Days in between	23	27	9	8	33	8
IV 1	586	169	324	370	373	227
IV 2	482	269	357	324	293	161
IV 3	406	169	314	433	299	138
IV load 200%						
IV 4	759	329	387	522	624	281
IV 5	367	281	353	475	619	295
IV 6	332	244	374	511	574	198
IV 7	336	170	457	634	514	134
Days in between	0	46	0	0	9	0
V 1	410	470	551	623	699	201
V 2	335	326	527	517	556	96
V 3	542	309	569	572	422	123
V load 200%						
V 4	729	553	685	561	744	173
V 5	454	416	625	630	641	156
V 6	421	418	730	625	552	322
V 7	483	408	706	720	370	373

Table 3 Descriptive statistics, blood Phe concentration (mean and SD) per individual in µmol/L

Period		Patient					
		1	2	3	4	5	6
I	days 1-3	352(84)	122(23)	162(18)	303(14)	509(59)	98(6)
	days 4-7	297(56)	148(33)	143(25)	420(23)	577(93)	249(81)
	total	321(69)	137(30)	151(23)	370(65)	548(83)	184(99)
II	pre-load	425(127)	207(23)	153(18)	369(35)	672(18)	263(11)
	post-load	485(83)	194(43)	228(37)	600(33)	657(51)	416(56)
	total	459(99)	199(34)	196(49)	501(127)	663(38)	350(91)
III	pre-load	335(66)	106(19)	237(12)	566(40)	458(66)	372(73)
	post-load	446(61)	234(23)	279(70)	478(73)	464(35)	388(25)
	total	398(83)	179(71)	261(55)	515(73)	462(45)	381(46)
IV	pre-load	491(90)	202(58)	332(23)	376(55)	322(45)	175(46)
	post-load	449(208)	256(67)	393(45)	536(69)	583(51)	227(75)
	total	467(157)	233(65)	367(47)	467(103)	471(146)	205(66)
V	pre-load	429(105)	368(88)	549(21)	571(53)	559(139)	140(55)
	post-load	522(140)	449(70)	687(45)	634(65)	577(159)	256(108)
	total	482(126)	414(83)	628(81)	607(65)	569(138)	206(103)

DISCUSSION

The most important finding of the study was that the individual blood Phe concentration was not greatly influenced by an incidental additional Phe intake of 100% of the individual daily Phe intake. Even 200% additional Phe intake was tolerated in some patients, provided that the blood Phe concentration was stable and well below the upper limit of the target range.

Possibly methodological factors might have influenced the outcome of the study. The study was performed while patients were at home to obtain blood Phe concentrations in the normal day-to-day situation, and to enable a longitudinal study. All participants were able to complete all five study periods and to collect all data that were asked for. We were aware of the disadvantages of performing such a study at home but by keeping intake as usual and performing loading easily with capsules of free L-Phe, the instruction was simple. All participants were known to be compliant to their diet. Results of the registration of intake and body weight suggest that patients were not in a catabolic state during the study periods. Absorption and utilisation of free L-Phe is known to be different from natural protein that normally will be taken as incidental extra intake of Phe[13-15]. However, in this study it was important to keep dietary intake similar in energy and protein except for the Phe. Patients in this study had greatly different Phe tolerances. Therefore the magnitude of the Phe-load was adapted to the individual Phe tolerance. Furthermore sampling time was set before breakfast, thus preventing differences in blood Phe concentrations due to the diurnal variations as shown in previous studies[5-7,9].

Our results showed unpredictable changes in blood Phe concentrations in all

individuals and in all study periods, where the individual differences from week-to-week were more outspoken than from day-to-day, irrespective of the differences in the time periods between the study periods (Table 2). Studies on variation in blood Phe concentrations concern the diurnal variations, and not the day-to-day or week to week variation[5-9]. These variations with larger time periods in between (days to weeks) are used as a parameter of dietary control and also as a parameter in intervention studies.

The effect of the Phe load on the variation in blood Phe concentration was surprisingly small compared to the variation without intervention. We also observed that the intra-individual effect in the similar loading periods (II –III and IV-V) was sometimes very different in magnitude. Only in period V a significant difference in mean blood Phe concentrations in day 1-3 compared to day 4-7 was observed. We expected that rise in blood Phe concentration would occur shortly after loading with free L-Phe. In our results the individual maximum Phe concentration varied considerably, with less than 50% of the individual maximum blood Phe concentrations in the different study periods on the morning after loading in period II-V. In this study only patient three showed a continuous effect of loading, as blood Phe concentrations did not return to base-line after the 200% load. No or little effect of loading was also seen in patients in the preceding study by Huijbregts et al[16]. In this study of Huijbregts, a considerable rise in blood Phe concentrations could not be effectuated in a short period of time with 100% extra Phe intake. Similarly, van Spronsen et al reported only small differences between pre-and post-load blood Phe concentration by giving 33 %, 50% or 75% of the total daily intake of Phe in one meal[9]. The reasons for this lack of effect of higher oral Phe intake of short duration are unknown. In the publications of MacDonald 1996 and van Spronsen 1993 a more pronounced effect of intake at a younger age is seen[8,17]. This may be explained by the larger plasma Phe pool at older age when compared to the relatively small oral intake. Langenbeck et al propose a theoretical framework to predict plasma Phe concentrations[18]. In this report the effect of protein synthesis, Phe catabolism, residual PAH activity, capacity of transamination of Phe to phenylpyruvic acid and the steady state blood Phe concentration were taken into account. Further research on the fate of ingested Phe is necessary to be able to give a more precise prediction of blood Phe concentration resulting fom a certain Phe intake.

