Fetal Food – Preemie's Prerequisite?

Studies on human fetal and neonatal protein metabolism

Foetale voeding. Een blauwdruk voor prematuur geborenen?

Studies naar humaan foetaal en neonataal eiwit metabolisme

The studies as presented in this thesis were financially supported by the Sophia Children's Hospital Fund (SSWO; grant 459; institutional grant, Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands) and the Nutricia Research Foundation (grant 2006-10; independent charity, Nutricia, Wageningen, the Netherlands). Both grant suppliers had no involvement whatsoever in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the reports for publication.

ISBN: 978-90-8559-462-8

Layout: C.H.P. van den Akker.
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Printed by: Optima Grafische Communicatie, Rotterdam.

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Foetale voeding. Een blauwdruk voor prematuur geborenen?

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Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
donderdag 11 december 2008 om 13:30 uur

door

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geboren te Zevenaar

PROMOTIECOMMISSIE

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PART I

CHAPTER

1

General introduction, outline, and aims of the thesis

Partly based on:

CHP van den Akker, FWJ te Braake, and JB van Goudoever
Nutrition in the neonatal intensive care unit
Hospital Pharmacy Europe 2005;19:49-51

&

FWJ te Braake, CHP van den Akker, MA Riedijk, and JB van Goudoever
Parenteral amino acid and energy administration to premature infants in early life
Seminars in Fetal and Neonatal Medicine 2007;12:11-18

PREMATURITY

In 1960, the terms ‘neonatology’ and ‘neonatologist’ were first coined in a textbook on newborns [1]. In hindsight, that decade turned out to be the start of modern care for prematurely born babies. Since then, survival chances for premature infants improved dramatically: for 1-kg-weighing infants from hardly any to approximately 90% nowadays. In addition, due to ongoing research [2], many infants born too early now have good health outcome, although there is also a large group facing mild handicaps and a smaller group facing more severe handicaps.

In the Netherlands, the incidence of all live births delivered preterm – that is before 37 weeks of gestation – was 7.3% in 2004 [3]. Infants born alive very preterm (<32 weeks) make up 1.1% of the 194.007 births in the Netherlands that same year [3]. These very preterm infants spent on average 28 days on a neonatal intensive care unit (NICU) [3]. In the United States, the current incidence of births delivered preterm amounts 12.8% and is thus higher than in the Netherlands. Moreover, the percentage is on the rise: between 1981 and 2005 it increased with 35% (Figure 1) [4]. Although we are aware of several factors responsible for this increase (see below), prematurity is becoming a problem affecting society more and more.

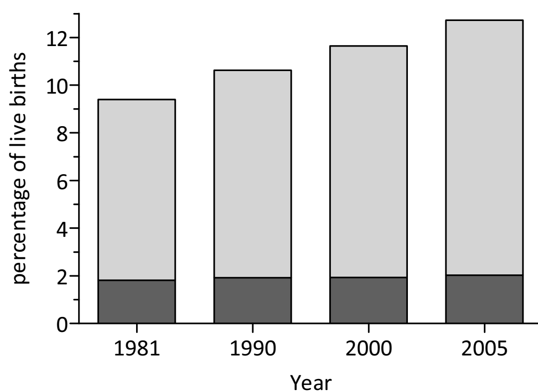

FIGURE 1: Percent distribution for all live births according to gestational age (dark-gray bars under 32 weeks gestation; light-grey bars from 32 to 37 weeks gestation) in the United States in 1981, 1990, 2000, and 2005 [4].

Besides the direct high costs of neonatal (intensive) care, one should also take in account the consequences of possible lifelong handicaps these infants sometimes face. Apart from social responsibilities, research to improve the outcome in these infants is thus a good investment. As can be seen in Figure 1, the percentage of very prematurely born infants (below 32 completed weeks of gestation) has remained relatively stable at about 2%. The incidence of late prematurity (32-36 weeks gestation) is thus responsible for the overall increase. More assisted reproduction techniques available to more people have resulted in an increase in the incidence of multiplets which tend to be born earlier than singletons. In the Netherlands, 1.1% of all births in 1980 were multiplets; this increased to 1.8% in 2006 [5]. In addition, there is a trend with multiple pregnancies towards earlier delivery. In 1990,

47.9% of all twin births in the United States were born preterm; in 2005 this increased to 60.5% [4].

However, the increase in the incidence of prematurity is not solely due to multiple pregnancy as the frequency has also increased in singletons, albeit less than that of multiplets (Figure 2) [4]. Improved and more intensive obstetrical management and neonatal interventions have probably resulted in a trend towards earlier induction of labor and cesarean delivery. Decreasing fetal mortality rates after 28 weeks gestation or more since 1990 are probably a reflection of improved obstetric care. Fetal mortality rates between 20 and 27 weeks, however, did not change during this period [6]. The shift towards earlier delivery has probably been of greater influence than improvements in antenatal care to prevent premature birth.

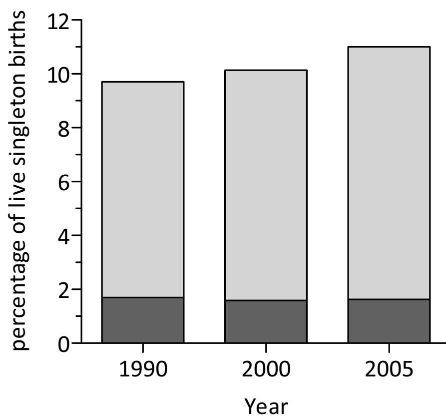

FIGURE 2: Percent distribution of gestational age (dark-grey bars under 32 weeks gestation; light-grey bars from 32 to 37 weeks gestation) for live singleton births only in the United States in 1990, 2000, and 2005 [4].

BIRTH WEIGHT

Although many (very) prematurely born infants will also be of low birth weight (LBW; <2500 g), infants born at term can also be LBW because of growth-restriction. Other classifications in birth weight include very LBW (VLBW; <1500 g) and extremely LBW (ELBW; <1000 g). In 2004, 6.4% of all live birth in the Netherlands were LBW infants; 1.0% was being born alive with a VLBW [3].

Just as prematurity has increased in the United States during the last decades, (V)LBW has also (Figure 3) [4]. However, there are some ethnic differences. Mothers of Non-Hispanic Black origin still have a 2 to 3 times higher risk to deliver a (V)LBW infant than Caucasian mothers. Yet the incidence of prematurity in Non-Hispanic Black mothers remained constant from 1989 on, whereas the incidence in Caucasian mothers increased with 30% since 1989.

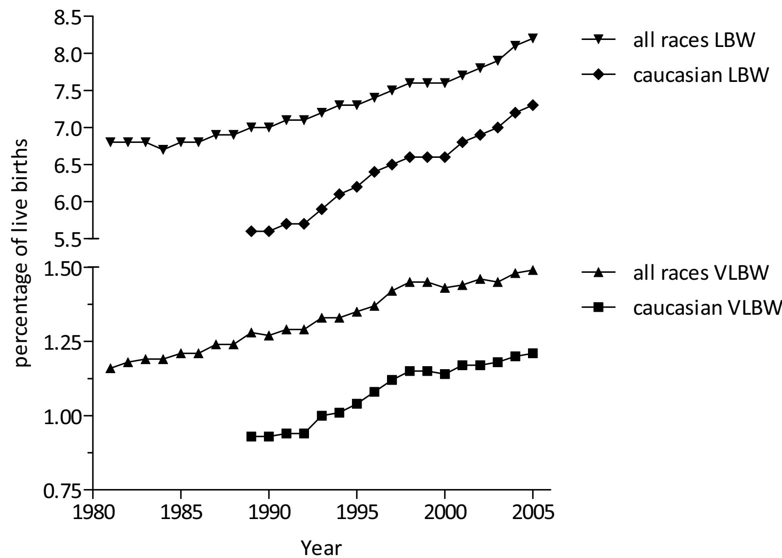

FIGURE 3: Percentage of live births of very low birth weight (VLBW; <1500 g) and low birth weight (LBW; <2500 g) in mothers of all races (1981-2005) and of Caucasian origin (1989-2005) in the United States [4].

INFANT MORTALITY

Since 1940, neonatal and infant mortality (below 28 and 365 days of age, respectively) has decreased dramatically until about 1995 after which it plateaud (Figure 4). In the Netherlands, neonatal and infant mortality rates in 2006 were 3.3 and 4.4 per 1000 live births, respectively [5]. The 10 leading causes of infant death in the Netherlands are shown in Table I. In total, over one-third of infant deaths in the United States are related to prematurity [7]. In fact, there is an exponential rise in survival chances with increasing birth weight (Figure 5).

OUTCOME & NUTRITION

The outcome of (V)LBW and/or prematurely born infants is of course something neonatologists care and worry about. The general trend is that among premature infants, outcome improves with increased birth weight and each week of intrauterine gestation [9-17]. Functional long term outcome will not be discussed here in detail, but many reviews provide extensive insight [18-20]. Based upon data collected by Tyson and colleagues on 4446 premature infants [12], the outcomes of infants born under 26 weeks gestation can be estimated using a web-tool by filling in several clinical birth characteristics of the infant [21].

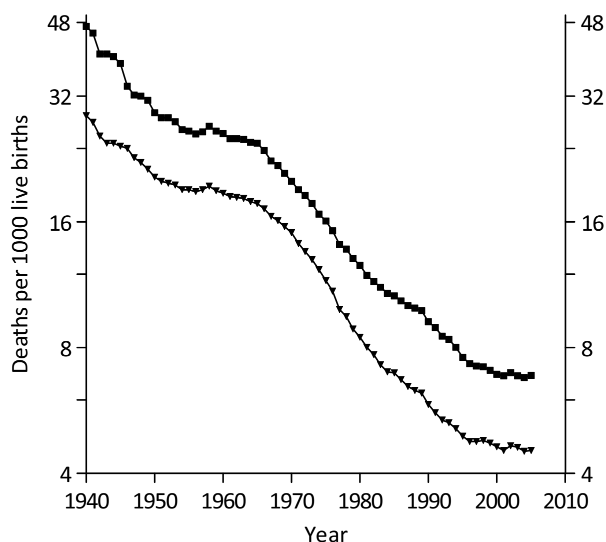

FIGURE 4: Neonatal (triangles) and infant (squares) mortality rates. Rates are neonatal (under 28 days) and infant (under 1 year) deaths per 1000 live births in the United States from 1940 to 2005.

TABLE I: Percentage of total infant deaths for the 10 leading causes of infant death (under 1 year) in the Netherlands in 2006 (n=820) [5] and between brackets in the United States in 2005 (n=28440) [8].

Rank	Cause of death [Based on the WHO *International Classification of Diseases, Tenth Revision, ICD-10,* 1992]	Percent of total deaths
1 (1)	Congenital malformations, deformations and chromosomal abnormalities [Q00-Q99]	35.1 (19.5)
2 (2)	Disorders related to short gestation and low birth weight, not elsewhere classified [P07]	10.7 (16.6)
3 (12)	Intrauterine hypoxia and birth asphyxia [P20-P21]	6.1 (1.9)
4 (4)	Newborn affected by maternal complications of pregnancy [P01]	4.9 (6.2)
5 (5)	Newborn affected by complications of placenta, cord and membranes [P02]	4.4 (3.9)
6 (8)	Bacterial sepsis of newborn [P36]	3.7 (2.9)
7 (9)	Neonatal hemorrhage [P50-P52,P54]	3.2 (2.3)
8 (10)	Necrotizing enterocolitis of newborn [P77]	1.6 (1.9)
9 (?)	Spinal muscular atrophy and related syndromes [G12]	1.5 (?)
10 (3)	Sudden infant death syndrome [R95]	1.3 (7.8)
…	All other causes [Residual]	27.5 (37.0)

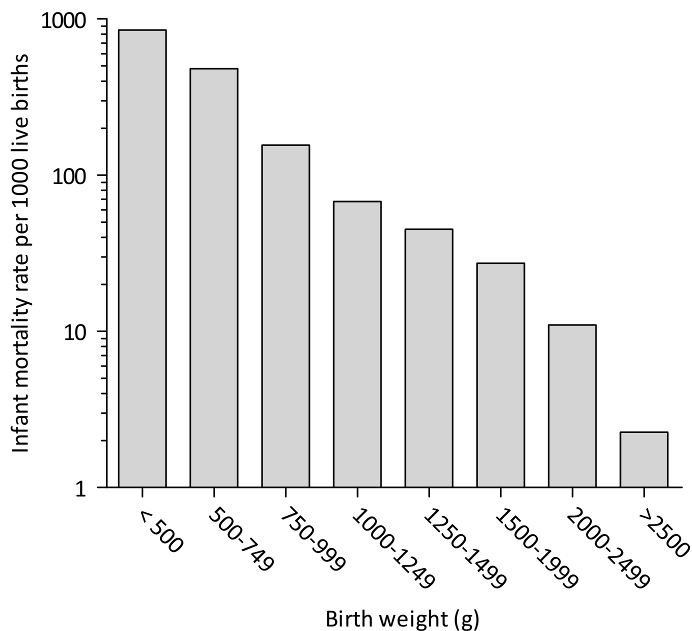

FIGURE 5: Infant mortality rates per 1000 live births by birth weight in the United States in 2004 [7].

The problem, however, when studying long-term outcome, is that today's outcome is the result of yesterday's practice.

Several factors, such as cerebral hemorrhages, increased oxidative stress, and inappropriate nutrition all may impair functional outcome. Since certainly the latter factor is iatrogenic, more research should be performed in this area. Nutrition is one of the key factors for normal cell growth. The fact that a fertilized oocyte undergoes approximately 47 cycles of cell divisions until it reaches adult tissue, but that only 5 of these divisions take place after term birth, stresses the importance of normal intrauterine development [22]. Thus, when the placenta cannot control normal metabolic supply to the fetus anymore after premature birth, neonatologists take over the role of the placenta and the function of the fluid filled amnion cavity.

Considering the many cell divisions during normal fetal life, it is not surprising that suboptimal growth during early life has a long-lasting influence on further development, even into adulthood. Providing the right amount and quality of nutrients could therefore prove essential in stimulating normal development. Although for normal growth and brain development a wide range of macro and micro nutrients is necessary, we will focus in this thesis only on proteins as these are the main functional component of organs and tissue as is also discussed further below.

'DEVELOPMENTAL ORIGINS' HYPOTHESIS, 'THRIFTY PHENOTYPE' HYPOTHESIS, AND EPIGENETICS

Starting already in the 1970s, reports started to appear that linked adverse nutritional development during early life to late onset disease, especially regarding coronary heart disease [23,24]. With David Barker as a major contributor of numerous later studies, many observations lead to the formation of the so-called 'developmental origins of adult disease' hypothesis [25,26]. This epidemiological hypothesis proposed that late onset disorders such as cardiovascular disease and type 2 diabetes may be a consequence of metabolic programming in response to poor nutrition in utero or during early postnatal life. Well-known are the controlled experiments in female rats receiving a low protein diet during pregnancy or the lactation period [27,28]. Not only did offspring have reduced birth weight, they also suffered from elevated blood pressure and glucose intolerance during adult life. In humans, epidemiologic evidence originates in the Dutch famine during the Second World War. Offspring of those that encountered severe nutrient deprivation during pregnancy is now faced during adult life with increased risk of insulin resistance and obesity [29-32].

The current thought behind these observations is that the fetus forecasts the nutritional environment it will receive after birth so that its growth trajectory, whole body physiology, and metabolism can be modified appropriately to maximize survival chances [33]. An example is that during intrauterine hypoglycemia resulting from maternal undernutrition, the fetus reduces insulin secretion and increases peripheral insulin resistance, thereby directing more glucose to the heart and brain and less to tissue such as skeletal muscle which are more insulin sensitive [34,35]. However, these adaptations, including metabolic and endocrine changes that may lead to life-long changes in the function and structure the body, can become detrimental if the postnatal conditions mismatch those experienced during fetal life [33]. A reduced number of pancreatic β-cells and sustained peripheral insulin resistance can then lead to type two diabetes in later life. This concept is known as the 'thrifty phenotype' hypothesis.

However, the molecular mechanisms of this programming effect by which a phenomenon that takes place in utero has a phenotypic consequence during adult life are largely unknown. A potential concept, however, is that transient exposure to a variety of insults during early life leads to permanent alterations in gene expression. A mechanism that allows for the stable propagation of gene activity-states from one generation of cells to the next is thereby required. Epigenetic mechanisms are one such possibility [36]. Epigenetics refers to modifications that regulate gene activity by affecting the DNA itself (methylation) or the proteins that package DNA (histone modification), but without altering the actual nucleotide sequence of DNA. Because DNA methylation is maintained and copied after cell division, it is an attractive candidate mechanism for fetal programming as the effects are lasting. Thus, during critical time windows during early life, environmental insults might trigger epigenetic modifications in susceptible 'developmental programming' genes in

the DNA of certain organs. Altered expression of these critical genes during development could result in aberrant organ growth and differentiation with altered metabolic rates. Consequently, these organs might have reduced plasticity and be predisposed to stress by unusual circumstances or aging, so that diseases part of the metabolic syndrome might develop [36].

Lastly, the risk of disease might even be transgenerational, because environmentally induced epigenetic effects are unlikely to be reprogrammed in the germ line [33]. Epigenetic inheritance with concomitant diseased phenotype has been shown up to generation four in rats [37,38]. In addition, grandchildren of women that were pregnant in the Dutch famine also still seem to be affected by reduced birth weight [39].

WHAT ARE PROTEINS AND AMINO ACIDS?

The word protein was first mentioned in a letter sent by the Swedish chemist Jöns Jakob Berzelius to his Dutch research associate Gerhardus Johannes Mulder on July 10, 1838. He wrote: “The name protein that I propose for the organic oxide of fibrin and albumin, I wanted to derive from the Greek word πρωτειος (proteios, meaning of primary quality), because it appears to be the primitive or principal substance of animal nutrition” [40].

Proteins are defined as polymers of amino acids, which are nitrogen-containing molecules (Figure 6), and can be divided according to their dynamic or structural function. Enzymes are typical examples of the dynamic group of proteins, but also hemoglobin, albumin, immunoglobulin, fibrin, and many hormones join this category. Structural proteins

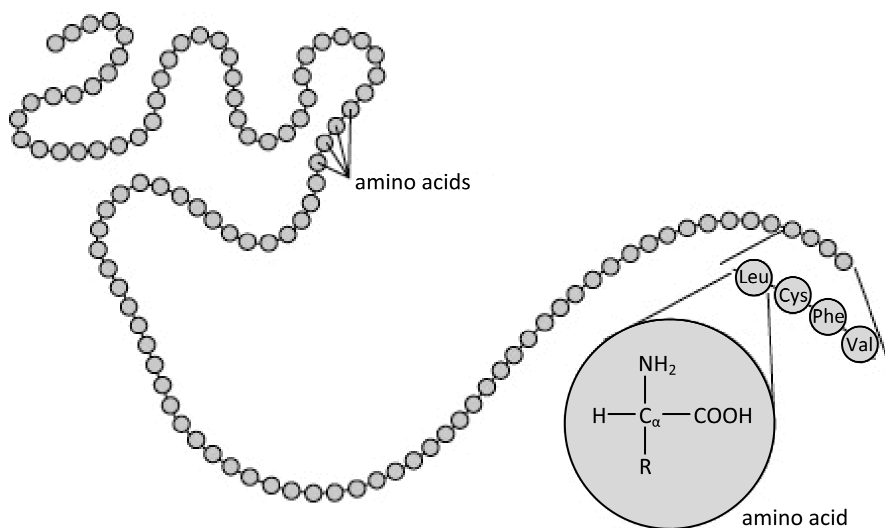

FIGURE 6: The primary protein structure is a chain of amino acids. All amino acids contain an amino (NH_2), carboxyl (COOH), and rest (R) group attached to the central α-carbon atom.

are usually incorporated into tissue (e.g. muscle or splanchnic organs) and are thus mainly responsible for growth. Every protein is a polymer of only 20 different amino acids, which we call α-amino acids and for which the order is defined after translation from the DNA. β- and γ-amino acids (e.g. taurine and hydroxy-proline, respectively) cannot be incorporated into proteins but have important intracellular functions as individual acting amino acids. The a-amino acids can be grouped into essential (indispensable) or non-essential (dispensable) ones in that the former can only be derived through dietary intake and not produced de novo (Table II). In the fetus and during early neonatal life, some amino acids are considered as conditionally or semi-essential, as their metabolic system might not have developed completely, yet, to fulfill the demands for these amino acids just by de novo synthesis.

TABLE II: Subdivision of α-amino acids in essential, non-essential, and in supposedly conditionally essential amino acids (for premature infants).

Essential	Non-essential	Conditionally essential
Leucine	Alanine	Tyrosine
Isoleucine	Serine	Cysteine
Valine	Asparagine	Glutamine
Methionine	Aspartate	Arginine
Phenylalanine	Glutamate	Glycine
Threonine	Tryptophan	Proline
Histidine		
Lysine		

Tyrosine, for example, is suggested to be conditionally essential due to impairment of hydroxylation of phenylalanine to tyrosine [41,42]. Some others did however show premature infants to be able to hydroxylate phenylalanine to a certain extent [43-45]. However, the question remains whether these hydroxylation rates are sufficient to meet tyrosine demands in the first days after birth. Tyrosine is poorly soluble and, therefore, it is difficult to provide adequate intake in infants receiving parenteral nutrition. Currently used solutions contain <1% of tyrosine, which is far below the needs of the parenterally fed neonate. Roberts et al. found the mean tyrosine requirement of the parenterally fed neonate to range from 66 to 88 mg/(kg·d), representing 2.8% to 3.8% of total amino acids [46].

Similarly, the transsulphuration pathway of converting homocysteine (derived from methionine) to cysteine might also be impaired during early life due to suboptimal activity of the responsible enzyme called cystathionase [47,48]. Nevertheless, others have recently shown that the trans-sulphuration pathway in VLBW neonates is active 48 hours after birth [49]. Cysteine is not stable in solution and oxidizes easily to cystine, which is insoluble; most

standard parenteral solutions therefore contain little cysteine or are cysteine free, which puts parenterally fed infants at risk for cysteine deficiency. Providing cysteine in its acetylated form (*N*-acetyl-L-cysteine), which is stable in parenteral solutions, does not aid premature infants as high concentrations of acetylated cysteine can be found in urine, indicating a low bioavailability [50]. The other conditionally essential amino acids will not be discussed here since they are not studied in this thesis, but more information can be found elsewhere [51]. Other ways of grouping amino acids are by their chemical characteristics: leucine, valine, and isoleucine are called branched chain amino acids; phenylalanine, tyrosine, and tryptophan form the aromatic group; methionine and cysteine are sulfur-containing amino acids; serine and threonine contain a hydroxy (or alcohol) group; glutamine and asparagine are acidic and lysine, histidine, and arginine are basic amino acids.

Almost all proteins undergo a constant process of synthesis and breakdown. Halftime differs from minutes (many enzymes) to weeks or months (muscle tissue) or hardly ever (eye lens and brain tissue). Anabolism, or growth, is defined as the net balance between protein synthesis and breakdown rates of a specific protein or whole body protein in general, respectively. The process of breakdown serves to release amino acids during fasting, to remove defective proteins after erroneous translation or after oxidative damage. Protein breakdown can also occur when there is a high need of certain scarce amino acids, so that the needed individual amino acids become free for synthesis of other proteins.

For individual amino acids there exists no storage pool, like there is for for example glucose and fatty acids in the form of glycogen and fat, respectively. Also muscle protein is formed from a predefined (DNA) and fixed ratio of selected amino acids that form a polymer. Therefore, individual amino acids that are in surplus of those needed for protein synthesis cannot be stored separately, nor can amino acids that are in short for synthesis of a certain protein molecule selectively be degraded from some other protein molecule. Neither can the kidney selectively excrete or retain a single amino acid. Therefore, to avoid aminoacidemia, the only metabolic fate for an amino acid in excess is to be degraded into ammonia (later converted into urea) and a carbon skeleton which can either be oxidized in the citric acid cycle thereby yielding energy or used for glucose synthesis.

Preterm infants are known to have very high rates of both protein synthesis and proteolysis as compared to older individuals [52]. Especially protein synthesis is an energy demanding process. It might be hard to imagine why growing individuals put so much energy in breaking down and resynthesizing proteins. Several amino acids kinetics studies have shown a marked difference in protein metabolism between preterm and term infants. There is a negative correlation between gestational age and protein loss, resulting in a doubling of the protein losses in ELBW infants as compared to term infants [44]. During protein administration, term infants [53] and adults [54] respond by decreasing proteolysis, whereas human preterm infants [55-57] and ovine fetuses [58] increase protein synthesis, rather than suppress breakdown. Only one study in premature infants found a concomitant decrease in proteolysis [43]. A developmental change seems responsible. A very high

turnover rate is also found in adults during illness or after injury, again a situation in which at first sight high breakdown and synthesis rates seem contradictory to energy scarceness. Presumably, a high turnover rate aids in fast damage repair and amino acid redistribution. One other possible benefit could be that if a positive stimulus (e.g. a hormone) increases the synthesis rate, leaving degradation unaltered, the net effect on growth is amplified when turnover rates in general are already raised [59].

METHODS OF PROTEIN INVESTIGATION

Nitrogen balances form the classic method of nitrogen-related research in individuals. It is no more than a simple comparison of nitrogen intake through diet and the rate of nitrogen excretion over a period of time [60,61]. Nitrogen is mainly excreted by the urinary tract (80% as urea, the remainder being ammonia and free amino acids), whereas fecal losses as well as breathing, sweating, hair, nails, and skin losses form only minor contributions [62]. However, obtaining a nitrogen balance is not informative on how a particular nutritional state is reached. As mentioned above, growth is defined as a balance between catabolism and anabolism and different combinations of the two can lead to the same conclusion with a nitrogen balance. Research tools that can aid in quantifying protein synthesis and proteolysis, like stable-isotope-labeled amino acids, provide an excellent and safe method when studying metabolism [63-73]. Stable isotopes are atoms with an extra neutron in the nucleus, and thus, slightly heavier. This difference in mass can be distinguished with mass-spectrometry techniques. Nowadays, stable isotopes are widely available and applicable for numerous clinical and research purposes. Stable isotopes are not radioactive, their biological behavior is assumed to be the same as their parent compound, and they are already naturally occurring in the human body and in normal nutrition (the natural abundance for ^{13}C is approximately 1.11%).

Two of the most used models in pediatric stable isotope research include quantification of whole body amino acid turnover or the synthesis rate of a specific protein. In whole body turnover studies, an isotopically labeled amino acid is intravenously infused at a constant rate for several hours. The tracer mixes with the amino acids that enter the system after proteolysis, dietary intake, or if possible *de novo* synthesis. The sum is called the rate of appearance (Ra) and must equal the rate of disappearance (Rd) to avoid accumulation or depletion of an amino acid. The Rd consists of incorporation into proteins, oxidation, or conversion into another amino acid (Figure 7 and Figure 8). After tracer equilibrium, one can calculate from the tracer enrichment the dilution and thus Ra in the system. If [1-^{13}C]leucine is used as a tracer for example, proteolysis can be quantified after subtraction of the dietary leucine intake from the Ra since leucine cannot be produced *de novo*. After collection of expiratory air, the $^{13}CO_2$ content can be analyzed, and amino acid oxidation can be quantified. The non-oxidative disposal (NOD), which is assumed to be equal to incorporation

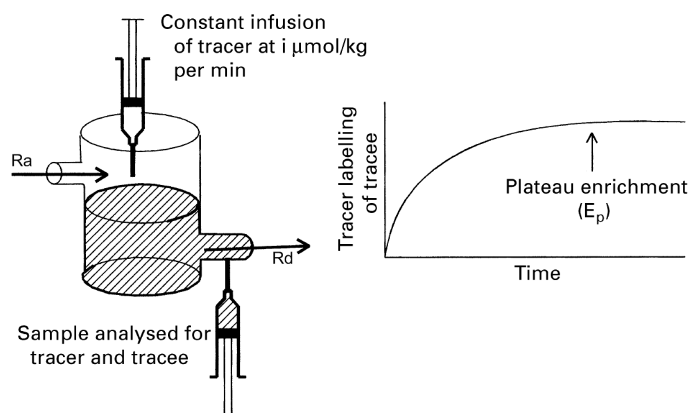

FIGURE 7: The tracer dilution principle. Flux through the pool (µmol/kg per min) = i/Ep, where i is infusion rate of the tracer (µmol/kg per min) and Ep is the plateau enrichment. (From Rennie [68], with permission Cambridge University Press)

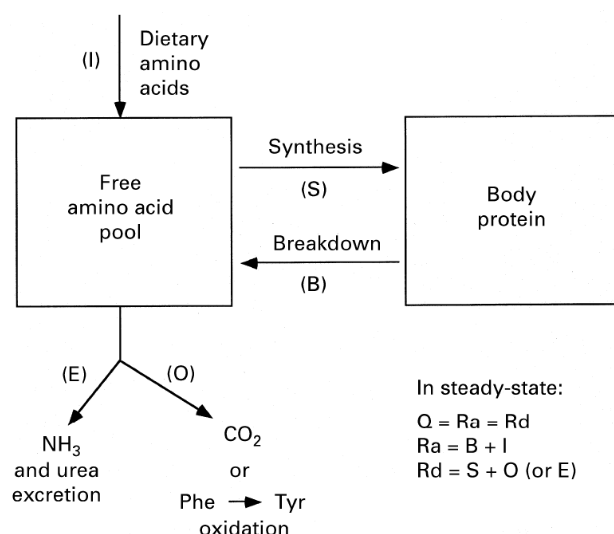

FIGURE 8: General model of protein metabolism used in the whole-body turnover methods. Q, whole-body nitrogen turnover; Ra, rate of appearance in the free amino acid pool; Rd, rate of disappearance from the free amino acid pool; Phe, phenylalanine; Tyr, tyrosine. (From Wagenmakers [69], with permission Cambridge University Press)

into proteins, can be calculated from the subtraction of oxidation from the Rd. The difference in proteolysis and protein synthesis then gives information on the net rate of protein, or more precisely amino acid, growth.

However, a potential disadvantage of these kinds of turnover experiments is that they are an average of protein metabolism in the whole body. Counteracting changes in for example muscle versus liver metabolism will not be noted. From an ethical point of view, however, it is hardly ever possibly to obtain organ biopsies. Yet, to obtain insight in liver metabolism, one can study one of the liver proteins like albumin that are excreted into the accessible plasma compartment. After continuous tracer administration and subsequent multiple plasma sampling, one can calculate the albumin synthesis rate from the increase of tracer enrichment that is incorporated into albumin over time.

When quantifying amino acid kinetics in the human fetus, however, the two models described above cannot be used since the tracer cannot be directly infused into the fetus,

and we cannot take multiple blood samples from the fetus. Therefore, alternative models must be used. Since the fetus is provided with nutrients through the umbilical cord, one can use a veno-arterial balance model to calculate fetal uptake by using the Fick principle (net uptake = blood flow × veno-arterial concentration difference). In combination with tracer administration to the pregnant women and Doppler ultrasound measurements of the umbilical cord prior to delivery, one can sample blood from both the vein and arteries in the umbilical cord after birth and calculate fetal whole body amino acid kinetics after measurement of the amino acid concentrations and enrichments in both the umbilical vein and arteries. These kinds of balance models are often used when studying leg or splanchnic organ metabolism in animals or adult humans, but can, with some small adaptations also be used for studying fetal kinetics.

Quantification of the fetal synthesis rate of specific proteins like albumin is also complicated due to the fact that only one blood sample can be taken from the fetus or umbilical cord, which is immediately after delivery whereas at least two subsequent samples are needed. To overcome this problem, we modified the staggered infusion protocol proposed by Dudley and colleagues [74] into a simplified multiple tracer infusion model. In one of the following chapters we will elaborate more on this new type of model.

NUTRITION FOR PREMATURE INFANTS IN THE 20TH CENTURY

Pierre Budin, a famous French obstetrician and together with his mentor the founder of modern perinatal care (by then obstetricians usually took care of newborns until aged about two years), stated already in 1907: “The path of pleasure, for adults, is drinking. May it not be the same for weaklings? I increased their absorption of milk with, as you have seen, the happiest of results” [75]. Thus, during these days, premature infants received high volumes of human milk by tube feeding (up to 200 mL/(kg·d)) to stimulate rapid growth [76]. But from the 1940s on, worries about aspiration pneumonias and kidney failure resulted in withholding all fluids for up to 72 hours after birth. Until approximately 1965, hardly any attention was paid on nutrition, but from then on, after the recognition of adverse neurodevelopmental outcome attributed to low initial fluid and nutrient intake, early provision of fluids/feedings was advocated again [76]. Since then, several small adaptations to formulas or breast milk fortifiers resulted in the way we now treat our babies with enteral feeding [77].

But also intravenous nutrition already has a long history. The first report on intravenous amino acid administration to young infants in 1939 described many complications [78]. More triumphant was the report that appeared in 1944 where a marasmic suckling received solely total parenteral nutrition (TPN) for five consecutive days [79]. Almost 25 years later, a LBW neonate with near total small bowel atresia received for 44 consecutive days TPN without any enteral feedings; her weight had increased with 80% during the study period

[80]. However, these first solutions containing hydrolyzed amino acid residues also caused significant problems such as hyperammonemia [81]. The first generation of the synthetic crystalline solutions, however, also caused problems such as acidosis [82]. Studies reporting these adverse effects had, and still have, a profound effect on nutritional policies. Although it was recognized that withholding amino acids resulted in a catabolic state, they were withheld during early life under the assumption that the preterm infant was 'intolerant' to amino acids solutions. We have come to realize that both the method of manufacture and the composition of the amino acids solutions were likely to have caused complications such as hyperammonemia and metabolic acidosis, rather than the amino acids solutions *per se*. Nevertheless, fear of metabolic derangements is still firmly rooted in clinical practice.

Guidelines, such as those presented in 1977 by the Committee on Nutrition of the American Academy of Pediatrics, have stressed the importance of amino acid administration to preterm neonates [83]. The goal stated at that time remains valid today: a postnatal growth rate that duplicates fetal growth rate. An additional aim is to strive for a similar body composition at term corrected age to that of a healthy term-born infant. Considering that nutrition is extremely important for normal cell function and development, ultimately we hope of course that optimal nutritional strategies will also result in a functional outcome in ex-premature infants that is comparable to term born infants. However, even today, international pediatric guidelines hardly state anything on the initiation and amounts of administrating TPN during the first week of life after very preterm birth. We might even conclude that compared to other innovations in neonatology, such as artificial ventilation, progress in the field of nutrition is lagging behind, probably because malnutrition or even complete absence of nutrients is not immediately life threatening at first sight. On the other hand, we have come to realize that even a single day of starvation can be detrimental to the preterm infant [77].

PLACENTAL NUTRIENT TRANSPORT

Prior to birth, the fetus receives its nutrients through the umbilical cord from the placenta. The maternal facing microvillous membrane and the fetal facing basal membrane of the syncytiotrophoblast are the functional barrier between the fetal and maternal blood circulations at which nutrients, water, respiratory gases, ions, and waste products are exchanged. Transport of fatty acids, glucose, and amino acids all occurs through different processes which have not all completely been unraveled. Contrary to the transport of respiratory gases which diffuse freely through the placental membranes and which rate is mainly dependent on concentration gradients and uterine and umbilical blood flow rates, the transport of nutrients is more complicated. Successively, we will shortly discuss the placental transport of lipids, glucose, and finally amino acids.

Lipid transfer to the fetus has been studied the least, partly because of its technical

difficulties, partly because the lipid content of most research animal tissue is far less than that of human tissue (Table III) [84-87]. In most mammals, fat is thus transported across the placenta at a much lower rate than in the human feto-maternal dyad. What we do know however, is that maternal plasma free fatty acids, lipoproteins and triacylglycerols are bound to appropriate receptors and lipases, respectively, on the microvillous membrane [88 -90]. Released free fatty acids that are not used for placental metabolism are transported over the basal membrane either by simple diffusion or by facilitated transport proteins.

TABLE III: lipid content in different species at term birth [84-87].

Species	g fat/100 g body weight at term birth
Black bear	0.9
Pig	1.1
Rat	1.1
Cat	1.8
Mouse	2.1
Horse	2.6
Baboon	3.0
Sheep	3.3
Rabbit	3.9
Seal	4.0 – 9.0
Guinea pig	10.0
Human	16.0

Glucose transport on both membranous layers of the placenta is mediated by facilitated glucose transporters of the GLUT family, mainly GLUT 1 [91,92]. The materno-placento-fetal concentration gradient is the main driving force for the glucose transport rate. In sheep, approximately 75% of glucose taken up by the placenta is not transported further to the fetus, but used for placental metabolism, consisting largely of oxidative purposes [93,94]. One-third of oxidized glucose occurs anaerobically, however, thereby forming lactate which is taken up by either the maternal or fetal circulation [95].

Since the primary driving force for glucose transfer is the fetal concentration, the fetus tries to control it tightly to ensure a constant supply [96]. During maternal hyperglycemia, the fetal concentration might also rise to decrease the materno-fetal glucose gradient thereby preventing an increased transplacental overload of glucose. On the other hand, during maternal hypoglycemia, the fetus will decrease its glucose concentration to maintain a gradient in concentration across the placenta [96]. Fetal gluconeogenesis is therefore only induced in an ultimate situation to prevent severe hypoglycemia as endogenous glucose production indeed raises the fetal glucose concentration, but at the same time reduces the materno-fetal glucose concentration gradient. Fetal gluconeogenesis is therefore only a very short-term survival mechanism and is probably only seen in experimental situations [97]. The fact that fetal glucose concentrations decrease physiologically towards the end of

gestation is probably also a mechanism to increase the feto-maternal glucose gradient to increase placental glucose transport to meet the increasing fetal glucose demands [96].

Protein transport across the placenta plays only a very minor role. Immunoglobulin class G (IgG) is probably the only protein transported in considerable amounts. Yet, only the hemochorial placenta (humans, guinea pigs, rats, and rabbits) is permeable so that just these fetal mammals receive passive immunization. Besides, the primary function of this placental protein transfer is probably not nutritionally. Also albumin, the main plasma protein is not transported to the fetus, although it is approximately four times smaller in size than IgG.

Amino acids, however, are transported in large quantities to the fetus by active transport. Substrates for active transport include adenosine triphosphate (ATP) or sodium, but many amino acids are also transported inwardly in exchange for another amino acid that is transported outwardly [98]. At least 15-20 different amino acid transporters exist, each with their own, yet overlapping specificity for certain amino acids. At the microvillous membrane, amino acids are actively pumped into the trophoblast, where concentrations are up to four times higher than in maternal plasma [99,100]. The exact mechanisms how amino acids are then transported to the fetus are largely unknown. Previously, this was thought to occur through simple passive diffusion across the basal membrane into the fetal plasma where concentrations are approximately two times lower. However, since a few years, this thought seems to be too simplistic. Efflux transporters with specificity for only a few amino acids work together with many exchange transporters with broad specificity to create a net amino acid transport rate towards the fetus [101].

