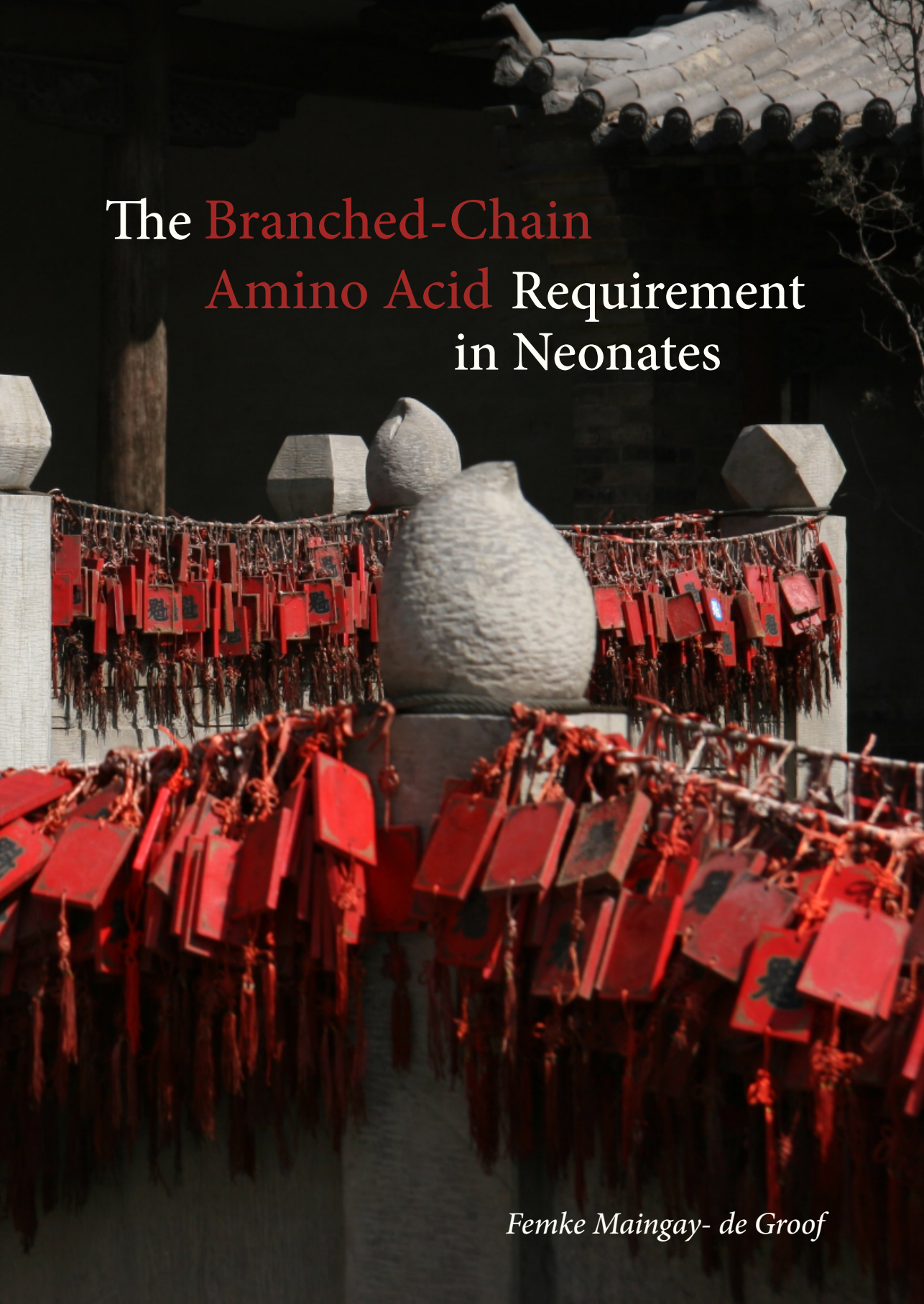

The Branched-Chain Amino Acid Requirement in Neonates

Femke Maingay- de Groof

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De behoefte van de pasgeborene aan vertakte keten aminozuren

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties

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	Prof.dr. A.P. Bos

"Let food be your medicine"
Hippocrates (460-377 BC)

Voor mijn ouders,
Gijs, Ella & Fleur

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Introduction

CHAPTER 1

General introduction, outline and aims of the thesis

INTRODUCTION

Growth during the earliest stages of life is an important determinant of an individual's later health and risk of chronic disease(1). Substantial evidence shows that growth in the first 2 years of life, especially high early weight gain, is associated with adverse health outcomes later in life, including increased blood pressure (2), increased weight gain and body fat deposition (3-6) and increased risk of diabetes (7). Higher protein intake for infants who are formula fed may play a role with these health outcomes because formula-fed children reach a higher body weight and weight for length at one year of age compared to those fed breast milk (8). A lower protein intake in infant formula is associated with lower weight up at 2 years of age (9) which might be beneficial because the slower pattern of growth in breastfed infants might be protective for the development of the metabolic syndrome (10). In preterm infants, however, a higher protein intake in the first month of life correlates with improved neurodevelopment (11, 12). Preterm infants have higher protein turnover rates and protein losses than terms and thereby higher protein requirements (13, 14). Excessive intake of amino acids has been shown to reduce (brain) growth and to influence neurotransmitter concentrations in the brain of rats which might put the developing brain at risk (15). Inadequate amino acid intake impairs protein synthesis, which is pivotal for growth. The growth rate of the preterm infant should mimic at least the growth rate of the intrauterine fetus at the same gestational age (16). Achieving appropriate growth and nutrition accretion of preterm neonates is often difficult during hospitalization because of metabolic and gastrointestinal immaturity and other complicating medical conditions. Many preterm neonates require total parental nutrition for their initial nutritional support but this is associated with several complications, including the increased risk of infection, mucosal atrophy and cholestatic jaundice. Therefore, transition to full enteral feeding and the cessation of TPN are accomplished as soon as feasible and safe, taking in consideration that enteral feeding is associated with a detrimental morbidity like necrotising enterocolitis (17). Enteral nutrition can be initiated immediately after birth by introducing small amounts to enhance the development of the gastro-intestinal tract and for this reason is referred to as "trophic", "priming" or "minimal enteral feeding".

Protein is an important component of adequate nutrition as it provides essential amino acids required for critical protein synthesis and growth. The goal in feeding preterm neonates is to provide the quantity and quality of protein needed to achieve foetal rates of tissue growth and nitrogen accretion. This goal should be accomplished in the context of physiological and metabolic development of the infant in order to avoid accumulation of potentially harmful protein metabolic products (18, 19). Current understanding of the

nutritional needs for early growth and development is fragmentary and inadequate to provide answers that are needed (20).

NUTRIENT NEEDS AND DEFENITIONS

Historically, nutrients are categorized in micronutrients and macronutrients. The micronutrients are minerals and vitamins. The macronutrients include protein, carbohydrates, fat, fiber and water. They supply energy to sustain the body's various functions, including respiration, circulation, physical activity and protein synthesis. Carbohydrates provide energy to cells in the body, particularly the brain which uses glucose as the main energy source. Fat is a major source of fuel for other parts in the body and contributes to the absorption of fat-soluble vitamins and other food components as carotenoids. Via dietary fat intake the essential fatty acids linoleic acid and alfa-linoleic acid are absorbed which the body cannot synthesize. Proteins form the major structural components of all the cells of the body. Along with amino acids, they function as enzymes, transporters and hormones. In addition, rotein binds all kinds of toxic metabolites.

Definitions of optimal intakes of all individual macronutrients and their relative concentrations in complete formulas are areas of active research. A generic model for the protein dietary requirement defines the requirement of the needs of the organism, i.e. metabolic demands, and the dietary amount which will satisfy those needs, i.e. efficiency of utilization, thus: dietary requirement = metabolic demand/ efficiency of utilization (21).
The *metabolic demand* is determined by the nature and extent of those metabolic pathways that consume amino acids and are conventionally identified in most factorial models of requirement as maintenance and special needs (growth, pregnancy and lactation). The *dietary requirement* is the amount of protein or its constituent amino acids, or both, that must be supplied in the diet in order to satisfy the metabolic demand and achieve nitrogen equilibrium. The *recommended dietary intake* is the dietary requirement plus 2 SD: the intake that will satisfy the metabolic needs of 97.5 % of the population.

An other way of defining the optimal intake is to determine the recommended dietary allowance, adequate intake, tolerable upper intake level and estimated mean requirement (22). *The estimated average requirement (EAR*) is the average daily nutrient intake level estimated to meet the requirement of half of the healthy individuals in a particular life stage and gender group. The *recommended dietary allowance (RDA)* is the average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97-98 percent) health individuals in a particular life stage and gender group. The RDA

is intended to be used as a goal for daily intake by individuals as this value estimates an intake level that has a high probability of meeting the requirement of a randomly chosen individual (about 97.5%). The *adequate intake (AI)* is used when a RDA cannot be determined. The AI is the recommended average daily intake based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate. The *tolerable upper intake level (UL)* is the highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the potential risk of adverse effects may increase (Figure 1).

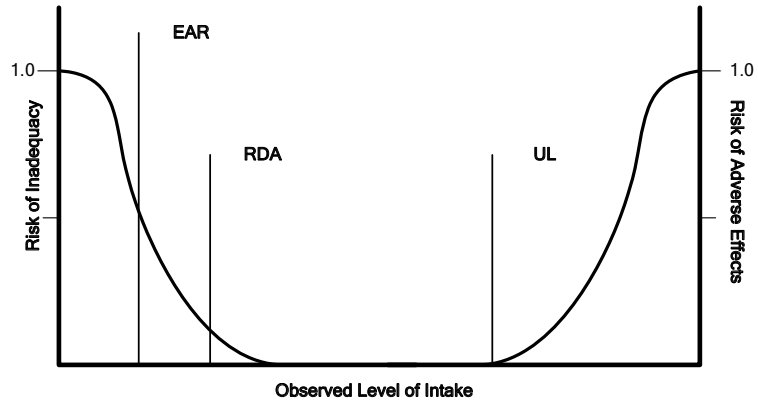

Figure 1: This figure shows that the estimated average requirement (EAR) is the intake at which the risk of inadequacy is estimated to be 50% to an individual. The recommended dietary allowance (RDA) is the intake at which the risk of inadequacy would be very small, only 2-3%. At intakes between the RDA and the tolerable upper intake level (UL), the risk of inadequacy and excess are both estimated to be close to zero. At intakes above the UL, the potential risk of adverse effects may increase (23).

When assessing nutrient intakes for groups, it is important to consider the variation in intake in the same individual, as well as underreporting. The EAR is the appropriate reference intake to use in assessing the nutrient intake in groups, whereas the RDA does not. Assuming a normal distribution of requirements, the percentage of individuals whose intakes is less than the EAR equals the percentage of individuals whose diets are considered inadequate based on the criteria of inadequacy chosen to determine the requirement (22).

The biological basis of protein or amino acid requirements should be defined on the basis of experimental studies. Are a few direct data on the protein requirements for children and infants. For term children in the first 6 months the assumption is made that human milk from a well-nourished mother can be regarded as providing an optimal

intake for the infant (24). The average content of human milk is used to determine an adequate intake (AI). These data can provide some guidance for the composition for infant formula, but gross compositional similarity is not an adequate determinant or indicator of the safety and nutritional adequacy of infant formula (25). Human milk composition shows markedly variation and breastfed infants have a variable milk consumption rate, i.e. they largely regulate the intake they require themselves (26-28). For preterm infants, infants > 6 months and adults, dietary needs have been estimated by the factorial approach which we will discuss next.

METHODS TO DETERMINE REQUIREMENTS

Dietary protein requirements can be estimated by 2 different methods. The first method calculates the daily requirement theoretically, by a factorial approach which is based on estimating the nitrogen (obligatory) losses that occur when a person is fed a diet that meets energy needs but is essentially protein free and, when appropriate, also relies on estimates of the amount of nitrogen that is accreted during periods of growth or lost to mothers during lactation. The second, the empirical approach, measures biochemical or physiological responses to graded intakes. It includes anthropometry, chemical indices like serum albumin, total protein, immunoglobulin, retinol-binding protein and transthyretin and biochemical markers of protein excess such as blood urea nitrogen (BUN), pH, pCO_2 and bicarbonate. They also include nitrogen balance and isotopic studies of whole-body nitrogen kinetics.

Factorial Approach

The factorial approach is based on the assumption that the basal requirements of a component are the same throughout the life cycle and requirements in infants and children are higher than those in adults due to growth. The factorial approach considers the total requirement for a nutrient as the sum of obligatory losses (e.g. urine, faeces, skin), plus the amount incorporated into newly formed tissues (22, 29). This method, originally described for use in humans by Hegsted (30), has been useful for estimating requirements and designing experiments for obtaining definitive empirical data. In this method the obligatory losses (maintenance requirement) are added to the nutrients needed for protein synthesis (growth requirement). The factorial model to define the protein requirements for preterm infants is based on foetal body composition during normal intrauterine development. For the foetus and the preterm infant, protein is the predominant component of the requirements for developing new tissue. Compositional analysis of foetal issues has been a valuable source of data for our understanding of the nutrient needs of the foetus, and by extension, those of the growing preterm infant

(18). Foetal accretion rates of protein have been compiled from compositional analyses of aborted foetuses or stillborn infants (31). In these analyses, foetal nitrogen accretion has been expressed in terms of gestational age (GA) (24 weeks to term) and birth weight (BW) (700-2500 gram). This foetal nitrogen accretion model is limited because of the large variations in the rates of foetal weight gain, particular at early time points.

Nitrogen balance

Historically, descriptive or gross measures like growth and nitrogen balance have been used. In this method an increasing intake of the test amino acid results in an increase in growth rate (usually weight gain) or nitrogen balance until the requirement level is met for the test amino acid, after which there is no further increase in either growth rate or nitrogen balance. Nitrogen balance is the difference between nitrogen intake and the amount excreted in urine, feces, skin, and miscellaneous losses such as breath and sweat. A limitation of the method is the need for a seven day adaptation time to the diet since the body urea pool needs 7 to 10 days to adapt. In addition, intakes of nitrogen are often being overestimated and excretion is often underestimated (32). Since it is considered unacceptable to maintain children or infants on either deficient of excessive intakes for longer periods nowadays, alternative methods were needed. The availability of isotopically labelled tracers made it possible to determine the metabolic fate of a labelled amino acid at varying dietary intakes as will be discussed next.

Isotopic tracer methods

Isotopic tracer methods measure amino acid oxidation, and are based on the principle that the oxidative losses are reversible and need daily replenishment, and that the amino acids provided in excess of the needs of protein synthesis, are preferentially oxidized. A ^{13}C-labelled amino acid is administered enterally or parentally and is distributed throughout the body's free nitrogen pool. Stable isotopes are used: atoms with an extra neutron in the nucleus which slightly increases the mass which can be detected by mass spectrometry techniques. Different techniques using stable isotopes have been developed to determine the requirements of the indispensable amino acids like the direct and indirect amino acid oxidation methods. Isotopically labelled tracers are used to determine the metabolic rate of a labelled amino acid at varying dietary intakes. Amino acids consumed in excess of requirements for protein synthesis are oxidized. Deficient intake of an indispensable amino acid will result in only minimal obligatory losses. As intake increases above the requirement level, the oxidation of the amino acid will also increase linearly. In the *direct amino acid oxidation (DAAO)* technique a known dose of labelled indispensable amino acid, for example [1-^{13}C] lysine is provided to subjects fed graded dietary intakes of lysine with all other dietary factors being constant. The inflection point at which oxidation begins to increase rapidly over the obligatory minimum is referred to as the breakpoint and represents the dietary requirement for that individual (33) (Figure 2).

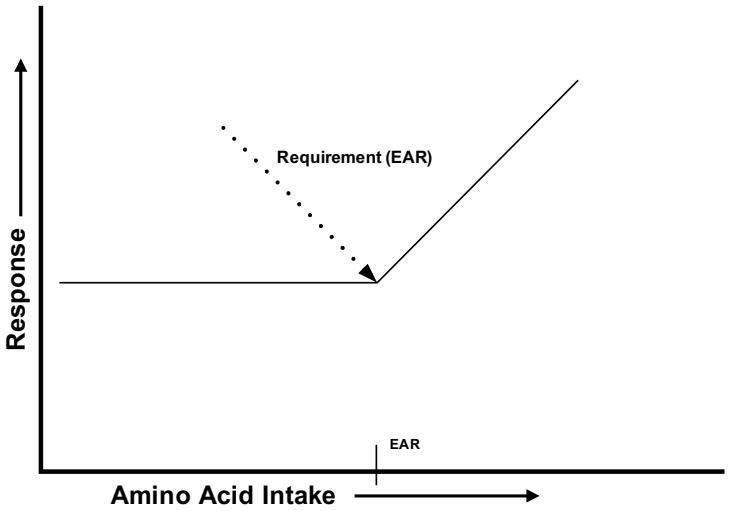

Figure 2: Breakpoint estimation using the direct indicator amino acid oxidation (DAAO) method. Adapted from (37).

A refinement of the direct oxidation technique is the *24h tracer balance (24-h DAAB)* (34, 35). This method uses a daily balance of the amino acid that is measured and determines the requirement level of the test amino acid as the minimum intake at which the estimate of daily balance is zero (36). El- Khoury et al. showed that the short (3-6 h) direct oxidation method was within 5% on the 24 h estimate and thus is a valid representation of daily amino acid requirements (35). Since a 6 d adaptation to the study diet is needed, this method is not applicable to study vulnerable populations like infants.

An alternative approach of estimating indispensable amino acid requirements using amino acid oxidation is the *indirect amino acid oxidation (IAAO)* technique. The main principle of this method is that because there is no storage of free amino acids, deficiency of one essential amino acid will limit protein synthesis. If the tested amino acid is deficient in the diet, this will limit protein synthesis and the indicator amino acid will be oxidized. If the dietary intake of the test amino acid increases, the oxidation of the indicator will decrease until requirement of the test amino acid is met. When intake meets the requirement then protein synthesis occurs at an optimum capacity and the oxidative degradation of all other essential amino acids plateau. The mean requirement or estimated average requirement (EAR) of the test amino acid is identified by this breakpoint (Figure 3) (37-39).

The advantage of the DAAO and IAAO method are the short adaptation time needed to the study diet which makes them applicable to study vulnerable groups such as infants.

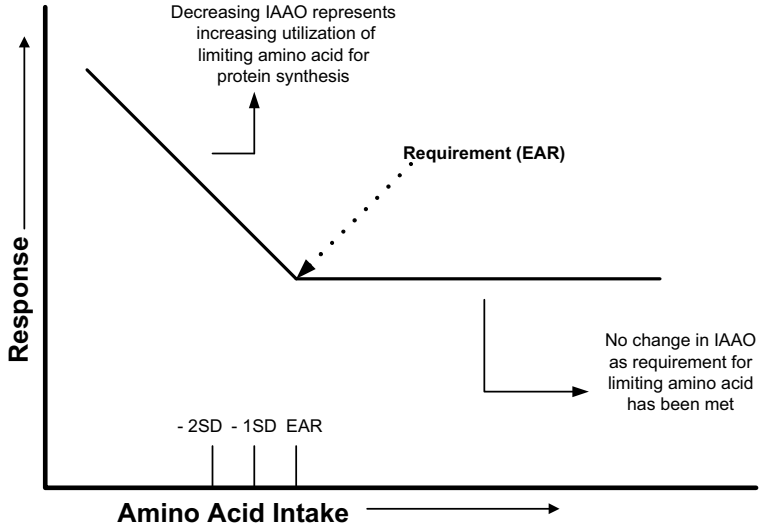

Figure 3: Breakpoint estimation by using the indicator amino acid oxidation (IAAO) method. Adapted from (40).

A limitation of the DAAO method is that it is not applicable to all indispensable amino acids whereas with the IAAO method the requirements of any essential amino acid or conditionally indispensable amino acid can be studied. Thereby there is no dietary restriction on intake of the test amino acid in the IAAO technique as a result of the fact that the requirement of one amino acid is determined by the oxidation of another amino acid. This makes it possible to study all possible dietary levels of essential amino acids.

PROTEIN AND AMINO ACIDS

Protein is the major functional and structural component of all the cells of the body. The defining characteristic of protein is its requisite amino nitrogen group. Proteins are macromolecules consisting of a long chain of amino acid subunits. In the protein molecule, the amino acids are joined together by peptide bonds. In biological systems, the chains formed might be anything from a few amino acids (di-, tri- or oligopeptide) to thousands of units (polypeptide). The sequence of amino acids in the chain is known as the primary structure. A critical feature of proteins is the complexity of their physical structures. Polypeptide chains do not exist as long chains but they fold in a three-dimensional structure. The chains of amino acids tend to coil into helices (secondary structure). Sections of the helices may fold on each other due to hydrophobic interactions between non-polar side chains and, in some proteins, to disulfide bonds so that the overall molecule might be

globular or rod-like (tertiary structure). Their exact shape depends on their function and for some proteins, their interaction with other molecules (quaternary structure) (23).
Amino acids that are incorporated into mammalian protein are α-amino acids, with the exception of proline which is an α-imino acid. This means that they have a carboxyl group, an amino nitrogen group and a side chain attached to a central α-carbon (Figure 4). Functional differences among the amino acids lie in the structure of their side chains. In the body there are 20 amino acids, nine of which are essential. The essential or indispensable amino acids are those which cannot be synthesized by the body. Clas-

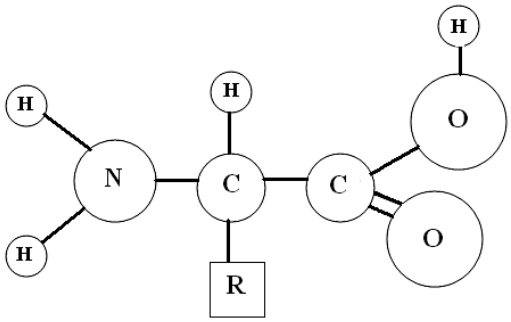

Figure 4. An amino acid: an amino (NH_2) group, a carboxylgroup (COOH) and a side chain attached to a central α-carbon (R).

sically nine amino acids are regarded as dietary essential; if these amino acids are not administered in the right proportions the protein synthesis will be reduced (33, 40). The classically defined essential amino acids have been supplemented by a group deemed conditionally essential in preterm infants because the temporarily metabolic and physiologic immaturity of these infants often leads to a delayed onset of adequate endogenous synthesis. The infant is unable to make sufficient amounts of that amino acid and hence all or a part of the daily needs for that amino acid have to be provided by the diet. The non-essential or dispensable amino acids can be synthesized by the body from intermediates of the tricarboxylic acid cycle (TCA) and other metabolic pathways. An overview of these 3 groups is shown in Table 1. Recently our group determined cysteine to be a non-essential amino acid in stead of a conditionally essential amino acid in preterm neonates (41).

Protein Turnover

Most proteins in the body are constantly being synthesized from amino acids and degraded back to them. There is a continual turnover of protein since there is no storage of proteins. There are two main reactions essential to amino acid metabolism: trans-

Table 1: Categorizing dietary amino acids by their degree of indispensability: essential = indispensable. Adapted from (42).

Essential	Non-Essential	Conditionally Essential
Leucine	Alanine	Tyrosine
Isoleucine	Serine	Glutamine
Valine	Asparagine	Arginine
Methionine	Aspartate	Glycine
Threonine	Glutamate	Proline
Lysine	Cyste(i)ne	
Phenylalanine		
Histidine		
Tryptophane		

amination and oxidative deamination. Transamination converts one amino acid into another by catalysing the transfer of the α-amino (NH_3^+) group from an amino acid to an α-keto-acid. All transamination reactions are fully reversible. The second reaction, which is irreversible, is the oxidative deamination. It removes the amino group, leaving behind the carbon skeleton. The ammonia (NH_4^+) formed enters the urea cycle and the carbon skeletons are all glycolytic and TCA intermediates. These products can, depending on the energy status of the cell, either be oxidized in the citric acid cycle to generate energy or used to synthesize glycogen or fat. Tyrosine is formed by the hydroxylation of phenylalanine, this is an irreversible reaction. Serine, glycine and cysteine are formed from glycolytic intermediates.

In the body is a "pool" of amino acids present in dynamic equilibrium with tissue protein as depicted in Figure 5. Amino acids are continually taken from the pool for protein synthesis and replaced by the hydrolysis of dietary and tissue protein.

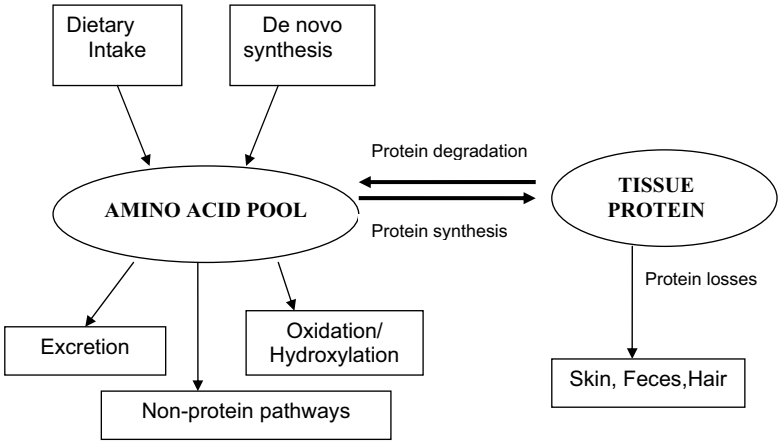

Figure 5. Exchange between body protein and free amino acid pools. Adapted from (23).

METABOLISM OF AMINO ACIDS

The branched-chain amino acids (BCAAs)

The branched-chain amino acids, leucine, valine and isoleucine have been extensively studied in vivo and in vitro because of the unique role of leucine in protein synthesis. Leucine is unique because it promotes protein synthesis by regulating translation initiation, inhibits protein degradation, and stimulates the secretion of insulin (43, 44). The BCAAs are the only amino acids that share common metabolic steps and have degradative metabolic pathways in the extrahepatic tissues, primarily muscle. They share common enzymes for the first 2 degradative steps, which are transamination and decarboxylative oxidation. The transamination is catalyzed by the branched-chain aminotransferase (BCAT) and yields α-ketoisocaproate (KIC, from leucine), α-keto-β-methylvalerate (KMV, from isoleucine) and α-ketoisovalerate (KIV) from valine. Its activity depends on the concentrations of the enzymes and substrates. Each of these branched-chain keto-acids (BCKAs) than undergoes an irreversible oxidative decarboxylation catalyzed by a branched-chain keto acid dehydrogenase (BCKAD). This is a multienzyme system located in the mitochrondial membranes. Keto-leucine (KIC) or α-ketoisocaproate is metabolized in isovaleryl-CoA, keto-isoleucine (KMV) or α-keto-β-methylvalerate is metabolized in 3-methylbutyryl-CoA, and keto-valine (KIV) is metabolized in iso-butytryl-CoA. The activity of the BCKAD is controlled by a phosphorylation-dephosphorylation system analogous to that of the pyruvate dehydrogenase complex. Thereafter, the pathways resemble those for fatty oxidation and leads to end products that can enter the tricarboxylic acid cycle. The end products of isoleucine catabolism are propionyl-CoA and acetyl-CoA; hence it is both glucogenic and ketogenic. Leucine yields acetoacetate and acetyl-CoA; it is therefore ketogenic. Valine yields succinyl-CoA: it is therefore glucogenic (45). The different steps are summarized in Figure 6.

BCAAs account for 35-40% of the dietary essential amino acids found in body protein and 14% of the total amino acids in skeletal muscle. Their main metabolic fate is incorporation into body protein, although utilization by the intestine and splanchnic tissues (first pass utilization) is also high (47). The BCAAs compete with other large neutral amino acids (LNAA), particularly tryptophan and tyrosine, for membrane transport. Although BCAAs do not act as direct precursors for neurotransmitters, they can affect the transport of certain LNAAs across the blood-brain barrier and thereby can influence central nervous system concentrations of neurotransmitters. Among the BCAAs, leucine can act independently as a nutrient signal and stimulates protein synthesis by the activation of translation initiation factors. Leucine stimulates insulin secretion (48) and when given in larges doses, it causes a transient but significant increase in serum insulin concentrations (49).

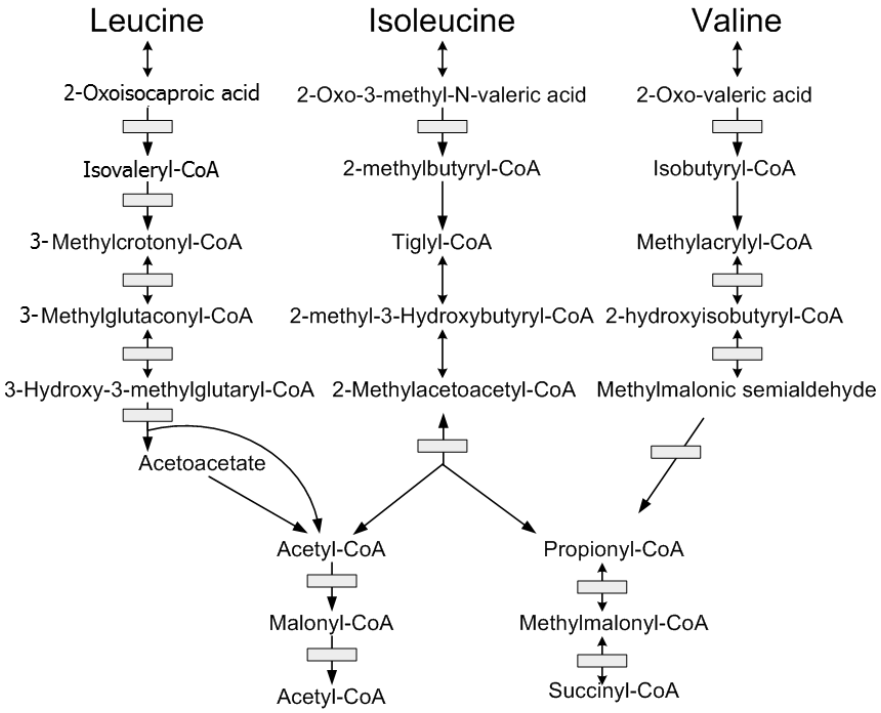

Figure 6: Metabolic pathway of the branched-chain amino acids. Adapted from (46).

Lysine

Lysine is an essential amino acid which is used primarily for protein synthesis: however is also acts as a precursor for carnitine synthesis which is used in obligatory fatty acid oxidation. In addition, lysine plays a major role in calcium absorption and in the formation of collagen. In cereals, such as rice and wheat, lysine concentrations are significantly lower compared to animal foods. A negative association was found in infants fed cereals at 3-6 mo of age on both length and head circumference, probably as a result of a reduction in breast milk intake (8). The catabolism of lysine is predominantly located in the liver. The main catabolic pathway for lysine in mammals is the sacchropine-dependent pathway leading to acetyl-CoA. Lysine-α-ketogluratate reductase converts L-Lysine into saccharopine which activity is dependent on the dietary intake (50). Because lysine is oxidized in the gut, intestinal lysine catabolism is also an important factor in the nutrition of preterms. Using stable isotope tracer techniques, van der Schoor et al. determined the fractional first pass lysine uptake to be high during enteral feeding in preterm infants and parenteral lysine to be not as effective as dietary lysine in promoting protein deposition in preterm infants (51).

Methionine

Sulfur is an essential element for cells and it plays an important role in membrane stabilization. The sulfur-containing amino acids are methionine, cysteine and taurine. Methionine is the single essential sulfur-containing amino acid. The metabolism of methionine is characterized by 2 components. The first way is the ubiquitous transmethylation cycle, in which methionine is transmethylated to homocysteine and remethylated back to methionine. Homocysteine is an intermediate in methionine metabolism and is highly correlated with cardiovascular events in neonates (52). The second, catabolic (transsulfuration) pathway of methionine involves the condensation of homocysteine with serine to form cystathionine. Cystathionine is converted to cysteine, α –ketobutyrate and ammonia and is oxidized in the TCA cycle. Methionine plays a major role as a methyl donor in several methylation processes, thereby affecting DNA and RNA translation, proteins, phospholipids, hormones, and neurotransmitters (53). Furthermore, it serves as a precursor for cysteine synthesis through the transsulfuration pathway. The splanchnic tissues play a major role in the transsulfuration pathway and thus in cysteine production (54). In the human fetus an absence of the transsulfation pathway activity has been shown, thereby suggesting that cysteine is a conditionally essential amino acid in the foetus. This appearance of transsulfation is probably stimulated by the birth-associated decrease in plasma insulin, increase in glucagon and thyroid-stimulated hormone (55). Recent data on healthy newborns and preterms show that the human neonate develops the capacity to metabolize methionine via transsulfuration rapidly after birth (56).

Threonine

Threonine is an essential amino acid that is does not participate in transamination reactions. It is a major component of the protein backbone of intestinal mucin constituting as much as 30% of its amino acid content. In addition, it is a significant (12-14%) component of 4E-binding protein-1, an important component of the translation initiation pathway. It may also play an important role in the immunological defense system (57). The liver and pancreas are the major sites of catabolism of threonine in vivo. Van der Schoor et al. showed that in preterm infants the splanchnic tissues extract 70-82% of dietary threonine, which indicates a high need for threonine by the gut (57). The absorbed threonine is used for synthesis of secretory glycoproteins, for the synthesis of mucosal cellular proteins or for oxidative purposes (58-60).

Three independent pathways for threonine degradation in mammals are known which primarily take place in the liver. It is either metabolized in the glycine-independent pathway or the glycine-dependent pathway. The glycine-independent pathway is catalyzed by serine/threonine dehydratase (STDH), which converts threonine in different steps to Propinoyl-CoA (61). The activity of STDH is regulated by dietary protein, insuline, glucagon and cortisol (62). Glycine-dependent oxidation, which converts threonine into

glycine and acetyl-CoA, is regulated by threonine dehydrogenase (TDH) and 2-amino-3-oxobutyrate-CoA ligase enzymatic reaction. Threonine can either be converted to 2-amino-3-oxobutyrate by the mitochrondial enzyme threonine dehydrogenase, and subsequently to glycine by 2-amino-3-oxobutyrate CA ligase, or it can be directly converted by glycine by cytosolic threonine aldolase. Studies show different results regarding the dominant pathway as discussed by Kalhan et al. (63). The different pathways are shown in Figure 7.

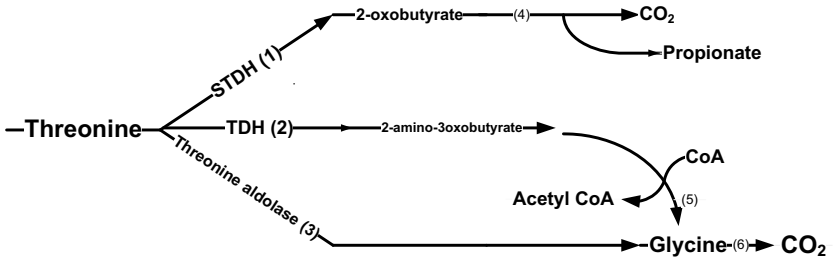

Figure 7. Hepatic metabolism of threonine. Enzymes 1 and 3 are cytosolic; the remaining enzymes are localized in the mitochondria. Adapted from (63).
(1) Serine/threonine dehydratase (STDH)
(2) Threonine dehydrogenase (TDH)
(3) Threonine Aldolase
(4) Pyruvate dehydrogenase
(5) 2-amino-3-oxybutyrate CoA ligase
(6) Glycine cleavage system

AMINO ACID REQUIREMENTS FOR NEONATES

The determination of dietary requirements in infants and children has proven to be a challenging task. Whatever method is used, graded levels of the amino acid of interest have to be fed to the subjects, ranging from below to above the requirement level (37, 64), and changes determined in a biological response. Over fifty-five years ago, Snyderman and colleagues have determined individual amino requirements for infants by means of the nitrogen balance method and weight gain (65, 66). In 1985, a modified factorial approach was adopted by the World Health Organization (WHO) which calculated a maintenance value derived from protein intakes of breastfed children and added a growth requirement. Short term nitrogen balance data were used to select a maintenance value (120 mg nitrogen $\cdot kg^{-1} \cdot d^{-1}$), rounded up from the highest range of values (80-118 mg nitrogen$\cdot kg^{-1} \cdot d^{-1}$). The growth requirement, calculated as mean nitrogen increment plus 50% to account for day-to-day variation in growth, was added to this maintenance value to give the average requirement, assuming a 70% efficiency of efficiency of conversion from dietary protein to body protein during growth. It was

thought that this 1985 report overestimated the requirement since it used the average intake of protein in breastfed children as the mean requirement thereby implying that half of breastfed infants have "deficient" intakes. Dewey et al. reviewed the 1985 data and re-examined the assumptions and evidence for the derivation of factorial estimations of protein requirement for the breastfed infants from birth to 6 months, and suggested that the requirement for breastfed children should be 10-25 % lower than those of the 1985 report (67). For infants aged 0 to 1 month, they calculated that breast milk (at intakes of 800 mL) provided on average a 45% excess of indispensable amino acids and a 61% excess at 1-3 months. Since intakes of breast milk from a healthy well-nourished mother are considered to satisfy protein requirements for the first 6 months of life, current recommendations from the WHO are based on the breast milk contents (68). The requirements for the essential amino acids determined by these different methods for neonates aged 0 to 1 month are summarized in Table 2.

Table 2: Requirement for neonates aged 0 to 1 month determined by the different methods in $mg \cdot kg^{-1} \cdot day^{-1}$.

Amino Acid ($mg \cdot kg^{-1} \cdot d^{-1}$)	Nitrogen balance and weight gain in infants (65)	DRI: human milk (22) Average Intake 0-6 months	WHO: (68) Human milk content Average requirement 0-1 month	Factorial approach by Dewey (67) Minimum requirement 0-1 month
Isoleucine	119	88	95	59
Leucine	150	156	165	109
Valine	105	87	95	72
Lysine	103	107	119	116
Methionine	45[a]			
Sulfur AA		59	57	64
Threonine	87	73	76	63
Phenylalanine	90[c]			
Aromatic AA		135	162	114
Histidine	34	36	36	-
Tryptophan	22	28	29	22

[a] in presence of cysteine
[c] in presence of tyrosine
Aromatic amino acids: phenyalanine and tyrosine
Sulfur amino acids: methionine and cysteine

USE OF THE IAAO METHOD IN THE PEDIATRIC POPULATION

The carbon oxidation methods had previously been developed in animals by Kim and Waterlow (69, 70). Vernon Young and co-workers pioneered the application of carbon oxidation methods to determine essential amino acids in adults. They used the direct oxidation approach, in which the test amino acid was also used as the tracer (71-73). The IAAO was introduced by Zello et al. to determine amino acid requirements in adults (33,

74). Recently, the IAAO and IAAB were accepted as the most appropriate to determine amino acid requirements in humans (21, 22).
To use the technique in infants and children, the dietary manipulations and study protocol most be short and non-invasive. Controversy exists over the necessity for an adaptation period to a specific dietary intake of test amino acid. Evidence suggest that a previous adaptation did not influence the requirement estimate (33). Thereby, the protein intake did not influence requirement of lysine (75) suggesting that amino acid requirements are not influenced by habitual protein intake. No studies are performed in children or neonates to the time needed to adapt to a study diet.

To avoid unnecessary invasive handling, non-invasive methods have been incorporated into the stochastic model for the study of protein and amino acid metabolism. An oral or intra-gastric infusion of isotope was used in infants (76) and an urine analysis of isotope enrichment was used in infants to measure amino acid kinetics (77). A minimally invasive protocol was developed which used oral tracers and isotopic enrichment in urine and breath as a viable alternative for the measurement of amino acid kinetics in vulnerable populations. This minimally invasive IAAO was used to determine amino acid requirements in school-aged children and recently in parenterally fed neonates (78-80). No studies have been performed in enterally fed term or preterm neonates to determine essential amino acid requirements.

AIMS OF THIS THESIS

The overall purpose of the work presented in this thesis is to determine the requirement of the individual essential amino acids in the term infant aged 0 to 1 month of age. In adults it was demonstrated using the factorial approach and studies with isotopic labelled amino acids that the nitrogen balance method underestimated the requirements of adults by two- to threefold (81). No studies have been performed using stable isotope techniques to determine essential amino acid requirements for enterally fed neonates. Because of the great importance of nutrition in preterm and term neonates with regard to long term effects as described in the introduction, determination of the amino acid requirements in these children is of high priority. Thereby, it is of great importance to use a minimal invasive protocol in this vulnerable group.

The overall aims of the work presented in this thesis are:

1. To determine whether adaptation to the study diet in the IAAO method influences the indicator oxidation rate.

2. To determine the tracer washout time and baseline adaptation to the study diet in the IAAO method.

3. To determine whether the use of only expired air is sufficient to determine amino acid requirements by comparing the breakpoint determined in air, urine and plasma using the IAAO method.

4. To determine the requirement for the branched-chain amino acids isoleucine, leucine and valine in the term infant 0 to 1 month of age using the IAAO method.

5. To determine the optimal BCAA ratio in term infants aged 0 to 1 mo of age using the IAAO method.

6. To compare current recommendations based on the different methods with the mean requirements determined by the IAAO method. We will compare these mean requirements with the amount of amino acids in current formulas and discuss the possible new recommendations based on our studies.