CONCLUSION

The results of the study suggest that an incidental extra intake of 100% of the usual individual daily Phe intake does not result in a larger variation of blood Phe concentrations than normally seen without intervention. Even if a higher blood Phe concentration occurred after loading, it rapidly vanished and blood Phe concentrations reverted to values normally observed in well controlled adult patients. In order to be able to predict what acceptable fluctuations in Phe intake are, more fundamental research concerning the fate of the ingested Phe in PKU patients is warranted.

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CHAPTER 5

General discussion, summary and future perspectives

Dietary treatment of PKU was first described in 1954. Dr. Horst Bickel, a German physician, working in England, was the first person who, in consultation with the British scientist, Dr. Woolf, prescribed a low phenylalanine formula to treat a child with PKU. A positive effect of dietary treatment in PKU patients on the outcome of the infants became undeniable. At the same time it became clear that the diet had to be adjusted for each patient. The optimal Phe intake was shown to be dependent on the individual Phe tolerance, age, and the aimed blood Phe concentration. Dietary treatment turned out not to be just "a certain amount of Phe", Phe tolerance varied from child to child, and from time to time in the same individual. A better comprehension of the biochemistry, genetics and molecular basis of the disease, as well as the need for easier treatment led to the development of additional or replacing treatments, but these are still experimental.

The basic principals of the dietary treatment have not changed since the first description. The amount of Phe containing natural protein in the diet is reduced markedly, while amino acid mixtures with all amino acids but Phe are given as supplements to meet protein requirement. Important improvements have been made in the composition of the supplements regarding micronutrients, fatty acids and other components. The amino acids have been made more user-friendly by choice of flavouring and shape, e.g. bars, drink mix, tablets etc. The range of low protein food items and recipes has increased but it is still difficult to meet the consumer's wishes in the present ready-to-eat culture.

Although the basic principles of the diet in PKU are clear, restriction of Phe while simultaneously assuring sufficient intake of all essential nutrients is not very user-friendly and many questions remain. This thesis focuses on different aspects of the dietary treatment of patients with PKU. The first question concerns the total protein need of patients with PKU. It was unclear if patients with PKU have the same protein requirements as healthy controls. The second question concerns daily and weekly variation of blood Phe concentration, and the influence of Phe intake on the blood Phe concentration. This information is necessary when evaluating a single Phe concentration. In daily practice changes in dietary advice are based on single measurements of the blood Phe concentration. In order to prevent unnecessary changes in the diet it is important to have an understanding of the variation in blood Phe concentrations as well as the effect of the intake on these concentrations in PKU patients.

In **chapter 2a** we investigated the question whether the protein requirements in adults with PKU are different from those of healthy controls. We studied whole body protein metabolism in adult PKU patients and healthy controls at the RDA (recommended daily allowance) rate of protein intake. Stable isotopic technique was applied to measure protein turnover, with [1-^{13}C]-valine as a tracer. Normal protein was given to controls whereas PKU patients received a combination of neutral protein and Phe-free amino acid mixture. No significant differences were observed between PKU patients and controls in preprandial and prandial rates of valine appearance

and oxidation, protein breakdown, protein synthesis and net protein balance. We concluded that whole body protein metabolism in adult PKU patients is comparable to healthy controls at the RDA level of protein intake. Our results do not support the recommendation to prescribe a protein intake in adult PKU patients higher than the RDA, as suggested from studies in children with PKU and reflected in the Guidelines of the Medical Research Council at the Ross Metabolic Nutrition Support System. Our results are important for the increasing group of adult patients, as amino acid supplements are difficult to take and very expensive.

Protein intake is a main determinant for growth. Restriction of protein intake during infancy carries the risk of negative effects on growth of height and head circumference. Protein intake of PKU patients consists of two components, i.e. the natural protein and the Phe-free amino acid substitute. In **chapter 2b** we evaluated the relation between growth and the two components of protein intake. Data from 174 Dutch PKU patients, born between 1974 and 1996, were collected from the metabolic units of 7 University Medical Centres in The Netherlands. Growth was assessed during the first three years of life. We found that the total protein intake and the natural protein intake had a significant relation with the increase in head circumference, but not with increase in height during the first three years of life. Head circumference growth was affected by the intake of natural protein rather than total protein, and not by the intake of protein from the protein substitute. Head circumference growth is a reliable reflection of brain growth and is therefore an important indicator for later psychomotor development. The finding that increase in head circumference was not influenced by total protein intake might indicate that the total amount of protein given to these patients was sufficient. The natural protein, part of natural foodstuffs given to these patients, only contributes to a small amount of total protein intake. That the head circumference growth was related to the intake of natural protein might have two explanations. Firstly, the amino acid supplement might be deficient in a particular essential amino acid. Secondly, it might be that a specific component of the natural food itself and not the natural protein was essential for growth. Food contains more than only protein, and therefore other factors in the natural nutrition such as essential fatty acids, or minerals might have caused the relation with increase in head circumference. More studies are needed, to investigate whether the protein substitute is deficient in specific essential amino acids and to ascertain whether the protein substitute does not provide all other essential components of nutrition. At the same time caution is warranted with regard to increasing the total protein intake in patients with PKU as excessive protein intake in early life is related to a number of disorders in later life like obesity, renal dysfunction and even possibly reduced intellectual outcome.

