Nutrition, Diet, and Infant Development: Long-Chain Polyunsaturated Fatty Acids in Infant Neurodevelopment

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Lipids are the predominant source of dietary energy for infants and young children and constitute the major energy stores in the body. There is a growing interest in the quality of dietary lipid supply in early childhood as a major determinant of infant growth, development, and long-term health. Thus, the selection of dietary lipid supply during the early stages of postnatal life is considered of great importance.

Lipids are structural components of all tissues and are indispensable for cell membrane synthesis; the brain, retina, and other neural tissues are particularly rich in long-chain polyunsaturated fatty acids (LC-PUFA). These fatty acids serve as specific precursors for eicosanoid production (prostaglandins, prostacyclins, thromboxanes, and leukotrienes). Eicosanoids are powerful autocrine and paracrine regulators of numerous cell and tissue functions (for example, thrombocyte aggregation, inflammatory reactions and leukocyte functions, vasoconstriction and vasodilation, blood pressure, bronchial constriction, uterine contraction). Dietary lipid intake also affects cholesterol metabolism at an early age and is associated with cardiovascular morbidity and mortality in later life. More recently, lipid supply, especially the provision of LC-PUFA, has been shown to affect neural structural development and function.

BRIEF SUMMARY OF ESSENTIAL FATTY ACID METABOLISM, LC-PUFA SYNTHESIS, AND BASIS FOR ESSENTIALITY

George and Mildred Burr in 1929 introduced the concept that specific components of fat may be necessary for proper growth and development of animals and possibly humans (1). They proposed that three fatty acids be considered essential: linoleic acid (18:2 n - 6), arachidonic acid (20:4 n - 6) and α-linolenic acid (LNA; 18:3 n - 3) (1). The essentiality of n - 6 and n - 3 fatty acids for humans is best explained by the inability of animal tissues to introduce double bonds in positions
De Novo Synthesis and Diet

before carbon 9, counting from the methyl or n terminus. Essential fatty acids (EFA) were considered of marginal nutritional importance for humans until the 1960s, when signs of clinical deficiency became apparent in infants fed skimmed-milk-based formula and in those given lipid-free parenteral nutrition (2,3). These infants presented with dryness, desquamation, and thickening of the skin and growth faltering as frequent manifestations of linoleic acid deficiency. More subtle clinical symptoms appear in n - 3 essential fatty acid deficiency. They include skin changes unresponsive to linoleic acid supplementation, abnormal visual function, and peripheral neuropathy (4).

Parent EFA (linoleic acid and LNA) are further elongated and desaturated by mammals, generating a family of compounds for each (Fig. 1). As shown in the figure, arachidonic acid can be formed from linoleic acid; it becomes essential only if the capacity for elongation and desaturation of linoleic acid is limited. This occurs in the cat and other felines. Further information can be found in recent reviews (5,6). The competitive inhibition of the desaturation step of the respective precursors (for n - 3, LNA; for n - 6, linoleic acid; and for n - 9, oleic acid) by Δ⁶-desaturase is of major significance because this is the controlling step of the pathway. If n - 3 fatty acids are absent or deficient in the diet, the elongation/desaturation of

FIG. 1. Long-chain polyunsaturated fatty acid (LC-PUFA) and essential fatty acids (EFA) metabolism. Parent EFA are derived from dietary sources for both n - 3 (18:3, LNA) and n - 6 series (18:2, LA). De novo synthesis is able to produce only n - 9 LC-PUFA. Elongation occurs two carbons at a time and Δ desaturases introduce double bonds at 9, 6, and 5 carbons from the carboxylic end of the fatty acid chain. The final step in the formation of n - 3 and n - 6 end products is catalyzed by a peroxisomal β-oxidation. PUFA of interest include 18:3 n - 6 (GLA), 20:4 n - 6 (AA), 22:5 n - 6 (DPA), 20:3 n - 9 (ETA), 20:5 n - 3 (EFA), and 22:6 n - 3 (DHA). EPA, AA, and 20:3 n - 6 are immediate precursors of prosta glandins (PG) and other eicosanoids.
LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN INFANTS

n – 6 compounds generates a significant increase in docosapentaenoic acid (DPA; 22:5 n – 6); if both EFA are lacking, eicosatrienoic acid (ETA; 20:3 n – 9) accumulates (5). The triene/tetraene (ETA/arachidonic acid) ratio may be used as an index of essential fatty acid deficiency but is not valid as a marker of isolated n – 3 deficit. An increased ratio of DPA to docosahexaenoic acid (DHA) has been suggested by us and others to serve as a useful index of n – 3 deficiency.