Part II: Validation of the minimally invasive protocol in preterm/term neonates

In **chapter 2** we determine whether an adaptation to the study diet for 1, 2, 4 or 6 days has an effect on the indicator amino acid oxidation rate. Preterm infants are adapted for 6 days to one of the leucine intakes and we determine the fractional oxidation of the [1-^{13}C] phenylalanine at days 1, 2, 4 and 6. We also determine the tracer washout time; the minimal time needed to perform the next tracer study. **Chapter 3** describes the study design of the study we recently started in the High Care Centers of the Erasmus Medical Center in Rotterdam to the requirement for threonine in the preterm infant. In **chapter 4** we compare the mean requirement for lysine determined in urine, plasma and air to show whether the sampling of $^{13}CO_2$ in expired air is sufficient to determine the mean requirement in term neonates.

Part III: The branched-chain amino acid requirement in term neonates

These three amino acids share a common membrane system for their transamination and decarboxylative oxidation. In **chapter 5** we determine the mean requirement of the essential amino acid isoleucine using the IAAO method in term neonates. In **chapter 6**

the mean requirement of valine is determined in term males in the first month of life, and the time needed to allow background adaptation to the study diet. In **chapter 7** the mean requirement of leucine in term neonates is determined using the IAAO method. In **chapter 8** we describe the optimal BCAA ratio and compare our estimated mean requirements with the current recommendations and current contents of infant formulas for term infants.

Part IV: West meets East/East meets West

This chapter describes the western versus the eastern view considering starting up a research project in China. In **chapter 9** the practical and ethical issues are explained by the western group , in **chapter 10** the eastern view is described by our Chinese colleagues.

Part V: Other essential amino acid requirement in term neonates

Chapter 11 describes the methionine requirement for term infants neonates in the first month of life.

Part VI: General discussion, overview of the studies, future perspectives and summary

In the general discussion, **Chapter 12,** we compare the requirements determined by the different methods with the requirements determined by our studies using the IAAO method. We compare these mean requirements with the amount of amino acids and protein in current formulas. Finally we will discuss the possible new recommendations for infant formulas for infants aged 0 to 1 month based on our studies and will give directions for future research. The results of the studies are summarized in **chapter 13** (English version) and **Chapter 14** (Dutch version).

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Part II

Study Design and Validation of the minimally invasive protocol

CHAPTER

2

New insights in the methodological issues of the IAAO method in preterm neonates

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ABSTRACT

Background: Infants and children are known to have higher turnover rates than adults, possibly reducing the required adaptation period. We aimed to determine the effect of adaptation to the study diet on the oxidation of the indicator amino acid and the required tracer washout time.

Methods: Subjects received a study diet for 6d that entailed a 50% reduction in leucine. Tracer studies using enterally infused [^{13}C]bicarbonate and [1-^{13}C]phenylalanine were performed on days 1, 2, 4 and 6. Breath samples containing $^{13}CO_2$ were collected during isotopic plateau, measured by infrared isotope analysis and $F^{13}CO_2$ was calculated.

Results: Preterm infants (n=11, birth weight 1.9 ± 0.1 kg, gestational age 32.6 ± 1.5 wks) received 166 mg·kg^{-1}·d^{-1} of leucine. Baseline enrichment changed significantly at day 1 of the study diet. $F^{13}CO_2$ did not change significantly between days 2 and 4 but was significantly lower at day 6. The tracer washout time was determined 7.5 h using a biphasic regression analysis.

Conclusion: One day of adaptation to a new diet is necessary before starting infant requirement studies. Adaptation for a period of 5 d results in a protein sparing response. The minimal time between two studies within the same subject is 7.5 h.

INTRODUCTION

Adequate nutrition is essential for optimal growth and development in preterm and term neonates. Protein is an important component, as it provides essential amino acids required for protein synthesis and growth. Nitrogen balance has long been used as the method to determine the requirement of the essential amino acids. An adaptation time of 7 d was needed to achieve equilibrium in the urea body pool and in urinary urea excretion (1). Because it is considered unethical to administer a deficient diet to an infant for 7-10 d, alternative methods were needed. The oxidation (or hydroxylation) of essential amino acids by the liver and peripheral tissues is dependent on the activity of the degrading enzymes, which in turn are dependent on the intake of the essential amino acids. Consequently, it is necessary to have sufficient time to adapt to a changing dietary intake (2, 3).

Studies have been performed using tracer oxidation methods in adults and pigs to examine whether protein intake or the adaptation time to the study diet influenced the amino acid kinetics (Table 1). Following a 2 d strictly controlled normal protein intake, an adaptation time of 8 h appears to be sufficient for adult subjects (4, 5). In premature or term infants, no studies have been performed to determine the necessary adaptation time. Amino acid and protein turnover studies in infants are 2 to 3 times higher than in adults on average, potentially reflecting a higher adaptive system to changes in the diet (6, 7). The adaptation to the study diet should be as short as possible to avoid restrictions in growth and development in this vulnerable population. This is especially true when considering preterm infants because protein intake in the first 4 weeks of life has a major influence on later cognitive function (8, 9) and blood pressure (10).

Therefore, we performed a study to determine the period of adaptation in infants who receive a study diet with decreased leucine content. We measured the oxidation rate of the indicator amino acid [1-^{13}C]phenylalanine to $F^{13}CO_2$. In addition, we determined the minimal time needed to perform the next tracer study by determining the tracer washout time.

Table 1: Overview of studies performed to define the time needed to adapt to a study diet using stable isotopes

IAAO/DAAO Studies	Intake	Objects	Days of adaptation	Indicator Oxidation /Flux Changes?	Conclusion
Thorpe (35), IAAO	0.8 -1.4- 2.0 g·kg^{-1}·d^{-1} protein	Adult humans	2 d	Flux↓[a] Oxidation↓[a] in 1.4 g/kg/d	2d adaptation to protein intake
Moehn (25), IAAO	Deficient and excess lysine	Growing pigs	2-6 d	Oxidation↑[b] No differences 2-6 d	Adaptation < 2 d
Moehn (25), IAAO	100/50 % protein	Adult pigs	<1-10	Oxidation↑[c] No differences 1-10 d	Adaptation < 24 h
Motil (36), IAAO	1.5- 1.0 -0.4 g·kg^{-1}·d^{-1} protein	Adult women	#6 d	Oxidation↓↑[d],89% of changes within 24 h	Adaptation < 2 d
Duncan (27), IAAO	0.8 or 1.0 g·kg^{-1}·d^{-1} protein	Adult men	3-9 d	No	No effect on requirements
Elango (5), IAAO	5,20,35,70 mg Lysine	Adult humans	#8 h, 3 d, 7 d	No	8h adaptation is sufficient
Young (22), DAAO	7, 14, 30 mg Leucine	Adult humans	7 d, 21 d	Flux↓[e] Oxidation↓[e]	Downregulation in lower intakes after 1 and 3 wks
Castillo (28), DAAO	Arginine-rich/ free diet	Adult humans	6 d	Flux↓ Oxidation↓	Downregulation of arginine catabolism
Tharakan (23), DAAO	Arginine-free/control diet	Adult humans	28 d	Flux↓ Oxidation↓	Similar downregulation of arginine catabolism
Zello (29), DAAO	4.2 or 14 mg·kg^{-1}·d^{-1} phenylalanine	Adult humans	3,6,9 d	No differences	Adaptation < 3 d Requirement independent of prior intake
Kurpad (37), DAAO	4 intakes lysine	Adult men	7-21 d	No differences	Adaptation < 7 d

after a controlled protein intake of 2 days prior to the study day

[a] 1.4 g·kg^{-1}·d^{-1} was significantly lower than 0.8 and 1.6 g·kg^{-1}·d^{-1} in flux and oxidation, respectively

[b] Oxidation was significantly increased in the low intake compared to the adequate level

[c] Oxidation was significantly increased in the 50% and 100% intakes compared to 200%

[d] Oxidation was significantly increased in the 0.4 compared to 1.0 and significantly decreased in the 1.5 compared to 1.0 g·kg^{-1}·d^{-1}

[e] Oxidation and flux were significantly decreased in the fed state in the 7-mg, 14-mg and 30-mg diets compared to an intake of 80 mg. Flux was significantly decreased in the 7- and 14-mg diets at 3 wks compared to 1 wk, and oxidation was decreased in the 7- and 30-mg diets at 3 wks compared to 1 wk

SUBJECTS AND METHODS

Subjects

Subjects eligible for the study were preterm infants admitted to the Neonatology Department of the Fudan Children's Hospital in Shanghai, China. Their gestational ages ranged between 28 to 37 wks, and their birth weights were less than 2.2 kg. The children had

to be clinically stable and in a growth state, defined as gaining weight at a rate greater than 10 $g \cdot kg^{-1} \cdot d^{-1}$ over the previous 5 d. All subjects tolerated full enteral feeding well and had no congenital or gastrointestinal diseases. The study protocol was approved by the Medical Ethical Committee of the Fudan Children's Hospital, and a statement of no objection was obtained from the Medical Ethical Committee of the Erasmus MC-Sophia Children's Hospital. Similar studies, including those determining cysteine requirements, have been performed previously in the Erasmus MC-Sophia Children's Hospital (11,12). Written informed consent was obtained from one or both parents for all participants after a Mandarin-speaking researcher provided a precise explanation of the study.

Experimental design

Tracer washout time

The tracer washout time was determined once in all 11 subjects on day 2. Breath samples were collected directly after the [1-^{13}C]phenylalanine was stopped (T0) and subsequently every hour during the first 9 h. Over the following 6 h, the breath samples were collected every 2 h. Subsequently, samples were collected every 3 h for 9 h until a 24-hour period had passed since the beginning of the tracer washout study. These samples were compared with the baseline samples of the study day to determine the time needed between 2 tracer study days. Subjects were weighed daily, and a head circumference was measured at the days that the IAAO study was performed. The study protocol is shown in Figure 1.

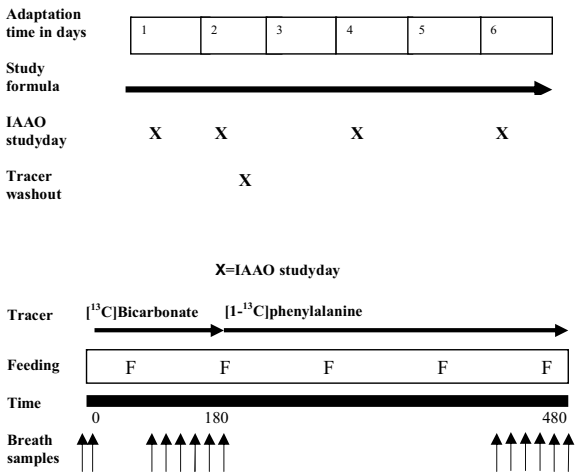

Figure 1: Study Design

F time of oral feeding (given every hour)

↑ time that breath samples were taken

Adaptation study

Tracer studies were performed on days 1, 2, 4 and 6. On the study days, baseline samples were obtained 15 and 5 min before starting the tracer infusion. Directly after the first study formula was administered, subjects received a primed (10 μmol·kg^{-1}) continuous (10 μmol·kg^{-1}·h^{-1}) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 2.5 h to quantify individual CO_2 production. The labelled sodium bicarbonate infusion was directly followed by a primed (30 μmol·kg^{-1}) continuous (30 μmol·kg^{-1}·h^{-1}) enteral infusion of [1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 5 h by an infusion pump via the nasogastric tube. Breath samples were collected on all study days and were obtained using the direct sampling method described by van der Schoor et al. (13). During the experiment duplicate, ^{13}C-enriched breath samples were collected every 15 min during the isotopic steady state, beginning 1.75 h after start of [^{13}C]bicarbonate administration and 3 h after the start of [1-^{13}C]phenylalanine administration, as depicted in Figure 1.

Study Formula

The study formula contained 166 mg·kg^{-1}·d^{-1} leucine, which is 50% below the current recommended intake for preterm neonates (14). Subjects received the leucine-low study formula for 6 d. All of the other essential amino acids were present at levels above the current recommendations and are presented in Table 2.

These amino acid concentrations were obtained by mixing a leucine-free formula, which also contained decreased amounts of valine and isoleucine (Analog, Danone/SHS International, Liverpool, United Kingdom) with regular Neocate, an amino acid based formula designed to fulfill the amino acid requirements of infants (Danone/SHS International, Liverpool, United Kingdom). Before the start of the study formula, subjects received Chinese formula from different brands. All subjects received the study formula in 8-12 boluses for 6 d. On these days, subjects received a fluid intake of 170 ml ·kg^{-1}·d^{-1}, which provided 135 kcal ·kg^{-1}·d^{-1} and a protein intake of 3.7 g ·kg^{-1}·d^{-1}, according to the current recommendations for preterm infants (14).

Analysis and Calculations

Samples were sent from Shanghai to Rotterdam every three weeks by air transport.

The $^{13}CO_2$ isotopic enrichment in expired air was measured using infrared isotope analysis (Helifan, Analytic Fischer Instruments, Leipzig, Germany) and expressed as atom percent excess (APE) above baseline. APE was plotted relative to time. Steady state was defined as three or more consecutive points with a slope not significantly different from zero ($p \geq 0.05$).

Table 2: Intakes of essential amino acids compared to current recommendations

	Minimum amino acid recommendations for preterm infants (mg· 135 kcal^{-1}) (14)	Study formula containing leucine 166 mg·kg^{-1}·d^{-1} (mg·135 kcal^{-1})
Leucine	340	166
Histidine	72	191
Isoleucine	174	174
Lysine	246	307
Phenylalanine & tyrosine	265	Phe: 224 Tyr: 220
Sulfur amino acid (methionine & cysteine)	115	Met: 83 Cys:121
Threonine	153	214
Tryptophan	51	82
Valine	178	194

Estimated body CO_2 production (mmol ·kg^{-1}·h^{-1}) was calculated for each infant as described previously (11). The fraction of $^{13}CO_2$ recovery from [1-^{13}C]phenylalanine oxidation as a percentage ($F^{13}CO_2$) was calculated using the following equation:

$$F^{13}CO_2\ (\%) = [IE_{PHE} \times i_B]/[\ i_{PHE} \times IE_B] \times 100 \quad (15)$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (µmol ·kg^{-1}·h^{-1}), i_{PHE} is the infusion rate of [1-^{13}C]phenylalanine (µmol ·kg^{-1}·h^{-1}) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.

Statistical analysis

Descriptive data were expressed as mean ± SD. Steady state of $^{13}CO_2$ in expired breath during the [1-^{13}C]phenylalanine was achieved when the linear factor of the slope was found to be not significantly different from zero ($p \geq 0.05$). Biphasic linear regression analysis was performed to analyze oxidation rates as described below. Statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA). A p-value ≤ 0.05 was considered significant. For the washout time, a biphasic regression analysis was determined on the breath enrichment values. In this model, a breakpoint is estimated using non-linear regression. With the biphasic linear regression analysis, the regression equation was split into 2 parts. For the first part, an intercept and slope were estimated. For the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit on the basis of the highest r^2 was selected. A correction was made to account for the fact that the measurements were performed within the same patients. A paired t-test was used to determine the differences before and during the study day and to compare measurements from day 1

to those from days 2, 4 and 6. The effect of growth during the study and the $F^{13}CO_2$ were tested with Pearson's correlation coefficient analysis.

RESULTS

Eleven patients were enrolled in the study, and the patient characteristics are shown in Table 3. Weight gain rates tended to decrease during the study when compared to rates before the study, but this trend did not reach significance. In addition, the study was not designed or powered to detect differences in weight gain rates. No correlation was observed between growth during the study and $F^{13}CO_2$.

Table 3: Characteristics of subjects (n=11)

	Mean ± SD
Birth weight (g)	1860 ± 143
Gestational age (wks)	32.6 ± 1.5
Postnatal age (d)	17.4 ± 8.2
Postconceptional age (wks)	35.0 ± 1.1
Study weight (g)	1948 ± 150
Growth before study ($g \cdot kg^{-1} \cdot d^{-1}$)	15.0 ± 3.8
Growth during the 6 d study diet ($g \cdot kg^{-1} \cdot d^{-1}$)	12.5 ± 5.5
Kcal before study	109.6 ± 15.4
Kcal during study	134.4 ± 2.9 *
Protein intake 1 day before study ($g \cdot kg^{-1} \cdot d^{-1}$)	3.03 ± 0.36
Protein intake during study ($g \cdot kg^{-1} \cdot d^{-1}$)	3.67± 0.08 *

*$p < 0.05$ between before study and during study using paired t-test (2-tailed)

Tracer washout time

The tracer washout time studies were performed on day 2. In 1 patient, two tracer washout studies were performed at day 2 and day 4; therefore, a total of 12 tracer washout studies were performed. After the end of the study protocol, the ^{13}C enrichment decreased, and stable background enrichment was determined using a biphasic regression analysis. From the two-phase regression analysis with time as the independent variable and ^{13}C enrichment in APE as the dependent variable, the breakpoint was determined to be 7.5 h ($r^2 = 0.43$, $p < 0.000$) (upper CI: 8.1 h; lower CI: 6.8 h). The return to baseline enrichments was determined to be 7.5 h. These results are depicted in Figure 2.

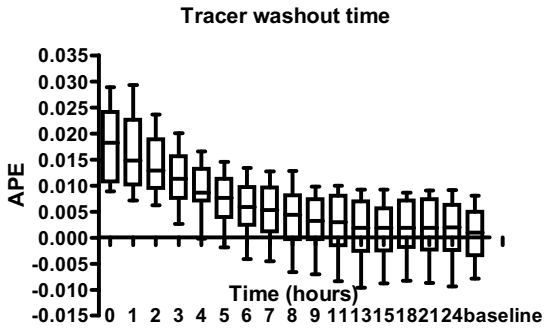

Figure 2: Tracer washout-time: From the two-phase regression analysis with time as the independent variable and ^{13}C enrichment in atom percent excess (APE) as the dependent variable, the breakpoint was determined to be 7.5 h (upper CI: 8.1 h; lower CI: 6.8 h)

Baseline enrichments

Baseline enrichments were significantly decreased on the first day when compared to all other study days (Table 4). The enrichment increased on the second day on the study formula. A small, statistically significant but not relevant difference of 1 PDB was observed between the baseline enrichments measured on day 4 and that measured on day 6. The large change in the baseline enrichment on the first day on the study formula made it impossible to calculate the whole body CO_2 for that day. The body CO_2 production estimated by the infusion of [^{13}C]bicarbonate did not differ between days 2, 4 and 6, indicating that energy expenditure did not change over the study period.

Table 4: Comparison between days 1, 2, 4 and 6 using paired t-tests

	Day 1	Day 2	Day 4	Day 6
Baseline PDB	-23.4 ± 1.1	-17.2 ± 1.3*	-16.1 ± 1.2*, **	-16.3 ± 1.1 *, ***
Fractional oxidation (%)		14.6 ± 6.6	13.0 ± 5.9	11.1 ± 5.6 ***,****
Body CO_2 production ($mmol \cdot kg^{-1} \cdot h^{-1}$)		26.7 ± 1.8	28.0 ± 2.4	28.9 ± 1.8

* $p < 0.05$ between day 1 and 2/4/6 (2-tailed)
** $p < 0.05$ between day 2 and 4 (2-tailed)
*** $p < 0.05$ between day 2 and 6 (2-tailed)
**** $p < 0.05$ between day 4 and 6 (2-tailed)

Oxidation rate of the indicator

No difference was observed in $F^{13}CO_2$ levels in infants receiving the study formula between days 2 and 4 (Figure 3). However, interestingly, day 6 $F^{13}CO_2$ was significantly lower than that measured on the preceding days (days 2 and 6, p= 0.033; days 4 and 6, p= 0.043).

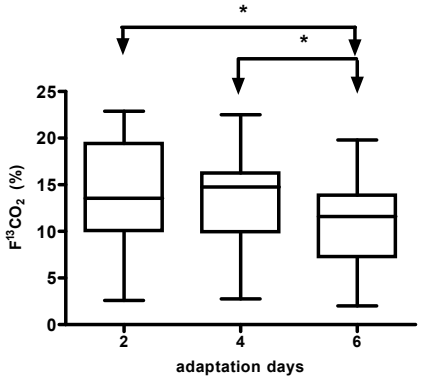

Figure 3: Fractional oxidation rate ($F^{13}CO_2$) after adaptation to the study diet for 2, 4 and 6 d. A significant decrease was observed in $F^{13}CO_2$ between days 2 and 6 (p= 0.033) and between days 4 and 6 (p= 0.043). * $p < 0.05$ determined by paired t-test (2-tailed).

DISCUSSION

Several interesting issues emerge from the present studies. First, energy expenditure is remarkably stable over a period of slightly less than a week. Secondly, the washout time of tracer is not a rate-limiting step in performing subsequent studies in infants because $F^{13}CO_2$ in the expired air returns to baseline levels within 8 h.

However, the present data do not allow us to draw firm conclusions about the time to adapt to a study diet. On the one hand, we did not observe differences in fractional oxidation rates between days 2 and 4. On the other hand, we did observe a significantly lower oxidation rate after 5 d of adaptation. This coincided with a lower weight gain rate observed after a period of 6 d. These results can be interpreted in two ways. First, a period of at least 5 d of adaptation to a study diet is necessary in IAAO studies determining the amino acid requirements in preterm infants. Secondly, the body adapts to a reduced essential amino acid intake by decreasing the growth rate, which again is reflected by a new equilibrium. In the new equilibrium, there is a decreased requirement for essential amino acids because growth is reduced. Therefore, a requirement determined under those circumstances would not reflect the optimal intake. If this theory were true, this would reflect the remarkable flexibility of the human body to adapt to changing circumstances. However, one would expect a higher oxidation rate of the indicator amino acid, whereas we observed the opposite. For example, Moehn et al. observed higher oxidation rates of the indicator in pigs fed a low protein diet after a 1-2 d adaptation to the study diet using phenylalanine flux and enriched expired CO_2 (24). The only possible explanation for our results is that the plasma phenylalanine concentration (the indicator amino acid) increased, resulting in a lower intestinal absorption of the dietary phenylalanine, including the enterally administered ^{13}C phenylalanine. Subsequently,

plasma enrichment would have been lower, resulting in a lower $^{13}CO_2$ production rate. Unfortunately, we did not collect either blood or urine samples to enable us to test this hypothesis.

Adaptation to a specific diet is widely studied. A decrease in the intake of protein or a specific indispensable amino acid intake results in a reduced rate of amino acid oxidation (16-19) and subsequently decreases the rate of nitrogen loss (18). When intakes of energy and other nutrients are adequate but the protein level is very low, this decline in N-excretion reaches, within a few days to 1 wk, a new, lower and relatively steady state (1). Harper described the enzymatic changes that occurred after ingestion of low- and high-protein diets (2). Enzymes involved in the metabolism of dispensable amino acids responded to the amount of protein consumed, whereas enzymes involved in the catabolism of indispensable amino acids adapted to changes in protein intake (and indispensable amino acid intake) in relation to the amino acid needs of the body. Thus, the capacity to degrade amino acids depends to a considerable extent on the diet. A change in the intake of an amino acid is promptly (within a few h) followed by a parallel change in its oxidation rate (20, 21). Young and co-workers observed a decrease in leucine oxidation in adult humans fed a leucine-restricted diet for 1 and 3 wks (22) and a decrease in arginine oxidation in adults receiving an arginine-free diet for 1 and 4 wks (23), indicating a decrease in catabolism to maintain the body's equilibrium. In an elegant study in which adults received a proline-free diet for 4 wks, a marked difference was observed in proline kinetics, but only small differences were noted in leucine kinetics. Therefore, long-term adaptation to a specific amino acid-deficient diet does not result in changes of other amino acids in adults (24). Because these studies used the DAAO, which is proposed to produce low oxidation rates in response to lower intakes of the test amino acid, these results are not comparable to our results using the IAAO method, in which deficient intakes are proposed to give high oxidation rates. Therefore, these direct oxidation studies involved a change in the pool size of the amino acid being oxidized due to the change in the dietary intake of the same amino acid. In contrast, because the IAAO technique employs an unchanging intake and pool size for the oxidized amino acid, it should be expected that the adaptation period for the IAAO method would be at least as short as that for the DAAO method and possibly shorter (25). The IAAO method is based on the partitioning of the indicator amino acid in either protein synthesis or oxidation, which occurs primarily at the acyl-t-RNA level. The adaptation needed does not relate to the urea pool, which needs 7 d to achieve an equilibrium, but does relate to the turnover of acylated t-RNAs, which adapt in less than 4 h (26). Because the Michaelis-Menten constant (K_m) for various amino acid degrading enzymes are relatively high and are not saturated under the physiological range of plasma or tissue amino acid concentrations, the oxidation will respond quite rapidly to a change in intracellular amino acid concentrations (2). Whether amino acid requirements for growth

and maintenance have already changed in these few hours is not known, but it does not seem likely. A recent IAAO study demonstrated no differences in lysine requirements in adults adapted to lower protein intakes with the remark that the test formulas were given only on the study day (27). To clear this controversy, a study should be performed that compares the requirement of an essential amino acid after 1 and 5 or more days of adaptation and meanwhile takes the reduced growth into account. Because we consider it unethical to maintain neonates for long periods on a deficient diet, we did not test adaptation longer than 6 d and consequently did not resolve the question of whether more than 6 d has an additional effect on the tracer oxidation. Further, in adult humans fed a 4-wks arginine-free diet, the decrease in oxidation was similar to a 1-wk restriction alone (23, 28).
Previous studies demonstrated that the carry-over effect of the isotope did not affect the background enrichment after 2 d (29). Most studies used a latency of 2 d (30, 31) or 7 d (32, 33) between 2 measurements. We determined a tracer washout time of 7.5 h. This makes it possible to measure the same patient on two consecutive study days, as we did in the present study.

A significant increase was observed between the baseline ^{13}C enrichment at day 1 compared to days 2, 4 and 6, implying that time is needed to adapt to the ^{13}C enrichment of the study diet. Because every diet differs in naturally enriched ^{13}C depending on the carbohydrate source used (34), a period of time is necessary to allow background adaptation to the experimental diet. This is probably a result of the fact that we used European formulas, which might be based on different sources of carbohydrates and proteins than the Chinese formula that the infants received before the adaptation day. A limitation of the present study is that because of the large increase in baseline enrichments after the start of the study formula on day 1, the $F^{13}CO_2$ on day 1 was not comparable to that measured on the other days.

In conclusion, our study indicates that a period of time is necessary to adapt to the ^{13}C level of the study formula, this adapts within 24h. Furthermore, if adaptation to a specific deficient diet has occurred, a tracer study as described here can be performed at daily intervals. No conclusive evidence has been generated as to how long an adaptation period on a deficient diet should last. No differences in metabolism were shown at day 2 or day 4, whereas at day 6, significant changes in metabolism were observed, most likely as a result of a protein-sparing adaptive response.

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CHAPTER

3

Threonine requirement in the enterally fed preterm infant: study design of the multicenter study started in the Netherlands

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ABSTRACT

Background: The exact requirement of essential amino acids for term and preterm neonates is not known. So far, requirements have been estimated either from the composition of human milk or are derived using nitrogen balance studies which are known to be imprecise. By using a new method, the indicator amino acid oxidation (IAAO) method, we are able to determine the exact individual requirement for all essential amino acids in both term and preterm infants. This will improve our knowledge on how to feed infants and might improve functional outcome in these vulnerable patient groups.

Design: The objective of the study is to quantify the mean requirement of threonine in preterm infants. We hypothesize that the current formulas provide too much threonine.

Study design: This will be a randomized, unblinded, non-therapeutic intervention study. We will perform the study in fully enterally fed preterm infants. Inclusion criteria are: preterm infants with a gestational age of 30-35 weeks, a postnatal age of 28 days and a birth weight between -2 SD and + 2 SD for the gestational age.

Intervention: Fully enterally fed preterm infants will randomly receive graded intakes of threonine (5-206 $mg \cdot kg^{-1} \cdot d^{-1}$) as part of an elemental formula. After 1 day adaptation to the study formula, [^{13}C]bicarbonate and [1-^{13}C]lysine tracers will be given enterally. Breath samples containing $^{13}CO_2$ will be collected during [1-^{13}C]lysine infusion, measured by infrared isotope analysis, and analyzed using a biphasic regression model. We will test the mean requirement for threonine by breakpoint estimation. This study will be the first to be conducted in a research program that will define the requirement of all 9 essential amino acids in preterm infants. These can be compared to the current recommendations based on the factorial approach and compared to what current preterm formulas provide. New recommendations can be made for preterm infant formulas.

BACKGROUND

Appropriate nutrition is essential for optimal growth and development in the preterm and term neonate. Protein is an important component of adequate nutrition as it provides essential amino acids required for critical protein synthesis and growth. Nutrition is especially important during the early phase of life since protein intake in the first 4 weeks of life has a major influence on later cognitive function (1) and blood pressure (2). Classically nine amino acids are regarded as dietary essential; if these amino acids are not administered in the right proportions, protein synthesis will be reduced (3, 4). The requirement of the indispensable amino acids have been determined by a number of different methods. Historically, descriptive or gross measures like growth and nitrogen balance have been used. New techniques using stable isotopes have shown that adult requirements have been underestimated using the balance techniques, and that the requirements for adults are 2-3 times higher than the current international recommendations which were based on balance data (5-9). No studies have been performed using stable isotope techniques to measure amino acid requirements for enterally fed infants properly until recently. Riedijk et al determined the minimal cysteine requirement for infants born at gestational age 32-34 wks to be < 18 $mg \cdot kg^{-1} \cdot d^{-1}$. They found that cysteine is not a conditionally essential amino acid and that the cysteine requirement does not depend on the gestational age or postmenstrual age in enterally fed preterm infants born < 29 wks (10, 11). More recently, we determined the requirement of methionine and lysine in enterally fed term infants (12, 13), i.e. determined that some of the currently used formulas provide 2 times too much essential amino acids. This study is the first to conduct in a research program that will define the requirement of all 9 essential amino acids in preterm infants to optimize infant nutrition. These requirements can be compared to the current recommendations based on the factorial approach and compared to what current preterm formulas provide. New recommendations can be made for preterm infants based on our mean requirements. The objective of the study is to quantify the mean threonine requirement for preterm infants. We hypothesize that the current preterm formulas provide too much threonine.

STUDY DESIGN

This will be a multi-centre, non-therapeutic, randomized, unblinded, investigator-initiated intervention study. The study will be performed in the Erasmus MC-Sophia and in the High Care Centers of the Sint Franciscus Gasthuis Hospital, Maasstad Hospital (both Rotterdam), Albert Schweitzer Hospital in Dordrecht, Amphia Hospital in Breda and the HAGA Hospital in the Hague, the Netherlands. Oral and written information about the study will be provided to the parents or legal guardians by one of the investigators. Writ-

ten and signed informed consent will be obtained of both parents or legal guardians prior to study initiation. The study started March 2012 and will last for 1-2 yrs.

Subjects

We will perform the study in fully enterally fed preterm infants. Eligible for the study are preterm infants with a gestational age of 30-35 wks and a birth weight between the -2 SD and +2 SD for the gestational age.

Inclusion criteria are:

- fully enterally fed preterm infants as described above
- weight gain rate > 10 g $\cdot kg^{-1} \cdot d^{-1}$ in preceding 5 d
- postnatal age < 28 d

Exclusion criteria are:

- congenital anomalies
- sepsis
- gastrointestinal pathology

Study formula

The study formula will be identical to regular Neocate, an amino acid based formula designed to fulfil the amino acid requirements for infants (SHS, Liverpool, UK), but without threonine and with less lysine. The amount of threonine will be adjusted separately as L-threonine. L-lysine will be supplied during the adaptation time and during the infusion of [^{13}C]bicarbonate to obtain a stable total intake of 166 $mg \cdot kg^{-1} \cdot d^{-1}$ during the entire study. L-alanine will be added separately to make the formula isonitrogenous.

The present minimum recommendations for threonine in preterm infants range from 113 - 128 $mg \cdot 100\ kcal^{-1}$ (14, 15), this is 138-192 $mg \cdot kg^{-1} \cdot d^{-1}$. In regular Neocate the concentration is 180 $mg \cdot 100\ kcal^{-1}$. This supplies 206 $mg \cdot kg^{-1} \cdot d^{-1}$ threonine when an intake of 170 $mL \cdot kg^{-1} \cdot d^{-1}$ is given. We will use study formulas containing the following graded threonine intakes in $mg \cdot kg^{-1} \cdot d^{-1}$:

5,10,15,20,25,30,35,40,45,50,55,60,65,70,75,80,85,90,100,110,120,130,140,150,160,170, 180,190,200,206.

Tracer protocol

30 min before start of the oxidation study the feeding regimen will be changed into continuous drip-feeding via a nasogastric tube. An elemental diet (Neocate®, Danone) will be used to provide the infants with different amino acid intakes. After the adaptation period of 1 d, subjects will receive a primed (15 $\mu mol \cdot kg^{-1}$) continuous (10 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% ^{13}C; Cambridge Isotopes, Woburn, MA) by the nasogastric tube for 150 min to quantify individual CO_2 production.

The IAAO technique uses as indicator a labelled essential amino acid that is different from the test amino acid. The indicator is an amino acid labelled with ^{13}C, a stable isotope which is safe and non-radioactive. The indicator is independent of the different intake levels of the test amino acid and has an oxidative pathway distinct from and unrelated to the test amino acid. We have chosen [1-^{13}C]lysine as the indicator which is a safe and good indicator as previously shown in (preterm) infants (16, 17). Taking into account the first-pass uptake of 18% for lysine [13], and the need to have a substantial tracer infusion rate to detect the label in expired air, we chose an amount of 40 μmol ·kg^{-1}·h^{-1} infusion rate. We will infuse the tracer enterally (by the nasogastric tube) to minimize the invasiveness of the experiment in preterm infants.

The labelled sodium bicarbonate infusion will be directly followed by a primed (50.0 μmol μmol ·kg^{-1}) continuous (40 μmol ·kg^{-1}·h^{-1}) enteral infusion of [1-^{13}C]lysine (99% ^{13}C; Cambridge Isotopes, Woburn, MA) for 4 h. Enterally infused tracer will be mixed with the study formula and infused continuously by an infusion pump via the nasogastric tube. All infants will be breathing spontaneously but are allowed to need supplemental oxygen by a nasal prong.

Sample collection

Breath samples will be obtained using the direct sampling method described by van der Schoor et al. (18) Briefly, a 6 Fr gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) will be placed 1 to 1.5 cm into the nasopharynx and end-tidal breath will be taken slowly with a syringe connected at the end. Collected air will be transferred into 10 ml sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis. Baseline samples will be obtained 15 and 5 min before starting tracer infusion. During the experiment duplicate ^{13}C-enriched breath samples will be collected every 10 min during the last 45 min of the [^{13}C]bicarbonate infusion and the last hour of the [1-^{13}C]lysine infusion.

Randomisation and blinding

Individuals will randomly receive one of the 30 intakes of threonine, randomization will be obtained by using sealed envelopes. It will be non-blinded study since the investigator will be the person that calculates and prepares the formulas based on the actual weight the day before the study day. It is not necessary to blind this process since it cannot influence the results.

Analysis and calculations

We will test the mean requirement of threonine by breakpoint estimation. This will be determined by applying a two-phase linear regression crossover model as discussed below.

Analysis of $^{13}CO2$ isotopic enrichment in expired air will be performed in the Academic Medical Center Amsterdam, where we have ample experience (18-21). Baseline samples will be obtained 15 and 5 min before starting tracer infusion. During the experiment duplicate ^{13}C-enriched breath samples will be collected every 10 min during the last 45 min of the [^{13}C]bicarbonate infusion and the last hour of the [1-^{13}C]lysine infusion. The $^{13}CO_2$ isotopic enrichment in expired air will be measured using infrared isotope analysis (Helifan, Analytic Fischer Instruments, Leipzig, Germany) and expressed as atom percent excess (APE) above baseline. APE will be plotted relative to time. Steady state will be defined as three or more consecutive points with a slope not significantly different from zero ($p \geq 0.05$).
Estimated body CO_2 production (mmol $\cdot kg^{-1} \cdot h^{-1}$) is calculated for each infant as described previously (19). The rate of fractional [1-^{13}C]lysine oxidation is calculated as follows:

$$\text{fractional lysine oxidation (\%)} = [IE_{lys} \times i_B]/[i_{lys} \times IE_B] \times 100\%$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]lysine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (µmol $\cdot kg^{-1} \cdot h^{-1}$), i_{lys} is the infusion rate of [1-^{13}C]lysine (µmol $\cdot kg^{-1} \cdot h^{-1}$) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.(16)

Univariate analysis

Descriptive data will be expressed as mean ± SD. Steady state of $^{13}CO_2$ in expired breath during the [1-^{13}C]lysine is achieved when the linear factor of the slope is found to be not significantly different from zero ($P \geq 0.05$). The test amino acid requirement is determined by using the IAAO method. The rate of oxidation of the indicator amino acid is plotted against varying dietary intakes of a test amino acid. The inflection or breakpoint in the rate of indicator oxidation represents the physiological requirement of the test amino acid (3). Biphasic regression analysis will be performed to analyze oxidation rates. All statistical analyses will be performed using SPSS (SPSS, Chicago, IL, USA).
The power analysis cannot be performed. We aim to study 20 to 35 infants, more than studies in parenterally fed infants using the same approach with intravenous administration of the tracer (22-24).

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CHAPTER

4

Lysine requirement of the enterally fed term infant in the first month of life

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ABSTRACT

Background: Infant nutrition has a major impact on child growth and functional development. Low and high intakes of protein or amino acids could have a detrimental effect.
Objective: The objective of the study was to determine the lysine requirement of enterally fed term neonates by using the indicator amino acid oxidation (IAAO) method. L-[1-^{13}C]phenylalanine was used as an indicator amino acid.
Design: Twenty-one neonates were randomly assigned to lysine intakes that ranged from 15 to 240 mg·kg^{-1}·d^{-1}. Breath, urine and blood samples were collected at baseline and during the plateau. The mean lysine requirement was determined by using biphasic linear regression crossover analysis on the fraction of $^{13}CO_2$ recovery from L-[1-^{13}C] phenylalanine oxidation ($F^{13}CO_2$) and phenylalanine oxidation rates calculated from the L-[1-^{13}C]phenylalanine enrichment of urine and plasma.
Results: The mean (± SD) phenylalanine flux calculated from urine and plasma L-[1-^{13}C] phenylalanine enrichment data were 88.3 ± 6.9 and 84.5 ± 7.4 μmol·kg^{-1}·h^{-1}, respectively. Graded intakes of lysine had no effect on phenylalanine fluxes. The mean lysine requirement determined by $F^{13}CO_2$ was 130 mg·kg^{-1}·d^{-1} (upper and lower CIs: 183.7 and 76.3 mg·kg^{-1}·d^{-1}, respectively). The mean requirement was identical to the requirement determined by using phenylalanine oxidation rates in urine and plasma.
Conclusions: The mean lysine requirement of enterally fed term neonates was determined by using $F^{13}CO_2$ and phenylalanine oxidation rates calculated from L-[1-^{13}C] phenylalanine enrichment of urine and plasma. These methods yield a similar results of 130 mg·kg^{-1}·d^{-1}. This study demonstrates that sampling of $^{13}CO_2$ in expired air is sufficient to estimate the lysine requirement by using the IAAO method in infants.

INTRODUCTION

Lysine is an essential amino acid that is primarily used for protein synthesis (1). In addition, lysine, together with methionine, is required for the biosynthesis of carnitine, which is essential for fatty acid metabolism (2). Lysine is the first limiting amino acid in the all cereal-based diet consumed by a large proportion of the world's population (3). A deficiency in the intake of lysine limits protein synthesis and causes weight loss in infants (4). In contrast, excess lysine intake also reduces the growth rate of animals caused by an imbalanced diet (5, 6). Thus, the dietary intake of amino acids is important for the rate of protein synthesis and growth.

Only a few studies have been performed in infants to determined enteral lysine requirements (4, 7). The criteria for adequacy of a diet were nitrogen balance and growth rates, which may not be the most sensitive methods. Thereby, the number of infants (n = 6-13) studied was relatively small. Because breast milk is considered to be the optimal nutrition for infants ≤ 6 months of age, the joint WHO/FAO/United Nations University expert consultation (8) recommended a lysine intake of 119 $mg \cdot kg^{-1} \cdot d^{-1}$ on the basis of the average intake of exclusively breastfed infants rather than on the available experimental evidence. Recently, the indicator amino acid oxidation (IAAO) method has been developed to estimate essential amino acid requirements (9).

Our aim was to determine the lysine requirement of enterally fed neonates by using the IAAO method. Furthermore, we aimed to test whether requirement estimates on the basis of the fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) yielded similar results compared twith the phenylalanine oxidation rates measured in urine and plasma. In addition, to shorten our study protocol, we compared the lysine requirement derived from $F^{13}CO_2$ data from a short-term (420 min) tracer infusion protocol with the results derived from a 900-min infusion protocol.

SUBJECTS AND METHODS

Subjects

Twenty-one neonates admitted to the Neonatal Ward in the Children's Hospital of Fudan University in Shanghai participated in the study. Each subject was selected for study by the following criteria: fully enterally fed infants with a gestational age of ≥ 37 weeks, birth weight ≥ 2500 grams, and clinically stable with a weightgain rate ≥ 5 $g \cdot kg^{-1} \cdot d^{-1}$ in the preceding 3 days. Subjects were excluded if they had congenital anomalies, gastrointestinal pathology, or sepsis.

The study was approved by the Institutional Review Boards of the Children's Hospital of Fudan University, and a statement of no objection was obtained from the Sophia

Children's Hospital, Erasmus Medical Center Rotterdam. Written consent was obtained from at least one of the parents by a Chinese-speaking researcher.