The studies described in chapter 2 were conducted while giving a combination of natural protein and Phe-free amino acid substitute in each feeding. Distributing amino acid substitution over all feedings during the day is very cumbersome for patients. The amino acid substitute has a very bad taste and smell. It makes taking part in a normal daily life complicated. The question therefore arose if it is necessary to divide

the amino acid substitution over different feedings, or if it might be given in one or two feedings a day. Previous studies indicated that an equal distribution of the amino acid supplements is of more importance than equal daily distribution of the natural protein [1-7]. However, the results of these studies were not unanimous, as is also clear from the Cockrane review of 2000 [8]. In the chapters **3**, therefore, we studied if other strategies of amino acid substitution negatively affects outcome variables including blood Phe concentrations.

In **chapter 3a** we studied a new approach to breast-feeding in infants with PKU. We analysed the effect of two different feeding regimes, bottle feeding and a combination of breast-feeding and bottle feeding, on metabolic control and growth during the first 6 months of life in infants with PKU.
It was previously assumed that it is not safe to breast-feed the newly diagnosed PKU infant without monitoring the amount of human milk given by weighing the expressed milk or by weighing the baby before and after drinking. We studied two groups of infants: one completely bottle fed and one breast-fed in combination with Phe-free bottle feeding. In the breast-fed group, feedings alternated between breast-feeding and Phe-free bottle-feeding. The numbers of breast-feedings were adapted to the blood Phe concentrations. We observed no significant differences in metabolic control or growth. A combination of breast-feeding and Phe-free bottle-feeding, given in alternation can therefore be advised. Using this strategy, all mothers were encouraged to continue breast-feeding their child. At four week of age the number of breast-feedings in all infants was 50% or more of the total amount of feeding, probably due to the low Phe-content in breast-milk. Given the important impact of the continuation of breast-feeding, especially in a child with a chronic disease, we recommend the continuation of breast-feeding up to 6 months in infants with PKU.

In **chapter 3b** we further analyzed the influence of the distribution of the Phe intake on the diurnal fluctuations in blood Phe concentrations in infants. Seven infants, aged 3-8 months, were studied. The results showed no difference in blood Phe concentrations taken before each feeding with either feeding regime, i.e. natural protein and Phe-free protein supplement, distributed either equally or unequally over the feeding moments. Therefore, the recommendation to give the breast-feeding and the Phe-free substitute in alternating feedings is warranted, thereby making the breast- feeding a more natural process. The study also showed that the fasting Phe concentration taken in the morning is not the best indicator of metabolic control. Samples taken in the early morning are the highest of the day. If the concentration in the morning is around or below the minimum target of 120 micromol/L, almost all concentrations over the day are likely to be below the minimal target concentration. Therefore, a higher minimal concentration in the morning should be advised, or another sampling time at the end of the day should be chosen.

In **chapter 4** the relation between diet and metabolic control in older patients with PKU was analysed. The amount of phenylalanine that can be consumed by patients

with PKU while keeping the blood concentrations within the target range varies considerably. Most likely this is largely due to the residual activity of the enzyme phenylalanine hydroxylase.
It is unknown how Phe tolerance (i.e. the amount of Phe per kg body weight per day that a patient can tolerate without generating blood Phe concentrations above the highest target concentration) changes with age. Since the paper of Güttler in 1980 the dietary Phe tolerance at 5 years is taken as an important indication of PAH enzyme capacity. In **chapter 4a** we investigated whether Phe tolerance at an earlier age could predict the Phe tolerance at a later age. The results showed that the tolerance at 2 years of age is a good predictor of the tolerance of Phe at 10 years of age. As expected, the results also showed that Phe tolerance decreased logarithmically with age, where the correlation of the pre-treatment Phe concentration with Phe tolerances at any age was weak. Further study of patients ≥ 20 years of age is advisable in order to ascertain whether tolerance changes after 20 years of age due to cessation of growth, hormonal changes or changes in body composition.

During the last two decades the target ranges for blood Phe concentrations in PKU treatment have been lowered and the frequency of blood sampling has been increased. In our centre the frequency of outpatient visits and blood sampling varied with the age of the patients from fortnightly during the first year of life to once every 3 months in adults. In 2001 we made three changes in the treatment schedule. We decreased the number of outpatient visits, introduced blood sampling at home and transferred responsibility for blood sampling and interpretation of the results to the patient (parents). Up until then, the measured blood Phe concentration resulted in a dietary advice of the dietician to the patient and/or caretaker. From that time onwards, patients (parents) were informed about the measured blood Phe concentrations by the nurse or secretary of the PKU treatment team, but were not given advice as to how to adapt the diet. The dietician was available for consultation, upon request by the patient or parents themselves. In **chapter 4b** we evaluated the effect of these changes in treatment on metabolic control. At the beginning of the study the dietician instructed the patients and caretakers how to interpret the results and how to adjust the diet. A small increase in blood Phe concentration was observed at almost all ages when the self management was introduced. The frequency of concentrations outside the target range however was not significantly different. At the same time parents and caretakers were pleased with the reduced number of hospital visits. Blood taking at home was well accepted. It is worthwhile to further evaluate this type of self management.