Many reviews have appeared in recent years regarding placental nutrient transport to which I refer for further reading [96,101-110].

INTRAUTERINE NUTRITION

The fetus receives a continuous supply of nutrients through the umbilical vein [104]. Nutritional uptake in utero is large, not only for accretion of new tissue and a high oxidation rate, but also for replacement of body water with protein and fat. The water content of fetal tissue drops from 89% at 24 weeks to 74% at 40 weeks gestation. This drop is counterbalanced by a rise in lipid content from 0 to 11% in the last trimester, and a rise in protein content from 8.8% to 12% [111]. Impressive though this increase in lipid content appears, a linear 11% increase over 16 weeks needs a constant uptake of only 1.0 g lipids/(kg·d) (calculated as 110 g/kg in 112 days). Apart from this change in body composition towards relatively leaner body mass and more fat tissue, the fetus also grows at a rate of about 15 g tissue/(kg·d) during mid-gestation, tapering off to 10 g/(kg·d) at term. Lipid need for new tissue increases from negligible during mid-gestation to 11% of 10 g tissue/(kg·d) (equaling 1.1 g lipids/(kg·d)) at term. A third component of uptake – oxidation – is rather

unimportant in fetal life as hardly any fatty acids are oxidized. Thus, the total need for fatty acids increases from about 1.0 g/(kg·d) at mid-gestation to a little over 2.0 g/(kg·d) at term (Figure 9).

After term birth, the lipid content further increases to about 20% at 2 months of age [112]. This implies that an increase of 90 g lipids/kg in 60 days (1.5 g/(kg·d)) is necessary for tissue replacement. The 1.1 g lipid/(kg·d) needed for new tissue at birth will increase to 1.5 g/(kg·d) (at a growth rate of 7.5 g tissue/(kg·d) at 2 months of age). Furthermore, significant lipid oxidation after birth is physiological, accounting for 2.5 g/(kg·d) [113]. Total lipid need at 2 months of age after term birth would therefore be 5.5 g/(kg·d) (Figure 9).

A similar calculation for protein yields an amino acids demand of almost 0.3 g/(kg·d) (32 g/kg in 112 days) to meet intrauterine changes in body composition (water replacement by protein). Demands for normal growth stabilize at 1.2 g amino acids/(kg·d) throughout the second half of pregnancy due to the opposite effect of a slower growth rate at term with increased tissue protein content at this time. Amino acid contribution to human fetal oxidation is largely unknown and the few available data are contradictory. Obligatory nitrogen excretion in fasting premature neonates is approximately 0.6-1.0 g/(kg·d) [57,114]. Animal fetal research under physiological conditions demonstrates that intrauterine amino acids oxidation is much higher, and that uptake is far beyond amino acids requirements for body accretion [115,116]. Human studies have also showed considerable intrauterine urea production [117]. Total amino acid uptake can be estimated to be between 3 and 4 g amino acids/(kg·d) (figure 10). Because tissue protein content does not increase any further after birth, protein requirements at 2 months of age after a term birth decrease slightly, to approximately 2.0-2.5 g/(kg·d) (Figure 10).

It must be noted that the factorial approach described above uses the tissue composition of deceased fetuses or newborns, whose growth might also have been affected; thus these figures might be underestimates. Nevertheless, we can still assume that the fetus receives a diet rich in protein and poor in fat. Compared to fetal nutrition, present postnatal nutritional strategies dictate the preterm infant be given a high-fat and

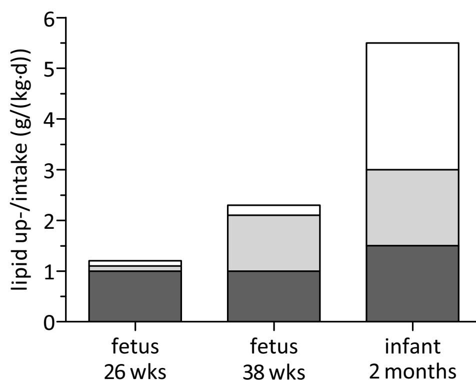

FIGURE 9: Stacked bar graph of the total estimated lipid uptake in the fetus at 26 and 38 weeks gestation and lipid intake in a term born healthy infant fed breast milk at two months of age. Bars indicate whether lipid will be used for replacement of water by tissue (dark-grey), net tissue accretion (light grey), or energy generation (oxidation) (open).

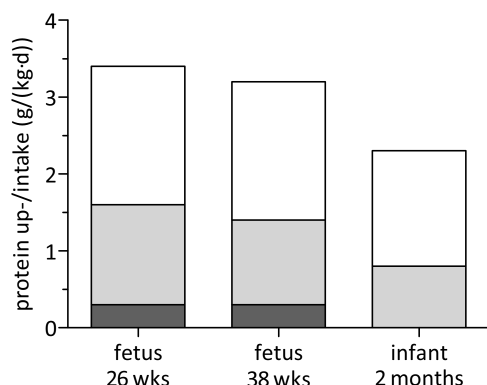

FIGURE 10: Stacked bar graph of the total estimated amino acid uptake in the fetus at 26 and 38 weeks gestation and protein intake in a term born healthy infant fed breast milk at two months of age. Bars indicate whether protein will be used for replacement of water by tissue (dark-grey), net tissue accretion (light grey), or energy generation (oxidation) (open).

moderately high-protein diet. Although a high-caloric diet does indeed stimulate a preterm infant's growth, mass accretion would have been different in composition had the infant still been *in utero*. Indeed, preterm infants were found to show a larger than desirable fat deposition after birth, especially around the visceral organs [118,119]. Achieving a body composition that more closely resembles fetal body composition usually implies a larger amino acid intake. This should be accomplished as soon as possible after birth.

PARENTERAL AMINO ACID ADMINISTRATION IN THE EARLY POSTNATAL PHASE

Whereas the supply of nutrients delivered from the placenta to the infant ceases unanticipatedly after preterm birth, the ongoing delivery in the age-matched fetus is vital for normal growth and neurodevelopment. Yet, preterm infants often do not receive any amino acids during the first postnatal days; or they receive insufficient amounts. Clinical complications such as respiratory distress and patent ductus arteriosus with a subsequent strict fluid management can complicate adequate nutrient provision. In the absence of total parenteral nutrition, and not being able to receive enteral feedings in the direct postnatal period, an infant is dependent on its own protein stores for obligatory protein catabolism. For an infant receiving only glucose, this requires approximately 1.0 g/(kg·d) or 1% of the endogenous protein stores each day [120]. An age-matched fetus accretes protein at a rate of approximately 1.5 g/(kg·d) and it is this growth rate that should be the goal when feeding preterm infants; it is often not achieved [121,122]. Many infants born appropriate for gestational age will leave the hospital too small [123]. Note however, that the initial weight loss in the first postnatal days also represents the rearrangement of body fluids necessary for adapting to extrauterine life, rather than solely catabolism.

In early studies, parenteral amino acids were initiated not until after 1 week in the smallest infants [124] or after 3 days in 1700-gram-weighing infants [125]; the infants were dependent on exogenous glucose for their metabolism during the intervening period. With

the introduction of solutions specifically designed for neonates [126], researchers began to study the effects of shortening time span of withholding amino acids [56,57,127,128]. In two separate studies, Van Goudoever et al. and Murdock et al. administered amino acids immediately after birth, although they used only 1.15 and 1.35 g/(kg·d) in infants weighing a mean 1400 g and 1500 g, respectively [114,129]. Neither these, nor the other early initiation studies, reported metabolic acidosis, hyperaminoacidemia or, hyperammonemia when measured. Beneficial effects – improvement in nitrogen balance, stable isotope balance, or plasma amino acids profile – were observed in all studies. Nevertheless, up till the advent of this thesis, clinicians were still reluctant to start giving amino acids to premature infants in the immediate postnatal phase. Compared to the in 2000 recommended daily intake of 3.0 g protein/(kg·d) and 120 kcal/(kg·d), premature infants born before 31 weeks gestation accumulated a protein and energy deficit during the first 7 weeks after life of more than 25 g/kg and 1000 kcal/kg, respectively [121]. Besides the cumulative protein deficit, Figure 11 also shows the dramatic change in *z*-score in these infants during the same period. Stepwise regression analysis indicated that 45% of the variation in z scores could be explained by the reduced intake.

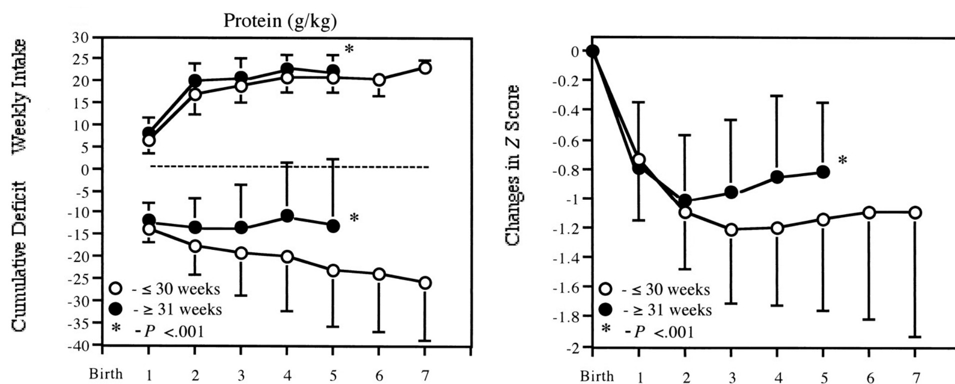

FIGURE 11: Protein intake, cumulative protein deficit, and change in weight *z*-score (standard deviation score) during hospital stay in premature infants in first seven postnatal weeks. (From Embleton *et al.* [121])

The current guidelines, however, such as those proposed by the ESPGHAN, prescribe even 3.5 g protein/(kg·d) [77], which would double the cumulative protein deficit in the previously mentioned study by Embleton et al. to approximately 50 g protein/kg for the infants born under 31 weeks gestation. Two other recent reports show similar results [130,131]. The main reason for the protein deficit is that not only most NICU's do not start immediately after birth with intravenous amino acids, but in addition also a stepwise increase in intake over several days is common. The motivation for the stepwise increase of amino acids intake is however not empirically based. Fluid limitations, concerns of intolerance and fear of hyperglycemia in case of mixed glucose/amino acids solutions might

provide some explanations.

However, over the years, quality of intravenous amino acids solutions has improved, and so has the general condition of the very low birth weight (VLBW) infant before and immediately after delivery. This provides a starting point for new nutritional strategies that must be explored.

AIMS OF THIS THESIS

The ultimate goal in neonatology is to achieve an outcome in premature infants that is comparable to healthy term born infants. Part hereof, but probably also directly related is to achieve a growth rate in premature infants that is comparable to that of intrauterine counterparts of the same gestational age. In addition to fetal growth rate, the fetal tissue composition should be mimicked.

As can be read in this introductory chapter, nutritional intake in premature infants is not optimal, especially during the first weeks of life. Yet, the impact of nutrition for normal development is huge, not only for normal growth and brain development, but even beyond through epigenetics. A first simple recognition that many infants are catabolic in the first week of life and leave the hospital with growth failure indicates room for improvement of nutritional therapies. Therefore, in this thesis we will first focus on nutrition in the immediate postnatal phase as the sudden unanticipated transition from intra- to extrauterine life is critical and might set the further course of the infant on the NICU. Our specific hypotheses were that high dose parenteral amino acid administration to premature infants immediately after birth:

- is safe and results in anabolism,
- promotes protein synthesis rather than decreases proteolysis, and
- stimulates liver activity by means of increased albumin synthesis rates.

Then, we will try to unravel several aspects of fetal amino acid and protein metabolism. This information might give us new perspectives on how to feed premature infants in the near future. Our specific objectives were to quantify the fetal:

- albumin synthesis rate,
- protein accretion rate by means of phenylalanine kinetics,
- hydroxylation rate of phenylalanine into the semi-essential amino acid tyrosine, and
- metabolic pathways of leucine, valine, and methionine.

Additionally, we were able to quantify these objectives in the pregnant woman and we could determine the synthesis rate of placental structural protein. All data obtained from the fetal studies can then be compared to those obtained postnatally to determine if postnatal nutrition can be improved.

OUTLINE OF THIS THESIS

The first clinical part of this thesis describes a large clinical trial in premature infants where half of the group received relatively high dose parenteral amino acids next to glucose from birth onwards whereas the control group only received glucose during the first few postnatal days. **Chapter 2** describes the safety and efficacy (in terms of nitrogen balance) of early amino acid administration to these infants. **Chapter 3** then determines the mechanisms behind any change in metabolism by using leucine and glucose stable isotopes. **Chapter 4** describes effects of early amino acid administration on the albumin synthesis rate in these infants. The outcome at age two of the infants that participated in the large trial is described in **chapter 5**.

The next part of this thesis describes several studies in which pregnant women received multiple stable isotope infusions in the hours prior to cesarean section. After birth, umbilical cord blood was sampled and analyzed for the amino acid concentrations and enrichments. In **chapter 6** the fetal albumin synthesis rate is quantified using a relatively novel stable isotope model. In **chapter 7**, the fetal metabolic pathways of phenylalanine and tyrosine are quantified and in **chapter 8** the same is done for leucine, valine, and methionine. **Chapter 9** describes, using the same model as in chapter 6, the synthesis rate of mixed structural placental proteins.

Finally, **chapter 10** provides a general discussion of the results found here and in **chapter 11** all results are summarized in both the English and Dutch language.

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PART II

CHAPTER

2

Amino acid administration to premature infants directly after birth

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Published in:
The Journal of Pediatrics, Volume 147, October 2005, Pages 457-461

ABSTRACT

Objectives
To test the hypothesis that the administration of 2.4 g amino acids (AA)/(kg·d) to very low birth weight infants is safe and results in a positive nitrogen balance.
Study design
We conducted a randomized, clinical trial. Preterm infants with birth weights <1500 g received either glucose and 2.4 g AA/(kg·d) from birth onward (n = 66) or solely glucose during the first day with a stepwise increase in AA intake to 2.4 g AA/(kg·d) on day 3 (n = 69). Blood gas analysis was performed daily during the first 6 postnatal days; plasma urea concentrations were determined on days 2, 4, and 6; AA plasma concentrations and nitrogen balances were determined on days 2 and 4. Student *t* tests, Mann-Whitney tests, and χ^2 tests were performed to compare groups.
Results
Infants supplemented with AA had no major adverse side effects. Their plasma urea concentrations were higher, nitrogen balance turned positive upon AA administration, and more AA concentrations were within reference ranges.
Conclusion
High-dose AA administration to very low birth weight infants can be introduced safely from birth onward and results in an anabolic state.

INTRODUCTION

After birth, very low birth weight (VLBW) infants are dependent on externally administered nutrients, as hardly any stored energy is at their disposal [1]. Both fat tissue and glycogen levels are limited, especially in small-for-gestational age (SGA) VLBW infants. Consequently, without adequate exogenous nutrient supply, protein breakdown will increase in these infants, resulting in a catabolic state.

Despite a growing body of literature regarding the safety and efficacy of early amino acid (AA) administration, there is still wide variability in practice. Often, carbohydrates are still the only exogenous nutrients administered in the immediate postnatal period. In the past, AA were often withheld since formerly used AA mixtures were found to result in metabolic acidosis and hyperammonemia [2,3]. In utero, fetuses are supplied with large amounts of AA, which not only are used for protein synthesis but also serve as an important fuel source [4-7]. It seems logical, therefore, to supply newborn infants with adequate amounts of both energy and growth substrates to meet energy requirements and to promote protein accretion for ongoing growth. Indeed, several studies indicate that the currently used crystalline solutions seem well suited for the preterm infant, who may benefit from the anabolic effects [8-14]. However, in most of these studies, either low amounts of AA were administered, administration started only after the first day of life, infants with higher birth weights were studied, or the number of infants studied was small.

Hypothesizing that premature infants may benefit from the anabolic effects of AA without metabolic derangement, we investigated the safety and efficacy of relatively large amounts of AA supplied postnatally to a large group of VLBW infants.

METHODS

SETTING

A randomized, blinded trial was performed in the neonatal intensive care unit (NICU) of the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. For logistic reasons, it was not possible to perform the study using a double-blinded design. The trial was investigator-initiated, with no funding from the pharmaceutical industry. The study protocol was approved by the Erasmus MC Medical Ethical Committee, and parental consent was obtained before random assignment and subsequent enrollment in the study.

STUDY DESIGN

Prematurely born infants with birth weights equal or less than 1500 g born between March 2003 and September 2004 in the hospital and admitted to the NICU were randomly assigned to receive one of two parenteral nutritional schemes, as indicated in Table I. The amount of 2.4 g AA/(kg·d) was chosen because that was the amount that resulted in a positive nitrogen balance in an earlier study [14].

TABLE I: Targeted intravenous macronutrient intake in mg/(kg·min) (glucose) or g/(kg·d) (AA and lipids).

		Day			
		1	2	3	4
Intervention	Glucose [1]	5.5	5.6	5.7	7.1
	AA [2]	2.4	2.4	2.4	2.4
	Lipids [3]	0	1.4	2.8	2.8
Control	Glucose [1]	5.5	5.6	5.7	7.1
	AA [2]	0	1.2	2.4	2.4
	Lipids [3]	0	1.4	2.8	2.8

[1] If enteral feedings were tolerated, parenteral glucose intake was decreased.
[2] Primene 10%, Baxter, Clintec Benelux NV, Brussels, Belgium.
[3] Intralipid 20%, Fresenius Kabi BV, 's Hertogenbosch, the Netherlands.

After the third day of life, all nutrient intakes, including enteral feedings, were the decision of the attending neonatologist. Minimal enteral nutrition (6 to 12 feedings of 1.0 mL) was whenever possible started on postnatal day 2 to day 3 and advanced to full enteral nutrition in the subsequent days if tolerated. We recorded birth weight, gestational age, percentage of SGA infants (< -2 SD) [15], sex ratio, number of prenatal corticosteroid doses (0, 1, or 2), and severity of illness at entry to the study with Apgar and CRIB scores [16]. Exclusion criteria were known congenital abnormalities, chromosome defects, metabolic diseases, and endocrine, renal, or hepatic disorders.

SAFETY ANALYSIS

We analyzed blood gas and glucose concentrations 12 hours after delivery, followed by daily measurements at 8 am until day 6. Plasma urea concentrations were monitored on days 2, 4, and 6 (Roche Hitachi 912, Roche Diagnostics, Basel, Switzerland). On days 2 and 4, we determined plasma AA concentrations (Biochrom 20, Biochrom Ltd, Cambridge, England) in a subset of patients (intervention group n = 17, control group n = 14) to identify possible hyperaminoacidemia (i.e., above reference ranges, as defined in Reference [17]). We also recorded fluid intakes and medications.

EFFICACY ANALYSIS

Efficacy of early AA administration was studied by quantifying the nitrogen balance in both groups on postnatal days 2 and 4. Because most nitrogen leaves the body in urine, we collected urine during a 12-hour period on both study days. Urinary nitrogen content was measured with a CHN elemental analyzer (ANA 1500; Carlo Erba Strumentazione, Milan, Italy). By subtracting the calculated nitrogen excretion rates from the precisely recorded nutritional intakes, nitrogen balances could be defined under the assumption that 1 g of nutritional AA equals 160 mg of nitrogen. Although 24-hour collections of urine are preferable, 12-hour or even 6-hour collections can be used to establish reasonable

estimates of nitrogen excretion [18]. Many investigators used 12-hour urine collections accordingly [8,11,12,19]. Finally, to express efficacy in terms of a measurable clinical variable, we recorded on which postnatal day infants regained their birth weight.

STATISTICS

Differences between groups were tested by Student *t*-tests, Mann-Whitney tests, and χ^2 tests, using SPSS version 11.0 (SPSS Inc, Chicago, IL). Depending on distribution and type of test, values are expressed as mean ± SD, as median (min-max), or as percentage, respectively. Significance level was set at $P < 0.05$. However, because of multiple variables assessed on single samples, differences in AA concentrations were considered to be statistically significant at $P < 0.01$. From previous findings, we calculated that with a power of 0.80, group size needed to be at least 26 to detect a difference in the nitrogen balance of 150 mg N/(kg·d), with a standard deviation of 120 mg N/(kg·d). However, as we intended to study safety aspects as well, we continued to include patients for the full 18 months.

RESULTS

We included 66 infants in the intervention group and 69 in the control group; all infants were included on the basis of intention to treat (Table II). Despite random assignment, infants in the intervention group were more frequently exposed to prenatal corticosteroids ($P = 0.017$). According to study design, the infants in the intervention group received AA within 2 hours after birth (median, 1 hour, 33 minutes). Non-protein energy intakes did not differ between groups, except on day 5 (68 ± 14 [intervention] vs. 63 ± 14 [control] kcal/(kg·d); p = 0.033) (Figure 1).

SAFETY

Results of blood gas analysis and whole blood glucose levels 12 hours after birth and on the second day are shown in Table III. Between postnatal days 3 and 6, there were no differences. Plasma urea concentrations are shown in Table IV. Table V shows individual

TABLE II: Clinical characteristics of the infants in the intervention and control groups.

	Intervention	Control
N (male/female)	66 (34/32)	69 (31/38)
Birth weight (g)	1039 ± 235 [1]	989 ± 252
Gestational age (wk)	28.4 ± 2.0	28.4 ± 1.9
SGA infants (<−2 SD)	20%	29%
CRIB score	3 (0–13) [2]	4 (0–14)
Apgar (5’) score	9 (1–10)	8 (2–10)
Prenatal corticosteroids (% 0/1/2 doses)	18/18/64	39/19/42

[1] Mean ± SD (all such values).

[2] Median (min - max) (all such values).

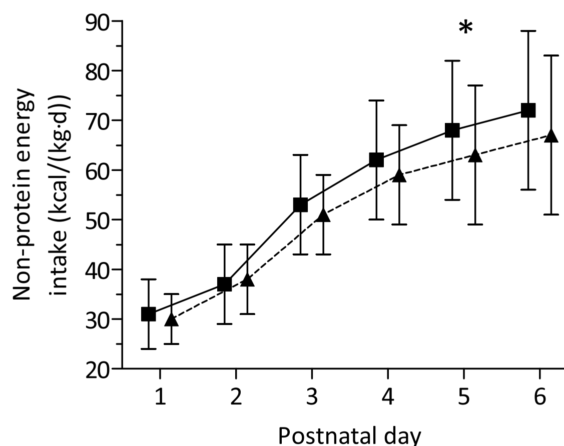

FIGURE 1: Total non-protein energy intakes (parenteral and enteral) during the first postnatal week in the intervention group (squares) and the control group (triangles). * Statistically significant; $p < 0.05$ (Student t-test).

TABLE III: Blood gas analysis and whole blood glucose concentrations in the intervention and control groups 12 hours postnatally and on postnatal day 2. [1]

	12 h		Day 2	
	Intervention	**Control**	**Intervention**	**Control**
pH	7.33 ± 0.08	7.34 ± 0.08	7.31 ± 0.06	7.32 ± 0.07
Base excess (mmol/L)	−4.8 ± 3.1	−3.7 ± 3.3	−5.7 ± 2.4 *	−4.4 ± 2.4
Bicarbonate (mmol/L)	20.5 ± 2.6 *	21.5 ± 2.6	20.3 ± 2.5 *	21.4 ± 2.2
Glucose (mmol/L)	5.7 ± 3.2	6.1 ± 2.4	4.4 ± 1.9 *	5.3 ± 2.1

[1] All values are mean ± SD and differences between groups are tested with Student t-test.

* Statistically significant; $p < 0.05$.

TABLE IV: Plasma urea concentrations in mmol/L and plasma urea nitrogen concentrations in mg N/dL between brackets in both groups on postnatal days 2, 4, and 6. [1]

	Intervention	**Control**
day 2	9.6 ± 2.8 (27.0 ± 7.8) *	6.0 ± 1.8 (16.7 ± 5.2)
day 4	9.4 ± 3.5 (26.4 ± 9.8) *	6.0 ± 3.3 (16.8 ± 9.2)
day 6	8.4 ± 3.8 (23.6 ± 10.7) *	6.7 ± 3.1 (18.7 ± 8.7)

[1] All values are mean ± SD and differences between groups are tested with Student t-test.

[*] Statistically significant; $P < 0.05$.

plasma AA concentrations on the second day of life. No statistical differences between the two groups were found on the fourth postnatal day. Medications, including sodium bicarbonate for metabolic acidosis, were not different between groups.

EFFICACY

As follows from study design, nitrogen intake on the second day was higher in the intervention group (Figure 2). On the fourth day, intakes were similar between groups. Nitrogen excretion rates in the intervention group exceeded excretion rates in the control

TABLE V: Plasma AA concentrations (in µmol/L) in the intervention and control groups on postnatal day 2 (mean ± SD) and reference values from healthy term breast-fed infants on postnatal day 11 [reference 17].

	Intervention	Control	Reference range
Leucine **	148 ± 43	47 ± 13	86 – 171
Isoleucine **	88 ± 33	18 ± 8	31 – 124
Valine **	281 ± 90	88 ± 23	56 – 154
Threonine	125 ± 48	123 ± 63	67 – 143
Lysine **	345 ± 144	98 ± 34	65 – 282
Histidine **	103 ± 53	52 ± 19	25 – 126
Methionine *	42 ± 22	22 ± 9	21 – 55
Phenylalanine **	92 ± 31	58 ± 10	35 – 112
Cystine	31 ± 79	22 ± 12	33 – 55
Tyrosine	83 ± 43	122 ± 57	48 – 122
Alanine **	265 ± 139	124 ± 67	137 – 362
Proline *	175 ± 89	102 ± 56	
Serine *	186 ± 89	116 ± 49	79 – 227
Glycine	282 ± 161	205 ± 70	66 – 432
Arginine **	70 ± 19	29 ± 12	11 – 88
Glutamine	507 ± 296	313 ± 153	147 – 623
Glutamate **	64 ± 34	22 ± 9	76 – 551
Asparagine	39 ± 23	49 ± 24	16 – 21
Aspartate *	35 ± 16	18 ± 14	5 – 46
Taurine	150 ± 87	106 ± 112	
Citrulline	54 ± 67	31 ± 44	20 – 84
Ornithine **	180 ± 87	40 ± 13	39 – 386
OH-Proline	47 ± 26	46 ± 28	

* Statistically significant; $P < 0.01$.
** Statistically significant; $P < 0.001$.

group on both day 2 and day 4. Furthermore, within the intervention as well as within the control group, rates of excretion did not change between days 2 and 4. Consequently, nitrogen balance was higher in the intervention group on day 2 as compared with the control group, which had a negative nitrogen balance. On the fourth day, nitrogen balances in both groups were positive. However, in the control group, the balance was more positive than in the intervention group. There was no correlation between antenatal steroid administration and nitrogen excretion or balance. Fluid intakes were higher in the intervention group on both postnatal day 1 and day 2 due to the administration of AA. On

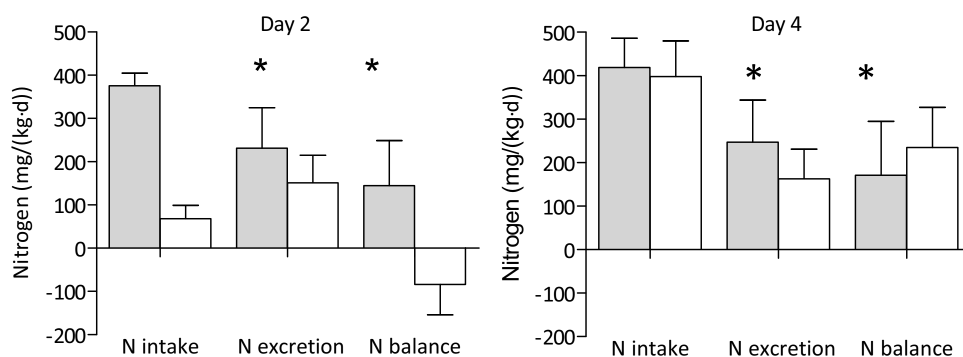

FIGURE 2: Nitrogen balances on postnatal days 2 and 4. Grey bars represent the intervention group; open bars the control group. * Statistically significant; $p < 0.05$ (Student t-test).

all other days, fluid intakes were similar. Fluid balances, determined on postnatal days 2 and 4, did not differ between groups. Age to regain birth weight was not statistically different; newborn infants in the intervention group regained their birth weight at day 8 (2-25) (median and [min-max]), those in the control group at day 10 (2-26) ($p = 0.286$).

DISCUSSION

The currently available AA solutions are safe and can be administered to premature infants during the first few days of life [8-14]. We performed the largest study to date confirming the safety and anabolic effects of early AA administration beginning within 2 hours after birth. Unlike most other reports, we did find modestly altered blood gas values and increased plasma urea concentrations with early AA administration. This could be due to the inclusion of fewer infants in other studies, with subsequently the possibility of reduced statistical power. Another explanation could be the early start of AA administration in our study, which was within 2 hours instead of 24 hours after birth [10,12] or even later [14]. In addition, others used a smaller amount of AA (≤1.5 g/(kg·d)) [8,13] or included infants with higher birth weights [13,14].

We found that early AA administration normalized the plasma concentrations of most AA and that nitrogen balance was positive on day 2 of life, despite a relatively low energy intake (<40 kcal/(kg·d)). Plasma urea concentrations were higher in the intervention group, which theoretically could have increased urine production but in fact did not (data not shown). Besides, fluid balance is usually tightly controlled in NICUs. To our knowledge, no other potential side effects of increased urea concentrations have been reported. The higher urea concentrations are a reflection of a higher AA oxidation rate. This resembles the intrauterine situation in which AA seem to be a key nutrient for energy generation [5,7] and where plasma urea reference values for human umbilical cord blood are 7.5 to 14.3 mmol/L

(21.0 to 40.1 mg/dL) [20]. In conjunction with the higher urea concentrations, the higher amounts of excreted nitrogen in the intervention group also indicate a higher oxidation rate. Higher urea concentrations should, therefore, not be interpreted as a sign of AA intolerance but rather as a reflection of AA oxidation, just like in utero, where the AA are partly oxidized and partly used for protein synthesis.

Many of the infants in the intervention group had on average less hyperglycemia than did the control group, which might be explained by higher insulin concentrations triggered by relatively higher plasma arginine and leucine concentrations [12,21,22]. In addition to these two AA, all essential AA levels, except for threonine and most of the nonessential AA concentrations, were higher and were within the reference range in the intervention group on the second day of life [17]. Although the plasma concentrations of valine, lysine, and asparagine exceeded the reference values measured postnatally in term breast-fed infants, the former two AA concentrations fit within intrauterine reference ranges [23].

The nitrogen balance was calculated by subtracting nitrogen excretion from nitrogen intake. However, nitrogen excretion is often modestly underestimated, because of incomplete urine collections and stool, breath, and skin losses, which are not accounted for [24]. Furthermore, although nitrogen balance measurements demonstrate net loss or accretion of protein, they do not reveal the mechanisms underlying these conditions. Previously performed studies using stable isotope techniques showed that premature infants supplied with AA have an improved balance, which is due to increased protein synthesis, while proteolysis is not suppressed [8,12,14,25].

Inasmuch as premature infants cannot survive without growth, we conclude that the administration of AA soon after birth with the aim of promoting anabolism is safe and effective.

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CHAPTER

3

Effects of early amino acid administration on leucine and glucose kinetics in premature infants

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Published in:
Pediatric Research, Volume 59, May 2006, Pages 732-735

ABSTRACT

Introduction

We previously showed that, in prematurely born infants, an anabolic state without metabolic acidosis can be achieved upon intravenous amino acid (AA) administration in the immediate postnatal phase, despite a low energy intake. We hypothesized that the anabolic state resulted from an increased protein synthesis and not a decreased proteolysis. Furthermore, we hypothesized that the energy needed for the higher protein synthesis rate would be derived from an increased glucose oxidation.

Methods

To test our hypotheses, 32 ventilated premature infants (<1500 g) received intravenously either solely glucose or glucose and 2.4 g AA/(kg·d) immediately postnatally. On postnatal day 2, each group received primed continuous infusions of either [1-^{13}C]leucine or [U-$^{13}C_6$] glucose. $^{13}CO_2$ enrichments in expiratory air and plasma [1-^{13}C]α-KICA (as an intracellular leucine precursor) and [U-$^{13}C_6$]glucose enrichments were measured by mass spectrometry techniques.

Results

The AA administration resulted in an increased incorporation of leucine into body protein and a higher leucine oxidation rate, whereas leucine release from proteolysis was not affected. Glucose oxidation rate did not increase upon AA administration.

Conclusions

The anabolic state resulting from AA administration in the immediate postnatal period resulted from increased protein synthesis and not from decreased proteolysis. The energy needed for the additional protein synthesis was not derived from an increased glucose oxidation.

INTRODUCTION

A series of studies on AA administration in premature infants within the first few postnatal days show a positive effect on nitrogen retention or plasma AA concentrations starting immediately after birth [1,2], within or at 24 h postnatally [3-5], or later [6]. In our latest study regarding early AA administration, we administered 2.4 g AA/(kg·d) to one half of 136 VLBW infants within 2 h postnatally [1]. This resulted in a positive nitrogen balance and converted plasma AA concentrations to levels fitting reference ranges. Furthermore, there were no major metabolic disturbances in comparison with the group receiving solely glucose.

However, nitrogen balance calculations provide no information on how a particular nutritional status was reached. An anabolic state can arise from increased protein synthesis, decreased protein breakdown, or a combination of both. To clarify the mechanism by which an anabolic state is reached in VLBW infants, we conducted in a first trial a stable isotope study using L-[1-^{13}C]leucine. We speculated that the anabolic state would have been induced by an increased protein synthesis, a phenomenon also observed in other studies, none of which, however, started AA supplementation immediately after birth [3,5,6]. Furthermore, by collecting $^{13}CO_2$ we were able to quantify leucine oxidation rates.

We hypothesized that the extra energy required for protein synthesis would be derived from additional glucose oxidation. Therefore, we studied glucose metabolism in a second trial in which infants also received either solely glucose or glucose and AA, using D-[U-$^{13}C_6$] glucose as a tracer.

METHODS

The included infants were a subset of the patients included earlier by Te Braake et al. [1] in a study determining safety and efficacy of high-dose early AA administration. The present study was designed as a randomized open trial and was performed in the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Committee and parental consent was obtained before the study.

PATIENTS

Thirty-two prematurely born infants with a birth weight <1500 g, who were born in the Erasmus MC – Sophia Children's Hospital, were mechanically ventilated, had an arterial catheter, and were expected to be completely dependent on parenteral nutrition for the first 2 d of life, were directly after birth randomly assigned to receive either i) only glucose during the first 2 d (control group, n = 16) or ii) glucose and 2.4 g of protein/(kg·d) as AA (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) within 2 h postnatally

(intervention group, n = 16).

AA and/or glucose solutions were constantly infused without interruptions during the study. Lipids and/or (minimal) enteral feedings were not administered until after the study period. Exclusion criteria were known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders. For all infants, we recorded birth weight, gestational age, SD scores for weight [7], antenatal corticosteroid usage, and severity of illness at entry of the study by means of Apgar and CRIB scores [8]. We also assessed blood gases and nitrogen balances as described previously [1].

The control and intervention groups were each subdivided into two cohorts (n = 8 each). In one cohort (A), we studied the effects of early AA administration on leucine kinetics on postnatal d 2. In the other cohort (B), we determined glucose kinetics on d 2 upon early AA administration.

STABLE ISOTOPES

[^{13}C]Sodium hydrogen carbonate (NaH^{13}CO$_3$) (99% enriched), L-[1-^{13}C]leucine (99% enriched), and D-[U-^{13}C$_6$]glucose (99% enriched) were purchased from Cambridge Isotope Laboratories (Andover, MA) and were diluted with a 0.9% saline solution by the hospital's pharmacy after which it was tested on sterility and pyrogenicity. For the leucine study, the bicarbonate pool was initially enriched with a primed (10 µmol/kg) continuous NaH^{13}CO$_3$ infusion (10 µmol/(kg·h)). After 2 h, the infusion was replaced by a primed (15 µmol/kg) continuous L-[1-^{13}C]leucine infusion (15 µmol/(kg·h)) lasting for 5 h (Figure 1A).

In the second cohort (the glucose study), the bicarbonate pool was also enriched with a primed (15 µmol/kg) continuous NaH^{13}CO$_3$ infusion (15 µmol/(kg·h)). After 2 h, the infusion was replaced by a primed (10 µmol/kg) continuous D-[U-^{13}C$_6$]glucose infusion (5 µmol/(kg·h)) lasting for 6 h (Figure 1B). Tracers were infused with a Perfusor fm infusion pump (B|Braun Medical B.V., Oss, the Netherlands) along the same infusion route as the parenterally administered nutrients.

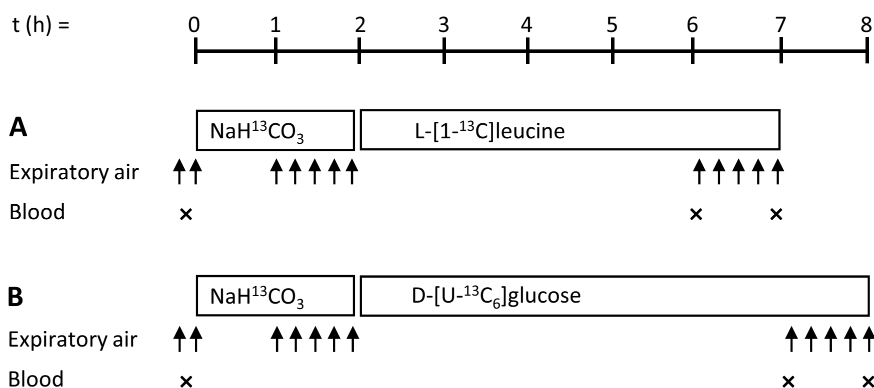

FIGURE 1: Study design. Infants in both the control and intervention group were subjected to either the labeled leucine (A) or the labeled glucose (B) protocol on postnatal day 2.

MEASUREMENT OF ISOTOPIC ENRICHMENTS IN PLASMA.

Arterial blood samples were drawn once before the isotope infusions (baseline) and twice during the last hour of the leucine or glucose tracer infusion. After collection, the samples were put on melting ice immediately and centrifuged, after which the plasma was aspired and stored at -80°C until analysis.

Within the cell, leucine is reversibly transaminated to its keto-analogue, α-KICA. The plasma enrichment of [1-^{13}C]α-KICA is very close to intracellular [1-^{13}C]leucine enrichment. Measurement of the enrichment of [1-^{13}C]α-KICA after L-[1-^{13}C]leucine infusion will, therefore, reflect both the site of incorporation of leucine in protein and the site for the irreversible decarboxylation of [1-^{13}C]α-KICA to isovaleryl-CoA and $^{13}CO_2$ [9,10].

Samples (50 µL plasma) were treated and analyzed as previously described [2,11]. The ^{13}C enrichment of α-KICA was, after derivatization to butyldimethyl-silylquinoxalinol derivatives, determined with a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV, Breda, the Netherlands) by measuring the intensity of the 259 and 260 fragments in electron impact ionization mode.

