The Essential Amino Acid Requirements of Infants

De behoefte aan essentiële aminozuren van zuigelingen

新生儿对必需氨基酸的需求量

Lisha Huang

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	Prof.dr. I.K.M. Reiss
	Prof.dr. A.P. Bos

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CHAPTER 1

Introduction

The importance of nutrition during early life

Infancy is a period characterized by a high growth velocity and a high rate of physiological development. Nutrition plays a major role in determining health during this critical period of life. Moreover, early nutrition has a programming effect on health in later life (1). Early nutrition has been found to be associated with obesity, coronary heart disease, neurodevelopmental outcome, cardiovascular and allergic diseases in later life (2-5). These associations are supported by evidence from experimental studies in infants (6-7).

Protein and amino acids

Protein receives particular attention, because protein intake during infancy has been postulated as one of the main early nutritional factors that may have a lifelong effect on obesity risk (8-11), cardiovascular health (7), and kidney volume and function (12-13). The underlying mechanisms are still poorly understood. High protein intake stimulates insulin and insulin-like growth factor 1 (IGF-1) secretion (14-17). Elevated levels of growth factors during infancy may have programming effect on the IGF-1 axis, and this may have a relevant effect on the later risk of adult diseases.

Twenty α-amino acids serve as building blocks of proteins. Apart from protein synthesis, amino acids have many functions in metabolism (e.g. precursors of hormones, precursors of low-molecular weight nitrogenous substances, cell signalling molecules, regulators of gene expression and regulators of protein phosphorylation cascade (18)). Amino acid composition is an important determinant of protein quality. α-Amino acids can be divided into nutritionally essential and non-essential amino acids. Nine are classified essential since they cannot be synthesized *de novo* by the human body, and must be provided by the diet (table 1). The following amino acids are classified as conditionally essential in infants, because they cannot be produced in sufficient amount endogenously: arginine, glutamine, glycine, proline, and tyrosine. Cysteine used to be defined as conditionally essential. However, recent clinical studies demonstrated the capability of transsulfuration of methionine to cysteine in (pre)term neonates (19-21), which supports the classification as non-essential. Providing infants with sufficient amount of amino acids is essential for growth, since the protein synthesis rate is limited by the first limiting essential amino acid in the

diet. On the other hand, excess of amino acid intake is a burden on the immature kidneys and liver. Increased kidney size in formula-fed infants is an adaptive response to high renal solute load as a consequence of higher protein intake compared with breastfed infants (12-13). Moreover, elevated plasma levels of amino acids and their metabolites are pathogenic (e.g. homocysteine in cardiovascular disease, ammonia in neurological diseases, phenylalanine in metabolic disease, etc.). Thus, it is crucial to supply a balanced amount of amino acids in an optimal composition to the infants.

Current recommendations of amino acids

Since breastfeeding is considered to be the optimal nutrition for term infants, the current recommended essential amino acid intakes in the first 6 months of life are based on average intakes from breast milk rather than on experimentally derived requirement values (22). These recommended intakes are calculated from the average protein intake of breastfed infants multiplied by the average amino acid content of human milk protein (table 1) (22). However, human milk shows enormous variations in protein concentrations during the lactation period; the protein content in human milk declines from 23 g/L on post partum day 3 to 14 g/L on day 28, to 7 g/L at 6 months (23-24). The recommendations do take into account the decline in protein intake by the breastfed infant, but not the change in whey casein ratio and thus the change in amino acid composition (25). The accuracy of the estimation is also challenged by the daily and between feeds variations in protein/amino acid concentrations of mothers and between mothers, the day-to-day variation in protein/amino acids intake of infants and between infants (26). The recommendations may slightly underestimate the actual intake, since the amino acid intakes from non-protein nitrogen component of breast milk is not included.

The recommended essential amino acid contents in infant formulas should at least be equal to those contained in the human milk protein (27-28). Human milk is rich in essential amino acids. Standard infant formulas are made from cow's milk, which has a different amino acid profile than human milk. To compensate for the difference, infant formulas have higher protein content than human milk in order to provide sufficient quantities of essential amino acids. As a consequence of the higher protein intake, infants fed classical infant formulas have higher plasma levels of certain amino acids and elevated plasma urea levels compared with breastfed infants (29).

Higher urea levels in formula fed infants indicate excessive protein/amino acid intake, which must be catabolised through the amino acid degradation pathway. Moreover, higher protein intake is associated with an increased infant weight gain (30). Upward weight percentile crossing has been associated with increased risk for obesity and cardiovascular diseases later in life (10-11, 31-32). A challenge of infant formula research is to reduce the total protein concentration, while meeting all the essential amino acid requirements of the infant. One of the recent approaches is to add more bovine α-lactalbumin to the infant formula to increase the protein quality (33-35). Concomitantly, the protein content has been decreased. The nutritional safety and compositional adequacy of the infant formula has been monitored by plasma essential amino acids, serum albumin and growth rate. However, these methods are not sensitive enough to determine a small imbalance in the diet. A better approach is to determine the requirements of the individual amino acid to ensure adequate intakes of all amino acids and to provide a balance diet with regards to the essential amino acids.

TABLE 1

The average essential amino acid intakes in exclusively breastfed infants from 1-6 months of age (22).

Essential amino acids	Average amino acid intakes in $mg \cdot kg^{-1} \cdot d^{-1}$				
	Age 1 month	Age 2 months	Age 3 months	Age 4 months	Age 6 months
Histidine	36	26	23	21	20
Isoleucine	95	69	60	54	52
Leucine	165	121	105	95	90
Lysine	119	87	75	68	65
Methionine	28	20	18	16	15
Phenylalanine	72	53	46	42	39
Threonine	76	55	48	44	41
Tryptophan	29	21	19	17	16
Valine	95	69	60	54	52

These values are based on the estimated milk protein intakes (75% of crude protein) of breastfed infants (36) multiplied by the average amino acid content of the mixed human milk proteins (37-39). These values probably underestimate the actual amino acid intakes since the non-protein nitrogen also includes some free amino acids.

Methods to estimate amino acid requirements

To estimate the amino acid requirements, various amount of the test amino acid has to be fed to the study subject irrespective the method used. The distinction of different methods is the determination of the change in biological response to the different test intakes.

1. Nitrogen balance

The nitrogen balance method has been recognized as the classic method to determine the amino acid requirements in the 1950s (40). Rose (40) was the first to determine the essentiality and the requirements of the essential amino acids in men using this method. Nitrogen balance is based on the concept that protein is the major nitrogen containing substance in the human body. Gain or loss of nitrogen can be regarded as gain or loss of body protein. Nitrogen balance studies measure the amount of nitrogen intake from the diet and the amount excreted; this includes losses via faeces, urine and miscellaneous losses such as via skin, hear, sweat, and breath. In adults, diet adequacy is reflected by the maintenance of nitrogen equilibrium. In growing individuals, adequacy is reflected by increasing body nitrogen retention adequately.

Errors of nitrogen balance experiments from practical aspects are overestimation of the intake as a consequence of spillage/vomiting and underestimation of the nitrogen losses as miscellaneous nitrogen losses are rarely measured (41). As a result, the nitrogen balance method tends to underestimate the requirement. In addition, there are unexplained findings. For example, increasing nitrogen retention was measured in infants with increasing nitrogen intake while the weight gain was not different compared with the infants with less nitrogen retention (42). Another example was the experiments by Rose et al. (43-44) with histidine. Histidine has large body storage. A positive nitrogen balance was maintained with a histidine deficient diet, while the body storage got depleted slowly. Hence, a nitrogen balance does not necessarily imply amino acid equilibrium (45).

Another drawback of this method is that it requires 7-10 days consumption of the test diet before nitrogen excretion will reflect to the new intake (46). This is due to the slowly changing body urea pool, which needs to be stabilized before urea nitrogen excretion is indicative to the test diet. For this reason, it is not suitable for use in vulnerable populations such as infants.

2. Growth

Infancy is characterized by a high weight gain rate. Therefore growth can be used to monitor adequacy of the diet. In the 1950s, Snyderman and colleagues (47) performed a series of experiments using growth and nitrogen balance to determine the amino acid requirements in infants. These studies were performed using a full synthetic diet. The test amino acid was removed completely from the diet and gradually increased. Each test intake was fed for a period of ~1 week to monitor the growth and to perform the nitrogen balance studies. Each infant received 2-5 intakes. The studies includes small amount of infants ($n \leq 8$) with a wide range of ages at the time of the study (from 2 weeks to 9 months old). Their results are shown in table 2. For comparison purposes, the ranges of intakes of breastfed infants from 1 to 6 months of age are also shown in the table. For histidine, leucine, lysine, threonine, and tryptophan, the estimated requirements by Snyderman (47) are within the range of intakes of breastfed infants. However, the requirement estimates for isoleucine, methionine and phenylalanine are higher than the intakes of breastfed infants. The essential amino acid intakes of breastfed infants, therefore, do not necessarily reflect the requirements of infants.

The method is invasive due to the relative long period whereby the study diet needs to be fed to the infant. It is not considered ethical anymore to maintain infants on either deficient or excess amino acid intakes for a long period of time. Moreover, the requirement of growing subjects during the study period might change and the method is not sensitive enough to detect small differences between diets.

TABLE 2

The estimated essential amino acid requirements by Snyderman et al. (47) using weight gain and nitrogen balance method, and the average intakes of breastfed infants from 1 to 6 months of age (22). Both expressed as mg·kg^{-1}·d^{-1}.

Essential amino acids	requirement estimates by Snyderman et al. (47)	Ranges of intakes of breastfed infants from 1 to 6 months of age[a]
Histidine	34	20-36
Isoleucine	119	52-95
Leucine	150	90-165
Lysine	103	65-119
Methionine	45 [b]	15-28
Phenylalanine	90 [c]	39-72
Threonine	60 [d]	41-76
Tryptophan	22	16-29
Valine	105	52-95

- [a] Values from Table 1
- [b] In presence of cysteine
- [c] In presence of tyrosine
- [d] Pratt et al. (48)

3. Plasma amino acid levels

Plasma amino acid concentrations of infants have also been used to asses the adequacy of the diet. The plasma amino acid concentrations of formula-fed infants have been compared with those of breastfed infants (49-50). The plasma concentration of a certain amino acid rises when the intake exceeds the rate of its disposal. However, this method is not sensitive enough to yield more than supportive information.

4. Factorial approach

Dewey et al. (36) used the factorial approach to calculate the amino acid requirements, which is the maintenance needs plus an additional component for growth. This method is based on the assumption that the pattern of maintenance needs for infants (0-6 months) is equal to that for adults and the needs for growth are given by the body amino acid composition. The estimation of the maintenance requirement in adults is based on the nitrogen balance method. The drawbacks of the nitrogen balance method have been discussed above. Furthermore, no adjustments for digestibility or efficiency of utilization of the milk amino acids (either human or

cow's) are made. Yet, the results of these calculations suggest that the average amino acid intakes of breastfed infants of 0-6 months of age exceed their requirements.

5. Carbon oxidation methods

Carbon oxidation methods are based on the concept that there is no body storage of amino acids. When amino acids are liberated after absorption of dietary proteins, they are either oxidized for energy, or incorporated in proteins, or used in the formation of a number of other N-containing compounds (51). Carbon oxidation methods measure the response in oxidation to the test amino acid intake. To measure the oxidation in expired air (as $^{13}CO_2$), the test amino acid (in the direct amino acid oxidation method) or another essential amino acid (in the indicator amino acid oxidation method) is labelled with a stable isotope. Both methods are described below.

5.1. Direct Amino Acid Oxidation (DAAO)

The DAAO is one of the carbon oxidation methods (52). A carbon atom of the test amino acid is labelled with a stable isotope. Changes in oxidation in response to graded alterations in test amino acid intake have been studied. An increase in the oxidation occurs when the dietary intake exceeds requirement levels. The inflection point in the oxidation response curve is the requirement level (figure 1). This method has been used to determine essential amino acid requirements in adults (53-56).

There are a number of limitations of the DAAO method (57-58). First, DAAO is only applicable in amino acids where the major route of loss is oxidation and the carboxyl group is directly released to the bicarbonate pool and appears in breath as isotopically labelled $^{13}CO_2$. The essential amino acids of this group include phenylalanine (with excess of tyrosine), methionine (with excess of cysteine), lysine and the branched chain amino acids.

Second, a substantial amount of the labelled test amino acid has to be used to obtain a detectable amount of $^{13}CO_2$ enrichment in expired air. This amount may be nutritionally significant. It is therefore not possible to study low test amino acid intakes.

Third, the pool size in which oxidation takes place increases with increasing intake test amino acid. As a consequence, the dilution of labelled with unlabelled amino acid in

this pool changes with varying test intakes. Corrections based on plasma enrichment of the test amino acid have to be applied to calculate the oxidation rate of the test amino acid. However, corrections based on plasma amino acid enrichments may not necessarily reflect the precursor pool where the oxidation takes place intracellular. This is due to the lack of a suitable marker of intracellular enrichment except for leucine, which is the ketoisocaproate.

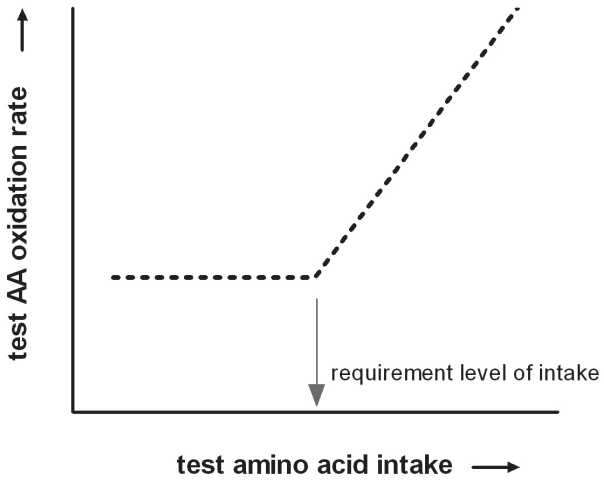

FIGURE 1
Direct amino acid oxidation method: the amino acid oxidation rate is plot against different test amino acid intakes. The inflection point of the line indicates the requirement of the test amino acid. The oxidation rate of the labelled test amino acid starts increasing at intakes above the requirement. Solid line: IAAO - The oxidation rate of the indicator amino acid decreases with increasing test amino acid intake until the requirement is met. Then the oxidation rate stabilized.

5.2. Indicator Amino Acid Oxidation (IAAO)

In the IAAO method the requirement of one amino acid is determined by measuring the oxidation of another amino acid. The method is based on the fact that when the diet is deficient in one essential amino acid (the test amino acid), protein synthesis will be limited and relative excesses of the other amino acids will be oxidized, including the indicator amino acid which is labelled with a stable isotope (59). 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 further increase in its intake will have no further effect on the oxidation rate of the indicator amino acid. Oxidation of the indicator amino acid can be measured in expired air as $^{13}CO_2$ (figure 2).

One of the advantages of the IAAO method is that the labelled amino acid is a different amino acid than the test amino acid. Therefore, all the other essential amino acids can be tested with the indicator, except the indicator amino acid itself. In that case, another amino acid is used to be the indicator amino acid. An indicator amino acid must meet several requirements: it has to be an essential amino acid; the main fates of the indicator amino acid must be either incorporation into protein or oxidation to CO_2; and the labelled carboxyl group must be irreversibly oxidized and detectable in expired air (58). Phenylalanine and lysine were the most suitable indicators (60-61). Phenylalanine has a small stable pool. If tyrosine is supplied in excess, phenylalanine would be directly channelled to oxidation. Lysine has a larger pool than phenylalanine, but it has no other metabolic pathways than protein synthesis or oxidation. Taking together, the IAAO allows us to select the most suitable indicators to determine the essential amino acid requirements.

Another advantage of this method is that the precursor pool of the indicator amino acid does not change with altering intakes of the test amino acid and the lowest test intake can be as low as zero. Testing a wide range of intakes below and above the requirement level is important in order to increase the power of the statistical model (the biphasic linear regression crossover model) used (62).