The diet of PKU patients consists of a strongly limited amount of natural protein and protein substitution with a Phe-free amino acid supplement. The amount of natural protein i.e. of Phe is crucial, and patients and/or caretakers have to know the amount of Phe they consume when eating protein containing food. There are two ways to realize a certain Phe intake, measuring and estimating. In measuring, the patient and/or caretaker weighs all protein-containing food items, checks the Phe content of each

product and so calculates the Phe intake as exactly as possible. Another frequently practised method is estimating the weight and Phe content of a food product, based on previous experiences. This second method is less cumbersome for the patient, but may be less accurate. In **chapter 4c** we investigated the relationship between the method applied to realize a certain Phe intake and the metabolic control in PKU patients. A questionnaire was sent to 327 Dutch patients with PKU (age 0-29 years) asking which method was used to realize a certain Phe intake and also documenting the resulting blood Phe concentration. For the analysis we divided the patients into three groups according to age, below 10, from 10 to 16 and older than 16 years. Of the 188 patients who returned the questionnaire, 75 used the exact measurement, 75 estimated the intake and 38 used both methods. The number of patients that estimated Phe intake increased with age. During childhood exact measurement was used more frequently, while from adolescence on estimation was the preferred method. The method of realizing a certain Phe intake did not result in a statistically significantly different Phe concentration in any of the age groups. Not surprisingly, the mean blood Phe concentration increased with age. From this study we concluded that both estimation and exact measurement of Phe intake are reliable methods to obtain optimal metabolic control. Therefore, patients should be instructed both to use the exact measurement method and the measurement of estimation. Patients and/or caretakers can then use the method they feel most comfortable with, in different situations. Having adequate knowledge of the method of estimation will make it easier for PKU patients to take part in outdoor activities.

The metabolic control of PKU patients is almost always based on infrequently taken single measurements of blood Phe concentrations. The dietary changes are based on these measurements. Little is known about the day-to-day and week-to-week fluctuation of Phe concentrations in adult PKU patients. It is important to know the fluctuations as dietary adaptations might either be made too frequently or not frequently enough. In **chapter 4d** we performed a longitudinal study in 6 adult PKU patients. The study was divided into 5 periods of 7 days. In period 1 patients kept a constant diet with regard to energy, protein and amino acid substitute. A fasting blood sample was taken for determination of the Phe concentration every morning in all study periods. In the four following 7 days periods the diet was equal to the first week of the study, but on the third day an extra dose of 100% (period 2 and 3) or 200% (period 4 and 5) of the daily Phe intake was added to the evening meal. The extra dose was taken as encapsulated Phe powder. The results of this study show that Phe concentrations vary considerably from day to day and from week to week, without any change in the diet. An incidental 100% extra intake of the allowance did not affect Phe concentrations in the days thereafter. In week 4 the 200% extra intake of Phe did not have a significant effect on the blood concentration, but in week 5 there was a significantly higher Phe concentration the days after the second extra dose. The results of this study clearly indicated a rather wide variation of Phe concentration, even if the dietary intake is constant. At the same time, patients with reasonably good metabolic control had all their Phe measurements within the target range. In patients

at the border of the target range a number of samples showed values outside the target range. The study also showed that an incidental doubling of the Phe intake does not lead to disturbed metabolic control. Intake of twice the allowance might lead to increased Phe concentrations, especially when initial concentrations were in the higher zone of the target range.
Our results are important for a number of reasons. First, when a concentration is within the target range, possibly only with the exception of the upper zone of the target range, adaptations in the diet are not necessary. Secondly, an incidental extra intake of Phe has no consequences for the blood Phe concentration. Therefore it is not necessary when an incidental extra intake is consumed for the patient to reduce Phe intake the day thereafter. This makes dietary self management for PKU patients more acceptable.

The aim of this thesis was to provide additional new evidence to the merits of the dietary treatment and to give tools to the patient to obtain better outcomes with less effort and more individual choices. Fine tuning a treatment that has been applied successfully for over half a century was and is challenging but essential to improve the quality of life of PKU patients. Many aspects of treatment can be still improved. Measuring the effects is crucial to evaluate changes in treatment strategies. Evaluation of metabolic control is an important part of evaluation but quality of life is equally important to measure. For this purpose we recommend validated instruments, specially adapted to this disorder.

New developments and future research concern a variety of topics and those of importance for the dietary treatment, quality of life as well for improvement of metabolic control will be mentioned in the following paragraphs.
In 2005 Bilginsoy investigated several options that are potential improvements for better metabolic control [9]. One of the options that was mentioned, is the possibility of measuring blood samples at home with a device capable of measuring blood Phe concentrations. This makes possible more often and timely feedback at moments that a patient wishes to know the blood Phe concentration. Currently, the protocol for monitoring blood Phe concentrations requires periodic blood sampling by the patient, followed by laboratory analysis of the sample and a report back to the patient, generally within four to five days. In the publication of Wendel in 1996 the development of a home-monitoring device for blood phenylalanine is mentioned, but such a device is still not available at this moment [10].
The aim of treatment in PKU is to achieve normal neuro-psychological outcome together with optimal socio-psychological outcome, resulting in a good quality of life. In research and daily care most attention has focused on the technical aspects of the treatment and the effect on intellectual and neuro-psycological outcome. Less attention has been paid to quality of life related issues. Only a few studies have dealt with emotional well-being, the psychological aspects of the treatment. Daily care is very often characterized by the lack of support of psychologists. Evaluation of the advantages of specialists (psychologists) participating in the PKU treatment

team, in terms of quality of life and economically is warranted. More attention should also be paid to the fact that only few adult physicians are dedicated to continuing care of the adult patients. There is an urgent need for physicians who will deal with centralized care of adults with Phenylketonuria[11]. Attention for adult PKU patients is still scarce and mostly focused on the care for the women with PKU in child baring age to prevent maternal PKU.