The [U-$^{13}C_6$]glucose enrichment of the glucose aldonitril pentaacetate derivatives was monitored, after combustion to carbon dioxide at mass 44 for $^{12}CO_2$ and mass 45 for $^{13}CO_2$, using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) (Delta XP, Thermo Electron, Bremen, Germany).

OXIDATION MEASUREMENTS

To determine the fractions of leucine or glucose oxidized, approximately 15 mL of expiratory air was collected in a vacuum tube at the outlet of the ventilator: two times in duplicate before the isotope infusion (baseline), five times in duplicate during the last hour of the $NaH^{13}CO_3$ infusion, and five times in duplicate during the last hour of the labeled leucine or glucose infusion. We assumed an equal CO_2 production and retention during the sodium bicarbonate and leucine or sodium bicarbonate and glucose infusions. Breath samples were analyzed for $^{13}CO_2$ enrichment on an isotope ratio mass spectrometer (IRMS) (ABCA, Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands).

CALCULATIONS

The turnover rates were calculated by measuring tracer dilution in plasma at steady state with standard isotope equations, as previously described for leucine [2] and glucose [12] studies.

STATISTICS

Based on previous findings from our study group, we calculated that with an α of 0.05, a power of 0.80, and a difference in protein synthesis rate of 1.4 g/(kg·d) with an SD of 0.8, group size in the leucine study needed to be at least six to detect a difference [6]. A statistically detectable increment in glucose oxidation of 2.0 mg/(kg·min) with an SD of 1.0 would also require six infants in each group to be studied [12]. However, both in the leucine and the glucose studies, we included eight infants in the intervention and control groups and the control groups to increase power.

One-way ANOVA was used to detect differences between group characteristics, clinical laboratory measurements, and nutritional intakes between the four subgroups. Differences between intervention and control groups were tested by t-tests, Mann-Whitney tests, and χ^2 tests, as appropriate, using SPSS version 11.0 (SPSS Inc., Chicago, IL). Depending on distribution and type of test, values are expressed as mean ± SD, as median (25th to 75th percentile), or as percentage, respectively. Significance level was set at $p < 0.05$.

RESULTS

We included 32 infants, of whom 7 were small for gestational age (<-2 SD) [7]. Patient characteristics are provided in Table I. Overall, the infants in the intervention group had, by coincidence, received antenatal steroids more often than those in the control group. AA administration to the infants in the intervention group started within 2 h postnatally. The stable isotope study was started on the second postnatal day, i.e., between 20 and 44 h after birth. Isotopic steady state in $^{13}CO_2$ excretion in expiratory air was reached in all infants during the last hour of each infusion (Figure 2).

The actual protein intakes at time of study were 0 ± 0 and 2.32 ± 0.08 g/(kg·d) ($p < 0.001$) and the non-protein energy intakes (solely glucose) were 34 ± 8 and 30 ± 6 kcal/(kg·d) ($p = 0.103$) in the control and intervention groups, respectively. Other relevant patient data are provided in Table II. Inasmuch as we only performed a power calculation on protein synthesis and glucose oxidation rates, all other outcomes should be regarded as hypothesis-generating data.

Leucine kinetic data are displayed in Figure 3. Infants in the intervention group had a higher leucine flux, NOLD rate (indicative of protein synthesis), and oxidation rate. The LRP rate (indicative of protein breakdown) was not altered. Leucine balance improved significantly in the infants receiving AA ($p < 0.001$). The control group had a negative

TABLE I: Clinical characteristics of the infants in the control and intervention groups.

	Control	Intervention
N (male/female)	16 (9/7)	16 (8/8)
Birth weight (kg)	0.949 ± 0.231 [1]	0.923 ± 0.192
Gestational age (wks)	27.4 ± 1.4	27.3 ± 1.8
SD score for weight	-0.9 ± 1.4	-1.0 ± 1.3
CRIB score [2]	6 (3-9) [3]	5 (2-7)
Apgar score (5 min)	8 (7-9)	9 (8-9)
Antenatal corticosteroids (0/1/2 doses)	8/6/2	2/4/10

[1] Mean ± SD (all such values).

[2] CRIB is clinical risk index for babies [8].

[3] Median (25th – 75th percentile) (all such values).

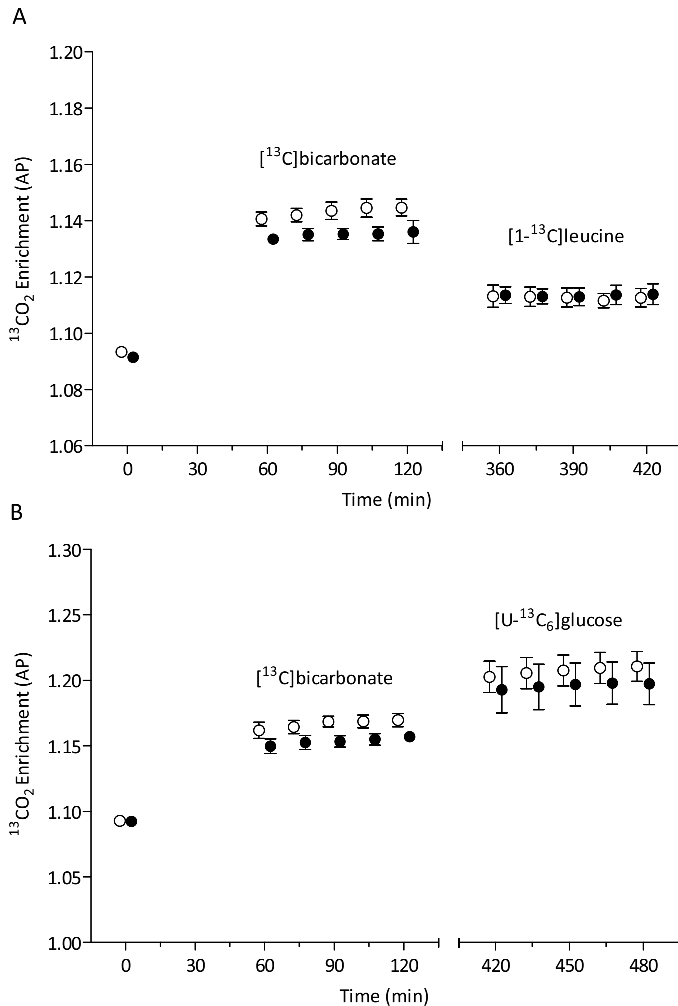

FIGURE 2: $^{13}CO_2$ excretion curve during the leucine (A) and glucose (B) experiments. Enrichments are represented in atom percent (AP) as mean ± SD in the control (open circles) and intervention (grey circles) groups.

balance, whereas the balance in the intervention group was not different from zero.

The glucose kinetic data are outlined in Figure 4. AA administration did not have any significant effects on glucose metabolism with regard to GPR, flux, oxidation, and NOGD rate. In both groups, GPR (gluconeogenesis or glycogenolysis) was not completely inhibited, despite a mean intake of 5.7 ± 1.2 mg glucose/(kg·min) during the study period. In the control group, 75% of the glucose flux was oxidized; in the intervention group, this fraction amounted to 84% ($p = 0.185$). The absolute amount of oxidized glucose did not differ significantly between groups (5.3 ± 1.3 mg/(kg·min) and 5.8 ± 1.4 mg/(kg·min), respectively; $p = 0.462$).

TABLE II: Study parameters on the second day of life in the control and intervention groups.

	Control	Intervention
Glucose intake (mg/(kg·min))	5.9 ± 1.3 [1]	5.2 ± 1.0
Blood glucose concentration (mmol/L)	6.1 (4.2 – 6.9) [2]	4.6 (3.7 – 5.4)
Nitrogen balance (mg N/(kg·d))	-99 ± 42	151 ± 105 *
pH	7.31 ± 0.05	7.30 ± 0.07
Base Excess (mmol/L)	-4.4 ± 1.3	-5.4 ± 2.0
Plasma urea concentration (mmol/L)	6.2 ± 1.5	9.7 ± 2.6 *

[1] Mean ± SD (all such values).

[2] Median (25th – 75th percentile) (all such values).

* Statistically significant; p<0.05.

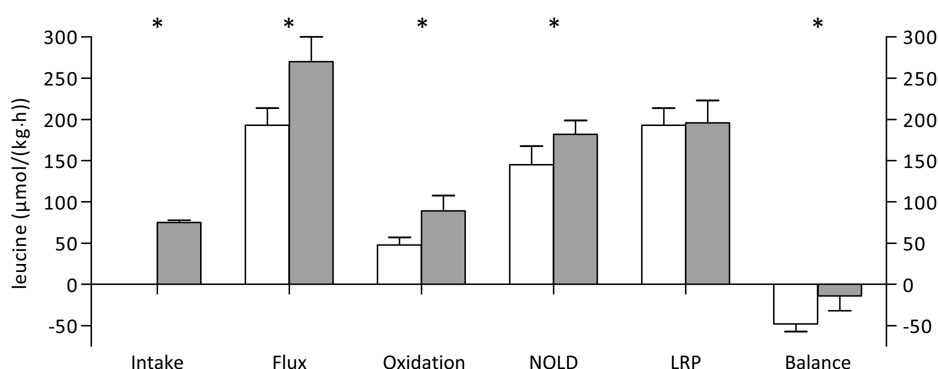

FIGURE 3: Leucine kinetics. Data from the [1-^{13}C]leucine infusion protocol (A) on postnatal day 2 in infants in the control (open bars, n = 8) and intervention (filled bars, n = 8) groups. Bars represent mean ± SD. NOLD represents protein synthesis. LRP represents protein breakdown. *Statistically significant, p < 0.05.

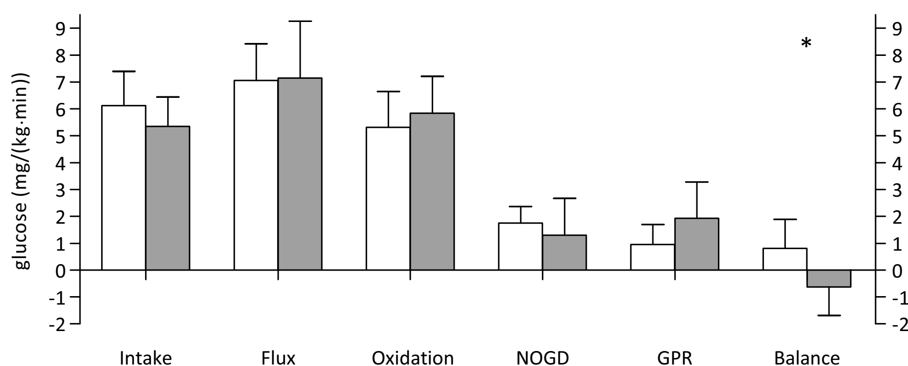

FIGURE 4: Glucose kinetics. Data from the [U-$^{13}C_6$]glucose infusion protocol (B) on postnatal day 2 in infants in the control (open bars, n = 8) and intervention (filled bars, n = 8) groups. Bars represent mean ± SD. *Statistically significant, p < 0.05.

DISCUSSION

We found that AA administration at a relatively high dose from birth onward exerted its anabolic effect through increased protein synthesis and not decreased proteolysis. The additional energy needed was not derived from glucose, but could, at least partially, be derived from a concomitant increase in AA oxidation.

Most studies in premature infants, including the present, show a positive effect of AA administration on protein accretion caused by an increased synthesis rate [3,5,6,13]. Also, in the ovine fetus, AA administration does have a beneficial effect on protein accretion by increasing protein synthesis while leaving proteolysis unaltered [14]. One study, however, investigating a short-term change in nutritional regimen, found a simultaneous decrease in proteolysis [15].

Anabolism in adults [16,17] and healthy term infants [18,19], unlike in preterm infants and ovine fetuses, is predominantly achieved by suppression of proteolysis instead of protein synthesis. Possibly, a new balance between protein breakdown and synthesis is developing during early life, explaining this observed discrepancy.

We found a positive nitrogen balance, not only in the intervention group of the 135 infants studied earlier [1], but also in the intervention group of the leucine cohort in this study. However, the leucine balance was not significantly different from zero. Nevertheless, there is still a significant correlation between both balance methods ($r^2 = 0.47$, $p = 0.003$). This discrepancy, which we and others noted before [2,3], might be explained by the relative abundance of leucine in parenteral AA solutions relative to the occurrence of leucine in body protein. Because the rate of protein deposition is controlled by the rate-limiting AA in the AA solution, all excess AA are oxidized. Leucine in particular might be oxidized pro rata more than other AA, explaining a negative leucine balance despite a positive nitrogen balance. On the other hand, the difference between the stable isotope and nitrogen balance techniques could also be partially due to the tendency to overestimate nitrogen retention [20].

In our study, early AA administration had hardly any effect on glucose metabolism. Glucose oxidation, NOGD, and GPR were unaltered. In adults, AA supplementation, and thus provision of gluconeogenic substrates, was found to result in an increased endogenous GPR [21]. This contrasts with findings in premature neonates from the study by Poindexter et al. [13] and the present study. Like in adults, we expected to find a higher glucose oxidation rate for generating the energy needed for extra protein synthesis after AA administration. Poindexter et al. [13], using indirect calorimetry, also suggested a higher glucose oxidation rate after AA had been introduced, inasmuch as the respiratory quotient increased. Surprisingly, we could not detect any difference in oxidation rate. The source of the needed extra energy remains, therefore, speculative. Nevertheless, the higher leucine oxidation rate might reflect that AA oxidation itself might provide some of the energy needed. Other sources of energy could include fatty acids and ketone bodies.

In conclusion, AA administration to premature infants from birth onward reverses the catabolic state that is otherwise obtained when AA are withheld. Particularly protein synthesis rate especially is increased. The additional energy needed for this process is not derived from glucose oxidation.

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CHAPTER

4

Albumin synthesis in premature neonates is stimulated by parenterally administered amino acids during the first days of life

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Published in:
The American Journal of Clinical Nutrition, Volume 86, October 2007, Pages 1003-1008

ABSTRACT

Background
Recently we demonstrated that parenteral administration of amino acids (AA) immediately after birth to premature infants is safe and results in a positive nitrogen balance and increased whole body protein synthesis. However, we did not determine organ specific effects; Albumin, produced by the liver, is an important protein, but its concentration is often low in premature neonates during the first few days after birth.
Objective
To test the hypothesis that the albumin fractional and absolute synthesis rates would increase upon AA administration following birth, even at low non-protein energy intake.
Design
Ventilated premature infants (<1500 g) received from birth onwards either solely glucose (control group, n=7) or glucose and 2.4 g AA/(kg·d) (intervention group, n=8). On postnatal day 2, all received a primed continuous infusion of [1-^{13}C]leucine and mass spectrometry techniques were used to determine the incorporation of the leucine into albumin. Results are expressed as median (25th – 75th pctl).
Results
Albumin fractional synthesis rates in the intervention group were significantly higher than those in the control group (22.9 (17.6 – 28.0) %/d versus 12.6 (11.0 – 19.4) %/d; p=0.029). Likewise, the absolute synthesis rates in the intervention group were higher than those in the control group (228 (187 – 289) mg/(kg·d) versus 168 (118 – 203) mg/(kg·d); p=0.030).
Conclusion
Amino acid administration increases albumin synthesis rates in premature neonates even at low energy intake.

INTRODUCTION

Plasma albumin concentration is a routinely measured parameter on the neonatal intensive care unit (NICU) and is often found to be low in ill premature infants [1,2]. Albumin, produced by the liver, has several important roles in neonatal physiology [3,4]. It is the main preserver of the colloid osmotic pressure in plasma (~75%), functions as an anticoagulant, and is an important binding transporter of certain metabolites, e.g. bilirubin, free fatty acids, and drugs. Moreover, albumin is an important antioxidant because it has specific binding sites for copper ions and a free sulfhydryl group, which can scavenge harmful reactive oxygen species [5]. The free sulfhydryl group can also bind nitric oxide (NO) forming a reservoir for this regulator of vascular tonus [6]. Furthermore, albumin synthesis probably provides for temporary 'storage' of amino acids (AA) so as to spare them from oxidation [7-9]. Albumin consists of 585 AA and is the most abundant plasma protein [2], though about 60% of the total albumin pool is in the interstitial space [10].

Albumin metabolism has been studied mainly in healthy adults and in adults during various stages of renal or liver disease. Most studies in neonates are limited to static properties such as concentrations. Measuring albumin synthesis rates would no doubt provide more insight into the dynamics of albumin metabolism and its response to nutrition, for example.

Several authors have described relations between low albumin concentration and morbidity and mortality rates among premature neonates [11,12]. In the fasting state, albumin concentrations drop 2 to 3 g/L in the first 24 hours after birth [2]. As there is discussion about benefit and safety of exogenous albumin infusions in premature infants [13 -15], stimulating endogenous synthesis via adequate nutritional support might be an attractive alternative. The latter strategy requires good knowledge of premature infants' protein metabolism. Studies using stable isotopes have provided insights into anabolism and catabolism in general [16-18]. For one, AA administration directly after birth stimulates whole body protein synthesis rather than depressing protein breakdown [16]. Studying whole body metabolism is limited, however, in that it only provides information on the average of all metabolic processes in the body rather than organ specific changes. It is unknown whether exogenous administration of AA also stimulates organ specific protein synthesis, e.g. albumin, in premature infants. Albumin synthesis can be quantified by measuring the incorporation rate of a stable isotope labeled AA into plasma albumin.

We report a study in preterm infants aimed at determining the effect of AA administration starting directly after birth on subsequent albumin synthesis. We hypothesized that AA added to glucose would increase albumin synthesis rates.

SUBJECTS AND METHODS

SUBJECTS

Patients were eligible for the study when they were in the Sophia Children's Hospital, had a birth weight less than 1500 g, and required an arterial line for clinical purposes. Exclusion criteria were known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal or hepatic disorders. The cohort here described is the same as in an earlier study on whole body leucine metabolism [16]. It is a subset of infants participating in a large trial on AA administration in the immediate postnatal phase [19] and were selected if born within a predefined time span during that study and if they met current inclusion criteria. The study was designed as a randomized open trial and was performed in the NICU of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Review Board and written parental consent was obtained prior to study.

EXPERIMENTAL DESIGN

Within two hours after birth, infants were randomly assigned to receive during the first two postnatal days either glucose only (control group, n=7) or glucose and 2.4 g protein/(kg·d) as AA (Primene 10%, Baxter, Clintec Benelux NV, Brussels, Belgium) (intervention group, n=8).

The administration of glucose solution or the AA and glucose solution was accomplished by continuous infusion. Lipids and/or (minimal) enteral feedings were withheld during the study period. None of the infants received exogenous albumin infusions during the study. The hospital's pharmacy dissolved L-[1-^{13}C]leucine (99% enriched, Cambridge Isotope Laboratories, Andover, MA, USA) in normal saline and tested it for sterility and pyrogenicity. We infused it (prime: 15 µmol/kg; continuous 15 µmol/(kg·h)) with the use of an infusion pump (Perfusor fm; B Braun Medical B.V., Oss, the Netherlands).

Arterial blood samples (0.4 mL) were drawn before the isotope infusion (baseline) and after 4 and 5 hours of infusion. The blood samples were immediately put on melting ice and centrifuged (2500 × *g*, 10 min, 4 °C), after which the plasma was stored at -80°C until analysis.

ANALYTICAL METHODS

To isolate plasma albumin [20], samples (50 mL plasma) were deproteinized and washed with 10% trichloroacetic acid. To the protein pellet, water and 1% trichloroacetic acid in 96% ethanol were added and the sample was centrifuged. The supernatant was mixed with 26.8% ammonium sulfate to precipitate albumin overnight. The pellet was then dissolved in 0.3 mol NaOH/L and again precipitated with 2 mol perchloric acid/L. After washing, the new pellet was redissolved in 6 mol HCl/L and hydrolyzed for 24 hours, after which the acid was dried under nitrogen and dissolved in water. AA were isolated using a cation-exchange column, derivatized with ethylchloroformate, and enrichment was measured on a gas

chromatograph – combustion – isotope ratio mass spectrometer (GC-C-IRMS; Delta XP; Thermo Electron, Bremen, Germany) as previously described [21].

As albumin precursor we used the plasma [1-^{13}C]α-ketoisocaproate (α-KIC, the keto acid of leucine) enrichment at plateau which had already been measured as described in our preceding paper [16]. While liver amino acyl-tRNA enrichment forms the true precursor, its use requires tissue biopsies and technically demanding assays. Nevertheless, α-KIC enrichment adequately represents leucyl-tRNA enrichment and is valuable in this type of research [22,23]. Plasma albumin concentrations were routinely measured on a Roche Hitachi 912 (Roche Diagnostics, Basel, Switzerland).

CALCULATIONS

The FSR reflects the fraction of the intravascular albumin pool that is renewed per unit of time (%/d) and can be calculated as follows:

$$FSR = \frac{E_{leu\text{-}alb,t_2} - E_{leu\text{-}alb,t_1}}{E_{KIC}} \times \frac{24}{t_2 - t_1} \times 100\%$$

where $E_{leu-alb}$ is the enrichment in mole percent excess (MPE) of incorporated leucine in albumin at t_2 and t_1 (at 5 and 4 hours after the start of infusion, respectively) and E_{KIC} is the mean enrichment in MPE of the precursor, i.e. plasma α-KIC, at both time points.

The ASR represents the absolute amount of albumin that is produced per unit of time (mg/(kg·d)), and can be calculated as follows:

$$ASR = FSR \times C_{alb} \times vol_{bl} \times (1 - Ht) \times weight^{-1}$$

where C_{alb} is the plasma albumin concentration in g/L, vol_{bl} is the infant's total blood volume in mL (assumed to be 75 mL/kg body weight [24,25]), (1 – Ht) is the fraction of blood which is plasma, and weight is birth weight in kg.

We also calculated the contribution (%) of albumin ASR in relation to whole body protein synthesis in percentage based on previously measured leucine turnover data according to the following formula:

$$contribution = \frac{ASR \times 0.104}{NOLD \times 131.2 \times 24 \times 0.001} \times 100\%$$

where NOLD is the non oxidative leucine disposal representing whole body protein synthesis in µmol/(kg·h) and which was calculated in an earlier study by our group [16]. Furthermore, 0.104 represents the fraction of leucine residues in albumin on a weight basis, 131.2 is the mole mass of leucine, and 24 and 0.001 convert to day and milligram, respectively.

STATISTICS

Calculations were made with Microsoft Office – Excel software (version 2000; Microsoft Corp, Redmond, WA, USA) and all statistical tests were done with GraphPad Prism software (version 4.0; GraphPad, San Diego, CA, USA). Differences between control and intervention groups were tested by Mann-Whitney tests unless otherwise stated. Values are expressed as median (25th – 75th percentile) or as mean (SD) and significance level was set at $p<0.05$.

RESULTS

Fifteen premature infants were studied: seven in the control group and eight in the intervention group. All infants were mechanically ventilated. Birth weight, gestational age, SD score for weight [26], sex, disease scores (Apgar and CRIB [27]), and antenatal steroid use to improve lung maturation were not significantly different between the groups (Table I). The birth weights of one infant in the control group and two infants in the intervention group were below 2 SD when related to gestational age. Blood gas parameters, whole blood glucose concentrations, and non-protein calorie intakes (only glucose) on the second day of life did not differ between groups (Table II). Because the intervention group received AA, the plasma urea concentration and the nitrogen balance were higher in these infants.

The mean leucine enrichments in albumin in the control group were 0.243 (0.12) and 0.289 (0.13) mole percent excess (MPE) after 4 and 5 hours of infusion, respectively. In the intervention group, enrichments were 0.201 (0.050) and 0.249 (0.050) MPE. The mean α-KIC enrichments at plateau were 7.16 (0.56) MPE in the control group and 5.18 (0.46) MPE in the intervention group.

The intervention group showed significantly higher albumin FSR than the control group (Figure 1). Albumin plasma concentration was measured in 5 out of 7 infants in the control group and in 6 out of 8 infants in the intervention group; it was significantly higher in the intervention group (Figure 2). The calculated ASR was also higher in the intervention group (Figure 3). Because we had also obtained leucine turnover data [16], we were able to compare the albumin ASR with the whole body protein synthesis rate. The median NOLD (a measure of protein synthesis) increased from 130 (122 – 172) to 185 (169 – 203) μmol leucine/(kg·h) upon AA administration (p=0.030). The proportion of leucine used for albumin synthesis relative to whole-body NOLD was approximately 4% in both subject groups (Figure 4), which implies that AA administration stimulates albumin synthesis and whole-body protein synthesis at a similar rate.

TABLE I: Clinical characteristics. [1]

	Control	Intervention
No. (male/female) (n)	7 (2/5)	8 (4/4)
Weight (kg)	0.960 (0.780 – 1.080) [2]	0.940 (0.770 – 1.070)
Gestational age (wks)	26.7 (26.4 – 28.9)	26.9 (26.6 – 29.9)
SD-score for weight [26] (SD)	-0.53 (-0.75 – -0.11)	-1.14 (-2.22 – -0.19)
CRIB score [27]	5 (2 – 8)	3 (1.3 – 5)
5 min Apgar score	9 (7 – 9)	9 (8 – 10)
Antenatal corticosteroids (no/yes) (n)	(2/5)	(1/7)

[1] There were no statistical differences between groups (Mann-Whitney).

[2] Median (25th – 75th percentile) (all such values).

TABLE II: Study parameters on the second day of life. [1]

	Control (n=7)	Intervention (n=8)
Non-protein energy intake (kcal/(kg·d))	33.3 (30.7 – 36.9)	31.2 (26.0 – 32.9)
AA intake (g/(kg·d)) *	0	2.3 (2.3 – 2.4)
Nitrogen balance (mg N/(kg·d)) *	-110 (-133 – -56)	156 (116 – 226)
Plasma urea concentration (mmol/L) *	6.2 (5.8 – 6.9)	9.7 (7.6 – 11.8)
Glucose concentration (mmol/L)	4.9 (3.1 – 6.2)	3.9 (3.0 – 4.8)
pH	7.31 (7.25 – 7.33)	7.31 (7.28 – 7.38)
Base excess (mmol/L)	-5 (-6 – -4)	-6 (-6 – -3)

[1] All values are median (25th – 75th percentile).

* Statistically significant, p<0.05 (Mann-Whitney).

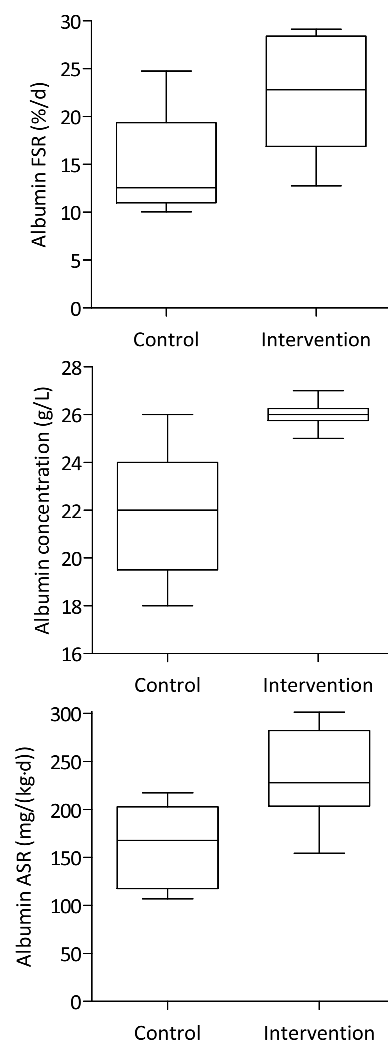

FIGURE 1: Albumin FSR in %/d in the control (n=7) and intervention (n=8) groups. Presented as median and interquartile range. The intervention group had a significant higher albumin FSR (p=0.029; Mann-Whitney).

FIGURE 2: Albumin plasma concentration in g/L in the control (n=5) and intervention (n=6) groups. Presented as median and interquartile range. The intervention group had a significant higher plasma albumin concentration (p=0.030; Mann Whitney).

FIGURE 3: Albumin ASR in mg/(kg·d) in the control (n=5) and intervention (n=6) groups. Presented as median and interquartile range. The intervention group had a significant higher albumin ASR (p=0.030; Mann Whitney).

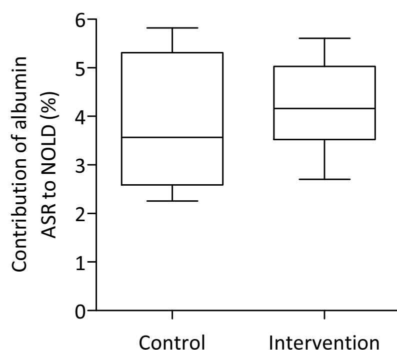

FIGURE 4: Contribution in % of albumin synthesis (ASR) to whole body protein synthesis (NOLD) in the control (n=5) and intervention (n=8) groups. Presented as median and interquartile range. There was no difference between groups (Mann-Whitney).

DISCUSSION

Our data are consistent with the assumption that parenteral AA administration in premature neonates stimulates albumin synthesis. For on the second day of life albumin FSR, ASR, and plasma albumin concentrations were significantly higher in premature infants who had received parenteral AA from birth onwards.

Plasma albumin concentration is governed by four processes; changes in synthesis, degradation, intravascular space, and transcapillary escape. In the present study, we were not able to measure the three latter processes, but we speculate that increased synthesis is the primary cause for the higher plasma concentration noted. Alternatively, a rise in albumin synthesis does not automatically coincide with a parallel rise in concentration. Albumin is a negative acute-phase protein, which means that its concentration will fall during an inflammatory event. Such falls have been described during cholecystitis [28], hemodialysis [29], cancer [30], and in head trauma patients [31], despite a coinciding rise in albumin FSR. Cytokines might be responsible for this paradoxical increase [29,32]. The lowered concentrations probably result from concomitant increases in catabolic rate and transcapillary escape.

The albumin FSR in healthy adults is about 6-8 %/d [8,9,32-36] and seems unresponsive to intravenous nutrients [37]. Meals, however, will increase albumin synthesis [7,8,38]. A recent study showed that the protein portion of meals is the effective component responsible for this increase [9]. Adults suffering from chronic hemodialysis were also found sensitive to nutrition, as albumin FSR improved after intradialytic administered nutrition [20]. Overall, it seems that albumin synthesis in adults is more responsive to gastro-intestinal nutritional uptake than intravenous nutrition, as was also demonstrated after surgery [39] and in rats [40]. Our findings in human neonates and findings in young piglets suggest that other metabolic mechanisms might be regulating albumin synthesis in younger individuals, whose albumin synthesis is also responsive to parenteral nutrition [41,42]. Consistent with the general finding that younger individuals have higher metabolic rates than adults, higher albumin FSR, ranging from 15 to 20 %/d, have been found in 12-month-old infants [43,44]. Bunt et al found values of 14 %/d in fasted premature infants with a

gestational age of 28 weeks on the first postnatal day [21]. Yudkoff et al calculated a mean albumin FSR of 12%/d in parenterally fed, premature neonates with appropriate size-for-gestational-age (which was 28 weeks), after approximately 1 week of life [45]. These figures correspond well with the synthesis rates we observed in this study. Yet, unlike Bunt et al [21], we did not find clear correlations between the FSR and SD-scores for weight related to gestational age. This might have been the result of reduced power in our study or interference by our nutritional intervention.

We calculated that albumin constitutes about 4% of all proteins synthesized in the body. Besides, in healthy humans and rats, it was estimated that of all proteins synthesized in the liver, including those not excreted but produced for intrahepatic maintenance, 15% was albumin [34,38]. Combining these figures reveals that the liver would contribute over 25% to whole body protein synthesis. Normal hepatic functioning would therefore seem to be of vital importance. Apart from all the important roles of albumin mentioned in the introduction, increasing the albumin FSR is also interesting from a nutritional point of view. A higher albumin ASR makes premature infants less vulnerable to catabolic insults through the temporary storage of AA in albumin, preventing excess AA from being oxidized. Later, during low protein intake or increased demands, body protein stores can be spared albeit at the cost of albumin breakdown, thereby releasing free AA.

Especially in the first 24 hours after premature birth, non-protein energy intake is usually very low (~30-35 kcal/(kg·d)) and less than desirable. Recently, energy expenditure was measured in premature infants during the first few days after life having comparable caloric intakes [46]. Energy expenditure was estimated at 29-35 kcal/(kg·d), thus leaving, at an intake of approximately 30 kcal/(kg·d), no calories for net energy storage or growth. As a consequence, AA efficacy in terms of anabolism is usually moderate in that a large fraction will be irreversibly oxidized [16]. Carbohydrate intake is limited due to potential hyperglycemia and fluid restrictions. Parenteral lipids are also often withheld in the first 24 hours after premature birth, as neonatologists fear pulmonary disease, hypertriacyl-glycerolemia, and high free fatty acid concentrations leading to competition with bilirubin binding on albumin [47]. Albumin is the main transport vehicle for fatty acids to and from the tissues according to metabolic demands. Notwithstanding the fact that lipids in blood are largely in the form of triacylglycerols, the turnover and utilization of fatty acids bound to albumin is high, thus making fatty acids an important contributor to lipid metabolism [48]. Providing AA directly after birth increases albumin synthesis and subsequent binding capacities, which theoretically improve the tolerance of intravenous lipids. Apart from the advantage of delivering essential fatty acids, the high caloric content makes immediate commencement of lipids after birth beneficial by improving the energy balance. This in turn might stimulate protein synthesis even more. A recent study with high dose AA and lipids initiated immediately after birth, demonstrated high anabolic use of AA, probably due to a higher energy availability [49]. We would like to speculate that an increased albumin synthesis rate was at least partially responsible for the increased tolerance of lipids. More

clinical trials are required to determine efficacy and safety of starting parenteral administration of lipids together with high dose AA immediately after birth to premature infants.

A potential limitation of this study is that the hydrolyzed protein pellet may not have contained pure albumin. Jacobs et al earlier reported that after simple ethanol extraction about 8% of proteins were contaminants [50]. By adding ammonium sulfate we aimed to eliminate some of the contaminating proteins [20]. However, even if purification of the protein pellet was still incomplete, the vast majority must have been albumin.

In conclusion, we have shown that introducing AA immediately after birth to premature neonates stimulates not only whole body protein synthesis, but also albumin synthesis. This finding might have important implications in view of the vital roles of albumin, among which serving as an antioxidant and binding bilirubin and free fatty acids. Improving albumin synthesis might, therefore, have major impact on later outcome.

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CHAPTER

5

Two year follow-up of early postnatal amino acid administration to premature infants
- short report -

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Published in:
Submitted

INTRODUCTION

Since the early 1970s the routine availability of parenteral nutrition has enabled the administration of lipids and amino acids (AAs) to preterm infants in the neonatal phase. However, administration of first generation AA solutions resulted in serious metabolic disturbances, impeding their use in preterm infants, and impacting on nutritional regimens up till the present day. A large number of studies has been conducted since, on what is described as *aggressive* early AA administration. In this journal in 2005, we published data of a randomized clinical trial, in which we investigated safety and efficacy aspects of early AA administration in a group of 135 very low birth weight (VLBW) infants [1]. Our results reconfirmed metabolic safety of early AA administration while a catabolic state was converted into anabolism.

Although it is known that postnatal growth restriction may have long-lasting adverse effects, such as short stature and compromised neurodevelopmental [2,3], evidence for improved long-term outcome as a result of early AA administration is limited [4]. In fact, recently some concerns have been raised about safety and efficacy of early aggressive AA administration [5-8].

In this short report, we describe neurodevelopmental outcome at two years of age in our previous cohort of 135 very low birth weight infants. We hypothesized that early AA administration at a dose of 2.4 g/(kg·d) does not negatively affect neurologic development.

METHODS

In our initial study we included 66 infants with a birth weight below 1500 g who received AAs (2.4 g/(kg·d)) directly from birth onwards (intervention group), and 69 infants who received glucose only for the first two days of life (control group) [1]. Of the 135 infants, 132 had a gestational age less than 32 weeks and were therefore considered eligible for neurodevelopmental follow-up and anthropometric assessment at 2 years of corrected age. All parents gave written permission to use the follow-up data for statistical analyses.

The prevalence of handicaps, such as cerebral palsy, visual and hearing impairments was documented. The Mental Developmental Index (MDI) was assessed by the Bayley Scales of Infant Development (BSID), 2nd edition. Furthermore, anthropometric data were recorded.

Data were analyzed using the statistical package SPSS version 15.0 (SPSS Inc, Chicago, USA). Data are described as mean ± SD. A p-value <0.05 was considered statistically significant.

RESULTS

In the initial study we included 132 infants with a gestational age less than 32 weeks. Eighteen infants died in the neonatal phase (ten infants in the control group; eight infants in the intervention group). Twelve of the 114 surviving children were lost to follow-up (eight in the intervention and four in the control group). Consequently, in 102 children (89%, 47 in the intervention and 55 in the control group), follow up data were available. There were no differences in demographic data except for a trend towards more males in the intervention and more females in the control group (Fisher's Exact Test p=0.05).

Table I shows neurologic outcome at two years of age. No differences were observed between the intervention and control group as a whole. In boys but not in girls, the percentage of handicaps (e.g. visual/hearing deficiency, cerebral palsy and/or MDI<70) in the intervention versus the control group tended to be lower (p=0,10) (Figure 1). Table II shows anthropometric data of the included children. Again, no differences were observed between groups.

DISCUSSION

In the past decade, a large number of studies have been published on early AA administration and its beneficial effects in terms of promoting anabolism in the early postnatal phase. Recently we demonstrated additional benefits upon early AA

TABLE I: Neurodevelopmental outcome at two years of age in infants in the control and intervention groups.

		Control (n=55)	**Intervention (n=47)**	**Total (n=102)**
Any handicap [1]	Absent	44 (80%)	42 (89%)	86
	Present	11 (20%)	5 (11%)	16
Neurology	Normal	47 (86%)	38 (81%)	85
	Mildly abnormal	4 (7%)	8 (17%)	12
	Cerebral palsy	4 (7%)	1 (2%)	5
Visual deficiency	Absent	53 (96%)	47 (100%)	100
	Present	2 (4%)	0 (0%)	2
Hearing deficiency	Absent	54 (98%)	46 (98%)	100
	Present	1 (2%)	1 (2%)	2
Mental Developmental Index (BSDI II) [2]	mdi >85	36 (77%)	34 (76%)	70
	mdi 85-70	6 (13%)	8 (18%)	14
	mdi <70	5 (11%)	3 (7%)	8

[1] visual/hearing deficiency, cerebral palsy or MDI<70.

[2] visual/hearing deficiency and cerebral palsy (n=8) and refusals (n=2) excluded.