The LC-PUFA arachidonic acid, eicosapentaenoic acid (EPA; 20:5 n – 3), and DHA (22:6 n – 3) are important membrane components and precursors of potent bioactive oxygenated products. Eicosanoids such as prostaglandins, leukotrienes, and epoxides derived from arachidonic acid and EPA modulate or are required in numerous physiological processes; a myriad of clinical correlates associated with deficient or excessive essential fatty acid intake have been observed. The conversion of parent EFA to LC-PUFA is under active regulation; therefore, the effects of providing arachidonic acid, EPA, or DHA are not replicated if the equivalent amount of linoleic acid or LNA is provided (7,8). The uniqueness of feeding human milk relative to essential fatty acid metabolism is based on the direct supply of LC-PUFA bypassing the regulatory step of the Δ⁶-desaturase. Excess dietary linoleic acid associated with some vegetable oils, particularly safflower, sunflower, and corn oils, may decrease the formation of DHA from LNA because the Δ⁶-desaturase is inhibited by excess substrate. In addition, arachidonic acid formation is reduced when excess linoleic acid or LNA is provided (5–8). The inhibitory effect of EPA on Δ⁵-desaturase activity has been considered in part responsible for the lower arachidonic acid observed when marine oil is consumed. Excess linoleic acid, as seen in infants receiving corn oil or safflower oil as the predominant fatty acid supply, will inhibit the elongation/desaturation of the parent EFA and thus lower the LC-PUFA supply necessary for membrane synthesis. Marine PUFA provide minimal preformed arachidonic acid and substantial amounts of preformed n – 3 LC-PUFA such as EPA and DHA (9).

The biochemical and functional evidence indicates that in early life, C₁₈ n – 3 precursors are not sufficiently converted to DHA. Thus, not only linoleic acid and LNA but also DHA should be considered essential nutrients for normal eye and brain development in the human.

ASSESSMENT OF CLINICAL TRIALS ON LC-PUFA SUPPLEMENTATION

The dry weight of the human brain is predominantly lipid; 22% of the cerebral cortex and 24% of white matter consist of phospholipids. Studies of several animal species and recent evidence from humans have established that brain phospholipid arachidonic acid and DHA decrease, whereas n – 9 and n – 7 mono- and polyunsaturated fatty acids increase, when linoleic acid and LNA are deficient in the diet (10–12). Typically, n – 3 fatty acid-deficient cells have decreased DHA and increased levels of the end product of n – 6 metabolism, DPA. Within the subcellular
organelles, synaptosomes and mitochondria seem to be more sensitive to a low dietary \( n - 3 \) supply, as evidenced by the relative abundance of DHA and the changes in composition of these organelles in response to dietary deprivation (10–12). The animal data accumulated over several decades strongly support the essential nature of EFA for humans, and particularly a need for LC-PUFA in early life. Direct information from humans is limited because human investigation in this area is just a decade old. In this chapter we review randomized clinical trials addressing the putative effects of LC-PUFA on neurodevelopment.

How to Define and Design the Diet to be Tested (the Independent Variable)

The Human Milk Model

A good starting point is to mimic the composition of human milk. Unfortunately, human milk does not have a uniform lipid composition; the type of fatty acid in human milk is affected by mother’s diet during pregnancy and lactation and varies according to postpartum age, preterm or term delivery, and maternal diseases affecting lipid metabolism such as diabetes, cystic fibrosis, and abetalipoproteinemia. Arachidonic acid is the main \( n - 6 \) and DHA is the most significant of the \( n - 3 \) series of LC-PUFA found in human milk. The ratio of total \( n - 6 \) to \( n - 3 \) is 5:1 to 10:1, ranging up to 18:1 if oils high in linoleic acid are consumed. The ratio of arachidonic acid to DHA is most commonly 1.5:1 to 2:1. The variability in LC-PUFA in human milk is high and determined mainly by diet. Eicosapentaenoic acid is found in minimal amounts except in populations consuming high intakes of fish; it is always lower than the DHA content (13,14). The DHA levels range from 0.1% in Germany to 1.4% in Inuits of North America. Typical values range from 0.3% to 0.4%; however, higher concentrations are found in human milk from women consuming non-Western diets (13). Recently, Gibson et al. reported a longitudinal reduction in the DHA content of human milk from Australian women on Western diets from 0.32% in 1981 to 0.21% in 1995 (15).

The question of what amounts of specific fatty acids to feed is not answered just by deciding to follow the human milk model. Should one select a value in the upper range of LC-PUFA content, the lower range, or the midpoint? If the effort is focused primarily on demonstrating functional efficacy, selecting a value in the upper range is preferable. On the other hand, if safety concerns are the main objective of study, selecting a value in the lower range might be more appropriate. In our initial studies, we did not have access to pure LC-PUFA sources; thus, in order to provide DHA, we were obligated to include EPA because the available marine oil had a ratio of EPA/DHA of 2:1. No sources of arachidonic acid were available for commercial use; thus, we could not include this fatty acid, which is recognized as of potentially critical importance. We chose to provide 0.35% DHA in both the preterm and term infant studies (16–18). This value is in the mid- to upper range of the mean DHA content derived from combined data on human milk composition of omnivorous
TABLE 1. Observational studies of dietary LC-PUFA intake and neurodevelopmental function