Future developments in the dietary treatment concerning the amino acid supplements might aim at a reduction of the number of times that the patient has to take the supplement. More slow releasing products that have to be taken only twice a day are necessary to achieve this. While the taste and smell of the amino acid supplements have been improved, they are still unmistakable to others, with all the social drawbacks that this entails. The development of new products (formula, flavoured shakes, bars, tablets) for the amino acid supplementation is aimed to facilitate dietary adaptations for special needs such as age and pregnancy, and to improve user friendliness and taste. In this regard it is remarkable that these flavoured products are not suitable for the young age group due to legislative restrictions. However studies on development of taste in infancy have shown that an important factor influencing this process of flavour preferences is early and repeated exposure[12-14]. Most taste preferences are acquired early in life. The study of Owada 2000 concluded that in some children with PKU the characteristic taste of the amino acid formula encountered in early life is considered to be imprinted and remains as a preference for a long time [15]. This could explain why older patients are not capable of switching to new amino acid supplements. Changes in legislation are therefore very important as it is difficult to convince parents that a product is suitable at an earlier age than that recommended by the manufacturer. The development of a product free of taste and smell will be a valuable advance for the group of patients who encounter difficulties with the amino acid supplementation because of its taste and smell.
In the ready-to-eat generation, cooking skills are minimized and basic knowledge of the composition of food products is in general poor. This makes instruction for parents and patient time consuming, but crucial to provide the PKU patient with attractive and varied menu. Better availability of a wide variety of low protein food products will contribute to this. Adequate resources to support dietary instruction, product knowledge and diet cooking skills are essential in order to make a patients and parents more capable and improve self treatment from both a metabolic and quality of life point of view.

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Nederlandse samenvatting voor de geïnteresseerde buitenstaander

Dieetbehandeling van Phenylketonurie:
van ervaringsdeskundigheid tot wetenschappelijke onderbouwing

Inleiding

Wat is Phenylketonurie (PKU)?

Een stukje geschiedenis

In 1934 beschreef de Noorse arts en scheikundige Følling twee kinderen die zich niet goed ontwikkelden, vooral geestelijk niet. Deze twee kinderen (een broertje en een zusje) hadden beiden blond haar, blauwe ogen en een muf ruikende urine. De urine vertoonde een hoog gehalte aan phenylketonen, de afbraakproducten van phenylalanine (Phe). De oorzaak hiervan bleek een stofwisselingsziekte te zijn waarbij Phe onvoldoende wordt afgebroken. Dit leidt tot een te hoog Phe-gehalte in het bloed, met ernstige hersenbeschadiging resulterend in sterk achterblijvende geestelijke ontwikkeling. Phe is een van de 23 aminozuren waaruit eiwitten zijn opgebouwd. Na jaren van onderzoek lukte het de Duitse kinderarts Bickel in 1954 een behandeling te ontwikkelen, gebaseerd op een nieuw dieetpreparaat. De aanpak is eenvoudig en doeltreffend: beperk het aanbod van de stof Phe in de voeding. Dit betekent dat de hoeveelheid gewoon eiwit in de voeding beperkt moet worden omdat Phe voorkomt in alle voedingseiwitten, en worden vervangen door een eiwitpreparaat zonder Phe. Deze beperking van het zogenaamde "natuurlijke eiwit" met de toevoeging van het eiwitpreparaat zonder Phe, zorgt ervoor dat er minder Phe in het bloed komt. Nu kon men de ziekte wel behandelen, maar de ziekte werd in die tijd pas vastgesteld als het kind al onherstelbare schade had ondervonden van de ziekte.

De ziekte

Bij gezonde kinderen en volwassenen zorgt het enzym phenylalanine-hydroxylase ervoor dat het aminozuur Phe in het lichaam wordt omgezet in het aminozuur tyrosine. Bij patiënten met PKU gebeurt niet of nauwelijks door een tekort aan dit enzym met als gevolg een sterk verhoogde Phe concentratie in het bloed. PKU is een erfelijke stofwisselingsziekte die zoals de meeste erfelijke stofwisselingsziekten een autosomaal recessief overervingspatroon kent. Dit betekent dat beide ouders drager zijn van de ziekte, maar de ziekte zelf niet hebben. Met een kans van 1 op 4 bij elke zwangerschap zullen hun kinderen de stofwisselingsziekte wel hebben, door van allebei de ouders de eigenschappen voor de ziekte te erven. De kans om de ziekte te krijgen is voor jongens en meisjes gelijk.

Screening

Sinds 1974 wordt in Nederland, in navolging van veel westerse landen bij alle zuigelingen door middel van de hielprik gescreend op PKU. Daardoor is het mogelijk deze stofwisselingsziekte te behandelen vóórdat het teveel aan Phe de geestelijke ontwikkeling van het kind blijvend schadelijk heeft kunnen beïnvloeden. Wanneer de behandelingsvoorschriften goed kunnen worden opgevolgd zal het kind zich als ieder ander kind ontwikkelen.