To study amino acid requirements by means of the IAAO method in infants, the period of dietary manipulations have to be short. This method is based on the partitioning of the amino acids between either protein synthesis or oxidation by measuring the degree of oxidation rate of another essential amino acid other than the test amino acid. Since the amino acid degrading enzymes (high Km) and the aminoacyl-tRNA synthetases (low Km) are set to response in metabolism to an influx of amino acid rapidly (63-64). Therefore, the time required to adjust to the changing amino acid intakes is regarded to be short, within hours (65).

Initially, the IAAO method, developed in animal experiments, 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 indicator amino acid oxidation in expired air ($F^{13}CO_2$) (66-68). The method has been further modified to make it less invasive and is therefore suitable to use in vulnerable populations such as children and neonates (69-77).

However, the essential amino acid requirements of enterally fed infants has never been determined using the IAAO method on the basis of $^{13}CO_2$ data and flux data of the indicator amino acid. Data of amino acid kinetics are very limited in this group of population. Therefore, it is important to establish a non-invasive IAAO study protocol including amino acid kinetic data in infants.

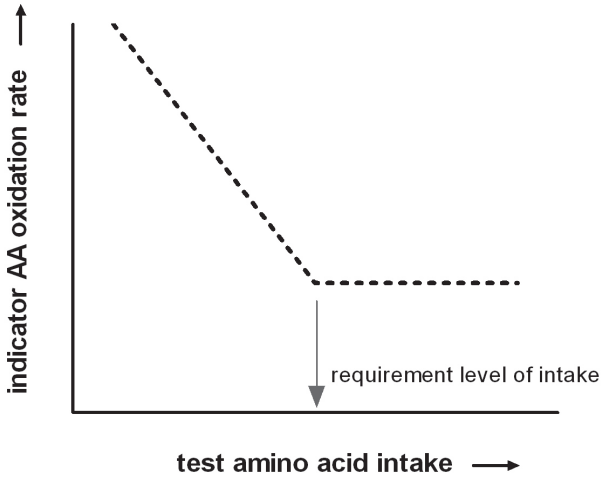

FIGURE 2
Indicator amino acid oxidation method: the indicator amino acid oxidation rate is plot against different test amino acid intakes. The inflection point of the line indicates the requirement of the test amino acid. The oxidation rate of the indicator amino acid decreases with increasing test amino acid intake until the requirement is met, a further increase in the test amino acid intake will have no further effect on the oxidation rate of the indicator amino acid.

Aims of the thesis

Knowledge of amino acid requirements is needed to optimise infant nutrition. The existing data is derived from nitrogen balance and growth studies with only a relative small number of infants studied in a wide range of age (47). As discussed earlier, these methods have many limitations and it is therefore important to use the recently

developed more accurate IAAO method to determine the amino acid requirements. The studies described in this thesis use this method to determine the essential amino acid requirements of term infants up to one month of age.

The studies presented in this thesis were performed in Shanghai, China. **Chapter 2** describes the relevant issues of setting up a collaborative study in Shanghai in both eastern and western points of view.
We determine the lysine requirement and validate the IAAO method by calculations based on the sampling of expired air compared with urine and blood (**chapter 3**).
Chapter 4 describes the study of the minimal obligatory methionine requirement in presence of excess of cysteine.
Chapter 5 describes the study of tryptophan requirement.
Chapter 6.1, 6.2 and 6.3 describe the studies of the branched chain amino acids (BCAAs) requirements. These three studies provide information of the exact requirement of each BCAA individually and the ideal ratio of these BCAAs, which will be discussed in **chapter 6.4**.
In the general discussion in **chapter 7**, the results obtained from this thesis are reviewed and discussed. The implications and suggestions for future work are proposed.

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CHAPTER 2

A Sino-Dutch collaborative research project in China

CHAPTER 2.1

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 (10). The Chinese government invested approximately 1 billion Renminbi (EUR €100 million) in medical research in 2010 (9). 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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CHAPTER 2.2

Western view

Femke de Groof
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 committee

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. Furthermore, 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 to conduct a study on nutrition. The study involved two days of a specific infant formula and sampling of expiratory air (4). In term neonates, 113 approved (58%) and 82 (42%) rejected. In preterm neonates, 43 parents approved (56%) and 34 (44%) rejected. These values are considerably lower than the approval rates we obtain in the Netherlands, where

almost 80% of parents approve for a study concerning nutrition 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 who 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.

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. Significant urban/rural differences and inequities in services between the rich and insured people and the poor people are the resultant of the fact that only 10% of the people in rural sites are covered by the cooperative medical system, compared to 45% in the cities (5). 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%. 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 (6). 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) (7), 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"(8).

When you enter a hospital in China, the waiting line is the first thing you see. No treatment is started before payment is received, both for outpatient and inpatient clinics. This situation disturbs the patient-doctor relationship; some parents do not trust doctors because the doctor's 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 (9).

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 (10). 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 (11). Sex ratios are further skewed by widespread abortion, that 1 of 4 women in their twenties undergo, and 55% of them more than once (12). This occurs despite the fact that conducting an ultrasound to identify fetal sex is illegal in China.

In the clinical practice, the neonatal department admission rate 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. The large difference most likely results 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 any female after the inclusion of the first 15 patients.

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 conduct clinical research in China, 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. In 2007, 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. For research purposes, one must find a system that does not require individual handling by nurses or nurse-assistants to avoid mistakes. All additional procedures have to be taken care of by the research team.

Study requirements

A problem we experienced and did not expect before we went to China was the difficulties importing our study materials. China exports a lot but imports only a few products. Import requires a large amount of forms and stamps, and thus advanced preparation. The required forms must be prepared with the correct stamps from the government of the country where the products are produced. At many occasions, the products were hold up during the customs process; a situation that sometimes

took months to resolve. 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 preparations are finished and all 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 3

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

Lisha Huang
Jacomine E. Hogewind-Schoonenboom
Femke de Groof
Jos W.R. Twisk
Gardi J. Voortman
Kristien Dorst
Henk Schierbeek
Günther Boehm
Ying Huang
Chao Chen
Johannes B. van Goudoever

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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 minutes) tracer infusion protocol with the results derived from a 900-minutes 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, gastro-intestinal 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 Centre 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-hour 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 $mg \cdot kg^{-1} \cdot d^{-1}$ (8). 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 channelled 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-hour period whereby the study formula was fed to the neonates. After 24 hours of study formula consumption, tracers were administered on day 2 for 15 hours. Infants were bottle fed every 3 hours 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 3 hours 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 hours. The duration of [1-^{13}C]phenylalanine infusion was 12 hours, 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.

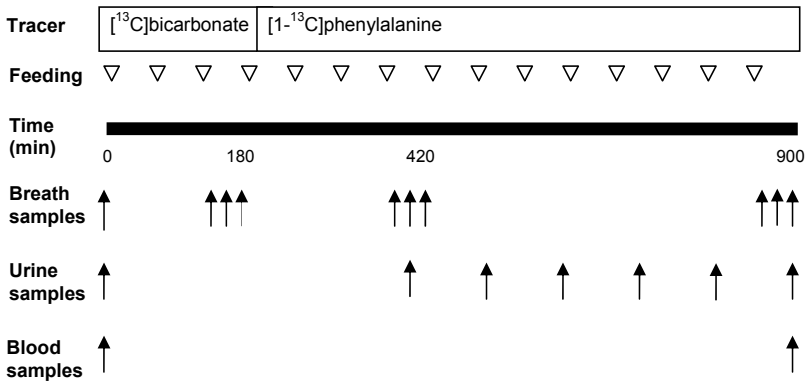

FIGURE 1

Schematic overview of tracer infusion day. Arrows indicate times that breath, urine and plasma samples were taken.

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 infusion. Duplicated breath samples were obtained at 15 minute intervals during the period of 105-180 minutes after the tracer infusion,

and duplicated samples were obtained at 10 minute intervals during the period of 360-420 minutes (the first plateau period). Another set of duplicated samples were obtained at 10 minute 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 minutes was chosen because the isotopic steady state of L-[1-^{13}C]phenylalanine in expired air was obtained after 360 minutes of tracer infusion in our pilot study, which was 180 minutes 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 void frequencies of the infants from 360 minutes 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:

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

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 minutes 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 minutes. 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 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.

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

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.

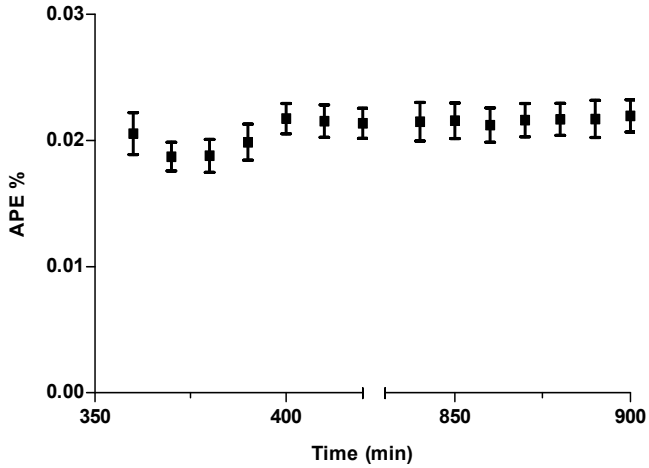

FIGURE 2
Mean ± SEM ^{13}C enrichments in atom percent excess (APE) in expired air during the first (period: 360-420 minutes) and the second (period: 840-900 minutes) isotopic plateaus of the [1-^{13}C]phenylalanine infusion (n = 21).

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 minutes) and the second plateau (the period 840-900 minutes), are shown in Figure 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).

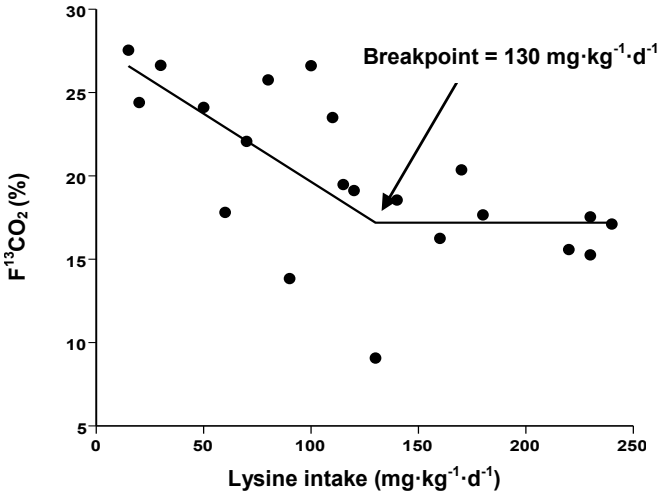

FIGURE 3A
Fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the first isotopic plateau (period: 360-420 minutes) at different lysine intakes (n = 21). With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($P < 0.0001$, $r^2 = 0.46$). Upper and lower 95% CIs of the breakpoint estimate were 188.4 and 71.6 mg·kg^{-1}·d^{-1}, respectively.

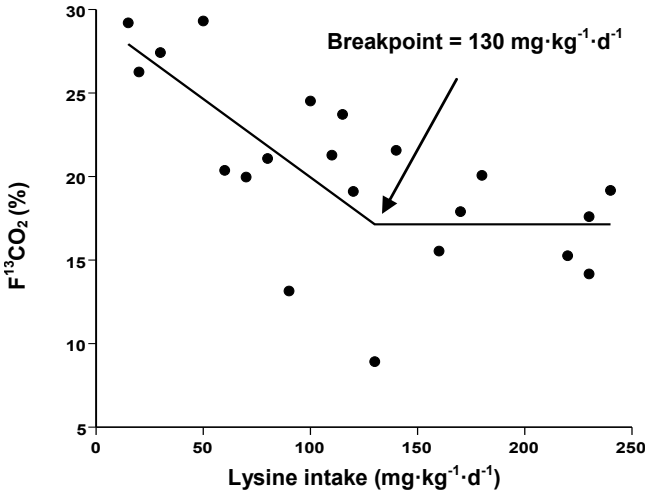

FIGURE 3B
$F^{13}CO_2$ during the second isotopic plateau (period: 840-900 minutes) at different lysine intakes (n = 21). With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($P < 0.0001$, $r^2 = 0.51$). Upper and lower 95% CIs of the breakpoint estimate were 183.7 and 76.3 mg·kg^{-1}·d^{-1}, respectively.

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 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 of phenylalanine oxidation. The breakpoints in the urinary and plasma phenylalanine oxidation data are shown in Figures 6, A and B, 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}.

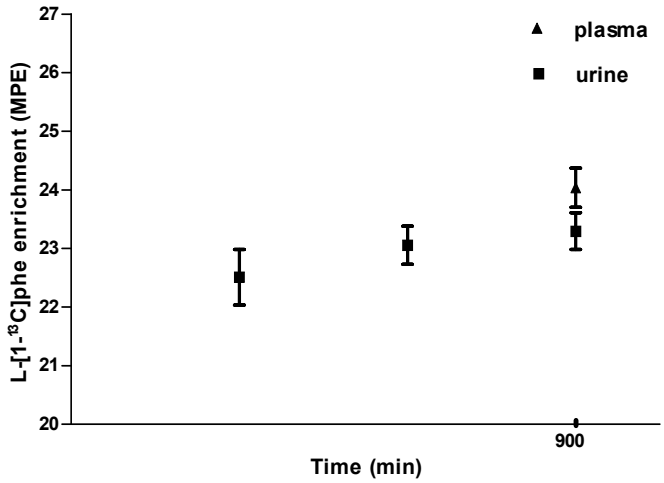

FIGURE 4
Mean ± SEM L-[1-^{13}C]phenylalanine enrichments in mole percent excess (MPE) in urine during isotopic plateau and the L-[1-^{13}C]phenylalanine enrichments in plasma at 900 minutes.

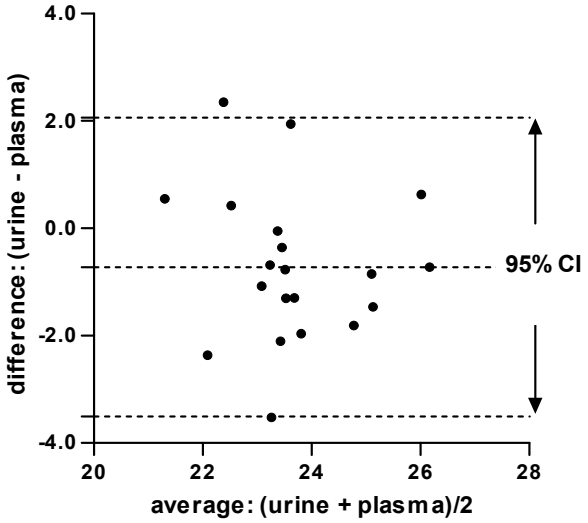

FIGURE 5

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

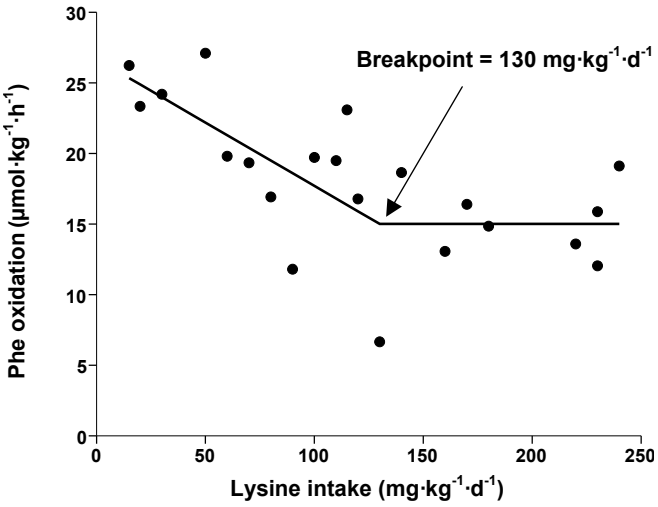

FIGURE 6A

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

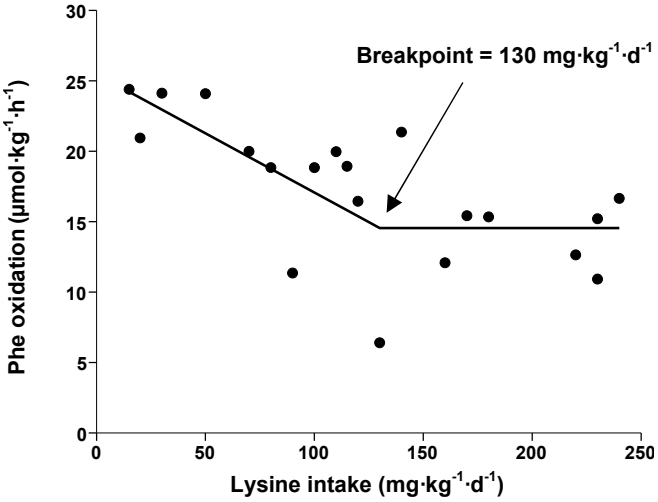

FIGURE 6B
Phenylalanine (Phe) oxidation calculated from the plasma enrichment data at different lysine intakes (n = 20). With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 130 mg·kg^{-1}·d^{-1} ($P < 0.0001$, $r^2 = 0.49$). Upper and lower 95% CIs of the 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·kg^{-1}·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·kg^{-1}·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·kg^{-1}·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 postsurgical 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 need 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]phenylalanine 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 minutes 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 hours), 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 in 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 nonoxidative 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 labeled 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 gastro-intestinal 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 of 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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CHAPTER 4

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

Lisha Huang
Jacomine E. Hogewind-Schoonenboom
Mariska J.A. van Dongen
Femke de Groof
Gardi J. Voortman
Henk Schierbeek
Jos W.R. Twisk
Andras Vermes
Chao Chen
Ying Huang
Johannes B. van Goudoever

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

Design: Fully enterally fed term infants < 1 month of age were randomly assigned to methionine intakes that ranged from 3 to 59 mg·kg^{-1}·d^{-1} as part of an elemental formula. After 1 day of 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. The nean methionine 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 means ± SDs.