<table>
<thead>
<tr>
<th>Author, year (ref.)</th>
<th>Subjects, age at evaluation</th>
<th>Main outcome variables</th>
<th>Test diets</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birch, 1993 (22)</td>
<td>Term infants, 3 years</td>
<td>DHA plasma PL &lt;1 year</td>
<td>HM or term formula</td>
<td>HM better stereoaucity and better recognition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OPL visual acuity</td>
<td></td>
<td>DHA at 4 months + correlated with stereoaucity at 3 years</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OPL stereoaucity, HOT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>recognition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bjerke, 1993 (23)</td>
<td>VLBW infants, 1 year</td>
<td>Bayley MDI and PDI</td>
<td>Not characterized</td>
<td>DHA + correlated with PDI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DHA and EPA serum PL</td>
<td></td>
<td>EPA-correlated with MDI</td>
</tr>
<tr>
<td>Makrides, 1993 (24)</td>
<td>Term infants, 5 months</td>
<td>VEP visual acuity DHA and LA in RBC PL</td>
<td>HM or term formula</td>
<td>HM better acuity</td>
</tr>
<tr>
<td>Innis, 1994 (25)</td>
<td>Term infants, 3 months</td>
<td>TAC visual acuity FAs in plasma and RBC PL</td>
<td>HM or term formula</td>
<td>DHA + correlated with acuity</td>
</tr>
<tr>
<td>Courage, 1995 (26)</td>
<td>Term infants, 18 months</td>
<td>TAC visual acuity</td>
<td>HM or cow's milk formula</td>
<td>No differences</td>
</tr>
<tr>
<td>Jorgensen, 1996 (27)</td>
<td>Term infants, 4 months</td>
<td>TAC visual acuity DHA, AA, EPA in RBC PL</td>
<td>HM or term formula</td>
<td>HM better acuity at 3, 6, and 18 months</td>
</tr>
</tbody>
</table>

women. Other investigators have studied premature infants given 0.2% to 0.5% DHA (19,20) and term infants given 0.1% to 0.36% (21), as shown in Tables 1 through 3.

What Sources of LC-PUFA to Use in the Test Formulas

Vegetable oils derived from maize, safflower, and sunflower contain predominantly linoleic acid and little or no LNA. Oils derived from soybean and linseed contain ample LNA. This latter fatty acid has higher concentrations in green leaf vegetables than in vegetable seeds. Thus, products from animals fed in the wild have higher n – 3 fatty acids than grain-fed animals. This is of interest in terms of the higher DHA content of eggs from range-fed chickens. The use of evening primrose oil or black current oil provides 18:3 n – 6 γ-linolenic acid (GLA), bypassing the controlling step, Δ^5-desaturase, necessary for arachidonic acid formation. γ-Linolenic acid has been added by some manufacturers as an alternative to arachidonic acid, with limited benefits. The main source for the de novo synthesis of n – 3 fatty acids in the aquatic environment is marine autotrophic bacteria,
microalgae, and protozoa, which constitute the zooplankton and phytoplankton (38,39). Fish, higher in the food chain, incorporate the $n - 3$ PUFA and further elongate them to form EPA and DHA. Thus, fish will concentrate EPA and DHA as triglycerides, mainly in the adipose tissue and in the fat of muscle and visceral organs. The higher the fat content of fish, the higher its content of $n - 3$ fatty acids (40,41).

Another important source of LC-PUFA used in infant diets is egg yolk phospholipid. The concentrations of PUFA are different depending on the feed given to

**TABLE 2. Randomized controlled trials of biochemical and functional effects of LC-PUFA supplementation**

<table>
<thead>
<tr>
<th>Author, year (ref.)</th>
<th>Subjects, age at evaluation</th>
<th>LC-PUFA source</th>
<th>LA (% total)</th>
<th>LNA (% total)</th>
<th>AA (% total)</th>
<th>EPA (% total)</th>
<th>DHA (% total)</th>
<th>Main outcome variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uauy, 1992 (16–18)</td>
<td>VLBW infants, bw 1000–1500 g, 57 weeks PCA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Marine oil</td>
<td>20–21</td>
<td>2.7</td>
<td>0.1</td>
<td>0.65</td>
<td>0.35</td>
<td>FA composition plasma, RBC, and cheek cells Rod and cone ERG, VEP, and FPL visual acuity Growth, bleeding time Membrane fluidity</td>
</tr>
<tr>
<td>Carlson, 1992 (19, 28, 29)</td>
<td>VLBW infants, bw 748–1396 g, 9 months</td>
<td>Marine oil</td>
<td>19–33</td>
<td>3.2–4.9</td>
<td>0.3–0.7</td>
<td>0.2–0.4</td>
<td>FA composition plasma and RBC TAC visual acuity Growth Development (Bayley)</td>
<td></td>
</tr>
<tr>
<td>Agostoni, 1995 (30)</td>
<td>Term infants, 4 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Egg phospholipids</td>
<td>11</td>
<td>0.3</td>
<td>0.44</td>
<td>0.05</td>
<td>0.3</td>
<td>FA composition Cholesterol Development (Brunet–Lezine)</td>
</tr>
<tr>
<td>Makrides, 1995 (31)</td>
<td>Term infants, 30 weeks&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Marine oil, primrose oil</td>
<td>17</td>
<td>1.6</td>
<td>0.01 and 0.27</td>
<td>0.58</td>
<td>0.36</td>
<td>FA composition VEP visual acuity</td>
</tr>
<tr>
<td>Carlson, 1996 (32, 33)</td>
<td>Preterm infants, bw 747–1275 g, 12 months</td>
<td>Marine oil</td>
<td>21</td>
<td>2.4</td>
<td>GLA</td>
<td>0.06</td>
<td>0.2</td>
<td>FA composition Growth TAC visual acuity</td>
</tr>
<tr>
<td>Mena, 1996 (34)</td>
<td>Preterm infants, bw 1000–1500 g, 18 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Animal phospholipids</td>
<td>11–15</td>
<td>1.1–1.4</td>
<td>0.8–0.9</td>
<td>0.5</td>
<td>FA composition Growth Rod and cone ERG, VEP, and FPL visual acuity BAER Sleep–wake cycle, heart rate variability</td>
<td></td>
</tr>
<tr>
<td>Carlson, 1996 (35)</td>
<td>Term infants, 12 months&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Egg phospholipids</td>
<td>22</td>
<td>2.2</td>
<td>0.43</td>
<td>0.0</td>
<td>0.1</td>
<td>FA composition Growth TAC visual acuity</td>
</tr>
</tbody>
</table>