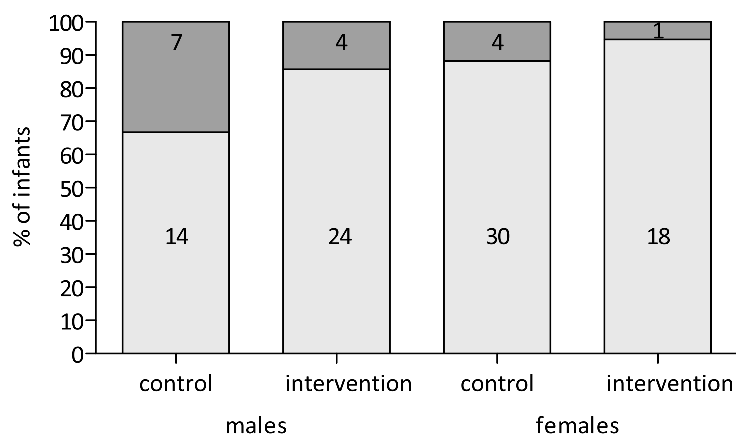

FIGURE 1: Absence (light-grey) or presence (dark-grey) of handicaps (visual/hearing deficiency, cerebral palsy and/or MDI<70) in boys and girls in the control versus the intervention group at two years of age. Numbers inside bars indicate the total number of infants evaluated.

TABLE II: Weight and head circumference at two years of age in infants in the control and intervention groups.

	Control (n=50)	Intervention (n=44)
Weight (kg)	11.4 ± 1.68	11.6 ± 1.83
Head circumference (cm)	48.2 ± 1.74	48.5 ± 1.85
ΔSD weight 2 y minus birth (SD)	-0.112 ± 1.39	0.122 ± 3.10
ΔSD head circumference 2 y minus birth (SD)	-0.30 ± 1.52	-0.04 ± 1.58

administration in terms of increased albumin and glutathione synthesis rates [9,10]. In our study, however, there was no benefit in terms of growth at two years of age. In most studies anthropometric measurements improve at discharge after early or high AA administration [11-14]. Measured effects at later age are more sparse. Poindexter and colleagues, however, for example found that head circumference measured in 1000 infants at 18 months corrected age was larger in boys in the high dose amino acid supplemented group, although no effects could be observed in mental or psychomotoric indices [13]. In addition, favorable effects of a rapid postnatal weight gain on neurodevelopment, as assessed by BSID, were found in a study of Latal-Hajnal et al. in 219 VLBW infants [3].

In the exploratory study described here, we demonstrate that providing 2.4 g AAs/(kg·d) seems safe with respect to the handicap rate at two years of age. On the other hand, no statistically significant clinical benefits were demonstrated as yet, although there were many theoretical advantages of being anabolic during the early postnatal phase. Neurodevelopmental outcome in preterm infants is, however, dependent on multiple variables, of which only one is nutrition. It is therefore very difficult to determine whether

deprivation of two days of AAs will have a major clinically measurable effect on long-term outcome.

In general, girls tend to have a more favorable neurodevelopmental outcome than boys, which is also observable in our data. Furthermore, the handicap rate in boys in the intervention group tended to be lower than in boys in the control group. The number of studied children was relatively small, however and the age of 24 months is too young for definitive conclusions regarding neurocognitive outcome at a later age.

In conclusion, in this exploratory study, no negative effects of early AA administration on postnatal growth and handicap rate were found. Nevertheless, we strongly encourage a nutritional strategy including early AA administration, seeing its beneficial effects in the early neonatal phase. Long term follow-up is, however, mandatory to define whether early aggressive AA administration will have a, perhaps subtle, effect on long-term, neurocognitive outcome.

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PART III

CHAPTER

6

Human fetal albumin synthesis rates during different periods of gestation

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Published in:
The American Journal of Clinical Nutrition, Volume 88, October 2008, Pages 997-1003

ABSTRACT

Background
Despite nutritional intervention, albumin concentrations are often low in critically ill premature neonates.
Objective
Our aim was to quantify albumin synthesis rates during early life under physiologic circumstances. Human fetuses thereby reflect the developmentally related optimal condition.
Design
Pregnant women undergoing elective cesarean section received three different labeled amino acid infusions starting at different times prior to surgery. Using mass- spectrometry techniques, this novel model enabled us to quantify fetal albumin synthesis from a single blood sample taken from the umbilical cord after cesarean delivery. The fractional synthesis rate reflects the fraction of the albumin pool that is daily renewed. The absolute synthesis rate is the absolute amount of albumin that is daily synthesized. Results are expressed as median (25th-75th percentile).
Results
We studied 8 fetuses at 29.9 (28.4-35.4) weeks gestation and 8 fetuses around term. Fractional synthesis rates in premature fetuses (17.5 (12.1-24.4) %/d) were higher (p=0.02) than in mature fetuses (10.4 (9.1-13.7) %/d). Absolute synthesis rates were also higher (p=0.02) in premature than in mature fetuses: 280 (227-365) versus 205 (184-238) mg/(kg·d).
Conclusions
On a weight basis, albumin synthesis rates in premature fetuses were higher than in fetuses at term and higher the rates previously found in neonates after preterm birth. Considering that the premature fetal liver has the capacity to synthesize albumin at a high rate, the observed hypoalbuminemia in premature infants therefore would seem to suggest that current (nutritional) therapies fail to meet requirements necessary to sustain an optimum in albumin synthesis rates.

INTRODUCTION

Albumin concentrations are considered a marker of nutritional status; albumin synthesis rates a measure of liver activity. Albumin is the major export protein produced by the liver and forms more than half of the total plasma protein mass. Albumin has been described as "the body's tramp steamer, shuttling cargo of various kinds between ports of call" [1]. Its load includes bilirubin, cysteine, free fatty acids, calcium, and drugs. Besides, albumin preserves the colloid osmotic pressure and is an important antioxidant.

Recently, we determined albumin synthesis rates in premature infants immediately after birth and who received only glucose [2]. These rates almost doubled in response to additional intravenous amino acid administration [3]. Despite this increase, plasma albumin concentrations were still very low. However, having knowledge of albumin synthesis rates during early life under physiologic circumstances, i.e. pregnancy, would enable us to relate the intrauterine with the extrauterine synthesis rates. To this aim, we employed a stable isotope model allowing measurements on the human fetal albumin synthesis rates.

It has been long known that animal [4,5] and human [6] fetuses are capable of endogenous albumin synthesis from early pregnancy on. Besides, all albumin in the fetus is from fetal origin since albumin does not cross the hemochorial placenta as demonstrated in the rat [7], guinea pig [8], and the in vitro dually perfused human placenta [9]. But also after intravenous injection of radioiodinated albumin to pregnant women, only trace amounts were found in umbilical cord blood [10,11]. Furthermore, fetal plasma albumin concentrations at term are often higher than in maternal plasma [12,13], which suggests no passive materno-fetal transport. In addition, normal concentrations of fetal plasma albumin were found during mild or severe maternal hypoalbuminemia [13,14].

The only available kinetic information on albumin synthesis, however, is in the ovine fetus, where the albumin fractional synthesis rates (FSRs) were determined [15,16]. The albumin FSR reflects the fraction of the intravascular albumin pool that is renewed per unit of time. The FSR is usually calculated by infusing one stably labeled amino acid and obtaining multiple blood samples at consecutive time points. From the increase of tracer incorporation in albumin over time, one can calculate its synthesis rate. In humans, however, the insertion of catheters in the fetus or umbilical cord for repetitive blood sampling is impossible on ethical grounds. Obtaining blood from both the umbilical vein and artery is only possible at birth. We therefore modified the staggered infusion protocol proposed by Dudley and colleagues [17] into a simplified model enabling us to measure the synthesis rate of albumin from a single blood sample taken at birth.

SUBJECTS AND METHODS

SETTING AND SUBJECTS

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by the Dutch (CCMO, The Hague) and the institutional medical ethical review board. Pregnant women scheduled to undergo elective cesarean section (repeat, breech, or multiple pregnancy) were eligible. We aimed to include fetuses who were close to term as well as fetuses who were still premature. Exclusion criteria were obesity (preconceptional body mass index > 30 kg/m^2), diabetes, or known fetal anomalies. Participants gave written consent after having been fully informed about the study.

EXPERIMENTAL DESIGN

L-[1-^{13}C,^{15}N]leucine, L-[1-^{13}C]phenylalanine, and L-[U-$^{13}C_5$]valine were bought from Buchem BV, Apeldoorn, The Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA) (all 99% enriched and tested for sterility and pyrogenicity). Our hospital pharmacy dissolved the isotopes in 0.9% saline after which the solution was filtered (0.2 µm) and sterilized. Tests were performed to reassure the correct identity, concentration, and a sterile and pyrogen free product.

Pregnant women received primed continuous stable isotope infusions of L-[1-^{13}C,^{15}N] leucine (8 µmol/(kg·h)), L-[1-^{13}C]phenylalanine (5 µmol/(kg·h)), and L-[U-$^{13}C_5$]valine (5 µmol/(kg·h)), starting at least 4, 3, and 2 hours prior to planned surgery, respectively. The priming doses were half of the hourly doses. Tracers were given in a forearm vein with three separate Perfusor® fm infusion pumps (B|Braun Medical B.V., Oss, the Netherlands) until surgery was completed.

Maternal blood was sampled before the tracer infusions had begun (baseline) and from a contralateral maternal forearm vein immediately before anesthesia started. Fetal blood was sampled from both the vein and arteries of a doubly clamped segment of the umbilical cord immediately after delivery. After collection, blood samples were centrifuged (2000×g) in heparin tubes and plasma was frozen at -80°C until analysis.

BLOOD SAMPLE ANALYSES

To isolate albumin from plasma, we used anti-human serum albumin affinity resin kits (Vivascience – Sartorius Group, Hannover, Germany). Fetal and adult albumin are indistinguishable [18]. Enclosed spin columns were filled with 400 µL affinity resin and 25 µL of thawed plasma. According to the included protocol, the column was washed three times with a tris-buffer and albumin was thereafter eluted from the affinity resin with 0.1 mol glycine/L (acidified to pH 2.5 with HCl). Eluted albumin was precipitated with 750 µL of 2 mol $HClO_4$/L. A washing step was performed with 0.2 mol $HClO_4$/L by resuspending and precipitating the pellet again. The protein pellet was then hydrolyzed in 140 µL of 6 mol HCl/L for 22 hours at 110°C. Following hydrolyzation, the acid was evaporated using a speedvac, after which the dried amino acids were dissolved in H_2O. Samples were derivatized using propylchloroformate (commercial kits: Phenomenex for hydrolysates, EZ:Faast, Bester BV,

Amstelveen, The Netherlands) and measured in triplicate on a gas chromatograph – combustion – isotope ratio mass spectrometer (Delta XP, Thermo Electron, Bremen, Germany) [2].

The enrichments of the true albumin precursors (intrahepatic amino-acyl tRNA) can obviously not be measured in the human fetus or mother. Because keto acids are intracellularly derived metabolites of amino acids, their enrichment has been advocated as a surrogate precursor [19,20]. However, keto acids are also transported transplacentally and it is thus not possible to discriminate whether the keto acids have undergone intracellular metabolism in the maternal, placental, or fetal compartment. Therefore, we chose to use plasma amino acid enrichments as the albumin precursors. As keto acid enrichment can only be lower than amino acid enrichment, the use of the latter results in a slight underestimation of synthesis rates.

Amino acids were extracted from plasma and derivatized using the same Phenomenex kits which were also used for product (albumin) sample preparation. Enrichments of plasma leucine, phenylalanine, and valine were measured in triplicate on a gas chromatograph – combustion – isotope ratio mass spectrometer as well. Plasma albumin concentrations in maternal and umbilical plasma were measured on a Roche Hitachi 917 (Roche Diagnostics, Basel, Switzerland). Hematocrit was measured on an Advia 120 (Bayer Diagnostics, Leverkussen, Germany).

CALCULATIONS

Baseline enrichment in the fetus could not be measured but was considered to be identical to that in the pregnant woman since the fetus consists of what the mother eats.

The fetal liver is perfused with blood directly from the umbilical vein (70%) and with blood which first passes the ductus venosus and then reenters the liver through the portal vein (20%) and hepatic arteries (10%) [21,22]. Blood from the portal vein and hepatic arteries has theoretically the same composition as in the umbilical arteries. Thus, the fetal liver is perfused with blood from both umbilical cord vessels. However, plasma amino acid enrichment in the umbilical arteries is slightly lower than that in the umbilical vein due to isotopic dilution by unlabeled amino acids released from fetal protein breakdown. Therefore, we calculated the precursor enrichment as the mean of umbilical venous and arterial plasma enrichment.

The enrichment of amino acids incorporated in fetal albumin was very similar in blood from the umbilical vein and arteries, which indicates no materno-fetal albumin transport. Nevertheless, we averaged the values. In each subject, the separate leucine, phenylalanine, and valine product/precursor enrichment ratios were plotted in a graph against the moment the corresponding infusion was started (Figure 1). Using computer software, the slope and the correlation coefficient of the linear trend line were calculated. The FSR was then derived using the following equation:

FSR (%/d) = slope of trend line × -1 × 24h × 100%

The absolute synthesis rate (ASR) represents the absolute amount of albumin that is

produced per unit of time and can be calculated with the following equation:

$$ASR\ (mg/(kg \cdot d)) = FSR \times C_{alb} \times vol_{pl} \times weight^{-1}$$

where C_{alb} is the plasma albumin concentration in g/L, vol_{pl} is the plasma volume in mL, and weight is the maternal actual weight or infant's birth weight in kg. Maternal plasma volume was estimated from data by Whittaker et al. [23] according to the following equation: plasma volume (mL) = 36.1 × height (cm) + 11.0 × weight (kg) – 3029. Fetal plasma volume (including placental and umbilical blood) was calculated by multiplying (1-hematocrit) with an estimated 105 mL blood/kg fetal body weight [24].

In our model, the use of one single amino acid with three different isotopomers (e.g. [1-^{13}C]leucine, [D_7]leucine, and [^{18}O]leucine) could theoretically be preferred over infusing three different labeled amino acids as in our study. However, since the enrichment of incorporated amino acids in albumin is very low (ranging from 0.01 mole percent enrichment (MPE) for valine to 0.17 MPE for leucine in our study), enrichments can only be analyzed accurately by using GC-C-IRMS. Measuring hydrogen and oxygen on a GC-C-IRMS is technically very challenging. Besides, leucine with an oxygen label was at time of the study prohibitively expensive. Unfortunately, [^{15}N]leucine could not be used because of label loss due to transamination. Owing to these technical difficulties and financial constraints, the

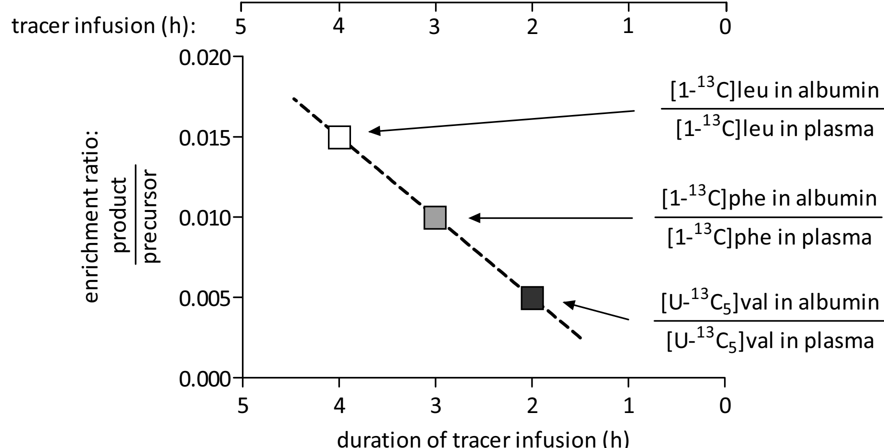

FIGURE 1: Study design. Pregnant women received three different stable isotopically labeled amino acid infusions starting at different times prior to cesarean section. In maternal and umbilical cord blood, sampled at t=0, we measured the product/precursor enrichment ratio of each of the three infused amino acids. These ratios were plotted in a graph against the moment the corresponding isotope infusion was started. As labeled leucine had the longest infusion time, its incorporation into albumin will be highest. The slope of the trend line determines the albumin FSR.

single amino acid strategy could not be utilized. We thus have chosen to use carbon labels only, which necessitates using different amino acids when measuring with a GC-C-IRMS. In addition, because of anticipated low enrichment in the last infused amino acid, valine was uniformly labeled to increase measurement accuracy.

STATISTICS

Calculations were made with Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA) and statistical tests were done in GraphPad Prism software (version 4.0; San Diego, CA, USA). Because of our small groups, normality distribution of our data could not be determined or assumed. Therefore non-parametric data analysis was performed. Consequently, values are expressed as median (25th – 75th percentile) and Mann-Whitney tests were used to detect statistical differences. Significance level was set at $p<0.05$.

RESULTS

We included eleven pregnant women, of whom eight delivered at term, one at 31 weeks gestation, one delivered a triplet at 35 weeks (two identical, one non-identical), and one delivered a quadruplet at 28 weeks (all non-identical). We thus studied 16 fetuses, classified into two groups: premature (<37 weeks gestation) and mature. Maternal age, preconceptional and current body mass index, and parity are shown in Table I. Descriptive characteristics of fetuses/neonates, which include birth weight, gestational age, birth weight Z-score [25], sex, umbilical pulsatility index, and Apgar score are shown in Table II.

Table III and Table IV show the enrichments of the three infused labeled amino acids both incorporated in albumin and free in plasma, respectively. Figure 2 displays the trend lines through the leucine, phenylalanine, and valine product/precursor enrichment ratios in each studied subject. The median linear regression coefficients (r^2) of these trend lines were 0.995 (0.985 – 0.999) in pregnant women, 0.988 (0.981 – 0.993) in premature fetuses, and 0.996 (0.985 – 0.998) in mature fetuses. In Figure 3, the maternal, premature fetal, and mature fetal albumin FSRs are outlined. They were all significantly different from each other; pregnant women had the lowest FSRs, premature fetuses the highest.

Maternal albumin concentrations were 32.0 (29.5 – 34.5) g/L. Concentrations in

TABLE I: Maternal characteristics (n=11).

Characteristic	**Value**
Age (y)	35.0 (27.5 – 36.5) [1]
Preconceptional BMI (kg/m^2)	22.8 (20.3 – 24.6)
Actual BMI (kg/m^2)	29.4 (25.2 – 31.6)
Parity (0:1:2:3) (n)	(6:1:2:2)

[1] Median (25th – 75th percentile) (all such values).

TABLE II: Characteristics of the premature (<37 weeks gestation) and mature group of fetuses.

	Premature (n=8)	Mature (n=8)
Gestational age (wks)	29.9 (28.4 – 35.4) [1]	38.5 (37.6 – 38.9)
Birth weight (kg)	1.3 (1.2 – 1.9)	3.3 (2.7 – 3.4)
Birth weight Z-score (SD) [2]	-0.19 (-0.70 – 0.22)	-0.11 (-0.86 – 0.52)
Sex (M:F) (n)	3:5	4:4
P.I. [3]	1.28 (1.18 – 1.36)	0.89 (0.78 – 0.96)
Apgar score at 5 min [4]	9 (9 – 10)	10 (10 – 10)

[1] Median (25th – 75th percentile) (all such values).

[2] Birth weight corrected for gestational age (reference 28).

[3] The umbilical pulsatility index (P.I.) is a Doppler ultrasound derived index on the blood stream velocity profile through the umbilical arteries and is a marker of fetal well-being. A normal P.I. decreases slightly over gestation.

[4] The Apgar score is a postnatal scoring scale ranging from 0-10.

TABLE III: Enrichments of the infused amino acids incorporated into albumin (product enrichments) in the maternal and fetal (mean of arterial and venous umbilical cord plasma) compartment. [1]

	Pregnant women (n=11)	Premature fetuses (n=8)	Mature fetuses (n=8)
[1-^{13}C]leucine	0.075 (0.061 – 0.083)	0.105 (0.097 – 0.119)	0.096 (0.089 – 0.112)
[1-^{13}C]phenylalanine	0.063 (0.054 – 0.078)	0.103 (0.089 – 0.116)	0.095 (0.087 – 0.108)
[U-$^{13}C_5$]valine	0.015 (0.013 – 0.022)	0.019 (0.017 – 0.024)	0.025 (0.023 – 0.030)

[1] Enrichment is expressed in mole percent excess (MPE). All values are median (25th – 75th percentile).

TABLE IV: Enrichments of the infused amino acids in plasma (precursor enrichments) in the maternal and fetal (mean of arterial and venous umbilical cord plasma) compartment. [1]

	Pregnant women (n=11)	Premature fetuses (n=8)	Mature fetuses (n=8)
[1-^{13}C]leucine	8.89 (8.27 – 9.27)	5.40 (4.84 – 6.12)	6.55 (5.98 – 7.24)
[1-^{13}C]phenylalanine	12.7 (11.1 – 13.0)	8.55 (8.27 – 8.96)	10.1 (9.19 – 11.4)
[U-$^{13}C_5$]valine	6.73 (6.02 – 7.06)	3.75 (3.24 – 4.34)	4.97 (4.56 – 5.20)

[1] Enrichment is expressed in mole percent excess (MPE). All values are median (25th – 75th percentile).

premature fetuses were 28.8 (27.3 – 30.8) g/L and in mature fetuses 33.5 (32.6 – 34.6) g/L, which is significantly different (p=0.003). Hematocrit in umbilical cord blood (mean of venous and arterial blood) was 0.43 (0.40 – 0.50) in the premature fetuses and 0.46 (0.46 –

0.48) in the mature group. The albumin ASRs are shown in Figure 4. Similar to the fractional values, premature fetuses had the highest ASRs, followed by the mature fetuses and the pregnant women.

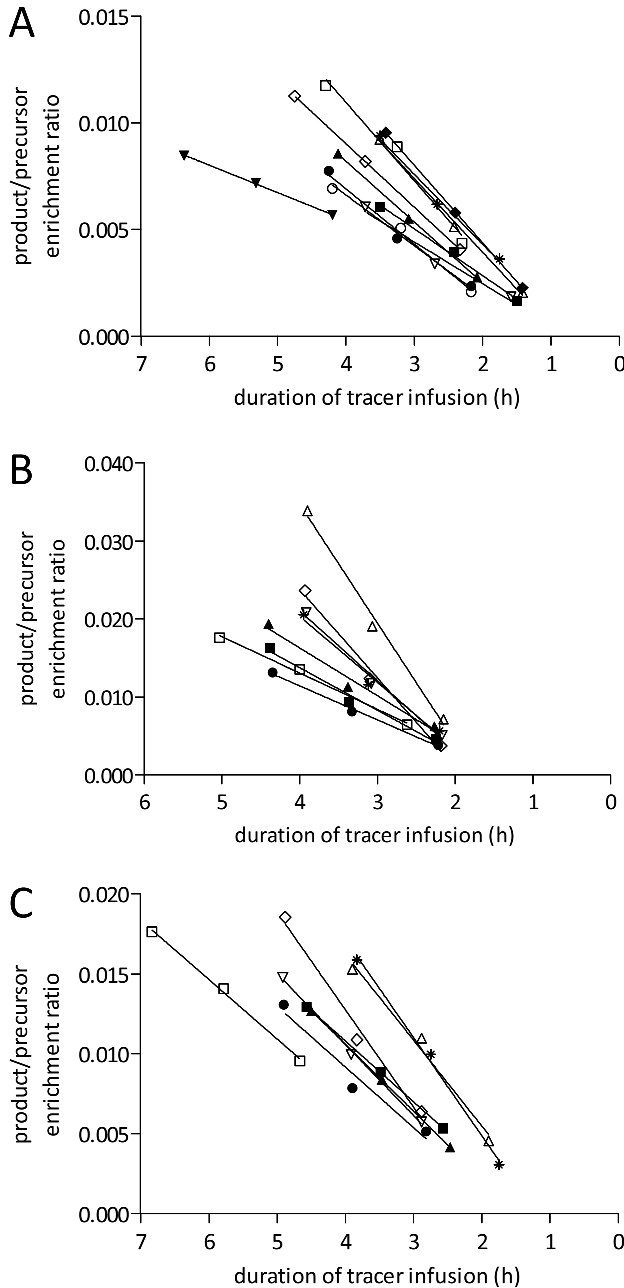

FIGURE 2: Individual trend lines through the three product/ precursor trend lines in (A) pregnant women (n=11), (B) premature fetuses (n=8), and (C) mature fetuses (n=8). In each case, leucine had the longest infusion time, followed by phenylalanine and valine, respectively.

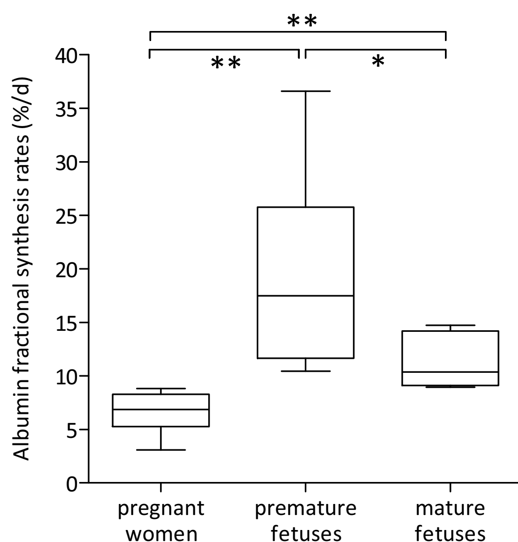

FIGURE 3: Albumin fractional synthesis rates in pregnant women (n=11), premature fetuses (<37 weeks gestation, n=8), and mature fetuses (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. * Significantly different (Mann-Whitney), $p<0.05$; ** Significantly different (Mann-Whitney), $p<0.001$.

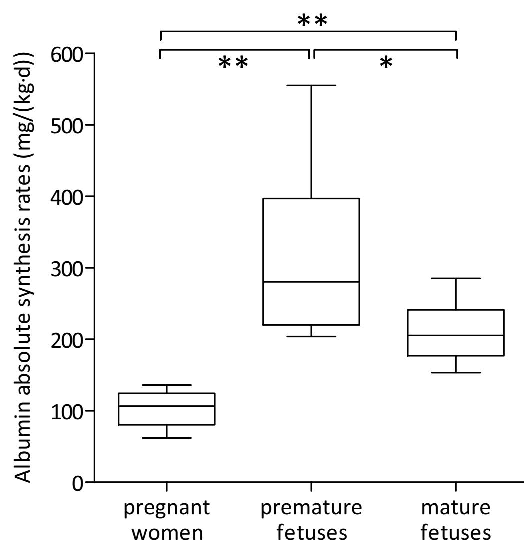

FIGURE 4: Albumin absolute synthesis rates in pregnant women (n=11), premature fetuses (<37 weeks gestation, n=8), and mature fetuses (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. * Significantly different (Mann-Whitney), $p<0.05$; ** Significantly different (Mann-Whitney), $p<0.001$.

DISCUSSION

This is the first study addressing albumin synthesis rates in human fetuses. These values are of great importance since they give guidance as what to strive for in aiming optimal nutrition for premature infants. The fetal measurements were possible due to a relatively novel multiple stable isotope infusion method. This enabled us to measure a protein's synthesis rate from a single blood sample. Given the high correlation coefficients, our method proves to be valid.

In this study, we compared albumin synthesis between pregnant women, fetuses at term, and fetuses that were still premature. In mothers of the latter group, however, elective cesarean sections are rarely performed as these are usually in the acute setting because of sudden (worsening of) fetal or maternal distress. Thus, there is usually no time for obtaining informed consent followed by a four-hours-lasting infusion protocol for research purposes. Still, we were able to include three women who underwent a planned cesarean section before term, and whose infants were assumed to be in relatively good condition. One woman had to give early birth because of maternal cervical carcinoma, the two other women because of anticipated complications due to triplet and quadruplet pregnancy. Whether the results in the premature group of fetuses were influenced the effects of multiple pregnancy itself or by genetic relationships remain unknown. However, a common genetic background does not imply having equal fetal metabolic nutrient availability. In normal pregnancy, the latter depends more on placental activity in each individual than on maternal nutrient availability. Thus, amongst multiplets, it is likely that the intrauterine metabolic environments are different, which was also reflected by different synthesis rates between siblings.

As the maternal blood sample used for calculation of the albumin FSR was taken before spinal anesthesia was initiated and surgery had started, the latter two procedures could not have influenced our results. It is unknown, however, to what extent maternal surgery influences fetal metabolism. Yet, surgery until the infant was born only lasted some ten minutes, which is only a short period relative to the total infusion time. Thus, potential effects of maternal surgery would only minimally influence fetal synthesis rates.

The maternal plasma albumin concentrations in this study are low as compared with those in non-pregnant individuals, but a 10 g/L drop in concentration starting early in pregnancy is common [23]. However, rather than simple dilution because of a pregnancy-associated plasma volume expansion, actual alterations in albumin metabolism during pregnancy have been observed. During late gestation, albumin FSRs and ASRs as well as the total intravascular albumin pool were found to be higher than those in non-pregnant women [23,26]. Our measured maternal synthesis rates were very similar to the rates in those studies. Increased synthesis could be necessary to compensate for the albumin loss caused by placental uptake and subsequent degradation, thereby releasing free amino acids available for transport to the fetus [27,28].

Two of the mature fetuses had birth weights that were only on the 5th percentile. These two small for gestational age infants had the lowest two albumin FSRs and ASRs. When nutrient availability is compromised, ultimately leading to reduced growth, oxygen and nutrient rich blood entering the fetus through the umbilical vein is shunted away from the liver through the ductus venosus towards the upper body half [29]. Bypassing the fetal liver ensures a more or less constant supply of essential substrates to the myocardium and brain. Underperfusion of the fetal liver, however, results in diminished liver growth. Small for gestational age infants are known to have smaller liver volumes, also when corrected for

total body weight [30,31]. Interestingly, these two fetuses did not upregulate albumin synthesis rates so as to compensate their supposedly smaller liver size. In fact, the opposite was true as the albumin synthesis rates were the lowest. This could have important implications as impaired liver functioning might have lifelong effects on metabolism. Summarized as the 'fetal origins of adult disease' or 'Barker hypothesis', compromised growth during early life of organs such as the liver, pancreas, spleen, kidneys and adrenal glands, predisposes an individual to cardiovascular disease, stroke, and type two diabetes [32,33].

Considering the functions of albumin, which include acting as an antioxidant and transporting bilirubin and free fatty acids, one may wonder why normally grown fetuses, especially earlier in gestation, have such high synthesis rates. During intrauterine life, oxygen tension in blood is low, thereby generating only low amounts of radicals, which could damage albumin. The low oxygen tension is compensated for by the increased oxygen affinity of fetal hemoglobin. After birth, fetal hemoglobin is rapidly broken down, thereby releasing large amounts of bilirubin that should be transported off by albumin. Also, during the beginning of the third trimester, fatty acid concentrations are low and will be of no burden to albumin. The surge in albumin synthesis would therefore be expected just prior to term birth, as a preparation against an elevated radical exposure and for a higher transport load consisting of hemoglobin breakdown products and fatty acids, the latter found in high amounts in postnatal nutrition (breast milk). In addition, all mothers of the prematurely born infants had received corticosteroids in the two days prior to their planned cesarean section. Antenatally given steroids accelerate fetal lung maturation in preparation for postnatal life. These stress hormones, however, can also elicit a catabolic response in the fetus. Albumin synthesis might therefore even have been downregulated in the premature group at time of the measurements.

The reason for a decreasing albumin synthesis rate during gestation could either be functional or depend on the general metabolic rate. During ovine pregnancy, fetal whole body protein synthesis rates decrease significantly throughout gestation [34]. Oxygen consumption by the ovine fetal liver has also been shown to decrease [35]. In human preterm infants, whole body protein metabolic rates are also higher when compared to infants born at term [36]. However, human fetal liver volume as percent of body weight does not decrease as much throughout gestation as it does in fetal sheep [37].

The albumin ASR in intravenously fed premature babies (27 wks gestation) was 228 (187 – 289) mg/(kg·d) [3]. The ASRs of premature fetuses measured in the current study are higher than the postnatal values from premature infants. Having low albumin concentrations and ASRs after birth is an unfortunate situation considering that sick premature infants experience more oxidative stress after high oxygen pressure ventilation and have to deal with increased bilirubin and drug transport. In our previous study, we showed that albumin synthesis in premature neonates is responsive to parenteral nutrition [3]. Yet, the current recommended nutrient intakes for premature infants still not appear to

be sufficient to increase the albumin synthesis rates to levels observed in fetuses. This can be speculated since premature infants should however, theoretically, be able to synthesize albumin in larger quantities as they also did while still in utero. Although the traditional method of measuring an FSR used in premature infants is different from our employed infusion model, the two should theoretically give comparable results.

In conclusion, we showed that mature fetuses produce twice as much albumin as their mothers per kg bodyweight and premature fetuses three times as much. Premature fetuses have higher albumin synthesis rates than parenterally fed premature neonates indicating that postnatal synthesis capacity is reduced or that recommended nutrient intake is not sufficient. Our employed method is not only applicable in fetal research, but could be of benefit in all situations where multiple sampling is impossible or inconvenient to a subject. In organ protein metabolism studies (for example liver, bowel, or muscle protein synthesis) the required number of tissue biopsies can be reduced to one, instead of two or three with many currently used models [38,39]. In addition, our single sample method shortens sample preparation and analysis time and reduces risk on measurement artifacts.

ACKNOWLEDGEMENTS

Most of all, we would like to thank all participating women. Furthermore, Willemijn Corpeleijn, Frans te Braake, Ad de Bruijn, and all staff from the obstetrical, and anesthesiological departments were a great helping hand in collecting all material and providing the facilities.

Financial support was kindly provided by the Sophia Children's Hospital Fund (SSWO) and the Nutricia Research Foundation, but they had no influence in study design, results, publication, or whatsoever.

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CHAPTER

7

Human fetal amino acid metabolism at term gestation: phenylalanine and tyrosine kinetics

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Published in:
The American Journal of Clinical Nutrition, Volume 89, 2009, In press

ABSTRACT

Background

Knowledge on human fetal amino acid (AA) metabolism, largely lacking so far, is pivotal in improving nutritional strategies for prematurely born infants. Phenylalanine kinetics are of special interest since there is debate as to whether neonates will adequately hydroxylate phenylalanine to the semi-essential AA tyrosine.

Objective

Our aim was to quantify human fetal phenylalanine and tyrosine metabolism.

Design

Eight fasted, healthy pregnant women undergoing elective cesarean section at term received primed continuous stable isotope infusions of [1-^{13}C]phenylalanine and [*ring*-D_4] tyrosine starting prior to surgery. Umbilical blood flow was measured by ultrasound. Maternal and umbilical cord blood was collected and analyzed by gas chromatography mass spectrometry for phenylalanine and tyrosine enrichments and concentrations. Data are expressed as median (25th – 75th percentile).

Results

Women were in catabolic state for which net fetal AA uptake was responsible for at least one quarter. Maternal and fetal hydroxylation rates were 2.6 (2.2 – 2.9) and 7.5 (6.2 – 15.5) µmol phenylalanine/(kg·h), respectively. Fetal protein synthesis rates were higher than breakdown rates: 92 (84 – 116) vs. 73 (68 – 87) µmol phenylalanine/(kg·h), respectively, indicating an anabolic state. The median metabolized fraction of available phenylalanine and tyrosine in the fetus was less than 20% for both AA.

Conclusions

Around term gestation, fetuses still show considerable net AA uptake and AA accretion (converted to tissue ~12 g/(kg·d)). The low metabolic uptake (AA usage) implies a very large nutritional reserve capacity of nutrients delivered through the umbilical cord. Fetuses at term are quite capable of hydroxylating phenylalanine to tyrosine.

INTRODUCTION

These days, many premature infants survive – yet sometimes at the cost of impaired outcome [1,2]. Inappropriate nutrition is at least partially responsible for suboptimal outcome, as it negatively affects neonatal growth and brain development [3,4]. Since several decades, the international pediatric nutritional goals are to feed premature infants so that they grow at the same rate they would have had while in utero and thereby mimicking the fetal tissue composition or quality. Many infants do not reach these targets, however, as growth lags behind. Moreover, the body composition of preterm-born infants is often more adipose at term corrected age [5]. Seeing that hardly anything is known about human fetal metabolism itself, it is not surprising that fetal accretion rates are often not met. It would seem, therefore, that better mimicking of fetal growth could be achieved by putting more effort in unraveling human fetal metabolism. The obtained knowledge could then lead to improved nutritional strategies.

Current knowledge on fetal metabolism is mostly derived from animal data. Technical difficulties and ethical issues are of course causal to the lack of knowledge on human fetal metabolism. However, the use of stable isotopes to study protein metabolism during human pregnancy provides a safe research tool.

The quantification of fetal phenylalanine and tyrosine kinetics is of particular importance. It does not only give information on fetal protein breakdown and synthesis rates in general, but also quantifies the metabolic conversion (hydroxylation) rate of the essential amino acid phenylalanine to tyrosine. Hydroxylation occurs in the liver and kidneys [6]. It is important for two reasons: it disposes of excess phenylalanine, and provides an alternative source of tyrosine if tyrosine is absent in the diet, for example due to poor tyrosine solubility in parenteral nutrition. Parenterally fed neonates thus depend on hydroxylation for their tyrosine requirements necessary for net protein accretion. Yet, the enzymatic activity of phenylalanine hydroxylase might be suboptimal in neonates and even older infants, making tyrosine a conditionally essential amino acid [7]. Tyrosine that is not incorporated into proteins can be degraded and oxidized through the formation of fumarate and acetoacetate. The amount of tyrosine used as a precursor of the catecholamines dopamine, norepinephrine, and epinephrine, is quantitatively negligible.

In this study, our aim was to investigate several aspects of fetal phenylalanine and tyrosine kinetics by analyzing umbilical cord blood after having infused pregnant women with stable isotopically labeled amino acids prior to elective cesarean section at term.

SUBJECTS AND METHODS

SETTING AND SUBJECTS

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by both the institutional medical ethical review board and the Dutch central committee on research involving human subjects (CCMO, The Hague). Pregnant women undergoing elective cesarean section (repeat or breech pregnancy) under spinal anesthesia at term were eligible. Exclusion criteria were maternal obesity (preconceptional BMI >30), preeclampsia, diabetes, severe fetal growth restriction (< -2 SD), or known fetal anomalies. Participating women gave written consent after having been fully informed about all study details.

EXPERIMENTAL DESIGN

To determine the blood flow necessary for our calculations (see below), blood flow velocity and vessel diameters were measured in the umbilical vein with an ultrasound machine (iU22, Philips Medical Systems, Eindhoven, the Netherlands) as previously described [8]. Ultrasound measurements were made in the late afternoon on the day preceding the cesarean section; sections were all performed at approximately 8.00 a.m. after an overnight fast.