Results: Thirty-three neonates (gestational age 39 ± 1 wk) were studied at 13 ± 6 days. 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 $F^{13}CO_2$. The 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 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 consumed a methionine-fortified formula with a methionine content of 788 mg/L or a high protein formula that provided 9 g protein per kg per day (5-6). Extreme hypermethioninemia may cause cerebral edema (5). Hyperhomocysteine has been shown to be associated with increased risk of neonatal stroke (7). Because both a deficient and excess intake of methionine have detrimental effects, it is important to determine the methionine requirement to optimize infant nutrition.

Experimental evidence of methionine requirement of enterally fed infants is scarce. In previous studies with a relatively small number of infants (n = 7 -13), the methionine requirement was estimated to be between 27 and 49 $mg \cdot kg^{-1} \cdot d^{-1}$ (8-11). Because breast milk is considered to be the optimal nutrition for infants ≤ 6 months of age, the joint WHO/FAO/UNU expert consultation recommended a methionine intake of 28 $mg \cdot kg^{-1} \cdot d^{-1}$ on the basis of the average intake of breastfed infants (12). Human milk is known to vary in protein content, whereas the volume ingested varies 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 of term infants by 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 days. Subjects were excluded if they had congenital anomalies, gastro-intestinal 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 formula (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.

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 ≥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.

The formula provided cysteine intake of 91 $mg \cdot kg^{-1} \cdot d^{-1}$. This amount was considered to be in excess because it is > 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 formula (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} (12). 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 could 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 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; thiamine: 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 was based on the minimally invasive IAAO method (13), which was recently modified to apply in enterally fed infants (14). The advantages of this method are the short adaptation period to the test intake (1 day), 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 labeled 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 additional 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 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 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) 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.

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) 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) 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 breath samples was analyzed by an infrared isotope analysis technique (Helifan, Analytic Fischer Instruments) (19). The ^{13}C enrichment was expressed as the atom percent excess above baseline (APE).

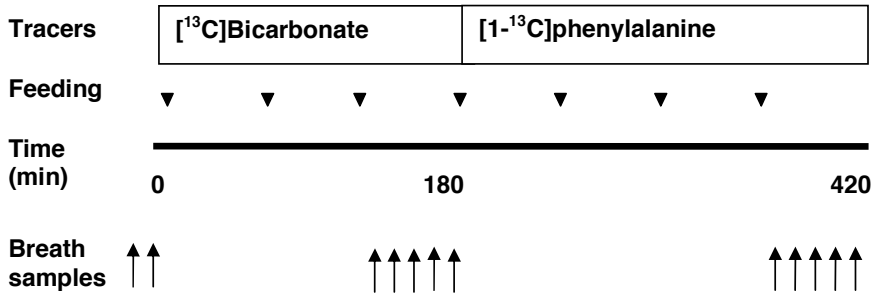

FIGURE 1
Schematic overview of tracer infusion day. Arrows indicate times that breath samples were taken.

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 ($F^{13}CO_2$) as a percentage was calculated by using the following equation (20):

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

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 (14).

Statistical analysis

Descriptive data are expressed as means ± SDs. Determination of the methionine requirement (ie, the breakpoint) was performed by using a biphasic linear regression crossover model (21). With the biphasic linear regression analysis, the regression equation was split into 2 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. A value of $P < 0.05$ was considered significant. Analyses were performed in STATA software (version 11; StataCorp LP).

The power analysis could not be performed. We aimed to study 20 to 35 infants, which was greater than the number of infants used in studies in parenterally fed infants.by using the same approach with an 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 days 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. 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 that was 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 before the study of infants who participated in the study (n = 33).

	Values
Birth weight (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 ($g \cdot kg^{-1} \cdot d^{-1}$)	13 ± 5
Sex (F:M)	9:24
Protein intake before the study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.5 ± 0.4
Energy intake before the study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	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 ± 0.0032 APE. The corresponding mean CO_2 production rate was 23.44 ± 2.04 $mmol \cdot kg^{-1} \cdot h^{-1}$. The mean $^{13}CO_2$ enrichment at the isotopic plateau and their corresponding CO_2 production rate of each infant were plotted against the methionine intake (Figure 2).

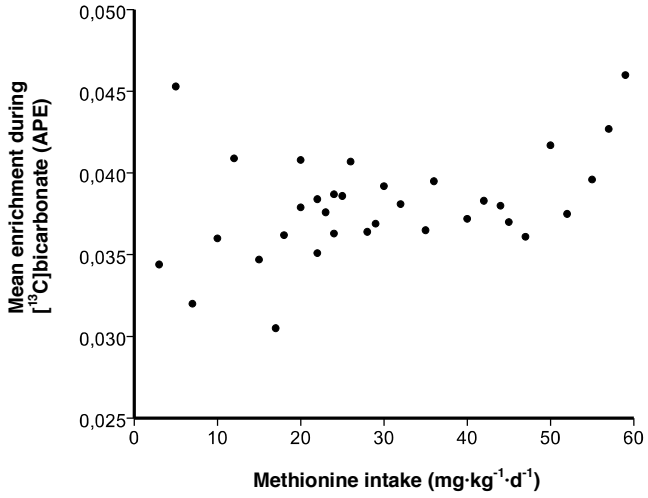

FIGURE 2A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral [^{13}C]bicarbonate infusion of each infant plotted against the methionine intake (n=33). APE, atom percent excess.

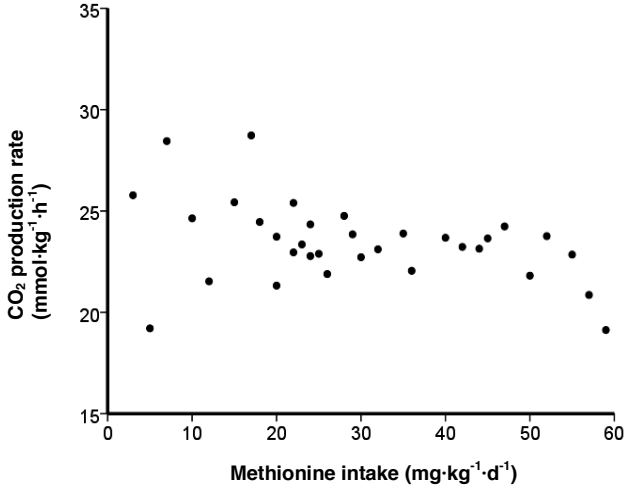

FIGURE 2B
CO_2 production rate of each infant plotted against the methionine intake (n=33).

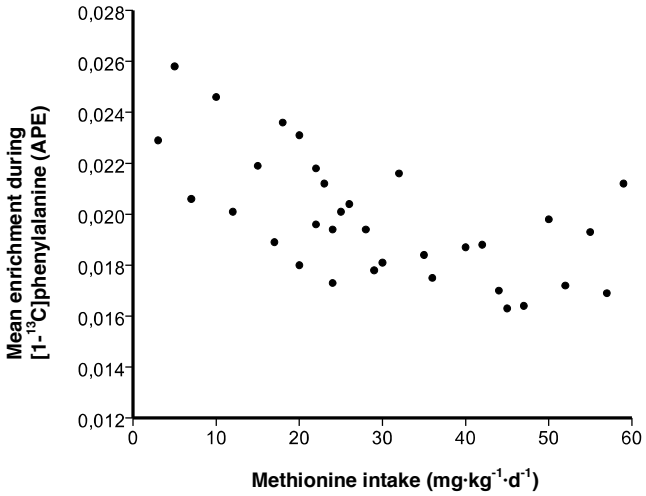

FIGURE 3A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral L-[1-^{13}C]phenylalanine infusion of each infant plotted against the methionine intake (n=33). APE, atom percent excess.

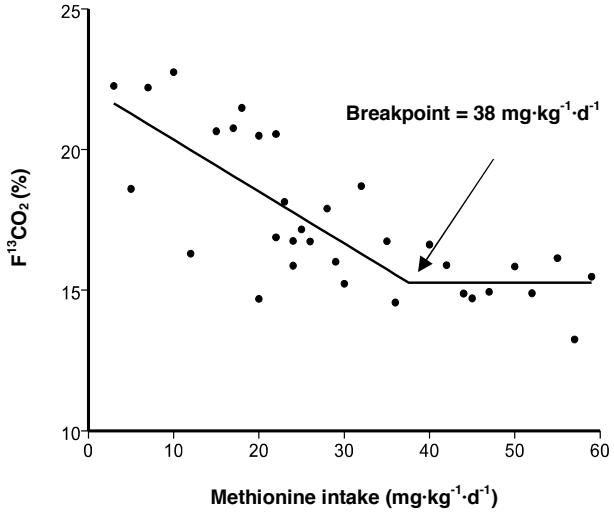

FIGURE 3B
The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the isotopic plateau at different methionine intakes (n = 33). Each infant received a different methionine intake. With the use of a biphasic linear regression crossover model, the breakpoint 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 ± 0.0024 APE. These $^{13}CO_2$ enrichment values and the $F^{13}CO_2$ were plotted against methionine intakes, as shown in Figure 3. As the methionine intake increased, $F^{13}CO_2$ decreased. This negative correlation was shown between $F^{13}CO_2$ and methionine intakes ≤38 mg·kg^{-1}·d^{-1}; additional increases in methionine intake did not affect the $F^{13}CO_2$. With the use of a biphasic linear regression crossover model, the mean methionine requirement 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 of enterally fed term infants was estimated to be 38 mg·kg^{-1}·d^{-1} by 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 the study of Snyderman et al. (8), the methionine requirement was determined in 7 infants with postnatal age between 2 weeks and 2 months by using weight gain rates and the nitrogen retention. The study diet used by Snyderman et al. (8) was an elemental diet that provided 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 13 infants were included in each study diet. The adequacy of the diet and, thus, the adequacy of sulfur amino acids intakes were estimated by measurement of growth, serum chemical indexes, and nitrogen retention. Fomon et al. (9-11) 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 for male infants < 56 days old. Although our study was not designed to detect sex differences in the methionine requirement, the average requirement estimates by Fomon et al. (9-11) were consistent with our results. Limitations of previous studies were the relative small numbers 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.

Courtney-Martin et al. (22) determined the methionine requirement in parenterally fed postsurgical neonates by 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), which is the enzyme involved in the final step in the cysteine synthesis pathway. However, clinical studies showed the capability of transsulfuration of methionine to cysteine in term and preterm neonates (27-29) and, thereby, support the evidence that cysteine has a sparing effect on methionine requirement. The amount 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. The authors showed that the parenteral methionine requirement was ~70% of the enteral requirement, and the dietary cysteine reduces the methionine requirement by an equal proportion in both feeding routes of ~40%. With the use of these fractions, the parenteral methionine requirement of 49 mg·kg^{-1}·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 an excess of cysteine as in our study. The predicted requirement would be 42 mg·kg^{-1}·d^{-1}. This amount is nearly the same as our estimated requirement of 38 mg·kg^{-1}·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·kg^{-1}·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, whereas the recommendations do not discriminating between whole-protein-based, partially hydrolyzed, or elemental formulas. All of these formulas are on the market for infants. A recent report showed that an elemental diet provides an average of 17% less protein substrate per gram of free amino acids than does a protein bound diet. This diiference is due to the release of a water molecule when a peptide bond is formed (36).

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

Third, the composition of human milk shows remarkable variation and is influenced by many factors, such as the gestational age at parturition, stage of lactation, and nutritional status of the mother. The protein content in human milk declines remarkably during lactation (39-40). The recommendations 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).

Last, the protein digestibility and amino acid bioavailability in human milk are different from that in formula. Therefore, the gross amino acid composition of human milk may not necessarily reflect the amino acid-requirement profile of infants who consumed 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 of 33 infants were in a (possible) post-infectious state. Because 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 would 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.

In conclusion, the minimal obligatory methionine requirement is determined to be 38 $mg \cdot kg^{-1} \cdot d^{-1}$ for 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 of infants fed an infant formula, which is necessarily to improve infant nutrition.

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CHAPTER 5

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

Lisha Huang*
Jacomine E. Hogewind-Schoonenboom*
Li Zhu
Juliette V.S. Kraaijenga
Nicky P.C. van Haren
Gardi J. Voortman
Henk Schierbeek
Jos W.R. Twisk
Ying Huang
Chao Chen
Johannes B. van Goudoever

*Huang and Hogewind-Schoonenboom contributed equally to this paper.

ABSTRACT

Background: Tryptophan is not only an essential amino acid, it also serves as a precursor in the serotonin and the kynurenine pathways. While it is considered to be the first limiting amino acid in artificial feeding, animal studies have shown that excess tryptophan can depress growth and impair brain development.

Objective: The objective was to determine the tryptophan requirement of term infants using the Indicator Amino Acid Oxidation (IAAO) method with L-[1-^{13}C] phenylalanine as the indicator.

Design: Fully enterally fed infants were randomly assigned to tryptophan intakes ranging from 0.5 to 73 mg·kg^{-1}·d^{-1} as part of an elemental diet. 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. The mean tryptophan requirement was determined by using the 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 is presented as mean ± SD.

Results: Thirty term neonates (gestational age 39 ± 1 wk) were studied at 9 ± 4 days. $F^{13}CO_2$ decreased until a tryptophan intake of 15 mg·kg^{-1}·d^{-1}; additional increases in tryptophan intake did not affect $F^{13}CO_2$. Mean tryptophan requirement was determined to be 15 mg·kg^{-1}·d^{-1} with an upper CI of 31 mg·kg^{-1}·d^{-1} (r^2 = 0.17).

Conclusions: The mean tryptophan requirement for elemental formula fed term infants is 15 mg·kg^{-1}·d^{-1}. This requirement is lower than the current recommended intake of 29 mg·kg^{-1}·d^{-1}, which is based on the average intake of a breastfed infant.

INTRODUCTION

Tryptophan is an important amino acid in infant nutrition (1). It is not only one of the essential amino acids used for protein synthesis, it is also a precursor in two important metabolic pathways: the serotonin pathway and the kynurenine pathway. The main metabolites of the serotonin pathway are the neurotransmitter serotonin and the neurosecretory hormone melatonin; both are involved in almost every physiological process, including the regulation of appetite, digestive processes, gastrointestinal motility, circadian rhythm, cognition, learning, memory and affective reaction control (2). Tryptophan is mainly degraded through the kynurenine pathway, which supplies a number of important metabolites, such as kynurenine, niacin and nicotinamide adenine dinucleotide (NAD) (3).