<sup>a</sup> Study includes a human-milk-fed reference group.
chickens: ample use of fish meal in chicken feed results in increased egg yolk DHA (42,43). LC-PUFA products for blends in infant formulas can be successfully produced if chicken feed is carefully monitored and if refined lipid extraction procedures are used. This is presently an important LC-PUFA source used in some infant formulas. Bacterial strains and microalgae isolated from the intestinal content of some fish have a remarkably high content of EPA and DHA (41). Efforts to grow these microorganisms in natural or artificial seawater to obtain DHA for nutritional or pharmacologic use have been successful. In addition, selected fungal strains produce concentrated arachidonic acid that is suitable for human consumption. The industrial production of arachidonic acid, EPA, and DHA from strains of these single-cell organisms has been successful; their expanded use will depend on price and demand relative to the concentrates obtained from marine oils. Single-cell oils offer a promising new source of LC-PUFA provided mass production becomes commercially feasible (38).

Rigorous purity and toxicologic testing should be conducted on fatty acid sources intended for use in commercial infant formula. Currently there is insufficient knowledge on the implications of using these novel LC-PUFA sources in infant formula. Initial studies used a mixture of vegetable oils to supply linoleic acid and LNA, and marine oil as a source of $n-3$ LC-PUFA (17,19). More recent studies, including our own, have used nearly pure DHA from marine oil fractions or DHA and arachidonic acid from single-cell oils. Most published work to date is based on infant formulas enriched with marine oil, marine oil fractions, or egg phospholipids as LC-PUFA sources.

*Defining the Correct Balance Between Fatty Acid Species for Optimal Endogenous LC-PUFA Biosynthesis*

Metabolism of LC-PUFA is greatly affected by the interaction of various fatty acids. This may determine the final effect of the diet being fed. Excess linoleic acid, a high linoleic acid/LNA ratio, and possibly other fatty acids may affect the
endogenous synthesis of DHA and arachidonic acid. Because the choice of oil mixes
to be used in formulas was traditionally based on fat digestibility, a high linoleic
acid was considered beneficial. Furthermore, LNA was usually not included in order
to decrease peroxidative potential. The linoleic acid/LNA ratio in the older formul-
tions was often 50:1 and sometimes as high as 100:1. These ratios are in sharp
contrast with human milk, where the linoleic acid/LNA ratio is close to 5:1 and at
most 10:1. In addition, when marine oil sources are used, EPA is also included. If
the concentration of EPA is sufficiently high, it will interfere with arachidonic acid
biosynthesis and compete for incorporation into phospholipids.

The alternative fates of dietary linoleic acid and LNA are (a) mitochondrial oxida-
tion to yield energy, (b) esterification and incorporation into membranes or circulat-
ing lipid moieties, and (c) serving as precursors for arachidonic acid and DHA
biosynthesis. The relative effects of these alternate pathways on the size of the LC-
PUFA pool are difficult to quantify, yet it can be anticipated that several dietary
factors will affect them. In addition, even if preformed arachidonic acid and DHA
are provided, they can also be oxidized as fuels or undergo peroxidation before
incorporation into phospholipid or other functionally relevant cellular pools. Thus,
in evaluating the effect of lipid composition of the diet, one should consider not
only the arachidonic acid and DHA content of the formulation but also the balance
of LC-PUFA precursors, antioxidants, and other fatty acids that may affect LC-
PUFA metabolism.

Controlling Major Diet-Related Confounders

Energy Balance

Energy balance and the provision of energy substrates are major determinants of
fatty acid oxidation rates. If the infant is in negative energy balance, a greater propor-
tion of the absorbed linoleic acid and LNA will be oxidized. The provision of me-
dium-chain triglycerides may be critical to spare EFA and LC-PUFA from oxidation.
The full extent of this phenomenon has not been evaluated, but the available informa-
tion indicates that LNA is preferentially spared from oxidation, while C16 and
shorter-chain fatty acids are readily oxidized (5).

Overall Organ Function

The elongation and desaturation pathway is dependent on intact function of several
cellular organelles. The endoplasmic reticulum is responsible for the desaturation
steps, whereas elongation requires availability of activated acetate from mitochon-
drial energy metabolism, and the final partial β-oxidation necessary to form DHA
occurs in peroxisomes. Potential physiological or pathologic conditions that affect
the function or maturation of these organelles may also affect the biological response
to a given essential fatty acid or LC-PUFA intake. In addition, desaturation and
elongation enzyme systems are dependent on the availability of a variety of cofactors. Metals such as iron, zinc, and copper and cofactors such as coenzyme A, cytochrome C, NADP, and NADPH are all necessary for the endogenous synthesis of arachidonic acid and DHA from linoleic acid and LNA. The selection of human subjects in terms of gestational age, energy balance, micronutrient status, disease condition, and liver and other organ function may all be critical in determining the biological response to dietary essential fatty acid supply.