At least 3 hours to planned surgery, the women received a priming dose of L-[1-^{13}C] tyrosine (0.5 mmol/kg) directly followed by a primed continuous infusion of L-[1-^{13}C] phenylalanine (2.5 mmol/kg; 5 mmol/(kg·h)) through a forearm vein. One hour later a primed continuous infusion of L-[*ring-2,3,5,6*-D_4]tyrosine (1.5 mmol/kg; 3 mmol/(kg·h)) was started along. Isotopes (all >99% enriched and tested for sterility and pyrogenicity) were bought from Buchem BV, Apeldoorn, the Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA). Our hospital pharmacy dissolved the isotopes in 0.9% saline and the solutions were filtered (0.2 µm) and sterilized. Tests were performed to ensure the correct identity, concentration, and a sterile and pyrogen free product. Tracers were given using Perfusor® fm (B|Braun Medical B.V., Oss, the Netherlands) and Graseby 3000 (Graseby Medical Ltd, Watford, UK) infusion pumps for the phenylalanine and tyrosine tracers, respectively. Maternal blood was sampled before the tracer infusions started (baseline), then immediately before anesthesia and, if possible (n=4), also about 20 minutes later just before surgery started. Fetal blood was sampled after birth from both the vein and arteries of a doubly clamped segment of the umbilical cord. The fact that there are two arteries in the umbilical cord does not affect our results as the concentrations of the amino acids and their enrichments in the blood of both arteries should be equal. After collection in heparin tubes, blood was centrifuged and plasma was frozen at -80°C until analysis.

BLOOD SAMPLE ANALYSIS

As calculations in a veno-arterial balance model (as on the umbilical cord in the fetus, see below) largely depend on the small differences in concentration and enrichment between the vein and arteries, rather than on the absolute values, measurements must be

extremely precise. To minimize the effects of potential analytical measurement errors, samples were prepared for analysis twice using two different derivatization methods (PCF and MTBSTFA, see below). Each derivatized sample was analyzed in triplicate on two different gas chromatography mass spectrometers (GCMS) (see below). Enrichments were calculated from the mean of all twelve analyses; concentrations could be calculated from the mean of the six analyses using the MTBSTFA derivative only.

PCF (propylchloroformate) derivatization on samples was performed using commercial kits (EZ:Faast for hydrolysates, Phenomenex, Bester BV, Amstelveen, the Netherlands) according to the enclosed protocol. As internal standards for concentration determinations, $[D_8]$phenylalanine and $[U\text{-}^{13}C_9,^{15}N]$tyrosine were added to the samples to be derivatized with MTBSTFA. Concentration calibration curves were prepared using MTBSTFA as well. Two different enrichment calibration curves were made with either PCF or MTBSTFA derivates. Samples and calibration curves were analyzed with a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, the Netherlands) on a VF-17ms, 30m x 0.25mm ID capillary column (Varian Inc., Middelburg, the Netherlands) and a Thermo DSQ GCMS (Thermo Fisher, Breda, the Netherlands) on a VF-1701ms, 30m x 0.25mm ID capillary column (Varian Inc., Middelburg, the Netherlands).

CALCULATIONS

For the calculation of maternal whole body phenylalanine and tyrosine kinetics, including hydroxylation rates, we used the Clarke and Bier model [9], in combination with the adjustments proposed by Thompson et al. [10]. To control for pregnancy, we added an extra parameter to the rate of disappearance. In our model, amino acids disappear not only through hydroxylation (or oxidation) or incorporation into protein synthesis, but also through net transport to the fetus. The latter is calculated as the umbilical veno-arterial concentration difference multiplied with the umbilical blood flow per kg maternal weight. If maternal blood was sampled twice prior to surgery, enrichments were averaged.

We quantified fetal whole body kinetics by using the concept of an umbilical veno-arterial balance model. To do so, we rewrote the leucine arteriovenous balance model by Tessari et al. [11] and the phenylalanine hydroxylation equation proposed by Nair et al. [12] into a phenylalanine and tyrosine model suitable for fetal studies. The model is outlined in Figure 1 and its determinants are calculated using the following equations, where kg in all units denotes fetal weight (= birth weight):

Rate of phenylalanine delivery from umbilical vein to the fetus in µmol/(kg·h):

$$\text{delivery} = [\text{phe}]_{\text{vein}} \times \text{BF} \qquad (1.1)$$

where [phe] is the total (labeled + unlabeled) phenylalanine concentration (µmol/L) and BF the umbilical blood flow (L/(kg·h)). Subscripts indicate whether blood was sampled from the umbilical vein or arteries (as below).

Rate of phenylalanine release from fetus to umbilical artery in µmol/(kg·h):

$$\text{release} = [\text{phe}]_{\text{art}} \times \text{BF} \qquad (1.2)$$

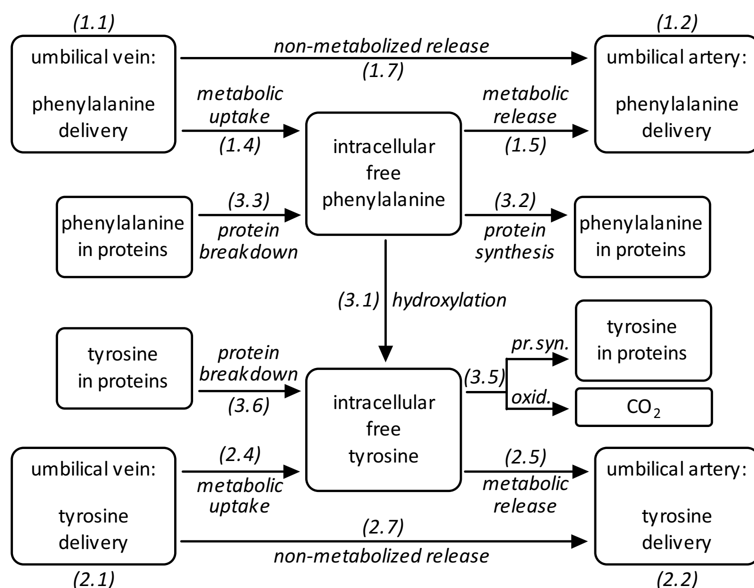

FIGURE 1: Schematic model of fetal phenylalanine and tyrosine metabolism. Phenylalanine and tyrosine are delivered to the fetus through the umbilical vein (1.1) and (2.1). Part of these amino acids are taken up from the fetal intravascular system into the fetal cells (1.4) and (2.4), whereas theremainder of the intravascular amino acids are transported back to the placenta through the umbilical arteries (1.7) and (2.7). Amino acids are constantly released from proteins due to proteolysis (3.3) and (3.6). Part of the available phenylalanine is hydroxylated to tyrosine (3.1), incorporated in proteins (3.2), or released into the vascular system (1.5). Tyrosine is either used for protein synthesis or oxidation (3.5), or also released into the vascular system (2.5). Finally phenylalanine and tyrosine are transported back to the placenta through the umbilical arteries (1.2) and (2.2). Numbers in brackets also correspond to the equations in the methods section and the fluxes outlined in table 5.

Fraction of phenylalanine in the umbilical vein that is metabolized intracellularly in %:

$$\text{metabolized fraction} = \left(1 - \frac{[^{13}\text{C}\bullet\text{phe}]_{art}}{[^{13}\text{C}\bullet\text{phe}]_{vein}}\right) \times 100\% \qquad (1.3)$$

where $[^{13}\text{C}\cdot\text{phe}]$ is the labeled phenylalanine concentration (µmol/L).

Rate of phenylalanine inflow from umbilical vein into intracellular compartment in µmol/(kg·h):

$$\text{metabolic inflow} = \text{Eq}(1.1) \times \text{Eq}(1.3) \qquad (1.4)$$

Rate of phenylalanine outflow from intracellular compartment into umbilical artery in µmol/(kg·h):

$$\text{metabolic outflow} = \text{Eq}(1.2) + \text{Eq}(1.4) - \text{Eq}(1.1) \qquad (1.5)$$

Net fetal phenylalanine uptake in µmol/(kg·h):

$$\text{net uptake} = Eq(1.1) - Eq(1.2) = Eq(1.4) - Eq(1.5) \quad (1.6)$$

Rate of phenylalanine directly released from umbilical vein to artery without being metabolized in μmol/(kg·h):

$$\text{non-metabolized release} = Eq(1.1) - Eq(1.4) \quad (1.7)$$

Equations (1.1) through (1.7) can also be used for calculations of tyrosine kinetics (using tyrosine concentrations and the [D_4]tyrosine enrichments), yielding equations (2.1) through (2.7).

Rate of phenylalanine hydroxylation to tyrosine in μmol/(kg·h):

$$H_{PT} = Eq(2.1) \times \left(\left(\frac{D_4 \bullet tyr \bullet E_{vein}}{D_4 \bullet tyr \bullet E_{art}} \times \frac{^{13}C \bullet tyr \bullet E_{art}}{^{13}C \bullet phe \bullet E_{art}} \right) - \frac{^{13}C \bullet tyr \bullet E_{vein}}{^{13}C \bullet phe \bullet E_{art}} \right) \quad (3.1)$$

where $D_4 \cdot tyr \cdot E$ is the [D_4]tyrosine enrichment (in MPE). Other enrichments are abbreviated accordingly.

Rate of intracellular phenylalanine incorporation into protein (synthesis) in μmol/(kg·h):

$$S_{phe} = Eq(1.4) \times \frac{^{13}C \bullet phe \bullet E_{vein}}{^{13}C \bullet phe \bullet E_{art}} - Eq(3.1) \quad (3.2)$$

Rate of phenylalanine release from proteolysis (breakdown) into the intracellular space in μmol/(kg·h):

$$B_{phe} = Eq(3.1) + Eq(3.2) - Eq(1.6) \quad (3.3)$$

Rate of net phenylalanine accretion in μmol/(kg·h):

$$\text{accretion}_{phe} = Eq(3.2) - Eq(3.3) \quad (3.4)$$

In our model, we could not discriminate between the two major intracellular pathways of tyrosine metabolism, i.e. incorporation into protein and oxidation. This is why we used the sum of the latter two rates in μmol/(kg·h):

$$S_{tyr} + O_{tyr} = Eq(2.4) \times \frac{D_4 \bullet tyr \bullet E_{vein}}{D_4 \bullet tyr \bullet E_{art}} \quad (3.5)$$

Rate of tyrosine release from proteolysis into the intracellular space in μmol/(kg·h):

$$B_{tyr} = Eq(3.5) - Eq(3.1) - Eq(2.6) \quad (3.6)$$

Phenylalanine and tyrosine protein synthesis and breakdown rates can be converted from molar rates into grams of protein and grams of tissue under the assumption that one gram protein contains 246 μmol phenylalanine and 158 μmol tyrosine [13] and new tissue contains 14% protein [14].

Hydroxylation rates in several previously performed whole body experiments have also been calculated without tyrosine tracer infusion in order to measure tyrosine kinetics or proteolysis rates [10,15]. The latter are then estimated by multiplying the actual phenylalanine proteolysis rate with an average tyrosine/phenylalanine breakdown ratio

($^{Btyr}/_{Bphe}$) measured in similar studies or with the theoretical tyrosine/phenylalanine molar content ratio of total body protein. No equations were as yet available for an arteriovenous balance model, and these were therefore developed ourselves, using analogous derivations to the whole-body model by Thompson et al. [10]. These equations are outlined beneath and enable to compare our hydroxylation rates with those of Chien et al. [16] in spite of the fact that this group did not infuse labeled tyrosine to their subjects.
The rate of phenylalanine released from proteolysis (equation 3.3) can also be calculated as follows (mathematically the same):

$$B_{phe} = Eq(1.1) \times \left(\frac{^{13}C{\bullet}phe{\bullet}E_{vein}}{^{13}C{\bullet}phe{\bullet}E_{art}} - 1 \right) \quad (4.1)$$

The total rate of tyrosine appearance (Ra_{tyr}), defined as Eq(2.1)+Eq(3.1)+Eq(3.5), can normally be calculated after labeled tyrosine infusion according to the following equation:

$$Ra_{tyr} = Eq(2.1) \times \frac{D_4{\bullet}tyr{\bullet}E_{vein}}{D_4{\bullet}tyr{\bullet}E_{art}} \quad (4.2)$$

Equation (3.1) can then be rewritten into:

$$H_{PT} = Ra_{tyr} \times \frac{^{13}C{\bullet}tyr{\bullet}E_{art}}{^{13}C{\bullet}phe{\bullet}E_{art}} - Eq(2.1) \times \frac{^{13}C{\bullet}tyr{\bullet}E_{vein}}{^{13}C{\bullet}phe{\bullet}E_{art}} \quad (4.3)$$

However, if no labeled tyrosine is infused, Eq(4.2) cannot be used so that Ra_{tyr} has to be calculated alternatively using a known ratio $^{Btyr}/_{Bphe}$ (in our case the mean of the other seven fetuses):

$$Ra_{tyr} = H_{PT} + Eq(2.1) + Eq(4.1) \times {}^{B_{tyr}}/_{B_{phe}} \quad (4.4)$$

Equations (4.3) and (4.4) can then be combined and rewritten into:

$$H_{PT} = \frac{Eq(4.1) \times {}^{B_{tyr}}/_{B_{phe}} \times {}^{13}C{\bullet}tyr{\bullet}E_{art} + Eq(2.1) \times \left({}^{13}C{\bullet}tyr{\bullet}E_{art} - {}^{13}C{\bullet}tyr{\bullet}E_{vein} \right)}{^{13}C{\bullet}phe{\bullet}E_{art} - {}^{13}C{\bullet}tyr{\bullet}E_{art}} \quad (4.5)$$

Thus, using the latter equation it is possible to calculate hydroxylation rates in a balance model if no labeled tyrosine has been infused.

In our model, we make the following assumptions: 1, a labeled molecule will not be discriminated from an unlabeled molecule; 2, the labeled molecule will trace the movement of the unlabeled molecules; 3. the administration of the labeled molecules will not affect the kinetics of the unlabeled molecules.

STATISTICS

Calculations were made using Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA). Statistical analysis was performed using GraphPad Prism software (version 4.0; San Diego, CA, USA). Because the number of included subjects was relatively small (n=8), normality distribution of our data could not be determined or assumed. All results are therefore expressed as median (25th – 75th percentile). Consequently, however, by presenting our data as medians, all rates as outlined in Table 5 do not add up correctly as outlined in our model (Figure 1). The fluxes of each individual subject still do, nonetheless.

RESULTS

We included eight feto-maternal dyads. Maternal age, preconceptional and actual body mass index (BMI), and parity are shown in Table I. Five of the cesarean sections were performed because of breech presentation; three because of a cesarean section in the patient's medical history. There were no complications during any of the cesarean sections. Visual inspection of the placentas and umbilical cords showed no abnormalities. Fetal characteristics in terms of gestational age, birth weight, birth weight *z*-score [17], sex, umbilical blood flow, pulsatility index (P.I.), umbilical arterial pH and base excess, and Apgar score are shown in Table II. None of the infants had congenital anomalies and all were discharged from the hospital in good health together with the mother at the fifth day of life.

In four women, we obtained two blood samples before surgery had started at an approximately 20-minutes interval. Phenylalanine and tyrosine enrichments had not changed over this time interval, even though spinal anesthesia had been started meanwhile. Therefore, steady state was assumed. Only one blood sample could be withdrawn from the other four women, which was before anesthetics were initiated. Concentrations and enrichments of phenylalanine and tyrosine in maternal and fetal plasma are shown in Table III. The median coefficients of variance for all measurements of the phenylalanine and tyrosine concentrations were 0.007 (0.002 – 0.011) and 0.012 (0.007 – 0.019), respectively. The coefficients of variance for the enrichments of [1-^{13}C]phenylalanine, [1-^{13}C]tyrosine, and [*ring*-D_4]tyrosine amounted 0.022 (0.011 – 0.040), 0.091 (0.060 – 0.123), and 0.026 (0.011 –

TABLE I: Maternal characteristics (n=8).

Characteristic	Value
Age (y)	33.0 (28.8 – 38.0) [1]
Preconceptional BMI (kg/m^2)	21.9 (20.3 – 24.5)
Actual BMI (kg/m^2)	30.5 (23.3 – 31.6)
Parity (0:1:2:3) (n)	(4:1:2:1)

[1] Median (25th – 75th percentile) (all such values).

TABLE II: Fetal characteristics (n=8).

Characteristic	Value
Gestational age (wks)	38.5 (37.6 – 38.9) [1]
Birth weight (kg)	3.3 (2.7 – 3.4)
Birth weight *z*-score (SD) [2]	-0.11 (-0.86 – 0.52)
Sex (m:f) (n)	4:4
Umbilical blood flow (mL/(kg·min))	101 (90 – 110)
P.I. [3]	0.89 (0.78 – 0.96)
Umbilical arterial pH	7.30 (7.28 – 7.32)
Umbilical arterial Base Excess (mmol/L)	-1.5 (-2.0 – -1.0)
Placental weight (kg)	0.590 (0.558 – 0.649)
Apgar score at 5 min [4]	10 (10 – 10)

[1] Median (25th – 75th percentile) (all such values).

[2] Birth weight corrected for gestational age (reference 17).

[3] The umbilical pulsatility index (P.I.) is a Doppler ultrasound derived index on the blood stream velocity profile through the umbilical arteries and is a marker of fetal well-being.

[4] The Apgar score is a postnatal scoring scale ranging from 0-10.

TABLE III: Phenylalanine (phe) and tyrosine (tyr) concentrations (μmol/L) and enrichments (MPE) measured in the maternal vein, umbilical vein, and umbilical artery (n=8 for each). [1]

	Maternal vein	Umbilical vein	Umbilical artery
phe concentration	64.7 (58.5 – 67.2)	87.0 (83.4 – 93.6)	84.1 (78.6 – 88.4)
tyr concentration	48.0 (42.7 – 49.4)	65.8 (61.5 – 76.2)	66.9 (65.2 – 71.6)
$[1\text{-}^{13}C]$phe enrichment	11.5 (10.9 – 11.9)	9.8 (9.0 – 10.2)	8.4 (7.6 – 8.7)
$[1\text{-}^{13}C]$tyr enrichment	0.94 (0.89 – 1.08)	1.04 (0.97 – 1.12)	1.02 (0.97 – 1.13)
$[D_4]$tyr enrichment	8.3 (7.7 – 8.6)	5.8 (5.3 – 6.1)	4.9 (4.7 – 5.4)

[1] All values are median (25th – 75th percentile).

0.058), respectively. The feto-maternal enrichment ratios across the maternal and umbilical veins were 0.90 (0.80 – 0.92) for phenylalanine and 0.72 (0.67 – 0.75) for tyrosine.

Table IV shows maternal phenylalanine and tyrosine kinetics. Since women were in fasting state, phenylalanine released from protein breakdown equaled the total flux. The tyrosine/phenylalanine breakdown ratio ($^{Btyr}/_{Bphe}$) was 0.75 (0.74 – 0.79). The fraction of the maternal net catabolic state that could be explained by net fetal uptake was 26 (23 – 40)%.

Figure 1 and Table V display fetal phenylalanine and tyrosine kinetics. Although gravidae were catabolic, their infants had a positive phenylalanine accretion balance. The fetal $^{Btyr}/_{Bphe}$ was 0.75 (0.70 – 0.81), slightly higher than the theoretical ratio of 0.64 (=158/246) calculated from the molar expressed amino acid content of protein in deceased fetal bodies [13]. Conversion to protein turnover rates from phenylalanine kinetics reveals a protein

TABLE IV: Maternal phenylalanine and tyrosine kinetics (n=8). [1]

Flux	Value
Phenylalanine released from proteolysis	38.2 (36.6 – 40.5)
Phenylalanine used for protein synthesis	34.2 (33.4 – 37.8)
Net phenylalanine balance	-3.8 (-4.7 – 2.8)
Phenylalanine hydroxylation	2.6 (2.2 – 2.9)
Tyrosine released from proteolysis	29.5 (28.5 – 31.2)

[1] All values are expressed in µmol/(kg·h) as median (25th – 75th percentile).

TABLE V: Fetal phenylalanine and tyrosine kinetics (n=8).

Flux	Phenylalanine	Tyrosine
Umbilical vein delivery (1.1 & 2.1) [1]	559 (456 – 603) [2]	454 (325 – 492)
Umbilical artery output (1.2 & 2.2)	509 (443 – 567)	449 (310 – 472)
Metabolized fraction (%) (1.3 & 2.3)	18 (17 – 20)	16 (12 – 17)
Metabolic uptake from umb. vein (1.4 & 2.4)	91 (80 – 111)	69 (55 – 78)
Metabolic output into umb. artery (1.5 & 2.5)	59 (57 – 66)	55 (49 – 67)
Net uptake (1.6 & 2.6)	23 (16 – 47)	2.4 (-3.9 – 9.2)
Non-metabolized release (1.7 & 2.7)	449 (371 – 499)	374 (253 – 422)
Hydroxylation (3.1)	7.5 (6.2 – 15.5)	
Protein synthesis (3.2 & 3.5)	92 (84 – 116)	77 (63 – 93) [3]
Release from protein breakdown (3.3 & 3.6)	73 (68 – 87)	52 (47 – 75)
Net accretion (3.4)	17.5 (7.8 – 30.6)	

[1] Numbers between brackets indicate the used equation and flux for phenylalanine and tyrosine kinetics, respectively, as they are also outlined in figure 1.

[2] All values are expressed in µmol/(kg·h) as median (25th – 75th percentile) unless indicated otherwise.

[3] Includes tyrosine oxidation.

synthesis rate of 9.0 (8.2 – 11.3) g/(kg·d) and a proteolysis rate of 7.1 (6.7 – 8.5) g/(kg·d). Accretion rates are 1.7 (0.8 – 3.0) g protein/(kg·d) or 12.2 (5.4 – 21.3) g tissue/(kg·d). Conversion from tyrosine kinetics yield a proteolysis rate of 7.9 (7.2 – 11.4) g/(kg·d).

DISCUSSION

In this study, we described several aspects of human maternal and fetal phenylalanine and tyrosine metabolism. We aimed to add to the minute knowledge on fetal amino acid metabolism, so as to stimulate and aid the development of better nutrition for premature

infants.

Whether the fetus is a significant drain on maternal substrate availability during fasting is a semantic issue. Umbilical phenylalanine uptake per kilogram maternal weight was 1.0 (0.6 – 1.9) µmol/(kg·h). This amount can easily be realized through a small increase in the maternal proteolysis rate or a reduction in protein synthesis as these rates are approximately 30 times higher than net umbilical uptake. On the other hand, when considering the effects on the gravida's net catabolic state, one-fourth is attributable to net umbilical uptake. This fetal attribution to maternal catabolism is even underestimated since it does not include the extra amino acid consumption of other conceptus tissues such as the placenta.

How the metabolic load of the total conceptus is handled by the gravida is not exactly known. In rats, maternal protein stores initially increase during early pregnancy, and are later catabolized to sustain fetal demands necessary for rapid growth [18,19]. In humans, there is no such evidence. However, since protein intake does not seem to be increased substantially in pregnant women, other mechanisms probably also account for the total accumulation of 925 gram protein in various tissues and fetus during pregnancy [20]. Some studies showed unchanged oxidation [21-23], but others showed reduced amino acid oxidation and urea synthesis rates [24,25], or reduced nitrogen excretion rates [22,24,26], probably all to spare nitrogen necessary for fetal growth [26]. Maternal phenylalanine hydroxylation rates in our subjects were lower than those found in non-pregnant individuals, but comparable to those in other pregnant women [15,21,27]. Relating the other kinetic rates measured in this study to non-pregnant women is difficult. For one thing, the differences are probably more subtle. Moreover, changes in body weight and composition during pregnancy, and the contribution of the feto-placental compartment to maternal metabolism, distort comparisons to non-pregnant women.

Whereas net umbilical uptake of all essential amino acids was considerable in the hereafter cited studies, tyrosine uptake in the term human fetus has been shown to be small or even slightly negative [16,28-30]. During the second trimester of gestation, fetal tyrosine uptake was also found to be absent [31], or small at most [29]. Although placental amino acid transporters are capable of transporting tyrosine to the fetus, the in vitro measured tyrosine influx is strongly inhibited by the presence of several other amino acids, even at physiological concentrations [32,33]. The K_i value (giving half-maximal inhibition) of tyrosine transport across the maternal facing trophoblastic membrane was found to be 68±4.0 µM with phenylalanine [32]. This value does not deviate much from the observed maternal phenylalanine concentrations (Table 3). In this light, it is interesting that mothers of growth-restricted fetuses have elevated plasma amino acid concentrations, including phenylalanine [34]. Whether this could result in further inhibition of materno-fetal tyrosine transport remains speculative.

Because net fetal tyrosine uptake is probably low, it thus seems that the fetus is largely dependent on endogenous tyrosine synthesis from phenylalanine. Some early in vitro

studies reported substantial enzymatic activity in liver extracts from first or second trimester aborted human fetuses [35-37]. Tyrosine formation in premature neonates immediately after death has also been described [38]. One other study describes disappearing phenylalanine hydroxylase capacity during the second half of pregnancy [39] and the absence was confirmed in a deceased premature infant [40]. Next to these in vitro studies, of which none measured renal hydroxylating capacity, only Chien and colleagues attempted to quantify in vivo phenylalanine hydroxylation rates in fetuses at term [16]. They did not simultaneously infuse labeled tyrosine, however, and Nair et al. [12] had not published their balance model by then, resulting in a highly simplified model to determine hydroxylation rates. But when using their enrichment results in combination with our equation (4.5), hydroxylation rates are very high, i.e. 42.6 µmol/(kg·h) using our median $^{Btyr}/_{Bphe}$ of 0.75, or 40.8 µmol/(kg·h) using the theoretical breakdown ratio of 0.64 from deceased fetuses [13]. As these rates are much higher than the net umbilical phenylalanine uptake, they seem improbable. Hydroxylation rates in premature and term infants have been measured in several studies during the last 15 years. Mean rates in fasting premature infants receiving only glucose range from 6 to 17 µmol/(kg·h) [41-45]. If amino acids are also supplemented, some studies report no change [41], other show a small increase with means ranging from 11 to 22 µmol/(kg·h) [42-44]. Shortland et al. even measured hydroxylation rates of 48 µmol/(kg·h) after amino acid supplementation [45], but these rates appear to be overestimated [46]. Rates in healthy term infants do not seem to be different from rates in preterm infants and range from 8 to 13 µmol/(kg·h), irrespective of nutrient administration [42,47]. Our observed hydroxylation rates of 7.5 (6.2 – 15.5) µmol/(kg·h) are in concordance with the postnatal values.

Fetal growth velocities around 38 weeks gestation are around 8 to 10 g/(kg·d). Our observed growth rates (12.2 (5.4 – 21.3) g tissue/(kg·d)), calculated from the accretion rate of one amino acid, are not much different. Potential errors in the conversions to proteins and body weight and measurement errors are probably responsible for the small difference.

Much to our surprise, the metabolized fraction of available fetal phenylalanine and tyrosine was only approximately 20% for both amino acids. Calculations on data from Chien et al. in term human fetuses as well [16], reveal a metabolic uptake of 26% for phenylalanine and 36% for leucine. In ovine fetuses, we calculated from available data [48] a fraction of approximately 25% for leucine, which does not seem to differ between normally grown and growth-restricted fetal animals. This implies that most amino acids entering the fetus through the umbilical cord remain intravascular before returning to the placenta through the umbilical arteries. It thus seems that the placenta provides the fetus with an enormous reserve capacity of these amino acids. Intrauterine growth restriction would, therefore, not seem to be primarily caused by a lack of amino acids. The cause of fetal growth restriction could then lie in a reduced potential or necessity to internalize amino acids from the fetal tissue arterioles into the tissue or organ, because of a lack of secondary metabolites necessary for cellular inward transport or growth (e.g. ATP, oxygen, or sodium),

or through hormonal influences. Insufficient oxygen supply to the ovine fetus, for example, decreases protein synthesis more than breakdown, so that net protein accretion becomes compromised [49]. Fetal amino acid concentrations increased during this four-hours lasting experiment, whereas placental leucine transport decreased. Whether the latter is primarily the result of hypoxemia too or a compensatory mechanism in order to avoid hyperaminoacidemia is speculative.

On the other hand, growth-restricted fetuses show reduced umbilical plasma concentrations [34]. Besides, placentas of fetuses with intrauterine growth restriction (IUGR) have reduced transporter activity [50,51] which has also been described in human in vivo research [52]. Although there seems to be a large overcapacity of phenylalanine and tyrosine available for fetal growth, protein accretion is determined by the first limiting amino acid. It might well be that for one of the other essential amino acids, the surplus is less abundant. Reduced placental functioning might then induce a lower availability of this first limiting amino acid and consequently slower growth. Further studies on other amino acids and studies in growth-restricted fetuses are necessary to support these hypotheses.

All our concentration and enrichment measurements were done in the plasma compartment, rather than in whole blood, due to analytical advantages. Many studies, however, reported rapid equilibrium between erythrocyte and plasma concentrations of various amino acids, including phenylalanine and tyrosine [53-55]. The role of erythrocytes in organ amino acid delivery is thus as important as the role of the plasma compartment. Compared to normal organ balance studies, the circulation time of blood in fetal balance studies is relatively long since blood from the umbilical vein flows through the whole fetus before returning to the umbilical arteries. By then complete mixing can be expected. Even in single organ balance studies, many groups chose to use plasma sampling in combination with whole blood flows rather than plasma flows [56-59]. The latter would reduce all kinetic rates by approximately 40% (~ hematocrit) and yield improbably low kinetic rates.

Whether maternal anesthesia and surgery would have any consequences for fetal metabolism remains speculative. Spinal anesthesia might result in maternal hypotension and blood flow redistribution, but these effects can be prevented by using a lateral wedge. Furthermore, blood pressure monitoring allows for prompt correction if necessary. Besides, the pulsatility index of the umbilical artery does not seem to be influenced by spinal anesthesia [60]. Konje et al. measured flow using a transonic time flowmetry technique on a exteriorized loop of the umbilical cord during cesarean surgery [61]. Their flow values halfway during surgery correspond well to our flow measurements. Besides, umbilical blood flow after vaginal delivery has been reported to be stable for the first 100 postnatal seconds [62]. We thus assume that umbilical blood flow is fairly constant during surgery. The fetal metabolic response to maternal surgery remains speculative. A maternal noradrenalin surge after an invasive procedure did not seem to reach the human fetus [63]. In mice, however, noradrenalin was suggested to have transferred the placenta [64].

To conclude, we showed that the fetus at term receives considerable amounts of

phenylalanine from the placenta. Nevertheless, the fetus actively uses only a relatively small part; two-thirds of which are used for net protein synthesis and one-third for hydroxylation to the semi-essential amino acid tyrosine. Whether these findings would also hold true for the growth restricted fetus or a fetus earlier in gestation remains to be elucidated.

ACKNOWLEDGEMENTS

Most of all, we would like to thank all participating women. Furthermore, Frans te Braake, Willemijn Corpeleijn, and all personnel from the obstetrical, and anesthesiological departments were a great helping hand collecting all material and providing all facilities.

Financial support was kindly provided by the Sophia Children's Hospital Fund (SSWO) and the Nutricia Research Foundation, but they had no influence in study design, results, publication, or whatsoever.

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CHAPTER

8

Amino acid metabolism in the human fetus at term: leucine, valine, and methionine kinetics

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Published in:
Submitted

ABSTRACT

Introduction

Human fetal metabolism is a largely unexplored field. However, increased knowledge might play a pivotal role in improving nutritional strategies for prematurely born infants to ameliorate long-term outcome. Therefore, our aim was to quantify human fetal leucine, valine, and methionine metabolism.

Methods

Eight fasted, healthy pregnant women undergoing elective cesarean section at term received continuous stable isotope infusions of [1-^{13}C,^{15}N]leucine, [U-$^{13}C_5$]valine, [1-^{13}C] methionine, and [*methyl*-D_3]methionine starting prior to surgery. Umbilical blood flow was measured using ultrasound. Maternal and umbilical cord blood was collected and analyzed for amino acid enrichments and concentrations using gas chromatography mass-spectrometry. Data are expressed as median (25th – 75th percentile).

Results

Fetuses showed considerable leucine, valine, and methionine uptake (90 (79 – 145), 71 (68 – 123), and 16 (11 – 20) µmol/(kg·h), respectively). These three amino acids were released from fetal protein breakdown at rates of 242 (220 – 306), 194 (168 – 216), 42 (35 – 48) µmol/(kg·h), respectively. Conversion from these molar rates reveals a protein breakdown rate of approximately 10 g protein/(kg·d). After reversible leucine transamination to α-ketoisocaproate, the latter was transported to the placenta at a rate of 18 (32 – 15) µmol/(kg·h), reaminated to leucine (77 (67 – 99) µmol/(kg·h)), or oxidized. Oxidation rates could not be quantified directly, but our data indicate that a large part of leucine and valine is ultimately oxidized.

Conclusions

Collectively, our data show that the fetus around term gestation has considerable amino acid uptake in combination with high protein breakdown rates. Probably, amino acids are used in large amounts for oxidation to yield energy.

INTRODUCTION

Historical wise, the fact that the design of current nutrition for premature neonates are merely step-by-step alterations of the original composition of breast milk [1] is not surprising. Years ago, gestational viability was closer to term than it is currently and the analysis of normal neonatal nutrition was easier than the study of fetal nutrition. But with increased survival of less mature infants, metabolic demands of these young individuals probably deviate much more from those who receive breast milk or regular formula after term birth. Besides, we know from several decades of animal fetal research that intrauterine nutrient supply delivers more amino acids and less fat than is supplied during breast feeding [2]. Today, however, the exact composition of human fetal nutrient supply still remains unknown. Current common practice for a premature infant dependent on parenteral nutrition, is to supply 2 to 3 g/(kg·d) of amino acids and 2 to 3 g/(kg·d) of lipids [3]. With the current preterm formula, infants even have a higher lipid intake. However, since protein is the main functional component of tissue gain, this macronutrient has gained most attention and, over time, recommended protein intake and quality have often changed. Yet, most of the prematurely born infants still show significant postnatal growth restriction which can affect growth and development over a long period.

With the availability of harmless tracer studies, human fetal metabolism can be unraveled. This knowledge could prove pivotal in further ameliorating nutritional strategies for the premature infant. Therefore, our aim was to investigate several aspects of human fetal essential amino acid metabolism, specifically leucine, valine, and methionine kinetics. These could be determined by analyzing umbilical cord blood after the infusion of stable isotopically-labeled amino acids into pregnant women undergoing elective cesarean section at term. These kinds of studies provide normative data against which amino acid metabolism in the neonate after, for example, fetal growth restriction or prematurity can be judged.

METHODS

PATIENTS

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by both the institutional medical ethical review board and the Dutch central committee on research involving human subjects (CCMO). Pregnant women undergoing elective cesarean section (repeat or breech presentation) at term were eligible. Exclusion criteria were obesity (preconceptional body mass index (BMI) >30), preeclampsia, diabetes, known fetal anomalies, or severe intrauterine growth restriction (< -2SD). Participating women gave written consent after having been fully informed about all study details.

EXPERIMENTAL DESIGN

To determine the umbilical blood flow, blood flow velocity and vessel diameters were measured in the umbilical vein using an ultrasound machine (iU22, Philips Medical Systems, Eindhoven, the Netherlands) as previously described [4]. Ultrasound measurements were made in the late afternoon on the day preceding surgery; cesarean sections were scheduled at approximately 8.00 a.m. after an overnight fast.

At least 4 hours prior to planned surgery, the women received a primed continuous infusion of L-[1-^{13}C,^{15}N]leucine (8 mmol/(kg·h)) through a forearm vein. Two hours later primed continuous infusions of L-[U-$^{13}C_5$]valine, L-[1-^{13}C]methionine, and L-[*methyl*-D_3] methionine (5, 2, and 2 mmol/(kg·h), respectively) were started along. Priming doses were half the hourly doses. Isotopes (all >98% enriched and tested for sterility and pyrogenicity) were obtained from Buchem BV, Apeldoorn, the Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA). Our hospital pharmacy dissolved the isotopes in 0.9% saline and the solutions were filtered (0.2 µm) and sterilized. Tests were performed to ensure the correct identity, concentration, and a sterile and pyrogen free product. Tracers were given using Perfusor® fm (B|Braun Medical B.V., Oss, the Netherlands) infusion pumps. Maternal blood was sampled before the tracer infusions started (baseline), once immediately before anesthesia (spinal) and, if possible (n=4), about 20 minutes later just before surgery started. Fetal blood was sampled after birth from both the vein and arteries of a doubly clamped segment of the umbilical cord. After collection in heparin tubes, blood was centrifuged and plasma was frozen at -80°C until analysis.

ANALYSIS

As calculations in a veno-arterial balance model (as on the umbilical cord in the fetus, see below) largely depend on the small differences in concentration and enrichment between the vein and arteries, rather than on the absolute values, measurements must be extremely precise. To minimize the effects of potential analytical measurement errors, samples were prepared for analysis twice using two different derivatization methods (PCF and MTBSTFA, see below). Each derivatized sample was analyzed in triplicate on two different gas chromatography mass spectrometers (GCMS) (see below). Enrichments of valine and methionine were calculated from the mean of all twelve analyses; enrichments of leucine isotopomers (m+0, m+1: [1-^{13}C]leucine (without ^{15}N), and m+2: [1-^{13}C,^{15}N]leucine) were calculated from the MTBSTFA samples only (due to the fragmentation pattern after PCF analysis); amino acid concentrations were calculated from the mean of the six analyses using the MTBSTFA derivative.

PCF (propylchloroformate) derivatization on samples was performed using commercial kits (EZ:Faast for hydrolysates, Phenomenex, Bester BV, Amstelveen, the Netherlands) according to the enclosed protocol. Samples to be derivatized with MTBSTFA were added with [D_{10}]leucine, [D_8]valine, and [U-$^{13}C_5$,^{15}N]methionine as internal standards for concentration determinations. Concentration calibration curves were prepared using MTBSTFA as well. Two different enrichment calibration curves were derivatized with PCF

and MTBSTFA for analysis. Samples and calibration curves were analyzed with a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, the Netherlands) on a VF-17ms, 30m x 0,25mm ID capillary column (Varian Inc., Middelburg, the Netherlands) and a Thermo DSQ GCMS (Thermo Fisher, Breda, the Netherlands) on a VF-1701ms, 30m x 0,25mm ID capillary column (Varian Inc., Middelburg, the Netherlands).

Samples intended to be analyzed for the enrichments and concentrations of the ketoacids αKIC and αKIV were added with [*methyl*-D_3]αKIC and [*dimethyl*-$^{13}C_2$]αKIV as internal standards. Enrichment and concentration calibration curves were also prepared. Samples were derivatized with quinoxanol-silyl, phenylenediamine, and MTBSTFA and measured in triplicate on the same Thermo DSQ GCMS as described above.

CALCULATIONS

Maternal leucine fluxes (Q) were calculated from the tracer dilution resulting from the rate of leucine appearance [5,6]. In short, carbon leucine flux (Q_C) is made up of leucine appearing from proteolysis only, whereas nitrogen leucine flux (Q_N) is made up of both proteolysis and αKIC reamination. Thus, the difference between Q_N and Q_C yields the rate of αKIC reamination to leucine. Fluxes were calculated as $I \times [(E_i/E_p) - 1]$, where I is the [1-^{13}C,^{15}N]leucine infusion rate and E_i and E_p represent the enrichments in mole percent excess (MPE) of the infusate and of either plasma [1-^{13}C,^{15}N]leucine or [1-^{13}C]αKIC yielding Q_N or Q_C, respectively. Because [1-^{13}C,^{15}N]leucine enrichment in plasma is no more than the site of infusion and not the site where the majority of metabolism will take place, the plasma enrichment will be slightly overestimated leading to an underestimated Q_N. The [1-^{13}C]αKIC enrichment is the resultant from intracellular metabolism and thus gives a good reflection of intracellular metabolism because of rapid exchange.