Experimental data in infants have shown that dietary tryptophan modulates sleep patterns (4-5). In animal models dietary modification of tryptophan supply causes changes in the brain concentrations of tryptophan and serotonin (6-7); altered serotonin levels during early brain development have been suggested to influence neuronal circuitry and plasticity (8-9). Because of tryptophan's numerous physiological functions and the potential risks of unbalanced intake, it is important to know the exact tryptophan requirement.

Current recommendations for essential amino acid intakes in infants up to 6 months are based on the average intakes of breastfed infants. The recommended tryptophan intake for infants at one month of age is 29 $mg \cdot kg^{-1} \cdot d^{-1}$ (10). Experimental data of tryptophan requirement in infants are very limited. In the 1960s, Snyderman et al. (11) attempted to define the tryptophan requirement of 7 infants using the nitrogen balance technique and measurement of growth, resulting in a much lower tryptophan requirement of 13 to 16 $mg \cdot kg^{-1} \cdot d^{-1}$.

The indicator amino acid oxidation (IAAO) method is a minimally invasive method that has recently been modified to determine the essential amino acid requirements in vulnerable populations (12). It has been used successfully to determine several essential amino acid requirements in enterally fed infants (13-14). The aim of this study was to determine the tryptophan requirement of term infants using this method.

METHODS

Subjects

Thirty neonates admitted to the Neonatal ward in the Children's Hospital of Fudan University, Shanghai, P.R. China, participated in the study. Each subjects was selected for study by the following criteria: fully enterally fed infants with a gestational age of ≥ 37 weeks, a birth weight of ≥ 2500 grams 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, gastro-intestinal pathology or sepsis. All subjects were formula fed every 3 hours according to the feeding regime of the hospital.

The study was approved by the Institutional Review Boards of the Children's Hospital of Fudan University and obtained a statement of no objection from Erasmus MC-Sophia Children's Hospital. 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 based on free amino acid. 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 tryptophan and phenylalanine content. Tryptophan, which was completely withdrawn from the study formula, was separately added to obtain different amounts of intake. Niacin is an essential nutrient and can be replaced by tryptophan. The study formula provided the recommended niacin intake of 1.02 $mg \cdot kg^{-1} \cdot d^{-1}$ (15), and therefore should minimize the tryptophan required for niacin synthesis. 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 (10). 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 will be directly channelled to oxidation into $^{13}CO_2$, which can be measured in expired air (16).

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)	0.26
L-Phenylalanine (g) [2]	0.20
L-Proline (g)	1.16
L-Serine (g)	0.71
L-Threonine (g)	0.8
L-Tryptophan (g) [3]	0
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 tryptophan 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] 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.
[3] L-tryptophan was added separately, depending on the test tryptophan level.

The nitrogen intake was kept constant for all of the subjects during the study by the substitution of L-alanine for the tryptophan 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; thiamine: 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 was based on the adapted minimally invasive IAAO model (12), that has recently been modified to determine the essential amino acid requirements in enterally fed infants (13). 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 labeled 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 $^{13}CO_2$. We used L-[1-^{13}C]phenylalanine as the indicator.

During the study, all of the infants received fluid intake of ~150 mL·kg^{-1}·d^{-1}, amino acid intake equivalent to the protein intake of ~2.96 g·kg^{-1}·d^{-1} and energy intake of ~108 kcal·kg^{-1}·d^{-1}. Infants were randomly assigned to one of the graded test intake of tryptophan, ranging from 0.5 to 73 mg·kg^{-1}·d^{-1}. Each study took place over a 31-h period. After 24 h of the study formula consumption, tracers were administered on day 2 for 7 h. Infants were bottle fed every 3 h during the adaption period. Subsequently, the feeding regimen was changed to hourly bottle feeding during the tracer infusion until the end of the study. On the tracer infusion day, a nasogastric tube was placed for tracer infusion. Infants received, a primed (14 μmol·kg^{-1}) continuous (9 μmol·kg^{-1} per hour) 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). After the [^{13}C]bicarbonate infusion was stopped, a primed (34 μmol·kg^{-1}) continuous (27 μmol·kg^{-1} per hour) enteral infusion of L-[1-^{13}C]phenylalanine (99% ^{13}C APE; Cambridge Isotopes) was started and lasted for 4 h. The tracer infusion day is

depicted in Figure 1. Syringes were weighted before and after the study to determine the exact amount of the tracers that were given to the infants.

Sample collection and analysis

Breath samples were obtained by using the direct nasopharyngeal sampling method described by Van der Schoor et al. (18). Two duplicated baseline samples were obtained before the start of [^{13}C]bicarbonate, and 2 sets of breath samples were obtained during isotopic plateaus of [^{13}C]bicarbonate and L-[1-^{13}C]phenylalanine. The first set of six duplicated breath samples were obtained every 15 minutes during the period of 105 to 180 minutes after initiation of [^{13}C]bicarbonate and second set of 7 duplicated samples were obtained every 10 minutes during the period of 360 and 420 minutes (Figure 1). Breath samples were stored in room temperature until analysis.

^{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 percentage excess above baseline (APE).

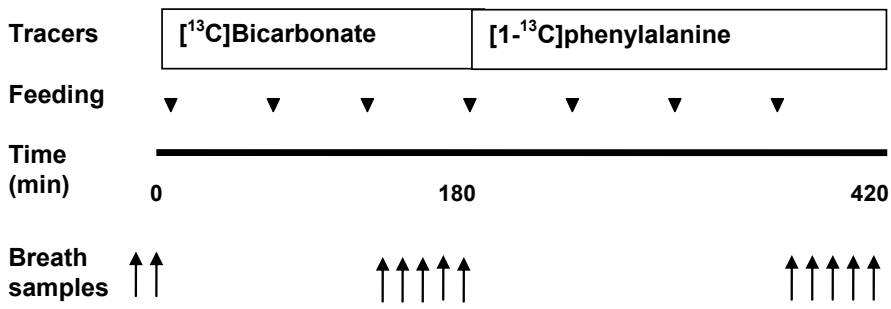

FIGURE 1
Schematic overview of tracer infusion day. Arrows indicate times that breath samples were taken.

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 ($mmol \cdot kg^{-1} \cdot h^{-1}$) was calculated as described previously (13, 17).

The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) as a percentage was calculated by using the following equation (19):

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

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 L-[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, test amino acid intake has no effect on the phenylalanine flux (13).

Statistical analysis

Descriptive data are expressed as means ± SDs. Determination of the tryptophan requirement (i.e. the breakpoint) was performed using a biphasic linear regression crossover model (20). With the biphasic linear regression analysis, the regression equation was split into 2 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 lines was equal to the breakpoint. The model with the best fit on the basis of the highest r^2 was selected. The analyses were performed in STATA version 11. A value of $p < 0.05$ was considered significant.

RESULTS

Subject characteristics

Thirty neonates were studied at a tryptophan intake that ranged between 0.5 and 73 $mg \cdot kg^{-1} \cdot d^{-1}$. Subject characteristics are summarized in Table 2. All subjects were growing well before entering the study. The mean (± SD) weight gain rate 3 days before the study was 12 (± 5) $g \cdot kg^{-1} \cdot d^{-1}$. The mean (± SD) energy intake was 108.9

± 0.5 kcal·kg^{-1}·d^{-1}. The total amino acid intake was 2.98 ± 0.01 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 reasons for admission were unconjugated hyperbilirubinemia (n = 12), pneumonia with a negative blood culture (n = 7), fetal distress (n = 4), infection suspicion with a negative blood culture (n = 3), wet lung (n = 2), meconium stained amniotic fluid (n = 1) and peripheral cyanosis (n = 1). Intravenous antibiotics (penicillins and/or cephalosporins) were given to 29 of the 30 infants.

TABLE 2

Subject characteristics, and protein and energy intake prior to the study of infants who participated in the study (n = 30).

	Values*
Birth weight (kg)	3.3 ± 0.4
Gestational age (wk)	39 ± 1
Age on study day (d)	9 ± 4
Weight on study day (kg)	3.4 ± 0.5
Weight gain rate before study (g·kg^{-1}·d^{-1})	12 ± 5
Sex (F:M)	13:17
Protein intake prior to the study (g·kg^{-1}·d^{-1})	2.2 ± 0.4
Energy intake prior to the study (kcal·kg^{-1}·d^{-1})	105 ± 17

*Mean ± SD

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

The baseline $^{13}CO_2$ enrichment was -17.77 ± 1.69 Pee Dee Belemnite (PDB). The mean $^{13}CO_2$ enrichment at isotopic plateau during [^{13}C]bicarbonate infusion was 0.0385 ± 0.0036 APE. The corresponding mean CO_2 production rate was 23.20 ± 2.46 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 tryptophan intake (Figure 2).

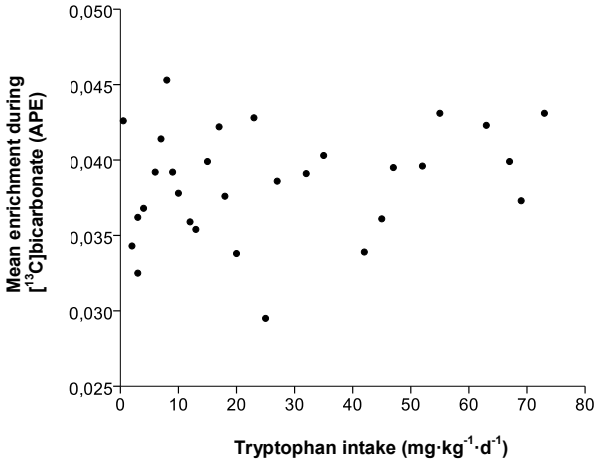

FIGURE 2A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral [13C]bicarbonate infusion of each infant plotted against the tryptophan intake (n=30). APE, atom percent excess.

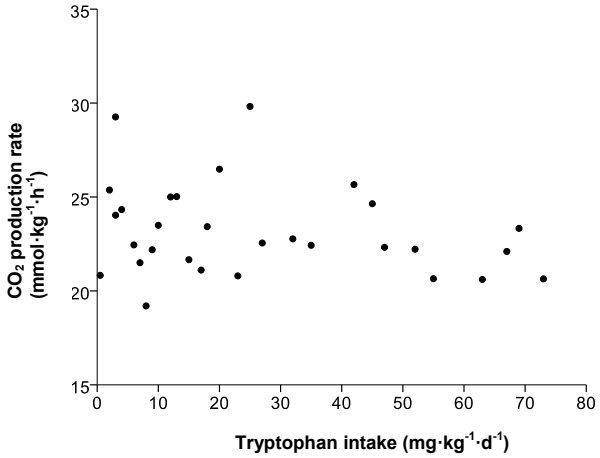

FIGURE 2B
The CO_2 production rate of each infant plotted against the tryptophan intake (n=30).

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

The mean $^{13}CO_2$ enrichment at isotopic plateau during L-[1-^{13}C]phenylalanine infusion was 0.0189 ± 0.0044 APE. These $^{13}CO_2$ enrichment values and the $F^{13}CO_2$ are plotted against tryptophan intakes in Figure 3. As the tryptophan intake increased, $F^{13}CO_2$ rates decreased, this negative correlation was shown between $F^{13}CO_2$ rates and

tryptophan intakes up to 15 mg·kg^{-1}·d^{-1}; a further increase of tryptophan intake did not affect the $F^{13}CO_2$ rates. Using a biphasic linear regression crossover model, the mean tryptophan requirement was determined to be 15 mg·kg^{-1}·d^{-1} with an upper CI of 31 mg·kg^{-1}·d^{-1} (r^2 = 0.17).

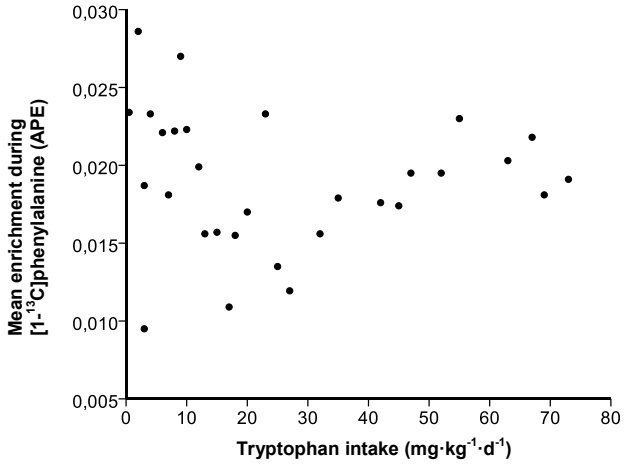

FIGURE 3A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral L-[1-^{13}C]phenylalanine infusion of each infant plotted against the tryptophan intake (n=30). APE, atom percent excess.

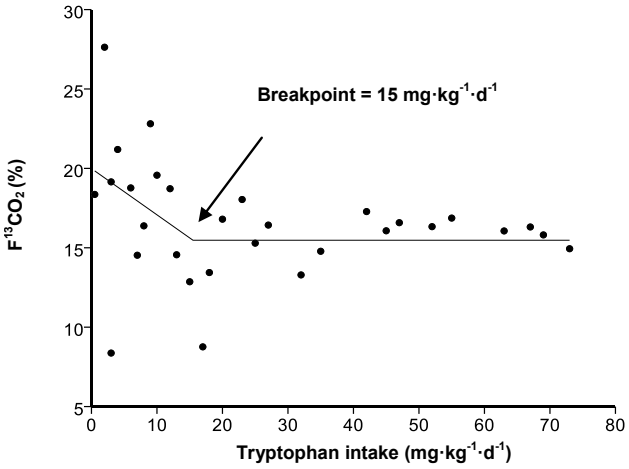

FIGURE 3B
The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the isotopic plateau at different tryptophan intakes (n = 30). Each infant received a different tryptophan intake. With the use of a biphasic linear regression crossover model, the breakpoint was estimated to be 15 mg·kg^{-1}·d^{-1} (r^2 = 0.17). The upper CI was 31 mg·kg^{-1}·d^{-1}.

DISCUSSION

In the present study, using the IAAO method, the mean tryptophan requirement of 30 term infants fed an elemental formula was estimated to be 15 mg·kg^{-1}·d^{-1}. Since breast milk is considered to be the optimal nutrition for infants, current recommended essential amino acid intakes are based on the average intakes of breastfed infants, which for tryptophan is estimated to be 29 mg·kg^{-1}·d^{-1} (10). In general, infant formulas feeding are produced from cow's milk and they must provide at least an equal amount of amino acids as that provided by human milk (15). The tryptophan content of cow's milk proteins is about half of that of human milk proteins, which makes tryptophan the first limiting amino acid in artificial feeding (21). In order to meet this minimum tryptophan content, formulas generally have a higher protein content than breast milk (22). However, increased protein intake during infancy has been found to be associated with increased obesity risk (23-26) and increased kidney size (27-28).

Although human milk is assumed to meet all the amino acid requirements of the infant, this amount may not necessarily reflect the requirement of formula-fed infants since the digestibility and bioavailability of breast milk proteins/amino acids are different to those in formula. In addition, the accuracy of the estimation is challenged by the day-to-day and within-feed variation in the protein/amino acid intake of breastfed infants, the variation in milk content between mothers, and the change in protein content during lactation (29). More accurate knowledge of the essential amino acid requirements is needed to provide infants with a formula that meets their requirement profile and prevents the potential risks of an unbalanced diet.

In the past, Snyderman et al. (11) studied the tryptophan requirement of 5 term and 2 preterm infants with a postnatal age ranging between 12 days and 3 months old, on the basis of nitrogen retention and weight gain. Each infant received 2-4 test intakes. Three infants showed inadequate weight gain at intakes of about 13 mg·kg^{-1}·d^{-1} and all infants did well on intakes in the range of 16 to 22 mg·kg^{-1}·d^{-1}. They concluded that the tryptophan requirement in these infants lies between 13 and 16 mg·kg^{-1}·d^{-1}, which is similar to our estimated mean requirement. One of the limitations of the study by Snyderman et al. (11) is the relatively small number of subjects studied.

Another limitation is their use of nitrogen retention and growth gain, which are not the most sensitive methods for yielding an accurate estimation.

The IAAO method is both accurate and reliable. It has been validated in animal experiments (30) and successfully used in humans to determine amino acid requirements (31-32). We modified the method to enable measurements in enterally fed infants (13). However, the method does have a number of limitations as discussed below.