De behandeling

De eerder genoemde behandeling met speciale voeding naast de strenge beperking van het zogenaamde "natuurlijke eiwit" is erg belastend. Het ligt voor de hand om

als alternatief het enzym, dat bij PKU niet of onvoldoende werkt, als medicijn te gaan geven. Een dergelijk medicijn is echter niet voorhanden. Er zijn weliswaar alternatieven voor de behandelingen in onderzoek, zoals gentherapie, maar deze zijn nu nog niet toepasbaar.
De enig mogelijke behandeling blijft vooralsnog de dieetvoeding, waarin de aanwezigheid van het aminozuur Phe, onderdeel van elk eiwit, wordt beperkt. Het menselijk lichaam heeft Phe echter ook nodig voor eigen eiwitopbouw voor celweefsel. Het dieet kan daarom ook niet Phe vrij zijn en moet zo zijn samengesteld, dat het kind een geringe, maar precies de juiste hoeveelheid binnenkrijgt. Of deze hoeveelheid goed is wordt steeds gecontroleerd door het meten van de Phe-concentraties in het bloed. De hoeveelheid Phe die een PKU patiënt kan verdragen is individueel sterk verschillend. Dit wordt beïnvloedt door de ernst van de PKU, groeisnelheid, zwangerschap, lactatie en ziekte. De Phe-concentratie kan zowel uit een buisje bloed als een "bloedspot" op filtreerpapier worden bepaald. Ouders worden geïnstrueerd zelf thuis bloedmonsters af te nemen en per post te versturen. De Phe-concentraties kunnen op deze wijze frequent gecontroleerd worden en de dieetbehandeling wordt hierop afgestemd. Het dagelijkse menu van de PKU patiënt bevat naast het Phe vrije eiwitpreparaat, dat minimaal drie maal per dag moet worden gebruikt, veel eiwitarme dieetproducten zoals eiwitarm brood, eiwitarme biscuits en koekjes, eiwitarme pasta's en rijst. Dit is de basis voor een volwaardig menu. De kleine hoeveelheid Phe bevattend natuurlijk eiwit wordt in het menu grotendeels gebruikt als fruit, groentes, aardappelproducten en een beperkte hoeveelheid versnaperingen. De hoeveelheid natuurlijk eiwit die verdragen wordt is individueel verschillend. Het varieert tussen de 5 en 15 gram eiwit per dag, dit is slechts 5 – 10 % van wat de gemiddelde Nederlander gebruikt. Eiwitrijke voedingsmiddelen zoals vlees, kaas, ei, melkproducten en gewone graanproducten kunnen niet of nauwelijks in het dagelijks menu gebruikt worden.

Het onderzoek
De dieetbehandeling die direct na de diagnose wordt gestart, bestaat uit het zeer sterk beperken van Phe en het voorschrijven van een Phe-vrij eiwitpreparaat (aminozurenmengsel). Dit wordt verrijkt met vitamines en mineralen om zo de tekorten uit de voeding te compenseren. De hoeveelheid gewoon eiwit die verdragen wordt is individueel sterk verschillend. Het wordt vastgesteld door de voeding steeds aan te passen aan de gemeten Phe-concentratie in het bloed. Het streven is deze concentratie binnen bepaalde grenzen te houden, waarbij geen schade wordt verwacht. Wanneer de gemeten concentratie boven die grens ligt word de hoeveelheid Phe houdend natuurlijk eiwit verminderd en de hoeveelheid Phe vrij eiwitpreparaat verhoogd. Ligt de gemeten concentratie onder de ondergrens gebeurt het omgekeerde.

Voorspelling van de tolerantie
Wanneer de diagnose PKU is gesteld willen ouders weten wat dit voor het latere leven van hun kind gaat betekenen. In een studie onder Nederlandse PKU patiënten hebben we gekeken of je al op jonge leeftijd een voorspelling kan doen over de

tolerantie (de hoeveelheid Phe die wordt verdragen met goede Phe concentraties in het bloed) op latere leeftijd. In eerdere studies is de leeftijd van 5 jaar naar voren gekomen. Uit onze studie bleek dat op de leeftijd van 2 jaar een goede voorspelling kan worden gedaan voor de tolerantie op de leeftijd van 10 jaar.

Borstvoeding
De afgepaste hoeveelheid Phe die het kind kan verdragen wordt bij de zuigeling gegeven in de vorm van moedermelk of normale flesvoeding. Daarnaast wordt als voeding een Phe-vrije zuigelingenvoeding gegeven. De keus de hoeveelheid Phe in het dieet exact af te meten heeft aanvankelijk geleid tot het advies geen borstvoeding te geven bij PKU. Later werd dit bijgesteld: de toegestane hoeveelheid moedermelk kan worden afgemeten door af te kolven of door het kind voor en na de voeding te wegen. Een andere methode is een afgepaste hoeveelheid Phe-vrije flesvoeding te geven, direct gevolgd door drinken aan de borst tot het kind verzadigd is. Wij onderzochten een methode waarbij de borstvoeding en de Phe-vrije zuigelingenvoeding afwisselend worden gegeven, niet gecombineerd binnen één voeding. In dit onderzoek werden de groei en de wekelijks gemeten Phe-concentraties van de groep gevoed volgens de nieuwe methode vergeleken met een even grote groep kinderen gevoed met uitsluitend flesvoeding. Deze flesvoeding bestond uit een combinatie van Phe-vrije voeding en normale zuigelingenvoeding. Het onderzoek liet geen verschillen zien in groei en de gemeten Phe-concentraties.