Maternal valine and methionine kinetics were studied regarding their carbon skeletons only; Q_C was calculated analogous to leucine using the [U-$^{13}C_6$]αKIV enrichment. Maternal methionine transmethylation kinetics were not quantified as the tracer infusion would be too short to achieve [1-^{13}C]homocysteine steady state [7].

We quantified fetal whole body kinetics by using the concept of an umbilical veno-arterial balance model. To do so, we rewrote the leucine arteriovenous balance model by Tessari et al. [8] to suit fetal studies. The model is outlined in Figure 1 and its determinants are calculated using the following equations, where kg in all units denotes fetal weight (= birth weight):

Rate of leucine delivery from umbilical vein to the fetus:

$$\text{delivery} = [\text{leu}]_{\text{ven}} \times \text{BF} \quad (1.1)$$

where [leu] is the total (labeled + unlabeled) leucine concentration (µmol/L) and BF the umbilical blood flow (L/(kg·h)). Subscripts indicate whether blood was sampled from the umbilical vein or arteries (as below).

Rate of leucine release from fetus to umbilical artery:

$$\text{release} = [\text{leu}]_{\text{art}} \times \text{BF} \quad (1.2)$$

CHAPTER VIII

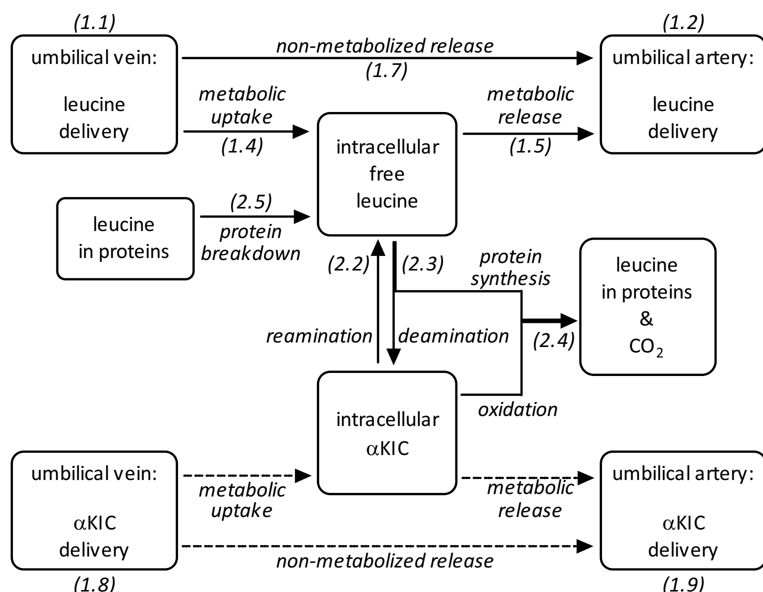

FIGURE 1: Schematic model of fetal leucine metabolism. Numbers in brackets correspond to the equations in the methods section and the fluxes outlined in table 5. Dashed lines indicate fluxes that could not be quantified. Fluxes 2.3 and 2.4 are the sums of protein synthesis and either deamination or oxidation, respectively.

Fraction of leucine that is metabolized intracellularly:

$$\text{metabolized fraction} = 1 - \frac{[^{13}\text{C}, ^{15}\text{N}\bullet\text{leu}]_{art}}{[^{13}\text{C}, ^{15}\text{N}\bullet\text{leu}]_{ven}} \tag{1.3}$$

where $[^{13}C,^{15}N{\cdot}leu]$ is the labeled $[1\text{-}^{13}C,^{15}N]$leucine concentration (µmol/L).

Rate of leucine inflow from umbilical vein to intracellular compartment:

$$\text{metabolic inflow} = \text{Eq}(1.1) \times \text{Eq}(1.3) \tag{1.4}$$

Rate of leucine outflow from intracellular compartment to umbilical artery:

$$\text{metabolic outflow} = \text{Eq}(1.2) + \text{Eq}(1.4) - \text{Eq}(1.1) \tag{1.5}$$

Net fetal leucine uptake:

$$\text{net uptake} = \text{Eq}(1.1) - \text{Eq}(1.2) = \text{Eq}(1.4) - \text{Eq}(1.5) \tag{1.6}$$

Rate of leucine directly released from umbilical vein to artery without being metabolized:

$$\text{non-metabolized release} = \text{Eq}(1.1) - \text{Eq}(1.4) \tag{1.7}$$

Rate of α-ketoisocaproate delivery from umbilical vein to the fetus:

$$\text{delivery} = [\text{KIC}]_{ven} \times \text{BF} \tag{1.8}$$

where [KIC] is the total (labeled + unlabeled) αKIC concentration (µmol/L).

Rate of α-ketoisocaproate release from fetus to umbilical artery:

$$\text{release} = [\text{KIC}]_{art} \times \text{BF} \quad (1.9)$$

Net fetal α-ketoisocaproate uptake:

$$\text{net uptake} = \text{Eq}(1.8) - \text{Eq}(1.9) \quad (1.10)$$

Fraction of [1-^{13}C]leucine that is metabolized intracellularly:

$$\text{metabolized fraction} = 1 - \frac{[^{13}\text{C}\bullet\text{leu}]_{art}}{[^{13}\text{C}\bullet\text{leu}]_{ven}} \quad (2.1)$$

where [^{13}C·leu] is the total labeled [1-^{13}C]leucine concentration (µmol/L). The total [1-^{13}C]leucine enrichment was calculated as the sum of the [1-^{13}C]leucine (without ^{15}N) enrichment and the [1-^{13}C,^{15}N]leucine enrichment.

Rate of intracellular α-ketoisocaproate reamination to leucine:

$$\text{reamination} = \frac{\text{BF} \times [^{13}\text{C}\bullet\text{leu}]_{art} \times (\text{Eq}(1.3) - \text{Eq}(2.1))}{^{13}\text{C}\bullet\text{KIC}\bullet\text{E}_{art}} \quad (2.2)$$

where ^{13}C·KIC·E is the [1-^{13}C]αKIC enrichment (in MPE).

In our model, we could not discriminate between leucine being incorporated into protein and leucine being deaminated to αKIC. Thus, we calculated the sum of the latter two rates in µmol/(kg·h):

$$\text{S} + \text{deamination} = \text{Eq}(2.2) + \frac{\text{BF} \times \left([^{13}\text{C}\bullet\text{leu}]_{ven} - [^{13}\text{C}\bullet\text{leu}]_{art}\right)}{^{13}\text{C}\bullet\text{KIC}\bullet\text{E}_{art}} \quad (2.3)$$

In our model, we could not discriminate between leucine being incorporated into protein and αKIC being oxidized to CO_2. Thus, we calculated the sum of the latter two rates in µmol/(kg·h):

$$\text{S} + \text{O} = \text{Eq}(2.3) - \text{Eq}(2.2) + \text{Eq}(1.10) \quad (2.4)$$

Rate of leucine release from proteolysis into the intracellular space:

$$\text{B} = \text{Eq}(1.2) - \text{Eq}(1.1) + \text{Eq}(2.3) - \text{Eq}(2.2) \quad (2.5)$$

The above outlined model can also be used for calculations on valine kinetics. However, because the nitrogen group of valine was not labeled, equations (1.3, 1.4, 1.5, 1.7, 2.2, and 2.3) could not be determined. Equations (1.1, 1.2, 1.6, 1.7, 1.8, 1.9, 1.10, 2.1, 2.4, and 2.5) can be resolved by replacing leucine by valine and αKIC by αKIV.

Since a balance model does not require steady state assumptions (e.g. for [1-^{13}C]homocysteine), such as in the whole body model, methionine kinetics were calculated using the same model as the leucine kinetics by replacing leucine, αKIC, [^{13}C,^{15}N·leu], deamination, and reamination by methionine, homocysteine, [D_3·met], demethylation, and remethylation, respectively. However, homocysteine concentration and enrichment were not actually measured because its plasma concentration and uptake are very low [9]. The denominator in equations 2.2 and 2.3 was therefore formed by the [1-^{13}C]methionine

instead of homocysteine enrichment, thereby slightly underestimating transmethylation and proteolysis fluxes.

Proteolysis rates of these three amino acids can be converted from molar rates into grams of protein under the assumption that one gram of fetal protein contains on average 562 µmol leucine, 395 µmol valine, and 130 µmol methionine [10].

STATISTICS

Calculations were made using Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA). Statistical analysis was performed using GraphPad Prism software (version 4.0; San Diego, CA, USA). Because the number of included subjects was small (n=8), normality of data could not be assumed. All results were therefore expressed as median (25th – 75th percentile). Consequently, by presenting our data as medians, all rates in Table 6 do not add up correctly as outlined in our model (Figure 1). Nonetheless, the fluxes of each individual subject do.

RESULTS

We included eight feto-maternal dyads. These patients are the same as those described in an earlier study by our group on fetal phenylalanine and tyrosine kinetics [11]. Maternal age, preconceptional and actual BMI, and parity are shown in Table I. Fetal characteristics in terms of gestational age, birth weight, birth weight *z*-score [12], sex, umbilical blood flow, umbilical pulsatility index, and Apgar score are shown in Table II.

From four women, we obtained two blood samples before surgery had started with an interval of approximately 20 minutes. Enrichments did not differ during this time interval, despite spinal anesthesia being applied in between. Therefore, steady state was assumed. The other four women had only one blood sample taken, which was before anesthetics were initiated. Achieving steady state is important in whole body modeling; calculating kinetics in a balance model like we did on the fetus, however, does not depend on steady state. The maternal and umbilical leucine, αKIC, valine, αKIV, and methionine concentrations and enrichments are shown in Table III. The feto-maternal enrichment and concentration ratios across the maternal and umbilical veins are outlined in Table IV.

Table V shows maternal leucine, valine, and methionine kinetics. Since the women were fasting, amino acids released from protein breakdown equaled the total rate of appearance.

Table VI displays fetal kinetics of the 3 amino acids. However, the data of the leucine and valine kinetics from one patient were excluded in this table as the findings deviated greatly from the other 7 subjects. Results from this particular patient are outlined in the discussion.

TABLE I: Maternal characteristics. Results are expressed as median (25th – 75th percentile), except for parity (n).

Characteristic	Value
Age (y)	33.0 (28.8 – 38.0)
Preconceptional BMI (kg/m^2)	21.9 (20.3 – 24.5)
Actual BMI (kg/m^2)	30.5 (23.3 – 31.6)
Parity (0:1:2:3) (n)	(4:1:2:1)

TABLE II: Fetal characteristics. Birth weight *z*-scores are corrected for gestational ages [12]. The pulsatility index (P.I.) is a Doppler ultrasound derived index on the blood stream velocity profile through the umbilical arteries and is a marker of fetal well-being. The Apgar score is a postnatal scoring scale from 0-10. All results are expressed as median (25th – 75th percentile), except for sex.

Characteristic	Value
Gestational age (wks)	38.5 (37.6 – 38.9)
Birth weight (kg)	3.3 (2.7 – 3.4)
Birth weight *z*-score (SD)	-0.11 (-0.86 – 0.52)
Sex (m:f) (n)	4:4
Umbilical blood flow (mL/(kg·min))	101 (90 – 110)
P.I.	0.89 (0.78 – 0.96)
Placental weight (kg)	0.590 (0.558 – 0.649)
Apgar score at 5 min	10 (10 – 10)

TABLE III: Plasma leucine (leu), αKIC, valine (val), αKIV, and methionine (met) concentrations (µmol/L) and enrichments (MPE) measured in the maternal vein (n=8), umbilical vein (n=8), and umbilical artery (n=8). Results are expressed as median (25th – 75th percentile).

	Maternal vein	Umbilical vein	Umbilical artery
leu concentration	108 (99.7 – 119)	145 (134 – 148)	125 (111 – 133)
αKIC concentration	28.1 (25.5 – 31.2)	36.5 (35.0 – 41.2)	39.3 (36.4 – 40.9)
val concentration	149 (138 – 157)	191 (179 – 221)	179 (167 – 186)
αKIV concentration	11.0 (10.4 – 11.9)	13.0 (12.1 – 14.7)	12.6 (11.6 – 14.0)
met concentration	28.3 (24.7 – 30.5)	36.7 (34.3 – 39.0)	34.8 (32.0 – 36.7)
[1-^{13}C,^{15}N]leu enrichment	4.1 (3.2 – 4.5)	2.6 (2.6 – 2.8)	2.0 (1.8 – 2.1)
[1-^{13}C]leu (w/o* ^{15}N) enrichment	5.5 (5.0 – 5.8)	5.5 (5.0 – 6.1)	5.0 (4.5 – 5.6)
[1-^{13}C]leu (total) enrichment	9.4 (8.7 – 9.8)	8.1 (7.4 – 8.8)	7.0 (6.3 – 7.8)
[1-^{13}C]αKIC enrichment	6.0 (5.8 – 6.2)	5.7 (5.2 – 5.9)	4.8 (4.3 – 5.2)
[U-$^{13}C_5$]val enrichment	6.6 (6.2 – 7.0)	5.0 (4.7 – 5.1)	4.4 (4.2 – 4.7)
[U-$^{13}C_5$]αKIV enrichment	4.5 (4.0 – 4.8)	3.9 (3.7 – 4.3)	3.6 (3.2 – 3.8)
[1-^{13}C]met enrichment	9.8 (9.5 – 10.2)	6.9 (6.6 – 7.5)	5.9 (5.5 – 6.2)
[*methyl*-D_3]met enrichment	9.6 (8.9 – 10.4)	6.4 (6.1 – 6.9)	5.2 (4.8 – 6.0)

* without

TABLE IV: Feto-maternal ratios of the concentrations and enrichments across the umbilical vein and maternal vein. Results are expressed as median (25th – 75th percentile).

Result	Ratio
Leucine concentration	1.3 (1.3 – 1.4)
αKIC concentration	1.4 (1.2 – 1.5)
Valine concentration	1.4 (1.3 – 1.5)
αKIV concentration	1.2 (1.1 – 1.3)
Methionine concentration	1.3 (1.2 – 1.5)
[1-^{13}C,^{15}N]leu enrichment	0.70 (0.60 – 0.81)
[1-^{13}C]leu (total) enrichment	0.92 (0.81 – 0.95)
[1-^{13}C]αKIC enrichment	0.92 (0.90 – 0.95)
[U-$^{13}C_5$]val enrichment	0.79 (0.70 – 0.82)
[U-$^{13}C_5$]αKIV enrichment	0.87 (0.84 – 0.92)
[1-^{13}C]met enrichment	0.74 (0.69 – 0.83)
[*methyl*-D_3]met enrichment	0.70 (0.61 – 0.78)

TABLE V: Maternal leucine, valine, and methionine kinetics (n=8). All results are expressed in µmol/(kg·h) as median (25th – 75th percentile).

Flux	Value
Leucine released from proteolysis	125 (119 – 129)
Valine released from proteolysis	104 (98 – 118)
Methionine released from proteolysis	19 (18 – 20)
αKIC reamination to leucine	63 (49 – 107)

DISCUSSION

This report is the third in a series exploring fetal amino acid and protein metabolism by our group [11,13]. In this study several metabolic pathways of the essential amino acids leucine, valine, and methionine were quantified.

Previously, we found that the fetal net protein accretion rate in the same subjects as here was 1.70 g/(kg·d) when measured with phenylalanine and tyrosine tracers [11]. This can be converted to a net accretion rate of 40 µmol leucine/(kg·h), 28 µmol valine/(kg·h), and 9.2 µmol methionine/(kg·h). Yet, the net TLC, TVC, and methionine uptakes (table 6) are much higher than the net accretion rates. This would therefore seem to suggest that a large proportion of these amino acids is being oxidized, or in the case of methionine, follows the transsulfuration pathway. This is especially true for valine, where 60% of the net TCV uptake is oxidized. Leucine oxidation would contribute for approximately 40% of total TLC uptake. Chien et al. showed fetal leucine oxidation rate to be one-third of TLC uptake [14], a value close to our estimation. In the ovine fetus around term, valine has the highest net fetal

TABLE VI: Fetal leucine (leu), valine (val), and methionine (met) kinetics. Numbers in brackets indicate the equation used and flux as they are outlined in the methods section and in figure 1. Data are expressed as median (25th – 75th percentile) in µmol/(kg·h) unless otherwise indicated.

	leu (n=7)	αKIC (n=7)	val (n=7)	αKIV (n=7)	met (n=8)
Umbilical vein delivery (1.1 & 1.8)	837 (644 – 935)	229 (171 – 245)	1067 (956 – 1249)	82 (57 – 90)	220 (165 – 250)
Umbilical artery output (1.2 & 1.9)	765 (569 – 817)	247 (185 – 272)	997 (798 – 1170)	76 (60 – 90)	202 (164 – 231)
Metabolized fraction (%) (1.3)	40 (27 – 41)	—	—	—	20 (19 – 23)
Metabolic uptake from umbilical vein (1.4)	248 (231 – 327)	—	—	—	48 (33 – 57)
Metabolic output into umbilical artery (1.5)	155 (130 – 208)	—	—	—	32 (25 – 38)
Net fetal uptake (1.6 & 1.10)	90 (79 – 145)	-18 (-32 – -15) [1]	71 (68 – 123)	0 (-6 – 2) [1]	16 (11 – 20)
Net TLC/TVC uptake	67 (51 – 118)		69 (65 – 128)		—
Non-metabolized release (1.7)	533 (424 – 600)	—	—	—	164 (136 – 193)
Metabolized fraction of carbon skeleton (%) (2.1)	30 (20 – 31)	—	19 (15 – 22)	—	19 (19 – 24)
αKIC reamination to leu (2.2)	77 (67 – 99)	—	—	—	1.9 (-2.1 – 4.5) [2]
Protein synthesis + deamination to αKIC (2.3)	402 (381 – 523)	—	—	—	61 (45 – 71) [3]
Protein synthesis + αKIC/αKIV oxidation (2.4)	314 (292 – 399)		279 (228 – 379)		58 (48 – 69) [4]
Release from protein breakdown (2.5)	242 (220 – 306)	—	194 (168 – 216)	—	42 (35 – 48)
Proteolysis rates (g protein/(kg·d))	10.3 (9.4 – 13.1)	—	11.8 (10.2 – 13.2)	—	7.7 (6.4 – 8.9)

[1] a negative value indicates net fetal release towards the placenta
[2] homocysteine remethylation to met
[3] protein synthesis + demethylation to homocysteine
[4] protein synthesis + transsulfuration

uptake of all essential amino acids [15-18]. Yet, only a relatively small part is necessary for protein deposition [16]. These findings, in combination with the fact that especially valine plasma concentrations are much higher during fetal life [19] than postnatally in healthy term breast-fed infants [20], could confirm that valine is largely oxidized during intrauterine life. Direct measurements of fetal valine oxidation, however, are unavailable in humans or animals. Since we were using several ^{13}C tracers simultaneously, we were not able to directly quantify the oxidation rates of the specific amino acids.

In our study and in the human study by Chien and colleagues [14], fetuses showed a net output of αKIC towards the placenta. Contrary are the findings in studies sheep, where fetuses demonstrate a net uptake of αKIC [21-23]. A species-related difference could explain this phenomenon. However, the ketoacid of valine, i.e. αKIV, did not show any significant net uptake or output. To our knowledge, studies on ovine fetal metabolism have not described αKIV concentrations.

As addressed in the results section, one fetus showed different results, especially reflected by a very high positive net uptake of αKIC (92 μmol/(kg·h)). The uptake of αKIV was also higher (30 μmol/(kg·h)) than in the other seven fetuses. Given the surprising result in this infant, measurements on αKIC and αKIV concentrations were repeated, but showed comparable results. Interestingly enough, this fetus also had a very high reamination rate (307 μmol/(kg·h)), probably to dispose the high αKIC uptake into leucine. All other fetal leucine and valine kinetic parameters were comparable to the other fetuses. The maternal reamination rate in this feto-maternal dyad was comparable to the other women. As can be seen in equation (2.2) under methods, the quantification of the fetal reamination rate occurs mathematically independent of fetal αKIC uptake or concentrations. We do not have any explanation for this finding. Additionally, mother and infant were in good clinical condition and the postnatal course of the infant was normal. Yet, the fact that a high fetal αKIC uptake was counterbalanced by a mathematically independent high reamination rate, gives us confidence in our methods, analyses, and thus overall results in all other subjects. As shown in table 3, the enrichment of [1-^{13}C]leucine (without ^{15}N) was very similar to the enrichment of [1-^{13}C]αKIC. This also confirms the model as a [1-^{13}C]leucine molecule (without the ^{15}N label) can only be formed through the reamination of an [1-^{13}C]αKIC molecule.

The pregnant women in our study showed higher reamination rates than previously measured in pregnant women [6] but were still lower than the protein breakdown rates. Maternal leucine proteolysis rates in studies using similar methodology have yielded rates that were 10% lower [6,14,24], similar [25], or 10% higher [26,27] than those reported here.

Ovine premature fetuses showed reamination rates approximately twice the rate of leucine appearance from protein breakdown [22,28]. This is in contrast with our results, where reamination rates are only one-third of the proteolysis rates. Apart from species differences, the sheep fetuses were also studied earlier in gestation. A higher growth rate in these premature sheep could demand fast transamination rates in order to shuttle nitrogen

between various tissues.

Reamination rates in enterally-fed growing premature neonates at 35 weeks corrected gestational age were with ~250 μmol/(kg·h) [29] much higher than the rates we observed in fetuses at term. In term-born healthy neonates on postnatal day two, however, reamination rates amounting ~140 μmol/(kg·h) were closer to our values, although still higher [30]. Nonetheless, proteolysis rates in these two studies by Parimi and colleagues and in a study by our group in premature neonates [31] were comparable to the fetal protein breakdown rates in the current study. Thureen et al, however, found lower proteolysis rates in premature infants [32].

Taken together, it seems that our observed fetal reamination rates are lower than the postnatal values in human neonates. A reason, partially explaining the higher postnatal values, is that the placenta cannot take up substrates anymore. Thus, in order to avoid irreversible αKIC oxidation, neonates reaminate at high rates. Another reason for a lower reamination rate in human fetuses compared to fetal sheep could be that αKIC is being transported towards the placenta, whereas in ovine fetuses the opposite is true, which necessitates a higher reamination rate.

The activity of BCAA aminotransferases (deamination) was reported to be very high in human first trimester placentas [33] and ovine placentas at term [34,35]. The feto-maternal enrichment ratios as reported in table 4 provide qualitative information on transplacental amino acid transport in relation to proteolysis rates in the placenta or fetus (and endogenous synthesis in non-essential amino acids). The fact that the feto-maternal enrichment ratio of [1-^{13}C,^{15}N]leucine was much lower than the total [1-^{13}C]leucine enrichment ratio, indicates that the placenta indeed reaminates at a high rate. The total [1-^{13}C]leucine enrichment ratio we found was comparable to the ratio observed by Marconi et al. [27], but higher than the one by Chien et al. [14]. Umbilical cord manipulation to perform flow measurements could theoretically have caused the lower enrichment ratios in the latter study. The enrichment ratio of another branched chain amino acid, valine, showed very good agreement, whereas the ratio for methionine was slightly lower for unknown reasons.

Surprisingly, we found on average very low remethylation rates. Besides, in some individuals, calculations even revealed small negative values. Of course, the latter is physiologically impossible. A relatively low methionine turnover rate, which can hamper accurate kinetic calculations, in combination with measurement or modeling errors, could be causal for these negative values. Therefore, although we must interpret our average remethylation rate with care, in the end we believe it is low and that most homocysteine undergoes transsulfuration ultimately leading to cysteine. In several recent studies, transsulfuration pathways have indeed been suggested to be active in both preterm and term neonates [36-38].

As the drawbacks of this study, including plasma versus whole blood measurements and potential effects of maternal surgery on fetal metabolism, have already been addressed

previously [11], they will not be elaborated upon here. In short, however, the amino acids studied here also show very rapid exchange between the erythrocyte and plasma compartments [39-41], implicating that plasma measurements as performed in this study should suffice. Several reports show in various ways that umbilical blood flow does not seem to be influenced by maternal anesthesia or surgery [42-44].

To conclude, we have described an explorative study on several metabolic pathways of three essential amino acids in human fetuses at term. Our data suggest high protein breakdown and synthesis rates, comparable with, or even slightly higher than in premature infants. The relatively large uptakes of leucine and valine total carbon suggest high fetal oxidation rates of these branched chain amino acids.

ACKNOWLEDGEMENTS

Most of all, we would like to thank all the participating women. Furthermore, Willemijn Corpeleijn, Frans te Braake, and all the personnel from the Obstetrics and Anesthesiological departments were very helpful in the collection of material and provision of facilities.

Financial support was kindly provided by the Sophia Children’s Hospital Fund (SSWO) and the Nutricia Research Foundation, but they had no influence in study design, results, publication, or whatsoever.

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CHAPTER

9

Placental protein synthesis rates during different periods of gestation

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Published in:
Submitted

ABSTRACT

Background

The fetus is highly dependent on the placenta for its nutrient and oxygen supply. Further knowledge on placental metabolism helps us understand the critical role of the placenta in ensuring high rates of placental nutrient transport necessary for fetal growth. Therefore, our objective was to study the in vivo placental protein synthesis rate in human pregnancies during different periods of gestation.

Methods

Pregnant women received three different stable isotope infusions (1-^{13}C,^{15}N]leucine, [1-^{13}C] phenylalanine, and [U-$^{13}C_5$]valine) starting at different times prior to elective cesarean section. After delivery, placental tissue samples were collected. Using mass spectrometry techniques, we determined the enrichment of the incorporated tracer amino acids in placental proteins. From the three product/precursor enrichment ratios, we calculated the fractional synthesis rate, which is the fraction of mixed placental proteins that is daily renewed. Results are expressed as median (25th-75th percentile).

Results

We analyzed placentas from preterm (n=8) and term-delivered pregnancies (n=8). Fractional synthesis rates were higher in premature than in term placentas. (24.5 (21.6 – 26.0) vs. 18.0 (15.6 – 22.8) %/d; p=0.028). Concordantly, more placental proteins were synthesized daily in the premature group (1.7 (1.5 – 1.8) vs. 1.2 (0.9 – 1.3) g/d per 100 g placenta; p=0.005).

Conclusion

The placenta has a very high protein turnover rate, which, however, decreases with advancing gestational age.

INTRODUCTION

Imagining the placenta as a passive organ or a simple materno-fetal conduit, solely functioning as a barrier between maternal and fetal circulations for some substances, yet passing nutrients and oxygen, is an outdated thought. In fact, the placenta is a metabolically very active organ. For example, placental intracellular amino acid concentrations are higher than in maternal or fetal plasma indicating active energy demanding transport [1-3]. To support placental aerobic metabolism, half of the total uterine oxygen uptake during ovine late pregnancy is retained within the placenta, whereas the other half is transported further to the fetus [4,5]. Glucose consumption by the placenta accounts even up to 60% of the total glucose uptake by the conceptus [6]. In addition, the relative placental consumption rates of oxygen and glucose are even higher during midgestation than at term [7].

Furthermore, if the pregnant ewe is subjected to chronic hypoglycemia, importance of undisturbed placental metabolism is even more stressed. Although under prolonged maternal hypoglycemia, the absolute glucose consumption rates by the fetus and placenta together are reduced, the uteroplacental tissues retain relatively even more glucose than under normoglycemic circumstances, thus, at the expense of glucose available for the fetus [4,6]. Measurements in human pregnancies at term yield similar data: 40% of oxygen uptake by the total conceptus is retained by the placenta, whereas only 60% is transferred further to the fetus [8].

Large part of the generated energy by the placenta is probably necessary for optimal amino acid transporter functioning at the expense of ATP either directly or indirectly by maintaining the electron gradients which drive the sodium dependent transporters. This in addition to the normal energy costs of cellular metabolism [9] which include continuous tissue remodeling and during midgestation also placental growth.

Placental protein and cellular turnover is physiological and supports optimal placental functioning. Apoptosis rates are twice as high at term as in first trimester placentas [10]. Apoptosis rates even further rise in a series of complications, amongst which intrauterine growth restriction [11,12]. Whether higher programmed cell death in pathologic states or towards the end of pregnancy is a direct result or a compensatory mechanism is unknown.

However, the mixed placental protein turnover rate might be a more specific indicator of its metabolic activity. Under the hypothesis that the protein turnover would decrease during gestation we measured the human in vivo placental protein fractional synthesis rate (FSR) using a stable isotope model.

PATIENTS AND METHODS

SETTING AND SUBJECTS

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by both the institutional medical ethical review board and the Dutch central committee on research involving human subjects (CCMO, the Hague). Pregnant women scheduled to undergo elective cesarean section (repeat, breech, or multiple pregnancy) under spinal anesthesia were eligible. We aimed to include pregnancies which were close to term as well as pregnancies which were premature. Exclusion criteria were maternal obesity (preconceptional body mass index > 30 kg/m^2), diabetes, severe fetal growth restriction (< -2SD), or known fetal anomalies. Participants gave written consent after having been fully informed about all study details.

EXPERIMENTAL DESIGN

L-[1-^{13}C,^{15}N]leucine, L-[1-^{13}C]phenylalanine, and L-[U-$^{13}C_5$]valine were purchased from Buchem BV, Apeldoorn, The Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA) (all 99% enriched and tested for sterility and pyrogenicity). Our hospital pharmacy dissolved the isotopes separately in 0.9% saline after which the solutions were filtered (0.2 µm) and sterilized. Tests were performed to ensure the correct identity, concentration, and a sterile and pyrogen free product.

Pregnant women received primed continuous stable isotope infusions of L-[1-^{13}C,^{15}N] leucine (8 µmol/(kg·h)), L-[1-^{13}C]phenylalanine (5 µmol/(kg×h)), and L-[U-$^{13}C_5$]valine (5 µmol/(kg·h)), starting at least 4, 3, and 2 hours prior to planned surgery, respectively. The priming doses were half of the hourly doses. Tracers were given in a forearm vein with three separate Perfusor® fm infusion pumps (B|Braun Medical B.V., Oss, the Netherlands) until surgery was completed. After delivery of the neonate, the placenta was weighed and put on melting ice. Fetal blood was sampled from both the vein and arteries of a doubly clamped segment of the umbilical cord. After collection, blood samples were centrifuged (2000´g) in heparin tubes and plasma was frozen at -80°C until analysis. During centrifugation of the cord blood, three tissue samples (± 1.5×1.5×1,5 cm) were cut from the placenta in different areas around the insertion of the umbilical cord. Samples were washed several times in fresh and chilled saline and vigorously shaken until largely devoid of visible blood. Tissue was then quickly frozen at -80°C until analysis.

SAMPLE ANALYSES

Of each frozen placental tissue sample (n=3 per placenta, 48 samples in total), approximately 1.2 g tissue was cut and subsequently freeze-dried for at least 15 hours. Exact wet and dry weights were measured. Dried placental material was dissolved in H_2O (1:20 m/m) before homogenization and sonification. The material was treated as cold as possible during all procedures. Portions of two-hundred µL of homogenate were used to determine the placental protein concentration in sextuplicate according to the photospectrometrical method of Lowry et al. [13]. The remaining homogenate (1.5 mL) was

deproteinized using 1.5 mL of 2 mol perchloric acid (PCA)/L. After centrifugation, supernatant was separated and treated further as described below. To the protein pellet, 2 mL of 0.2 mol PCA/L was added as a washing step. After complete mixing and centrifugation, supernatant was discarded. The washing steps were repeated another two times. To the protein pellet, 200 µL internal standard solution (10 g norvaline/L and 10 g norleucine/L) and 1.2 mL of 7 mol HCl/L were added. Samples were then hydrolyzed for 22 hours at 110°C. After cooling down, a portion of 100 µL of hydrolysate was taken and added with sodium hydrogen carbonate to direct the pH between 1.5 and 3.0 and subsequently filtered through 0.22 µm nylon filters to remove ashes. Samples were then derivatized using ethylchloroformate and measured in triplicate on a gas chromatograph – combustion – isotope ratio mass spectrometer (Delta XP, Thermo Electron, Bremen, Germany) [14]. Enrichment calibration graphs were created by mixing known amounts of enriched and nonenriched amino acids; these were derivatized and analyzed similar to the samples. Baseline enrichments of leucine, phenylalanine, and valine in placental proteins could of course not be measured, but were assumed to be equal to those amino acids that were not administered and do not share common metabolic pathways. Therefore, we measured the natural enrichment of lysine and alanine (~0) in the placental samples taken after birth. The averaged values were used to correct for the natural abundance of the leucine, phenylalanine, and valine tracers.

As a precursor for placental protein synthesis, we measured the placental intracellular free amino acid enrichment. However, the intracellular free amino acid enrichment turned out to be approximately one-fourth to one-half of the enrichments that were measured in the maternal and fetal plasma [15]. Probably, placental protein started to degrade already very soon after placental removal from the uterus thereby diluting the intracellular amino acid enrichment. Free intracellular tissue amino acid enrichments could therefore not be used. Yet, Watt et al. found that the enrichment of aminoacyl-tRNA, which is the true site of protein incorporation, was not only very close to free intracellular enrichment, but also to the plasma enrichment in the umbilical arteries [16]. We therefore took the previously measured free amino acid enrichments in umbilical arterial plasma as precursors [15].

CALCULATIONS

In each subject, the separate leucine, phenylalanine, and valine product/precursor enrichment ratios were plotted in a graph against the moment the corresponding infusion was started (Figure 1) [15]. Using computer software, the slope and the correlation coefficient of the linear trend line were calculated. The FSR was then derived using the following equation:

$$FSR\ (\%/d) = \text{slope of trend line} \times -1 \times 24h \times 100\%$$

The absolute synthesis rate (ASR) represents the absolute amount of placental protein that is produced per unit of time and can be calculated with the following equation:

$$ASR\ (g/d \text{ per } 100\ g \text{ placenta}) = FSR \times C_{protein} \times weight^{-1}$$

where $C_{protein}$ is the placental protein content in g protein/placenta, and weight is the

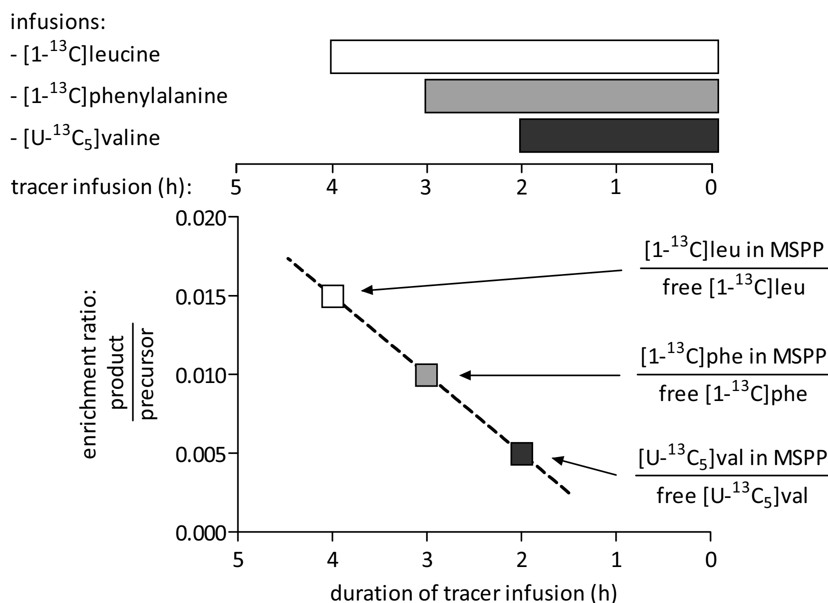

FIGURE 1: Study design. Pregnant women received three different stable isotopically labeled amino acid infusions starting at different times prior to cesarean section. At time=0 (i.e. birth), placental tissue was collected and the enrichments of the three infused amino acids that were incorporated in mixed structural placental protein (MSPP) was measured. The product/precursor enrichment ratio of each of the three infused amino acids was then plotted in a graph against the moment the corresponding isotope infusion was started. As labeled leucine had the longest infusion time, its enrichment ratio will be highest. The slope of the trend line determines the fractional synthesis rate of MSPP.

trimmed placental weight in g. The latter was calculated as follows [17,18]:

trimmed placental weight = gross placental weight × 0.86.

The half life time was calculated as follows:

$t_{½}$ (d) = 100 × ln 2 × FSR $^{-1}$

Finally, the results of all three placental tissue samples per placenta were averaged.

STATISTICS

Calculations were made with Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA) and statistical tests were done in GraphPad Prism software (version 5.0; San Diego, CA, USA). Because of our small groups, normality distribution of data could not be determined or assumed. Therefore, non-parametric data analysis was performed. Consequently, values are expressed as median (25th – 75th percentile) and Mann -Whitney tests were used to detect statistical differences. Significance level was set at $p<0.05$.

RESULTS

We included eleven pregnant women, of whom eight delivered at term, one at 31 weeks gestation, one delivered a triplet at 35 weeks (two identical, one non-identical), and one delivered a quadruplet at 28 weeks (all non-identical). The placenta of the monozygotic twin was regarded as two separate placentas as the insertions of the umbilical cord were far apart and surrounding placental tissue for each infant might have different metabolic rates. We thus studied 16 placentas, classified into two groups: premature (<37 weeks gestation) and term. Table 1 shows the clinical characteristics of the studied subjects. Term infants were born in good health and premature infants were, considering their prematurity, in good clinical condition. Placental characteristics in terms of dry weight and protein content are displayed in Table 2. Water and protein content of placentas did not differ between the premature and term group.

TABLE I: Clinical characteristics of included women and their infants divided in a prematurely-born group (< 37 weeks gestation; n=3 mothers, n=8 neonates) and a term-born group (n=8 mothers, n=8 neonates). Data are displayed as median (25th – 75th percentile), except if indicated otherwise.

	Premature group (n=3/8)	**Term group (n=8/8)**
Maternal age (y)	35.0 (29.5 – 35.0)	33.0 (28.8 – 38.0)
Preconceptional BMI (kg/m^2)	23.9 (21.9 – 26.6)	21.9 (20.3 – 24.5)
Actual BMI (kg/m^2)	29.4 (27.9 – 33.6)	29.7 (23.6 – 31.4)
Parity (0:1:2:3) (n)	(2:0:0:1)	(4:1:2:1)
Gestational age (wks) ***	29.9 (28.4 – 35.4)	38.5 (37.6 – 38.9)
Birth weight (wks) ***	1.3 (1.2 – 1.9)	3.3 (2.7 – 3.4)
Birth weight Z-score (SD) ref [42]	-0.19 (-0.70 – 0.22)	-0.11 (-0.86 – 0.52)
Gross placental weight (g) **	396 (362 – 467)	590 (558 – 649)
Sex (m:f) (n)	3:5	4:4
Apgar score (0-10) *	9 (9 – 10)	10 (10 – 10)

* Significantly different (Mann-Whitney), p<0.05

** Significantly different (Mann-Whitney), p<0.01

*** Significantly different (Mann-Whitney), p<0.001

TABLE II: Morphometric characteristics of studied placentas in the premature group (n=8) and the term group (n=8). Data are displayed as median (25th – 75th percentile).