Study formula

The study formula used was an elemental formula that was based on free amino acids. The composition was the same as Neocate® (SHS International) except for the lysine and phenylalanine content. Lysine, which was completely withdrawn from the study formula, was separately added in the form of L-lysine to obtain different amounts of intake. The phenylalanine intake was kept constant during the study by separately adding L-phenylalanine during the 24 h adaptation period to obtain the same amount as in the Neocate (SHS International), and this amount of phenylalanine was given as stable isotope L-[1-^{13}C]phenylalanine on the tracer infusion day. The phenylalanine intake during the study was 166 mg·kg^{-1}·d^{-1}, which was above the recommended amount of 72 mg·kg^{-1}·d^{-1} (8). A generous amount of tyrosine (166 mg·kg^{-1}·d^{-1}) was provided to ensure that the newly formed [1-^{13}C]tyrosine hydroxylated from [1-^{13}C]phenylalanine would be directly channeled to oxidation into $^{13}CO_2$, which can be measured in expired air (10). This amount of tyrosine was almost twice the amount ingested by exclusively breastfed infants (8). The nitrogen intake was kept constant for all of the subjects by the substitution of L-alanine for the lysine that was withdrawn. The caloric intake was kept constant during the study period in all infants.

Experimental design

The study was designed to determine the lysine requirement of term neonates by using the minimally invasive IAAO method (9). The IAAO method is based on the concept that, when the test amino acid intake is insufficient to meet the requirement, protein synthesis will be limited and all of the amino acid will be oxidized, including the indicator amino acid, which is labeled with a stable isotope. As the dietary intake of the test amino acid increases, the oxidation rate of the indicator amino acid will decrease until the requirement of the test amino acid is met. Once the requirement of the test amino acid is met, a additional increase in its intake will have no further influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as $^{13}CO_2$. To use the IAAO method in infants and children, the study protocol must be non-invasive. Initially, the IAAO method was used in adults to determine amino acid requirements by measuring the amino acid kinetics in plasma and the rate of release of $^{13}CO_2$ from the oxidation of the indicator amino acid in expired air (11-13). Because a good correlation between [1-^{13}C]phenylalanine enrichment in urine and plasma has been shown in adults (14, 15) and in neonates (16), the IAAO method has been used in vulnerable populations, such as parenterally fed neonates (17-19). The method has been additionally adapted to make it minimally invasive by using enteral

instead of intravenous isotope administration (20, 21) and additionally simplified by using the direct nasopharyngeal sampling method of the expired air (22).

During the study, all infants received a fluid intake of ~150 mL·kg^{-1}·d^{-1}, a caloric intake of 108 kcal·kg^{-1}·d^{-1}, and an amino acid intake equal to the protein intake of ~2.96 g·kg^{-1}·d^{-1}. Infants were randomly assigned to one of the graded test intakes of lysine, which ranged from 15 to 240 mg·kg^{-1}·d^{-1}. Each study took place over a 39-h period whereby the study formula was fed to the neonates. After 24h of study formula consumption, tracers were administered on day 2 for 15 h. Infants were bottle fed every 3h during the adaptation period. Subsequently, the feeding regimen changed to hourly bottle feeding during the tracer infusion until the end of the study. On the tracer day, a nasogastric tube was placed for tracer infusion. Infants received a primed (14 μmol·kg^{-1}) continuous (9 μmol·kg^{-1}·h^{-1}) enteral infusion of [^{13}C]bicarbonate (sterile, pyrogen free, 99% ^{13}C APE; Cambridge Isotopes) for 3h to quantify individual CO_2 production rates. Phenylalanine was used as the indicator amino acid. After the [^{13}C]bicarbonate infusion was stopped, a primed (34 μmol·kg^{-1}) continuous (27 μmol·kg^{-1} ·h^{-1}) enteral infusion of L-[1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes) was started and lasted for 12 h. The duration of [1-^{13}C]phenylalanine infusion was 12 h, to ensure achievement of the steady state in urine and to ensure adequate urine sample collection during the steady state. Syringes were weighted before and after the study to determine the exact amount of the tracers that were given to the infants. The tracer infusion day is depicted in Figure 1.

Breath samples were obtained by using the direct nasopharyngeal sampling method described by van der Schoor et al. (22). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical) was placed 1-1.5 cm into the nasopharynx and the end-tidal breath was taken slowly with a syringe. Collected air was transferred into 12 mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments) and was stored at room temperature until analysis. Baseline breath samples were collected before the start of tracer infu-

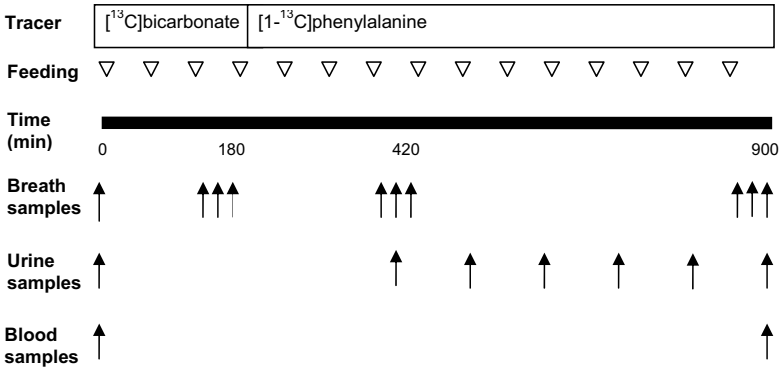

Figure 1. Schematic overview of tracer infusion day. Triangles indicate time that bolusfeeding was given. The arrows indicate the times the breath, urine and plasma samples were taken.

sion. Duplicated breath samples were obtained at 15 min intervals during the period of 105-180 min after the tracer infusion, and duplicated samples were obtained at 10 min intervals during the period of 360-420 min (the first plateau period). Another set of duplicated samples were obtained at 10 min intervals during the last hour of L-[1-^{13}C] phenylalanine infusion (the second plateau period). To validate the short term study protocol, the requirement estimated during the first $^{13}CO_2$ enrichment plateau was compared to the requirement estimated during the second plateau. The period of 360-420 min was chosen because the isotopic steady state of L-[1-^{13}C]phenylalanine in expired air was obtained after 360 min of tracer infusion in our pilot study, which was 180 min after L-[1-^{13}C]phenylalanine infusion.

Urine samples were collected by using urine bags. One urine sample (1 mL per sample) was collected at the baseline, and 4 to 10 samples were collected depending on the voiding frequencies of the infants from 360 min onward until the end of the study. Urine samples were kept at -80˚C until analysis.

Blood samples (0.5 mL per sample) were collected by venipuncture. One blood sample was taken at the baseline and one blood sample at the end of the study. Blood samples were collected in anticoagulant tubes and were immediately centrifuged; the plasma was stored at -80˚C until analysis.

Analytical procedures

$^{13}CO_2$ isotopic enrichment in breath samples was analyzed by an infrared isotope analysis technique (Helifan, Analytic Fischer Instruments). The ^{13}C enrichment was expressed as the atom percentage excess above baseline (APE).

Urine and plasma enrichment of L-[1-^{13}C]phenylalanine were measured by gas chromatography-mass spectrometry (MSD 5975C Agilent GCMS; Agilent Technologies) as their ethyl chloroformate ester derivatives. Briefly, amino acid fractions in 50 µL of urine and 30 µL plasma were isolated by a Dowex cation-exchange resin column (AG 50W-X8, hydrogen form, Bio-Rad Laboratories) and were eluted with 0.7 mL 6 M NH_4OH. The eluate was evaporated under vacuum at room temperature in a speedvac (GeneVac miVac, GeneVac Ltd). Ethyl chloroformate derivatization of the samples was performed according to a modified procedure of Hušek (23). A CP-Chirasil L-Val GC column (25 m x 0.25 mm id, 0.12 µm film thickness; Varian) was used for the separation of D-[1-^{13}C]phenylalanine and L-[1-^{13}C]phenylalanine. An enrichment calibration curve was made for the measurement of L-[1-^{13}C]phenylalanine in urine and plasma. Samples were measured by using a selected ion monitoring mode method by using the mass fragments with an *m/z* of 176 for the unenriched (*M*) and a *m/z* 177 for the enriched (*M* + 1) L-phenylalanine. Each sample was analyzed in triplicate by using gas chromatography-mass spectrometry. Enrichments were calculated from the mean of the 3 analyses. Isotopic enrichment was calculated at the isotopic steady state and was expressed as mole percent excess (MPE).

Calculations

The isotopic steady state was represented by plateaus in $^{13}CO_2$ and L-[1-^{13}C]phenylalanine enrichments in urine. The last plasma sample was considered to be at isotopic plateau. Plateaus were determined by visual inspection and were confirmed by regression analysis as a slope not significantly different from zero.

Phenylalanine flux (*Q*) was measured from the dilution of the administered L-[1-^{13}C] phenylalanine into the amino acid pool by using enrichments of L-[1-^{13}C]phenylalanine in urine or plasma once the isotopic steady state was reached by using the following equation:

$$Q_{\text{urine or plasma}} = i_{\text{PHE}} \times [(IE_i \div IE_{\text{urine or plasma}}) - 1]$$

where i_{PHE} is the infusion rate of [1-^{13}C]phenylalanine in μmol·kg^{-1}·h^{-1}, and IE_i is the isotopic enrichment of L-[1-^{13}C]phenylalanine in the infusate in MPE. $IE_{\text{urine or plasma}}$ is the isotopic enrichment of L-[1-^{13}C]phenylalanine of urine or plasma, respectively.

The estimated body CO_2 production rate (mmol·kg^{-1}·h^{-1}) was calculated as follows (20):

$$\text{Body } CO_2 \text{ production} = [(IE_i \div IE_B - 1) \times i_B] \div 1000$$

where IE_i is the ^{13}C enrichment of [^{13}C]bicarbonate in the infusate (APE), IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (μmol·kg^{-1}·h^{-1}). This equation does not correct for retention of labelled carbon within the body bicarbonate pool and will overestimate the CO_2 production rate. However, the same correction factor has to be applied to quantify the phenylalanine oxidation rate with the assumption of a constant CO_2 production rate during the [^{13}C]bicarbonate infusion and during the L-[1-^{13}C]phenylalanine infusion (24). Consequently, this correction factor can be diminished in the following equation, and there is no need to measure the exact CO_2 production rate.

The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation in percentage ($F^{13}CO_2$) was calculated by using the following equation (24):

$$F^{13}CO_2 = (IE_{PHE} \times i_B) \div (i_{PHE} \times IE_B) \times 100$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (μmol·kg^{-1}·h^{-1}), i_{PHE} is the infusion rate of L-[1-^{13}C]phenylalanine (μmol·kg^{-1}·h^{-1}) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.

Whole body phenylalanine oxidation by using urinary L-[1-^{13}C]phenylalanine enrichment or plasma L-[1-^{13}C]phenylalanine enrichment was calculated as follows:

Whole body phenylalanine oxidation = ($F^{13}CO_2 \div 100$) × $Q_{\text{urine or plasma}}$

Statistical analysis

Descriptive data are expressed as means ± SDs. The effect of lysine intake on phenylalanine was tested with Pearson's correlation coefficient analysis. The difference in L-[1-^{13}C] phenylalanine enrichment of urine during isotopic plateau and plasma at 900 min was evaluated by a paired *t*-test. Bland and Altman analysis (25) was used to assess the agreement of L-[1-^{13}C]phenylalanine enrichment of urine during isotopic plateau and plasma at 900 min. The determination of the mean lysine requirement (ie, the breakpoint) was performed by using a biphasic linear regression crossover model (26). With the biphasic linear regression analysis, the regression equation was split into two parts. For the first part an intercept and slope were estimated, whereas for the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit on the basis of the highest r^2 was selected. The 95% CIs were calculated. The analyses were performed in STATA (version 11; StataCorp LP). $p < 0.05$ was considered significant.

RESULTS

Subject characteristics

Twenty-one term neonates participated in the study. The neonates were studied at a lysine intake that ranged between 15 and 240 mg·kg^{-1}·d^{-1}. Subject characteristics are summarized in Table 1. All subjects were growing well before entering the study. The mean (± SD) weightgain rate 3 days before the study was 9 ± 4 g·kg^{-1}·d^{-1}. The mean (± SD) energy intake was 109.1 ± 0.8 kcal·kg^{-1}·d^{-1}. The nitrogen intake was equivalent to a protein intake of 2.99 ± 0.02 g·kg^{-1}·d^{-1}. The infants were clinically stable and were considered healthy because they were discharged on the study day or the day after. The primary

Table 1: Subject characteristics of infants participating the study (n = 21)

	Mean ± SD
Birthweight (kg)	3.3 ± 0.3
Gestational age (wk)	39 ± 1
Age on study day (d)	12 ± 6
Weight on study day (kg)	3.5 ± 0.4
Weight gain before study (g·kg^{-1}·d^{-1})	9 ± 4
Sex (F:M)	9:12

reasons for admissions were unconjugated hyperbilirubinemia (n = 15), pneumonia (n = 3), infection suspicion (n = 2) and skin infection (n = 1). Intravenous antibiotics (penicillins and/or cephalosporins) were given to 15 of the 21 neonates.

Phenylalanine kinetics

Complete data sets of breath and urine samples were obtained from all but one subject. We could not obtain the last blood sample from the one infant.

The mean (± SD) phenylalanine flux calculated from urinary enrichment and plasma enrichment was 88.3 ± 6.9 μmol·kg^{-1}·h^{-1} and 84.5 ± 7.4 μmol·kg^{-1}·h^{-1}, respectively. There were no significant correlations between urinary phenylalanine flux and lysine intake (p = 0.73) or plasma phenylalanine flux and lysine intake (p = 0.53).

The ^{13}C enrichments in expired air of the first and second plateaus during L-[1-^{13}C]phenylalanine infusion are shown in Figure 2. The breakpoints in $F^{13}CO_2$ data as analyzed by biphasic linear regression crossover analysis from $^{13}CO_2$ isotopic enrichment of the first plateau (the period 360-420 min) and the second plateau (the period 840-900 min), are shown in Figures 3, A and B, respectively. For the first and second $F^{13}CO_2$ plateau data, a negative correlation was shown between lysine intake (if the intake increased to 130 mg·kg^{-1}·d^{-1}) and $F^{13}CO_2$; additional increases in lysine intake did not affect $F^{13}CO_2$. The breakpoint represented the mean lysine requirement, which was 130 mg·kg^{-1}·d^{-1}, with 95% upper and lower CIs of 188.4 and 71.6 mg·kg^{-1}·d^{-1}, respectively, for the first plateau (p < 0.0001, r^2 = 0.46). The breakpoint of the second plateau was also 130 mg·kg^{-1}·d^{-1}, with 95% upper and lower CIs of 183.7 and 76.3 mg·kg^{-1}·d^{-1}, respectively (p < 0.0001, r^2 = 0.51).

As illustrated in Figure 4, the urinary L-[1-^{13}C]phenylalanine enrichment was significantly different from the plasma L-[1-^{13}C]phenylalanine enrichment (p = 0.04, 2-tailed). From

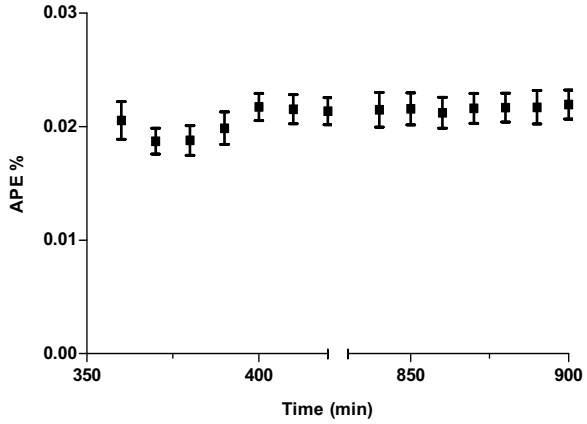

Figure 2. ^{13}C enrichments in APE (Mean ± SEM) in expired air during the first (period 360-420 min) and the second (period 840-900 min) isotopic plateaus of the [1-^{13}C]phenylalanine infusion (n = 21).

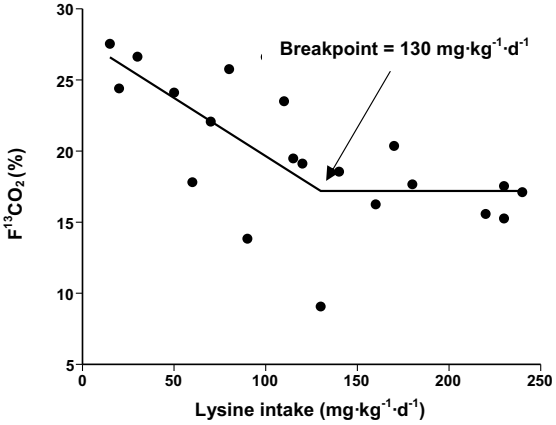

Figure 3A. The rates of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the first isotopic plateau (period 360-420 min), at different lysine intakes (n = 21). Using a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.46$). The upper and lower 95% confidence intervals of the breakpoint estimate were 188.4 and 71.6 mg·kg^{-1}·d^{-1}, respectively.

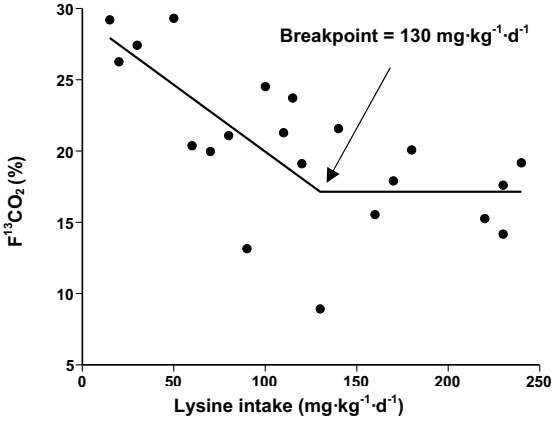

Figure 3B. The rates of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the second isotopic plateau (period 840-900 min), at different lysine intakes (n = 21). Using a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.51$). The upper and lower 95% confidence intervals of the breakpoint estimate were 183.7 and 76.3 mg·kg^{-1}·d^{-1}, respectively.

the Bland and Altman analysis, the mean (upper and lower 95% CIs) difference between urine and plasma enrichments was -0.72 (2.06, -3.51) MPE. There was a 5% probability that the measured enrichment using urine and plasma differed more than this amount (Figure 5). Phenylalanine oxidation calculated from the urine and plasma enrichment data also decreased with increasing lysine intake to 130 mg·kg^{-1}·d^{-1}, an additional increase of lysine intake more than 130 mg·kg^{-1}·d^{-1} did not result in an additional decrease

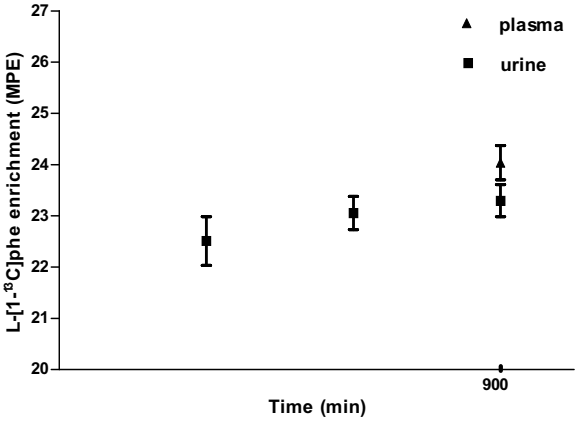

Figure 4. L-[1-^{13}C]phenylalanine enrichments in MPE (Mean ± SEM) in urine during isotopic plateau and the L-[1-^{13}C]phenylalanine enrichments in plasma at 900 min.

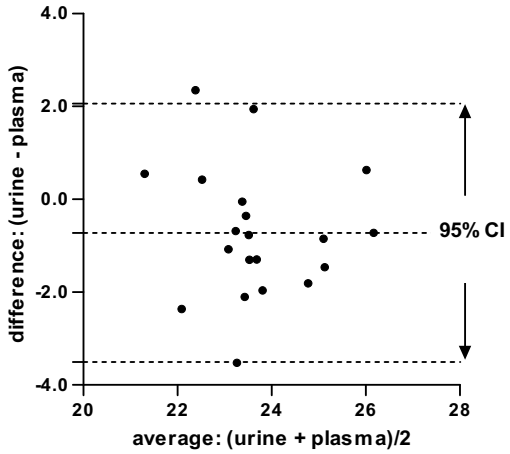

Figure 5. Bland-Altman analysis showing the difference between the mean L-[1-^{13}C]phenylalanine enrichments in urine during the isotopic plateau and the L-[1-^{13}C]phenylalanine enrichments in plasma at 900 min in 20 infants. The mean (upper and lower 95% confidence intervals) difference between urine and plasma enrichments was -0.72 (2.06, -3.51) MPE. There was a 5% probability that the measured enrichment in urine differs from the measured enrichment in plasma.

of phenylalanine oxidation. The breakpoints in the urinary and plasma phenylalanine oxidation data are shown in Figures 6A and 6B, respectively. Identical to the breakpoint determined by using $F^{13}CO_2$, the breakpoint determined by using phenylalanine oxidation rates in urine and plasma was 130 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.51$ and $p < 0.0001$, $r^2 = 0.49$, respectively). The 95% upper and lower CIs for urine was 183.2 and 76.8 mg·kg^{-1}·d^{-1}, respectively. The upper CI for plasma was 185.6 mg·kg^{-1}·d^{-1} and the lower CI was 74.4 mg·kg^{-1}·d^{-1}.

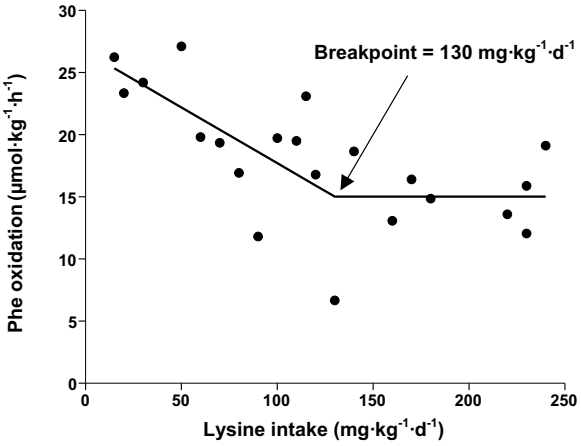

Figure 6A. Phenylalanine (Phe) oxidation calculated from urinary enrichment data at different lysine intakes (n = 21). Using a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.51$). The upper and lower 95% confidence intervals of the breakpoint estimate were 183.2 and 76.8 mg·kg^{-1}·d^{-1}, respectively.

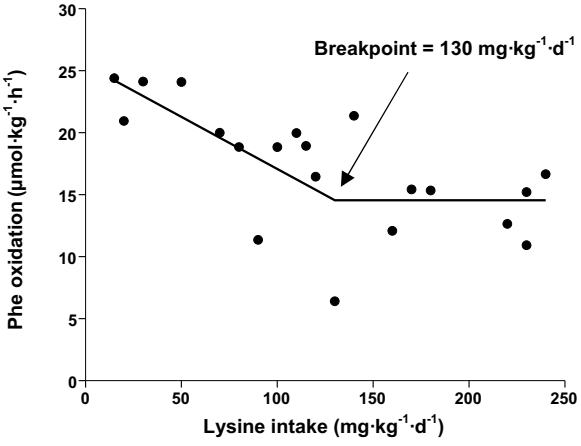

Figure 6B. Phenylalanine (Phe) oxidation calculated from the plasma enrichment data at different lysine intakes (n = 20). Using a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.49$). The upper and lower 95% confidence intervals of the breakpoint estimate were 185.6 and 74.4 mg·kg^{-1}·d^{-1}, respectively.

DISCUSSION

To our knowledge, this was the first study of the lysine requirement of fully enterally fed term neonates that used the IAAO method. The mean lysine requirement of enterally fed term neonates was estimated to be 130 $mg \cdot kg^{-1} \cdot d^{-1}$.

The experimental evidence for the lysine requirements of infants is very scarce. With the use of nitrogen balance and weight gain, Holt and Snyderman (27) estimated lysine requirements of 6 infants of postnatal ages between 1 and 5 months to be 90-105 $mg \cdot kg^{-1} \cdot d^{-1}$. The difference in estimated requirements with our study might have been due to the ages of the infants studied, the small number of infants studied, and the use of the nitrogen balance method, which may have underestimated the requirement. Fomon et al. (7) observed adequate growth in 13 normal full term female infants during ages of 8 to 41 days with an average lysine intake of 114 $mg \cdot kg^{-1} \cdot d^{-1}$, which was in the same range as our estimates. The infants in the study of Fomon et al. (7) were fed *ad libitum*, which meant that the infants can regulate their own intake, which resulted in a wide range of observed intakes.

Recently, Chapman et al. (17) estimated the lysine requirement of parenterally fed post-surgical neonates by using the IAAO method to be 104.9 $mg \cdot kg^{-1} \cdot d^{-1}$. Because the use of dietary essential amino acids by the intestine results in a lower systemic availability of these essential amino acids (28, 29), a higher amino acid requirement can be expected in fully enterally fed neonates. The first-pass lysine uptake in preterm infants with full enteral feeding was 18% (29). In our results, a requirement of 130 $mg \cdot kg^{-1} \cdot d^{-1}$ fit perfectly in the parenteral requirement determined by Chapman et al. (17) at a first pass use of 20%.

The current recommended lysine intake is based on human milk composition (8). Human milk has huge variations in protein concentrations; the protein content declines from 23 g/L on post partum day 3 to 14 g/L on day 28 (30, 31). This decline in protein content is accompanied by changes in the whey-casein ratio (32); consequently, the amino acid composition changes during the lactation period. However, the average lysine intake estimated in exclusively breastfed infants in the first month of life is 119 $mg \cdot kg^{-1} \cdot d^{-1}$ (8), which is comparable with our estimated requirement. The gross amino acid composition of human milk may not necessarily reflect the requirement profile of infants who consume infant formula because protein andamino acid digestibility and bioavailability are different in human milk from that in formula. Our study provided scientific knowledge of amino acid needs of infants fed an infant formula.

Raffii et al. (33) showed that change in phenylalanine hydroxylation, which is the first step in phenylalanine oxidation was better represented by apoB-100 instead of plasma phenylalanine. However, the requirement derived from $F^{13}CO_2$ data in our study was identical to the requirement estimated from the urine and plasma L-[1-^{13}C]phenylala-

nine enrichment data. The reason for the same estimates might have been due to the relative small range of distribution of phenylalanine flux in our study caused by the strict control of amino acid intake and the continuous tracer infusion. Because phenylalanine oxidation was calculated by multiplying $F^{13}CO_2$ with the flux, and the flux was constant, the phenylalanine oxidation rate consequently depended on the $F^{13}CO_2$. Moreover, by using the [^{13}C]bicarbonate method, which thereby determined the changes in $^{13}CO_2$ of each individual infant during both the [^{13}C]bicarbonate and L-[1-^{13}C]phenylalanine infusions (which corrected the bicarbonate retention individually), the $F^{13}CO_2$ can be measured more accurately.

Our second aim was to compare the lysine requirement from a short period tracer infusion protocol with a 900 min infusion protocol. Both protocols yielded identical requirement estimates. Therefore, we concluded that a short (and, thus, less invasive) IAAO protocol is valid for enterally fed infants.

We showed a small but significant difference of L-[1-^{13}C]phenylalanine enrichment in urine compared with plasma. Amino acid enrichment in urine is assumed to reflect the enrichment in arterialized blood. The difference might be because urine samples represent average enrichment values during the collection period, whereas plasma represents enrichment at a specific time and site of sampling. In our study, urine samples were collected in the period before the collection of the venous blood sample from the hand or foot. Another explanation might be that isotopic steady state had not yet been reached in urine of neonates who had relative long voiding intervals, which resulted in few urine samples at steady state. The lower urinary L-[1-^{13}C]phenylalanine enrichment compared with in plasma was also shown in the studies by Zello et al. (15) and Bross et al. (14) in adults. A possible explanation is the short tracer infusion time (4 h), which resulted in non steady-states. The lack of significance in the study by Bross et al. (14) was possibly the consequence of a small number of subjects (n = 4). Wykes et al. (16) observed a higher enrichment in urine compared with plasma. This observation might have been due to the contamination of D-[1-^{13}C]phenylalanine in the tracer. A recent study showed a significant confounding effect of D-phenylalanine in urine even when [1-^{13}C]phenylalanine was used with optical purity of 0.1% in neonates (34). We used a chiral column for the separation of the D- and L-phenylalanine to overcome this problem.

There were some limitations in our study design. The study was performed by using an amino acid formula. Metges et al. (35) have shown that leucine oxidation is higher and non-oxidative leucine disposal is lower when an amino acid diet is used compared with when a casein diet is used. These results suggest that leucine derived from an amino acid diet has a lower utilization rate. Their findings were supported by the study of Dangin et al. (36), which demonstrated that the protein digestion rate is an independent factor of protein retention. The effect of the decreased utilization rate of amino acids by

consuming an amino acid diet could result in higher requirement estimates compared with consumption of a protein diet. Therefore, our determined lysine requirement could have been an overestimation. Future studies with an intrinsically labelled protein that is the closest simulation to a normal dietary amino acid intake are required to evaluate this issue.

Another limitation of our study was the antibiotic used in our study population. Antibiotics are extensively prescribed to children who are admitted to the children's hospitals in China (37). As a result of this practice, 15 of 21 infants in our study received intravenous antibiotics. Antibiotic treatment has a major impact on the bacterial flora in the gastrointestinal tract (38), and it has been shown that microbial lysine can be made available to a human host (39, 40). Previous studies did not clarify the issue whether microbial lysine contributes to the dietary amino acid requirement estimates (41). To our knowledge, there are no data in the literature on antibiotic use and its effect on essential amino acid requirements.

In conclusion, this study was the first in a series of studies designed to determine the essential amino acid requirements of enterally fed neonates by using the adapted minimal invasive IAAO method. Under the conditions of this study, the lysine requirement of enterally fed term neonates was 130 $mg \cdot kg^{-1} \cdot d^{-1}$. Current term formulas provide an excess of lysine (172-256 $mg \cdot kg^{-1} \cdot d^{-1}$) according to our estimated mean requirement (42, 43). The lack of knowledge with regard to the optimal amino acid pattern in formula feeding is a reason to perform additional studies on the amino acid requirements of enterally fed infants to optimize nutrition for (preterm) infants.

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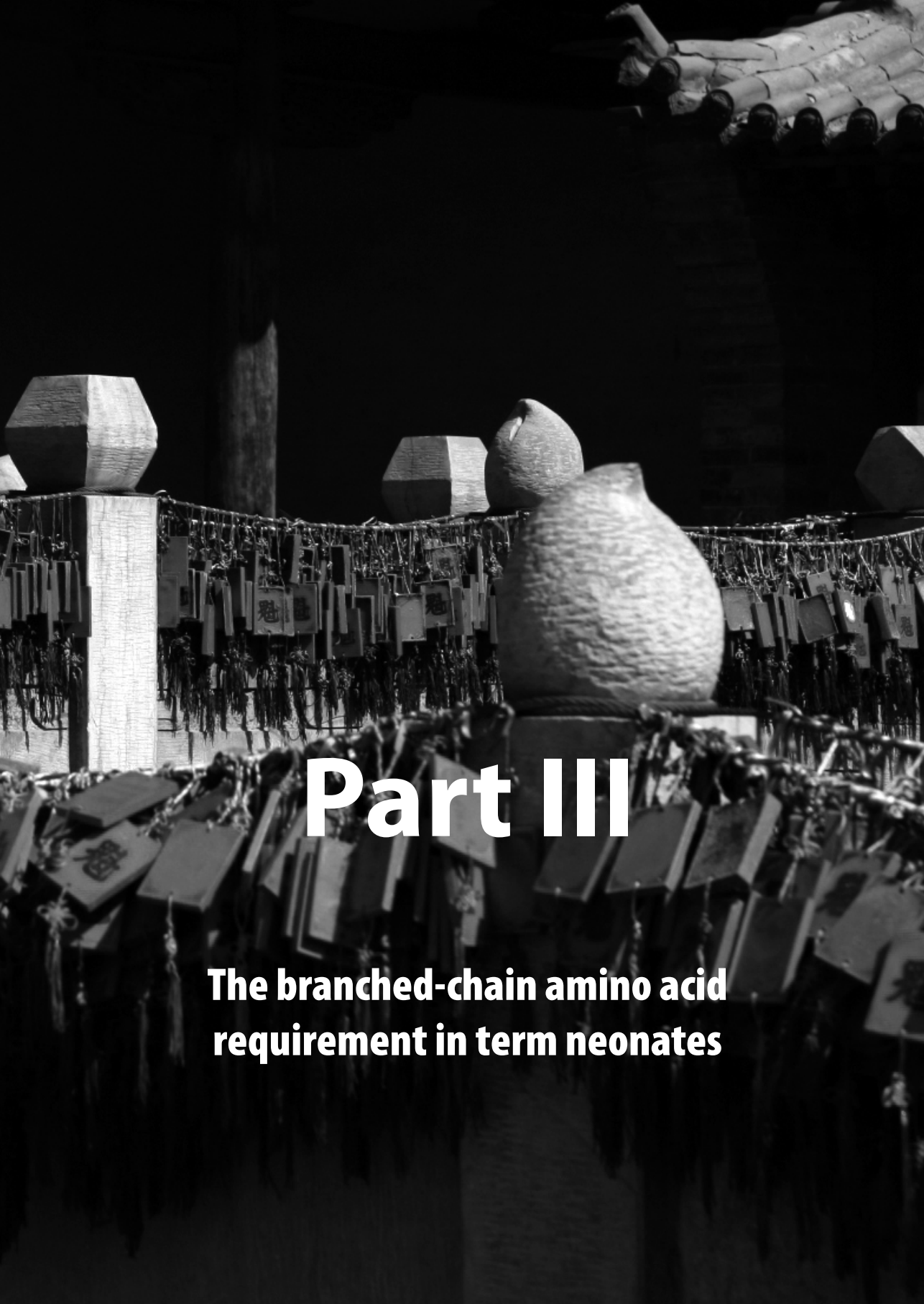

Part III

The branched-chain amino acid requirement in term neonates

CHAPTER

5

Isoleucine requirement for enterally fed term neonates in the first month of life

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ABSTRACT

Background: Knowledge of the essential amino acid requirements for infants is important because excessive intake can lead to increased long-term morbidity, such as obesity. A deficient intake can lead to suboptimal growth and impaired neurodevelopment. Current recommended isoleucine requirement for infants aged 0 to 1 month (95 $mg \cdot kg^{-1} \cdot d^{-1}$) is based on the amino acid content of human milk.

Objective: To quantify the isoleucine requirement for term neonates using the indicator amino acid oxidation (IAAO) method with [1-^{13}C]phenylalanine as the indicator.

Design: Fully enterally fed term infants received randomly graded amounts of isoleucine (5-216 $mg \cdot kg^{-1} \cdot d^{-1}$) as part of an elemental formula. After 1 day adaptation to the test diet [^{13}C]bicarbonate and [1-^{13}C]phenylalanine tracers were given enterally. Breath samples containing $^{13}CO_2$ were collected during [1-^{13}C]phenylalanine infusion, measured by infrared isotope analysis, and analyzed using a biphasic regression model. Data are expressed as mean ± SD.

Results: Twenty-two Asian term neonates (birth weight 3.22 ± 0.41 kg, gestational age 39.5 ± 1.2 wks) were studied at a postnatal age of 12 ± 5 d. The mean isoleucine requirement (at breakpoint) was 105 $mg \cdot kg^{-1} \cdot d^{-1}$ ($r^2 = 0.61$, $p < 0.001$). The upper and lower CIs were determined to be 150 and 60 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively.

Conclusion: Our study shows that the current human milk-based recommendation for isoleucine in term infants aged 0 to 1 month are correct. The IAAO method should be used to determine the requirements for valine and leucine as well to validate current recommendations.

INTRODUCTION

The increased prevalence and severity of obesity in children has renewed an interest in feeding patterns during infancy. High early weight gain in the first 1-2 years of life is associated with adverse health outcomes later in life, including increased blood pressure (1), increased weight gain and body fat deposition (2-5) and an increased risk of diabetes (6). The higher protein intake in infants who are fed formula may play a role with these health outcomes because formula-fed infants reach a higher body weight and weight for length at one year of age compared with infants who are fed breast milk (7, 8). However, early nutrition (especially protein intake) correlates with improved neurodevelopment in preterm infants (9, 10). Thus, protein intake should be strictly regulated early in life to result in the best possible neurodevelopment while reducing the risk of obesity.

The current recommended isoleucine requirement for infants aged 0 to 1 month (95 $mg \cdot kg^{-1} \cdot d^{-1}$) is based upon the amino acid content of human milk, which is considered to be the optimal nutrition for infants up to 6 months of age (11). Breastfed infants, however, have a variable milk consumption rate, which makes it difficult to provide an accurate estimation of the amino acid intake, and they largely regulate the intake they require themselves (12-14). Our group recently showed that the indicator amino acid oxidation (IAAO) method is a minimally invasive method that can be used to determine the amino acid requirement in neonates (15). To validate current recommendations based on human milk we will determine the requirement for all the nine essential amino acids in term neonates.

The branched-chain amino acids (BCAAs) valine (Val), isoleucine (Ile) and leucine (Leu) are similar in structure and share common enzymes for transamination and oxidative decarboxylation (16, 17). Considerable interaction has been reported in humans and animals in response to disproportional intake of BCAAs. In rats, the antagonism of BCAAs results in impaired growth, and BCAA supplementation has negative effects on fetal brain growth (17). BCAA-enriched total parental nutrition results in decreased apnea and an improvement of the respiratory pattern and function in premature neonates (18). A high BCAA concentration in plasma, which has been observed for infants who are fed formula with a high protein content, may affect insulin metabolism, carbohydrate metabolism, weight gain and the future incidence of diabetes (19, 20). Identifying the optimal isoleucine intake and the optimal BCAA ratio can benefit neonates. Therefore, the aim of the present study is to determine the mean isoleucine requirement for term neonates.

SUBJECTS AND METHODS

Subjects

Term infants (n= 22) admitted to the Neonatology Department of the Fudan Children's Hospital in Shanghai, China, between September 2008 and July 2009 were enrolled in this study. The infants' gestational age was 37 to 43 wks, their birth weight exceeded 2.5 kg and their postnatal age was ≤ 28 d. Each infant was clinically stable and in an anabolic growth state as shown by a weight gain ≥ 5 $g \cdot kg^{-1} \cdot d^{-1}$ over the previous 3 days. All tolerated full enteral feeding well and had no congenital or gastrointestinal diseases. The study protocol was approved by the Medical Ethics Committee of the Fudan Children's Hospital and a statement of no objection was obtained from the Medical Ethics Committee of the Erasmus MC- Sophia's Children's Hospital. Similar studies, such as those determining cysteine requirements, have been performed previously at the Erasmus MC-Sophia Children's Hospital (21, 22). Written informed consent was obtained from one or both parents for all participants after a Mandarin-speaking researcher provided a precise explanation of the study.

Experimental design

The study is based on the minimally invasive IAAO method that our group recently modified for use in enterally fed infants by using a short period of adaptation to the test diet (1 d), enterally infused isotopes and the sampling of expired air without the sampling of amino acid enrichments in urine of plasma (15). The IAAO technique (23) uses an indicator that is oxidized when one essential amino acid is limiting; typically, there is no storage of amino acids because they are incorporated into protein or metabolized by oxidation (24). If the tested amino acid is deficient in the diet, protein synthesis will be limited, causing the indicator amino acid to be oxidized. Upon the increase of the dietary intake of the test amino acid, indicator oxidation will decrease until the test amino acids' requirement is met. Once the intake meets a critical threshold (or requirement), protein synthesis can occur at an optimum capacity, and the oxidative degradation of all other essential amino acids reaches a plateau. The mean requirement for the test amino acid is identified by this breakpoint.

The subjects were randomly assigned to receive graded amounts of isoleucine ranging from 5 to 216 $mg \cdot kg^{-1} \cdot d^{-1}$. Each infant received a different intake and was studied one time with one intake. After adaptation to the study diet for 24 h, baseline breath samples were obtained, and a tracer protocol was initiated, as depicted in Figure 1. Subjects were weighed daily, before and at the end of the tracer protocol.

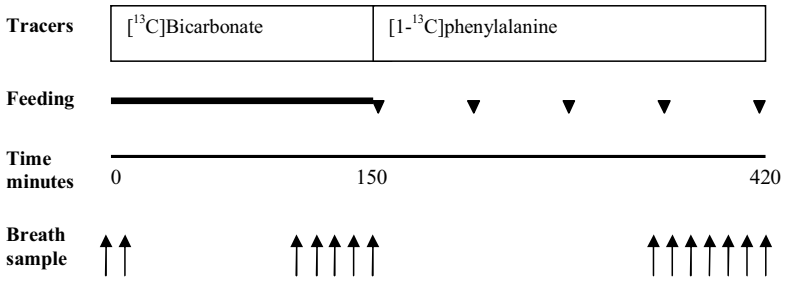

Figure 1: Study design after one day of adaptation to the study diet
▼ time of oral feeding (given every hour)
—— continuous feeding (dripfeeding)
↑ time that breath samples were taken

Study Formula

The study formula was based on an amino acid-based formula designed to fulfill infants' amino acid requirements of infants (SHS, Liverpool, United Kingdom), but without isoleucine and with reduced phenylalanine to compensate for the tracer. The amount of isoleucine was adjusted separately as L-isoleucine. L-phenylalanine was supplied during the adaptation period and during the infusion of [^{13}C]bicarbonate to obtain a stable total intake of 166 mg·kg^{-1}·d^{-1} throughout the entire study. L-alanine was added separately to make the formula isonitrogenous. The formula's amino acids, fat, carbohydrates and energy content are shown in Table 1. The osmolality of the study formula is 330 Osm/L. The minerals, trace elements and vitamins of the formula were described previously (25). Because phenylalanine, which is hydroxylated to tyrosine before oxidation can occur, served as indicator, we made sure that tyrosine intake exceeded present requirements. A tyrosine intake of 166 mg·kg^{-1}·d^{-1} was provided ,which is almost twice the human milk-based recommendated intake of 90 mg·kg^{-1}·d^{-1},to ensure that the newly formed [1-^{13}C] tyrosine hydroxylated from [1-^{13}C]phenylalanine would be directly channeled into $^{13}CO_2$ which could be measured in expired air (26).