Need for Antioxidant Protection

The need for antioxidant protection is increased according to the number of double bonds present in the fatty acid. Thus, the balance of PUFA + LC-PUFA to tocopherol and ascorbate is important to preserve the unsaturated bonds. This should be considered in designing the formula product as well as in the evaluation of biological effects. Sufficient antioxidants must be present in the formula and in the body to minimize peroxidation of the LC-PUFA. If peroxidation of LC-PUFA is not prevented, these compounds will be lost, and potential damage to membranes by the products of peroxidation may occur.

Digestibility of Fat Sources and Specific Fatty Acids

Further considerations need to include limited bioavailability of LC-PUFA in formula compared to human milk. Because human milk contains bile-acid-stimulated lipase capable of enhancing LC-PUFA digestion and assimilation into tissue phospholipids, addition of LC-PUFA in excess of that in human milk or addition of human milk lipase to formula may need to be considered (19). This issue also affects the source of LC-PUFA, where positional isomerization of the acyl group can influence metabolic utilization. Thus, overall digestibility and specific LC-PUFA utilization should be considered in evaluating the effect of diet on an infant’s LC-PUFA status. Recent evidence suggests that LC-PUFA in formula are better absorbed than in fortified pasteurized human milk (44).

Controlling Major Clinical Confounders

The selection of subjects is a critical issue in the design of studies to define the effects of LC-PUFA on neurodevelopment. Randomized controlled clinical trials using identical study formulas except for the addition of the test fatty acids are necessary to establish effects. The evaluation of human-milk-fed infants in comparison to formula-fed infants should not be used to support a relationship between a specific compound present in human milk, such as LC-PUFA, and developmental outcome.
Using "Healthy" or Truly Representative Experimental Subjects

Selection of relatively healthy infants has the advantage of providing results that are not confounded by intercurrent illness or morbidity, which may affect the developmental outcomes or metabolic handling of EFA. On the other hand, if the study is intended to address the needs of preterm infants, selecting a "healthy" subgroup may not reflect the population of interest, limiting the external validity of the study. If a representative sample of a clinically heterogeneous population is used, the variability in developmental outcomes will most likely be greater; this should be considered in the estimation of sample size. Initial studies to explore the relationship between diet and neurodevelopment may consider using select subgroups, but if the purpose is to modify feeding practices, a true representative sample with very few exclusion criteria is necessary to establish efficacy and safety of a new feeding regimen. Randomization methods need to assure that the chance to enter the experimental or control groups is truly random. Specific gestational age and possibly birth weight strata as well as gender should be considered to assure randomness in the distribution of these intervening variables. If in the course of a randomized controlled clinical trial, a new treatment that significantly modifies survival or complications becomes available, the randomization scheme should block subjects according to time of entry in order to assure that interactions are appropriately evaluated. The randomized controlled clinical trial is indeed the only way to scientifically define optimal feeding on the basis of safety and efficacy.

The Human-Milk-Fed Reference Group

It is important to define reference groups if the experimental diet is better, the same, or worse than the standard feeding regimen. The human-milk-fed infant appears to be an obvious reference control group to compare the relative effects of diet on growth and development. Unfortunately, it is not possible to randomize infants to human milk feeding because this practice requires the cooperation of the mother, and success is not always achieved. Most studies that use human-milk-fed reference controls match the groups by the main confounding variables of birth weight, sex, gestational age, and presence of morbidity. Maternal characteristics that affect growth and central nervous system (CNS) development, such as maternal height, socioeconomic status, and educational level, are recorded and used in post hoc analysis. Unfortunately, these confounders are usually biased in favor of the human-milk-fed group. Covariance adjustments are performed but cannot truly correct for group differences. A truly randomized trial of human milk feeding to define a reference population is ideal but not feasible.

Term Infants as a Reference Group in Studies of Preterm Infants

In evaluating developmental outcomes of premature infants, the use of controls born at term provides another valid reference group. The preterm group given the
Defining Outcome Variables

The selection of outcome variables is another key step in determining the effect of LC-PUFA on infant neurodevelopment. Biochemical and functional responses may be used to define biological effects. If both are compared in a correlation analysis, one can use biochemical effects as a proxy to predict functional outcomes. The timing of measurements is crucial because effects may be found only within a given developmental time interval. The duration of the effect should also be characterized; enhancing or accelerating a maturational or developmental milestone may not necessarily be better. The true validation requires either a reference group or a sufficiently long follow-up to assure that the final outcome is indeed modified. In the following sections, we analyze the most common outcome variables used in LC-PUFA clinical trials.