	Premature group (n=8)	**Term group (n=8)**
Estimated trimmed placental weight (g) **	341 (311 – 402)	507 (479 – 558)
Dry matter content (%)	12.3 (12.1 – 12.6)	11.8 (10.6 – 12.4)
Protein content (%)	6.9 (6.1 – 7.1)	6.6 (5.4 – 6.8)
Protein content of dry weight (%)	54.7 (50.8 – 57.4)	54.7 (50.5 – 57.3)

** Significantly different (Mann-Whitney), p<0.01.

Table 3 shows the enrichments of the three infused labeled amino acids both incorporated in placental protein and as free amino acids in umbilical arterial plasma. The median linear regression coefficients (r^2) of the trend lines through the three product-precursor enrichment ratios were 0.97 (0.90 – 0.99) and 0.94 (0.91 – 0.96) in the premature and mature groups, respectively.

The mixed protein FSRs and ASRs of placental tissue in placentas of the premature and term groups are displayed in Figure 2 and Figure 3, respectively. The half life times of placental protein were significantly shorter (p=0.028) in the premature group than in the term group (2.8 (2.7 – 3.2) days and 3.9 (3.1 – 4.5) days, respectively). Both the FSR and ASR decrease significantly during the third trimester of human pregnancy as is shown by a linear regression plot in Figure 4.

TABLE III: Enrichments of the infused amino acids incorporated (inc.) into mixed structural proteins of placentas (product enrichments) and enrichments free in umbilical arterial plasma (precursor enrichments) in the premature group (n=8) and term group (n=8). Enrichments are expressed in mole percent excess (MPE) and displayed as median (25th – 75th percentile).

	Premature group (n=8)	Term group (n=8)
[1-^{13}C]leucine (inc.)	0.163 (0.142 – 0.187)	0.174 (0.160 – 0.209)
[1-^{13}C]phenylalanine (inc.)	0.210 (0.188 – 0.239)	0.227 (0.199 – 0.278)
[U-$^{13}C_5$]valine (inc.)	0.042 (0.036 – 0.050)	0.060 (0.045 – 0.073)
[1-^{13}C]leucine (free)	5.17 (4.72 – 5.82)	5.93 (5.31 – 6.67)
[1-^{13}C]phenylalanine (free)	8.18 (8.05 – 8.58)	9.45 (8.63 – 10.76)
[U-$^{13}C_5$]valine (free)	3.61 (3.15 – 4.15)	4.66 (4.35 – 4.90)

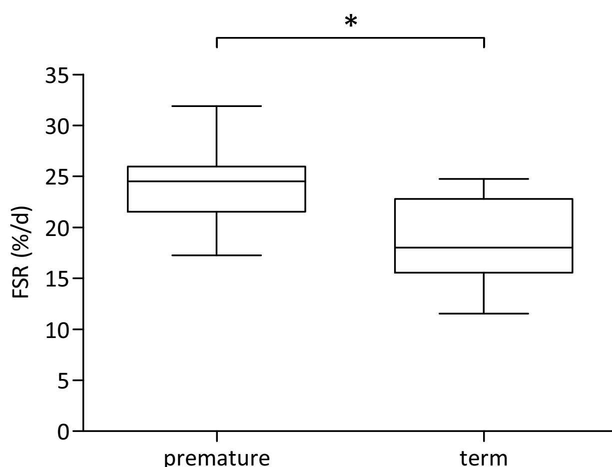

FIGURE 2: Fractional synthesis rates (FSR) of mixed structural placental proteins in placentas from infants born prematurely (n=8) and at term (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. * Significantly different (Mann-Whitney), p<0.05.

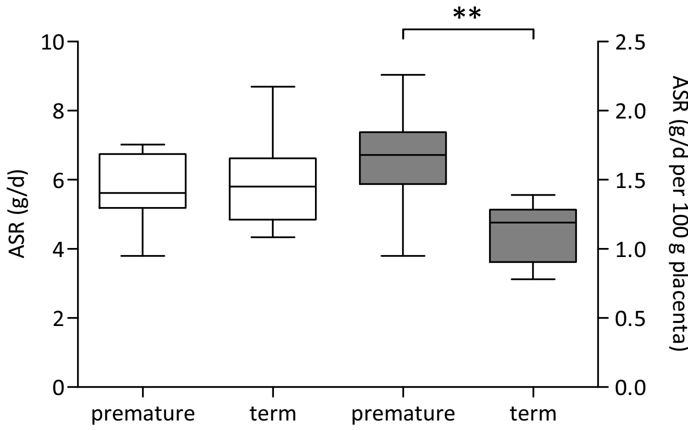

FIGURE 3: Absolute synthesis rates (ASR) of mixed structural placental proteins in placentas from infants born prematurely (n=8) and at term (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. Open boxes depict the ASR expressed per trimmed placenta (left ordinate), cross-hatched boxes depict the ASR expressed per 100 g trimmed placenta (right ordinate).

** Significantly different (Mann-Whitney), $p<0.01$.

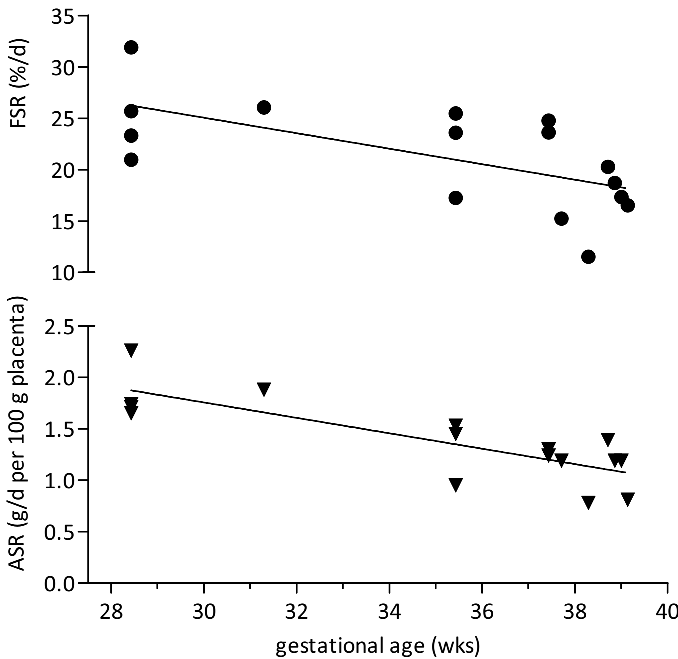

FIGURE 4: Linear regression plot of the gestational age at which placentas were delivered versus both the fractional synthesis rate (FSR; triangles) and the absolute synthesis rate (ASR; circles). Both synthesis rates are inversely correlated with gestation (r^2=0.40, p=0.008; r^2=0.66, p<0.001, respectively).

DISCUSSION

In this study, we measured the mixed structural protein synthesis rates of human placentas in pregnancies with delivery at term or at premature gestation. In mothers of the latter group, however, elective cesarean sections are rarely performed as these are usually in the acute setting because of sudden (worsening of) fetal or maternal distress. Thus, there is usually no time for obtaining informed consent followed by a four-hours-lasting infusion protocol for research purposes. Still, we were able to include three women who underwent elective cesarean section before term, and whose infants were assumed to be in relatively good condition. One woman had to give early birth because of cervical carcinoma, the two other women because of anticipated complications due to triplet and quadruplet pregnancies. Whether the results in the group of premature fetuses were influenced by the effects of multiple pregnancy itself or by genetic similarities remain unknown. However, the placenta is entirely of fetal origin and since most of the multiplet-fetuses were non-identical, it seems unlikely that a partially common genetic background was solely responsible for the observed differences.

In the past, the FSR of placental protein has been measured in sheep, but probably due to different techniques, a wide variety of results was found. FSRs ranged from 13 to 23 %/d when measured in vitro [19,20], or 60%/d under in vivo circumstances [21,22]. Maternal insulin infusion even led to a doubling of the in vivo placental protein FSR which could be an explanation for the large placentas often observed in diabetic pregnancies [22]. Interestingly, the placental FSR remained unchanged during maternal starvation, whereas the FSR of most fetal tissues significantly decreased [21]. This stresses again the importance of normal placental function, even at the expense of fetal metabolism. In guinea pigs, 12-16% of amino acids taken up from the uterine circulation were used for incorporation into placental proteins [23]. In humans, the protein FSR of in vitro perfused term placenta was found to be 12 %/d [24]. Using an improved perfusion medium, the same group later reported a FSR of 40 %/d [25]. To our knowledge, the only in vivo measured placental protein FSR in humans was 18 %/d at term [16], very similar to our values.

Gestational changes in FSR have only been studied in animals so far. Although a drop in placental protein FSR was observed during the first third of pregnancy in sheep, it remained constant in the second third of gestation [19]. In rat placental proteins, the FSR was found to decrease gradually during the last third of pregnancy from approximately 65 to 23 %/d [26,27], but in another experiment the FSR increased [28]. Whereas the maximum weight of the ovine placenta is reached halfway during pregnancy after which it even decreases [19], the human placenta is believed to grow continuously, even after 40 weeks of gestation [18,29]. Dry weight of term human placentas has been found to be 15 to 19% of total weight [29-33]. The fact that our observed values (~12%) are somewhat lower is probably due to the fact we washed the placentas to remove most blood. The placental protein content described in literature varies more, ranging from 6 to 15% [30,33-36]. Protein

concentrations in combination with cellular DNA and RNA content have been used to describe placental growth. As reviewed, most studies show that when approaching term, cell division ceases and cell hypertrophy is mainly responsible for further placental weight increments [37]. This seems to be in accordance with our observations.

Despite the fact that we found decreased placental protein synthesis rates at term when compared to early in the third trimester, Vatnick et al. demonstrated in sheep that the oxygen consumption per gram placental protein remains constant throughout gestation [38], which would suggest unaltered metabolic rates. Maintenance of the high placental oxygen and glucose consumption, which far exceeds those of the fetus on weight base, are probably necessary to fulfill the need to transport more nutrients according to increasing demands by the growing fetus. Throughout gestation, most relative placental growth precedes fetal growth [18]. To compensate for the decreasing placento-fetal weight ratio, the placenta probably undergoes continuous tissue remodeling, which includes increased surface exchange area, increased transporter density, and decreased barrier thickness. Since ammonia is a byproduct of protein turnover, large amounts of uterine ammonia excretion have been observed in sheep [39]. During midgestation, 44% of nitrogen uptake was released as ammonia back into the maternal or fetal circulation [40]. During late gestation the excreted amount of ammonia by the uterus is, however, reduced, which would seem to indicate less amino acid oxidation possibly due to less protein remodeling [40]. Of interest is that in humans the term placenta probably does not release ammonia into the fetal circulation, but instead extracts it from the fetal side [41].

A drawback of the here presented study is that the placentas in the premature group are mostly from multiplets; this issue has already been described above. Additionally, the well-known problem of choosing the correct precursor pool must be discussed. Most likely due to placental protein breakdown very soon after removal from the uterus, we did not successfully measure the true placental intracellular free enrichments. However, considering that the bound pool size (protein) is magnitudes larger than the free intracellular pool, an enrichment that is just one-fourth of maternal enrichment indicates that the proteolytic rate was not that large. Nevertheless, our used surrogate precursors (umbilical arterial plasma amino acid enrichments) were previously shown to be close to the intracellular aminoacyl tRNA enrichments [16]. Should we have chosen a faulty precursor, the results in the premature and term group would probably only be affected absolutely, whereas the relative differences would have been likely to persist.

So far, all in vivo and most of the in vitro measurements of placental FSRs were performed on placentas sampled at a single time point. Although we sampled at a single time point as well, our multiple staggered infusion protocol enables calculations on three separate enrichment ratios which theoretically should benefit the accuracy of the experiments. The great advantage of our employed model was previously demonstrated in measuring the fetal albumin synthesis rates. The model showed great accuracy as reflected by correlation coefficients (r^2) amounting approximately 0.99 [15]. Unfortunately,

the coefficients in this study are slightly lower, approximately 0.96. This is probably due to the fact we could not directly sample the precursor pool.

To conclude, we showed that placental activity in terms of protein turnover slightly decreases towards term. However, it remains speculative whether this is a result of diminished placental growth, ontogenetic changes in metabolic rate, or even placental exhaustion. Besides, since placentas of small-for-gestational-age infants are relatively smaller than those of appropriate-for-gestational-age counterparts [18], further research is necessary to explore potential differences in placentas of growth-restricted fetuses or affected by other diseases.

ACKNOWLEDGEMENTS

Most of all, we would like to thank all participating women. Furthermore, Frans te Braake, Willemijn Corpeleijn, Gardi Voortman, Janneke Bouma, and all staff from the obstetrical, and anesthesiological departments were a great helping hand in collecting all material and providing the facilities.

Financial support was kindly provided by the Sophia Children's Hospital Fund (SSWO) and the Nutricia Research Foundation, but they had no influence in study design, results, publication, or whatsoever.

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PART IV

CHAPTER

10

General discussion

Partly based on:

CHP van den Akker, FWJ te Braake, and JB van Goudoever
Nutrition in the neonatal intensive care unit
Hospital Pharmacy Europe 2005;19:49-51

&

FWJ te Braake, CHP van den Akker, MA Riedijk, and JB van Goudoever
Parenteral amino acid and energy administration to premature infants in early life
Seminars in Fetal and Neonatal Medicine 2007;12:11-18

INTRODUCTION

As stated in introductory **Chapter 1**, the problem of premature birth is significant as it not only affects the individual's health, but also his or her direct environment and society in several ways, both emotionally and economically. Whereas it not only has appeared to be extremely difficult to counteract the causes of premature birth, improved fetal monitoring has even led to a trend towards earlier delivery [1,2]. Although research has helped to improve the outcome of premature birth drastically during the last several decades, still too many infants end up with some form of disability.

Normally, a human fetus has nine months to develop from a fertilized oocyte to a healthy individual whose gastrointestinal and respiratory systems should be ready to extract and metabolize the right substances for normal further growth and development. After premature birth, the neonatologist is responsible to deliver the right metabolites, but is faced with underdeveloped digestive organs and lungs. Besides, a premature infant cannot be regarded just as an extrauterine fetus, as a newborn also has to cope with its own thermoregulation and fluid balance, increased muscle activity due to breathing and gravity, and an environment full of microorganisms. These processes and which all demand extra energy and oxygen. Yet, the most prominent function of intrauterine life, and thus also after premature birth, is to grow and develop normally for which the right amounts of nutrients and oxygen are necessary.

Because of the often unanticipated situation of premature delivery, the focus in care in the immediate postnatal phase is on immediate life threatening situations. For this reason, much research have been done and major advances have been made in the field of neonatal respiratory medicine, although still many infants end up having respiratory problems [3]. Nutrition has been studied much less than several other areas probably because not considered immediately life-threatening which is indeed true. However, considered from a more developmental perspective, optimal nutrition is a key element in accomplishing undisturbed lung, bowel, immune defense, and brain maturation. To minimize the transition from intrauterine tot extrauterine life, the right nutrition in the very early phase after premature birth could prove essential and might even set the clinical course on the NICU. One can easily imagine that being in a catabolic state in the first phase of life is not a good start.

EARLY AMINO ACID ADMINISTRATION

The sudden change from a usually well-fed intrauterine state to the extrauterine environment makes the sick premature very vulnerable and therefore in urgent need of optimal nutrition. Both growth and disease elicit very high protein turnover rates, necessary for continuous remodeling and net accretion. Although Van Goudoever et al. and Murdock

et al. were the first in the mid 1990s to show that low birth weight infants tolerated low dose amino acids from birth onwards with beneficial effects on growth, it did not result in clinical implementation in the NICUs for unknown reasons. In 2003, when we also started with our trial as described in **Chapter 2** [4], reports started to appear again that 1-kg weighing infants benefit from high dose amino administration soon after birth. Results of these and our trials are summarized in Table I (4-6).

These three studies indicate that a catabolic state during the very first stage of postnatal life can be prevented by infusing high dose amino acids. Additional benefits of early amino acid administration in these studies included increased plasma amino acid and decreased blood glucose concentrations that both better fit reference ranges [4,5]. A potential negative side effect in the study by Ibrahim et al. was an increased mean peak serum indirect bilirubin concentration (104 versus 132 μmol/L); this was without any clinical implications [6]. We found besides a lower base excess on the second day also slightly lower bicarbonate levels 12 hours after birth and on day two in the supplemented group. These findings did not have clinical implications because they did not require increased exogenous bicarbonate administration [4].

Although some groups did not find higher urea concentration in the high dose amino acid supplemented groups [5-7] and Ridout et al. did not find a correlation between urea concentrations and protein intake in premature infants [8], we found higher urea concentrations in the supplemented group (9.6 ± 2.8 mmol/L) [4]. In addition, Blanco et al. infused extremely premature infants (25.7 ± 2.0 wks gestation) with high dose amino acids soon after birth (up to 4 g/(kg·d) on day 3 of life) [9]. Whereas the mean peak urea concentration was already very high (19.6 mmol/L), it even ranged up to 36 mmol/L in the most immature infants (≤ 24 wks). Ammonia concentrations were elevated as well in these

TABLE I: Summary of recently published studies on the effects of supplemented amino acids shortly after birth on nitrogen balance [4-6].

Study	Number of infants	Birth weight (g) mean ± SD	Start of protein administration	Protein intake (g/(kg·d))	Study age (day)	N balance (mg/(kg·d)) mean ± SD
Thureen et al. (2003)	13	945 ± 187	24 h	0.85	2	−42 ± 63
	15	947 ± 232	24 h	2.65	2	186 ± 93
Ibrahim et al. (2004)	14	968 ± 244	-	0	1	−203 ± 78
	15	846 ± 261	<2 h	3.5	1	384 ± 78
Te Braake et al. (2005)	69	989 ± 252	36 h	0.4	2	−84 ± 70
	66	1039 ± 235	<2 h	2.4	2	145 ± 104

infants (~100 µmol/L), where normal values during early life in premature infants in the absence of parenteral nutrition are 70 ± 25 µmol/L [10]. The fact that very high levels of ammonia are neurotoxic is well established [11]. However, with normal liver functioning, ammonia should readily be transformed into urea, even in premature infants. On the other hand, whether uremia is problematic remains unsolved. Although to my knowledge urea toxicity has not been tested in infants, it has been studied in animals and men. In unilaterally nephrectomized dogs for example, high dose continuous intermittent urea injection (10 g/(kg·d)) for 45 consecutive days resulted in plasma concentrations ranging between 200 and 250 mmol/L [12]. Except for a mild drowsiness and increased diuresis, urea did not induce any severe toxic symptoms. Nor was bleeding time altered. In humans suffering from renal failure, however, deliberate loading with high dose urea for two to three months resulted in malaise, vomiting, weakness, lethargy, and bleeding if plasma concentrations started to rise above 50 mmol/L [13]. Another short-term (24h) experiment in healthy humans observed drastic reduction of platelet aggregation if serum urea concentrations were between 20 and 40 mmol/L already [14]. Whereas diuresis is closely monitored on a NICU, adverse blood coagulation is problematic in these infants who are for example very vulnerable to intraventricular hemorrhages. Furthermore, it is not known whether the very high urea concentrations directly affect the growing brains of neonates.

Lastly, when comparing urea concentrations between various studies, one should be careful as various measurements are performed, yet these are not always converted correctly. In Europe concentrations are usually measured in plasma and expressed as mmol/L. However, in the USA, the nitrogen content of urea is measured and accordingly expressed as mg N/dL. Converting to a concentration in mmol/L should thus be done with a conversion factor of 28 mg/mmol (equaling 2 times the molar mass of nitrogen), rather than taking the complete molar mass of urea, i.e. 60 mg/mmol. Besides, while in the past, urea was measured in whole blood, it is currently measured in plasma only. Yet, the terms blood urea nitrogen and plasma (or serum) urea nitrogen are used arbitrarily and thus often wrongly. Potentially, this gives some misinterpretation as the blood urea nitrogen concentration is on average 12% lower than the plasma concentration.

To conclude, the most prominent benefit of the early amino acid infusion studies is that an anabolic situation can be achieved soon after birth upon amino administration. A breakeven point is observed when approximately 1 g AA/(kg·d) are administered so that the nitrogen balance is neutral. This can be concluded if available data [4-7,15-18] are summarized in a graph (Figure 1). A major additional benefit was that most of the amino acid concentrations better resemble those of the healthy fetus or the healthy breast-fed term newborn. Furthermore, we could not detect any major disadvantages of amino acid administration starting the immediate postnatal phase. Yet, one should be cautious if the urea concentrations get too high as we do not know when these start to elicit disadvantageous side effects.

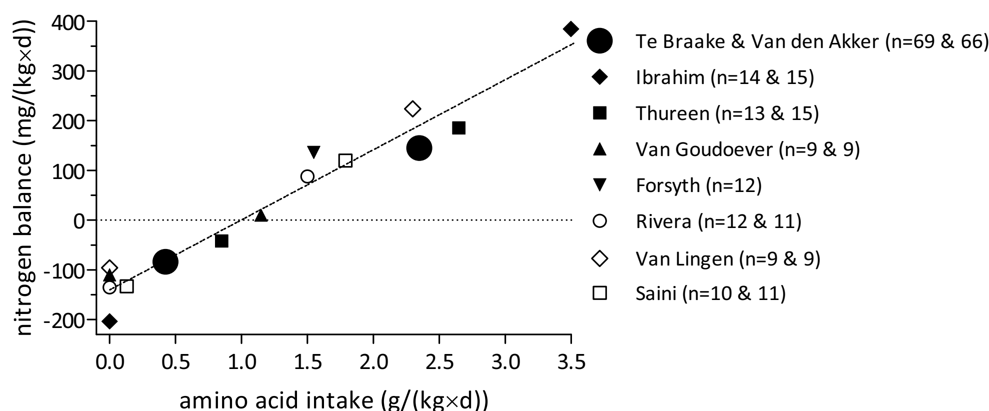

FIGURE 1: Summarizing graph of studies investigating the effects of different levels of parenteral amino acid administration starting during any of the first two postnatal days in premature neonates on the nitrogen balance [4-7,15-18]. Primary authors including the number of studied infants in the low and high dose amino acid groups are indicated by symbols.

ENERGY AND QUALITY OF PROTEINS

The ideal nutritional strategy provides for amounts of amino acids and energy that satisfy the needs for both growth and metabolism. There is ongoing controversy whether amino acids should be regarded as metabolic fuel or not. Their main function lies in protein synthesis and thus tissue growth, but it remains unsolved in neonatal medicine to what extent it is physiological for amino acids to be used as an energy source. Reasons for amino acids to be oxidized not only include energy generation in general but also are to avoid accumulation of amino acids in relative excess to other scarce amino acids so that ongoing protein synthesis is hampered.

As already discussed in this thesis, it is questionable whether large urea formation should be regarded as a sign of amino acid intolerance. More likely, it is the result of uncomplicated amino acid oxidation and subsequent ammonia disposal by the liver. Because urea concentrations are a rather crude measurement of amino acid oxidation, we aimed to measure it directly by using a stable isotope of the essential amino acid leucine **(Chapter 3)** [19]. Besides, we could determine whether the improvements in nitrogen balance were due to decreased proteolysis rates, increased protein synthesis, or a combination. It turned out that upon amino acid administration whole body protein breakdown remained unaltered, and synthesis was increased to an average of 182 μmol leucine/(kg·h). Under assumption that 1 gram of fetal protein at 28 weeks gestation contains on average 560 μmol leucine [20], this translates to 7.8 g of protein that are daily synthesized per kg body weight. These high rates of protein synthesis have also been observed by using other tracers. Yet if the body composition of a premature infant contains

on average 8.8 % protein [21], the fractional protein synthesis rate of a premature infant amounts 8.9 %/d.

However, only half of the administered amino acids was used for extra protein synthesis, whereas the other half was oxidized. Although we did not measure ammonia concentrations, the high leucine oxidation rate and urea concentrations suggest that amino oxidation occurs uncomplicated in premature infants. However, efficacy in terms of anabolic usage was thus rather low, which was in fact the primary purpose of the amino acid administration. The question therefore remains why not more amino acids were incorporated into proteins.

On the other hand, in the ovine fetus, the umbilical supply of amino acids also highly exceeds the amount deposited for tissue growth. Oxidation contributes 25–50% of fetal amino acid uptake [22,23]. To what extent this also holds true for the human fetus is largely unknown, although significant intrauterine urea production has been demonstrated [24]. Thus, although we should not consider postnatal amino acid oxidation or slightly elevated urea concentrations as an unphysiological situation, a more anabolic usage would be more desirable and we should thus investigate if we can increase the proportion of anabolic usage.

A first potential mechanism would be to improve the quality of the used amino acid solution. Currently commercially available solutions are not specifically designed for premature infants. Besides, tyrosine is in fact the only amino acid for which the requirements in parenterally fed premature infants are known [25]. It turned out that 2.4 g AA/(kg·d) with present-day solutions provide only 15% of total tyrosine demands, due to poor aqueous solubility. Therefore, the remaining sufficiency needs to be fulfilled through endogenous synthesis through hydroxylation of phenylalanine. Unfortunately, the latter metabolic conversion might be hampered due to metabolic immaturity. Requirements for all other individual amino acids in parenterally fed infants are unknown. Cysteine, one of the other amino acids, is also suspected to be too low in concentration in some parenteral solutions. In 2-week-old term-born post-surgical infants who were parenterally fed, total sulfur amino acid requirements (methionine + cysteine) were approximately 50 mg/(kg·d) [26]. Although these requirements are easily met through the infusion of 2.4 g amino acids/(kg·d) of the solution we use in Rotterdam (i.e. Primene 10%, Baxter), the needs of a premature infant, especially in the direct postnatal phase, might be completely different from those in term infants. Whereas in the trial by Blanco and colleagues [9] the amount of administered cysteine-HCl was 40 mg/(kg·d) and thus reasonable in total, it is only 10 mg cysteine-HCl per gram of amino acids. Assuming fetal porcine protein to be the same as human [27], 10 mg cysteine-HCl provides only half of the cysteine required for one gram of protein (cysteine content in human fetal protein has never been determined). Besides, especially during the very first postnatal phase while adapting to extrauterine life, cysteine requirements could be higher for example to increase glutathione synthesis to cope with increased postnatal oxygen exposure resulting in higher amounts of free radicals.

Any absent cysteine thus needs to be endogenously synthesized through conversion from methionine. Whereas Riedijk et al. showed cysteine should not be considered a (semi-) essential amino acid, their studies were performed in premature infants who were enterally fed [28,29]. This should thus not necessarily lead to the same conclusions in parenterally fed premature infants during early life. Although data obtained in the latter category of infants came out very recently, I doubt their conclusions. Thomas et al. observed that besides a cysteine-HCl supplementation of 124 mg/(kg·d), cysteine was produced from methionine with a rate of 25 µmol/(kg·h) [30]. Combined, this yields 168 mg of cysteine/(kg·d), which appears too high. Besides, methionine intake in these infants equaled the transsulfuration rate so that no methionine is left for net protein synthesis or growth. At an intake of 3 g amino acids/(kg·d), this does not seem plausible.

To conclude, we still must be careful to administer enough cysteine to parenterally fed infants as long as it has not been unequivocally demonstrated that these infants are able to synthesize cysteine endogenously. Insufficient amounts of cysteine, tyrosine, or any of the other (semi-)essential amino acids in parenteral solutions could thus be causative of increased oxidation of all other amino acids which will then result in the elevated urea concentrations.

Studies have demonstrated that amino acids administration together with as little as 30 non-protein kcal/(kg·d) can turn the nitrogen balance from negative to zero or even positive [15,31]. The energy to protein ratio in our study was however lower than in many other studies (~15 kcal of non-protein energy per gram protein). As this is not an ideal proportion of energy for optimal protein synthesis, a considerable amount of the amino acids will be oxidized. An intake of 25-40 kcal of non-protein energy per gram of protein will enhance optimal protein deposition [32], although this is not feasible with glucose alone in early preterm life at larger protein intakes. However, the effect of increasing energy intakes on protein deposition will be greatest below 50-60 kcal/(kg·d), above which the beneficial effect of extra energy ceases and the amount of administered amino acids itself will have a higher correlation with anabolism [33]. Early lipid administration, with its high caloric content of 9 kcal/g, might be beneficial in delivering calories for the cost of protein synthesis. Furthermore, the infant is dependent on essential fatty acids (mainly DHA (docosahexaenoic acid)) for its brain maturation. At the beginning of the third trimester the fetus receives only a small amount of lipid, which raises the question whether the very preterm infant is suited to metabolize lipids in large amounts. Supposed metabolic intolerance and a relationship with chronic lung disease have led to their delayed introduction, often beyond the first 24 h postnatally, although just as many positive and protective effects of lipids on lung development have been observed [34]. A recent meta-analysis on the effects of early as compared to late lipid administration could not detect any positive or negative effects of an early start [35]. However, for example in one of the included studies questioning the safety of early lipid administration, only lipids and glucose were infused during first few days, whereas amino acids were withheld [36]. Also, in the

other studies the amount of infused amino acids was either not mentioned [37] or lower than the amount of administered lipids [38]. Possibly, concomitant amino acid infusion is necessary to properly metabolize and dispose of the infused lipids. For example, albumin plays an important role in the transport of fatty acids to and from the various tissues and organs [39]. As we have shown, amino acids stimulate the synthesis of albumin and could thereby also improve tolerance to infused lipids [40].

If in combination with amino acid infusion premature infants indeed tolerate intravenous lipids, the latter delivers not only essential fatty acids, but also the urgent calories that can support protein synthesis even more. This prevents that amino acids have to be oxidized because of a lack of energy and the infant is not faced with a high urea production. Indeed, in the study by Ibrahim et al., infants were infused not only with high dose amino acids (3.5 g/(kg·d)), but also with 3.0 g lipids/(kg·d) within 2 hours after birth [6]. No elevated urea concentrations were observed and a very high proportion of nitrogen was retained within the body (Figure 1). On the other hand, Forsyth and colleagues did not observe any improvement in nitrogen balance upon the administration of an extra 15 kcal/(kg·d) in the form of glucose [16]. A cause for the lack in improvement could be that only 1.5 g amino acids/(kg·d) were administered in combination with an already reasonable caloric intake of 58 kcal/(kg·d) in the control group. Nevertheless, the many theoretical benefits of early lipid administration to premature infants warrant new large trials [41].

ALBUMIN

The first two studies we just described, measured metabolism at a 'whole body' level. In other words, it is the average of all metabolic processes in all organs. For example, changes in one or more of the splanchnic organs could be counterbalanced by opposite changes in muscles, so that on a whole body level no difference is seen. However, ethical constraints of course prevent direct tissue biopsies in the human neonate to study single organ kinetics and therefore we are limited to the plasma compartment which can be sampled more easily. Albumin is one of the few plasma proteins that is sufficiently high in concentration to permit research by obtaining small blood volumes. Measuring the albumin synthesis rate gives good indication of general liver activity and is more responsible to nutrition than simple concentrations are. Whereas albumin forms over half of the total plasma protein content, albumin plasma concentrations are an insensitive marker of nutritional status. Only 40% of the total albumin mass resides intravascular. However, during reduced albumin synthesis, which elicits a lowering effect in plasma albumin concentration, albumin from the interstitium increases its lymphatic return into the intravascular compartment, so that a measurable drop in albumin concentration will not be observed initially [39].

On the other hand, during inflammatory events, the transcapillary albumin escape rate might be well increased so that albumin concentrations decrease despite an increase in the

albumin production rate by the liver [42]. Correlations between albumin concentrations in premature neonates and mortality [43] or necrotizing enterocolitis [44] thus do not necessarily result from decreased liver activity. Studying the latter by means of the albumin synthesis rate provides more detailed information on the specific organ effects of supplemented nutrition. Amino acid administration did not only elicit positive effects on nitrogen and leucine as aforementioned, but also stimulated the albumin synthesis rate on the second postnatal day **(Chapter 4)** [40]. In fact, we also found that there was no preferential use of leucine for either albumin synthesis or any of the other anabolic processes in the body. In both the unsupplemented and supplemented groups, approximately 4% of the whole body protein synthesis rate was dedicated to albumin synthesis.

Unfortunately, the albumin concentrations remained low. This triggered our question whether the relative hypoalbuminemia was the result of the fact a maximum in synthesis rate was reached upon the amino acid administration already or that hypoalbuminemia was the result of a high transcapillary escape rate so that all synthesized albumin left the intravascular compartment. Therefore, despite a physiological situation is per definition not applicable to a premature infant, we aimed to measure the albumin synthesis rate under optimal circumstances. Intrauterine counterparts of similar gestational ages thereby provide good comparison. Although the intrauterine circumstances are entirely different from those on a neonatal intensive care unit, they give well insight into the metabolic capabilities of a young individual during undisturbed gestation.

Using a relatively new multiple tracer infusion protocol (modified from Dudley et al. [45]), we were able to measure the albumin synthesis rates in fetuses from different gestational ages in a single sample taken from the umbilical cord immediately after birth **(Chapter 6)** [46]. In Figure 2, these synthesis rates are depicted together with those from premature neonates as described in chapter 4. Although there was a wide range, especially premature fetuses showed surprisingly high albumin synthesis rates. As described in the concerning chapter, from a physiological developmental point of view we cannot figure out why premature fetuses have such high albumin synthesis rates. Many of the functions of albumin, such as fatty acid and bilirubin transport or anti-oxidant defense, pertain more to the postnatal than the prenatal phase. Nevertheless, the most important finding is that the liver of a premature fetus is capable of synthesizing albumin at very high rates. Thus, premature infants should theoretically also be able to achieve these high rates as they also did while still in utero. It seems however, that the postnatal values are lower than prenatally, and that it thus should be possible to raise the synthesis rates. Since albumin synthesis rate already have shown to be responsive to parenteral nutrition, it seems logic that more nutrition would indeed simulate the albumin synthesis rates even more.

As will also be described in the forthcoming section, were able to measure the protein synthesis rate in the feto-maternal dyads at term using a phenylalanine isotope. This enables us to calculate the percentage of protein synthesis that is spent on albumin, just as

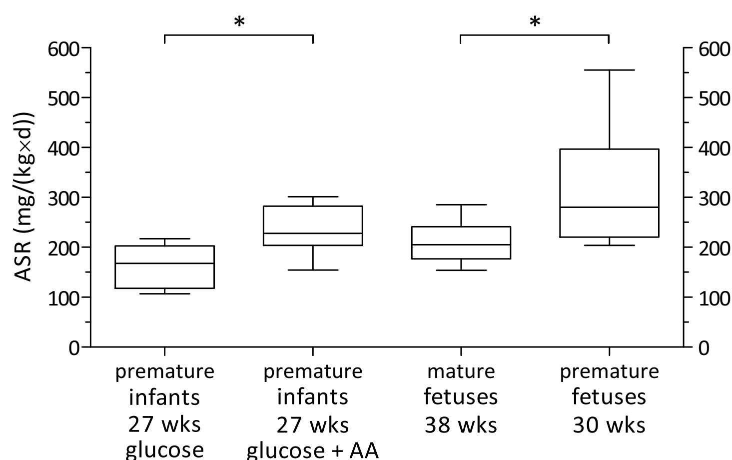

FIGURE 2: Albumin absolute synthesis rates (ASR) in premature infants with or without amino acid (AA) administration and in fetuses of different gestational ages [40,46]. * p<0.05 (Mann Whitney). Differences between neonates and fetuses were not statistically tested because of different methodology and different model assumptions.

we did in premature infants (chapter 4). Since albumin contains 6.87% phenylalanine on a weight basis, combination of the results in chapter 6 and 7 yields that 3.4 (3.1 – 4.4) and 5.2 (4.4 – 5.9) % of total protein synthesis is albumin synthesis in term fetuses and their mothers, respectively. These values are close to the values we found in premature infants (~4%). These figures are not only interesting from a metabolic point of view, but also give extra credence to our methods used in the fetal study as they are entirely different from those used in the neonate, yet give similar values.

FETAL AMINO ACID METABOLISM

Quantitative data on human in vivo fetal amino acid metabolism are very scarce [47], although Battaglia and colleagues provided a lot of qualitative data. By interpreting enrichment ratios, ideas were formed on intrauterine growth restriction and placental transport characteristics [48-51]. The large advantage of most of their study designs was that they did not require steady state assumptions and blood was often sampled in a relatively unstressed situation by cordocentesis from the umbilical vein. Quantitative balance studies such as those performed by Chien et al. [47] and us in **Chapter 7** and **Chapter 8**, however, require blood sampling both types of umbilical blood vessels, i.e. vein and arteries. Therefore, the type of research we performed is limited to the metabolism just prior to birth. Cesarean sections are thereby probably less stressful to the infant, but most of all, if elective, they are of course scheduled. At the same time, the electivity therefore

largely constraints to the study of infants at term. Most research in the fetal phase so far has therefore probably been limited to research animals. Whereas neonatal research in large animals is often performed in pigs because of the many similarities with human neonates, fetal research in non-rodents occurs predominantly in sheep. Reasons are that, contrary to pigs, ovine pregnancy rears mostly singletons and allows fetal surgical manipulation without instigating litter death. Besides, animal models allow the study in an (almost) completely unstressed and physiologic situation by the insertion of multiple catheters into various fetal and maternal blood vessels that allow, after a few days recovery, for blood sampling and flow measurements from all sites simultaneously. Even more so, this can be done during different periods of gestation and of course during numerous experimental settings, e.g. during fasting, hormonal infusion, and after priorly induced fetal growth restriction.

Nevertheless, despite all advantages of animal research, we wished to explore human fetal metabolism due to the many interspecies differences and the feasibility of direct comparisons to postnatal research results, just like we did with the albumin synthesis rates. In chapter 7 for example, we determined the fetal whole body protein synthesis rate on the basis of phenylalanine kinetics. In fetuses around term, we calculated that protein synthesis amounted 9.0 g/(kg·d). Therefore, under the assumption that the term fetus contains on average 120 g protein per kg body weight, a fractional synthesis rate of fetal proteins at term of 7.4 %/d can be calculated. This fractional protein turnover rate seems to be slightly lower than that of the premature infant on the second day of life (8.9%/d) as calculated earlier in this discussion.

Since in the fetuses, studied at 38.5 weeks gestation, protein synthesis was higher than breakdown, they were still accreting proteins at a median rate of 1.7 g/(kg·d). On average 1 gram of fetal term protein contains 158 μmol tyrosine [20], in order that ($1.7 \times 158 \times 24^{-1}$) 11 μmol tyrosine/(kg·h) would be necessary to fulfill this demand. From their mothers, however, they only received 2.4 μmol tyrosine/(kg·h), so that the remaining requirement must met by endogenous synthesis through the hydroxylation of phenylalanine. Often, it was questioned whether the required enzymes in the liver and kidneys of a newborn (premature) infant are fully capable of doing so, making tyrosine a conditionally essential amino acid [52-54]. Yet, we found an in vivo hydroxylation rate of 7.5 μmol/(kg·h), which yields together with the umbilical uptake approximately just enough of required tyrosine to be deposited in net protein synthesis.