We used [1-^{13}C]phenylalanine as a tracer, but because the tracer behaves identical to the tracee, phenylalanine intake was appropriate and constant for the complete duration of the study. On the adaptation day the subjects were fed every 3 h. On the study day the subjects were fed by a continuous dripfeeding during the [^{13}C]bicarbonate infusion to minimize the variance in CO_2 production which is dependent on the feeding regimen (27). We changed it into hourly feedings during [1-^{13}C]phenylalanine infusion to minimize the discomfort because the infants were used to drink their own bottles. This hourly feeding regimen has shown a steady state during 4h of [1-^{13}C]phenylalanine infusion in our previous study (15).

Table 1: Energy, carbohydrates, fat and amino acid content of the study formula[1].

Component	*Per 100g formula*
Energy (kcal)	475
Carbohydrates (g)	54
Fat (g)	23
Total amino acid (g)	13
L-Alanine (g)[2]	≥ 0.61
L-Arginine (g)	1.08
L-Asparagine (g)	1.01
L-Cyst(e)ine (g)	0.4
Glycine (g)	0.95
L-Histidine (g)	0.62
L-Isoleucine (g)[3]	0
L-Leucine (g)	1.63
L-Lysine (g)	1.11
L-Methionine (g)	0.26
L-Phenylalanine (g)[4]	0.20
L-Proline (g)	1.16
L-Serine (g)	0.71
L-Threonine (g)	0.8
L-Tryptophan (g)	0.32
L-Tyrosine (g)	0.73
L-Valine (g)	1.04
L-Carnitine (g)	0.01
Taurine (g)	0.03
L-Glutamine (g)	1.34

[1] The study formula was based on Neocate (Danone, United Kingdom), an amino acid based formula**.**

[2] Variable levels of L-alanine were added to the diet depending on the test isoleucine level of each infant to maintain an isonitrogenous diet. The study formula contained at least 0.61 g L-alanine per 100 g formula.

[3] L-isoleucine was added separately, depending on the test isoleucine level.

[4] 0.53 g L-phenylalanine per 100 g formula was added to the study diet on day 1. Equivalent amount of L-phenylalanine (0.52 g per 100 g formula) was given as isotope on day 2.

Tracer protocol

On the study day, the subjects received a primed (14 μmol·kg^{-1}) continuous (9 μmol·kg^{-1}·h^{-1}) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% ^{13}C atom percent excess (APE); Cambridge Isotopes, Woburn, MA) for 2.5 h to quantify individual CO_2 production. The labelled sodium bicarbonate infusion was directly followed by a primed (34 μmol·kg^{-1}), continuous (27 μmol·kg^{-1}·h^{-1}) enteral infusion of [1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 4.5 h by an infusion pump via the nasogastric tube. Our previous study showed that this short-term protocol of 420 min was sufficient to determine the lysine requirement when compared to a 900 min infusion protocol; they showed a similar requirement in breath, urine and plasma (15). Syringes with tracers were weighed before and after infusion to determine the exact amount of tracer administered during the study.

Sample collection

On the study day, baseline samples were obtained 5 and 15 min before starting the tracer infusion using the direct sampling method, as described by van der Schoor et al. (28). Duplicate ^{13}C-enriched breath samples were collected every 10 min starting after 1.75 h during the isotopic steady state of the [^{13}C]bicarbonate infusion and then every 15 min during the isotopic steady state of the [1-^{13}C]phenylalanine infusion starting after 3 h, as depicted in Figure 1.

Analysis and Calculations

Samples were sent from Shanghai to Rotterdam every 3 wks by air transport. The $^{13}CO_2$ isotopic enrichment in expired air was measured using infrared isotope analysis (Helifan, Analytic Fischer Instruments, Leipzig, Germany) and expressed as the APE above baseline. Steady state was defined as 3 or more consecutive points with a slope not significantly different from zero ($p \geq 0.05$). The estimated body CO_2 production ($mmol \cdot kg^{-1} \cdot h^{-1}$) was calculated for each infant as previously described (15, 29). The fraction of $^{13}CO_2$ recovery from [1-^{13}C]phenylalanine oxidation in percentage ($F^{13}CO_2$) was calculated by using this equation (30):

$$F^{13}CO_2\ (\%) = [IE_{PHE} \times i_B] \div [i_{PHE} \times IE_B] \times 100$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), i_{PHE} is the infusion rate of [1-^{13}C]phenylalanine ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.

Phenylalanine flux was not obtained. As shown in our previous study, the test amino acid intake has no effect on the phenylalanine flux (15). Regarding the potential interaction of the BCAAs; in enterally fed adults, valine kinetics were determined at different leucine intakes and leucine kinetics were determined at different valine and isoleucine intakes. Valine turnover did not change among the various intakes of valine and leucine. Leucine flux was also not affected by the valine or the isoleucine intakes. Valine and leucine requirements were not affected by the ratio of BCAA used when given within a physiological range (31, 32).

Statistical analysis

Descriptive data are expressed as the mean ± SD. The effect of weight gain on $F^{13}CO_2$ was tested with Pearson's correlation coefficient analysis. A paired t-test was used to test the difference between the weight for age z-score at birth and the weight for age z-score at the study day. The effects of isoleucine intake on mean $^{13}CO_2$ enrichment at the isotopic plateau during [^{13}C]bicarbonate infusion, and on CO_2 production rate were tested with

Pearson's correlation coefficient analysis. Mean isoleucine requirement was determined by applying a two-phase regression model (24, 33) on the $F^{13}CO_2$ values. In this model a breakpoint is estimated using non-linear regression. With the biphasic lineair regression analysis, the regression equation was split into 2 parts. For the first part, an intercept and slope were estimated. For the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit (based on the highest r^2) was selected. The 95% confidence intervals (CIs) were calculated. All statistical analyses were performed with STATA (version 11; Stata-Corp LP). A p-value < 0.05 was considered significant. The power analysis could not be performed. We aimed to study 20-25 infants which is a higher number of subjects than was studied in the IAAO studies that used intravenous administration of the tracer in parenterally fed infants (34-36).

RESULTS

Clinical characteristics

The clinical characteristics of the 22 subjects studied are presented in Table 2. A total of 22 oxidation studies were performed. The reasons for admission were unconjugated hyperbilirubinemia (n=8), pneumonia with a negative blood culture (n= 7), infection suspicion with a negative blood culture (n= 3), cardiac arrhythmia (n=2), asphyxia (n=1) and pneumothorax (n=1). The infants were in a clinically stable condition and considered healthy as demonstrated by their weight gain rates and the fact that they were discharged on the study day or the day after. The mean weight gain rate in the five days before the study was 12.6 ± 6 $g \cdot kg^{-1} \cdot d^{-1}$. The weight for age z-scores at birth and the weight for age z-scores at the study day were determined as shown in Table 2. The

Table 2: Subject characteristics, protein and caloric intake before and during the study (n = 22).

	Mean ± SD
Gestational age at birth (wks)	39.5 ± 1.2
Age at study (d)	12 ± 5
Birth weight (g)	3.22 ± 0.41
Weight for age z-score at birth	- 0.25 ± 1.04
Weight at study day (g)	3.36 ± 0.41
Weight for age z-score at study day	- 0.55 ± 1.00
Male:Female ratio	9:13
Intake during study ($mL \cdot kg^{-1} \cdot d^{-1}$)	150.2 ± 0.7
Intake during study day (g formula$\cdot kg^{-1} \cdot d^{-1}$)	22.8 ± 0.1
Caloric intake before study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	112 ± 8.3
Caloric intake during study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	108.2 ± 0.5
Protein intake before study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.53 ± 0.25
Protein intake during study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.96 ± 0.15

weight for age z-score at the study day was significantly lower than the weight for age z-score at birth ($p < 0.000$, 2-tailed).

Expired CO_2 enrichment

All subjects achieved an isotopic steady state (plateau) at both the [^{13}C]bicarbonate and the [1-^{13}C]phenylalanine infusion as shown in Figure 2. The baseline $^{13}CO_2$ enrichment was -18.45 ± 1.11 pee dee belemnite (PDB) (0.0000 APE). The mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion was 0.0370 ± 0.0043 APE. The corresponding mean CO_2 production rate was 24.45 ± 3.01 mmol·kg^{-1}·h^{-1}. No correlation was found between isoleucine intake and the mean $^{13}CO_2$ enrichment at isotopic plateau (p= 0.21) or between the isoleucine intake and the CO_2 production rate (p=0.13).
The mean $^{13}CO_2$ enrichment at isotopic plateau during [1-^{13}C]phenylalanine infusion was 0.0203 ± 0.0040 APE. The mean $^{13}CO_2$ enrichments during [1-^{13}C]phenylalanine infusion were plotted against isoleucine intakes and are shown in Figure 3A.
No correlation was found between weight gain before the study and F$^{13}CO_2$ (p = 0.35).

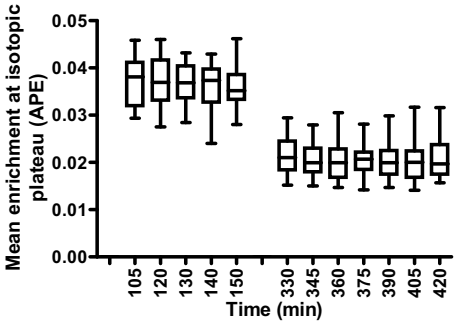

Figure 2: Mean ± SD ^{13}C enrichments in APE in expired air at isotopic plateaus: the first plateau is during [^{13}C]bicarbonate infusion (T105-T150) and second plateau is during [1-^{13}C]phenylalanine infusion (T330-420). APE: atom percent excess.

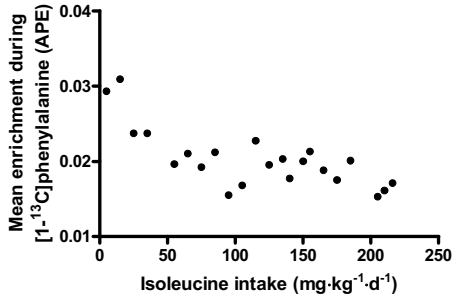

Figure 3A: Mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [1-^{13}C]phenylalanine infusion plotted against isoleucine intake (n=22).

Overall there was a significant decrease in fractional oxidation when isoleucine intake increased ($r^2 = 0.61$, $p < 0.001$). From the two-phase regression analysis with isoleucine intake as the independent variable and fractional oxidation of the [1-^{13}C]phenylalanine tracer as the dependent variable, the breakpoint was determined to be 105 mg·kg^{-1}·d^{-1} (Figure 3B). The upper and lower 95% CIs of the breakpoint estimate were determined to be 150 and 60 mg·kg^{-1}·d^{-1}, respectively.

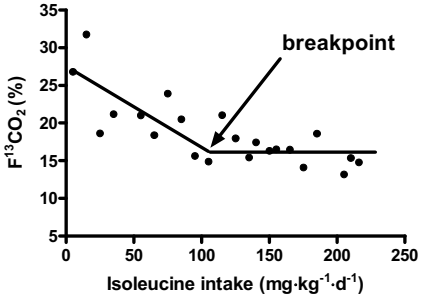

Figure 3B: $F^{13}CO_2$ during the isotopic plateau at different isoleucine intakes (n=22). Each infant received a different intake and was studied one time with one intake. With the use of a biphasic linear regression model the breakpoint (mean isoleucine requirement) was estimated to be 105 mg·kg^{-1}·d^{-1} ($r^2 = 0.61$, $p < 0.001$). The upper CI was 150 mg·kg^{-1}·d^{-1} and the lower was 60 mg·kg^{-1}·d^{-1}. APE: atom percentage excess; $F^{13}CO_2$: fraction of recovery from [1-^{13}C]phenylalanine oxidation.

DISCUSSION

The mean isoleucine requirement in term neonates fed an elemental diet using the IAAO method is 105 mg·kg^{-1}·d^{-1}. The current recommended isoleucine requirement based on human milk is 95 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month. The present data suggest that the current human milk-based recommendations for isoleucine are correct.

There are no isotopic data for individual isoleucine requirements in humans or animals. In 1964, Snyderman et al. (37) determined the isoleucine requirement in 6 healthy male infants to be between 79 and 126 mg·kg^{-1}·d^{-1} based on the nitrogen balance method and weight gain. The nitrogen balance method underestimated the amino acid requirements in adults (38) which was likely due to the failure to account for miscellaneous nitrogen losses. Because a limited amount of available data regarding amino acid requirements in infants and children, the use of a factorial approach was proposed to define dietary indispensable amino acid requirements (11, 39). This method uses the obligatory losses as maintenance requirement and adds the nutrients needed for growth. Using the factorial approach, Dewey et al. (40) determined the isoleucine requirement in infants aged 0 to 1 month to be 59 mg·kg^{-1}·d^{-1} and implied that breast milk provided on average a 45% excess of indispensable amino acids at 0 month of age. Because the fact that the intake

of breast milk from a healthy, well-nourished mother is considered to satisfy protein requirements in the first 6 months of life, the amino acid content of breast milk was considered to be the best estimate of amino acid requirement for this group. Recently, we determined the mean lysine requirement using the IAAO method in term neonates to be comparable to the recommendation based on human milk (130 $mg \cdot kg^{-1} \cdot d^{-1}$ vs. 119 $mg \cdot kg^{-1} \cdot d^{-1}$), and the mean isoleucine requirement determined in the present study was also similar to the recommendation based on human milk (105 $mg \cdot kg^{-1} \cdot d^{-1}$ vs. 95 $mg \cdot kg^{-1} \cdot d^{-1}$) (15). The mean methionine requirement determined by our group, however, was substantially higher than the estimations based on human milk (38 $mg \cdot kg^{-1} \cdot d^{-1}$ vs. 28 $mg \cdot kg^{-1} \cdot d^{-1}$) (25) but the requirement based on human milk was within the 95% confidence interval determined by the IAAO method. Our estimations might overestimate requirements because a higher amount of amino acids derived from intact casein are utilized for protein synthesis than the proportion derived from an equivalent intake of free amino acids (41). Because human milk contains approximately 25% of non- protein nitrogen and calculations on human milk are based on the 75% of total nitrogen in breastfed infants, the actual amino acid intake in breastfed children might be slightly higher than the current recommendations (i.e., because the non-protein content of milk includes some free amino acids) (11). Indeed, the actual mean isoleucine requirement for infants aged 0 to 1 month might be between 95 and 105 $mg \cdot kg^{-1} \cdot d^{-1}$.

Isoleucine requirements should be considered and compared with the requirements for the other BCAAs valine and leucine, which are similar in structure and share common enzymes for transamination and oxidative decarboxylation (16, 17). Recent studies have shown that certain characteristics (e.g., the ratio between individual BCAAs) influence protein synthesis (42, 43). A high intake of leucine by humans or animals enhances the activity of branched chain keto-acid dehydrogenase (BCKAD) in various tissues (which catalyzes the decarboxylation of the BCAAs) (17, 44), and decreases valine and isoleucine concentrations in the blood. As described earlier, in adults it was shown that individual BCAA requirements do not change when the BCAA ratio was changed within the physiological range (32). Because these studies were performed using the direct oxidation model, one could argue whether this is also true for the indirect method in which the test amino acid differs from the indicator amino acid and the potential for antagonism might be increased. Highly elevated leucine concentrations (four to six fold above normal concentrations) decreased the concentrations of other amino acids such as valine, isoleucine, phenylalanine, tyrosine and methionine, probably as a result of altering amino acid transport (45). Interestingly, the reduction in the amino acids concentrations was determined to be a leucine-specific effect, e.g. valine and isoleucine intake had limited effect on the concentrations of phenylalanine, tyrosine and methionine (46). Because phenylalanine is the indicator amino acid in our study, the phenylalanine flux

is not allowed to change. We did not study the phenylalanine flux in the present study but the upper amount of isoleucine was within normal ranges (max 216 $mg \cdot kg^{-1} \cdot d^{-1}$), as we used a commercial formula. So besides the fact that isoleucine is not known to influence phenylalanine kinetics, the amount of isoleucine in the highest ranges of our study is not likely to result in a significant effect on the phenylalanine plasma concentrations. However, no studies have been performed in human neonates who have higher turnover rates and might react differently. Pencharz et al. studied a wide range of BCAA intakes in neonatal piglets, without an effect on phenylalanine flux (43), while also no change in phenylalanine flux was noted at different BCAA intakes in five 20 year old male syrup disease patients (47).

Regular protein and amino acid based formulas provide a maximum isoleucine of approximately 100-230 $mg \cdot kg^{-1} \cdot d^{-1}$ at an intake of 150-180 $ml \cdot kg^{-1} \cdot d^{-1}$ (48-51). A previous study showed no correlation between urinary phenylalanine flux and lysine intake or plasma phenylalanine flux and lysine intake (15).

By determining the BCAA requirements individually we can determine the optimal BCAA ratio in enterally fed infants. The current recommendated Ile:Leu:Val ratio in enteral feeding is 1:1.8:1 (39). Different formulas use BCAAs in different Ile:Leu:Val ratios depending on their casein-whey ratio (i.e.,1 : 1.4 : 0.9 – 1 : 2.3 : 1.2) (48-51). Identifying the optimal BCAA ratio can optimize infant nutrition. It would also be interesting to determine the BCAA ratio in parenteral nutrition because a BCAA ratio of 1 : 1 : 1 in parentally fed piglets is considered optimal, and isoleucine is considered to be the most limiting BCAA (52).
Previous studies in enterally fed subjects have shown that intrasubject variation is the major source of variability in amino acid requirements and is a potential source of error in the estimation of amino acid requirements in humans (53, 54). Present study shows a wide variability, reflected in the 95% confidence interval range of the breakpoint estimation. We postulate that the variability is largely the result of interindividual differences, with a possible cause being the enterally infused tracer because its oxidation depends on the rate of gastric emptying (55). A wide variability is also shown in the mean weight gain in the 5 days before the study. This might be the result of catch-up weight gain after recovery since all infants were admitted at the neonatal ward for several reasons (as described in the results section), and might have had suboptimal intakes before admission to the hospital. At the study day they were considered healthy since they were discharged on the study day or the day after. For children during rapid recovery, a high value of 70% for the efficiency of protein utilization is assumed whereas a value of 58% is assumed for normal children (11). Because the mean weight for age z-score was above the -2 at the study day (i.e., there was no wasting) we postulate that it did not influence

the requirements. If there was a minimal effect, the mean requirement would be lower because of the more efficient protein utilization of the diet.

The present study shows that current recommendations based on the content of amino acids in breast milk are correct. The factorial approach in infants aged 0 month that was calculated by Dewey et al. seems to underestimate the requirements in these infants. The IAAO method should be used to determine the requirements for valine and leucine and the optimal BCAA ratio in term infants to validate current recommendations based on human milk.

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CHAPTER

6

Valine requirement for enterally fed term neonates in the first month of life

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ABSTRACT

Background: Knowledge of newborns' essential amino acid requirement is important to prevent long term morbidity. Excess amino acid intake can lead to obesity; deficient intake to reduced growth and cognitive development. The currently recommended valine requirement for infants aged 0 to 1 month (95 mg·kg^{-1}·d^{-1}) is based upon the amino acid content of human milk. Since human milk composition shows remarkable variation, studies are needed to validate these data.

Objectives: To quantify the valine requirements for term neonates using the indicator amino acid oxidation (IAAO) method with [1-^{13}C]phenylalanine as the indicator.

Design: Fully enterally fed term infants received randomly graded intakes of valine (5-236 mg·kg^{-1}·d^{-1}) as part of an elemental formula. After 1 day adaptation to the study formula, [^{13}C]bicarbonate and [1-^{13}C]phenylalanine tracers were given enterally. Breath samples containing $^{13}CO_2$ were collected during [1-^{13}C]phenylalanine infusion, measured by infrared isotope analysis, and analyzed using a biphasic regression model.

Results: Twenty-eight Asian term male neonates (birth weight 3.39 ± 0.44 kg, gestational age 39.5 ± 1.2 wks) were studied at a mean postnatal age of 15 ± 7 d. The mean requirement (at breakpoint) was 110 mg·kg^{-1}·d^{-1} ($r^2 = 0.35$, $p = 0.001$) (upper and lower CIs: 164 and 56 mg·kg^{-1}·d^{-1}).

Conclusion: Our study shows that the present recommendations for valine in term infants aged 0 to 1 month based on the amino acid content of human milk are close to our mean requirement determined with the IAAO method and are within our confidence interval.

INTRODUCTION

Knowledge of the essential amino acid requirements for (preterm) infants is important since excessive or deficient intake might lead to long term morbidity such as obesity (1, 2) or suboptimal growth and impaired neurodevelopment (3, 4). Protein intake should be strictly regulated early in life to result in the best possible neurodevelopment while reducing the risk of obesity. According to the World Health Organization, exclusive breastfeeding by a healthy mother is the feeding standard from birth to 6 months in healthy, term infants. The current recommended valine requirement for term infants aged 0 to 1 month (95 $mg \cdot kg^{-1} \cdot d^{-1}$) is based upon the amino acid content of human milk (5). Since breastfed children have quite variable milk intakes it is difficult to provide an accurate estimation based on human milk (6-8). Furthermore, the human milk composition varies over the different stages on lactation; the protein intake of breastfed infants decreases from 1.7- 2.09 $g \cdot kg^{-1} \cdot d^{-1}$ at 0-1 month to 0.9- 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ at 3-4 months (9, 10). Other methods are needed to validate current recommendations. The IAAO method is a minimally invasive method to determine amino acid requirement in neonates (11, 12) and children (13). Our group estimated the mean requirement for methionine and lysine in infants in the first month of life with the IAAO method. The mean requirement of methionine showed to be higher than current recommendations based on human milk, the lysine requirement we found was comparable to the recommendations based on human milk (11, 12). To validate current recommendations based on human milk, our objective was to determine the valine requirements for enterally fed neonates using the IAAO technique. Our second objective was to determine the time needed to allow background adaptation to the experimental diet.

SUBJECTS AND METHODS

Subjects

Term male infants (n=28) admitted between September 2008 and March 2009 to the Neonatology Department of the Fudan Children's Hospital in Shanghai, China, were enrolled in this study. Their gestational age was 37-43 wks, birth weight exceeded 2.5 kg and their postnatal age was ≤ 28 days. They were clinically stable and in a positive growth state as shown by a weight gain > 5 $g \cdot kg^{-1} \cdot d^{-1}$ over the last 5 days. All tolerated full enteral feeding well and had no congenital or gastrointestinal disease. The study protocol was approved by the Medical Ethical Committee of the Fudan Children's Hospital and a statement of no objection was obtained from the Medical Ethical Committee of the Erasmus MC- Sophia's Children's Hospital. Similar studies, such as those determining cysteine requirements, have been previously performed in the Erasmus MC-Sophia

Children's Hospital (14, 15). Written informed consent was obtained from one or both parents for all participants after precise explanation of the study by a Mandarin speaking researcher.

Experimental design

The study is based on the minimally invasive IAAO method which our group recently modified to apply in enterally fed infants by using a short adaptation period to the test diet (1 d), enterally infused isotopes and sampling of expired air without sampling of amino acid enrichments in urine of plasma (11). The IAAO technique (16) uses an indicator that is oxidized when one essential amino acid is limiting. Because there is no storage of amino acids and amino acids must be partitioned between incorporation into protein or oxidation (17). If the tested amino acid is deficient in the diet, this will limit protein synthesis and the indicator amino acid, which is in excess at low protein synthesis rates, will be oxidized. Upon the increase of the dietary intake of the test amino acid, oxidation of the indicator will decrease until the requirement of the test amino acid is met. Once intake meets a critical threshold (or requirement), protein synthesis can occur at an optimum capacity, and the oxidative degradation of all other essential amino acids reaches plateau. The mean requirement of the test amino acid is identified by this breakpoint.

Subjects were randomly assigned to receive graded amounts of valine ranging from 5 to 236 mg·kg^{-1}·d^{-1}. Each infant received a different intake and was studied one time with one intake except for one infant that was measured twice. After adaptation to the study diet for 24 h, baseline breath samples were obtained, and a tracer protocol was initiated as depicted in Figure 1. Subjects were weighed daily, before and at the end of the tracer protocol.

In our protocol the infants adapt to the study formula for 24 h. The natural enrichment of stable isotopes is normally accounted for by taking baseline samples prior to the start of

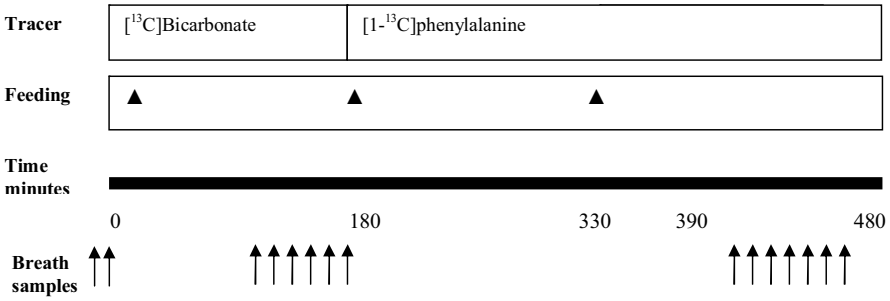

Figure 1: Study design after one day of adaptation to the study diet
▲: time of oral feeding (every 3 h)
↑: time that breath samples were taken

the isotope infusion, and then subtracting the enrichment of the baseline sample from all the samples obtained during and/or after the isotope infusion. Since every diet differs in naturally enriched ^{13}C (18) a period is necessary to allow background adaptation to the experimental diet. Because we used European formulas which might be based on different sources of carbohydrate and protein than the Chinese formulas the infants received before the adaptation day, we also determined the time needed to reach a stable background enrichment in the first 8 patients.

Study Formula

The study formula was based on an amino acid based formula designed to fulfil the amino acid requirements of infants (SHS, Liverpool, United Kingdom), but without valine and with reduced phenylalanine to compensate for the tracer. The amount of valine was adjusted separately as L-valine. L-phenylalanine was supplied during the adaptation time and during the infusion of [^{13}C]bicarbonate to obtain a stable total intake of 166 $mg \cdot kg^{-1} \cdot d^{-1}$ during the entire study. L-alanine was added separately to make the formula isonitrogenous. The amino acids, fat, carbohydrates and energy content of the study formula are shown in Table 1. The minerals, trace elements and vitamins of the formula were described previously (12). Since phenylalanine, which is hydroxylated to tyrosine before oxidation can occur, served as indicator, we made sure that tyrosine intake exceeded present requirements. Limited tyrosine intake reduces recovery of ^{13}C label in expiratory air. A tyrosine intake of 166 $mg \cdot kg^{-1} \cdot d^{-1}$ was provided which is almost twice the human milk-based recommendated intake of 90 $mg \cdot kg^{-1} \cdot d^{-1}$ to ensure that the newly formed [1-^{13}C] tyrosine hydroxylated from [1-^{13}C]phenylalanine would be directly channeled into $^{13}CO_2$ which could be measured in expired air (19).
We used [1-^{13}C]phenylalanine as a tracer, but because the tracer behaves identical to the tracee, phenylalanine intake was appropriate and constant for the complete duration of the study. Both on the adaptation day and study day subjects were fed every 3 h.

Tracer protocol

On the study day subjects received a primed (14 $\mu mol \cdot kg^{-1}$) continuous (9 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) enteral infusion of [$^{13}C$]bicarbonate (sterile pyrogen free, 99% ^{13}C atom percent excess (APE); Cambridge Isotopes, Woburn, MA) for 3 h to quantify individual CO_2 production. The labelled sodium bicarbonate infusion was directly followed by a primed (27 $\mu mol \cdot kg$), continuous (27 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) enteral infusion of [1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 5 h by an infusion pump via the nasogastric tube. The syringes with tracers were weighed before and after infusion to determine the exact amount of tracer given during the study.

Table 1: Energy, carbohydrates, fat and amino acid content of the study formula[1]

Component	*Per 100g formula*
Energy (kcal)	475
Carbohydrates (g)	54
Fat (g)	23
Total amino acid (g)	13
L-Alanine (g)[2]	≥ 0.61
L-Arginine (g)	1.08
L-Asparagine (g)	1.01
L-Cyst(e)ine (g)	0.4
Glycine (g)	0.95
L-Histidine (g)	0.62
L-Isoleucine (g)	0.95
L-Leucine (g)	1.63
L-Lysine (g)	1.11
L-Methionine (g)	0.26
L-Phenylalanine (g)[3]	0.20
L-Proline (g)	1.16
L-Serine (g)	0.71
L-Threonine (g)	0.8
L-Tryptophan (g)	0.32
L-Tyrosine (g)	0.73
L-Valine (g)[4]	0
L-Carnitine (g)	0.01
Taurine (g)	0.03
L-Glutamine (g)	1.34

[1] The study formula was based on Neocate (Danone, United Kingdom), an amino acid based formula

[2] Variable levels of L-alanine were added to the diet depending on the test valine level of each infant to maintain an isonitrogenous diet. The study formula contained at least 0.61 g L-alanine per 100 g formula.

[3] 0.53 g L-phenylalanine per 100 g formula was added to the study diet on day 1. Equivalent amount of L-phenylalanine (0.52 g per 100 g formula) was given as isotope on day 2.

[4] L-valine was added separately, depending on the test valine level.

Sample collection

Breath samples of the first 8 patients were collected before start of the study formula at the adaptation day and every 30 min for 8.5 h to determine the time needed to obtain a stable background enrichment, using the direct sampling method described by van der Schoor et al. (20). These samples were compared to the baseline samples at the study day (t= 1440) when 24 h of adaptation was achieved. At the study day, baseline samples were obtained 15 and 5 min before starting tracer infusion. Duplicate ^{13}C-enriched breath samples were then collected every 10 min during the isotopic steady state of the [^{13}C]bicarbonate infusion starting after 1.75 h, and next every 15 min during the isotopic steady state of the [1-^{13}C]phenylalanine infusion starting after 3 h as depicted in Figure 1.

Analysis and Calculations

Samples were sent from Shanghai to Rotterdam every three weeks by air transport. $^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (Helifan, Analytic Fischer Instruments, Leipzig, Germany) and expressed as APE above baseline. Steady state was defined as three or more consecutive points with a slope not significantly different from zero ($p \geq 0.05$). Estimated body CO_2 production ($mmol \cdot kg^{-1} \cdot h^{-1}$) was calculated for each infant as described previously (11, 21). The fraction of $^{13}CO_2$ recovery from [1-^{13}C]phenylalanine oxidation in percentage ($F^{13}CO_2$) was calculated as described previously (12, 22). Phenylalanine flux was not obtained. As shown in our previous study, the test amino acid intake has no effect on the phenylalanine flux (11). Regarding the potential interaction of the BCAAs; in enterally fed adults, valine kinetics were determined at different leucine intakes and leucine kinetics were determined at different valine and isoleucine intakes. Valine turnover did not change among the various intakes of valine and leucine. Leucine flux was also not affected by the valine or the isoleucine intakes. Valine and leucine requirements were not affected by the ratio of BCAA used when given within a physiological range (23, 24).

Statistical analysis

Descriptive data were expressed as mean ± SD. For the background enrichment a biphasic regression analysis was determined on the breath enrichment values as described below. An adjustment was made for the fact that repeated measurements within the same patients were used for analysis. The effect of valine intake on mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion, and on CO_2 production rate were tested with Pearson's correlation coefficient analysis. Mean valine requirement was determined by applying a two-phase regression model (17, 25) on the fractional oxidation rates. In this model a breakpoint is estimated using non-linear regression. With the biphasic lineair regression analysis, the regression equation was split into 2 parts. For the first part, an intercept and slope were estimated. For the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit on the basis of the highest r^2 was selected. The 95% confidence intervals were calculated. All statistical analyses were performed with STATA (version 11; StataCorp LP). A p-value < 0.05 was considered significant. The power analysis cannot be performed. We aimed to study 30 infants equal to our previous studies (11, 12) which is a higher amount of subjects than the IAAO studies performed in parenterally fed infants which used intravenous administration of the tracer (26, 27).

RESULTS

Clinical characteristics

Clinical characteristics of the 28 subjects studied are presented in Table 2. A total of 29 oxidation studies were performed, as one subject was measured twice with two different valine intakes. All subjects were male term Asian neonates. The reasons for admission were pneumonia with negative bloodculture (n=13), unconjugated hyperbilirubinemia (n=5), asphyxia (n=4), pneumothorax (n=2), infection suspicion with a negative blood culture (n=2), RS-bronchiolitis (n=1) and humerus fracture (n=1). Infants were studied just before discharge, when they were in a clinically stable condition and considered healthy as demonstrated by their weight gain rates and the fact that they were discharged on the study day or the day after. The mean weight gain in the 5 days before the study was 10.7 ± 4.3 $g \cdot kg^{-1} \cdot d^{-1}$.

Table 2. Subject characteristics, protein and caloric intake before and during the study (n = 28).

	Mean ± SD
Gestational age at birth (wks)	39.5 ± 1.2
Age at study (d)	15 ± 7
Birth weight (kg)	3.39 ± 0.44
Weight at study day (kg)	3.67 ± 0.53
Male:Female ratio	28:0
Intake during study ($mL \cdot kg^{-1} \cdot d^{-1}$)	150.2 ± 0.8
Intake during study day (g formula$\cdot kg^{-1} \cdot d^{-1}$)	22.8 ± 0.1
Caloric intake before study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	112.8 ± 13.8
Caloric intake during study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	108.1 ± 0.6
Protein intake before study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.43 ± 0.27
Protein intake during study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.96 ± 0.15

Expired CO_2 enrichment

All subjects achieved isotopic steady state (plateau) at both the [^{13}C]bicarbonate and [1-^{13}C]phenylalanine infusion. In the first 8 subjects, the mean background enrichment at the adaptation day before the start of the study formula was – 27.45 ± 1.15 pee dee belemnite (PDB). On the adaptation day, a stable background enrichment was achieved after 282 ± 9.6 min or the ingestion of two bottles of formula in the first 8 subjects as shown in Figure 2. The mean baseline $^{13}CO_2$ enrichment of the 22 subjects at the study day was -19.83 ± 1.42 PDB (0.0000 APE). The mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion was 0.0389 ± 0.0059 APE. The corresponding mean CO_2 production rate was 23.26 ± 3.57 $mmol \cdot kg^{-1} \cdot h^{-1}$. The mean $^{13}CO_2$ enrichment at isotopic plateau and the corresponding CO_2 production rate were plotted against the valine intake (Figure 3A and 3B respectively). No correlation was found between valine intake and the mean $^{13}CO_2$ enrichment at isotopic plateau (p= 0.46) and between the valine intake and the CO_2 production rate (p=0.37).

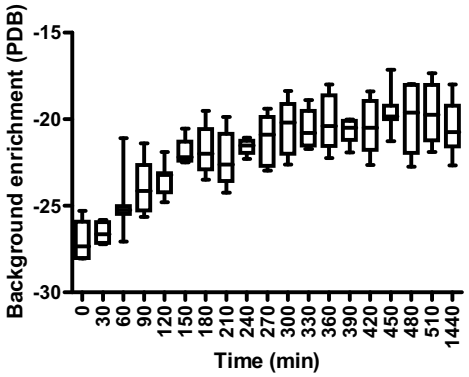

Figure 2: Background enrichment adaptation versus time (in PDB) to the study formula (n=8), expressed as box plots. PDB: pee dee belemnite.

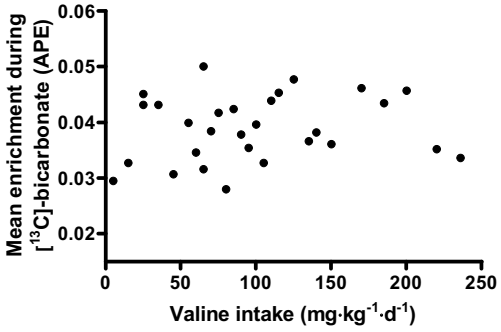

Figure 3A: mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [^{13}C]bicarbonate infusion plotted against valine intake (n=28).

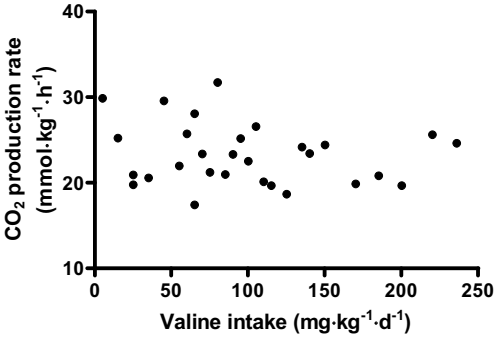

Figure 3B: The CO_2 production rate of each infant plotted against the valine intake (n=28).

The mean $^{13}CO_2$ enrichment at isotopic plateau during [1-^{13}C]phenylalanine infusion was 0.0216 ± 0.0065 APE. The mean $^{13}CO_2$ enrichments during [1-^{13}C]phenylalanine infusion are plotted against valine intakes which is shown in Figure 4A.

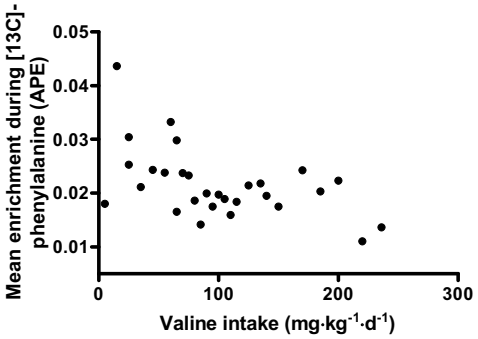

Figure 4A: Mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [1-^{13}C]phenylalanine infusion plotted against valine intake (n=28).

Overall there was a significant decrease in fractional oxidation when valine intake increased ($r^2 = 0.35$, $p = 0.001$). From the two-phase regression analysis with valine intake as the independent variable and $F^{13}CO_2$ as the dependent variable, the breakpoint was determined to be 110 mg·kg^{-1}·d^{-1} (Figure 4B). The upper CI was 164 mg·kg^{-1}·d^{-1} and the lower CI was 56 mg·kg^{-1}·d^{-1}.

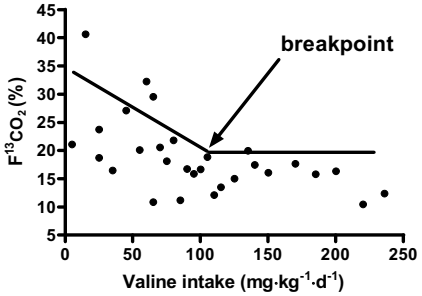

Figure 4B: $F^{13}CO_2$ plotted against increasing valine intakes (n=28). A biphasic linear regression model estimated the breakpoint (mean valine requirement) to be 110 mg·kg^{-1}·d^{-1} ($r^2 = 0.35$, $p = 0.001$). The upper CI was 164 mg·kg^{-1}·d^{-1} and the lower was 56 mg·kg^{-1}·d^{-1}. APE: atom percentage excess; $F^{13}CO_2$: fraction of recovery from [1-^{13}C]phenylalanine oxidation.

DISCUSSION

The mean valine requirement for term male neonates fed an elemental diet using the IAAO method is 110 mg·kg^{-1}·d^{-1}. Current recommended valine requirement for infants aged 0 to 1 month, based on human milk, is 95 mg·kg^{-1}·d^{-1}(5). Our data suggest that the current recommendations for valine, based on human milk, are correct.

In 1959, Snyderman et al. determined the valine requirement in five neonates to be between 85-105 mg·kg^{-1}·d^{-1} using the nitrogen balance and weight gain (28). Although

the nitrogen balance method underestimated the essential amino acid requirements in adults, our mean requirement is at the upper range of the requirement that Snyderman et al. determined and above the current recommendations based on human milk. Since we and Snyderman both used free L-amino acids in the study diet – rather than total proteins – our requirements might be overestimated since amino acids derived from intact casein are utilized in a higher proportion for protein synthesis than that from an equivalent intake of free amino acids (29). Most currently available infant formulas provide intakes of protein that markedly exceed the requirement and exceed the protein intakes from human milk in breast-fed infants. For example, the measured daily protein intake in infants aged 0 to 1 month is 1.7 – 2.09 $g \cdot kg^{-1} \cdot d^{-1}$ which declines to 0.9-1.0 $g \cdot kg^{-1} \cdot d^{-1}$ at 5-6 months (9, 10). Term neonates fed a formula with a protein content of 1.6 $g \cdot dL^{-1}$ received a protein intake of 2.5 $g \cdot kg^{-1} \cdot d^{-1}$ at 12 weeks (30) which is much higher than the intake of a breastfed infant at 3 months of age (0.9-1.2 $g \cdot kg^{-1} \cdot d^{-1}$) (9, 10) . The amino acid composition of the nutritionally available proteins from human milk differs from that found of bovine proteins (9). This results in different plasma amino acid profiles in formula fed infants compared to breastfed infants. For example, the concentrations of threonine, valine and total branched-chain amino acids are significantly higher in formula fed children fed a whey-dominant formula than in breastfed infants at 3 months of age (31) . At 6 months of age, children fed a casein-dominant formula which contained 2.7 $g \cdot dL^{-1}$ of protein, the concentrations of phenylalanine, methionine, leucine, valine and proline and isoleucine were more than 2 fold the values found in breastfed infants (32). Since high levels of branched-chain amino acids (BCAA) interfere with the transport of tryptophan (a serotonine precursor) and other large neutral amino acids (tyrosine) across the blood-brain-barrier, they influence central nervous system concentrations of neurotransmitters (33, 34). We speculate that current formulas provide too much protein and do not contain the optimal amino acid composition. The amino acid requirement of all essential amino acids should be determined to optimize infant nutrition. We postulate that formulas may contain lower amounts of protein if the quality of the milk protein can be modified.