Changes in Plasma and Red Blood Cell Membrane Composition

The effect of feeding lipids with specific fatty acid compositions will usually result in measurable differences in plasma lipid composition. For example, the fatty acid composition of triglycerides in plasma will reflect the fatty acid composition of recent lipid dietary intake, but plasma phospholipid composition will more probably reflect tissue pools with functional significance. The selection of plasma phospholipid or red blood cell biochemical indices of fatty acid intake is based on the fact that under deficiency conditions they have been found to be correlated to tissue composition and can readily be measured. The triene/tetraene ratio (that is, 20:3 \( n - 9 \) to 20:4 \( n - 6 \)) is useful for defining the overall essential fatty acid deficit. The DPA--DHA ratio (22:5 \( n - 6 \) to 22:6 \( n - 3 \)) has been related to overall \( n - 3 \) deficit. The use of absolute concentrations will better reflect true changes in blood lipid composition; on the other hand, values expressed as percentages of total lipids may be better indicators of available tissue pools. For example, the relative content of arachidonic acid and DHA blood lipids (percentage of total) drops markedly postnatally in formula-fed infants, yet the absolute concentrations per unit of volume fall only slightly after birth in formula-fed infants and rise in the human-milk-fed infants (45).
Composition of Neural Tissues

The composition of neural tissues such as the retina or brain cortex is clearly of greater interest but cannot be measured in humans. The study of infants who died suddenly of an unexplained cause has served to document a good correlation between the composition of the brain cortex and red blood cell total lipids (46). Two studies have examined neural tissue levels of LC-PUFA in formula-fed compared to human-milk-fed term infants (46,47). Farquharson and colleagues found 25% lower levels of DHA in cerebral cortex phospholipids from formula-fed infants than in those from human-milk-fed infants (47). Makrides et al. reported significantly lower levels of DHA in red blood cells and cortex but not in retina or basal ganglia of formula-fed term infants than in human-milk-fed infants. The DHA in cortex was dependent on age and duration of breast-feeding (46). The results indicate that DHA supply for brain accretion is critical during the first months of life: LNA conversion to DHA appears to be insufficient, although linoleic acid conversion to arachidonic acid is sufficient, based on compositional data (47,48).

Functional Response of the Retina and Brain Cortex

The functional response of the retina and brain cortex can be measured by electrophysiological or behavioral methods. The latter outcomes are potentially of greater interest but are more variable and difficult to measure. The selection of these indices should be based on the developmental stage being evaluated and the sensitivity and specificity of the response. If the wrong outcome measure is selected, no effect may be found. For example, behavioral assessment of visual acuity (by forced-choice preferential looking, FPL) is highly variable and is difficult to obtain before 40 weeks postconceptional age. On the other hand, visual evoked potential (VEP) acuity measurements can be obtained as early as 36 weeks; furthermore, these later acuity estimates are less variable. The VEP measurements may reveal a significant effect of diet, whereas the FPL assessment may yield no effect. Careful selection of specific times to conduct infant testing is critical; the test must be given at a time when rapid development is occurring, and nutritional influences will affect the function being examined. The need for normative data obtained with rigorously defined testing protocols is essential if subtle differences in visual outcome are to be detected. Most studies to date have been limited to retinal function and indices of visual development. Some studies have included measures of mental and motor development at 12 and 18 months. Studies of the effect of LC-PUFA on sleep–wake cycle development, sympathetic tone, auditory evoked responses, and activity levels are under way in our laboratories. In these investigations we have verified that sleep state, an often ignored factor, is an important determinant of electrophysiological responses such as electroretinography (ERG) and VEP (49,50).
SUMMARY OF EFFECTS

Recent clinical trials convincingly support modifications in the LC-PUFA composition of preterm infant formulations to reflect that of human milk. Comprehensive clinical studies have shown that dietary supplementation with marine oil results in increased blood levels of DHA as well as an associated improvement in visual function in formula-fed premature infants to match that of human-milk-fed infants (16–19).

Published observational studies comparing functional outcomes of human-milk- and un-supplemented formula-fed infants are summarized in Table 1. Randomized controlled studies that have included only biochemical measures are summarized in Table 2. We focus our discussion on published results of randomized controlled clinical trials that have included both biochemical measures and functional assessment of neurodevelopment, summarized in Table 3.

Preterm Infant Studies

In our studies, preterm infants received human milk or were randomized to one of three formulas. The formulas were based on: (a) corn (maize) oil, which provided 24% of total fat as linoleic acid (18:2 n – 6) and 0.5% LNA; (b) soybean oil, 21% linoleic acid and 2.7% LNA; and (c) soybean and marine oil, 20% as linoleic acid, 1.4% as LNA, 0.65% as EPA, and 0.35% as DHA. The corn oil and soybean oil diets provided no LC-PUFA, and the marine oil diet provided no arachidonic acid. Significant results of this study included a marked inability of the corn oil formula to support the necessary accumulation of LC-PUFA in plasma and red cell lipids. Supplementation of n – 3 LC-PUFA was sufficient to increase red cell DHA concentrations in the marine-oil-fed group to levels two- to fivefold higher than in the corn oil and soybean oil groups (17,18). The functional impact of this fatty acid modification included significant maturation of ERG responses in marine-oil-fed infants compared to the corn oil and soybean-oil-fed infants at 36 weeks postconceptional age (Fig. 2). Equivalent ERG thresholds (the minimum amount of light required to elicit a given retinal response) were found in the marine-oil- and human-milk-fed infant groups. The soybean-oil-fed infant group presented intermediate values. By 57 weeks postconceptional age, a time when retinal development is nearly complete, the corn-oil-fed group recovered in most indices of retinal function except in oscillatory potentials (Fig. 3). Visual acuity tests that measure higher neural activity such as cortical function (for example, pattern-reversal VEP) or cortical plus motor function (for example, FPL) were less mature in the corn oil and soybean-oil-fed groups throughout the 6-month study (17,18). The LC-PUFA-supplemented marine oil group had significantly better visual acuity as measured by VEP and FPL than the n – 3-deficient corn oil formula group, whereas visual acuity in the soybean-oil-fed group was intermediate (Figs. 2 and 3). Highly significant correlations were
found for both VEP and FPL visual acuity when compared to the level of DHA in multiple lipid fractions from study infants (Figs. 4 to 6). No significant differences in growth indices were evident among the three groups despite reduced levels of arachidonic acid in the marine oil group, probably attributable to EPA accumulation (two- to fourfold higher than other groups). Body length and weight in the LC-PUFA supplemented group were not reduced (16), in contrast to the observations of Carlson (28). The lower arachidonic acid levels, in part related to fish oil supplementation in her studies, were associated with poorer growth. Differences in subject selection criteria and in formula composition (higher linoleic acid and insufficient mineral and vitamin content), as well as the longer duration of LC-PUFA provision in her study (9 months versus 4 months), may explain the differences in the results.