Due to the insolubility of tyrosine in aqueous solutions, parenteral nutrition delivers per gram protein/(kg·d), only 1 μmol tyrosine/(kg·h). A desired tissue growth rate of 15 g/(kg·d) or 2.1 g protein(kg·d), necessitates 14 μmol tyrosine/(kg·h), so that parenteral nutrition only marginally attributes to tyrosine requirements and hydroxylation capacities in the premature infant are pulled. Fortunately, these hydroxylation rates have indeed been confirmed in premature infants [55-57]. Nevertheless, we must be precarious to definitively remove tyrosine from the list of conditionally essential amino acids as we do not have

information on hydroxylation rates in growth-restricted infants and in extremely premature neonates.

From animal research, mostly in sheep, we know that the fetus receives large amounts of amino acids that are well in excess of those necessary for protein deposition or growth [22,23]. Oxidation of the amino acids in excess can, together with oxidation of glucose, lactate, and fructose, provide the calories at which rapid ovine fetal growth is financed (4 times faster than in humans). Reliable quantitative data in humans lack although there are indeed indications that amino acids are oxidized to some extent as reflected by higher ammonia, urea, and leucine-derived CO_2 concentrations in the umbilical arteries than in the vein [24,47,58]. Our data in Chapter 8 also seem to suggest considerable oxidation of the branched-chain amino acids leucine and valine since their uptakes far exceed the amounts necessary for protein accretion. The great advantage of being intrauterine, however, is of course that the placenta can filter out ammonia and urea whereas postnatally only the liver and kidneys are responsible for doing so.

PLACENTA

The placenta is the most variable between different species of all mammalian organs [59]. Observation made in one species may or may not pertain to another. As described above, we experienced this ourselves in that it was well-known from ovine research that the keto-acid of leucine (α-ketoisocaproate) is transported towards the fetus, whereas we showed transport in the opposite direction. This example and many other differences must be taken into account when extrapolating results from animal research to the human situation. Moreover, it stresses the importance of research in humans. It is striking that we know so little about an organ that is available for research so easily. Although a lot of research has gone into for example all the different types of amino acid transporters that direct substrates from both the maternal as well as fetal membranes the trophoblast inwardly, we barely know how amino acids exit the placenta into the fetal circulation [60].

In our research on the placenta **(Chapter 9)**, we focused on its protein turnover rate. As outlined in the concerning chapter, the placenta is a highly metabolically active organ in order to ensure optimal nutrient transport to the fetus. Yet, the cost for doing so is high. The oxygen and glucose consumption far outweigh that of the fetus on a weight basis. Large part is undoubtedly used for the high rates of protein synthesis we observed. In the placentas of prematurely delivered babies, approximately one-fourth of all structural proteins was renewed daily. Towards the end of gestation this slightly decreased to about 18 %/d. In fact these protein turnover rates are almost three times as high as in the premature infant as calculated earlier in this discussion. These high rates are probably necessary for the maintenance of placental integrity and physiological function.

OUTCOMES OF EARLY AMINO ACID ADMINISTRATION

Ultimately, all studies regarding prematurity, including those described in this dissertation, are aimed to ameliorate the outcome of prematurely born infants to a level that is comparable to healthy term born infants. Regarding early amino acid administration, most studies have only investigated the effects in the direct postnatal phase; only a few considered medium- or long-term outcome parameters. Outcome criteria can be based on different aspects, such as growth based on intrauterine growth charts [61] or growth charts obtained from premature infants [62-64], incidence of a specific disease, hospital stay, neurodevelopmental outcome and so on. Suboptimal nutrient intake in preterm infants has many theoretical adverse consequences. Besides impaired growth, under- or malnutrition in premature infants can lead to for example an increased vulnerability to infectious disease arising from suboptimal immune defense, free-radical-mediated damage caused by impaired glutathione production, a greater need for ventilatory support, partially due to muscle weakness, and general underdevelopment of all organs including the brain.

Most of the long-term outcome effects are multifactorial and thus difficult to detect and ethically hard to test. Yet, some studies found favorable effects of early and/or high amino acids supplementation in the neonatal period on the incidence of bronchopulmonary dysplasia (BPD) [65,66] and retinopathy of prematurity [65]; others did not [67,68]. In almost all studies, anthropometric measurements at discharge had improved [67-69]. However, whether suboptimal nutrition is solely and primarily responsible for the impaired growth or potential adverse outcome is less clear. Disease itself is also likely to affect nutritional intake, growth, and perhaps also brain function independently. For long, we know that there is a significant relation between body growth and brain development. In fact, proteins appear to be the nutritional component most critical to development of neurological functions [70]. In infants who died of severe malnutrition during the first year of life, brain analysis showed decreased cellularity [71]. But also mild protein malnutrition in the prenatal phase induced altered neuronal density in rats [72]. In a study of premature infants, however, postnatal growth pattern during the first nine months, rather than SGA status at birth, was found to be significantly associated with adverse neurodevelopmental outcome at age two, even after statistical correction of certain diseases [73]. Also, the growth velocities during a premature infant's NICU hospitalization exerted a significant, and possibly independent, effect on neurodevelopmental and anthropometric outcome [74]. Famous is the trial by Lucas at al. in which four weeks of enriched formula versus standard formula supplementation to premature infants led to improved outcome 18 months post term [75]. Follow-up at age 7.5 years reconfirmed improved cognitive function in these same infants [76]. A recent reevaluation in a subset of the former premature infants at age 16 revealed larger volumes of the caudate nucleus measured using MRI and higher verbal IQs in the group that had received the enriched diet during a single month in early life [77]. In a recent study, premature and term neonates with significant perinatal brain damage

received during the first 12 months post term 20% extra energy and proteins as opposed to the control group [78]. After this year, not only weight and length were larger, but also occipitofrontal circumference and axonal diameters in the corticospinal tract.

Recently, a few studies appeared that questioned the safety of early amino acid supplementation. Clark et al. increased the amino acid intake in premature infants during the first 28 days with 0.6 g/(kg·d) on average in half their studied subjects [79]. No significant changes in growth rates could be observed between groups. Besides, serious concerns were expressed regarding slightly elevated urea concentrations and amino acid concentrations. It remains indeed true that we do not have appropriate reference ranges for several metabolites, including those aforementioned, simply because prematurity is not a physiological situation. Nevertheless, the study design Clark et al. used can be questioned [80] and we doubt whether the results obtained in this study validate the results the authors arrived at, although we agree that safety should always be closely monitored. Follow-up results of the trial by Blanco et al. [9] that was also earlier discussed, were recently presented at an international convention [81]. At birth and at hospital discharge, anthropometric measurements in both study groups were similar. However, after 18 months corrected age, cognitive scores as well as anthropometric measurements were worse in infants that had received early high dose amino acids after premature birth. Whether these results are directly related to the applied nutritional regimens, remains to be elucidated in larger trials.

In our trial regarding early amino acid administration, we did not encounter any negative effects in the intervention group or any measurable beneficial effect at age two **(Chapter 5)**. The difference in amino acid intake between both groups was only 4.8 g/(kg·d) in total divided over 2.5 days, and thus not very large in order to expect a long-term effect at first sight. However, the difference was realized immediately after birth, which might well be the most critical period in a premature infant's life. Therefore, considering both the many theoretical advantages of amino acid supplementation on organ development and the many short-term beneficial effects (more plasma amino acid concentrations between reference ranges, a positive nitrogen balance and improved albumin synthesis), we highly recommend the implementation of our protocol. Besides, Te Braake and colleagues recently showed beneficial increased glutathione synthesis in a very similar setting [82].

Poindexter et al. analyzed outcome of over 1000 premature infants stratified by whether they were provided more than 3.0 g amino acids/(kg·d) earlier than postnatal day six or not [67]. After day 8 of life, amino acid intakes were similar in both groups. At 36 weeks postmenstrual age, weight, length, and head circumference were larger in the high-dose amino acid group, also after adjustment for major neonatal morbidities. At 18 months corrected age, no differences could be observed anymore in term of weight and length. However, especially male infants in the low protein intake group were twice as likely to have head circumferences less than the tenth percentile. Yet, no differences in the mental and psychomotoric indexes as well as the occurrence of handicaps between both groups were

observed. Due to the retrospective nature of the study it remains questionable whether the observed anthropometric differences were entirely due to increased amino acid intake or not. Despite correction for major neonatal morbidities and several other variables, it could be that a lower amino acid intake was due to secondary variables, such as disease, so that these influenced outcome more than solely nutrition. Nevertheless, the major conclusion of the concerning study is that amino acids during early life do not influence neurodevelopment negatively.

FUTURE PERSPECTIVES

As research continues, we will learn more about energy and protein metabolism in neonates. However, we should acknowledge that most studies concern 'healthy' or stable preterm infants and that little is known about the metabolic impact of particular diseases, and how all this affects the needs of the infant [83-86]. Although some research has been done regarding the metabolic differences and capabilities between small- and appropriate-for-gestational-age infants this has not lead to a comprehensive nutritional strategy which suits their specific demands. In fact, pediatricians are in two minds. Indications exist that livers of growth-restricted infants are not able to metabolize large amounts of protein correctly [87-89], whereas on the other hand these infants are already small by definition and have limited body reserves so that anabolism should be advanced as soon as possible.

If conclusive studies about specific diseases or ailments will evolve in the future, this can fine-tune the current nutritional protocols that exist for premature infants in general. Nevertheless, present data provide strong evidence for the beneficial effect of rapid initiation of relatively high dose amino acid administration to the average premature infant. However, we should be precarious about the occurrence of very high urea concentration in the most immature group of infants (<26 wks) [9] and liver function in severely intrauterinely growth-restricted infants. Probably, improvement in the composition of the individual amino acids in the parenteral solutions (protein quality) should overcome these problems at least partially. Furthermore, hypothetically all premature infants will benefit from a lower protein/energy ratio in that higher amounts of energy are supplemented immediately after birth additionally to the amino acids. Perhaps then, we can even increase the amount of supplemented amino acids further. Lipids are an attractive candidate to provide these non-protein calories as they also prevent episodes of essential fatty acid depletion which might otherwise soon occur after birth and is detrimental to for example ongoing brain development [90]. An additional benefit of providing intravenous lipids after birth is that the binding of fatty acids to albumin protects the albumin from being broken down [39]. Completely fat-free albumin molecules are more susceptible to degradation than those bearing one or two long chain fatty acids.

Besides putting more effort in trying and testing new nutritional regimens, we must also

continue to follow the path of observational studies regarding intrauterine amino acid metabolism. If we are able to learn more on normal human fetal metabolism during different periods of gestation, then we might also better understand the capabilities and incapabilities of postnatal metabolism, growth, and development. Whereas most research regarding protein metabolism in the human fetus so far was of qualitative nature [49-51,91], we must also focus on quantitative results like we, and previously Chien et al. [47], did, despite all drawbacks. Apart from all stable isotope research, we still do not know the exact net umbilical uptakes of carbohydrates, most amino acids, and lipids in humans. With our very recently developed methods and new machinery, we are now able to very accurately measure for example all individual amino acids in umbilical cord blood so that the net uptake can be calculated. In our fetal studies, we have also shown that it is feasible to perform studies with many simultaneously administered stable isotope tracers. This strategy should also be implemented in the new trials in premature neonates. Several tracers are available to investigate whole-body protein metabolism. This should provide additional accuracy and information above the infusion of a single tracer as we did in our clinical trial. Liver function can well be studied by investigating urea metabolism, phenylalanine hydroxylation capabilities, and albumin synthesis rates. Hepatic function should be closely monitored now that higher amounts of nitrogen are administered to more immature infants. Unfortunately, studies on a whole-body level will predominate in neonates due to ethical restraints amongst which small blood volume limitations fall. Therefore, albumin and glutathione as studied more recently are very nice models in which nutritional manipulation can be studied more precisely.

To conclude, based on current knowledge it is probably too early to infer whether fetal food is the preemie's prerequisite or not. Yet, as more information is gained on normal and abnormal fetal metabolism during different periods of gestation, this might be incorporated in new nutritional strategies and tested in large trials.

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CHAPTER

11

Summary & Samenvatting

SUMMARY

CHAPTER 1

Growth and development is all about optimal nutrition. During normal fetal development an extensive interplay between the gravida, placenta, and fetus ensure an optimal substrate delivery. After premature birth, however, the neonatologist is responsible for doing so.

In the introductory chapter of this dissertation, an overview of epidemiology of premature birth and its consequences is given. Subsequently, some background is described on the metabolism of proteins and amino acids including applicable methods of research. Then, insight in nutrition for the fetus and nutrition for the premature infant throughout the last 100 years is given

Finally, the aims and outline of this dissertation are covered.

CHAPTER 2

Up to the advent of this dissertation, common practice was to delay the initiation of parenteral amino acids to premature infants, and to provide only carbohydrates until at least 48 hours after birth. This strategy evolved after the recognition that prompt administration of amino acids from so-called previous generations caused metabolic disturbances. During the absence of a exogenous source of nitrogenous compounds, infants lose 1% per day of their body protein stores where the daily intrauterine accretion would have been 1.5%.

Our hypothesis was that currently available amino acid solutions would, however, well be tolerated by premature infants from birth onwards. To test, we divided 135 infants with a birth weight less than 1500 gram into two groups; a control group receiving amino acids starting on postnatal day two at a rate increased stepwisely to 2.4 g/(kg·d) at day three of life and an intervention group receiving 2.4 g/(kg·d) within two hours after birth. During the first week of life, infants were biochemically closely monitored and nitrogen balances were constructed after urinary analysis.

Results showed that infants in the intervention group were clinically not more acidotic than infants in the control group. Plasma urea concentrations were higher in the amino acid supplemented group which indicated that amino acid oxidation had increased. Despite, nitrogen balances were still significantly higher in that same group and a catabolic state was prevented. In addition, plasma concentrations of most amino acids were higher in the intervention group and better fitted reference ranges. To conclude, providing parenteral amino acids from birth onwards to very premature infants is safe and effective in terms of anabolism during the first few days of life.

CHAPTER 3

The study described in this chapter shows the mechanism behind the increase in nitrogen retention that was demonstrated in the previous chapter. In a subset of infants who were ventilated and had an arterial catheter (n=8 in each group), [1-^{13}C]leucine was continuously administered on the second day of life and plasma was analyzed to measure the enrichment of αKIC (a leucine metabolite indicative of intracellular metabolism). In breath samples, $^{13}CO_2$ was measured. From these measurements revealed protein synthesis, breakdown, and oxidation rates could be quantified. In another subset of infants (also n=8 in each group), [U-$^{13}C_6$]glucose was infused to determine if the extra energy that increased protein synthesis costs was derived from increased glucose oxidation. Accordingly, plasma glucose was measured and breath samples were also analyzed for the relative amount of enriched carbon dioxide.

The protein breakdown rate turned out to be unaltered upon amino acid administration, so that the anabolic effect was primarily derived from an increased protein synthesis rate. In fact, the net effect of the administered amino acids was an almost similar absolute increase in both protein synthesis and amino acid oxidation. Glucose kinetics did not show a clear increase in glucose oxidation that could account for higher energy needs.

CHAPTER 4

In plasma of the same infants as studied in the labeled leucine study in the previous chapter, albumin was purified to measure the increase of incorporated tracer leucine over time. Doing so enabled the quantification of the albumin synthesis rate by the infant´s liver and thus gives information on organ kinetics instead of those on a whole-body level as described in chapter 3. Observing low albumin concentrations in these critically ill newborns, yet considering the many important roles of albumin, we speculated that albumin synthesis rates would rise upon amino acid administration.

Mass-spectrometry analysis revealed that the albumin synthesis rate had indeed increased and also led to higher concentrations. Yet, there was no preferential use by the infant´s metabolic system to increase the synthesis of albumin relatively more than that of all other proteins in the body.

CHAPTER 5

This chapter provides an exploratory overview of the outcome at age two of the infants that were included in chapter 2. Some other studies, that were not always well-designed, started to doubt the safety of so-called aggressive nutritional strategies for premature infants. For

as long as our power would be sufficient, we thoroughly assessed follow-up data at age two to detect any difference at a mid-long term outcome between both groups. No significant differences were observed, but a trend towards better neurological and anthropometric outcome could be observed in the early amino acid supplemented group, especially in boys.

CHAPTER 6

From this chapter until chapter 9, a series of studies is described where amino acid and protein metabolism is measured in feto-maternal dyads just prior to birth. These exploratory studies give insight in the physiological metabolic capabilities of an individual during early life.

In this chapter the fetal albumin synthesis rate was measured in a fetuses at term and preterm gestation (n=8 in each group). To do so, a relatively new model was applied whereby multiple tracers were administered to pregnant women starting at different times in the hours prior to cesarean section. From a single blood sample, taken at birth from the umbilical cord, the fetal albumin synthesis rates could be quantified. Whereas the functions of albumin during intrauterine life are not as clear as during postnatal life, fetuses synthesized very large amounts of albumin, especially earlier in gestation. The fact that a fetus of approximately 30 weeks gestation synthesizes under physiological circumstances large amounts of albumin but does not seem to continue this rate after birth (chapter 4), could indicate that postnatal nutritional strategies for premature infants do not provide enough substrates necessary for the high albumin synthesis rate.

CHAPTER 7

In the study described in this chapter, whole-body protein metabolism was measured in 8 fetuses that were at term gestation. By measuring the umbilical blood flow using ultrasound and infusing appropriate tracers of phenylalanine and tyrosine prior to cesarean section, several metabolic rates could be quantified amongst which the protein synthesis and breakdown rates. In addition, we could measure the conversion (hydroxylation) of phenylalanine into tyrosine, a process which is said to be hampered in young and critically ill individuals.

Where fetuses showed considerable net uptake of phenylalanine from the placenta, tyrosine uptake was negligible. Fetal phenylalanine uptake was even responsible for one-fourth of the net catabolic state the mother was at while fasting prior to cesarean section. The fetus used the amino acids for high protein synthesis rates. Converted to tissue, fetuses with a gestational age of 38 weeks had a net accretion rate of 12 g/(kg·d). Furthermore,

the fetuses showed considerable tyrosine production, indicating that phenylalanine hydroxylation occurs unproblematically.

CHAPTER 8

The same pregnant women as described in the previous chapter were also infused with stably labeled leucine, valine, and methionine to study several metabolic pathways of these essential amino acids in the fetus. A fairly surprising uptake by the placenta from the fetus was found of αKIC, a leucine metabolite, as one of the examples of the metabolic routes studied. Furthermore, results seemed to indicate that the fetus oxidizes large amounts of amino acids to generate energy besides the role of amino acids in protein synthesis.

CHAPTER 9

The placenta is a metabolically very active organ with high rates of oxygen and glucose consumption. On a weight basis, these are even larger than the utilization by the fetus. In this chapter we quantify the placental protein turnover rates in the same feto-maternal dyads as in chapter 6. This was possible after analysis of the amount of incorporated amino acid tracers in placental tissue samples collected after birth. It turned out that approximately 30 weeks gestation, one-fourth of all proteins in the placenta is broken down and resynthesized daily. At term, the turnover rate slightly decreased to about 20% per day. The high turnover rates are probably necessary to facilitate ongoing high nutrient transport to the fetus for optimal growth and development.

CHAPTER 10

This chapter provides a general discussion in which all results of this dissertation are again critically analyzed against the current literature. Furthermore, some considerations for future research are presented.

SAMENVATTING

HOOFDSTUK 1

Groei en ontwikkeling begint bij een optimale voeding. Gedurende de normale foetale ontwikkeling een uitgebreid samenspel tussen de zwangere vrouw, placenta, en foetus zorgt voor een optimale opname van voedingssubstraten. Na vroeggeboorte [prematuriteit], echter, draagt de neonatoloog die zorg.

In het inleidend hoofdstuk van dit proefschrift, wordt een overzicht gegeven in de statistieken en gevolgen van premature geboorte. Vervolgens wordt de achtergrond uiteengezet van stofwisseling van eiwitten en de bouwstenen daarvan (aminozuren), inclusief de toepasbare onderzoeksmethoden. Hierna volgt een inzage in voeding voor de foetus en het premature kind gedurende de laatste 100 jaar.

Ten slotte komen de doelstellingen en opbouw van dit proefschrift aan bod.

HOOFDSTUK 2

Voorafgaand aan de periode voordat de studies in dit proefschrift begonnen, was het in de kliniek gebruikelijk prematuur geboren kinderen te onthouden van aminozuren en alleen suikers te geven gedurende de eerste paar dagen na geboorte. Dit aangezien toediening van aminozuuroplossingen van mindere kwaliteit uit het verleden resulteerde in verstoringen bijvoorbeeld in het zuur-base evenwicht van deze kwetsbare groep kinderen. Echter, in afwezigheid van toediening van stikstofhoudende substraten, verliezen kinderen dagelijks 1% van hun eiwitvoorraad en dat terwijl de eigenlijke groeisnelheid in de baarmoeder 1.5% per dag zou bedragen.

Onze hypothese was echter dat de tegenwoordige generatie van aminozuuroplossingen goed verdragen zou worden bij toediening direct na geboorte in te vroeg geboren kinderen. Ter toetsing werden 135 kinderen met een geboortegewicht onder 1500 gram verdeeld in 2 groepen. De controle groep onderging het standaard voedingsprotocol waarbij aminozuren op dag 2 na geboorte gestart werden en daarbij stapsgewijs verhoogd werden to 2.4 gram/kg per dag en de interventiegroep kreeg vanaf direct na geboorte reeds 2.4 gram/kg per dag. Gedurende de eerste week na geboorte werden verscheidene biochemische bloedwaarden gecontroleerd en na analyse van urine twee keer een stikstofbalans geconstrueerd.

Uit de resultaten bleek dat kinderen in de interventiegroep klinisch gezien niet meer acidotisch waren dan kinderen in de controle groep. De plasma ureum concentraties waren hoger in de groep gesupplementeerd met aminozuren wat aangaf dat aminozuur oxidatie verhoogd was. Desondanks was de stikstofbalans hoger in de betreffende groep en een

katabole staat werd verkomen. Hiernaast waren de plasma concentraties van de meeste aminozuren hoger in de interventiegroep en pasten deze beter binnen de referentiewaarden. Concluderend, het geven van parenterale aminozuren aan premature kinderen vanaf de geboorte is veilig en effectief wat betreft anabolisme gedurende de eerste paar dagen na geboorte.

HOOFDSTUK 3

Dit hoofdstuk beschrijft het mechanisme achter de verhoging van stikstofretentie welk gedemonstreerd was in het vorige hoofdstuk. In een deel van de eerder geïncludeerde kinderen welke beademd werden en een arteriële lijn hadden (n=8 in iedere groep) werd een stabiele isotoop ([1-^{13}C]leucine) als tracer voor een aantal uren toegediend op dag twee na geboorte. Vervolgens werd in het plasma de verrijking van αKIC (een leucine metaboliet indicatief voor intracellulair metabolisme) geanalyseerd en in de uitademingslucht de verrijking van koolstofdioxide gemeten. Hieruit konden de eiwitsynthese, -afbraak, en -oxidatie berekend worden. In een ander deel van de eerder geïncludeerde kinderen (ook n=8 per groep), werd stabiel gelabeld [U-$^{13}C_6$]glucose gegeven om te bestuderen of er extra glucose verbrand werd als energiebron voor een verhoogde eiwitsynthese. Hiervoor werd de glucose plasma verrijking gemeten en in uitademingslucht eveneens de verrijking van $^{13}CO_2$.

De eiwitafbraaksnelheid bleek onveranderd te zijn als aminozuren direct na geboorte werden, zodat het anabole effect primair het effect was van verhoogde eiwitsynthese. In feite was het netto effect van de toegediende aminozuren een ongeveer vergelijkbare stijging van zowel eiwitsynthese als aminozuuroxidatie. Analyse van de glucose kinetiek liet geen duidelijke veranderingen zien die konden worden gewijd aan een verhoogde energiebehoefte als gevolg van extra eiwitsynthese.

HOOFDSTUK 4

Uit het plasma van dezelfde kinderen als die hiervoor meededen met de leucine studie werd albumine gezuiverd. Hierin kon vanuit de stijging van de hoeveelheid in albumine geïncorporeerde leucine-tracer gedurende de infusieduur, de albumine synthese snelheid berekend worden. Waar in hoofdstuk 3 de eiwitsynthese berekend kon worden als gemiddelde van alle lichaamsprocessen, is de albuminesynthese een maat voor alleen de leveractiviteit. Aangezien de albumine concentraties in te vroeg geboren kinderen vaak erg laag zijn, terwijl albumine talrijke functies heeft, was onze hypothese dat de synthese snelheid zou stijgen als gevolg van de aminozuurtoediening.

Massa-spectrometrie analyse liet zien dat de albumine synthese inderdaad was gestegen

en bovendien ook leidde tot hogere concentraties. Echter, er was geen preferentieel gebruik van aminozuren voor albuminesynthese ten opzichte van het gebruik voor alle andere eiwitten in het lichaam.

HOOFDSTUK 5

Dit hoofdstuk geeft een exploratief overzicht van de *outcome* op twee-jarige leeftijd van de kinderen die bestudeerd waren in hoofdstuk 2. Enkele andere studies, die niet altijd optimaal uitgevoerd waren, begonnen te twijfelen aan de veiligheid van de zogenoemde agressieve voedingsstrategieën voor prematuur geboren kinderen. Voor zover onze *power* groot genoeg is, hebben we de *follow-up* data op twee-jarige leeftijd grondig geanalyseerd om eventuele verschillen op middellange termijn tussen beide groepen te bestuderen. Er werden geen significante verschillen gevonden. Echter er was een trend, vooral in jongens, richting een betere *outcome* wat betreft zowel neurologische als groei parameters.

HOOFDSTUK 6

Vanaf dit hoofdstuk tot hoofdstuk 9 wordt een serie studies beschreven waar het aminozuur en eiwit metabolisme wordt gemeten in de foeto-maternale twee-eenheid vlak voor geboorte. Deze exploratieve studies geven inzicht in de fysiologische metabole precessen van een individu gedurende het vroege leven.

In dit hoofdstuk wordt de albumine synthese snelheid gemeten in foetussen na ongeveer driekwart en aan het einde van de normale zwangerschapsduur (n=8 in iedere groep). Dit was mogelijk met behulp van een relatief nieuw onderzoeksmodel waarbij verscheidene tracers werden geïnfundeerd aan zwangere vrouwen en welke startten op verschillende tijdstippen in de uren voor een keizersnede. Door bloed af te nemen na geboorte vanuit de navelstreng, kon de foetale albumine synthese snelheid berekend worden. Terwijl de functies van albumine gedurende het foetale leven niet zo duidelijk omschreven zijn als gedurende het leven na geboorte, werden zeer grote hoeveelheden albumine geproduceerd door de foetus, vooral in de maanden voor de uitgerekende datum. Het feit dat de foetus zoveel albumine synthetiseert bij ongeveer 30 weken zwangerschapsduur, maar deze snelheid niet lijkt te continueren na premature geboorte (hoofdstuk 4), kan een aanwijzing zijn dat de voedingsstrategieën voor prematuren niet genoeg substraten leveren voor een snelle albumine synthese.

HOOFDSTUK 7

In de studie beschreven in dit hoofdstuk werd het eiwitmetabolisme op geheel lichaamsniveau gemeten in 8 foetussen dicht bij de uitgerekende datum. Dit was mogelijk door zowel de bloedstroomsnelheid in de navelstreng te meten als de geschikte tracers van phenylalanine en tyrosine aan zwangere vrouwen te geven in de uren voor een keizersnede. Uit het navelstrengbloed konden we de eiwitsynthese en –afbraak snelheden berekenen. Tevens was het mogelijk de omzetting (hydroxylatie) van phenylalanine in tyrosine te meten, een proces dat mogelijk minder verloopt in jonge en zeer zieke individuen.

Terwijl de foetus een behoorlijke opname vanuit de placenta liet zien, was de opname van tyrosine verwaarloosbaar klein. De foetale phenylalanine opname was zelfs verantwoordelijk voor één vierde van de netto katabole toestand van de moeder terwijl zij aan het vasten was als voorbereiding op de keizersnede. De foetus gebruikte de aminozuren voor een hoge eiwitsynthesesnelheid. Omgerekend naar weefsel, foetussen hadden een netto groeisnelheid van 12 gram/dag per kilo lichaamsgewicht bij een zwangerschapsduur van 38 weken. Voorts lieten de foetussen een aanzienlijke tyrosine productie zien, welke indicatief was dat de phenylalanine hydroxylatie onproblematisch verliep.

HOOFDSTUK 8

Dezelfde vrouwen als beschreven in het vorige hoofdstuk werden ook geïnfundeerd met stabiel gelabeld leucine, valine, en methionine om verscheidene metabole *pathways* van deze essentiële aminozuren in de foetus te bestuderen. Een redelijk verrassende opname van αKIC (een leucine metaboliet) door de foetus werd waargenomen, als een van de voorbeelden van de bestudeerde metabole routes. Verder lijken de resultaten in dit hoofdstuk te wijzen op een hoge oxidatie van aminozuren in de foetus welke zo een alternatieve energiebron had.

HOOFDSTUK 9

The placenta is een metabool zeer actief orgaan met een hoog verbruik van zuurstof en glucose. Op gewichtsbase is dit gebruik zelfs groter dan dat van de foetus. In dit hoofdstuk kwantificeren we de eiwit-turnover van de eiwitten in de placenta in dezelfde personen als bestudeerd in hoofdstuk 6. Dit was mogelijk door analyse van de hoeveelheden ingebouwde tracers in stukjes placentaweefsel verzameld na geboorte. Het bleek dat bij een zwangerschapsduur van ongeveer 30 weken, één vierde van alle eiwitten in de placenta dagelijks afgebroken wordt en weer opgebouwd. Aan het einde van een normale zwangerschapsduur, daalt deze turnover snelheid naar ongeveer 20% per dag. De hoge

turnover snelheden zijn waarschijnlijk noodzakelijk om een continu hoog voedingstransport richting de foetus te waarborgen, welke nodig is voor optimale groei en ontwikkeling.

HOOFDSTUK 10

In dit hoofdstuk wordt een algemene discussie gegeven waarin alle resultaten uit deze dissertatie nogmaals kritisch bekeken worden in het licht van de huidige literatuur. Verder worden er enkele aanbevelingen gedaan voor toekomstig onderzoek.

dankwoord

De laatste punt in dit proefschrift was op de vorige pagina nog niet gezet. Aangezien velen hebben meegewerkt aan de totstandkoming van deze dissertatie, wil ik graag bij deze iedereen hier ontzettend voor bedanken. Echter, in het bijzonder mogen de volgende personen niet overgeslagen worden.

Allereerst natuurlijk de ouders van de te vroeg geboren kinderen; veel respect heb ik voor de beslissing jullie pasgeboren kind mee te laten doen aan het vele onderzoek op de afdeling neonatologie. Maar ook de ouders van de kinderen welke nog niet eens geboren waren en waarbij ik al onderzoek mocht doen, wil ik op deze plek van harte bedanken. Ongetwijfeld zullen veel van de kinderen die in de nabije toekomst te vroeg geboren worden veel baat hebben van de kennis die wij hebben opgedaan door jullie deelname.

En dan natuurlijk de initiator van dit onderzoek, Prof. dr. J.B. van Goudoever; beste Hans, de mogelijkheden die ik bij het onderzoek bij jou doen kreeg, zijn ongekend. Na een gesprek of overleg met jou was ik altijd weer meer geënthousiasmeerd, geïnspireerd, en optimistischer dan op het moment dat ik je kamer inliep. Ontzettend bedankt voor dit alles, voor de congressen, en voor alle kansen die je bood!

Ook mijn andere promotor, Prof. dr. E.A.P. Steegers ben ik erkentelijk; beste Eric, dank voor de hulp bij het opzetten van het foetale gedeelte van dit proefschrift en het welkom heten op de afdeling obstetrie.

Zonder een ‘akkoord’ van de leescommissie zou ‘11 december’ niet door hebben kunnen gaan. Prof. dr. H.J.G. Boehm, Prof. dr. A.J. van der Heijden, en Prof. dr. T.J.M. Helmerhorst, dank voor de snelle beoordeling van het manuscript. Prof. dr. G. Buonocore, Prof. dr. H.N. Lafeber, Prof. dr. H.P. Sauerwein, en Prof. dr. G.H.A. Visser dank ik bij deze voor hun bereidheid tot zitting in de grote commissie.

Vele uren zijn gespendeerd in het massa-spectrometrie lab. Gelukkig was ik daar niet alleen; niet alleen voor alle kennis die er aanwezig was, maar ook voor de gezelligheid! Gardi, Kristien, Trinet, Darcos, en bovenal natuurlijk Henk: ik kon jullie (ogenschijnlijk) niet vaak genoeg storen als ik weer eens iets niet wist of er weer eens wat verkeerd was gegaan. Dank voor al het geduld, meedenken, en integreren van alle pieken!

Ineke, zeker in het begin heb je me veel geholpen, waarvoor mijn dank; Alle METC administratie verliep door jou soepeltjes en het A4’tje statistiek voor dummies heb ik nog vaak kunnen gebruiken. Daniëlla, top dat je altijd wel weer een klein gaatje voor me in de baas zijn agenda wist te ritselen! Tevens vond ik het fijn dat je kamer altijd een warm welkom is voor een korte en gezellige stop by.

De verpleegkundigen en artsen op de afdeling neonatologie legden de basis voor de eerste hoofdstukken van dit proefschrift. Dank voor het tijdig aanhangen van de voeding en het verzamelen van alle gaasjes met urine.

Beste Andras, jij en je medewerkers in de apotheek zorgden voor de talloze tracer-flacons waarvoor zeer veel dank. Ik waardeer je striktheid, en prettige en zeer snelle communicatie.

Beste dr. Duvekot, beste Hans, dank voor het mee-enthousiastmeren van iedereen op de afdeling en van patiënten! Ook Joke en Wilma horen op deze plek genoemd te worden voor het meedenken over de praktische details. Voor het feit dat ik vaak op de meest onverwachte momenten belde, maar we toch altijd een geschikt moment voor een echo vonden, wil ik je bij deze nogmaals bedanken, Ernst!

Verder wil ik iedereen van OK 1 bedanken: de gynaecologen, anesthesiologen, arts-assistenten, en operatie-assistenten waren onmisbaar in hun soepele medewerking bij het verzamelen van alle monsters. Voor het feit dat de medewerkers in het AKC-Sophia uiterst behulpzaam waren bij de verwerking van een deel van de vele monsters, wil ik hen hartelijk bedanken.

Rogier, bij deze veel dank voor het op weg helpen met de lay-out van dit boekje; heeft een hoop puzzelen gescheeld!

Beste Frans, in 2003 samen als student op de afdeling begonnen en sindsdien 'het bureau gedeeld'. Vele pieken en dalen hebben we in het onderzoek meegemaakt waarbij als het even tegenzat, we met elkaar ook heerlijk konden dramatiseren, maar vooral door stevige discussies, humor, zangsessies, en andere hilariteiten elkaar een stuk verder konden helpen. Echter, that's what good colleagues do. De vele lol ook buiten het Sophia, bv ergens in de kroeg, squashbaan, of op congres is natuurlijk het mooist en zal zeker blijven. Thnx Mate!

Deels van bovenstaande geldt natuurlijk ook voor de rest van alle recente SK-2210 bewoonsters: Maaike & Maaike, Willemijn & Karien, en Hester, Denise & Anne. De SK is een topkamer! Maar ook elders gezelligheid met de collega's: met Carine, Heleen, Janine, Joanne, Nanda, en Patrycja voor een regelmatige lunch, met de bezoekers van de VOBS met als initiatoren natuurlijk Emile en Ralph, met de onderzoekers bij kerstdiners en op weekenden, en verder met Jan-Erik, Jeroen, Sascha, en alle andere artsen voor het grandioze vermaak op congres!

Paranimfen Maaike & Erik: een vaste steun en toeverlaat tijdens de afgelopen jaren. Het wel en wee gedurende de laatste jaren van mijn onderzoek hebben jullie vaak van me aan moeten horen. Ik heb jullie met veel plezier gevraagd dit nog één maal te doen, en wel op 11 december. Dank dat jullie de afsluiting van de afgelopen jaren met wij willen meevieren.

Ouders, zussen, broer en alle vrienden wil ik danken voor de betrokkenheid en steun gedurende de afgelopen jaren: eigenlijk hadden jullie allemaal prima mijn paranimf kunnen zijn!

Ik zie jullie allemaal graag verschijnen bij het promotie-slotfeest!

curriculum vitae

Chris van den Akker was born in Zevenaar, the Netherlands on the 16th of October, 1980. After having completed grammar school at Liemers College in Zevenaar in 1998, he went to Santa Barbara, CA, USA for one year to attend an international language school. After his return, he started his medical training at Erasmus University in Rotterdam, the Netherlands in 1999.

As a graduation project he started in 2003 to work on a study on early amino acid supplementation to premature infants under the supervision of Prof. dr. J.B. van Goudoever at the neonatal division of the department of pediatrics in the Erasmus MC – Sophia Children's Hospital in Rotterdam, the Netherlands. In 2004 he then postponed his rotations for his medical training to continue doing research as part of a PhD-project. The focus of his research was directed more towards amino acid and protein metabolism in the human fetus. Again under the supervision Prof. dr. J.B. van Goudoever, studies were performed in collaboration with Prof. dr. E.A.P. Steegers of the department of obstetrics and gynecology (division obstetrics and prenatal medicine) at the Erasmus MC, and have resulted in this dissertation.

In June 2008, he continued his medical training and started his rotations, which he will finish in 2010.

list of publications

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Submitted

portfolio

COURSES

- 2007: SPR-RC (Society for Pediatric Research – Research Conference) on health promoting effects of early nutrition, The Woodlands, TX, USA.
- 2006: Intensive Course in Tracer Methodology in Metabolism, Stockholm, Sweden.
- 2005: Nihes Statistics Course: Classical Methods for Data Analysis, Rotterdam, the Netherlands.

MEMBERSHIPS

- 2007: BASIS (BeNeLux Association for Stable Isotope Scientists).
- 2007: ASN (American Society for Nutrition), research interest section: Energy and Macronutrient Metabolism.

GRANTS

- 2005: Nutricia Research Foundation; € 50.000,00; Wageningen, the Netherlands.

AWARDS

- 2007: Travel award for the SPR-Research Conference in The Woodlands, TX, USA.
- 2005: NVVL (Network for Food Experts) award for best scientific thesis.
- 2004: 'Jan C. Molenaar' award for best presentation in the presence of the Scientific Advisory Council of the Sophia Foundation for Scientific Research.
- 2003: NVK (Dutch Society of Pediatrics) award for best scientific poster presentation.

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