Firstly, our estimated requirement may in fact overestimate the actual requirement for two reasons, which are related to the use of an elemental formula. In the first place, an elemental formula provides on average 17% less protein than does a protein-based formula due to the release of a water molecule when a peptide bond is formed (33). In the second place, the utilization and retention of amino acids depends on the digestion rate: a slowly digested protein source (i.e. protein formula) induces a higher retention rate than one digested more rapidly (i.e. elemental formula) (34-35). Future studies using intrinsically labeled proteins that closely resemble a whole protein diet are therefore required to investigate the effect of whole protein formula on the requirement estimates.

Secondly, due to ethical constraints, only one tryptophan intake was studied per infant. It was therefore not possible to determine the population variance when giving a recommendation for the dietary reference intake. In order to provide such variance information, a new approach needs to be developed that further minimizes the invasiveness of the method, thereby allowing repeated measures within the same infant.

Thirdly, we do not know whether the requirement estimated by the IAAO method includes tryptophan that is required for its metabolic pathways. One of these metabolic pathways is the serotonin/melatonin pathway, which regulates numerous neurobehavioral effects. Despite the small quantity required for this pathway, the synthesis of serotonin is influenced by dietary tryptophan intake (3, 5-7), and altering tryptophan intake in formulas has been demonstrated to modulate sleep patterns in infants (4-5). We did not measure the infants' behavioral states in relation to tryptophan intakes but it would certainly be of interest to apply such results and include neurobehavioral tests in future studies.

Another of tryptophan's metabolic pathways is the kynurenine pathway, the most significant pathway of tryptophan degradation. The complex kynurenine pathway not only yields energy, it also produces niacin, NAD, quinolinic acid and other metabolites. This pathway can be initiated by the enzymes tryptophan dioxygenase or indoleamine dioxygenase (36). Tryptophan dioxygenase is normally the main enzyme catabolizing the irreversible first step of tryptophan degradation (37). This enzyme has a high Km for tryptophan and is activated when tryptophan concentrations exceed basal requirements for protein and serotonin synthesis (36). The IAAO method indirectly determines the amino acid needs for protein synthesis. Since protein synthesis has a higher metabolic priority than the conversion to niacin (38), the tryptophan requirement determined here may not include the metabolic need for products of the kynurenine pathway.

Finally, the infants in our study were hospitalized patients. The second enzyme shown to be capable of initiating the kynurenine pathway is indoleamine dioxygenase, whose expression and activity is stimulated by cytokines during inflammation (36). Infants with infections may therefore have higher tryptophan requirements than healthy infants (3). Since the infants in the present study had all recovered from illnesses, as demonstrated by their growth rate and clinical status, this was not considered to be an important factor in relation to the estimated requirement value.

The phenylalanine flux of current study was not determined, since previous studies showed no change of the indicator amino acid flux with different test amino acid intakes (13, 39-40)

As determined under the conditions of our study, the mean tryptophan requirement is 15 $mg \cdot kg^{-1} \cdot d^{-1}$. The conditions of the study include consumption of an elemental diet and an adequate intake of niacin. While several studies have demonstrated the enteral requirements of several essential amino acids to be higher than the parenteral requirements (41-44), others have shown in neonatal piglets that the enteral and parenteral requirements of tryptophan appear to be similar to one another (45). This suggests that the gut is not using tryptophan to a great extent. Our estimate may therefore also extrapolate to the requirement of parenterally fed neonates, since many studies show large similarities of human and piglet gut amino acid metabolism(46). Our estimated requirement is lower than the current recommended

intake of 29 mg·kg^{-1}·d^{-1} (10), tryptophan should therefore not be considered a limiting amino acid in present artificial feeding. However, a population variance estimate is required to give a better recommendation for the dietary reference intake.

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CHAPTER 6

Branched-chain amino acids

CHAPTER 6.1

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

Femke de Groof
Lisha Huang
Ineke van Vliet
Gardi J. Voortman
Andras Vermes
Chao Chen
Ying Huang
Johannes B. van Goudoever

provisionally accepted

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 recommendations 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 hours, 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.

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 $mg \cdot kg^{-1} \cdot d^{-1}$ 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 $mg \cdot kg^{-1} \cdot d^{-1}$ 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 (26).

TABLE 1

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

Component	Per 100 g 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.

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 hours. On the study day the subject 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 4 hours of [1-^{13}C]phenylalanine infusion in our previous study (15).

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 labeled 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 hours 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 minutes before beginning 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 minutes starting after 1.75 h during the isotopic steady state of the [^{13}C]bicarbonate infusion and then every 15 minutes during the isotopic steady state of the [1-^{13}C] phenylalanine infusion starting after 3 h, as depicted in Figure 1.

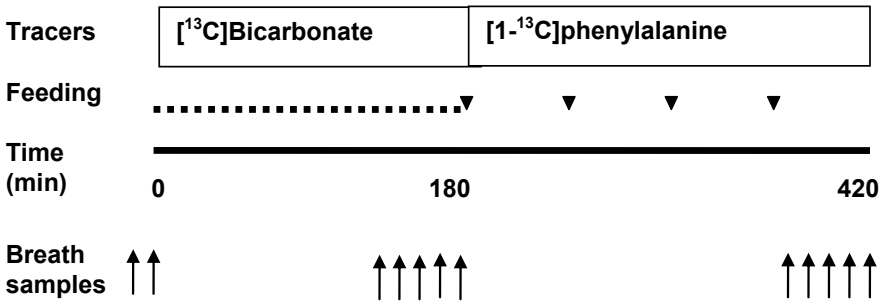

FIGURE 1
Schematic overview of tracer infusion day. Dashed line indicates the period of continuous intragastric feeding. Triangles indicate times that bolus feeding were given via a bottle. Arrows indicate times that breath samples were taken.

▼ time of oral feeding (given every hour)

→ continuous feeding (dripfeeding)

↑ time that breath samples were taken

Analysis and Calculations

Samples were sent from Shanghai to Rotterdam every 3 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 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 ($mmol \cdot kg^{-1} \times h^{-1}$) 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; StataCorp 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 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).

TABLE 2

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

	Mean ± SD
Gestational age at birth (weeks)	39.5 ± 1.2
Age at study (days)	12 ± 5
Birth weight (grams)	3.22 ± 0.41
Weight for age z-score at birth	- 0.25 ± 1.04
Weight at study day (grams)	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

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). 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).

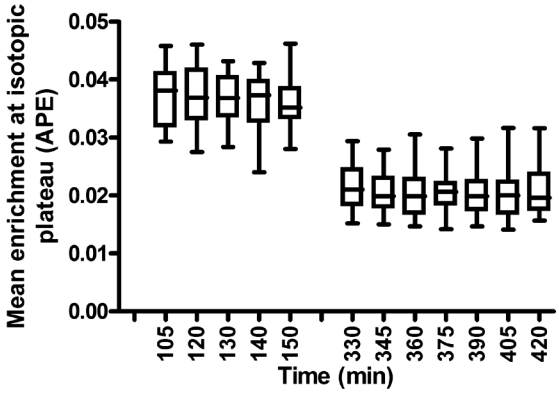

FIGURE 2
Mean ± SD ^{13}C enrichments in APE in expired air at isotopic plateaus: the first plateau is during [^{13}C]bicarbonate infusion (T105-T150) and second plateau is during [1-^{13}C]phenylalanine infusion (T330-420). APE: atom percent excess.

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). 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.

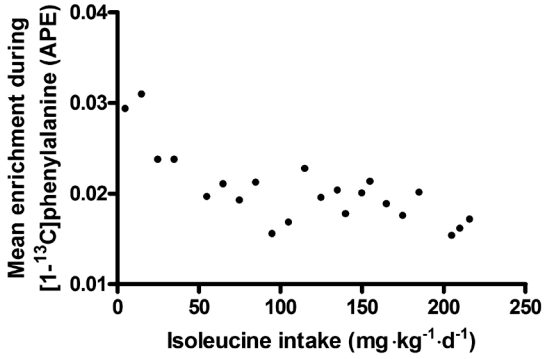

FIGURE 3A
Mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [1-^{13}C]phenylalanine infusion plotted against isoleucine intake (n=22).

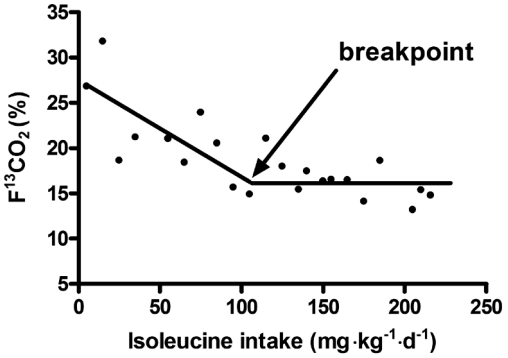

FIGURE 3B
$F^{13}CO_2$ during the isotopic plateau at different isoleucine intakes (n=22). Each infant received a different intake and was studied one time with one intake. With the use of a biphasic linear regression model the breakpoint (mean isoleucine requirement) was estimated to be 105 mg·kg^{-1}·d^{-1} (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 $^{13}CO_2$ 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 to1 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 to1 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 recommendations based on human milk (130 mg·kg^{-1}·d^{-1} vs. 119 mg·kg^{-1}·d^{-1}), and the mean isoleucine requirement determined in the present study was also similar to the recommendations based on human milk (105 mg·kg^{-1}·d^{-1} vs. 95 mg·kg^{-1}·d^{-1}) (15). The mean methionine requirement determined by our group, however, was substantially higher than the estimations based on human milk (38 mg·kg^{-1}·d^{-1} vs. 28 mg·kg^{-1}·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). 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 to1 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 of valine and leucine and the optimal BCAA ratio in term infants to validate current recommendations based on human milk.

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CHAPTER 6.2

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

Lisha Huang
Femke de Groof
Lodi W.C Roksnoer
Gardi J. Voortman
Henk Schierbeek
Jos W.R. Twisk
Andras Vermes
Chao Chen
Ying Huang
Johannes B. van Goudoever

provisionally accepted

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 $mg \cdot kg^{-1} \cdot d^{-1}$ 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 $mL \cdot kg^{-1} \cdot d^{-1}$.

Objective: The objective is to determine leucine requirement of 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 $mg \cdot kg^{-1} \cdot d^{-1}$, as part of an elemental formula. After 24 hours 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 $mg \cdot kg^{-1} \cdot d^{-1}$, with the upper 95% CI of 241 $mg \cdot kg^{-1} \cdot d^{-1}$ (P <0.01, r^2 = 0.26).

Conclusion: For term infants we propose a mean leucine requirement of 140 $mg \cdot kg^{-1} \cdot d^{-1}$, which is in the same range as the amount found in human milk. These data provide more evidence of 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. Firstly, 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 donor 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 acid 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 were 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 of 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, gastro-intestinal 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 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 $mg \cdot kg^{-1} \cdot d^{-1}$ (14). 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 (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.

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.

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 labeled 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-hour period whereby the study formula was fed to the infants. After 24 hours 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 hours to quantify individual CO_2 production rates (23). 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 hours. 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 hours during the adaptation period (the first 24 hours). 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.

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 minutes during isotopic plateau of [^{13}C]bicarbonate between 105 and 180 min. Seven duplicated samples were obtained every 10 minutes 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).

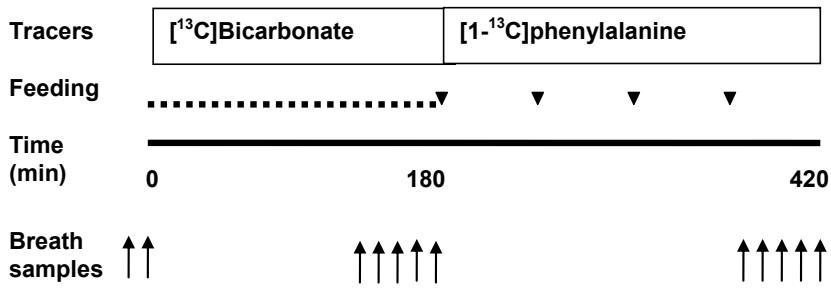

FIGURE 1
Schematic overview of tracer infusion day. Dashed line indicates the period of continuous intragastric feeding. Triangles indicate times that bolus feeding were given via a bottle. Arrows indicate times that breath samples were taken.

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 upper 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 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) $g \cdot kg^{-1} \cdot d^{-1}$. The mean (± SD) energy intake was 109 ± 1 $kcal \cdot kg^{-1} \cdot d^{-1}$. The nitrogen intake was equivalent to a protein intake of 3.0 ± 0.02 $g \cdot kg^{-1} \cdot d^{-1}$. 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.

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 (grams)	13 ± 6
Sex (F:M)	16:17
Protein intake before the study ($g \cdot kg^{-1} \cdot d^{-1}$)	2.8 ± 0.4
Energy intake before the study ($kcal \cdot kg^{-1} \cdot d^{-1}$)	117 ± 15

All values are means ± SDs.

$^{13}CO_2$ enrichments during [13C]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 $mmol \cdot kg^{-1} \cdot h^{-1}$. The mean $^{13}CO_2$ enrichment at isotopic plateau and their corresponding CO_2 production rate of each infant were plotted against the leucine intake (Figure 2).

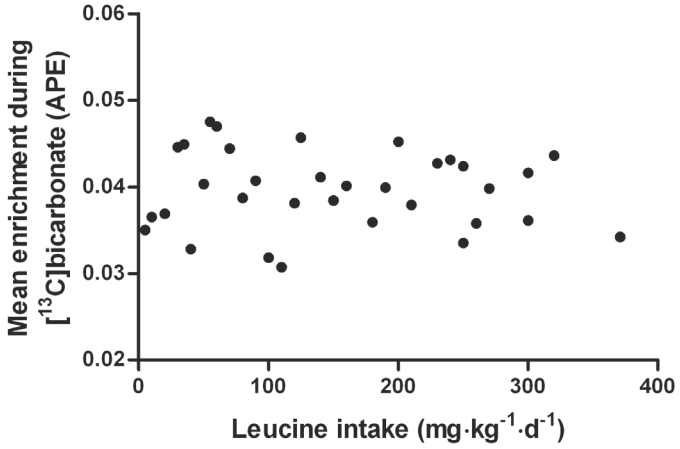

FIGURE 2A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral [^{13}C]bicarbonate infusion of each infant plotted against the leucine intake (n=33). APE, atom percent excess.

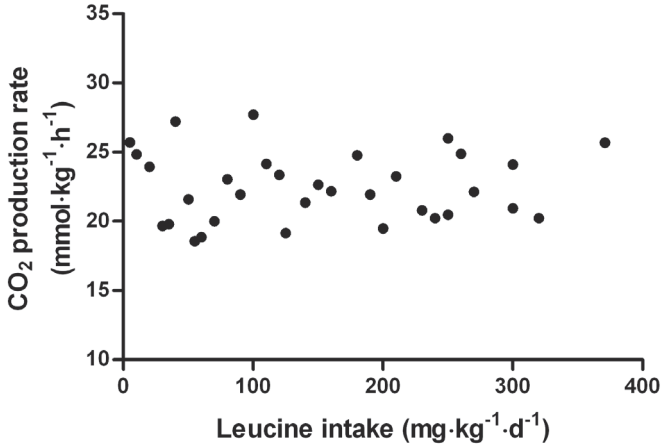

FIGURE 2B
CO_2 production rate of each infant plotted against the leucine intake (n=33).

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 3**. 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 increases in leucine intake did not affect the $F^{13}CO_2$. Using a biphasic linear regression crossover model, the breakpoint representing the mean 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}.