Carlson's randomized clinical study in preterm infants supplemented with 0.2% DHA/0.3% EPA showed that there was better visual acuity (measured by the Teller acuity card tests) in infants up to 4 months of age. After this, control infants "caught up" in visual function measures. These investigators also report evidence of more rapid visual processing, as measured by the Fagan test of visual recognition at 6 to

**FIG. 2.** Content of DHA in total red blood cell (RBC) lipids and plasma phospholipids (PL) of preterm infants on study diets at 36 weeks postconception. *Inset:* Rod ERG thresholds (intensity units are in log scotopic troland-seconds) and VEP visual acuity in Snellen equivalents in infants studied at 36 weeks. Arrows indicate significant differences between diet groups using Newman-Keuls multiple comparison analysis (\( p < 0.05 \)).
FIG. 3. Content of DHA in total RBC lipids and plasma phospholipids (PL) of preterm infants on study diets at 57 weeks postconception. Inset: Rod ERG thresholds (log scotopic troland-seconds) and VEP visual acuity (Snellen equivalents) measured in study infants at 57 weeks. Arrows indicate significant differences between diet groups using Newman–Keuls multiple comparison analysis ($p < 0.05$).

12 months of age in LC-PUFA-supplemented infants (29). The reduction in blood lipid levels of the $n = 6$ LC-PUFA, arachidonic acid, when fish oil was provided as a source of $n = 3$ fatty acids was a significant finding in this study. The reduction in arachidonic acid was associated with reduced weight and length growth ($r = 0.27$ to $0.53$, $p < 0.05$) (28). Similar correlations have been reported by others, yet no study has specifically tested this hypothesis prospectively (51,52).

The issue of a direct benefit to visual and cognitive function of specific DHA formula enrichment (using low-EPA marine oil) has been addressed in Carlson’s second preterm infant study, where infants were fed for up to 2 months corrected age (32). This study showed improved visual development at the 2-month follow-up and a 10-point IQ difference favoring the DHA-supplemented group at 12 months. No significant drop in arachidonic acid or deleterious effects on growth were observed when low-EPA marine oil was used. The DHA-supplemented group had shorter look times in the novelty preference test at 9 months, suggesting better visual processing. Weight-for-length indices and head growth were lower in the DHA-supplemented infants (33). We are presently completing a study using formulas
containing DHA and DHA plus arachidonic acid to confirm whether DHA supplementation by itself is sufficient or if DHA plus arachidonic acid is needed to optimize efficacy and prevent deleterious effects.

Additional preterm infant studies by other laboratories confirm the need for LC-PUFA enrichment of formula to maintain blood levels of \( n = 3 \) and \( n = 6 \) LC-PUFA in these "at-risk" infants (20,36,37). These data are summarized in Tables 1 through 3. Pending issues regarding LC-PUFA provision to preterm infants currently include defining the following:

1. The need to supplement with both DHA and arachidonic acid, and whether there are other fatty acids that could replace them.
2. The amount of LC-PUFA to include in the formula.
3. Duration of supplementation.
4. What sources of LC-PUFA should be used, considering safety and costs.

FIG. 4. Correlation coefficients of rod threshold measured at 36 weeks postconceptional age and DHA relative concentration in red blood cell and plasma lipid fractions. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI + PS, sum of phosphatidylinositol and phosphatidylserine; P.Lipid, total phospholipids; TG, triglycerides; CE, cholesterol esters; FFA, free fatty acids. Higher concentrations of DHA in plasma lipid fractions were associated with lower rod threshold values (light intensity required to elicit a 2-μV b-wave response)—that is, less light was needed.
FIG. 5. Correlation coefficients for visual evoked potential (VEP) acuity measured at 36 weeks postconceptional age and DHA relative concentration in red blood cell (RBC) and plasma lipid fractions. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI + PS, sum of phosphatidylinositol and phosphatidylserine; P.Lipid, total phospholipids; TG, triglycerides; CE, cholesterol esters; FFA, free fatty acids. Higher concentrations of DHA in RBC PC and in plasma lipid fractions were associated with lower values for minimum angle of resolution, that is, better visual acuity.