One difficulty in determining the requirement of the individual BCAA is the interaction between the three amino acids due to their common catabolic enzymes (35). In animals elevated rates of valine oxidation were seen when high intakes of leucine were given, and these high intakes also depressed the concentrations of free isoleucine and valine in plasma and tissue amino acids pools (36, 37). In elderly males receiving prolonged leucine supplementation the concentrations of valine and isoleucine decreased in blood, probably as a result of the BCAA antagonism (38). In adults fed BCAA intakes within the physiological range, however, no effect was seen on valine oxidation with different leucine intakes (23) and individual BCAA requirements did not change when the

BCAA ratio was changed (24). Because the upper intake level of the BCAAs in the present study is based on the amount supplied by a regular infant formula, we do not supply supraphysiological BCAA intakes. Thus, we postulate that the oxidation and requirement of the individual BCAAs will not be influenced by the BCAA ratio and that we can study the BCAAs individually.
Since breastfed boys consume 10% more human milk than girls in the first months after birth (10) and have a greater protein deposition in this period (39), the essential amino acids requirement in infants might be gender-specific. Gender-specific impaired neurodevelopmental outcomes have reported for preterm boys who consumed less protein in the first week of life (3) and were fed standard versus preterm formula in the first month of life (4). In China more boys than girls are born and admitted at the Neonatology Department (40). Following the inclusion of the first 15 children we noticed that only boys were included. Therefore we decided that we would proceed by including boys only for the present study. We speculate that the valine requirement for girls is slightly lower than our mean requirement in boys of 110 $mg \cdot kg^{-1} \cdot d^{-1}$.

One of the limitations of our study is the wide range of the 95% confidence interval. Intra-subject variation is the major source of variability in amino acid requirements and a potential source of error in the estimation of amino acid requirement in humans (13, 41). Thereby there might be large inter-individual differences between requirements in individuals as is shown in breastfed infants who have a variable milk consumption rate and largely regulate the intake they require themselves (6-8). Since we use enterally tracers whose oxidation depends on the rate of gastric emptying (42), this might be partially responsible for the variability in the fractional oxidation rates.
Every diet differs in naturally enriched ^{13}C. The natural abundance of ^{13}C in nature is approximately 1.1% ^{13}C. Atmospheric abundance differs: European breath CO_2 has a different percentage of natural abundance ^{13}C than North American breath CO_2 (18). ^{13}C abundances of formula differ dependent on the constituents: for example the source of protein, the source of lipids and carbohydrates all vary in ^{13}C abundance (18). Therefore, a period is necessary to allow background adaptation to the experimental diet. Since we used European formulas which might be based on different sources of carbohydrate and protein than the Chinese formulas the infants received before the adaptation day, we determined the time needed to reach a stable background enrichment in the first 8 patients. We found the time to obtain a stable background enrichment was two bottles of formula given every 3 h, or 4.7 h at the present feeding regimen used at the NICU of the Fudan Children's Hospital. These results are comparable to the study of Bross et al, who showed a stable background enrichment in breath between 225 and 255 min in adult humans fed hourly meals, and suggested that 5 hourly mails are required to achieve a constant $^{13}CO_2$ enrichment (13). This means that our protocol in which we

adapt 24 h to the study formula is sufficient to allow background enrichment to adapt to the new formula.

Concluding, our study shows that current human-milk based recommendations for the valine requirement in term male infants aged 0 to 1 month are correct. The IAAO method should be used to determine the requirements of isoleucine and leucine as well as the optimal BCAA ratio in term infants to optimize infant nutrition.

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CHAPTER

7

Leucine requirement for the enterally fed term infant in the first month of life

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ABSTRACT

Background: Leucine is a nutritionally essential amino acid for protein synthesis. Additionally, it regulates the protein turnover and serves as an important nitrogen donor for the brain glutamate synthesis. The present recommended leucine intake of 166 mg·kg^{-1}·d^{-1} for term infants is based on average human milk composition and the estimated volume intake. Marketed milk-based formulas provide 195 ± 15 mg leucine per kg per day at an intake of 150 mL·kg^{-1}·d^{-1}.

Objective: The objective is to determine the leucine requirement for fully enterally fed term infants using the indicator amino acid oxidation (IAAO) method. L-[1-^{13}C]phenylalanine was used as the indicator amino acid.

Design: Infants were randomly assigned to leucine intakes ranging from 5 to 370 mg·kg^{-1}·d^{-1}, as part of an elemental formula. After 24 h of study formula consumption, [^{13}C] bicarbonate and L-[1-^{13}C]phenylalanine tracers were given enterally. Breath samples were collected at baseline and during isotopic plateaus. Mean leucine requirement was determined by using biphasic linear regression crossover analysis on the fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$). Data are presented as mean ± SD.

Results: Thirty-three term neonates (gestational age at birth of 39 ± 1 weeks) were studied at 11 ± 4 days. The mean requirement was determined at 140 mg·kg^{-1}·d^{-1} (p <0.01, r^2 = 0.26), with the upper CI of 241 mg·kg^{-1}·d^{-1} and the lower CI of 40 mg·kg^{-1}·d^{-1}.

Conclusion: For term infants we propose a mean leucine requirement of 140 mg·kg^{-1}·d^{-1}, which is in the same range as the amount found in human milk. These data provide more evidence for leucine requirement in formula-fed infants.

INTRODUCTION

Leucine is one of the branched-chain amino acids (BCAA), along with isoleucine and valine, and is considered an essential amino acid in humans. The main metabolic fate of dietary BCAA is incorporation into body protein (1). Additionally, leucine has numerous metabolic effects. First, it plays an important role in regulation of protein turnover. Leucine inhibits protein degradation and acts independently as a nutrient signal stimulating protein synthesis via the activation of translation initiation factors (2-4). It is the most potent physiological insulin secretagogue among the amino acids (5). Anabolic effect of leucine has been shown in rats (6-7), neonatal pigs (8-9) and in men (10). Secondly, leucine is one of the main nitrogen donors for brain glutamate synthesis (11). Brain glutamate metabolism is crucial for the glutamatergic neurotransmission. Thirdly, dietary BCAA intakes influence the brain amino acid concentrations and consequently the neurotransmitter synthesis by competing with other large neutral amino acids for uptake into brain (1). Therefore, it is important to determine the leucine requirement.

Experimental evidence of essential amino acid requirements in infants is scarce. A series of essential amino acid requirement studies was performed in the 1960s using weight gain rate and nitrogen retention (12). For leucine, this amount has been found to be between 76 and 229 $mg \cdot kg^{-1} \cdot d^{-1}$ in 6 infants up to 5 months of age (13). However, the recommendations for essential amino acid requirements of infants are based on the average intakes of breastfed infants rather on experimentally derived requirement values (14). The estimated average leucine intake in exclusively breastfed infants is 166 $mg \cdot kg^{-1} \cdot d^{-1}$ (14).

The indicator amino acid oxidation (IAAO) method has been successfully used to determine BCAA requirements in adults (15) and children (16). The recently modified IAAO method has been used successfully to determine amino acid requirements in enterally fed infants (17-18). The aim of this study is to determine the leucine requirement in enterally fed term infants using the IAAO method.

METHODS

Subjects

Thirty-three neonates admitted to the neonatal ward in the Children's Hospital of Fudan University, participated in the study. Each subject was selected for study by the following criteria: fully enterally fed infants with a gestational age of ≥ 37 weeks, birth weight ≥ 2500 gram, and clinically stable with a weight gain rate ≥ 5 $g \cdot kg^{-1} \cdot d^{-1}$ in the preceding 3 days. Subjects were excluded if they had congenital anomalies, gastrointestinal pathology, or sepsis.

The study was approved by the Institutional Review Boards of the Children's Hospital of Fudan University, and a statement of no objection was obtained from the Erasmus MC-Sophia Children's Hospital. Written consent was obtained from at least one of the parents of each subject by a Chinese-speaking researcher.

Study formula

The study formula used was an elemental formula that was based on free amino acids. The amino acids, fat, carbohydrates, and energy content of the study formula are shown in Table 1. The composition was the same as Neocate (SHS International) except for the leucine, phenylalanine and alanine content. Leucine, which was completely withdrawn from the study formula, was separately added in the form of L-leucine to obtain different amounts of intake. The phenylalanine intake was kept constant during the study by separately adding L-phenylalanine during the 24 h adaptation period to obtain the

Table 1. Energy, carbohydrates, fat, and amino acids content of the study formula.

Component	Per 100 g formula
Energy (kcal)	475
Carbohydrates (g)	54
Fat (g)	23
Total amino acid (g)	13
L-Alanine (g) [1]	≥ 0.61
L-Arginine (g)	1.08
L-Asparagine (g)	1.01
L-Cyst(e)ine (g)	0.4
Glycine (g)	0.95
L-Histidine (g)	0.62
L-Isoleucine (g)	0.95
L-Leucine (g) [2]	0
L-Lysine (g)	1.11
L-Methionine (g)	0.26
L-Phenylalanine (g) [3]	0.20
L-Proline (g)	1.16
L-Serine (g)	0.71
L-Threonine (g)	0.8
L-Tryptophan (g)	0.32
L-Tyrosine (g)	0.73
L-Valine (g)	1.04
L-Carnitine (g)	0.01
Taurine (g)	0.03
L-Glutamine (g)	1.34

[1] Variable levels of L-alanine were added to the diet depending on the test leucine level of each infant to maintain an isonitrogenous diet. The study formula contained at least 0.61 g L-alanine per 100 g formula.

[2] L-leucine was added separately, depending on the test leucine level.

[3] 0.53 g L-phenylalanine per 100 g formula was added to the study diet on day 1. Equivalent amount of L-phenylalanine (0.52 g per 100 g formula) was given as isotope on day 2.

same amount as in the Neocate (SHS International) and this amount of phenylalanine was given as stable isotope L-[1-^{13}C]phenylalanine on the tracer infusion day. The phenylalanine intake during the study was 166 mg·kg^{-1}·d^{-1}, which was above the recommended amount of 72 mg·kg^{-1}·d^{-1} (14). A generous amount of tyrosine (166 mg·kg^{-1}·d^{-1}) was provided to ensure that the newly formed [1-^{13}C]tyrosine hydroxylated from [1-^{13}C] phenylalanine would be directly channeled to oxidation into $^{13}CO_2$, which can be measured in expired air (19). This amount of tyrosine was almost twice the recommended intake (14). The nitrogen intake was kept constant for all subjects by the substitution of L-alanine for the leucine that was withdrawn. The caloric intake was kept constant during the study period in all infants.

The minerals and trace elements supplied in 100 g formula were as follows: iron 7.0 mg, calcium 325 mg, phosphorus 230 mg, magnesium 34 mg, sodium 120 mg, chloride 290 mg, potassium 420 mg, manganese 0.38 mg, iodine 47 µg, selenium 11 µg, copper 380 µg, and zinc 5.0 mg.

The vitamin content of 100 g formula was as follows: vitamin A 528 µg retinol equivalent, vitamin D 8.5 µg, vitamin E 3.3 mg α-tocopherol equivalent, vitamin K 21 µg, thiamin 390 µg, riboflavin 600 µg, niacin 4.5 mg, vitamin B_6 520 µg, vitamin B_{12} 1.3 µg, pantothenic acid 2.3 mg, folic acid 38 µg, vitamin C 40 mg, and biotin 26 µg.

Experimental design

The study was designed to determine the leucine requirement in term infants using the minimally invasive IAAO (20-22), that has recently been modified to determine the essential amino acid requirements in enterally fed infants (17). The IAAO method is based on the concept that when the test amino acid intake is insufficient to meet the requirement, protein synthesis will be limited and all of the amino acids will be oxidized, including the indicator amino acid, which is labelled with a stable isotope. As the dietary intake of the test amino acid increases, the oxidation rate of the indicator will decrease until the requirement of the test amino acid is met. Once the requirement of the test amino acid is met, an additional increase in its intake will have no further influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as $^{13}CO_2$.

During the study, all infants received fluid intake of ~150 mL·kg^{-1}·d^{-1}, a caloric intake of ~108 Kcal·kg^{-1}·d^{-1}, and an amino acid intake of ~2.95 g·kg^{-1}·d^{-1}. Infants were randomly assigned to one of the graded test intake of leucine, ranging from 5 to 370 mg·kg^{-1}·d^{-1}. Each study took place over a 31 h period whereby the study formula was fed to the infants. After 24 h of study formula consumption, a nasogastric tube was placed. Infants received a primed (14 μmol·kg^{-1}) continuous (9 μmol·kg^{-1}·h^{-1}) enteral infusion of [^{13}C] bicarbonate (sterile pyrogen free, 99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 3 h to quantify individual CO_2 production rates (23). Phenylalanine was used as the indica-

tor. After the [^{13}C]bicarbonate infusion was stopped, a primed (34 μmol·kg^{-1}) continuous (27 μmol·kg^{-1}·h^{-1}) enteral infusion of L-[1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes) was started and lasted for 4 h. Syringes were weighted before and after the study to determine the exact amount of the tracers that were given to the infants. The tracer infusion day is depicted in Figure 1. Infants were bottle fed every 3 h during the adaptation period (the first 24 h). Subsequently, the feeding regimen changed to continuous feeding during [^{13}C]bicarbonate infusion and hourly bottle feeding during the L-[1-^{13}C]phenylalanine infusion until the end of the study.

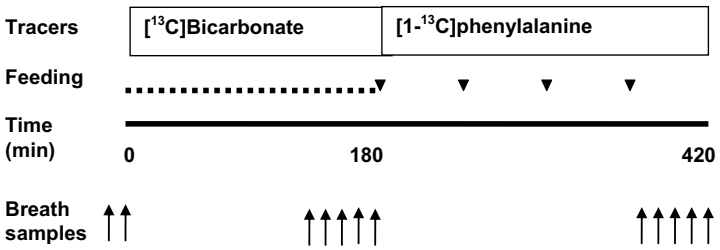

Figure 1. Schematic overview of tracer infusion day. The dashed line indicates the period of continuous intragastric feeding. Triangles indicate times that bolus feeding were given via a bottle. Arrows indicate times that breath samples were taken.

Sample collection and analysis

Breath samples were obtained by using the direct nasopharyngeal sampling method described by van der Schoor et al. (24). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical, Tullamore, Ireland) was placed 1 to 1.5 cm into the nasopharynx and the end-tidal breath was taken slowly with a syringe. Collected air was transferred into 12 mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and was stored at room temperature until analysis. Two duplicated baseline samples were obtained before the start of tracer infusion. Six duplicated breath samples were obtained every 15 min during isotopic plateau of [^{13}C]bicarbonate between 105 and 180 min. Seven duplicated samples were obtained every 10 min during isotopic plateau of L-[1-^{13}C]phenylalanine between 360 and 420 min (Figure 1).

^{13}C isotopic enrichment in the breath samples was analyzed by an infrared isotope analysis technique (Helifan, Analytic Fischer Instruments, Leipzig, Germany). The ^{13}C enrichment was expressed as the atom percent excess above baseline (APE).

Calculations

The isotopic steady state was represented by plateaus in $^{13}CO_2$. Plateaus were determined by visual inspection and were confirmed by regression analysis as a slope not significantly different from zero. The estimated body CO_2 production rate (mmol·kg^{-1}·h^{-1}) was calculated as described previously (17, 23). The fraction of $^{13}CO_2$ recovery from L-[1-

^{13}C]phenylalanine oxidation ($F^{13}CO_2$) in % was calculated with the following equation (25):

$$F^{13}CO_2 = (IE_{PHE} \times i_B) \div (i_{PHE} \times IE_B) \times 100\%$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (μmol·kg^{-1}·h^{-1}), i_{PHE} is the infusion rate of L-[1-^{13}C]phenylalanine (μmol·kg^{-1}·h^{-1}) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.
Phenylalanine flux was not obtained. As shown in our previous study, test amino acid intake has no effect on the phenylalanine flux (17).

Statistical analysis

Descriptive data are expressed as means ± SDs. Determination of the leucine requirement, the breakpoint, was performed using a biphasic linear regression crossover model (26). With the biphasic linear regression analysis, the regression equation was split into two parts. For the first part an intersept and slope were estimated, while for the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line is equal to the breakpoint. The model with the best fit, based on the highest r^2 was selected. The 95% confidence interval was calculated. A value of $p < 0.05$ was taken as significant. The analyses were performed in STATA software (version 11; StataCorp LP).

RESULTS

Subject characteristics

Thirty-three term neonates participated in the study. The neonates were studied at a leucine intake that ranged between 5 and 370 mg·kg^{-1}·d^{-1}. Subject characteristics are

Table 2. Subject characteristics and protein and energy intake before the study of infants who participated in the study (n = 33).

	Values
Birth weight (kg)	3.3 ± 0.3
Gestational age (wk)	39 ± 1
Age at study (d)	11 ± 4
Weight on study day (kg)	3.4 ± 0.4
Weight gain before study (g·kg^{-1}·d^{-1})	13 ± 6
Sex (F:M)	16:17
Protein intake before the study (g·kg^{-1}·d^{-1})	2.8 ± 0.4
Energy intake before the study (kcal·kg^{-1}·d^{-1})	117 ± 15

All values are means ± SDs.

summarized in Table 2. All subjects were growing well before entering the study. The mean (± SD) weight gain rate three days before the study was 13 (± 6) g·kg^{-1}·d^{-1}. The mean (± SD) energy intake was 109 ± 1 kcal·kg^{-1}·d^{-1}. The nitrogen intake was equivalent to a protein intake of 3.0 ± 0.02 g·kg^{-1}·d^{-1}. The infants were clinically stable and considered healthy supported by the discharge on the study day or the day after. The primary reason of admissions were unconjugated hyperbilirubinemia (n = 21), pneumonia with a negative blood culture (n = 6), asphyxia (n = 2), bloody stool (n = 1), wet lung (n = 1), constipation (n = 1) and urine tract infection (n = 1). Intravenous antibiotics (penicillins and/or cephalosporins) were given to 19 of the 33 infants.

$^{13}CO_2$ enrichments during [^{13}C]bicarbonate infusion

The baseline $^{13}CO_2$ enrichment was -17.18 ± 1.17 pee dee belemnite (PDB). The mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion was 0.0396 ± 0.0045 APE. The corresponding mean CO_2 production rate was 22.45 ± 2.50 mmol·kg^{-1}·h^{-1}. The mean $^{13}CO_2$ enrichment at isotopic plateau and their corresponding CO_2

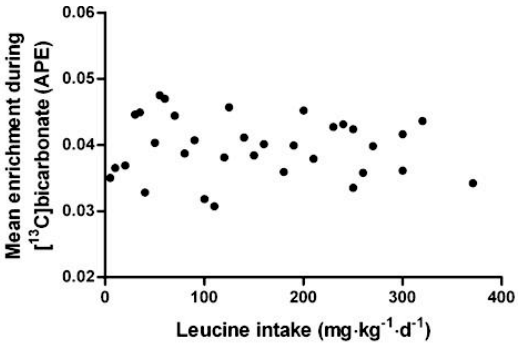

Figure 2A: Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral [^{13}C]bicarbonate infusion of each infant plotted against the leucine intake (n=33). APE, atom percent excess.

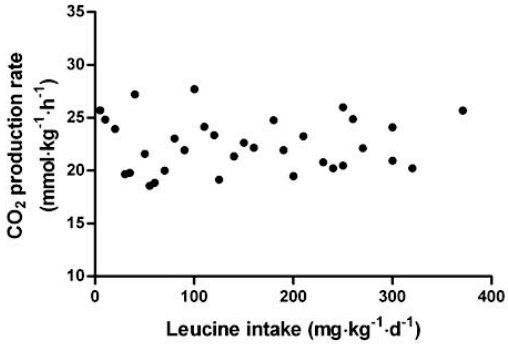

Figure 2B: CO_2 production rate of each infant plotted against the leucine intake (n=33).

production rate of each infant were plotted against the leucine intake (Figure 2A and 2B respectively).

L-[1-^{13}C]phenylalanine oxidation

The mean $^{13}CO_2$ enrichment at isotopic plateau during L-[1-^{13}C]phenylalanine infusion was 0.0173 ± 0.0039 APE. These $^{13}CO_2$ enrichment values and the $F^{13}CO_2$ are plotted against leucine intakes in Figure 3A and 3B. As the leucine intake increased, $F^{13}CO_2$ decreased. This negative correlation was shown between $F^{13}CO_2$ and leucine intakes up to 140 mg·kg^{-1}·d^{-1}; additional increase in leucine intake did not affect the $F^{13}CO_2$. Using a biphasic linear regression crossover model, the breakpoint representing the mean

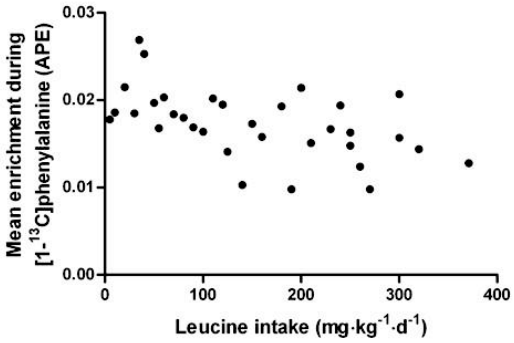

Figure 3A: Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral L-[1-^{13}C]phenylalanine infusion of each infant plotted against the leucine intake (n=33). APE, atom percent excess.

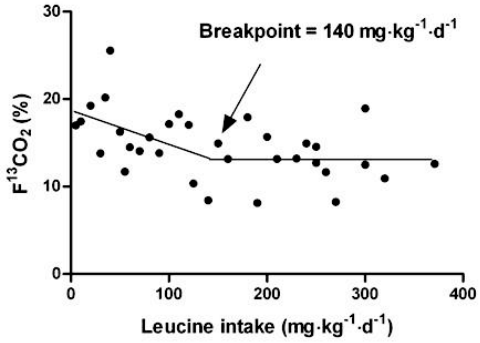

Figure 3B: The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the isotopic plateau at different leucine intakes (n = 33). Each infant received a different leucine intake. With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 140 mg·kg^{-1}·d^{-1} ($p < 0.01$, $r^2 = 0.26$). The upper CI was 241 mg·kg^{-1}·d^{-1} and the lower CI was 40 mg·kg^{-1}·d^{-1}

requirement was determined at 140 mg·kg^{-1}·d^{-1} (p <0.01, r^2 = 0.26). The upper CI was 241 mg·kg^{-1}·d^{-1} and the lower CI was 40 mg·kg^{-1}·d^{-1}.

DISCUSSION

Using the IAAO method, the mean leucine requirement for term infants less than one month of age is estimated to be 140 mg·kg^{-1}·d^{-1} with an upper CI of 240 mg·kg^{-1}·d^{-1}. Present recommendations for essential amino acid requirements by WHO/FAO/UNU (14) are estimated from the average intakes of breastfed infants. The accuracy of these estimations is complicated by many factors, including the change in milk composition over the course of lactation, the variation in milk composition between mothers, and the variation in milk intake between infants (27-28). The leucine intake of breastfed infants is estimated to be 166 mg·kg^{-1}·d^{-1} (14). Estimated amino acid composition of human milk may provide some guidelines for the composition of infant formulas, but it may not necessarily reflect the amino acid needs of infants. Current study provides experimental evidence of leucine requirement for infants fed an elemental formula.

In the present study, we observed a wide CI compared to the other amino acids studied thus far (17-18). Similar findings have been observed in the leucine requirement study by Snyderman et al. (13) in the 1960s. They attempted to estimate leucine requirement in 6 infants up to 5 months of age using growth rate and nitrogen retention. After complete withdrawal of leucine in the diet, it was then stepwise reintroduced. Each infant received 2-7 test intakes, and each for a period of ~1 week. They concluded that the requirement is in the range between 76 and 229 mg·kg^{-1}·d^{-1}, and also encountered a greater variation in the requirement for leucine than for any other amino acids they studied. In addition, they noticed a less obvious difference in nitrogen retention between inadequate and adequate periods. These findings might due to the potential interactions among BCAAs. BCAA use a common membrane-transport system and enzymes for their reversible transamination into α-ketoacids and irreversible oxidation into α-ketoacyl-CoA (1). A change in the dietary intake of leucine has been shown to decrease the plasma concentrations of valine and isoleucine (1, 29). However, whereas different BCAA ratios affect the requirement level for individual BCAA is not known. Studies by Pelletier et al. (30-31) observed no effect on valine oxidation at different leucine intakes, in addition, different valine-isoleucine ratios had no effect on leucine oxidation. Therefore, we speculate that different BCAA ratios have no effect on individual BCAA requirement.

We determined the requirements for each BCAA separately, since it enables us to determine the ideal BCAA ratio. In addition, current study design has many advantages. First, we have the ability to vary in leucine intakes while keeping the other nutrients constant, including valine, isoleucine and the total nitrogen intake. Secondly, the intersubject

variability in nutrient intakes is minimized due to the strict control of formula intake. Thirdly, the IAAO method is more accurate and less invasive than using the growth rate and nitrogen balance technique. These factors all contribute to a more accurate requirement estimate.

The efficiency of utilization of an elemental diet is less than a protein diet showed by a 20-35% higher oxidation rate by Metges et al. (32). Therefore, by postulating our estimate derived from infants fed an elemental infant formula to infants fed a protein formula may overestimate their requirement.

To give a recommendation on the safe level of intake, a level that nearly meets the requirement of all individuals, data of individual distribution of leucine requirement are needed. However, the distribution of leucine requirement is unknown. The safe level of leucine intake can be calculated based on the assumption that the safe level of amino acid intake is the same as the safe level of protein intake proposed by WHO (33), which is 125% of the average protein requirement. The calculated safe level of leucine intake is 140 $mg \cdot kg^{-1} \cdot d^{-1}$ (which is the mean requirement) when taking into account the less efficiency of utilization rate (~25%) by the use of an elemental diet. Current infant formulas provide 195 - 345 mg leucine per kg per day at an intake of 150 $mg \cdot kg^{-1} \cdot d^{-1}$ (34), which is 2-3 times our mean requirement. A higher amount than our mean requirement might be necessary with regards to the great individual variations in leucine requirement shown in the study by Snyderman et al. (13) and in the present study. Intakes above the requirement are well tolerated when an appropriate amount of protein with a balanced BCAA is consumed. So far, no evidence of toxicity was observed in human studies administering high dose of leucine (35-36). However, we have to be aware of the anabolic effect of leucine, which might contribute to the increase risk of obesity of formula fed infants later in life (37-38).

The current study determined the mean leucine requirement for infants up to one month of age to be 140 $mg \cdot kg^{-1} \cdot d^{-1}$. Current study provides more experimental evidence of amino acid needs for formula-fed infants, which is required to improve infant nutrition.

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CHAPTER

8

Enteral requirement for branched-chain amino acids in the neonate: search for the ideal dietary composition

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Submitted

ABSTRACT

Introduction: Higher protein intake for infants that are fed formula may play a role in the development of the metabolic syndrome. Leucine may affect muscle protein turnover and stimulates insulin release and tissue sensitivity. Human milk protein contains a isoleucine-leucine-valine (Ile:Leu:Val) ratio of 1: 1.74 : 1. Current recommendations of these branched-chain amino acids (BCAAs) for infants are based on this ratio. Different formulas use BCAAs in different ratios depending on the casein-whey ratio (milk-based formula: 1 : 1.6 : 1.1, whey-adapted formula: 1 : 2.3 : 1.1).

Objective: This review describes the requirement of the individual BCAAs and the optimal BCAA ratio in term neonates aged 0 to 1 month determined by the different methods and compares them with the mean requirement determined by using the indicator amino acid method. We compare these mean requirements with the amount of amino acids in current formulas and the current standards for infant formulas.

Results: The advised intake for isoleucine, leucine and valine based on the IAAO method is 105, 140 and 110 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively. The optimal Ile:Leu:Val ratio in enterally fed neonates aged 0 to 1 month, based on the IAAO method, is 1 : 1.3 : 1. This is comparable with the ratio in egg protein and with the ratio determined by Snyderman using the nitrogen balance and weight gain.

Conclusion: Our result imply that, regarding the BCAA ratio, egg protein might be a better alternative protein than cow milk protein for infants aged 0 to 1 month. Some of the currently used formulas provide 2-3 times too much BCAAs and might provide suboptimal BCAA ratios.

INTRODUCTION

It is becoming increasingly clear that the growth during the earliest stages of life can be an important determinant of an individual's later health and risk of chronic disease (1). There is now substantial evidence that growth in the first 2 years of life, especially high early weight gain, is associated with adverse health outcomes later in life, including increased blood pressure (2), increased weight gain and body fat deposition (3-6) and increased risk of diabetes (7). Higher protein intake for infants that are fed formula may play a role in these health outcomes because formula-fed children reach a higher body weight and weight for length at one year of age compared to those fed breast milk (8, 9). However, early nutrition (especially protein intake) correlates with improved neurodevelopment in preterm infants (10, 11). Current understanding of the nutritional needs for early growth and development is fragmentary and inadequate to provide answers that are needed (12).

Different methods have been developed to determine requirements in infants and adults. Over fifty-five years ago, Snyderman and colleagues have determined amino acid requirements for infants by means of the nitrogen balance method and weight gain (13-16). Because limited data on human infants and children were available, experts proposed a factorial approach to define dietary indispensable amino acid requirements in infants and children > 6 months (17, 18). The obligatory losses are used as maintenance requirement and the nutrients needed for growth are added to determine the requirement in children. The growth factor is calculated from the rate of protein mass gain in infants during the first two years of age as determined by Butte (19). Dewey et al. calculated the factorial approach for infants aged 0 to 1 month based on breast milk and implied that breast milk provided on average a 45% excess of indispensable amino acids at 0-1 month (20). They assumed that the average intake of breastfed infants does not approximate the mean requirement and stated that this would imply that half of the breastfed infants have deficient intakes. Because nearly all breastfed infants are meeting their protein intakes, their average intake should be above the safe level for protein intake, i.e. > 2 SD higher than the mean requirement.

Current recommended requirements for infants aged 0 to 1 month are based upon the amino acid content of human milk. These recommendations are based on the average amino acid content of breast-milk protein and multiplied by the milk protein intake (which is 75% of crude protein) (21). Estimating true protein intakes from breast milk is difficult because of the high proportion of non-protein nitrogen in human milk. The extend of utilization of this non-protein nitrogen (for example urea) is not entirely understood in any comparison of predicted requirements with human milk, and judgements of the amount of available nitrogen consumed as protein in breast milk must be made. These values also do not take into account that human milk shows remarkable variation

in protein and whey-casein ratio during different lactation stages and breastfed infants have a variable milk consumption rate (22-25). There might be a great variation in amino acid intake of breastfed children: they largely regulate the intake they require.
Recently it was shown by using stable isotopes that current Dietary Reference Intake (DRI) recommendations for protein intake in healthy school children determined by the factorial approach seems to underestimate the requirement by 71 and 63% (26). These results suggest that the IAAO method may be useful in re-evaluating amino acid and protein requirements in children and infants. This review describes the requirement of the branched-chain amino acids in term neonates 0-1 month determined by the different methods and compares them with the mean requirement determined using the indicator amino acid method. We compare these mean requirements with the amount of amino acids and protein in current formulas and the current standards for infant formula. Finally we will discuss which would be the new recommendations for infant formulas for infants aged 0 to 1 month.

Branched-chain amino acids: isoleucine, leucine, and valine

The essential branched-chain amino acids (BCAAs) differ from most other essential amino acids in that the enzymes initially responsible for their catabolism are found primarily in the extra-hepatic tissue. BCAAs account for 35-40% of the dietary essential amino acids found in body protein and 14% of the total amino acids in skeletal muscle. Their main metabolic fate is incorporation into body protein, although first pass utilization in neonates is also high (27). BCAAs are similar in structure and share common enzymes for transamination and oxidative decarboxylation. The BCAAs compete with other large neutral amino acids (LNAA), particularly tryptophan and tyrosine, for membrane transport. Although BCAAs do not act as direct precursors for neurotransmitters, they can affect the transport of certain LNAAs across the blood-brain barrier and thereby influence central nervous system concentrations of neurotransmitters (28, 29). BCAAs are both ketogenic and glucogenic, and their amino groups are used for the synthesis of alanine and glutamine in muscle, thereby providing a shuttle for the transfer of BCAA nitrogen from muscle to liver for urea formation. Among the BCAAs, leucine can act independently as a nutrient signal and stimulates protein synthesis via the activation of translation initiation factors (30). Leucine may affect muscle protein turnover (31) and stimulates insulin release and tissue sensitivity (32). High intakes of leucine by humans or animals enhances the activity of the branched-chain keto acid dehydrogenase in various tissues (33, 34), thereby decreasing valine and isoleucine concentrations in blood. An excess of leucine increases the oxidation of isoleucine and valine, thus limiting their availability as substrates for protein synthesis (33, 35-37).

The requirement for isoleucine in infants aged 0 to 1 month:

There are no isotopic data for individual isoleucine requirements in humans or animals. In 1964, Snyderman et al. used the nitrogen balance method to determine the isoleucine requirement in six healthy male infants to be between 79 and 126 mg·kg^{-1}·d^{-1} (14).
By using the factorial approach, Dewey et al. determined the isoleucine requirement in infants aged 0 to 1 month to be 59 mg·kg^{-1}·d^{-1} by using the rate of body mass, adjusted for the percentage of deposition as fat from the data of Fomon et al (38). They implied that breast milk provided on average a 45% excess of indispensable amino acids at 0-1-month. Because of this and given that intakes of breast milk of a healthy well-nourished mother are considered to satisfy protein requirements in the first 6 months of life, breast milk content of amino acids to be considered as the best estimate of amino acid requirement for this group. The current recommended isoleucine requirement, based on human milk is 95 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month (21). Using the IAAO method, our group recently determined the mean isoleucine requirement to be 105 mg·kg^{-1}·d^{-1} (all shown in Table 1). Current formulas provide 97-194 mg·kg^{-1}·d^{-1} isoleucine when an intake of 150 mL·kg^{-1}·d^{-1} is given (39-42). Since we and Snyderman both used free L-amino acids in the study diet – rather than total proteins – the requirement might be overestimated. A 20-35% higher first pass oxidation rate was seen in adults when an amino acid based diet was used compared to intact protein (43), so at least 20% of our mean requirement should be subtracted to correct for the amino acid based formula. The ESPGHAN advises an increase in protein content of infants formulas of 1.25 fold when the proteins used are based on hydrolyzed proteins in stead of intact proteins to correct for potentially less digestibility and biologic value of the nitrogen content (44). We therefore correct 25% for the use of amino acids in stead of intact proteins. Thereby, we determined a mean

Table 1: Previous studies using nitrogen balance techniques and current recommendations of the individual BCAA.

Requirement mg·kg^{-1}·d^{-1}	Nitrogen balance Infants 1-5 mo (13-15, 54)	Factorial Approach 0-1 mo[20]	IAAO 0-1 mo	Current Recomm Infants 0-1 mo[21]	Current Formulas Per 150 mL·kg^{-1}·d^{-1} (39-42)	Recomm intake Per 150 mL·kg^{-1}·d^{-1} IAAO
Isoleucine	119 (79-126)	59	105	95	97-194	**97**
Leucine	150 (76-229)	109	140	165	195-345	**130**
Valine	105 (85-105)	72	110	95	117-216	**102**
Ile:Leu:Val Ratio	**1 : 1.3 : 0.9**	**1 : 1.8 : 1.5**	**1 : 1.3 : 1**	**1 : 1.7 : 1**	**1 : 1.4 : 0.9-1 : 2.3 : 1.2**	**1 : 1.3 : 1**

requirement and not a safe level of intake in which a correction is made for individual variance in requirement. A safe level of intake is calculated as the mean requirement plus 2 SD of this mean requirement. Since the distribution of the amino acid requirement is not known, we use the safe level of protein intake as proposed by the WHO (21) which is 125% of the average protein requirement. If we correct our mean requirement for these two factors an intake of 105 mg·kg^{-1}·d^{-1} should be the advised intake for isoleucine in infants aged 0 to 1 month of age. The ESPGHAN recommends an isoleucine intake of 92 mg ·100 kcal^{-1} in infant formulas (44) which resembles our advised isoleucine intake of 97 mg·100 kcal^{-1} based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2).

The requirement for valine in infants aged 0 to 1 month

In 1959, Snyderman et al. determined the valine requirement in five neonates to be between 85-105 mg·kg^{-1}·d^{-1} using the nitrogen balance (13). Using the factorial approach, Dewey et al. determined the valine requirement in infants aged 0 to 1 month to be 72 mg·kg^{-1}·d^{-1} (20). The current recommended valine requirement, based on human milk is 95 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month (21). Our group determined the mean valine requirement in infants aged 0 to 1 month to be 110 mg·kg^{-1}·d^{-1} using the IAAO method. Current formulas provide an intake of 117-216 mg·kg^{-1}·d^{-1} valine when an intake of 150 mL kg^{-1}·d^{-1} is given (39-41) (Table 1). Since we used an amino acid based formula and because amino acids derived from intact casein are utilized in a higher proportion for protein synthesis than those from an equivalent intake of free amino acids (43), the true value of the requirement would be lower than our mean estimation. If we correct the mean requirement for the hydrolyzed proteins and correct for the safe protein intake which is 125% of the mean intake, an intake of 110 mg·kg^{-1}·d^{-1} should be the advised valine intake in infants aged 0 to 1 month. The ESPGHAN recommends an intake of 90 mg·100 kcal^{-1} (44) which is lower than our estimation of 102 mg·100 kcal^{-1} based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2).

The requirement for leucine in infants aged 0 to 1 month

In 1961, Snyderman et al. determined the leucine requirement in 1 preterm and 5 term infants to be 150 mg·kg^{1}·d^{-1} by using the nitrogen balance and weight gain (range 79-226 mg·kg^{1}·d^{-1}). Using a soy-isolate formula Fomon et al. determined the leucine requirement in normal female infants to be no greater than 132 mg·100 kcal^{-1}(45), lower intakes were not given . The factorial approach estimated the requirement for term neonates aged 0 month to be 109 mg·kg^{1}·d^{-1} (20). The current recommended isoleucine requirement, based on human milk, is 165 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month (21). Current formulas provide an intake of leucine of 195-345 mg·kg^{-1}·d^{-1} when an intake of

150 mL $kg^{1}\cdot d^{-1}$ is given (39-42) (Table 1). At discussed earlier, Metges et al. compared leucine oxidation rate, non-oxidative leucine disposal and net protein synthesis rate in adults that were fed an amino acid mixture and an intrinsically [1-^{13}C]leucine labelled casein (43). The oxidation rate was 22% higher, non-oxidative leucine disposal was 28% lower and the net protein synthesis rate was 35% lower in the amino acid mixture condition compared to the intrinsically labelled casein condition. Amino acid utilization might be lower from amino acid based or hydrolyzed formulas as also was shown in adults fed an elemental diet (46). The advised intake for leucine in infants aged 0 to 1 month based on the IAAO method corrected for hydrolyzed proteins and adapted for the safe level of protein is 140 $mg\cdot kg^{-1}\cdot d^{-1}$. The ESPGHAN recommends an intake of 169 mg · 100 · $kcal^{-1}$ (44) which is higher than our estimation of 130 mg ·100 $kcal^{-1}$ based on our study formula, which contains 108 kcal when an intake of 150 mL $kg^{-1}\cdot d^{-1}$ is given (Table 2). This indicates that current formulas may provide too much leucine which might be not beneficial for the neonate since leucine induces insulin release and sensitivity in the tissues which might be associated with diabetes mellitus later in life.