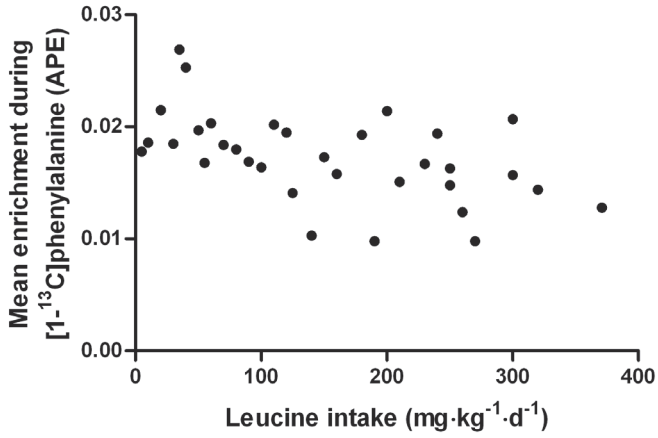

FIGURE 3A
Mean $^{13}CO_2$ enrichment at isotopic plateau during enteral L-[1-13C]phenylalanine infusion of each infant plotted against the leucine intake (n=33). APE, atom percent excess.

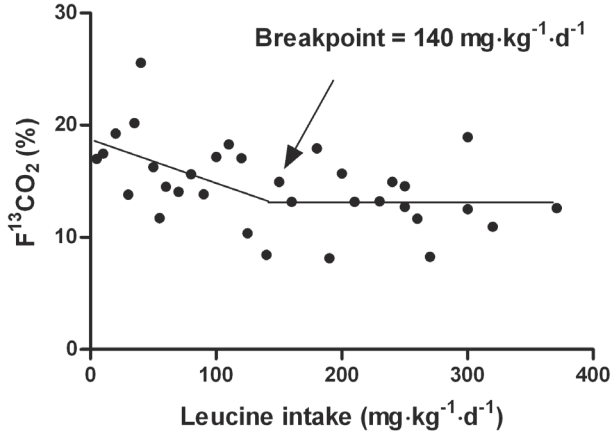

FIGURE 3B
The fraction of $^{13}CO_2$ recovery from L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$) during the isotopic plateau at different leucine intakes (n = 33). Each infant received a different leucine intake. With the use of a biphasic linear regression crossover model, the breakpoint was estimated 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}.

DISCUSSION

Using the IAAO method, the mean leucine requirement of 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 formulae, but it may not necessarily reflect the amino acid needs of infants. Current study provides experimental evidence of leucine requirement of 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 BCAA. BCAA use a common membrane-transport system and enzymes for their reversible transamination into α-ketoacids and irreversible oxidation into α-ketoacyl-CoA (1). Change in 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 of 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. Firstly, 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 is needed. However, the distribution of leucine requirement is unknown. Safe level of leucine intake can be calculated based on the assumption that the safe level of amino acid intake is the same as 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 protein based infant formulas provide 195 ± 15 mg leucine per kg per day at an intake of 150 $mg \cdot kg^{-1} \cdot d^{-1}$ (34), which is in the higher range to the needs. Such amount might be necessary with regards to the great individual variations in leucine requirement shown in the study by Snyderman et al. (13) and in present study. Intakes above the requirement are well tolerated when an appropriate amount of protein with a balanced BCAA are 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 of formula-fed infants, which is required to improve infant nutrition.

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CHAPTER 6.3

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

Femke de Groof
Lisha Huang
Gardi J. Voortman
Andras Vermes
Jos W.R. Twisk
Chao Chen
Ying Huang
Johannes B. van Goudoever

provisionally accepted

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 $mg \cdot kg^{-1} \cdot d^{-1}$) 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 $mg \cdot kg^{-1} \cdot d^{-1}$) as part of an elemental formula. After 1 day adaptation to the study formula, [^{13}C]bicarbonate and [1-^{13}C]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 $mg \cdot kg^{-1} \cdot d^{-1}$ ($r^2 = 0.35$, $P = 0.001$) (upper and lower CIs: 164 and 56 $mg \cdot kg^{-1} \cdot d^{-1}$).

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 of 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, since 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 hours, 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.

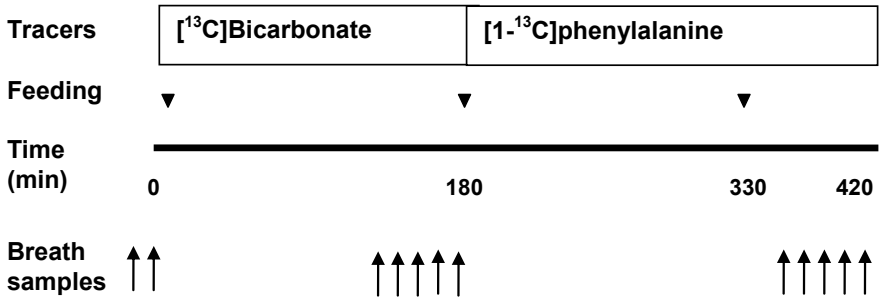

FIGURE 1
Schematic overview of tracer infusion day. Triangles indicate times that bolus feeding were given via a bottle. Arrows indicate times that breath samples were taken.
▲: time of oral feeding (every 3 hours)
↑: time that breath samples were taken

In our protocol the infants adapt to the study formula for 24 hours. The natural enrichment of stable isotopes is normally accounted for by taking baseline samples prior to the start of 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. 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 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·kg^{-1}·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·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 (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 hours.

TABLE 1

Energy, carbohydrates, fat and amino acid content of the study formula

Component	*Per 100g 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)	0.26
L-Phenylalanine (g) [2]	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)[3]	0
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 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.
[2] 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.
[3] L-valine was added separately, depending on the test valine level.
[4] The study formula was based on Neocate (Danone, United Kingdom), an amino acid based formula

Tracer protocol

On the study day 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 3 h to quantify individual CO_2 production. The labeled sodium bicarbonate infusion was directly followed

by a primed (27 µmol·kg), continuous (27 µmol·kg^{-1}·h^{-1}) enteral infusion of [1-^{13}C] phenylalanine (99% ^{13}C APE; Cambridge Isotopes, Woburn, MA) for 5 hours 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.

Sample collection

Breath samples of the first 8 patients were collected before start of the study formula at the adaptation day and every 30 minutes for 8.5 hours 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 hours of adaptation was achieved. At the study day, baseline samples were obtained 15 and 5 minutes before starting tracer infusion. Duplicate ^{13}C-enriched breath samples were then collected every 10 minutes during the isotopic steady state of the [^{13}C]bicarbonate infusion starting after 1.75 hours, and next every 15 minutes during the isotopic steady state of the [1-^{13}C]phenylalanine infusion starting after 3 hours 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·kg^{-1}·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 $mmol \cdot kg^{-1} \cdot h^{-1}$.

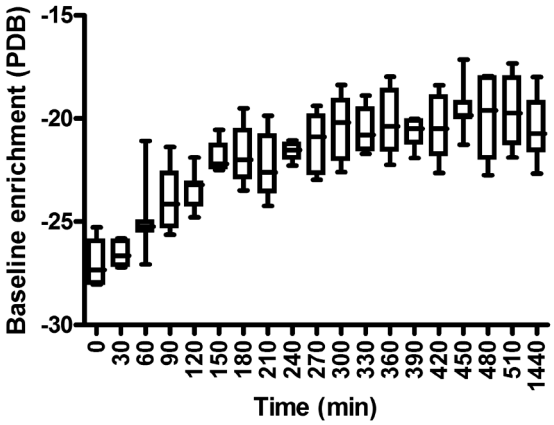

FIGURE 2
Background enrichment adaptation versus time (in PDB) to the study formula (n=8), expressed as box plots. PDB: pee dee belemnite.

The mean $^{13}CO_2$ enrichment at isotopic plateau and the corresponding CO_2 production rate were plotted against the valine intake (Figure 3). No correlation was found between valine intake and the mean $^{13}CO_2$ enrichment at isotopic plateau (P = 0.46, Figure 3A) and between the valine intake and the CO_2 production rate (P = 0.37, Figure 3B).

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.

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}.

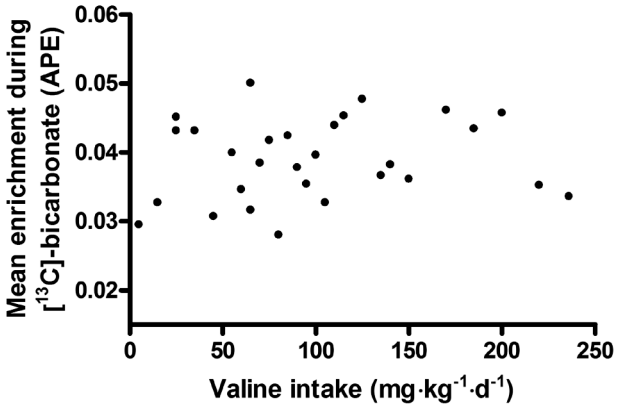

FIGURE 3A
Mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [^{13}C]bicarbonate infusion plotted against valine intake (n=28).

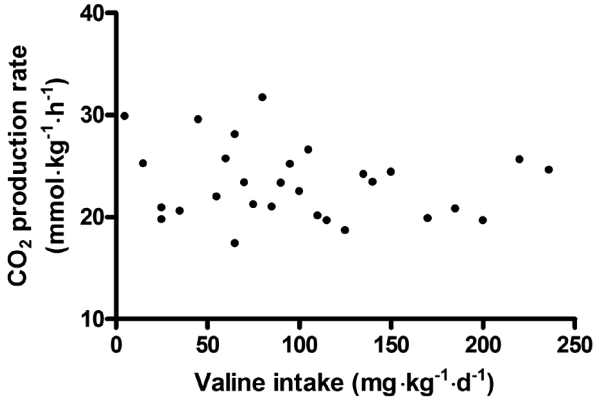

FIGURE 3B
The CO_2 production rate of each infant plotted against the valine intake (n=28).

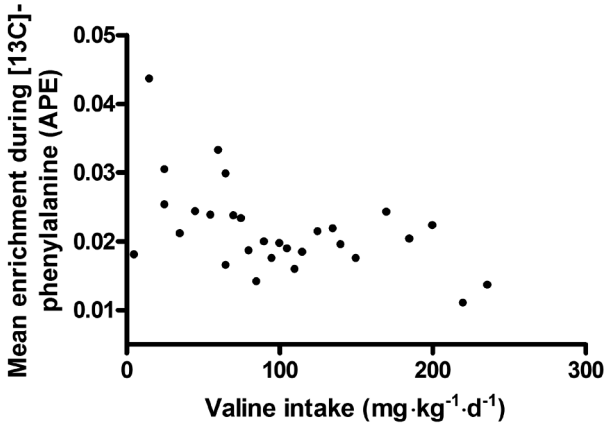

FIGURE 4A
Mean $^{13}CO_2$ enrichment of each infant at isotopic plateau during [1-^{13}C]phenylalanine infusion plotted against valine intake (n=28).

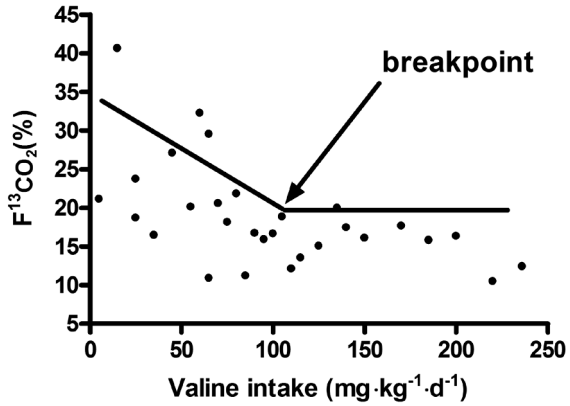

FIGURE 4B
$F^{13}CO_2$ plotted against increasing valine intakes (n=28). A biphasic linear regression model estimated the breakpoint (mean valine requirement) to be 110 mg·kg^{-1}·d^{-1} (r^2 = 0.35, *P* = 0.001). The upper CI was 164 mg·kg^{-1}·d^{-1} and the lower was 56 mg·kg^{-1}·d^{-1}. APE: atom percentage excess; $F^{13}CO_2$: fraction of $^{13}CO_2$ 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·kg^{-1}·d^{-1} which declines to 0.9-1.0 g·kg^{-1}·d^{-1} at 5-6 months (9, 10). 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 (30) 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}) (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 ·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 hours, or 4.7 hours 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 minutes 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 hours 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 6.4

The enteral requirement of branched-chain amino acids for the neonate: search for the ideal dietary composition

Femke de Groof
Lisha Huang
Johannes B. van Goudoever

submitted

ABSTRACT

Introduction: Higher protein intake for infants that are fed formula may play a role the development of 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 formula.

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 of age 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 results imply that egg protein might be a better alternative protein regarding the BCAA ratio for infants 0 aged 1 month than cow milk protein. 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) (4-6) and increased risk of diabetes (7). Higher protein intake for infants that are fed formula 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, 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 of infants by means of the nitrogen balance method and weight gain (13-16). Since 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 0-1 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 assume that the average intake of breastfed infants does not approximate the mean requirement and state 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 0-1 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 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 indicator amino acid oxidation method may be useful in re-evaluating amino acid and protein requirements in children and infants (Jackson, state-of the art). 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 by 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 0-1 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 of isoleucine for infants 0-1 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 $mg \cdot kg^{-1} \cdot d^{-1}$ (14).

By using the factorial approach, Dewey et al. determined the isoleucine requirement in infants 0-1 month to be 59 $mg \cdot kg^{-1} \cdot d^{-1}$ 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 was considered as the best estimate of amino acid requirement for this group. The current recommended isoleucine requirement, based on human milk is 95 $mg \cdot kg^{-1} \cdot d^{-1}$ in infants 0-1 months

(21). By 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 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 0-1 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.

The requirement for valine in infants 0-1 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). By using the factorial approach, Dewey et al. determined the valine requirement in infants 0-1 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 0-1 months (21). Our group determined the mean valine requirement in infants 0-1 month to be 110 mg·kg^{-1}·d^{-1} by 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). 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 0-1 month of age. 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 1

The estimated BCAA requirements by using different methods, the current recommended intakes for infants up to one month of age (21), the contents of current infant formulas per 150 mL·kg-1·d-1 (39-42) and the recommend intakes by using the IAAO method. Expressed in mg·kg-1·d-1.

Amino acid (mg·kg^{-1}·d^{-1})	Nitrogen retention and weight gain (13-15, 54)	Factorial approach (20)	IAAO	Current recommen-dations	Contents of current formulas	Recommended intakes by 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

The requirement of leucine in infants for 0-1 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}). 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}(45), lower intakes were not given. The factorial approach estimated the requirement for term neonates aged 0 to1 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 to1 months (21). 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 (39-42). 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 labeled 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 labeled 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 0-1 month of age 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} (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}·d^{-1} is given. 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 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 0-1 month of age 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 0-1 months 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 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 (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 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) 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 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 formula 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.

TABLE 2

The current recommendations based on the content in human milk (44)(59), the content in regular infant formulas (39-42) and the recommendations based on the IAAO method. Expressed in mg per 100 kCal.

Amino acid (mg per 100 kCal)	Human milk	Current Term formulas	IAAO
Isoleucine	90-92	105-190	97
Leucine	166-169	167-338	130
Valine	88-90	106-213	102
Ile:Leu:Val ratio	1: 1.9:1	1:1.4:0.9-1:2.3:1.2	1: 1.3:1

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 contain 2.5 time more BCAAs than current recommendations and provide too much leucine which could be associated with the development of 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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CHAPTER 7

General discussion

Breastfeeding is the optimal feeding for infants and the health benefits are well-established (1). Despite the increasing breastfeeding rates, less than 50% of infants are exclusively breastfed at 3 months of age (2-4). Infants who are not breastfed require infant formulas satisfying all their nutritional requirements during this critical period of life.

Protein is essential for growth and development. Amino acids are building blocks of proteins, as well as precursors of hormones and low-molecular weight nitrogenous substances, cell signaling molecules and regulators of gene expression. Because of the numerous physiological functions and the potential risks of unbalanced intake, it is important to know the exact essential amino acid requirements (2-3). In infants, data of amino acid requirements are very limited.

In the 1950s, Holt and Snyderman (4) attempted to define the essential amino acid requirements of healthy infants using the nitrogen balance technique and the measurement of growth. Infants in good health were fed a full synthetic diet. The test amino acid was removed completely from the diet and gradually increased. Each test intake was fed for a period of ~1 week. Each infant received 2-5 intakes. The number of infants studied per test amino acid is relatively small (n= 5-8). Their results are shown in table 1. As described in chapter 1, these methods have many drawbacks. As a result, these estimated requirement values might be unprecise. Because of these limitations, the data derived from these studies are not used in the recommendations of the essential amino acid requirements in infants by the FAO/WHO/UNU in 2007 (5).