Schommelingen in de Phe-concentratie
Hierna hebben we onderzocht of de schommelingen in de Phe-concentraties binnen 24 uur bij deze jonge kinderen groter was wanneer je de gewone voeding en de Phe-vrije voeding afwisselend gaf. Er bleek geen verschil te zijn met de methode waarbij de Phe-vrije en de Phe-bevattende voeding tegelijk werden gegeven.

Eiwitbehoefte
Een andere vraag waar, zowel nationaal en internationaal, nog niet eenduidig op kan worden geantwoord is: Wat is de optimale hoeveelheid eiwit voor de patiënt met PKU?
Het is niet bekend hoeveel eiwit, afkomstig uit het Phe vrije eiwitpreparaat, het beste gebruikt kan worden bij PKU. Een te hoge dosering van het synthetisch Phe-vrije eiwitpreparaat heeft twee grote nadelen: economisch (het mengsel is duur) en sociaal (het mengsel ruikt en smaakt slecht en moet meerdere keren per dag gebruikt worden). Met ons onderzoek naar het eiwitmetabolisme bij volwassen PKU patiënten, is er weer een stap gezet naar een antwoord op deze vraag. In de studie zijn onder vergelijkbare omstandigheden PKU patiënten en gezonde proefpersonen gemeten. De resultaten van de PKU patiënten zijn vergeleken met die van gezonde controle personen. In de opzet en uitvoering van de studie bestond het enige verschil tussen de twee groepen uit het soort voeding: gewoon eiwit voor de gezonde proefpersonen en Phe-vrij eiwitpreparaat voor de PKU patiënten. In de resultaten waren geen verschillen te zien tussen het basale eiwitmetabolisme van PKU patiënten

en dat van gezonde personen. Basaal is de eiwitbehoefte van PKU patiënten gelijk aan die van gezonde personen.

Groei
Omdat eiwit een belangrijke rol speelt bij de groei hebben we ook gekeken naar het verband tussen eiwitinname en groei. Hiervoor is een analyse gemaakt van de verschillen in groei en eiwitinname tussen een groep Nederlandse en een groep Amerikaanse kinderen met PKU. Voor het meten van de groei zijn zowel de lengte als de hoofdomtrek gegevens gebruikt. Uit het onderzoek bleek dat niet zozeer de totale hoeveelheid eiwit, maar vooral de hoeveelheid natuurlijk eiwit in het dieet invloed heeft op de hoofdomtrek.

In het kader van dit proefschrift is verder een drietal onderwerpen onderzocht dat de praktische uitvoering van de behandeling betreft:

Home monitoring
Het UMCG is in 2001 overgegaan op het thuis afnemen van bloedmonsters voor de Phe-bepaling. Op deze manier kan de Phe-concentratie frequenter gemeten worden. Ouders en patiënten is uitgelegd hoe ze zelf de Phe-uitslag kunnen beoordelen en het dieet kunnen aanpassen wanneer de uitslag niet binnen de gewenste range is. Eerder was het gebruikelijk voor de bloedafname naar het ziekenhuis te komen. Bij de uitslag werd dan ook informatie gegeven over de gewenste dieetaanpassing. Meer zelf sturen in de behandeling kan een manier zijn om beter ingesteld te zijn. Dit is van belang omdat de gemeten Phe- concentratie, met het ouder worden van het kind met PKU, vaker boven de gewenste range is. We hebben onderzocht of Phe-uitslagen van de Groningse patiëntengroep in de nieuwe situatie verschilden van de oude situatie. De resultaten gaven aan dat het aantal metingen veel groter was geworden maar dat het aantal buiten de gewenste range niet groter was geworden. Wel waren de uitslagen gemiddeld iets hoger.

Meer en minder
Effectmeting van dieetaanpassingen gebeurde tot nu toe op volwassen leeftijd weinig frequent. Hierdoor hadden de patiënt en de behandelaar ook weinig inzicht in de gevolgen van het afwijken van het dieet. Thuisprikken maakte het mogelijk een studie te doen naar de variatie van Phe in het bloed van dag tot dag, van week tot week en na het innemen van extra Phe, zoals gebeurt bij dieetoverschrijding. Uit de resultaten bleek dat vooral de schommelingen van week tot week groot kunnen zijn, ook als je niets aan het dieet verandert. Er is dus sprake van een natuurlijke schommeling. Ook bleek dat het voor PKU patiënten mogelijk is incidenteel extra Phe in te nemen, waarbij ze toch goed ingesteld blijven. Dat wil zeggen dat ze geen of slechts kort hogere Phe concentraties hebben na extra inname. Wel is het van belang dat de Phe concentratie gemiddeld steeds ruim onder de maximale concentratie is die wordt aanbevolen.

Meten, wegen en schatten
Voor de praktische uitvoering van het dieet leren ouders en patiënten gebruik te maken van een weegschaal en een maatbeker. Echter in de praktijk blijken ervaren patiënten te leren "schatten", zij meten op het oog de hoeveelheden zonder weegschaal en maatbeker. In de studie hebben we gekeken hoe ouders en patiënten in de praktijk het dieet uitvoeren. Daarbij was de vraag of er een verschil is in de Phe-concentratie, al naar gelang de patiënt de methode schatten of wegen gebruikte. Dit bleek niet te verschillen en we adviseren daarom patiënten beide methoden aan te leren.