The BCAA ratio

Considerable interaction has been reported in humans and animals in response to disproportional intakes of BCAAs. In rats, imbalanced BCAA concentrations result in impaired growth, and BCAA supplementation has negative effects on fetal brain growth (36). The difficulty about studying the different branched-chain amino acids is the potential interaction between the BCAA; by changing the intake of a single test amino acid, the dietary mixture may become imbalanced (47). Therefore different groups determined the total BCAA requirement in stead of individual branched-chain amino acids. The BCAA pattern used in these studies is based on egg protein (which has a Ile:Leu:Val ratio of 1: 1.3: 1.1) to minimize the potential interaction of the BCAA on assessment of the requirement. The optimal proportion for protein synthesis in children is assumed to

Table 2: The current recommendations for the BCAA in infant formulas in $mg\cdot 100\ kcal^{-1}$ compared to the amount in regular infant formula. The third column describes the new recommendations based on the IAAO method

	Human milk ($mg\cdot 100\ kcal^{-1}$) [59] [44]	**Current Term formulas ($mg\cdot 100\ kcal^{-1}$) [41] [39, 42]**	**IAAO ($mg\cdot 100\ kcal^{-1}$)**
Isoleucine	90-92	105-190	**97**
Leucine	166-169	167-338	**130**
Valine	88-90	106-213	**102**
Ile:Leu:Val Ratio	**1 : 1.7 : 1 - 1 : 1.8 : 1**	**1 : 1.4 : 0.9 - 1 : 2.3 : 1.2**	**1 : 1.3 :1**

be that present in egg protein (48, 49) and the total BCAA requirement was determined to be the same as in healthy men (50). The concentrations of the BCAA in human milk varies from 1 : 1.8 : 1.3 (51), 1 : 1.6 : 1 to 1 : 2.0 : 0.9 (44). Human milk protein contains a Ile:Leu:Val ratio of 1: 1.74 : 1 (21). Current recommendations are based on this ratio that is based on the content of the branched-chain amino acids in human milk, since exclusive breastfeeding by a healthy mother is the feeding standard from birth to 6 months in healthy, term infants (21). Different formulas use BCAAs in different ratios depending on the casein-whey ratio (milk-based formula: 1 : 1.6 : 1.1, whey-adapted formula: 1 : 2.3 : 1.1) (52). Milk protein contains a Ile:Leu:Val ratio of 1 : 1.6 : 1 (17), casein a Ile:Leu:Val ratio of 1 : 1.8 : 1.4 (39).

The difficulty in the development of infant formulas is that the amino acid content of cow milk proteins is different than that of human milk. Both milks are composed of two classes of proteins, casein or acid-preciptable proteins and whey or acid-soluble proteins. The whey-casein ratio in colostrum is 80:20 and changes to 55:45 in mature milk (52). Casein dominant cow's milk formulas are made with nonfat dry milk and contain about 82% bovine casein and 18% bovine whey proteins. During the manufacture of infant formulas whey is added to cow milk to obtain a whey-casein ratio of 60:40 which is more similar to human milk. However, human milk proteins differ from bovine proteins in concentrations and amino acid composition so adding bovine whey proteins does not make the formula identical to the amino acid composition of human milk. In casein-dominant formula, especially methionine and tyrosine are elevated. In whey-dominant formula, methionine, threonine and lysine are elevated. The sum of the BCAAs is much higher in formulas than human milk: infants fed formula have higher concentrations of BCAA than human milk fed infants suggesting that levels of these amino acids are more closely related to protein quantity than protein quality (51).
Besides the differences between human milk protein and cow milk protein it is important to note that the amino acid composition of human milk is not the same as that of body protein, and Dewey et al. stated in 1996 that the composition of human milk proteins is not necessary a definition of the biological amino acid requirement of the growing neonate (20). Whole body protein contains a Ile:Leu:Val ratio of 1 : 2.1 : 1.4 (53). Our group found an optimal Ile:Leu:Val ratio of 1 : 1.3 : 1 in neonates aged 0 to 1 month which is comparable with the ratio determined by Snyderman using the nitrogen balance and weight gain (13-15, 54). Our results imply that egg protein might be a better alternative protein regarding the BCAA ratio for infants aged 0 to 1 month than cow milk protein. Since egg proteins contain more sulfur amino acids than milk proteins, the concentration of methionine and cysteine should be monitored carefully.
The nutritional implications of these differences in amino acid content of different proteins or mixtures of proteins can be evaluated by comparing the amino acid com-

position of the protein source with a suitable reference amino acid pattern by use of an amino acid scoring pattern. These scoring systems use the amino acid requirement in humans to develop reference amino acid patterns for purposes of evaluating the quality of food proteins or their capacity to efficiently meet both the nitrogen and indispensable amino acid requirement of the individual (17). The scoring systems use the limiting essential amino acid in the test protein, divide it by the amount of amino acid in a reference protein and correct it for true digestibility. The indispensable amino acid composition of the specific protein source is compared to that of a reference amino acid composition profile. Earlier the amino acid composition of a good protein such as egg was used, which is regarded as being well balanced in amino acid content in relation to human needs (55). Later the amino acid content of human milk was used as reference pattern (56,57) because adequate growth and development are known to occur in infants provided human milk, and plasma amino acid profiles of infants have been shown to reflect the amino acid composition of human milk. The Life Sciences Research Office (LSRO) report concluded that the amino acid scoring pattern of human milk is an accurate and appropriate standard for assessing the protein quality of infants formulas (58). The difficulty in composing infant nutrition is that even if the amino acid composition of infant formulas could be made very similar to that of human milk, digestibility and absorption of amino acids and peptides would be quite different from that of breast milk, thus resulting in different plasma amino acid profiles. Hypothetically, one could develop an infant formula based on egg protein and determine the plasma amino acid profile in infants fed this formula to see if the plasma aminogram is more similar to that of breast-fed infants than normal formulas provide.

Concluding, the requirement of valine and isoleucine determined by the IAAO method showed that the recommendations based on human milk are correct. The factorial approach by Dewey et al. underestimates the requirement for all BCAAs. For leucine, the IAAO showed a lower requirement for term infants than the human milk data. This would imply that, based on the IAAO data, the optimal Ile:Leu:Val ratio in infant formula would be 1 : 1.3 : 1 which resembles the BCAA ratio of egg protein. Current formulas provide 2-3 times too much leucine which could be associated with the development of the metabolic syndrome later in life. The IAAO method should be used to determine the requirement of the other essential amino acids to determine the protein reference to optimize infant nutrition.

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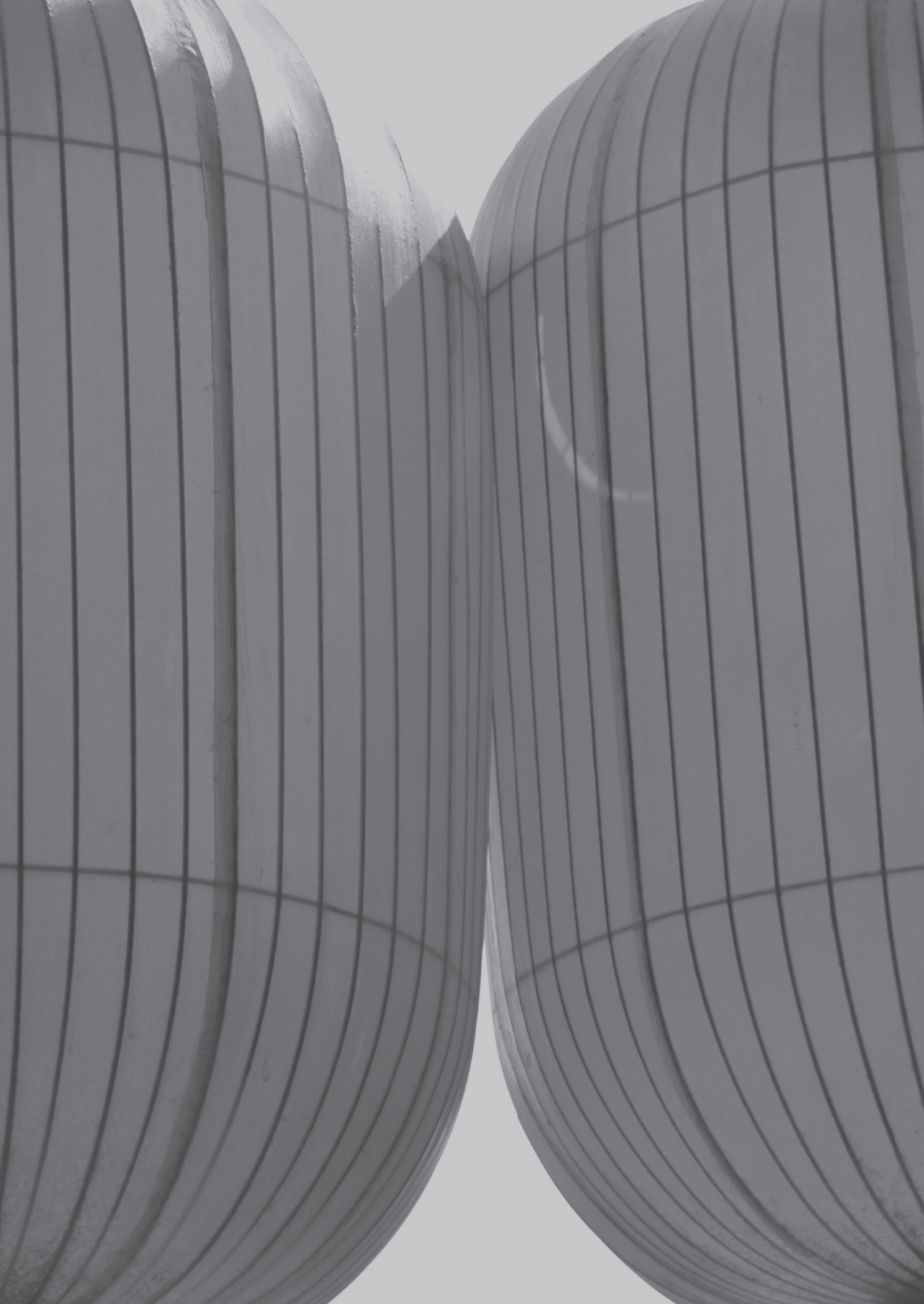
Part IV

West meets East/East meets West



CHAPTER

9

Considerations with regard to investigator initiated research in China: a western view

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Lisha Huang
Johannes B. van Goudoever

Submitted

INTRODUCTION

When you enter Shanghai, its immense structures that reflect the economic growth of the last 20 years force you to immediately realise you are in one of the cities of the world that matters. Economically, China has become a world power, while in other areas it is still a developing country. The Ping Pong diplomacy of Nixon in 1972 has led to China opening its doors to the world, with the organisation of The Olympic Games in 2008 as a major highlight. Several collaborations between China and the western world have developed in all types of fields. In our case, it led to a collaborative research program in the Children's Hospitals of our two universities: Rotterdam meets Shanghai, West meets East.

President Hu Jingtao's goal to be a superpower in medical research by 2020 has led to major investments in research and development. The Ministry of Health issued requirements for good clinical practice in 1999 that also included ethical review. The protection of human participants in international medical research collaborations in China, and in other developing countries, has been a focus of attention in the mass media and scientific literature of developed countries. The competition with China's enormous economic success has in some cases led to questions about the integrity of research conducted in China, and led to the " Declaration of Scientific Ideology" in 2006 by the Chinese Academy of Sciences (1). We collected our experiences from one year of medical research on a Neonatology Ward in China and exchanged them with our Chinese colleagues in an eastern view.

MEDICAL ETHICAL COMITTEE:

According to the World Medical Association's (WMA) Declaration of Helsinki (2), all medical research projects involving human subjects must undergo ethical review. In a developing country, such as China, not all hospitals have an ethics committee. In the institutions that have an institutional or independent local research ethics committee, it is very important that there is sufficient capacity and expertise to meet participants' needs.

In our hospital, a committee was formed during the preparation period of our research project. Although relatively inexperienced, the committee contributed many useful remarks. Three meetings in 6 months were required to have our protocol approved, which is similar to the time and process to obtain approval in the Netherlands.

Informed Consent

A fundamental principle of research ethics is that a participant that agrees to take part in research should do so voluntarily and with sufficient knowledge and understanding

of the procedures, risks and benefits involved. This is usually ensured through oral consultation and written consent, in our subjects, from one or both parents. In clinical trials, the International Conference on Harmonisation Guideline for Good Clinical Practice (3) is widely followed by both public and private sector researchers. These require detailed consent forms and a large amount of reading by the subjects or parents.

Because there was no experience in asking the parents at our Neonatology Department for consent, the informed consent process was a major challenge. Parents cannot visit their children because of the large number of patients per room and because of the supposed risk of infections. Parents have a telephone conversation with the doctor 1-2 times a week to receive information about their child. This is the only moment in which we can ask the parents for informed consent. The relationship between parents and doctors is not always optimal, as we will describe later. Additionally, many parents have negative associations with research and reject participation as soon as they hear the word research. Additionally, Chinese society is based on individual autonomy rather than social harmony. As a result, the argument that other people can benefit in the future from their participation does not have the desired effect; when there is no direct benefit for the child, no consent is given. In an observatory period of 12 months, we asked 272 parents for consent. In term neonates, 113 approved (58%) and 82 rejected. In preterm neonates, 43 parents approved (56%) and 34 rejected. These values are lower than the approval rates we obtain in the Netherlands, where almost 80% of parents approve for at least one study at our neonatal intensive care unit. Strikingly, in Shanghai, only fathers or grandfathers signed the informed consent form.

HEALTH CARE SYSTEM

Most developed countries have a universal health care system that provides partial or full compensation for medical costs depending on the system used. One of the major criticisms of performing research in a country where healthcare is not provided to children for free is that it is not ethical to perform free-of-charge intervention studies in patients that cannot pay for their own treatment. Because every country has its own medical system with its particular benefits and disadvantages, we will discuss only the Chinese system and the ethical issues that we experienced while conducting our research.

Inequity

The introduction of the Health Care Market System in the early 1980s led to the disappearance of universal, free basic healthcare in China. The responsibilities for the provision of health services have devolved to the provincial and county governments. Because only 10% of the people in rural sites are covered by the cooperative medical system, compared to 45% in the cities, this leads to significant urban/rural differences

and inequities in services between the rich and insured people and the poor people (4). In our daily practice, we observed patients not receiving optimal care or parents forced to stop treatment due to financial reasons. The government is aware of these problems and recently increased the health budget by 40.6%; the coverage percentage of the people of the rural sites has increased over the years. Thereby, inflation in medical costs that is greater than the economic growth will be banned to make health care more affordable in the future for all individuals (5). The mean cost for the treatment and hospitalisation of a term neonate is 4,235 RMB (440 euro). The mean cost for a preterm neonate is between 7,539 and 33,312 RMB (783-3,460 euro), depending on the gestational age and birth weight. These costs are almost 10 times lower than the costs in the Netherlands. Because the mean capital annual income in 2006 was 11,900 RMB (1,235 euro), and the average annual household income in Shanghai in that year was 20,668 RMB (2,148 euro) (6), these medical expenses are sometimes unaffordable and families are forced to abandon treatment.

Hopefully the loss of treatable patients will not occur in the future because the government's goal is to achieve universal access to basic health insurance by 2020. As quoted in 1999, "the Chinese experience shows that health development does not automatically follow economic growth"(7).

Prepayment Fee

When you enter a hospital in China, the waiting line is the first thing you see. No treatment is started before payment is received. This is the case for both outpatient and inpatient clinics. This situation disturbs the patient-doctor relationship; some parents do not trust doctors because their greatest concern appears to be the payment and not the patient. In 2007, the death of a boy led to protests against this so-called "Pay or Delay" system. Although the hospital was found not guilty of the accusations, statements blaming the hospital for the death were made during (illegal) protests (8).

One child policy*:*

To control the enormous population growth, the Chinese State of Council decided in 1979 to launch the one child policy. Deng Xiaoping, the acknowledged architect of the economic system that has made China an economic world power, approved this system to drive economic development and to raise the standard of living. This policy did have its desired effect when compared to the improvement of living standards in a country such as India that does not control population growth. However, the policy did have other unwanted effects. Because China has a long tradition of favouring boys, the ratio of male to female births is approximately 1.2 to 1.0 (9). Part of this difference lies in the under-registration of female births because females are sometimes left with relatives, given up for adoption or abandoned at orphanages (10). Sex ratios are further skewed

by widespread abortion, which 1 of 4 women in their twenties undergo, and 55% of them more than once (11). This occurs despite the fact that conducting an ultrasound to identify fetal sex is illegal in China.

In the clinical practice, our department admission rate in 2008 was 3376 patients per year, with 2096 males and 1280 females. Of these babies, 2236 were term, 65 were post-term and 1075 were preterm neonates. The male-to-female admissions ratio of Shanghai residents is 1.36 to 1. The ratio for infants that come from other provinces is 1.8 to 1. This large difference results most likely from the fact that ill or premature females are more frequently left untreated. We observed a tendency to abandon impaired babies by leaving them behind in the hospital. We also observed the male-to-female ratio difference in our study results; the first study we conducted in term infants did not contain one female after the inclusion of the first 15 patients.

OTHER ISSUES

Communication

What strikes you the most upon entering China is the fact that you cannot be understood if you do not speak Chinese. Even in a city such as Shanghai, most of the people do not speak any English. In the hospital, some of the doctors and nurses do speak English, but usually only the younger generation. To start clinical medical research in this country, a Chinese (-speaking) researcher is necessary, not only for communication with parents but especially for giving directions to avoid errors in your research setting.

Number of patients

Among the main reasons to perform research in China is the quantity of patients. There were 160 patients in our neonatology ward, which is 3-5 times higher than the western average. However, the numbers of nurses, doctors and nurse-assistants are similar to those in western hospitals. This results in medicine based on efficiency because every nurse has to take care of 15-20 babies and every neonatologist supervises 5 residents and is in charge of treatment for 55 patients. All procedures are performed at fixed time points in all patients at the same time. For research purposes, you must find a system that does not require individual handling by nurses or nurse-assistants to avoid mistakes. If individual handling is needed, you must have enough manpower to do it yourself.

Study requirements

One problem we experienced and did not expect before we went to China was the importation of our study materials. Because China has an enormous amount of exports, but few imported products and the regulations in China are based on forms and stamps,

importing items requires advanced preparation. The required forms must be prepared with the correct stamps from the government of the country where the products are produced. Because the list of required forms is not easy to acquire, at times the required forms are missing, the products are delayed in the customs process; this situation may take months to resolve. In our case, it took 4 months to pass the study formulas through customs, with help from a formula-producing company in China. It is also expensive to import items because the tax is 30%.

In short, to start up a medical research project in China takes time to learn about the culture and habits of the people and to become familiar with the logistics system; therefore, a Chinese-speaking researcher is a must. After all of the preparations are finished and all of the approvals and requirements are acquired, the amount of patients and the working spirit of the Chinese provide a stimulating research environment. The Chinese can learn from our ethical perspectives and our experience in asking for informed consent, whereas we can learn to manage large amounts of patients and learn from the Chinese efficiency. This is a win-win situation in a collaboration that has a large capacity for growth, which provides the opportunity to bring medical health care to a higher level in both eastern and western populations.

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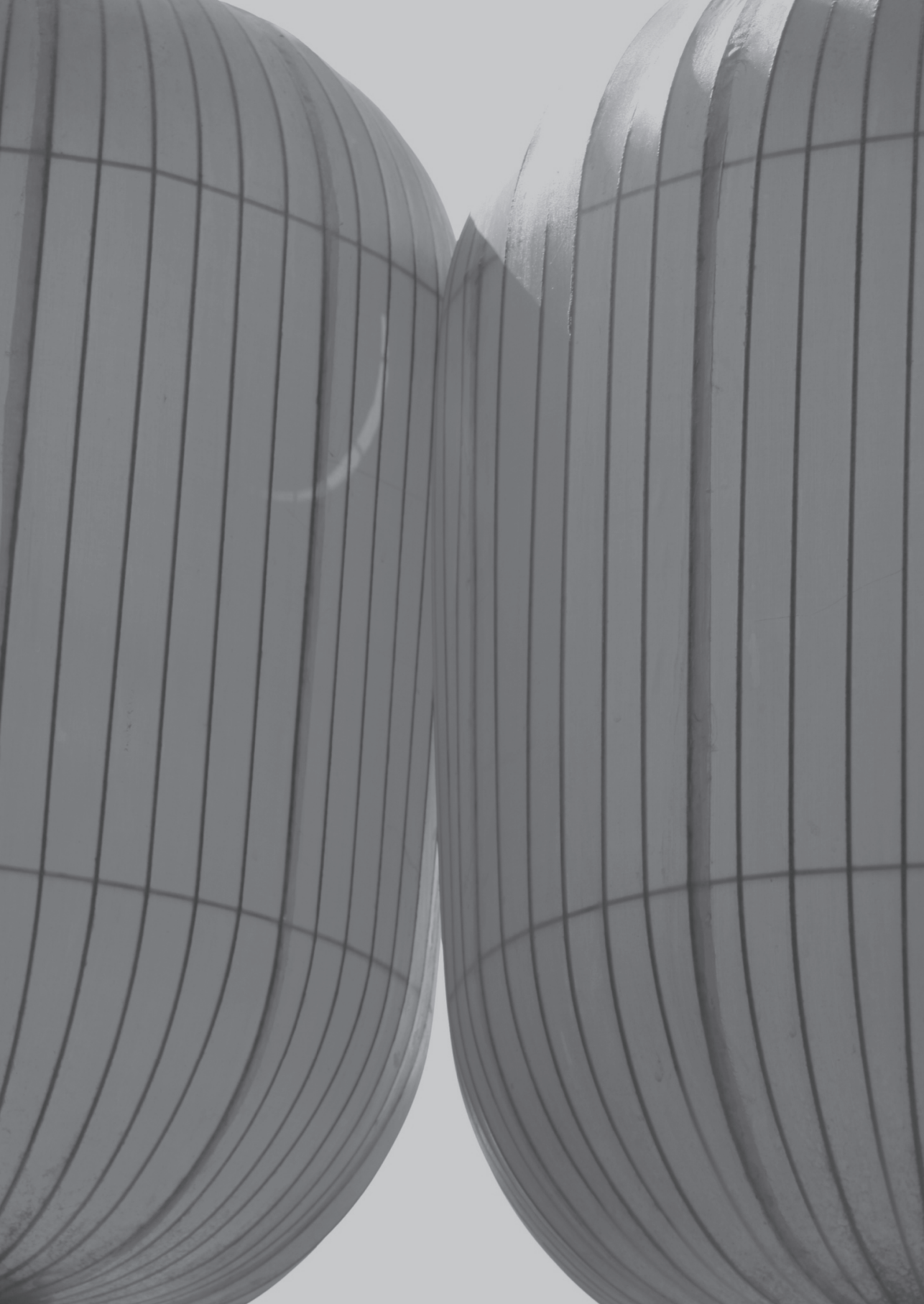

CHAPTER

10

A Sino-Dutch collaborative research project in China: an eastern view

Lisha Huang
Femke de Groof
Ying Huang

Submitted

INTRODUCTION

In January 2008, a Sino-Dutch research project started at the Children's Hospital of Fudan University in Shanghai. The aim of this research project was to determine essential amino acid requirements in Chinese neonates. It began in 2006, when I (Ying Huang, paediatric gastroenterologist of Fudan Children's Hospital) went to the Netherlands for an educational visit for 3 months. This was my first encounter with the neonatal nutritional studies by Prof. dr. van Goudoever. As a gastroenterologist, I view nutrition as one of the most important research fields. Due to the improvements in neonatal life support over the past decades, neonatal survival rates have increased tremendously, and the role of early nutrition is essential for this outcome.

WHY A COLLABORATION?

China is continually boosting its research potential. One of the opportunities to improve its innovativeness is through international collaborations. Erasmus MC-Sophia Children's Hospital, where Prof.dr. van Goudoever works, is one of the top institutions in the field of nutritional studies. To start the research project, two Dutch PhD students came to China for 1.5 and 3 years. They were assisted by a Chinese PhD student, several Dutch and Chinese medical students. The Chinese PhD student benefited from training during the design, implementation, and ethics of the trial and will perform her PhD defence according to the Dutch standards.

THE VALIDITY OF THE STUDY

As shown by WHO's new growth charts, given a healthy environment, children born in different regions of the world have the potential to grow and develop within the same range of height and weight for age (1). Therefore, the results from our research project (the essential amino acid requirements) performed in Chinese infants should be representative for infants across the world. This assumption is supported by the amino acid requirement studies performed in Canadian and Indian children (2-3). Therefore, we can make a contribution to optimising infant nutrition around the world.

CHILDREN'S HOSPITAL OF FUDAN UNIVERSITY

Fudan University is one of the nation's leading medical educational institutions; it represents the top level of clinical research in China. The neonatal department of the Children's Hospital of Fudan University is equipped with 50-70 neonatal intensive care unit beds and 200 medium-care beds, which makes our neonatal ward the largest in China. The hospital discharge rate of the neonatal ward is 4500 cases per year. Over 85% of the neonatologists and one-third of the nurses have been trained abroad.

RESEARCH ETHICS IN CHINA

In addition to the rapid development of medical technology in China, great progress has been made in the area of medical research ethics as well.

Medical research ethics is a relatively recent phenomenon in China. The basis of medical ethical committees has been imported from abroad. The development of ethical committees has been placed on a fast track. Since the acknowledgement of medical research ethics in 1987, 80 medical ethical committees were set up over 5 years. In 1998, China's Ministry of Health announced the establishment of "biomedical research ethics committees involving human subjects". Different steps have been taken to regulate the medical ethical committees across the country. By 2007, there were more than 400 institutional review boards across the country. During this process, the member composition and the legislation were improved. The Chinese guidelines for medical research ethics are greatly influenced by Euro-American medical ethics, especially by the US National Institutes of Health. The composition of ethical boards has been standardised. The institutional review board of the Children's Hospital of Fudan University includes 7 members: 1 lawyer, 1 ethicist, 2 administrators and 3 medical experts (a doctor of internal medicine, a surgeon and a pharmacist). The board's meetings are held once every 1-2 months.

For the current research project, additional focus has been placed on safety matters, the reasons behind setting up a study in China, and whether the same type of research has been performed in Europe. Additional documents, such as the review process documents from the Dutch institutional review board were required. Because there is no insurance company that offers insurance for studies which involve human subjects in China, a deposit from the research budget was needed for insurance funds.

INFORMED CONSENT

Informed consent is a cornerstone of ethics in medicine. In China, informed consent in medical handling has existed for a few decades. Parents must approve medical procedures and medical costs by written consent. Written consent improves human rights and the communication between patients and doctors.
Informed consent for research purposes is another developing area of medical ethics in China. Before the Sino-Dutch program started at the neonatal ward, there was not much experience in asking for informed consent for clinical studies in the NICU. This is not rare in China; at the time we started our collaboration in 2008, after studying all clinical trials published in the Chinese Journal of Pediatrics, informed consent procedures were mentioned in only 20% of the published clinical studies. A similar percentage was reported for randomised controlled trials conducted in 2004 (4). This amount is expected to increase in the near future because the majority of Chinese journals require ethics committee approval and informed consent from the participants.
In our studies, written informed consent was obtained from at least one of the parents by a Chinese-speaking researcher. Most Chinese informed consent forms are 1-2 pages in length. Therefore, we summarised the Dutch informed consent form of 8 pages into 1 page in Chinese. Low literacy among some of the parents and mistaking research for routine health care are common problems (5). An explanation of the research by a Mandarin-speaking researcher is necessary. The consent rate was approximately 50%, which is much lower than the 80% agreement rate observed in the Netherlands for nutritional studies in the neonatal intensive care unit.

MEDICAL INSURANCE

China has a high internal rural to urban migration rate. This rate is expected to increase in the coming decades. Migrants in Shanghai are largely excluded from urban services, including access to public health and public medical insurance. In general, the socioeconomic status of the migrants is below that of the urban population. Over 50% of the admissions at the neonatal ward of the Children's Hospital of Fudan University are new-borns of migrants (6). Of these 50%, the majority are new-borns of workers with low socioeconomic status who have to pay out-of-pocket for their new-born's medical services. In some cases, the unaffordable medical cost makes it necessary to take the sick infant to a rural hospital for further treatment. Shanghai residents have basic insurance that covers approximately 50% of medical costs. Since September 2011, certain funds exist for non-Shanghai household-registered patients who have incomes less than 100, 000 Renminbi per year per household.

The Chinese government is starting to focus on medical insurance coverage as a basic requirement for all citizens. In March 2009, an announcement made by the Chinese government brought hope for a national child insurance system. The proposal is to develop a national child insurance system to ensure that every child has the right to receive medical care. Moreover, the country has recently embarked on major health reform to achieve universal coverage of primary health services by 2020 (7).
Part of a doctor's duties is to inform parents about the admission and treatment costs for their new-borns. The doctor-patient relationship is not what it should be; mistrust of doctors and researchers is a common phenomenon.

USE OF EXTERNAL SUPPORT

The main logistical problem during the Sino-Dutch research program was the importation of study formulas. All sorts of issues had to be resolved to receive clearance from customs to import the study formulas to China. The main issues were the required import licenses and the continuously changing customs legislation, which made it impossible for a hospital to import study formulas directly. Industry sponsors, in our case Danone and Dumex China, were required to facilitate the transportation, but excessive costs were incurred to import the study formulas.

CONCLUSION

China has the largest scientific workforce in the world and has just started to bloom in the scientific field (8). The clinical research publication output has increased impressively at an average rate of 22% each year between 2000-2009 (9). The Chinese government invested approximately 1 billion Renminbi (EUR €100 million) in medical research in 2010 (10). This enormous boost is meant to make China's medical research more competitive internationally. There is enormous potential for collaborative scientific research work with China. As described in this article, there are many differences between conducting research in China and in a western country. However, the ethical principles for good clinical practice are universal. To achieve success in a Sino-European research collaboration, we should first follow the Western and Chinese ethical standards, and have co-investigators who can speak Mandarin and English and possess knowledge regarding the cultural settings.

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Part V

Other essential amino acid requirements in term neonates

CHAPTER

11

Methionine requirement for the enterally fed term infant in the first month of life in the presence of cysteine

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ABSTRACT

Background: The essential amino acid methionine can be used for protein synthesis, but also serves as precursor for homocysteine and cysteine.

Objective: The objective was to determine the minimal obligatory methionine requirement for infants in presence of excess cysteine (91 mg·kg^{-1}·d^{-1}) using the indicator amino acid oxidation (IAAO) method with L-[1-^{13}C]phenylalanine as the indicator.

Design: Fully enterally fed term infants less than one month of age were randomly assigned to methionine intakes ranging from 3 to 59 mg·kg^{-1}·d^{-1}, as part of an elemental formula. After 1 d adaptation to the test diet, [^{13}C]bicarbonate and L-[1-^{13}C]phenylalanine tracers were given enterally. Breath samples were collected at baseline and during isotopic plateaus. Mean methionine requirement was determined by using a biphasic linear regression crossover analysis on the fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$). Data are presented as mean ± SD.

Results: Thirty-three neonates (gestational age 39 ± 1 wk) were studied at 13 ± 6 d. With increasing methionine intakes, $F^{13}CO_2$ decreased until a methionine intake of 38 mg·kg^{-1}·d^{-1}; additional increases in methionine intake did not affect $F^{13}CO_2$. Mean methionine requirement was determined at 38 mg·kg^{-1}·d^{-1} and the upper and lower CIs were 48 and 27 mg·kg^{-1}·d^{-1}, respectively ($p < 0.0001$, $r^2 = 0.59$).

Conclusions: Although the current recommended methionine intake of 28 mg·kg^{-1}·d^{-1} is within the CIs of our study, the estimated mean requirement is substantially higher. However, most of the infant formulas provide a methionine intake of 49 to 80 mg·kg^{-1}·d^{-1}, which is above the upper CI of our study.

INTRODUCTION

Methionine is an essential amino acid required for protein synthesis. It is also needed for the biosynthesis of carnitine, which is essential for fatty acid metabolism (1). Methionine is the major methyl donor in mammalian cells and a precursor for polyamine synthesis (2). Transmethylation of methionine leads to homocysteine synthesis. Homocysteine can be remethylated to form methionine or catabolized via the transsulfuration pathway to form cysteine. Cysteine can be incorporated into protein and it is also involved in the production of glutathione, taurine, coenzyme A and inorganic sulfur. Cysteine, glutathione and taurine play a role in the defense mechanism against oxidative stress.

Deficient intake of methionine not only impairs growth, but has also an impact on the sulfur metabolic pathways in the synthesis of its key metabolic intermediates. In contrast, methionine is known as the most toxic amino acid in animals when supplemented in excess (3-4). Hypermethioninemia and hyperhomocysteinemia were observed in infants consuming methionine fortified formula with methionine content of 788 mg/L or a high protein formula providing 9 g protein per kg per d (5-6). Extreme hypermethioninemia may cause cerebral edema (5). Hyperhomocysteine is found to be associated with an increased risk for neonatal stroke (7). Because both a deficient and excess intake of methionine have detrimental effects, it is therefore important to determine the methionine requirement to optimize infant nutrition.

Experimental evidence for methionine requirement of enterally fed infants is scarce. In earlier studies with relatively small number of infants (n = 7 -13), methionine requirement was estimated to be between 27 and 49 $mg \cdot kg^{-1} \cdot d^{-1}$ (8-11). Since breast milk is considered to be the optimal nutrition for infants up to 6 months of age, the joint WHO/FAO/UNU expert consultation recommended a methionine intake of 28 $mg \cdot kg^{-1} \cdot d^{-1}$, based on the average intake of breastfed infants (12). Human milk is known to vary in protein content while the volume ingested varies as well on a daily basis. These factors all contribute to the difficulty of providing an accurate estimation of the intake of a breastfed infant.

The indicator amino acid oxidation (IAAO) method is minimally invasive and therefore suitable for determining the essential amino acid requirements in vulnerable populations including infants (13-14).

The aim of this study was to determine the minimal obligatory methionine requirement with excess intake of cysteine for term infants using the IAAO method.

SUBJECTS AND METHODS

Subjects

Thirty-three neonates admitted to the Neonatal Ward in the Children's Hospital of Fudan University participated in the study. Each subject was selected for study by the following criteria: fully enterally fed infants with a gestational age of ≥ 37 weeks, birth weight ≥ 2500 g, and clinically stable with a weight gain rate ≥ 5 $g \cdot kg^{-1} \cdot d^{-1}$ in the preceding 3 d. Subjects were excluded if they had congenital anomalies, gastrointestinal pathology or sepsis.

The study was approved by the Institutional Review Boards of the Children's Hospital of Fudan University, and a statement of no objection was obtained from the Erasmus Medical Centre-Sophia Children's Hospital, Rotterdam. Written consent was obtained from at least one of the parents of each subject by a Chinese speaking researcher.

Study formula

The study formula used was an elemental formula that was based on free amino acids. The amino acids, fat, carbohydrates, and energy content of the study formula are shown in Table 1. The composition was the same as Neocate (SHS International) except for the methionine, phenylalanine and alanine content. Methionine, which was completely withdrawn from the study formula, was separately added in the form of L-methionine to obtain different amounts of intake. The formula provided a cysteine intake of 91 $mg \cdot kg^{-1} \cdot d^{-1}$. This amount was considered to be in excess, since it is more than 3 times the intake of a breastfed infant (12). This amount should minimize the amount of methionine that is metabolized to cysteine via the transsulfuration pathway, which enables us to determine the minimal obligatory methionine requirement. The phenylalanine intake was kept constant during the study by separately adding L-phenylalanine during the 24 h adaptation period to obtain the same amount as in the Neocate (SHS International), and this amount of phenylalanine was given as stable isotope L-[1-^{13}C]phenylalanine on the tracer infusion day. The phenylalanine intake during the study was 166 $mg \cdot kg^{-1} \cdot d^{-1}$, which was above the recommended amount of 72 $mg \cdot kg^{-1} \cdot d^{-1}$ (12). A generous amount of tyrosine (166 $mg \cdot kg^{-1} \cdot d^{-1}$) was provided to ensure that the newly formed [1-^{13}C]tyrosine hydroxylated from [1-^{13}C]phenylalanine would be directly channeled to oxidation into $^{13}CO_2$, which can be measured in expired air (15). This amount of tyrosine was almost twice the recommended intake (12). The nitrogen intake was kept constant for all subjects by the substitution of L-alanine for the methionine that was withdrawn. The caloric intake was kept constant during the study period in all infants.

The minerals and trace elements supplied in 100 g formula were as follows: iron 7.0 mg, calcium 325 mg, phosphorus 230 mg, magnesium 34 mg, sodium 120 mg, chloride 290

Table 1. Energy, carbohydrates, fat, and amino acids content of the study formula.

Component	Per 100 g formula
Energy (kcal)	475
Carbohydrates (g)	54
Fat (g)	23
Total amino acid (g)	13
L-Alanine (g) [1]	≥ 0.61
L-Arginine (g)	1.08
L-Asparagine (g)	1.01
L-Cyst(e)ine (g)	0.4
Glycine (g)	0.95
L-Histidine (g)	0.62
L-Isoleucine (g)	0.95
L-Leucine (g)	1.63
L-Lysine (g)	1.11
L-Methionine (g) [2]	0
L-Phenylalanine (g) [3]	0.20
L-Proline (g)	1.16
L-Serine (g)	0.71
L-Threonine (g)	0.8
L-Tryptophan (g)	0.32
L-Tyrosine (g)	0.73
L-Valine (g)	1.04
L-Carnitine (g)	0.01
Taurine (g)	0.03
L-Glutamine (g)	1.34

[1] Variable levels of L-alanine were added to the diet depending on the test methionine level of each infant to maintain an isonitrogenous diet. The study formula contained at least 0.61 g L-alanine per 100 g formula.

[2] L-methionine was added separately, depending on the test methionine level.

[3] 0.53 g L-phenylalanine per 100 g formula was added to the study diet on day 1. Equivalent amount of L-phenylalanine (0.52 g per 100 g formula) was given as isotope on day 2.

mg, potassium 420 mg, manganese 0.38 mg, iodine 47 µg, selenium 11 µg, copper 380 µg, and zinc 5.0 mg.

The vitamin content of 100 g formula was as follows: vitamin A 528 µg retinol equivalent, vitamin D 8.5 µg, vitamin E 3.3 mg α-tocopherol equivalent, vitamin K 21 µg, thiamin 390 µg, riboflavin 600 µg, niacin 4.5 mg, vitamin B_6 520 µg, vitamin B_{12} 1.3 µg, pantothenic acid 2.3 mg, folic acid 38 µg, vitamin C 40 mg, and biotin 26 µg.

Experimental design

The study design is based on the minimally invasive IAAO method (13), which is recently modified to apply in enterally fed infants (14). The advantages of this method are the short adaptation period to the test intake (1 d), the enterally delivered isotopes, and the sampling of expired air without sampling of the amino acid enrichments in plasma or urine. The IAAO method is based on the concept that when the test amino acid intake

is insufficient to meet the requirement, protein synthesis will be limited and all of the amino acids will be oxidized, including the indicator amino acid, which is labelled with a stable isotope. As the dietary intake of the test amino acid increases, the oxidation rate of the indicator will decrease until the requirement of the test amino acid is met. Once the requirement of the test amino acid is met, an additional increase in its intake will have no further influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as $^{13}CO_2$.

During the study, all of the infants received a fluid intake of ~150 mL·kg^{-1}·d^{-1}, a caloric intake of 108 kcal·kg^{-1}·d^{-1} and an amino acid intake equal to the protein intake of ~2.96 g·kg^{-1}·d^{-1}. Infants were randomly assigned to one of the graded test intakes of methionine, which ranged from 3 to 59 mg·kg^{-1}·d^{-1}. To maximize the power of the breakpoint analysis used in our method, a wide range of methioinine intakes with approximately equal number of intakes above and below the expected breakpoint was chosen (16). Since the methionine requirement was expected to approximate the methionine content in human milk, we therefore studied an equal number of intakes above and below the expected requirement of 28 mg·kg^{-1}·d^{-1} (12). Each study took place over a 31-h period whereby each infant received one of the test intakes. After 24 h of study formula consumption, the tracers were administered on day 2 for 7 h. Infants were bottle fed every 3 h during the adaptation period. Subsequently, the feeding regimen changed to hourly bottle feeding during the tracer infusion until the end of the study. On the tracer day, a nasogastric tube was placed for tracer infusion. Infants received a primed (14 µmol·kg^{-1}) continuous (9 µmol·kg^{-1}·h^{-1}) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 3 h to quantify individual CO_2 production rates (17). Phenylalanine was used as the indicator. After the [^{13}C] bicarbonate infusion was stopped, a primed (34 µmol·kg^{-1}) continuous (27 µmol·kg^{-1}·h^{-1}) enteral infusion of L-[1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes) was started and lasted for 4 h. Syringes were weighted before and after the study to determine the exact amount of the tracers that were given to the infants. The tracer infusion day is depicted in Figure 1.

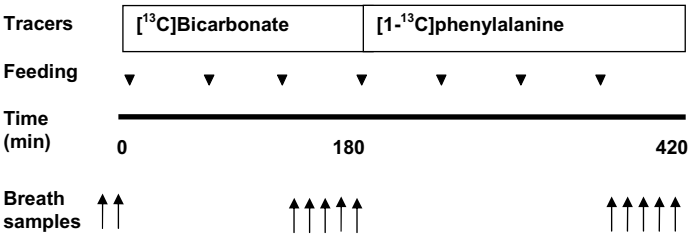

Figure 1. Schematic overview of tracer infusion day. Triangles indicate time that bolusfeeding was given. Arrows indicate times that breath samples were taken.

Sample collection and analysis

Breath samples were obtained by using the direct nasopharyngeal sampling method described by van der Schoor et al. (18). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical, Tullamore, Ireland) was placed 1 to 1.5 cm into the nasopharynx and the end-tidal breath was taken slowly with a syringe. Collected air was transferred into 12 mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and was stored at room temperature until analysis. Two duplicated baseline samples were obtained before the start of tracer infusion. Duplicated breath samples were obtained at 15 min intervals during isotopic plateau of [^{13}C]bicarbonate between 105 and 180 min. Seven duplicated samples were obtained every 10 min during isotopic plateau of L-[1-^{13}C]phenylalanine between 360 and 420 min (Figure 1).
^{13}C isotopic enrichment in the breath samples was analyzed by an infrared isotope analysis technique (Helifan, Analytic Fischer Instruments, Leipzig, Germany) (19). The ^{13}C enrichment was expressed as the atom percent excess above baseline (APE).