The current essential amino acid recommendations of infants in the first 6 months of life are based on human milk composition (5). These are shown in table 1. Although human milk is assumed to meet all the amino acid requirements of the infant, this amount may not necessarily reflect the requirement of formula-fed infants since the digestibility and bioavailability of breast milk proteins/amino acids are different to those in formula. In addition, the accuracy of the estimation is challenged by the huge variations in protein/amino acid concentrations during the lactation period and between mothers, the day-to-day variation in volume, and thus protein/amino acids intake of infants and between infants (6-8). Therefore, more accurate knowledge of the essential amino acid requirements is needed.

Stable isotope approaches are considered valid for the assessment of protein and amino acid kinetics in human (9-10). These approaches provide insight in the metabolic processes within the body rather than the static information by determination of concentrations. Carbon-13 (has one extra neutron in the nucleus) is already present at low levels in tissues and food, is not radioactive and considered safe. Carbon-13-labeled amino acids have been used in children, infants and pregnant women (11-13). The recently adapted stable isotope approach, termed the indicator amino acid oxidation method (IAAO) is minimally invasive, accurate and reliable (14). The IAAO method has been validated in a piglet experiment (15), in which the L-[1-14C] phenylalanine oxidation was shown to be inversely related to protein synthiesis. It has been successfully used to re-evaluate protein and amino acid requirements in adults, children and neonates (16-21). We have extended the knowledge of essential amino acid requirements in enterally fed term infants using the IAAO method. In the following section we discussed the findings presented in this thesis, how our findings integrate with the current recommendations, the limitations of current study design, and considerations for future research topics.

Essential amino acid requirements in infants determined using the IAAO method.
The lysine requirement is assessed (chapter 3) by measuring the oxidation rate of the indicator amino acid L-[1-^{13}C]phenylalanine in expired air as $^{13}CO_2$ ($F^{13}CO_2$). Additional measurements of the L-[1-^{13}C]phenylalanine flux in urine and blood support the validity of the adapted IAAO method in enterally fed infants. All three methods ($F^{13}CO_2$, urine and plasma L-[1-^{13}C]phenylalanine enrichments) yielded similar estimates. However, since L-[1-^{13}C]phenylalanine enrichment in plasma do not truly represent the precursor pool from which protein synthesis takes place, which is intracellular, $F^{13}CO_2$ is considered to be more accurately represent the lysine requirement. We therefore performed our following studies using only $F^{13}CO_2$.
The results of the IAAO studies (chapter 4, 5 and 6) are summarized in table 1 and they were compared with estimates by earlier studies (using weight gain and nitrogen retention technique) and the intakes of breastfed infants (4-5).

TABLE 1

Essential amino acid intakes in exclusively breastfed infants up to 1 month of age (5), the estimated requirements by Snyderman et al. (4) using nitrogen retention and weight gain, and by our group using the Indicator Amino Acid Oxidation method.

Amino Acid ($mg \cdot kg^{-1} \cdot d^{-1}$)	Based on human milk iontake[a]	Based on nitrogen retention and weight gain	Based on IAAO method (present thesis)
Lysine	119	103	130
Methionine	28	45[b]	38[b]
Isoleucine	95	119	105
Leucine	165	150	140
Valine	95	105	110
Threonine	76	60[c]	68
Tryptophan	29	22	15
Histidine	36	34	N.D.
Phenylalanine	72	90[d]	N.D.

- N.D. = not determined.
- [a] Amino acid intakes of exclusively breastfed infants at one month of age (5).
- [b] In presence of cysteine
- [c] Pratt et al. (22)
- [d] In presence of tyrosine

These data provide valuable information toward the development of an infant formula that better meets the requirement of a formula-fed infant. The accretion of body protein in infants is for an important part dependent upon the optimal intake of all of the essential amino acids. If the pattern of amino acids in the diet is not ideal, then the rate of protein synthesis will be suboptimal and determined by the first limiting essential amino acid. Deficiency will impair growth, physiological functions and development. On the other hand, excessive intake is a metabolic burden on the immature organ systems of the newborn and has been found to be associated with increased kidney size (23-24) and increased obesity risk (25-28).

Human milk of a healthy well-nourished mother is the ideal source of nutrition for infants. As showed in table 1, the gross amino acid composition of human milk does not reflect the amino acid requirement profile of formula-fed infants. Apart from the huge variations in composition in human milk, there are considerable differences in the bioavailability and physiological effects of similar contents of nutrients in human milk compared to infant formula (29-30).

Current studies present experimental evidence of essential amino acid requirements of Chinese infants. The derived values are expected to be valid for all infants independent of the ethnicity. As shown with the new growth charts of WHO, children born in different regions of the world have the potential to grow within the same range of height and weight for age (31). This assumption is supported by the amino acid requirement studies performed in Canadian and Indian children (13, 17). Lysine requirement of healthy school-aged Indian children was 33.5 $mg \cdot kg^{-1} \cdot d^{-1}$, which was similar to that of Canadian children (35 $mg \cdot kg^{-1} \cdot d^{-1}$), determined by using the IAAO method.

FUTURE PERSPECTIVES

(Conditionally) Essential amino acid requirements at different gestational ages and in different populations

The derived values of essential amino acid requirements of adults by the use of the IAAO method have led to a change in recommendations in the 2007 FAO/WHO/UNU report (5).

The adapted IAAO method is minimally invasive and therefore suitable for use in vulnerable populations. We simplified and shorten the method and applied it to enterally fed term infants to estimate the lysine, methionine, branched chain amino acids, tryptophan and threonine requirements. These studies will be extended to other (conditional) essential amino acids in the term and preterm infants by our group.

Several amino acid requirements are already successfully determined in other vulnerable groups, such as in enterally-fed preterm infants (21, 32), parenterally fed infants (13, 19, 33), children with metabolic diseases (34-36), and children with severe childhood undernutrition (37).

The population distribution of essential amino acid requirements

The current study design has allowed us to determine the mean requirement of the test amino acid. The mean requirement is defined as the estimated average requirement (EAR); it meets the requirement of 50% of test population. To give a

recommendation for the dietary reference intake (DRI); an intake that meets the requirement of ~97.5% of the population, the population variance has to be define. To determine the population variance, the requirement of each subject has to be determined using the repeated measures (38).

The length of adaptation to the test diet is a concern in repeated measures using current study protocol. Adaptation allows the metabolism to adjust to the change of diet. In case of the nitrogen balance technique, a balance approach measuring the nitrogen intake and nitrogen excretion, it required at least 7 days before nitrogen excretion reflected the dietary change of intake (39). The IAAO method, in contrast, measures the partitioning of an essential amino acid to protein synthesis or oxidation; therefore altered intake of essential amino acids has direct effect on the tissue amino acid concentrations and therefore on the rate of amino acid oxidation. This is due to the high Km of various amino acid degrading enzymes in combination with low Km of aminoacyl-tRNA synthetases. The amino acid degrading enzymes are rarely saturated. This results to the high efficiency of channeling amino acids into protein synthesis even at low amino acid intakes and higher oxidation rates at excess intakes (40-41). Recent studies show no adaptation is required except for the baseline enrichment of the diet, which is at equilibrium at the 6th hourly feeding (42-43). Thereafter, oxidation studies can be performed after the isotopic equilibrium has reached (4-5 hours determined in chapter 6.3). Taken together, future studies with repeated measures of each test intake every 4-5 h period within one subject will enables the determination of the dietary reference intake in vulnerable populations such as infants.

Ongoing effort to minimizing the invasiveness of the method should be taken. Infants of current studies were bottle-fed on hourly basis, while the tracer was administered continuously via an intragastric tube. Studies in children showed reliable results when tracers were ingested hourly. We might considered hourly bottle feed where the tracer is already dissolved in the formula.

Metabolic demand for amino acids

Metabolic demand for amino acids is the demand for maintaining the structure and function of the body (5). This includes protein synthesis and the conversion of certain

amino acids into important metabolites, such as tryptophan as a precursor for synthesis of the neurotransmitter serotonin, methionine as a precursor for synthesis of polyamines, and histidine for histamine synthesis (44). If protein synthesis of these amino acids has the priority over the synthesis of their metabolites, the IAAO method will only give an estimate of the requirement for protein synthesis. Therefore, the evaluation of the adequacy of a diet should also include functional outcomes, i.e. growth pattern, infection risks, neurodevelopmental outcome and chronic diseases in later life. Applying the results from the IAAO studies on long term trials are required to study the effect on growth, body composition, function and development.

Antibiotic use

The infants of the current studies had been hospitalized for illnesses. At the time of the study, all had recovered and were gaining weight. However, most of them still received antibiotics. Antibiotics use have an effect on the gut microbiota. Isotope studies in human have been shown that the gastrointestinal microflora was able to synthesized essential amino acids *de novo*, and which it can be made available to a human host (45-47). Raj et al. (47) attempted to quantify the microbial leucine contribution to body leucine input by measuring the change in oxidation before and after antibiotic treatment. The microbial leucine contribution is estimated to be between 19 and 22%. This estimated range is the decrease in leucine input into the body due to antibiotic treatment calculated from the relationship of leucine oxidation to leucine intake and the reduction in leucine oxidation after antimicrobial treatment. However, no data have been published in the change of essential amino acid requirement before and after antibiotic treatment.

Use of free amino acid formula

In the ideal experiment a single variable can be manipulated at a time. The use of free amino acid formula makes it possible to manipulate one amino acid intake while keeping the nitrogen intake and the other essential amino acid intakes constant. A disadvantage of an elemental diet is that the same amount in weight of an elemental mixture does not provide the same amount of proteins. An elemental diet provides on 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 (48). In addition, the absorption rate of amino acids from an elemental diet is different from a protein diet. Amino acids of an elemental diet are absorbed more rapidly and appear more quickly in the plasma compared to amino acids from intact proteins (49-50). Absorption rate is shown to be an independent regulating factor of postprandial protein/amino acid retention rate (49). Metges et al. (51) showed a 22% higher leucine oxidation rate and 38% lower non oxidative disposal (ie protein synthesis) when an elemental diet is ingested compared to a protein diet.
All these factors might contribute to the overestimation the actual requirement when using a free amino acid formula. Future IAAO study, using a protein bound diet with an intrinsic labelled indicator amino acid is required to evaluate the effect of free amino acid diet versus protein bound diet on the requirement estimates.

Conclusions

In conclusion, knowledge of amino acid requirements is of fundamental importance in infancy. Nutrition during this critical period of life not only influence growth and development, but also determines the long term health status. Current studies provide more scientific evidence of essential amino acid requirements in infants, which data are very limited. Ongoing effort is needed to extent the knowledge of amino acid requirements at different gestational age using the current method. The derived outcomes need to be applied in long term trials to study the effect on growth, body composition, function and development. In addition, research on methodology needs to include studies using different diet forms.

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SUMMARY

The growing body of evidence suggests that growth during early life is an important determinant of later health and the risk of chronic diseases. It emphasizes the importance of nutrition at this stage of life. To improve nutrition, it is critical to address the nutrient requirements. Amino acids are important nutrients. They are precursors for the synthesis of proteins and other biological important substances. **Chapter 1** describes the background of this thesis. We give an introduction to amino acids, its recommendations and the methods used to determine amino acid requirements.

This research project is a collaborative project between the Children's Hospital of Fudan University in Shanghai and the Erasmus MC-Sophia children's Hospital in Rotterdam. In **chapter 2**, we describe the realization of this collaborative research project in China, the difficulties of setting up this project and the Chinese versus the Dutch view of this collaboration.

Studies to determine the amino acid requrements in infants are difficult to perform due to ethical and practical considerations. The method must be safe and non-invasive. The indicator amino acid oxidation (IAAO) method, using stable isotopes is a safe method. In **chapter 3**, we validated the use of IAAO method in enterally fed full term infants in the first month of life by determining the lysine requirement of enterally fed neonates.
Lysine, an essential amino acid, is mainly required for protein synthesis. Moreover, lysine, together with methionine, is required for biosynthesis of carnitine, which is essential in fatty acid metabolism. We estimated the requirement by using isotopic enrichment in expired air, in urine and in plasma. These methods yielded similar mean requirement of 130 $mg \cdot kg^{-1} \cdot d^{-1}$. Therefore, we concluded that only sampling expired air was sufficient to estimate the requirement. Furthermore, the minimal tracers infusion time was determined to enable measurements over a shorter period of time and, therefore, more practical. These advances in methodology have allowed a less invasive and more practical approach to study essential amino acid requirements in enterally fed infants.

With the use of the minimally invasive IAAO method validated as described in chapter 3, a series of studies was performed to determine the requirements of the essential amino acids. These studies are described in the following chapters.

Chapter 4 describes a study of methionine requirement. Methionine is a protein substituent, a precursor required for carnitine synthesis, a major methyl donor in mammalian cells and a precursor for polyamine synthesis. Transmethylation of methionine leads to homocysteine. 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 are essential for host defense against oxidative stress.
Deficient intake of methionine impairs growth and has 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. We determined the methionine requirement in 33 infants to be 38 $mg \cdot kg^{-1} \cdot d^{-1}$. The estimated requirement was under the condition that an excess of cysteine (91 $mg \cdot kg^{-1} \cdot d^{-1}$) was provided in the diet.

In **chapter 5**, tryptophan requirement has been determined. Tryptophan is a precursor of the neurotransmitter serotonine and neurosecretory hormone melatonine. Dietary tryptophan intake has been shown to modulate sleep pattern in infants. The mean tryptophan requirement was estimated to be 15 $mg \cdot kg^{-1} \cdot d^{-1}$ when a niacin intake of 1.02 $mg \cdot kg^{-1} \cdot d^{-1}$ was provided.

Chapter 6.1-6.3 describes studies of isoleucine, leucine and valine requirement of enterally fed infants. Isoleucine, leucine and valine are the branched-chain amino acids (BCAAs). BCAAs account for an important part (35-40%) of the essential amino acids found in body protein. Their main metabolic faith is protein synthesis. In addition, BCAAs have metabolic effects that are unique. Leucine regulates the protein turnover by acting as a nutrient signal stimulating protein synthesis. BCAAs can affect the transport of large neutral amino acids, and thereby influence central nervous system concentrations of neurotransmitters.

The mean isoleucine, leucine and valine requirement were determined to be 105, 140 and 110 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively. **Chapter 6.4** gives a review how our result integrates in current recommendation based on BCAA ratio of human milk. The isoleucine:leucine:valine ratio of human milk is 1:1.7:1. The optimal isoleucine:leucine:valine ratio determined by the use of IAAO method is 1:1.3:1, which is comparable with the BCAA ratio in egg protein. Therefore, we concluded that egg protein has a BCAA ratio according to the requirement of bottle fed infants.

Chapter 7 provides a general discussion about the findings of our studies and how our findings integrate in the current recommendations based on human milk composition and the estimated amino acid intake of the breastfed infant. Our studies provide more experimental evidence of amino acid needs of infants fed an infant formula. The determined amino acid requirements differ partly from current recommendations. Factors such as protein digestibility, amino acid bioavailability, accuracy of estimation in breastfed infants may contribute to the differences. Therefore we conclude that current amino acid recommendations do not reflect the amino acid-requirement profile of infants who consumed an infant formula. The derived results from the IAAO studies need to be applied in long term trials.

SAMENVATTING

Er is toenemend wetenschappelijk bewijs dat de groei tijdens de vroege fase in het leven van een kind mede bepalend is voor de gezondheid op latere leeftijd en het risico op chronische ziekten. In deze fase van de groei is evenwichtige voeding essentieel. Kennis van behoefte aan voedingsstoffen is van noodzakelijk belang voor de samenstelling van evenwichtige voeding. Aminozuren zijn belangrijke voedingsstoffen. Het zijn bouwstenen van eiwitten en andere biologische belangrijke stoffen. **Hoofdstuk 1** begint met een inleiding over aminozuren. Verder zijn de aanbevelingen voor aminozuren en methoden om deze behoeften te bepalen besproken.