Toekomst
Een optie die vaak wordt genoemd ter verbetering van de behandeling van PKU, is de mogelijkheid om thuis niet alleen bloed af te nemen maar ook daadwerkelijk de Phe concentratie te meten. Naast het vaker kunnen meten zou zo'n meter het mogelijk maken om direct te meten op de momenten waarop de patiënt zelf zijn bloed Phe concentratie wil weten. De ontwikkeling van een meter om de bloed Phe concentratie thuis te meten is al lange tijd in ontwikkeling. In publicaties van meer dan 10 jaar geleden wordt het al genoemd. Het is technisch lastig gebleken om betrouwbaar te kunnen meten. Een financieel nadeel is dat er vergeleken met bijvoorbeeld bloedsuikermeters of bloeddrukmeters slechts weinig patiënten gebruik van zullen maken, PKU is een veel minder voorkomende ziekte, terwijl de ontwikkel- en productiekosten van zo'n meter hoog zijn.
Naast de zorg voor de fysieke gesteldheid van de PKU patiënt wordt er veel onderzoek gedaan naar de effecten van de ziekte op het psychosociaal functioneren. Het welbevinden van de patiënt, zijn toekomstverwachting en kwaliteit van leven zijn minstens zo belangrijk als zijn fysieke gesteldheid als maat voor de behandeling. Opmerkelijk is dat er slechts weinig geld beschikbaar is om de psychosociale ontwikkeling en functioneren regelmatig in kaart te brengen en zonodig met aanvullende zorg te ondersteunen als dit gewenst is. Het is bekend dat PKU patiënten op latere leeftijd in het psychosociale functioneren vaker problemen tegenkomen dan de gemiddelde leeftijdsgenoot. Een psycholoog zou daarom deel moeten uit maken van ieder PKU behandelteam, om dit tijdig te signaleren en de benodigde aansturing te geven.
De tijd is voorbij waarin PKU een "kinderziekte" was, 18 jaar na de start van de screening is de groep patiënten van 0-18 jaar stabiel in grootte geworden. Echter de groep volwassen PKU patiënten en ook andere aangeboren stofwisselingsziekten zal blijven groeien met een snelheid gemiddeld gelijk aan het aantal kinderen dat nieuw gediagnosticeerd wordt. Zo moeten zich nieuwe centra ontwikkelen waar internisten in plaats van kinderartsen deel uitmaken van het behandelteam.
Verder onderzoek zal gaan bijdragen aan de ontwikkeling van andere typen aminozuurmengsels. Het is nu nog nodig om meerdere malen, minimaal drie maal per dag dit supplement te gebruiken. Een product wat langzamer door het maagdarm kanaal wordt verwerkt kan het mogelijk maken dit te verminderen tot 2 maal per dag.

De slechte geur en smaak van het aminozurenmengsel is vergeleken met de eerst ontwikkelde producten al sterk verbeterd, maar blijft nog steeds erg opvallend voor de omgeving van de patiënt. Een belangrijke volgende stap in plaats van het toevoegen van zeer sterke smaak corrigens, is de ontwikkeling van een smaak- en geurloos product, dat binnenkort verkrijgbaar wordt.
In onze huidige fast food en kant-en-klaar maatschappij zijn basis kooktechnische vaardigheden niet meer vanzelfsprekend. Kennis over de samenstelling van onze voeding (waar is het van gemaakt?) ontbreekt steeds meer. Dit maakt dat het aanleren van kookvaardigheden en het verkrijgen van productkennis voor ouders en patiënten met PKU veel tijdsintensiever is geworden. Het is bij PKU echter cruciaal om in staat te zijn een aantrekkelijk en gevarieerd menu te kunnen samen te stellen. Het is een uitdaging om ziektekostenverzekeringsmaatschappijen te overtuigen van de zin om deze diëtaire kennisoverdracht financieel te steunen, evenals ook de benodigde eiwitarme dieetproducten. Kennis over de ziekte, middelen en vaardigheden om de dieetbehandeling optimaal uit te voeren stellen een patiënt in staat selfsupporting te zijn en zelf de keuzes te maken in de dieetbehandeling.

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ABOUT THE AUTHOR

The author of this thesis was born in Vlaardingen on the 16th of August 1951. Secondary school education (MMS) was completed in 1968 at the "Gemeente Lyceum Vlaardingen". She decided to start something different from her twin sister: in 1972 four years professional education at "Scholengemeenschap de Laan" in The Hague resulted in obtaining the certification of dietician.
Professional career started (and never ended) in the APSAZ: "Algemeen Provinciaal Stads en Academisch Ziekenhuis". Names of the hospital changed to Academisch Ziekenhuis Groningen and most recently to University Medical Centre Groningen. After eight years of working in different departments of the hospital, from 1980 onwards the focus narrowed to paediatrics, which became the most important part of her activities. Metabolic diseases offered great chances to get involved in high tech dietetics. The forum of information exchange expanded from national to international contacts and also became progressively more disease specific. Experience in clinical practice does not constitute scientific evidence, but rather is the source of research questions. The next step was a part time research project under the supervision of Dr. F.J. van Spronsen and Prof. P.J.J Sauer, resulting in this thesis.

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