Calculations

The isotopic steady state was represented by plateaus in $^{13}CO_2$. Plateaus were determined by visual inspection and were confirmed by regression analysis as a slope not significantly different from zero.
The estimated body CO_2 production rate (mmol·kg^{-1}·h^{-1}) was calculated as described previously (14, 17).
The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) in % was calculated with the following equation (20):

$$F^{13}CO_2 = [IE_{PHE} \times i_B] / [i_{PHE} \times IE_B] \times 100\%$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (μmol·kg^{-1}·h^{-1}), i_{PHE} is the infusion rate of L-[1-^{13}C]phenylalanine (μmol·kg^{-1}·h^{-1}) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion.
Phenylalanine flux was not obtained. As shown in our previous study, test amino acid intake has no effect on the phenylalanine flux (14).

Statistical analysis

Descriptive data are expressed as means ± SD. Determination of the methionine requirement, the breakpoint, was performed using a biphasic linear regression crossover model (21). With the biphasic linear regression analysis, the regression equation was split into two parts. For the first part an intersept and slope were estimated, while for the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second

line is equal to the breakpoint. The model with the best fit, based on the highest r^2 was selected. The 95% confidence intervals were calculated. A value of $p < 0.05$ was taken as significant. The analyses were performed in STATA version 11.
The power analysis cannot be performed. We aimed to study 20 to 35 infants, more than studies in parenterally fed infants using the same approach with intravenous administration of the tracer (22-24).

RESULTS

Subject characteristics

Thirty-three term neonates participated in the study. The neonates were studied at a methionine intake that ranged between 3 and 59 mg·kg^{-1}·d^{-1}. Subject characteristics are summarized in Table 2. All subjects were growing well before entering the study. The mean (± SD) weight gain rate 3 d before the study was 13 (± 5) g·kg^{-1}·d^{-1}. The mean (± SD) energy intake was 109 ± 1 kcal·kg^{-1}·d^{-1}. The nitrogen intake was equivalent to a protein intake of 3.0 ± 0.1 g·kg^{-1}·d^{-1}. The infants were clinically stable and considered healthy because they were discharged on the study day or the day after. The primary reasons for admissions were unconjugated hyperbilirubinemia (n = 15), pneumonia with a negative blood culture (n = 6), asphyxia (n = 4), infection suspicion with a negative blood culture (n = 5), wet lung (n = 1), observation due to uterine bleeding (n = 1) and pending results of TORCH (toxoplasmosis, other agents, rubella, cytomegalovirus, herpes simplex virus) (n = 1), which were negative. Intravenous antibiotics (penicillins and/or cephalosporins) were given to 28 of the 33 infants.

Table 2. Subject characteristics, and protein and energy intake prior to the study of infants who participated in the study (n = 33).

	Values
Birthweight (kg)	3.3 ± 0.4
Gestational age (wk)	39 ± 1
Age on study day (d)	13 ± 6
Weight on study day (kg)	3.5 ± 0.4
Weight gain before study (g·kg^{-1}·d^{-1})	13 ± 5
Sex (F:M)	9:24
Protein intake prior to the study (g·kg^{-1}·d^{-1})	2.5 ± 0.4
Energy intake prior to the study (kcal·kg^{-1}·d^{-1})	108 ± 14

All values are means ± SDs.

$^{13}CO_2$ enrichments during [^{13}C]bicarbonate infusion

The baseline $^{13}CO_2$ enrichment was -17.04 ± 0.94 pee dee belemnite (PDB). The mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion was 0.0380 ± 0.032 APE. The corresponding mean CO_2 production rate was 23.44 ± 2.04 mmol·kg^{-1}·h^{-1}. The mean $^{13}CO_2$ enrichment at isotopic plateau and their corresponding CO_2 production rate of each infant were plotted against the methionine intake (Figure 2A and 2B respectively).

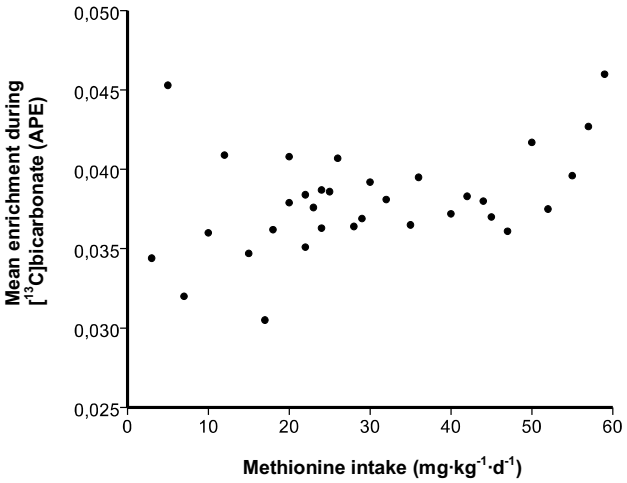

Figure 2A. Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral [^{13}C]bicarbonate infusion of each infant plotted against the methionine intake (n=33). APE, atom percent excess.

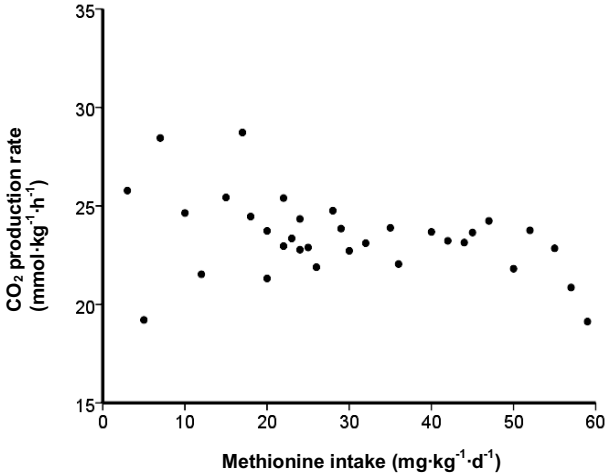

Figure 2B. The CO_2 production rate of each infant plotted against the methionine intake (n=33).

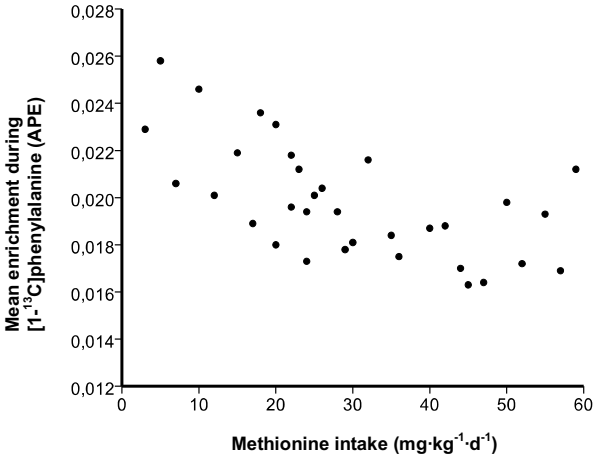

Figure 3A. Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral L-[1-^{13}C]phenylalanine infusion of each infant plotted against the methionine intake (n=33). APE, atom percent excess.

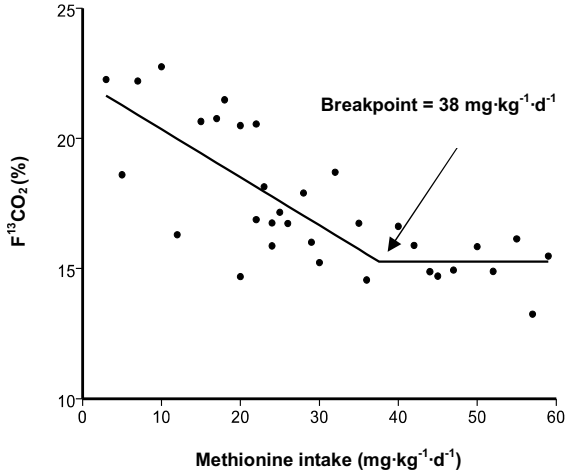

Figure 3B. The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the isotopic plateau at different methionine intakes (n = 33). Each infant received a different methionine intake. With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 38 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.59$). The upper CI was 48 mg·kg^{-1}·d^{-1} and the lower CI was 27 mg·kg^{-1}·d^{-1}.

L-[1-^{13}C]phenylalanine oxidation

The mean $^{13}CO_2$ enrichment at isotopic plateau during L-[1-^{13}C]phenylalanine infusion was 0.0198 ± 0.032 APE. These $^{13}CO_2$ enrichment values and the $F^{13}CO_2$ are plotted against methionine intakes in Figure 3A and 3B . As the methionine intake increased, $F^{13}CO_2$ decreased. This negative correlation was shown between $F^{13}CO_2$ and methionine intakes up to 38 mg·kg^{-1}·d^{-1}; additional increases in methionine intake did not affect the $F^{13}CO_2$. Using a biphasic linear regression crossover model, mean methionine require-

ment was determined to be 38 mg·kg^{-1}·d^{-1} ($p < 0.0001$, $r^2 = 0.59$). The upper CI was 48 mg·kg^{-1}·d^{-1} and the lower CI was 27 mg·kg^{-1}·d^{-1}.

DISCUSSION

The minimal obligatory methionine requirement for enterally fed term infants is estimated to be 38 mg·kg^{-1}·d^{-1} using the IAAO method. This value is comparable with the estimates of 32 to 49 mg·kg^{-1}·d^{-1} determined by Snyderman et al. (8). In their study, the methionine requirement was determined in 7 infants with postnatal age between 2 weeks and 2 months using weight gain rates and the nitrogen retention. The study diet used by Snyderman et al. (8) was an elemental diet providing a cysteine intake of 64 mg·kg^{-1}·d^{-1}. Fomon et al. (9-11) reported a series of studies with soy protein formulas with or without a methionine supplement fed to infants over a period of several months. Eight to thirteen infants were included in each study diet. Adequacy of the diet and thus adequacy of sulfur amino acids intakes was estimated by measurement of growth, serum chemical indices and nitrogen retention. They concluded that for female infants a diet with methionine content of 35 mg/100 kcal was considered adequate, however a methionine intake of 39 mg/100 kcal failed to meet the requirement of male infants under the age of 56 d. Although our study was not designed to detect gender differences in methionine requirement, the average requirement estimates by Fomon et al. (9-11) are consistent with our results. The limitations of earlier studies were the relative small number of subjects studied and the method used. Growth rates and nitrogen balance might not be the most sensitive and accurate methods for estimating the amino acid requirements.

Recently, Courtney-Martin et al. (22) determined the methionine requirement in parenterally fed postsurgical neonates using the IAAO method to be 49 mg·kg^{-1}·d^{-1}. The diet was devoid of cysteine. To compare their estimates with ours, the route of nutrition intake and the sparing effect of cysteine on methionine requirement need to be taken into account. Experiments with human fetal tissues demonstrated the lack of activity of cystathionase (25-26), the enzyme involved in the final step in the cysteine synthesis pathway. However, recent clinical studies showed the capability of transsulfuration of methionine to cysteine in (pre)term neonates (27-29) and thereby support the evidence of cysteine having a sparing effect on methionine requirement. The level of cysteine for sparing the dietary methionine requirement is ~33% in infants (30), ~55% in school-aged children (31), 60-89% in adults (32-33), ~40% in piglet studies (34). In a series of experiments, Shoveller et al. (34-35) compared the methionine requirement and cysteine sparing capacity in piglets that were parenterally and enterally fed. They showed that the parenteral methionine requirement was ~70% of the enteral requirement and the

dietary cysteine reduces the methionine requirement by equal proportion in both feeding routes of ~40%. Using these fractions, the parenteral methionine requirement of 49 $mg \cdot kg^{-1} \cdot d^{-1}$ with a diet devoid of cysteine in neonates determined by Courtney-Martin et al. (22) can be converted to enteral methionine requirement with excess of cysteine as in our study. The predicted requirement would be 42 $mg \cdot kg^{-1} \cdot d^{-1}$. This amount is nearly the same as our estimated requirement of 38 $mg \cdot kg^{-1} \cdot d^{-1}$.

The current essential amino acid recommendations are based on the average intake of an exclusive breastfed infant (12). The estimated methionine intake in the first month of life is 28 $mg \cdot kg^{-1} \cdot d^{-1}$, which is lower than our estimated mean requirement. At least four explanations might contribute to this finding.

First, a part of the difference may be caused by the elemental formula we used, while the recommendations are not discriminating between whole protein based formulas, partially hydrolyzed or elemental formulas. All these formulas are on the market for infants. A recent report demonstrated that an elemental diet provides in average 17% less protein substrate per gram of free amino acids than does a protein bound diet. This is due to the release of a water molecule when a peptide bond is formed (36).

Secondly, amino acids utilization and therefore retention were shown to be different depending on the protein/ amino acids digestion rates (37-38). Metges et al. (37) showed that the oxidation rate was 22% higher and the non-oxidative disposal is 38% lower when free amino acids are ingested compared to a protein diet. Therefore, we might overestimate the actual requirement. However, using a diet based on free amino acids provides us the ability to vary in test amino acid intake while keeping the other amino acid intakes constant. Future studies with an intrinsically labelled protein are required to evaluate this issue.

Thirdly, human milk composition shows remarkable variation, it is influenced by many factors, such as the gestational age at parturition, stage of lactation, nutritional status of the mother, etc. The protein content in human milk declines remarkably during lactation (39-40). The recommendations do take into account the decline in protein intake by the breastfed infant, but not for the change in whey-casein ratio and thus the change in amino acid composition (41).

Lastly, the protein digestibility and amino acid bioavailability in human milk are different from that in the formula. Therefore, the gross amino acid composition of human milk may not necessarily reflect the amino acid requirement profile of infants consuming infant formula. An ESPGHAN coordinated international expert group stated that "the composition of human milk can provide some guidance for the composition of infant formulae, but gross compositional similarity is not an adequate determinant or indicator of the safety and nutritional adequacy of infant formulae" (42). The results of our current study provide more scientific knowledge of amino acid needs of infants fed an infant formula, which is necessarily to improve infant nutrition.

A limitation of our study is that we performed the study in hospitalized infants. Although the infants were recovered from their illnesses, 11 out of 33 infants were in a (possible) post-infectious state. Since inflammation along with increased oxidative stress might deplete liver glutathione pool by increase glutathione usage, the liver glutathione pool might be depleted in these infants. The liver glutathione pool can be restored by increasing the cysteine content in the diet (43). We therefore assume that the current health status will not affect the estimated methionine requirement significantly because cysteine was supplied in excess and glutathione synthesis depends mainly on the availability of cysteine (44).

Another issue in our study is the extensive antibiotic use in our study population. Antibiotic treatment has a major impact on the bacterial flora in the gastrointestinal tract (45), and it is possible that the requirement is met not only by the diet but also by the de novo synthesis by the gastrointestinal bacterial flora (46). However, the bacterial contribution to amino acid requirements is still unclear. Therefore, the impact of antibiotic use on the estimated requirement is unknown.

The minimal obligatory methionine requirement is determined to be 38 $mg \cdot kg^{-1} \cdot d^{-1}$ of term neonates fed an amino acid based formula provided with an excess of cysteine. Current infant formulas provide excess methionine (49-80 $mg \cdot kg^{-1} \cdot d^{-1}$) when 150 $mL \cdot kg^{-1} \cdot d^{-1}$ is consumed (47). The results of our current study provide more scientific knowledge of amino acid needs for infants fed an infant formula, which is necessarily to improve infant nutrition.

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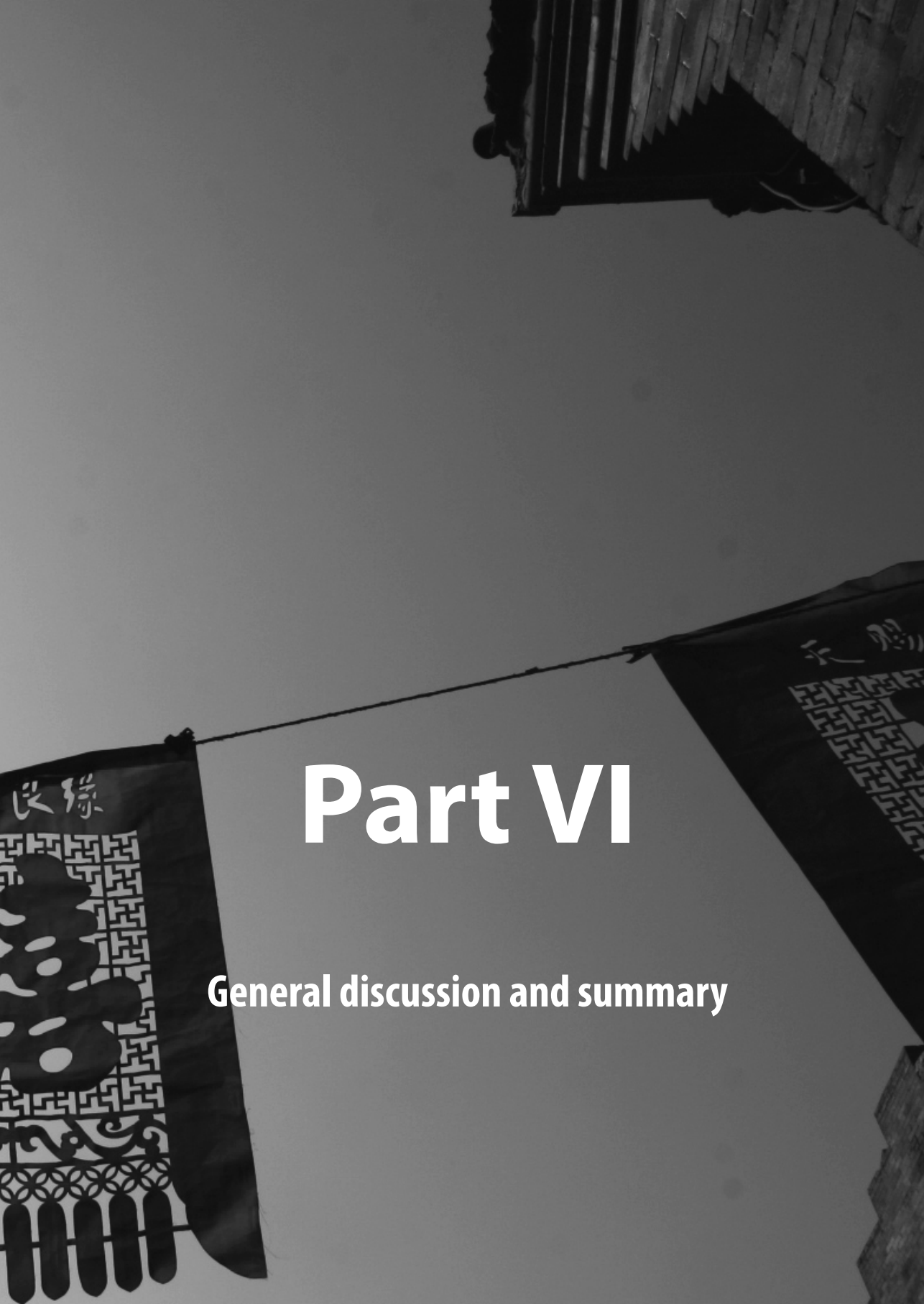

Part VI

General discussion and summary

CHAPTER

12

General discussion, overview of the studies and future perspectives

INTRODUCTION

Birth and adaptation to extrauterine life involve major shifts in the protein and energy metabolism of the human newborn. These include a shift from a state of continuous supply of nutrients including amino acids from the mother to cyclic periodic oral intake and a change in the mobilization and use of substrates. It is associated with marked surges in several hormones, lipolysis and initiation of endogenous glucose production. Although a vast majority of neonates' transition to the extrauterine environment uneventfully, a number of infants, in particularly those born prematurely, develop significant problems (1). Major advances in the care of these infants, such as ventilatory support, surfactant replacement therapy, and ante-partum glucocorticoids, have markedly improved their survival. At the same time it made their nutritional care a challenge for the physician; extremely low birthweight infants (ELBWI, birthweight < 1000 gram) survivors have an increased risk of growth failure (2, 3). The improvement in survival for ELBWI has not been accompanied by proportional reduction in the incidence of disability in this population (4, 5). In addition, many infants born with a weight between 1000 and 1500 g become growth restricted as well with parameters below the 10th percentile by 36 weeks' postconceptional age (6-8), and many remain small into childhood and adolescence (9, 10). The growth failure is attributable, at least in part, to inadequate nutrition in the first weeks of life. There is accumulating evidence that malnutrition during periods of vulnerability alters the growth of the developing brain and may have permanent negative consequences (11-13). A clear association between early enteral intake and neurodevelopmental outcome has been demonstrated in larger preterm infants. Lucas et al. demonstrated higher cognitive and motor scores at 18 months' corrected age, higher verbal IQs and lower rates of cerebral palsy at 7.5 to 8 years of age in preterm infants fed preterm formula versus term formula in the first 4 weeks of life (14, 15). More recently, Stephens et al. showed that increased first week protein and energy intakes are associated with higher mental developmental index (MDI) scores and lower likelihood of length growth restrictions at 18 months in ELBWI (16). In addition to the benefits, there may be adverse long-term effects of increased early protein and energy intake. Preterm infants randomly assigned to human milk versus formula, just for 4 weeks, have marked benefits 13-16 years later for lipid profile (17), blood pressure (18), leptin resistance (19) and insulin resistance (20). For the latter, a diet of human milk or standard formula was beneficial compared with nutrient-enriched formula (20).

In term neonates, weight gain in the first 1-2 years of life is associated with adverse health outcomes later in life, including increased blood pressure (21), increased weight gain and body fat deposition (22-25) and an increased risk of diabetes (26). The higher protein intake in infants who are fed formula may play a role with these health outcomes because formula-fed infants reach a higher body weight and weight for length at one

year of age compared with infants who are fed breast milk (27). Thus, protein intake should be strictly regulated early in life to result in the best possible neurodevelopment while reducing the risk of obesity.

METHODOLOGICAL ISSUES

Adaptation to the study diet

A decrease in the intake of protein or a specific indispensable amino acid intake results in a reduced rate of amino acid oxidation (28-31) and consequently a decrease in the rate of body-N loss (30). When intakes of energy and other nutrients are adequate but the protein level is very low, this decline in N-excretion reaches, within a few days to 1 week, a new, lower and relatively steady state (32). Harper (33) described the enzymatic changes that occurred after ingestion of a protein-low and protein-high intake. Enzymes involved in the metabolism of dispensable amino acids responded to the amount of protein consumed, whereas enzymes involved in the catabolism of indispensable amino acids adapted to changes in protein intake (and indispensable amino acid intake) in relation to the amino acid needs of the body. So the capacity to degrade amino acids depends to a considerable extent on the diet. A change in the intake of an amino acid is promptly, at least within a few hours accompanied by a parallel change in its oxidation rate (34, 35). Our study indicates that a period is necessary to adapt to the ^{13}C level of the study formula before a requirement study in infants can be performed, this adapts within 24 hours (**chapter 2**). Secondly, no differences were observed in fractional oxidation rates between day 2 and 4. However, and very interestingly, we did observe a significantly decrease in indicator oxidation rate after five days of adaptation. This coincided with a lower weight gain rate observed after a period of 6 days. These results can be interpreted in two ways. First, at least five days of adaptation to a study diet is necessary in IAAO studies determining the amino acid requirements in preterm infants. Secondly, the body adapts to a reduced essential amino acid intake to lower growth rate which again is reflected by a new equilibrium. In the new equilibrium, a lower requirement of essential amino acids is needed since e.g. growth is reduced. So a requirement determined under those circumstances would not reflect the optimal intake. If this theory is true, this would reflect the remarkable flexibility of the human body to adapt to changed circumstances.

In the minimally invasive protocol of Bross et al., subjects are adapted to a protein intake for 2 days followed by a study day adaptation to the test amino acid intake for 4 hours. Recently it was shown that adaptation to the study diet for 7 days, 3 days or 8 hours did not influence the oxidation of the indicator in healthy men (36) after a 2 day adaptation

to the protein intake. Duncan et al. showed no difference in lysine requirement in adults adapted to 2 different protein intakes (37). These studies indicate that 4 hours adaptation to the study intake should be sufficient to determine amino acid requirements using the IAAO method. The IAAO method is based on partitioning of the indicator amino acid in either protein synthesis or oxidation which occurs primarily at the acyl-t-RNA level. The adaptation needed does not relate to the urea pool which needs 7 days to achieve a equilibrium but does relate to the turnover of acylated t-RNA's, which adapts in less than four hours (38). Since the Michaelis-Menten constant (K_m) for various amino acid degrading enzymes are relatively high and not saturated under the physiological range of plasma or tissue amino acid concentrations, the oxidation will respond quite rapidly to a change in intracellular amino acid concentration (33). Whether amino acid requirement for growth and maintenance have already changed in these few hours is not known but it seems not likely. As mentioned above, the IAAO method is based on partitioning of the indicator amino acid in either protein synthesis or oxidation which occurs primarily at the acyl-t-RNA level which adapts in less than four hours. No conclusive evidence has been generated as how long an adaptation to a deficent diet should last. To solve this question the requirement of a test amino acid should be determined after 5 days adaptation to the study diet and compared to the requirement after 1 day adaptation to the study diet but this should be performed in the range of the requirement to avoid growth restrictions in these infants.

Sampling of air

To avoid unnecessary invasive handling, non-invasive methods have been incorporated into the stochastic model for the study of protein and amino acid metabolism. An oral or intra-gastric infusion of isotope was used in infants (39) and an urine analysis of isotope enrichment was used in infants to measure amino acid kinetics (40). An excellent correlation was found between plasma and urinary amino acid enrichment in both infants and adults (40, 41). The measurement of $^{13}CO_2$ in breath has shown to be a well method to determine the requirement without sampling the plasma pool in healthy adult males (37, 42). A minimally invasive protocol was developed which used oral tracers and determined isotopic enrichment in urine and breath. It was used to determine amino acid requirements in school-aged children and in parenterally fed neonates (46-48).

In **Chapter 4** we determined the lysine requirement in enterally fed infants. The requirement of lysine determined in air was identical to that determined in urine and plasma. We also showed that our short-period tracer protocol of 400 minutes is valid for determining amino acid requirements in enterally fed infants.

The use of an indicator amino acid to estimate protein synthesis and amino acid oxidation is the basic concept of the IAAO method. The IAAO is selected specifically because it has a carboxyl group that is irreversibly removed and excreted as carbon dioxide in

the earliest stage of degradation. The key concept behind the indicator amino acid method is the fact that due to the dietary change in the test amino acid from deficient to excess, the flux of the indicator does not change. The dietary concentration of the indicator remains constant with graded concentrations of the test amino acid, so there is no change in dilution of the tracer. In our lysine study, no correlation was found between urinary phenylalanine flux and lysine intake or plasma phenylalanine flux and lysine intake as was earlier shown in human adults (42). We thereby showed phenylalanine to be a good indicator in term infants. In the IAAO technique, oxidation of the indicator is inversely related to its uptake for protein synthesis, which in turn is determined by the concentration of the limiting amino acid. The effectiveness of all tracer studies depends upon an accurate assessment of the specific activity of the amino acid in the precursor pool. For phenylalanine, the specific activity of the liver free phenylalanine pool in pigs receiving phenylalanine in excess of their requirement, was not influenced by the supply of histidine, methionine, lysine and threonine in the diet (43, 44). The size of free phenylalanine pools in liver and plasma is closely regulated. Plasma phenylalanine showed the smallest change of the indispensable amino acids when piglets were fasted or fed protein-free diets. These data indicate that the size of the liver phenylalanine free amino acid pool is well controlled compared to other amino acids, thus making it a good choice as an amino acid indicator (45). Because phenylalanine hydroxylation occurs primarily in the cytosol of the hepatocytes, sampling the hepatic intracellular enrichments of amino acids might be more accurate in determining amino acid requirements than the plasma enrichments; plasma is not the true precursor pool from which protein synthesis takes place. In healthy young adults a well correlation was shown between $F^{13}CO_2$ and apo-B100, a hepatic export protein which is synthetized from intra-hepatocytic amino acids (46). Therefore, the use of $F^{13}CO_2$ circumvents the complex issue of measuring the true precursor pool enrichment in humans during routine studies where relative rates are being compared (47). Concluding, our results imply that in infants, sampling of $^{13}CO_2$ in expired air is sufficient to estimate amino acid requirements and our short protocol is valid to determine amino acid requirements.

Background adaptation and tracer washout time

Every diet differs in naturally enriched ^{13}C. The natural abundance of ^{13}C in nature is approximately 1.1% ^{13}C. Atmospheric abundance differs: European breath CO_2 has a different percentage of natural abundance ^{13}C than North American breath CO_2 (48). ^{13}C abundances of formula differ dependent on the constituents: for example the source of protein, the source of lipids and carbohydrates all vary in ^{13}C abundance (48). Therefore, a period is necessary to allow background adaptation to the experimental diet. Since we used European formulas which might be based on different sources of carbohydrate and protein than the Chinese formulas the infants received before the adaptation day,

we determined the time needed to reach a stable background enrichment in the first 8 patients of the valine requirement study (**chapter 6**). We found the time to obtain a stable background enrichment was two bottles of formula (when given every 3 hours) or 4.7 hours. These results are comparable to the study of Bross et al., who showed a stable background enrichment in breath between 225 and 255 minutes in adult humans fed hourly meals, and suggested that 5 hourly mails are required to achieve a constant $^{13}CO_2$ enrichment (49). This means that our protocol in which we adapt 24 hours to the study formula is sufficient to allow background enrichment to adapt to the new formula.
The possibility of isotope not clearing the body between two infusion studies and thus interfering with baseline isotope enrichment is an unwanted effect in tracer studies. Previous studies showed that the carry-over effect of the isotope did not affect the background enrichment after 2 days (50). Most studies used a latency of 2 days (51, 52) or 7 days (53, 54) between 2 measurements. We determined a tracer washout time of 7.5 hours (**chapter 2**). This makes it possible to measure the same patient on two consecutive study days, as we did in our adaptation study. The estimated body CO_2 production did not significantly differ between the different adaptation days; i.e. energy expenditure is remarkable stable over a period slightly less than a week.

AMINO ACID REQUIREMENTS IN TERM NEONATES

We will describe the requirement of the essential amino in term neonates 0 to 1 month of age determined by the different methods and will compare them with the mean requirement determined using the IAAO method. We compare these mean requirements with the amount of amino acids and in current formulas and the current standards for infant formula. An overview of all these studies is shown in Table 1 and 2 (pages 207 and 208).

Isoleucine requirement for infants 0 to 1 month of age

In 1964, Snyderman et al. (55) determined the isoleucine requirement in 6 healthy male infants to be between 79 and 126 $mg \cdot kg^{-1} \cdot d^{-1}$ based on the nitrogen balance method and weight gain.
The current recommended isoleucine requirement, based on human milk is 95 $mg \cdot kg^{-1} \cdot d^{-1}$ in infants aged 0 to 1 month (56). Using the factorial approach, Dewey et al. determined the isoleucine requirement in infants aged 0 to 1 month to be 59 $mg \cdot kg^{-1} \cdot d^{-1}$ (57). Using the IAAO method, we determined the mean isoleucine requirement to be 105 $mg \cdot kg^{-1} \cdot d^{-1}$ (**chapter 5**). Current formulas provide 97-194 $mg \cdot kg^{-1} \cdot d^{-1}$ isoleucine when an intake of 150 $mL \cdot kg^{-1} \cdot d^{-1}$ is given (58-61) (Table 1). Because we and Snyderman both used free L-amino acids in the study diet – rather than total proteins – the requirement might

be overestimated. A 20-35% higher first pass oxidation rate was seen in adults when an amino acid based diet was used compared to intact protein (67), so at least 20% of our mean requirement should be subtracted to correct for the amino acid based formula. The ESPGHAN advises an increase in protein content of infants formulas of 1.25 fold when the proteins used are based on hydrolyzed proteins in stead of intact proteins to correct for potentially less digestibility and biologic value of the nitrogen content (68). We therefore correct 25% for the use of amino acids in stead of intact proteins. Thereby, we determined a mean requirement and not a safe level of intake in which a correction is made for individual variance in requirement. A safe level of intake is calculated as the mean requirement plus 2 SD of this mean requirement. Since the distribution of the amino acid requirement is not known, we use the safe level of protein intake as proposed by the WHO (56) which is 125% of the average protein requirement. If we correct our mean requirement for these two factors an intake of 105 mg·kg^{-1}·d^{-1} should be the advised intake for isoleucine in infants aged 0 to 1 month. The ESPGHAN recommends an isoleucine intake of 92 mg ·100 kcal^{-1} in infant formulas (62) which resembles our advised isoleucine intake of 97 mg·100 kcal^{-1} based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2).

Valine requirement for infants 0 to 1 month of age

In 1959, Snyderman et al. determined the valine requirement in five neonates to be between 85-105 mg·kg^{-1}·d^{-1} using the nitrogen balance (63). Using the factorial approach, Dewey et al. determined the valine requirement in infants aged 0 to 1 month to be 72 mg·kg^{-1}·d^{-1} (57). The current recommended valine requirement, based on human milk is 95 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month (56). We determined the mean valine requirement in infants aged 0 to 1 month to be 110 mg·kg^{-1}·d^{-1} using the IAAO method (**chapter 6**). Current formulas provide an intake of 117-216 mg·kg^{-1}·d^{-1} valine when an intake of 150 mL kg^{-1}·d^{-1} is given (58-60) (Table 1). Since we used an amino acid based formula and because amino acids derived from intact casein are utilized in a higher proportion for protein synthesis than those from an equivalent intake of free amino acids (67), the true value of the requirement would be lower than our mean estimation. If we correct the mean requirement for the hydrolyzed proteins and correct for the safe protein intake which is 125% of the mean intake, an intake of 110 mg·kg^{-1}·d^{-1} should be the advised valine intake in infants aged 0 to 1 month. The ESPGHAN recommends an intake of 90 mg·100 kcal^{-1} (62) which is lower than our estimation of 102 mg·100 kcal^{-1} based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2).

Leucine requirement for infants 0 to 1 month of age

In 1961, Snyderman et al. determined the leucine requirement in 1 preterm and 5 term

infants to be 150 mg·kg^{1}·d^{-1} using the nitrogen balance and weight gain (range 79-226 mg·kg^{1}·d^{-1}) (64). By using a soy isolate formula Fomon et al. determined the leucine requirement in normal female infants to be no greater than 132 mg·100 kcal^{-1} (65), lower intakes were not given. The factorial approach estimated the requirement for term neonates 0 to 1 month of age to be 109 mg·kg^{1}·d^{-1} (57). We determined the mean leucine requirement in infants 0 to 1 month to be 140 mg·kg^{-1}·d^{-1} using the IAAO method (**chapter 7)**. The current recommended leucine requirement, based on human milk is 165 mg·kg^{-1}·d^{-1} in infants aged 0 to 1 month (56). Current formulas provide an intake of leucine of 195-345 mg·kg^{-1}·d^{-1} when an intake of 150 mL kg^{1}·d^{-1} is given (58-61) (Table 1). As discussed earlier, Metges et al. compared leucine oxidation rate, non-oxidative leucine disposal and net protein synthesis rate in adults that were fed an amino acid mixture and an intrinsically [1-^{13}C]leucine labelled casein (66). The oxidation rate was 22% higher, non-oxidative leucine disposal was 28% lower and the net protein synthesis rate was 35% lower in the amino acid mixture condition compared to the intrinsically labelled casein condition. Amino acid utilization might be lower from amino acid based or hydrolyzed formulas as also was shown in adults fed an elemental diet (67). The advised intake for leucine in infants aged 0 to 1 month based on the IAAO method corrected for hydrolyzed proteins and adapted for the safe level of protein is 140 mg·kg^{-1}·d^{-1}. The ESPGHAN recommends an intake of 169 mg · 100 · kcal^{-1} (62) which is higher than our estimation of 130 mg ·100 kcal^{-1}, which is based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2). This indicates that current formulas may provide 2-3 times too much leucine which might not be beneficial for the neonate. During feeding, leucine enhances the insulin sensitivity of protein synthesis, inducing a stimulation of muscle protein synthesis (68). Very high levels of leucine have the capacity to stimulate protein synthesis and inhibit protein degradation skeletal muscle in rats. This effect on protein synthesis may be enhanced by the transient but small increase in serum insulin that is induced by the insulin dose. A role for leucine as enhancer of insulin sensitivity also implies the possibility that prolonged very high intakes of leucine might lead to insulin resistance, in a similar way to insulin resistance resulting from prolonged hyperglycemia (68). A high protein intake in excess of metabolic requirements may enhance the secretion of insulin-like growth factor I (IGF-I) and insulin. High insulin and IGF-I levels can enhance growth during the first 2 years of life (69, 70) and adipogenic activity and adipocyte differentiation (71). More over, high protein intakes may also lead to decreased human growth hormone secretion and hence to reduced lipolysis. Epidemiological studies found that high protein intakes in early childhood, as opposed to high intakes of energy, fat or carbohydrates, was predictive of an early occurrence of the adiposity rebound and a high BMI in childhood corrected for parental BMI (72-75). Thus, the higher protein intake in infant formula feeding, as

compared to the protein supply in breastfed babies, may play a role in predisposing infants to an increased obesity risk later in life (the early protein hypothesis) (76).

Lysine requirement for the term infant

Only a few studies have been performed in infants to determine enteral lysine requirements. Snyderman et al. determined the mean requirement for lysine in enterally fed infants to be between 90 and 105 mg·kg^{-1}·d^{-1}(77). The factorial approach estimated the requirement for term neonates 0 to 1 month of age to be 116 mg·kg^{1}·d^{-1} (57). Current recommendated lysine intake based on human milk for infants 0 to 1 month is 119 mg·kg^{-1}·d^{-1} (78). We determined the mean lysine requirement in infants 0 to 1 month to be 130 mg·kg^{-1}·d^{-1} using the IAAO method (**chapter 4)**. Current formulas provide 158-244 mg·kg^{-1}·d^{-1} when an intake of 150 mL kg^{1}·d^{-1} is given (58-61). The advised intake for lysine in infants aged 0 month, based on the IAAO method, corrected for hydrolyzed proteins and adapted for the safe level of protein is 130 mg·kg^{-1}·d^{-1}. The ESPGHAN recommends an intake of 114 · 100 · kcal^{-1} (62) which is comparable with our estimation of 120 mg ·100 kcal^{-1} that is based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2). Because the main metabolic fate of lysine is protein synthesis, supplying sufficient intake of lysine is important in infants for growth. A deficient intake of lysine in infants limits protein synthesis and causes weight loss (77).

Methionine requirement for the term infant

Experimental evidence for the methionine requirement is scarce. Previous studies performed by Snyderman et al. determined a methionine requirement between 27 and 49 mg·kg^{-1}·d^{-1} (79). Fomon et al. studied the effects on growth, nitrogen balance and serum chemical values of soy isolated based formulas and concluded methionine to be the limiting nutrient in soy protein (65, 80, 81). Current recommendated methionine intake based on human milk for infants aged 0 to 1 month is 28 mg·kg^{-1}·d^{-1} (78). We determined the mean methionine requirement in infants aged 0 to 1 month to be 38 mg·kg^{-1}·d^{-1} using the IAAO method (**chapter 11)**. Current formulas provide 43-89 mg·kg^{-1}·d^{-1} when an intake of 150 mL kg^{1}·d^{-1} is given (58-61). The advised intake for methionine in infants 0 to 1 month of age, based on the IAAO method, corrected for hydrolyzed proteins and adapted for the safe level of protein is 38 mg·kg^{-1}·d^{-1}. The ESPGHAN recommends an intake of 24 mg · 100 · kcal^{-1} (62) which is lower than our estimation of 35 mg ·100 kcal^{-1} that is based on our study formula which contains 108 kcal when an intake of 150 mL kg^{-1}·d^{-1} is given (Table 2). This might be partially explained by the fact that our patients might be in a postinfectious state in which inflammation might deplete the liver glutathione pool, although cysteine was given in excess which should restore the glutathione pool (82). Methionine is known as the most toxic amino acid in animals when supplemented in excess (83, 84). Hypermethioninemia and homocysteinemia were observed in infants

who consumed a methionine-fortified formula or a high protein formula (85, 86). High levels of homocysteine are associated with stroke (87), increased levels of methionine can cause cerebral edema (85). We postulate that there is no need to supply intakes above our mean requirement of 35 mg ·100 kcal^{-1}.

OVERVIEW OF ALL STUDIES

Table 1: The requirement for the essential amino acids in mg·kg^{-1}·d^{-1} in term neonates 0 to 1 month of age determined by the different methods, and compared to the intake in term neonates when given 150 mL·kg^{-1}·d^{-1}. The last column discribes the new recommendations for infant formulas in mg·100 mL^{-1}

Requirement	Nitrogen balance* Infants 1-5 mo (36, 37, 66, 91)	Factorial Approach* 0-1 mo[60]	IAAO* Enterally fed infants (EAR)	Current Recomm* Infants (AI) 0-1 mo[59]	Current Formulas* per 150 mL·kg^{-1}·d^{-1} (61-64)	IAAO Recomm** (RDA)
Isoleucine	119 (79-126)	59	105	95	97- 194	**70**
Leucine	150 (76-229)	109	140	165	195- 345	**93**
Valine	105 (85-105)	72	110	95	117- 216	**73**
Lysine	103 (90-105)	116	130	119	158- 244	**87**
Methionine	45 (27-49)		38		43- 89	**25**
Sulfur AA		64		57		
Threonine	87 (45-87)	63		76	113- 173	
Phenylalanine	90 (63-91)				77- 143	
Aromatic AA		114		162		
Histidine	34 (17-35)	-		36	47- 85	
Tryptophan	22 (15-22)	22	15	29	28- 68	**10**
Ile:Leu:Val Ratio	**1 : 1.3 : 0.9**	**1 : 1.8 : 1.5**	**1 : 1.3 : 1**	**1 : 1.7 : 1**	**1 : 1.4 : 0.9- 1 : 2.3 : 1.2**	**1 : 1.3 : 1**

* mg·kg^{-1}·d^{-1}

** mg·100 mL^{-1}

Aromatic amino acids: phenyalanine and tyrosine

Sulfur amino acids: methionine and cysteine

RECOMMENDATIONS FOR INFANT FORMULAS AGED 0 TO 1 MONTH

Table 2: The current recommendations for infant nutrition in mg·100 kcal^{-1} compared to the amount in regular infant formulas. The third column describes the new recommendations based on the IAAO method.