Het onderzoek beschreven in dit proefschrift is een samenwerkingsproject tussen het kinderziekenhuis van de Fudan Universiteit in Shanghai en het Erasmus MC-Sophia kinderziekenhuis in Rotterdam. De realisatie van dit gezamenlijke onderzoeksproject, de uitdagingen van het opzetten van dit project en de Chinese en de Nederlandse visie van deze samenwerking zijn beschreven in **hoofdstukken 2.1 en 2.2**.

Het bepalen van de behoefte aan aminozuren bij kinderen is door ethische en praktische overwegingen moeilijk uitvoerbaar. De gebruikte methode moet veilig en non-invasief zijn. De indicator aminozuur oxidatie (IAAO)-methode is een veilige methode die gebruik maakt van stabiele isotopen. Dit wordt beschouwd als een veilige methode. In **hoofdstuk 3** is de validatie van de IAAO-methode in enteraal gevoed voldragen zuigelingen van minder dan een maand oud beschreven. In deze validatie-studie is de behoefte aan lysine bepaald. Lysine is een essentieel aminozuur; het kan niet door het lichaam gemaakt worden en moet dus in de voeding aanwezig zijn. Lysine is vooral nodig voor de eiwitsynthese. Bovendien is lysine samen met methionine vereist voor de biosynthese van carnitine. Carnitine is een essentieel element in het mitochondriale vetzuurmetabolisme ten behoeve van energievoorziening.
De lysine behoefte werd bepaald met de IAAO-methode door middel van isotopenverrijking in de uitademingslucht, in de urine en in het plasma te bepalen.

Alle drie bepalingen gaven een gemiddelde lysine behoefte van 130 $mg \cdot kg^{-1} \cdot d^{-1}$. Wij concludeerden dat het bepalen van de isotopenverrijking in uitademingslucht voldoende was om de behoefte te bepalen. Bovendien werd het studieprotocol verkort door de minimale isotopeninfusietijd te bepalen. Deze methodologische ontwikkelingen hebben tot gevolg dat het bestuderen van essentiële aminozuren behoefte bij enteraal gevoede zuigelingen minder invasief is. Met het gebruik van deze aangepaste IAAO-methode werd vervolgens een reeks onderzoeken gedaan naar de andere essentiële aminozuurbehoeften bij zuigelingen **(hoofstuk 4-6)**.

In **hoofdstuk 4** is het onderzoek naar methionine behoefte beschreven. Methionine is nodig voor eiwitsynthese. Tevens is methonine een precursor voor carnitine synthese, een methyl donor in zoogdiercellen en een precursor voor polyamine synthese. Door demethylering van methionine ontstaat homocysteïne. Homocysteïne kan worden geremethyleerd tot methionine of worden afgebroken tot cysteine. Cysteïne is betrokken bij de eiwitsynthese en bij de productie van glutathion, taurine, co-enzym A en anorganische zwavel. Cysteine, glutathione en taurine zijn cruciaal voor de anti-oxidatieve functies in het lichaam. Een tekort aan methionine vertraagt niet alleen de groei bij kinderen, maar heeft ook een impact op het zwavel metabolisme en de synthese van belangrijke metabole intermediairen. Anderzijds staat methionine bekend als het meest toxische aminozuur in dieren bij een overtollige inname. Daarom is het van belang dat de methionine behoefte van een zuigeling exact bepaald wordt. De methionine behoefte werd geschat bij 33 zuigelingen op 38 $mg \cdot kg^{-1} \cdot d^{-1}$. De schatting van methionine behoefte is gebaseerd op een meer dan voldoende cysteine intake in de voeding (91 $mg \cdot kg^{-1} \cdot d^{-1}$).

De behoefte aan tryptofaan werd in **hoofdstuk 5** beschreven. Tryptofaan is een precursor van de neurotransmitter serotonine en het hormoon melatonine, en is betrokken bij onder andere de regulatie van het slaap-waakritme. De hoeveelheid tryptofaan in de zuigelingenvoeding heeft een bewezen modulerend effect op de slaappatroon. De gemiddelde tryptofaanbehoefte werd geschat op 15 $mg \cdot kg^{-1} \cdot d^{-1}$ bij een niacine inname van 1.02 $mg \cdot kg^{-1} \cdot d^{-1}$.

Hoofdstukken 6.1-6.3 beschrijven de verrichte onderzoeken naar de behoefte aan isoleucine, leucine en valine. Isoleucine, leucine en valine zijn de vertakte ketenaminozuren, ofwel 'branched-chain amino acids' (BCAA's). BCAA's vormen een belangrijk aandeel (35-40%) van de essentiële aminozuren in de lichaamseiwitten. BCAA's dienen met name voor eiwitsynthese. Daarnaast hebben BCAA's unieke metabole effecten. Een voorbeeld is leucine, welke de eiwit turnover regelt. Daarnaast kunnen BCAA's het transport van andere grote neutrale aminozuren door de bloed-hersenbarrière beïnvloeden en daarmee de neurotransmitters concentraties in het centrale zenuwstelsel beïnvloeden. De gemiddelde behoefte aan isoleucine, leucine en valine werd geschat op respectievelijk 105, 140 en 110 $mg \cdot kg^{-1} \cdot d^{-1}$. **Hoofstuk 6.4** is een review waarin de BCAA's, de ideale BCAA verhouding volgens de IAAO-methode en de wijze waarop onze resultaten integreren in de huidige aanbeveling op basis van BCAA verhouding van de moedermelk werden beschreven. De isoleucine op leucine op valine-verhouding van de moedermelk is 1 op 1.7 op 1. De optimale isoleucine: leucine op valine verhouding die bepaald is aan de hand van de IAAO-methode is 1 op 1.3 op 1. Deze verhouding is vergelijkbaar met de BCAA verhouding in ei-eiwit. Hieruit kan worden geconcludeerd dat ei-eiwit een BCAA verhouding heeft die past bij de behoefte van zuigelingen die flesvoeding krijgen.

In **hoofdstuk 7** worden de bevindingen in dit onderzoek besproken in de context van literatuur en de huidige aanbevelingen. Onze studies bieden experimenteel bewijs voor de aminozuurbehoefte van zuigelingen die een zuigelingenvoeding krijgen. De vastgestelde aminozuurbehoefte verschillen van de huidige aanbevelingen. De huidige aanbevelingen zijn een schatting van de samenstelling van moedermelk en de hoeveelheid die door zuigelingen wordt ingenomen. Factoren zoals verteerbaarheid van het eiwit, de biologische beschikbaarheid en de accuratie van de inname schatting, kunnen bijdragen aan deze verschillen. De conclusie is dat de huidige aanbeveling voor een deel niet de aminozuurbehoefte van zuigelingen die zuigelingenvoeding krijgen vertegenwoordigt. De verkregen resultaten met de IAAO methode moeten worden toegepast in lange termijn studies.

章节简介

第1章

如今有越来越多的科学实验证据表明,儿童在早期生长状况与其今后的健康以及各种慢性疾病的发生有着极其重要的联系,而早期营养摄取至关重要。准确了解各种营养素的生理需求量是改善营养的关键所在。蛋白质是人体必需的重要营养成分之一,而氨基酸是蛋白质的基本组成单位,是构成营养所需蛋白质的基本物质。

本章主要介绍了本研究背景,各氨基酸的介绍,以及国际卫生组织对必需氨基酸需求量的建议和测量必需氨基酸需求量的不同方法。

第2章

此项研究是由上海复旦大学儿科医院和荷兰鹿特丹索非亚儿童医院合作进行的。本章主要介绍了次此合作在中国的实施过程和在实验准备阶段时所遇到的困难,以及中国研究者和荷兰研究者对这一合作的各自观点。

第3章

基于伦理和实际可操作性,婴儿氨基酸生理需要量方面的研究非常困难。实验方法必须为无创的、安全的。氨基酸标志物氧化法(IAAO)是一种使用稳定同位素的安全方法。本章节我们使用IAAO测定经肠道全量喂养的足月新生儿的赖氨酸的生理需求量。

赖氨酸是一种必须氨基酸,主要用于蛋白质的合成。此外赖氨酸和蛋氨酸为肉碱合成所必需,而肉碱是脂肪代谢过程中的关键物质。通过分别测定呼气、尿液和血浆中同位素的浓度计算氨基酸的生理需求量,这三种方式测得的新生儿对赖氨酸的需求量均为130mg/kg/d,所以只需采用呼气方法足以测定氨基酸的需求量。而且最少量标记物输注使得测量时间更短、更具操作性以及无创。

使用安全IAAO方法,我们测定了新生儿对一系列必需氨基酸需求量,具体见如下章节。

第4章

本章节描述了蛋氨酸生理需求量的研究。蛋氨酸是肉碱合成前体,哺乳动物细胞甲基的主要供体,多肽合成的前体。蛋氨酸去甲基化形成高半胱氨酸，高半胱氨

酸甲基化形成蛋氨酸,或通过反式硫化通路形成半胱氨酸。半胱氨酸参与组成蛋白,参与合成谷胱甘肽、辅酶A以及无机硫。半胱氨酸、谷胱甘肽是宿主抗氧化的必需物质。

蛋氨酸摄入不足影响生长,影响主要代谢中间产物的硫代谢通路。然而,过量蛋氨酸被证实为最具毒性氨基酸。通过本实验,我们认为新生儿对蛋氨酸的需求量是38mg/kg/d,低于目前奶粉中半胱氨酸的含量91 mg/kg/d 。

第5章

本章节测定了色氨酸的需求量,色氨酸是神经递质5-羟色胺和神经分泌激素褪黑素的前体物质。饮食中的色氨酸被认为与婴儿睡眠节律调节相关。当饮食中烟酸摄入量为1.02mg/kg/d时色氨酸需求量的平均值为15 mg/kg/d。

第6章

本章我们测定了新生儿对异亮氨酸的需求量是105mg/kg/d,亮氨酸的需求量为140 mg/kg/d。缬氨酸的需求量是110mg/kg/d。异亮氨酸,亮氨酸和缬氨酸为支链氨基酸（BCAA）。支链氨基酸是体内蛋白质的必需氨基酸的重要组成部分（35-40％）。它们主要生物功能为蛋白质合成,另具独特代谢功能。例如亮氨酸作为一种营养信号刺激蛋白质合成，调节蛋白质变形。此外,支链氨基酸协同大型中性氨基酸转运，从而影响中枢神经系统神经递质浓度。异亮氨酸，亮氨酸，缬氨酸平均需要量分别为105,140和110mg/kg/d。6.4章介绍了本研究结果如何与目前基于母乳中支链氨基酸比例的氨基酸推荐量相融合。母乳中异亮氨酸：亮氨酸：缬氨酸的比例为1:1.7:1,据IAAO法测得的比例为1:1.3:1,与鸡蛋中支链氨基酸的比例相似。

第7章

第7章对本实验结果进行总体讨论,以及如何与目前基于母乳成分的氨基酸推荐量相结合。本研究提供了人工喂养婴儿氨基酸需求量的试验证据。本研究估计的氨基酸所需量与目前推荐量有所差别,一些因素如蛋白消化率、氨基酸生物利用度和母乳喂养估计的准确性可能和此差别相关。因此我们认为目前的氨基酸的推荐量并不能反映人工喂养婴儿的需求量。本IAAO法测得的数据需要进一步长期试验。

AUTHOR'S AFFILIATIONS

Günther Boehm
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.
Danone Research, Centre for Specialised Nutrition, Friedrichsdorf, Germany.

Chao Chen
Department of Pediatrics, Division of Neonatology, Children's Hospital of Fudan University, Shanghai, P.R. China.

Mariska J.A. van Dongen
UMC st. Radboud Nijmegen, The Netherlands.

Kristien Dorst
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Femke de Groof
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Johannes B. van Goudoever
Department of Pediatrics, VU University Medical Centre, Amsterdam, The Netherlands.
Department of Pediatrics, Academic Medical Centre, Emma Children's Hospital, Amsterdam, The Netherlands.

Nicky P.C. van Haren
UMC st. Radboud Nijmegen, The Netherlands.

Jacomine E. Hogewind-Schoonenboom
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Lisha Huang
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Ying Huang
Department of Pediatrics, Division of Gastroenterology, Children's Hospital of Fudan University, Shanghai, P.R. China.

Juliette V.S. Kraaijenga
Academic medical center of the university of Amsterdam, The Netherlands.

Lodi W.C. Roksnoer
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Henk Schierbeek
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.
Department of Pediatrics, University of Amsterdam, Academic Medical Centre, Amsterdam, The Netherlands.

Jos W.R. Twisk
Department of Methodology and applied Biostatistics, VU University Medical Centre, Amsterdam, The Netherlands.

Andras Vermes
Hospital Pharmacy, Erasmus MC, Rotterdam, The Netherlands

Ineke van Vliet
Department of Pediatrics, VU University Medical Centre, Amsterdam, The Netherlands.
Department of Pediatrics, Academic Medical Centre, Emma Children's Hospital, Amsterdam, The Netherlands.

Gardi J. Voortman
Department of Pediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands.

Li Zhu
Department of Pediatrics, Division of Neonatology, Children's Hospital of Fudan University, Shanghai, P.R. China.

LIST OF PUBLICATIONS

L Huang, F de Groof, Y Huang
A Sino-Dutch collaborative research project in China: an eastern view.
Submitted

F de Groof, **L Huang**, JB van Goudoever
A Sino-Dutch collaborative research project in China: a western view.
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CURRICULUM VITAE

Lisha Huang was born on the 20th of September, 1980 in Dongguan, China. At the age of 10, she moved with her family from China to Suriname. At the age of 13, she moved to the Netherlands. After she completed her secondary school (VWO) at Christelijke College Henegouwen in Rotterdam, she started her medical training at the Erasmus University in Rotterdam in 2000.

After her medical training, she started to work as a resident at the paediatric department in Erasmus MC-Sophia Children's Hospital and Maasstad hospital in Rotterdam. From January 2008 until December 2011, she worked as a PhD student in the Sino-Dutch collaborative research project of Children's Hospital of Fudan University in Shanghai and Erasmus MC-Sophia Children's Hospital (supervised by professor J.B. van Goudoever) presented in this thesis. From January 2012, she worked as a resident of paediatrics in the VU Unversity Medical Centre in Amsterdam. From december 2012 on, she will work as a resident at the Internal Medicine at the Ikazia Hospital in Rotterdam.

Lisha is married to Hongwei Li. They have a son named Owen.

PHD PORTFOLIO

Name PhD student: Lisha Huang
Erasmus MC department: Pediatrics
PhD period: 2008-2011
Promotor: Prof.dr. J.B. van Goudoever

1. PhD training

	Year	Workload (ECTS)
General courses		
Basiscursus Regelgeving Klinisch Onderzoek	2011	1.0
The why and how of readable articles	2011	0.5
Biomedical English writing and communication	2011	4.0
Principles of research in medicine and epidemiology	2011	0.7
Biostatistical methods I: basic principles	2011	5.7
Regression analysis	2011	1.9
Survival analysis	2011	1.9
International conferences		
Young Investigator Meeting, Sanya, China	2008	1.0
2nd Shanghai Neonatal Forum, Shanghai, China	2009	1.0
The 50th Annual Meeting of the European Society for Paediatric Research, Hamburg, Germany	2009	1.0
The 3th Congress of the European Academy of Paediatric Societies, Copenhagen, Denmark	2010	1.0
Milanopediatria: nutrition, genetics-environment for health education, Milan, Italy	2010	1.0
The 44th Annual Meeting of ESPGHAN, Sorrento, Italy	2011	1.0
Presentations		
Oral: New insights in the methodological issues of the Indicator Amino Acid Oxidation method in preterm neonates	2008	1.0
Poster: Valine requirement of the enterally fed term infant	2009	1.0
Oral: Methionine requirement in presence of cysteine of the enterally fed term infant	2010	1.0
Oral: Daily eating habits of children of Shanghai	2010	1.0
Seminars and workshops		
Research Day Pediatrics	2010	0.3
Research seminars Pediatrics, Erasmus MC	2010-2011	0.3
2. Teaching activities		
Supervising master's thesis (C. Hau)	2008	2.0
Supervising master's thesis (L. Roksnoer)	2009	2.0
Supervising master's thesis (M. van Dongen)	2010	2.0

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