	Current Recomm based on Human milk (AI) (mg·100 kcal^{-1}) [92] [65]	Current Term formulas (mg·100 kcal^{-1}) [63] [63, 66]	IAAO (mg·100 kcal^{-1}) (RDA)
Isoleucine	90-92	105-190	**97**
Leucine	166-169	167-338	**130**
Valine	88-90	106-213	**102**
Lysine	113-114	165-243	**120**
Methionine	23-24	43-89	**35**
Threonine	77	99-170	
Phenylalanine	81-83	71-140	
Histidine	40-41	45-85	
Tryptophan	32-33	22-67	**14**
Ile:Leu:Val Ratio	**1 : 1.7 : 1 - 1 : 1.8 : 1**	**1 : 1.4 : 0.9 - 1 : 2.3 : 1.2**	**1: 1.3 : 1**

The optimal BCAA ratio in infants.

Considerable interaction has been reported in humans and animals in response to disproportional intakes of BCAAs. In rats, imbalanced BCAA concentrations result in impaired growth, and BCAA supplementation has negative effects on fetal brain growth (91). The difficulty about studying the different branched-chain amino acids is the potential interaction between the BCAA; by changing the intake of a single test amino acid, the dietary mixture may become imbalanced (92). Therefore different groups determined the total BCAA requirement in stead of individual branched-chain amino acids. The BCAA pattern used in these studies is based on egg protein (which has a Ile:Leu:Val ratio of 1: 1.3 : 1.1 (93) to minimize the potential interaction of the BCAAs on assessment of the requirement. The optimal proportion for protein synthesis in children is assumed to be that present in egg protein (94, 95) and the total BCAA requirement was determined to be the same as in healthy men (96). The concentrations of the BCAA in human milk varies from 1 : 1.8 : 1.3 (97), 1 : 1.6 : 1 to 1 : 2.0 : 0.9 (62). Human milk protein contains a Ile:Leu:Val ratio of 1 : 1.74 : 1 (56). Current recommendations are based on this ratio that is based on the content of the branched-chain amino acids in human milk,

since exclusive breastfeeding by a healthy mother is the feeding standard from birth to 6 months in healthy, term infants (56). Different formulas use BCAAs in different ratios depending on the casein-whey ratio (milk-based formula: 1 : 1.6 : 1.1, whey-adapted formula: 1 : 2.3 : 1.1) (98).

Besides the differences between human milk protein and cow milk protein it is important to note that the amino acid composition of human milk is not the same as that of body protein, and Dewey et al. stated in 1996 that the composition of human milk proteins is not necessary a definition of the biological amino acid requirement of the growing neonate (57). Whole body protein contains a Ile:Leu:Val ratio of 1 : 2.1 : 1.4 (99). Milk protein contains a Ile:Leu:Val ratio of 1 : 1.6 : 1 (100), casein a Ile:Leu:Val ratio of 1 : 1.8 : 1.4 (58). We found an optimal Ile:Leu:Val ratio of 1 : 1.3 : 1 in neonates 0 to 1 month of age (**chapter 8**), which is comparable with the ratio determined by Snyderman using the nitrogen balance and weight gain (55, 63, 88, 89). Our results imply that, regarding the BCAA ratio, egg protein might be a better alternative protein than cow milk protein for infants aged 0 to 1 month. Since egg proteins contain more sulfur amino acids than milk proteins, the concentration of methionine and cysteine should be monitored carefully. Thereby, in parenteral fed infants, the use of egg protein as nitrogen source showed higher concentrations of phenylalanine and tyrosine compared to an alternative with an amino acid profile more similar to breast milk (101). In parenteral fed piglets, the optimal BCAA ratio has been shown to be 1: 1: 1 (102), i.e. concentrations of amino acids in parenterally fed infants cannot be compared to enterally fed infants. The other essential amino acid requirements should be determined in term neonates to see whether modified egg protein could be an alternative for infant nutrition.

WEST MEETS EAST/ EAST MEETS WEST

One of the main reasons to start a collaborative study project in Shanghai is the high amount of infants admitted at the Neonatal Intensive Care Unit (NICU) in the Fudan Children's Hospital. The admission rate is 3376 patients per year, 2096 males and 1280 females. Of these babies 2236 are term, 65 post-term and 1075 were preterm neonates. There is a difference in male to female admissions: the ratio of Shanghai residences is 1.36 to 1, the ratio from infants that come from other provinces is 1.8 to 1. This big difference is probably a result of the fact that ill or premature females will be left untreated more frequently; at first because parents are allowed to have only one child because of the one child policy, and secondly because of the high costs of the medical treatment; parents have limited or no medical insurance. Research is still in a developing state, medical ethical committees are being formed and at the NICU in the Fudan Children's Hospital there was no experience in asking informed consent of the parents. We describe the western view of the European researchers who lived and worked 1.5-3 years

in Shanghai in **chapter 9**, and the eastern/Chinese view of the collaborative researchers in **chapter 10**.

INFANT FORMULAS COMPOSITION

The difficulty in the development of infant formulas is the fact that the amino acid content of cow milk proteins differs from that of human milk. Both milks are composed of two classes of proteins, casein or acid-preciptable proteins and whey or acid-soluble proteins. The whey-casein ratio in colostrum is 80:20 and changes to 55:45 in mature milk (98). Casein dominant cow's milk formulas are made with nonfat dry milk and contain about 82% bovine casein and 18% bovine whey proteins. During the manufacturing process of infant formulas, whey is added to cow milk to obtain a whey-casein ratio of 60:40, which is more similar to human milk. However, human milk proteins differ from bovine proteins in concentrations of the proteins present, and in amino acid composition of these proteins. So adding bovine whey proteins does not make the formula identical to the amino acid composition of human milk. In casein-dominant formula, especially methionine and tyrosine are elevated. In whey-dominant formula, methionine, threonine and lysine are elevated. The sum of the BCAAs is much higher in formulas than human milk: infants fed formula have higher concentrations of BCAAs than human milk fed infants suggesting that levels of these amino acids are more closely related to protein quantity than protein quality (97).

In studies of protein quality, the assessment of the protein and amino acid compositions of various formula preparations is usually conducted by comparing them to human milk. This assessment must consider that cow milk formula contains more protein per volume than human milk and that there are differences in the composition of both whey and casein from the two species (103). Whey-dominant infant formula produces concentrations of plasma free amino acids that are more like those in infants fed pooled human milk than does casein-predominant formula (104). According to Polberger et al., the standard for protein quality should be such that reflects the plasma amino acid pattern of optimally growing LBW infants fed only human milk proteins (103, 105). One could argue whether other standards like amino acid composition of human milk or plasma amino acid levels of healthy breast-fed term neonates should be the standard and that functional outcome measurements like neurodevelopmental outcome and development of metabolic syndrome should be taken more into account.

The nutritional implications of the differences in amino acid content of different proteins or mixtures of proteins can be evaluated by comparing the amino acid composition of the protein source with a suitable reference amino acid pattern by use of an amino acid scoring pattern. These scoring systems use the amino acid requirement in humans to

develop reference amino acid patterns for purposes of evaluating the quality of food proteins or their capacity to efficiently meet both the nitrogen and indispensable amino acid requirement of the individual (100). The scoring systems use the limiting essential amino acid in the test protein, divide it by the amount of amino acid in a reference protein and correct it for true digestibility. The indispensable amino acid composition of the specific protein source is compared to that of that of a reference amino acid composition profile. Earlier the amino acid composition of a good protein such as egg was used, which is regarded as being well balanced in amino acid content in relation to human needs (106). Later the amino acid content of human milk was used as reference pattern (107,108) since adequate growth and development are known to occur in infants provided human milk, and plasma amino acid profiles of infants have been shown to reflect the amino acid composition of human milk. The LSRO report concluded that the amino acid scoring pattern of human milk is an accurate and an appropriate standard for assessing the protein quality of infant formulas (109). The difficulty in composing infant nutrition is that even if the amino acid composition of infant formula could be made very similar to that of human milk, digestibility and absorption of amino acids and peptides could be quite different from that of breast milk, thus resulting in different plasma amino acid profiles. Hypothetically, one could develop an infant formula based on egg protein and determine the plasma amino acid profile in infants fed such a formula to see if the plasma aminogram is more similar to that of breast-fed infants than normal formulas provide.

Concluding, we speculate that current formulas provide too much protein and do not contain the optimal amino acid composition. We postulate that formulas may contain lower amounts of protein if the quality of the milk protein can be modified. The requirement of histidine and phenylalanine are currently determined in term infants. If all 9 essential amino acids are studied in term infants, new recommendations can be made for term infant formula. The effect on growth and plasma aminogram of alternative proteins like egg protein could be studied in pigs. Optimizing the amino acid composition might result in a decrease in protein intake in formula fed infants which might decrease the risk of the metabolic syndrome later in life.

FUTURE PERSPECTIVES

Most currently available infant formulas provide intakes of protein that markedly exceed the requirement and exceed the protein intakes from human milk in breast-fed infants. For example, the measured daily protein intake in infants aged 0 to 1 month is 1.7 – 2.09 $g \cdot kg^{-1} \cdot d^{-1}$ which declines to 0.9 – 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ at 5-6 months (97, 110). Term

neonates fed a formula with a protein content of 1.6 g ·dL^{-1} received a protein intake of 2.5 g·kg^{-1}·d^{-1} at 12 weeks (111) which is much higher than the intake of a breastfed infant at 3 months of age (0.9 – 1.2 g·kg^{-1}·d^{-1}) (97, 110) . The amino acid composition of the nutritionally available proteins from human milk differs from that found of bovine proteins (97). This results in different plasma amino acid profiles in formula fed infants compared to breastfed infants. For example, the concentrations of threonine, valine and total branched-chain amino acids are significantly higher in formula fed children fed a whey-dominant formula than in breastfed infants at 3 months of age (112) . At 6 months of age, children fed a casein-dominant formula which contained 2.7 g ·dL^{-1} of protein, the concentrations of phenylalanine, methionine, leucine, valine and proline and isoleucine were more than 2 fold the values found in breastfed infants (113). Because high levels of BCAA interfere with the transport of tryptophan (a serotonine precursor) and other large neutral amino acids (tyrosine) across the blood brain barrier, they influence central nervous system concentrations of neurotransmitters (114, 115). Current formulas might not contain the optimal amino acid composition and may provide too much protein which might be detrimental for the neonate.

Regarding the decrease in protein intake in human milk in the first months of life, one could question the use of one formula for infants 0 to 6 month of age. The largest difference in growth in a breastfed infant compared to a formula fed infant are observed between 3 and 6 months of life (27). The benefits of breastmilk for long term obesity might be due to a slower pattern of growth in breastfed infants compared to formula-fed infants, i.e. the growth acceleration hypothesis (116). This proposes that faster postnatal growth programmes components of the metabolic syndrome including insulin resistance, higher low-density lipoprotein cholesterol concentration, higher blood pressure and obesity (116). Most infant formulas have a slightly higher energy density than typical human milk, and energy intakes per kilogram of body weight in formula-fed infants aged 3-12 mo were reported to be 10-18% higher than those of breastfed infants (117). Even much larger is the difference in protein supply per kilogram of body weight, which is 55-80% higher in formula fed infants than breastfed infants (118). Protein requirements of infants 3-4 month of age are more similar to those of older infants than to those of infants less than 3 mo of age (110). The use of one high protein formula for 0-6 months is questionable. A formula for infants 3-6 months could be developed which might contain less proteins. Infants with a lower protein intake will use more of the protein for growth regardless of feeding mode (117). In the DARLING study, a formula with a reduced protein content normalized the weight gain during the first 24 months of age, making it more similar to breast-fed children (117).

Basing values for desirable amino acid intakes of preterm infants on values from the composition of preterm human milk is unadvisable. First, the absorption of amino acids present in human milk may be incomplete because of non-uniform hydrolysis of various

proteins by the preterm infant. Secondly, the total amino acid content of preterm milk is limiting for preterm infants after the first 2 weeks of life (103). For preterm infants, current recommendations are based on the factorial approach. A minimum and maximum protein intake is multiplied by the amount present in 1g of human milk protein (103). As discussed in **chapter 5**, the factorial approach underestimates the amino acid requirements in term infants. Optimizing early nutrition has been shown to effect neurodevelopmental outcome in preterm infants (15). Determining the requirements in this group is of high priority, especially because catch-up growth is associated with the development of metabolic syndrome later in life (20, 116). Based on their studies, Lucas et al. concluded that the first 2 weeks of life in the preterm infant may represent a critical growth window during which nutrition may have its greatest beneficial and adverse effects (15). To optimize infant nutrition in the first month of life, we recently started a study in the High Care Centers of the Erasmus MC-Sophia to determine the threonine requirement in preterm infants (**chapter 3**). This study will be the first to be conducted in a research program that will define the requirement of all 9 essential amino acids in preterm infants. These can be compared to the current recommendations based on the factorial approach and compared to what current preterm formulas provide. New recommendations can be made for preterm infant formulas like this thesis provides for term infant formulas.

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CHAPTER

13

Summary of the thesis

English Summary

Growth during the earliest stages of life is an important determinant of an individual's later health and risk of chronic disease. Substantial evidence shows that growth in the first 2 years of life, especially high early weight gain, is associated with adverse health outcomes later in life, including increased blood pressure, increased weight gain and body fat deposition, and increased risk of diabetes. Higher protein intake for infants who are formula fed may play a role in these health outcomes because formula-fed children reach a higher body weight and weight for length at one year of age compared to those fed breast milk, resulting in a higher body mass index (BMI). In preterm infants, however, a higher protein intake in the first month of life correlates with improved neurodevelopment. The goal in feeding preterm neonates is to provide the quantity and quality of protein needed to achieve foetal rates of tissue growth and nitrogen accretion. This goal should be accomplished in the context of physiological and metabolic development of the infant in order to avoid accumulation of potentially harmful protein metabolic products.

Because intakes of breast milk from a healthy well-nourished mother are considered to satisfy protein requirements for the first six months of life, current recommendations from the World Health Organization are based on the breast milk contents. Breastfed infants, however, have a variable milk consumption rate, which makes it difficult to provide an accurate estimation of the amino acid intake. Consequently, other methods were needed to validate these requirements. Over fifty-five years ago, Snyderman and colleagues have determined individual amino requirements of infants by means of the nitrogen balance method and weight gain. A limitation of this method is the need for a 7 day adaptation time to the diet. Thereby they studied only 5-6 infants per amino acid. More recently, the indicator amino acid method (IAAO) was developed. Isotopically labelled tracers are used to determine the metabolic rate of an amino acid at varying dietary intakes. In adults it was demonstrated using isotopic labelled amino acids that the nitrogen balance method underestimated the requirements of adults by 2 to 3 fold. No studies have been performed using stable isotope techniques to determine essential amino acid requirements for enterally fed neonates.

The major findings presented in this thesis will be discussed next. The thesis is divided into 6 parts. Part I describes the introduction and aims of the study. Part II describes the studies performed to validate our study protocol and the study design of the study recently started in the Netherlands. Part III describes the requirement of the branched-chain amino acids in term neonates. These are 3 amino acids that are similar in structure and share common degrading steps in metabolism. Part IV is a western versus eastern

view regarding the Sino-Dutch research program. Part V describes the requirement of another essential amino acid, methionine, in infants aged 0 to 1 month. Part VI provides the general discussion and reviews the requirements determined by the different methods. These are compared with the mean requirement determined in our studies using the indicator amino acid method. We compare these values with the current recommendations for infant formulas and the amount of amino acids in these formulas. Finally the new recommendations will be discussed for infant formulas for infants aged 0 to 1 month and what will be the future perspectives for further research.

In **Chapter 2** we determined whether the period of adaptation to a study diet with decreased intake of leucine has effect on the oxidation of the indicator amino acid [1-^{13}C]phenylalanine to F$^{13}CO_2$. To avoid accumulation of the tracer we determined the minimal time needed to perform the next tracer study by determining the tracer washout time. Our study indicates that a period up to 24 h is necessary to adapt to the ^{13}C level of the study formula. Furthermore, if adaptation to a specific deficient diet has occurred, a tracer study as described here can be performed at daily intervals. Regarding the time needed to adapt to the study formula, no difference was found in metabolism between day 2 and day 4. At day 6 significant changes were found in F$^{13}CO_2$, probably as a result of a protein sparing adaptive response. No conclusive evidence has been generated as how long an adaptation period on a deficient diet should last. To solve this question a study should be performed to compare the requirement of an essential amino acid after 1 and 5 days adaptation to the study diet but this should be performed within the range of the amino acid requirement to avoid growth restrictions in these infants.

In **Chapter 3** we describe the study protocol of the study we recently started in the High Care Centers of the Erasmus MC-Sophia in the Netherlands. We will determine the threonine requirement in preterm infants using the IAAO method. This study will be the first to be conducted in a research program a research program that will define the requirement of all 9 essential amino acids in preterm infants. These can be compared to the current recommendations based on the factorial approach and compared to what current preterm formulas provide. New recommendations can be made for preterm infant formulas like this thesis provides for term infant formulas.

In **Chapter 4** we showed that our short-period tracer protocol of 400 min is valid for determining amino acid requirements in enterally fed infants. The requirement of lysine in air was identical to that determined in urine and plasma. This implies that in both infants and adults, sampling of $^{13}CO_2$ in expired air is sufficient to estimate amino acid requirements. No correlation was found between urinary phenylalanine flux and lysine

intake or plasma phenylalanine flux and lysine intake as was earlier shown in human adults. We thereby showed phenylalanine to be a good indicator in term infants.
The requirement for lysine in term neonates determined by the IAAO method (130 $mg \cdot kg^{-1} \cdot d^{-1}$) corresponded well with the current recommendation based on human milk (119 $mg \cdot kg^{-1} \cdot d^{-1}$).

Part III: The branched-chain amino acid requirement in term neonates

In **Chapter 5** we determined the isoleucine requirement in term neonates 0 to 1 month of age. The mean requirement determined by the IAAO method (105 $mg \cdot kg^{-1} \cdot d^{-1}$) corresponded well with the current recommended isoleucine requirement, based on human milk (95 $mg \cdot kg^{-1} \cdot d^{-1}$). This was also the case for the requirement for valine that was determined in **chapter 6** (IAAO method: 110 $mg \cdot kg^{-1} \cdot d^{-1}$, current recommendation: 95 $mg \cdot kg^{-1} \cdot d^{-1}$). This value was also similar to the mean requirement determined by the nitrogen balance method in the 1950s by Snyderman et al. (105 $mg \cdot kg^{-1} \cdot d^{-1}$). In **chapter 7** we determined the requirement for leucine using the IAAO method. This was found to be lower than current recommendation (IAAO method: 140 $mg \cdot kg^{-1} \cdot d^{-1}$, current recommendation: 165 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively) but corresponded well with the nitrogen balance method (150 $mg \cdot kg^{-1} \cdot d^{-1}$). This indicates that current formulas may provide 2-3 times too much leucine which might not be beneficial for the neonate. Prolonged very high intakes of leucine might lead to insulin resistance, in a similar way to insulin resistance resulting from prolonged hyperglycemia. A high protein intake in excess of metabolic requirements may enhance the secretion of insulin-like growth factor I (IGF-I) and insulin. High insulin and IGF-I levels can enhance growth during the first 2 years of life and adipogenic activity and adipocyte differentiation. More over, high protein intakes may also lead to decreased human growth hormone secretion and hence to reduced lipolysis. Epidemiological studies found that high protein intakes in early childhood, as opposed to high intakes of energy, fat or carbohydrates, was predictive of an early occurrence of the adiposity rebound and a high BMI in childhood corrected for parental BMI. Thus, the higher protein intake in infant formula feeding, as compared to the protein supply in breastfed babies, may play a role in predisposing infants to an increased obesity risk later in life (the early protein hypothesis).
Different formulas use BCAAs in different ratios depending on the casein-whey ratio. We determined an optimal isoleucine-leucine-valine (Ile:Leu:Val) ratio of 1 : 1.3 : 1 in neonates 0 to 1 month of age (**chapter 8)** which is comparable with the ratio determined by the group of Snyderman using the nitrogen balance method and weight gain. Our results imply that egg protein might be a better alternative protein regarding the BCAA ratio for infants aged 0 to 1 month of than cow milk protein. Since egg proteins contain more sulfur amino acids than milk proteins, the concentration of methionine and cysteine should be monitored carefully. The other essential amino acid requirements should

be determined in term neonates to see whether egg protein could be an alternative for infant nutrition.

Part IV: West meets east/ East meets West
One of the main reasons to start a collaborative study project in Shanghai is the high amount of infants admitted at the NICU in the Fudan Children's Hospital. The admission rate during our studies in 2008 was 3376 patients per year, nowadays 4500 patients per year. Research is still in a developing state, medical ethical committees are being formed and at the NICU there was no experience in asking informed consent of the parents. We describe the western view of the Dutch researchers who lived and worked respectively 1.5 and 3 years in Shanghai in **chapter 9**, and the eastern view of the collaborative researchers in **chapter 10**.

Part V
In **Chapter 11** we determined the mean methionine requirement in infants 0 to 1 month of age to be 38 $mg \cdot kg^{-1} \cdot d^{-1}$ using the IAAO method (**chapter 11)** which is higher than current recommendations (25 $mg \cdot kg^{-1} \cdot d^{-1}$). Some of the current formulas, however, provide almost twice the recommended intake. This could be not beneficial for the neonate because methionine is known as the most toxic amino acid in animals when supplemented in excess.

Part VI:
In the general discussion, **Chapter 12,** the requirements determined by the different methods are compared with the requirements determined by our studies using the IAAO method. We compare these mean requirements with the amount of amino acids in current formulas. These results are summarized in Table 1 and Table 2 (pages 207 and 208). The current recommendations based on human milk seem to be correct for most of the essential amino acids. We speculate that current formulas provide too much protein and do not contain the optimal amino acid composition. We postulate that formulas may contain lower amounts of protein if the quality of the milk protein can be modified. The requirement of threonine, histidine and phenylalanine are currently determined in term infants. If all 9 essential amino acids are studied in term infants, new recommendations can be made for term infant formula. The use of egg protein as alternative protein for infant nutrition has to be studied. Optimizing the amino acid composition might result in a decrease in protein intake in formula fed infants which might decrease the risk of metabolic syndrome later in life.

CHAPTER

14

Nederlandse samenvatting voor niet-medici

Nederlandse samenvatting

Voeding speelt een belangrijke rol bij pasgeborenen. Te vroeg geboren (preterme) kinderen hebben een betere neurologische ontwikkeling als ze meer eiwitten in hun voeding krijgen in de eerste levensweken. Adequate groei is hierbij van groot belang; het doel is de kinderen volgens dezelfde groeicurve te laten groeien als ze in de baarmoeder zouden doen. De keerzijde hiervan is dat er een verband bestaat tussen inhaalgroei en de ontwikkeling van het metabool syndroom op latere leeftijd; hieronder vallen een hoge bloeddruk, suikerziekte, overgewicht en een verhoogd cholesterol.
Bij op tijd geboren (à terme) pasgeborene lijkt er een verschil in groei te bestaan afhankelijk van de soort voeding. Flesgevoede kinderen zijn op de leeftijd van 1 jaar zwaarder maar niet per se langer dan borstgevoede kinderen. Dit veroorzaakt een hogere body mass index (BMI) wat een risicofactor is voor het ontwikkelen van het metabool syndroom. De oorzaak van dit verschil in groei tussen flesgevoede kinderen en borstgevoede kinderen zou kunnen liggen in het feit dat flesvoeding meer eiwitten bevat dan borstvoeding. De huidige aanbevelingen voor à terme kinderen zijn gebaseerd op de hoeveelheid eiwitten in borstvoeding. Hierbij is de samenstelling van de aminozuren in borstvoeding als leidraad genomen; dit zijn de bouwstenen waaruit eiwitten zijn opgebouwd. Of deze aanname correct is, is nooit onderzocht. De enige studies naar de behoefte aan aminozuren van pasgeborenen (neonaten) dateren uit 1950-1965. Ze zijn gedaan door de groep van Snyderman in 5 tot 6 kinderen per aminozuur. Er werd gekeken naar groei en stikstofbalans bij verschillende hoeveelheden aminozuren in de voeding. Het nadeel van deze methode is dat de kinderen 7 dagen op een bepaald dieet moesten staan om een effect te kunnen meten. Recent is er een nieuwe methode ontwikkeld om naar de behoefte aan aminozuren te kijken door het meten van de stofwisseling. Dit wordt gedaan door de kinderen verschillende hoeveelheden aminozuren in een voeding te geven met "een vlaggetje" eraan, waarvan het effect op de stofwisseling gemeten kan worden in uitademingslucht. Deze methode heet de indirecte aminozuur oxidatie methode, afgekort IAAO methode. Met deze methode kan je de behoefte aan essentiële aminozuren meten, dit zijn aminozuren die het lichaam zelf niet kan maken en dus in het dieet moeten zitten.

Het proefschrift is opgebouwd uit 6 delen. Deel I is de inleiding met het doel van het onderzoek. Deel II beschrijft de onderbouwing van de methode bij à terme en preterme neonaten. Deel III beschrijft de behoefte aan vertakte keten aminozuren in de eerste levensmaand van à terme neonaten. Dit zijn 3 essentiële aminozuren die vergelijkbaar zijn in structuur en een gezamenlijk omzettingssysteem hebben. Deel IV is een West versus Oost uiteenzetting: het onderzoek is verricht als onderdeel van een Chinees-Nederlands

samenwerkingsproject en beide partijen geven hun visie op deze samenwerking. Deel V is de beschrijving van de behoefte aan een ander essentiëel aminozuur, methionine. In deel VI wordt het onderzoek samengevat in de discussie en worden er nieuwe aanbevelingen gedaan voor voeding voor à terme neonaten in de eerste levensmaand. Hieronder zullen we kort bespreken wat de belangrijkste uitkomsten waren van onze studies.

In **Hoofdstuk 2** hebben we gekeken naar het aanpassen aan het studiedieet bij preterme neonaten. Deze kinderen kregen 6 dagen een voeding met een lagere hoeveelheid leucine en op dag 2, dag 4 en dag 6 keken we naar de stofwisseling met behulp van de IAAO methode. We zagen dat het lichaam tijd nodig heeft om te wennen aan het dieet zelf, dit gebeurt binnen 24 uur. Als het lichaam eenmaal aan de dieetvoeding is gewend kan je dagelijks een meting doen aangezien de isotopen na gemiddeld 7.5 uur uit het lichaam verdwenen zijn. Daarbij was er geen verschil in stofwisseling tussen dag 2 en dag 4. Echter op dag 6 was er een lagere stofwisseling vergeleken met dag 2 en dag 4. Dit is waarschijnlijk het gevolg van het feit dat het lichaam in een nieuw evenwicht komt om zoveel mogelijk eiwitten te sparen. Er is geen duidelijke conclusie te trekken over hoe lang het lichaam van een neonaat zich moet aanpassen aan het dieet in de IAAO methode. Dit zou je kunnen onderzoeken door de behoefte aan een aminozuur op dag 2 te vergelijken met dag 6, hierbij moet de groei wel gewaarborgd blijven door de intake rondom de behoefte te houden.
Hoofdstuk 3 beschrijft de studie die we recent zijn gestart in de High Care Centers van het Erasmus MC-Sophia naar de threonine behoefte bij te vroeg geborenen. Dit is de eerste van de 9 essentiële aminozuren die onderzocht gaat worden. Het doel is om, net als in deze dissertatie gedaan is voor à terme pasgeborenen, nieuwe aanbevelingen te maken voor voeding voor de preterme neonaten.
In **Hoofdstuk 4** beschrijven we de studie bij à terme neonaten naar de behoefte aan lysine gemeten in uitademingslucht versus urine versus bloed. We zagen dat de behoefte aan lysine die je op de verschillende manieren meet niet verschilt. Het meten van alleen uitademingslucht is dus voldoende voor het bepalen van de behoefte aan een aminozuur. Het protocol van 400 minuten geeft hetzelfde resultaat als het 960 minuten protocol. De gebruikte tracer is een goede indicator gebleken bij à terme neonaten. De hoeveelheid lysine die een à terme pasgeborene nodig heeft volgens de IAAO methode (130 $mg \cdot kg^{-1} \cdot d^{-1}$) komt goed overeen met de huidige aanbeveling gebaseerd op borstvoeding (119 $mg \cdot kg^{-1} \cdot d^{-1}$).

In **Hoofdstuk 5** bepalen we de behoefte aan isoleucine in à terme neonaten. De waarde die we vinden met de IAAO methode (105 $mg \cdot kg^{-1} \cdot d^{-1}$) komt goed overeen met de huidige aanbevelingen gebaseerd op borstvoeding (95 $mg \cdot kg^{-1} \cdot d^{-1}$). Dit geldt ook voor de behoefte aan valine in de eerste levensmaand (IAAO methode: 110 $mg \cdot kg^{-1} \cdot d^{-1}$, huidige

aanbevelingen 95 mg·kg^{-1}·d^{-1}) zoals wordt beschreven in **Hoofdstuk 6**. Deze waarde komt ook goed overeen met de stikstofbalans studies van Snyderman zo'n 60 jaar geleden (105 mg·kg^{-1}·d^{-1}). Echter, de behoefte aan leucine bepaald met de IAAO methode (140 mg·kg^{-1}·d^{-1}) is lager dan de huidige aanbevelingen (165 mg·kg^{-1}·d^{-1}) maar is wel vergelijkbaar met de stikstofbalans methode (150 mg·kg^{-1}·d^{-1}) (**Hoofdstuk 7)**. Huidige voedingen voor kinderen van 0-1 maand bevatten mogelijk 2-3 maal te veel leucine. Een langdurig hoge leucine intake kan resulteren in een hoge insuline spiegel in het bloed. Bij langer bestaande hoge insuline spiegels kan de gevoeligheid voor insuline in de weefsels afnemen. Hypothetisch zou de hoge leucine intake negatieve effecten kunnen hebben bij de pasgeborene. Het zou een rol kunnen spelen bij het onstaan van insuline ongevoeligheid wat later in het leven een kans op suikerziekte zou kunnen geven. In **Hoofdstuk 8** wordt een overzicht gegeven van de verhouding tussen de vertakte keten aminozuren en wordt de optimale verhouding van deze aminozuren besproken. Op basis van de IAAO methode zou een vertakte keten aminozuurverhouding met minder leucine ten opzichte van isoleucine en valine, beter kunnen zijn voor pasgeborenen (isoleucine-leucine-valine (Ile:Leu:Val) verhouding: 1: 1.3 : 1 in plaats van 1: 1.7: 1 in de huidige aanbevelingen). De stikstofbalans methode vond zestig jaar geleden ook een verhouding van 1: 1.3 : 1. De Ile:Leu:Val verhouding gevonden in kippeneiwit komt dichter bij deze optimale verhouding voor een pasgeborene dan die in koemelkeiwit. Indien de behoefte van alle 9 essentiële aminozuren bepaald is, kan er gekeken worden of kippeneiwit als alternatief voor babyvoeding overwogen kan worden.

Een van de redenen om de Chinees-Nederlandse samenwerking te starten is het feit dat er in China veel meer pasgeborenen zijn opgenomen op de Intensive Care Neonatologie dan in Nederland: ten tijde van het onderzoek in 2008 werden er bijna 3400 kinderen opgenomen; in 2011 was dit bijna 4500. Aangezien China nog in ontwikkeling is op het gebied van informed consent en medisch ethische regelgeving is er voor beide partijen veel te leren en te doen. We beschrijven de westerse en oosterse visie op het samenwerkingsverband en bespreken de ervaringen van respectievelijk 1.5 jaar en 3 jaar Shanghai in **Hoofdstuk 9** en **Hoofdstuk 10**: West meets East, East meets West.

In **Hoofdstuk 11** bespreken we de behoefte aan methionine bij à terme neonaten. De behoefte aan methionine gevonden door middel van de IAAO methode (38 mg·kg^{-1}·d^{-1}) is hoger dan de huidige aanbevelingen gebaseerd op borstvoeding (25 mg·kg^{-1}·d^{-1}). Echter, sommige voedingen voor kinderen van 0-1 maand bevatten toch 2 maal de aanbevolen hoeveelheid methionine. De hoeveelheid methionine zou beperkt moeten worden gezien het feit dat in dierstudies methionine een van de meest toxische aminozuren is gebleken indien het in te hoge hoeveelheden gegeven wordt.

Hoofdstuk 12 geeft een overzicht van de belangrijkste onderwerpen en conclusies van deze dissertatie. De belangrijkste conclusies zijn dat de huidige aanbevelingen gebaseerd op borstvoeding grotendeels lijken te kloppen. Sommige voedingen blijken te veel aminozuren te bevatten en niet de optimale verhouding van deze aminozuren. De rol van kippeneiwit als alternatief voor babyvoeding zal onderzocht moeten worden. Hypothetisch zou, indien de verhoudingen aan aminozuren beter op elkaar afgestemd worden, er minder eiwit in deze voedingen nodig zijn. Dit zou gunstig kunnen zijn voor de pasgeborene, aangezien de hogere eiwitinname van flesgevoede kinderen kan leiden tot het ontwikkelen van het metabool syndroom later in het leven. Tabel 1 (bladzijde 207) geeft een overzicht van alle methoden en de huidige aanbevelingen voor neonaten en vergelijkt deze met de behoeften aan de verschillende aminozuren die wij gevonden hebben met de IAAO methode. Tabel 2 (bladzijde 208) geeft de nieuwe aanbevelingen voor babyvoeding voor à terme neonaten van 0-1 maand oud. Afsluitend worden er aanbevelingen gedaan voor toekomstig onderzoek, zoals het bepalen van de behoefte aan de essentiële aminozuren van preterme neonaten met als doel om ook nieuwe aanbevelingen te maken voor deze groep kinderen.

"Bij een goed einde hoort een goed begin"
Confusius, 孔夫子, (551-479 BC)

Dankwoord

List of publications

List of co-authors

List of abbreviations

Curriculum Vitae

PhD Portfolio

Dankwoord

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List of abbreviations

AI	adequate intake
APE	atom percent excess
BCAA	branched-chain amino acid
BCAT	branched-chain aminotransferase
BCKAs	branched-chain keto-acids
BCKAD	branched-chain keto-acid dehydrogenase
BMI	body mass index
BUN	blood urea nitrogen
BW	birth weight
CI	confidence interval
DAAO	direct amino acid oxidation
24-h DAAB	24h direct amino acid balance
DRI	Dietary Reference Intake
EAR	estimated average requirement
ELBWI	extremely low birthweight infants
GA	gestational age
IAAO	indicator amino acid oxidation
IAAB	indicator amino acid balance
IGF-I	insulin-like growth factor I
KIC	ketoisocaproate
KIV	ketoisovalerate
KMV	keto-β-methylvalerate
Ile : Leu : Val ratio	isoleucine-leucine-valine ratio
LNAA	large neutral amino acids
MDI	mental developmental index
MPE	mole percent excess
NICU	Neonatal Intensive Care Unit
PDB	pee dee belemnite
RDA	recommended dietary allowance
STDH	serine/threonine dehydratase
TCA	tricarboxylic acid cycle
TDH	threonine dehydrogenase
TORCH	Toxoplasmosis, Other agents, Rubella, Cytomegalovirus, Herpes virus
UL	tolerable upper intake level
WHO	World Health Organization
WMA	World Medical Associaton

Curriculum Vitae

Femke Maingay-de Groof was born on December 20th, 1975 in Eindhoven, the Netherlands. She received secondary education at the St. Joriscollege in Eindhoven and completed this in 1994. She started her medical training in 1994 at the Erasmus University in Rotterdam. In 1999 she participated in a project at the Pediatric Intensive Care Unit of the Erasmus MC- Sophia, Rotterdam and determined the Growth Hormone/ IGF-I axis in children with a meningococcal septic shock, supervised by Dr. K.F.M. Joosten and Prof. dr. A.C.S. Hokken-Koelega. After obtaining her medical degree in 2001, she worked as a pediatric resident (ANIOS) in the Albert Schweitzer Hospital in Dordrecht and the Maasstad Hospital in Rotterdam. She started as a pediatric resident (AIOS) in 2003 under supervision of Prof. dr. A.J. van der Heijden, Dr. M. de Hoog and Prof. dr. A.M. Oudesluys-Murphy. In the last year of her training (2008) she moved to Shanghai for 1.5 years to start up the research project "Essential amino acid requirement in term and preterm neonates" at the Fudan University Children's Hospital in Shanghai, China together with Lisha Huang, under supervision of Prof. dr. J.B. van Goudoever, Prof.dr. Y. Huang and Prof. dr. C. Chen. She started as a fellow Neonatology in 2009 under supervision of Prof. dr. J.B. van Goudoever and finished her fellowship last May 2012 under supervision of Prof. dr. I.K.M. Reiss. In this fellowship she started the research project in the High Care centers of the Sophia Children's Hospital for the "Threonine requirement in preterm infants" and after finishing her fellowship she worked on her dissertation " The Branched-Chain Amino Acid Requirement in Neonates". In August 2012 she started as a pediatrician-neonatologist in the Medisch Centrum Alkmaar. Femke is married to Gijsbert Wouter Maingay and they have 2 daughters Ella Josephine and Fleur Susanne.

PhD portfolio

Summary of PhD training and teaching

Name PhD student: Femke Maingay-de Groof

Erasmus MC department: Neonatal Intensive Care Unit

Research school: Erasmus MC

PhD period: 2008-2012

Promotor: Prof.dr. J.B. van Goudoever

1. PhD training		
	Year	Workload (hrs)
General courses		
Basiscursus Regelgeving Klinisch Onderzoek, Erasmus MC	2012	20
Biostatistics for Clinicians, NIHES, Erasmus MC	2012	20
Specific Courses		
ESN Course, Neonatal Respiration, Hamburg	2009	6
ESN Course , Neonatal Neurology, Hamburg	2009	6
Neonatal Update, Imperial College, London	2009	20
ESN Course, Resuscitation and Stabilization of Preterm Infants, Kopenhagen	2010	6
ESN Course, Neonatal Gastroenterology and Nutrition, Kopenhagen	2009	6
Ipokrates, Neonatal Hemodynamics, Bratislava, Slovakia	2011	12.5
ESN Course, Neonatal Infection, New Castle	2011	6
ESN Course, Neonatal Circulation, New Castle	2011	6
Newborn Life Support, Dutch foundation for Emergency Medical Care for Children, Riel	2011	6
Seminars and workshops		
Hands On Course Pediatric Echocardiography, Great Ormond Street Hospital, London	2011	12.5
Dutch Neonatal Fellow Day, Groningen	2011	7
Research Bespreking Moeder en Kind Centrum, Erasmus MC Rotterdam	2010-2012	10
Fellowdagen neonatologie	2010-2011	23
Interklinische en refereeravonden Erasmus MC	2011-2012	12
International Conferences		
Young Investigator Meeting, Sanya, China	2009	12.5
European Academy of Paediatric Societies, Kopenhagen	2010	20
European Society for Paediatric Gastroenterology, Hepatology and Nutrition, Sorrento	2011	20
Oral presentations		
Leucine Requirement in the Enteral Fed Asian neonate,Young Investigator Meeting, Sanya, China	2009	30
New insights in the methodological issues of the IAAO method in preterm neonates, Fudan University, Shanghai, China	2009	30
BCAA requirement in enterally fed term infants: Basis Isotopen Congres, Arnhem	2010	30
East meets West, Grand Round, Erasmus MC Sophia, Rotterdam	2010	60
BCAA requirement in term neonates, EAPS Kopenhagen	2010	60
BCAA behoefte in terme neonaten, interklinische avond Erasmus MC Sophia, Rotterdam	2010	30
BCAA behoefte in neonaten, Fellow Researchdagen Groningen	2011	30
BCAA requirement in term infants, ESPGHAN Sorrento	2011	60

2. Teaching activities

Lecturing activities		
ZO Onderwijsmodule " voeding bij neonaten" 3e jaars studenten Erasmus Universiteit	2010	40
"Necrotising enterocolitis" training paediatric residents IC Neonatology	2009-2012	150
"Nutrition" training paediatric residents IC Neonatology	2009-2012	150
Training nurses	2010	12
AKTE medical students Erasmus MC	2010	20
Supervising Master Theses		
Waheeda Joemai, medical student Erasmus MC	2009	25
Carmen Hau, medical student Erasmus MC	2009	25
Lodi Roksnoer, medical student Erasmus MC	2010	25
Fleur van der Meide, medical student Erasmus MC	2012	25
Nicole Schrier, medical student Erasmus MC	2012	25

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