Term Infant Studies

The controversy regarding the possible need for \( n = 3 \) and \( n = 6 \) LC-PUFA in human milk or term infant formula has gained increasing attention over the past year. The primary issue involves the putative need for LC-PUFA supplementation in the diet of an otherwise healthy term infant and whether this modification would promote optimal development of visual function.

Initial observational studies by us in Dallas were the first to compare visual outcomes of full-term infants fed exclusively on human-milk- or corn-oil-based formula. We documented significantly better VEP acuity (20/65 versus 20/83) and FPL acuity (20/107 versus 20/129) in the human-milk-fed group at 4 months of age (22). We also reported follow-up results on 3-year-old children in whom diets were controlled throughout the first year of life on a dietary regimen of human-milk- or corn-oil-based formula. In a series of near and far visual recognition tests and operant FPL acuity test, all human-milk-fed infants tended to have better acuity than formula-fed infants, but the results did not achieve statistical significance \( (p < 0.1) \). More
refined visual tests using random dot stereoacuity and HOT letter matching were significantly better ($p < 0.05$) for the human-milk group, indicating an association between early feeding and more advanced visual function later in life. A parallel observational study by Makrides et al. reported that full-term human milk-fed infants at 6 months of age had better VEP acuity (20/52 versus 20/110) than formula-fed infants (24). Similarly, red cell DHA concentrations were also higher in the human-milk-fed group (6.16% versus 3.31%) and were significantly correlated with VEP acuity ($r = -0.65$; $p < 0.01$).

Several research groups have recently reported results of controlled trials comparing infants fed formulas with and without LC-PUFA supplementation. Makrides et al. compared in a controlled randomized study infant groups that had received human milk for more than or less than 16 weeks or formula either with LC-PUFA supplementation or without LC-PUFA (24). Infants given formula supplemented with 0.36% DHA, 0.58% EPA, and 0.27% GLA had significantly higher red cell DHA concentrations than standard-formula-fed infants at 6, 16, and 30 weeks of age. The VEP acuity was significantly better in the supplemented group at 16 weeks (20/63
versus 20/110) and 30 weeks (20/18 versus 20/56), with no evidence of "catch up" by the LC-PUFA-deficient infants. Red cell DHA concentrations and VEP acuity were significantly correlated at 16 weeks ($R^2 = 0.23$) and 30 weeks ($R^2 = 0.12$). A dietary supply of DHA was recommended for the first 6 months of life because infants receiving less than 16 weeks of human milk had significantly poorer VEP acuity at 30 weeks of age than those fed human milk throughout their first 6 months (31).

In another controlled study of early cognitive function, Agostoni et al. compared full-term infant groups fed human milk, standard formula, or formula enriched with 0.3% DHA, 0.4% arachidonic acid, and 0.3% 18:3 $n-6$ (30). At 4 months of age, red cell DHA concentrations in the standard-formula-fed group were significantly lower than those of the human-milk and LC-PUFA-supplemented groups (1.8%, 4.1%, and 4.1%, respectively). These investigators found significantly worse scores at 4 but not at 12 months in the Brunet–Lezine psychomotor development test for the DHA-deficient formula-fed group (30,53). In this study, LC-PUFA added to the formula originated from egg yolk phospholipids; thus, formulas differed in cholesterol content and in choline phosphoglycerides. Carlson recently reported that infants receiving formula supplemented with 0.1% DHA and 0.43% arachidonic acid had better visual acuity than an unsupplemented formula group at 2 months (20/220 versus 20/315) but not at 4 months according to the Teller acuity card (TAC) procedure. Thus, the DHA-deficient infants are able to "catch up" with the LC-PUFA-supplemented infants. No differences in acuity were found at 6 and 12 months between groups (35).

Several other investigators have presented early results of observational studies of human milk versus formula-fed term infants. A consensus finding is that both DHA and arachidonic acid concentrations are reduced in formula-fed infants; however, there are conflicting results regarding the potential benefits to visual function of human milk feeding in full-term infants compared to formulas not containing LC-PUFA. Courage et al. report that infants receiving an evaporated cow's-milk-based formula had significantly poorer visual acuity as measured by the Teller acuity card test than human-milk-fed infants at 3, 6, and 18 months and that there was no evident catch-up phase by the $n-3$--deficient group (26). There was a trend toward lower acuity in standard formula-fed infants compared to human milk-fed infants in each age group. Jorgenson and colleagues report better Teller card acuity at 2 and 4 months but not at 1 month for human milk-fed infants compared to a formula-fed group (27). Higher red cell DHA concentrations in the human-milk group paralleled better visual acuity. Innis reported trials comparing Teller-card-derived acuity in human-milk-fed and standard-formula-fed groups (25,54). In a prospective non-randomized study, infants receiving a controlled formula had a reduction of DHA levels in red cell phosphatidylethanolamine of 47% compared to human-milk-fed infants at 3 months of age (25). Teller visual acuity was 20/152 in the human-milk group and 20/126 in formula-fed infants. In a second prospective study, term infants provided formulas with two levels of LNA (4.7% or 1.9%), with linoleic acid/LNA ratios of around 8:1, were compared to a human-milk-fed group. Although plasma
and red cell DHA concentrations were significantly lower in the formula-fed groups, visual acuity, as measured with the Teller acuity card procedure, was not influenced by diets at 3 months of age. In a third retrospective study, 327 infants receiving human milk in graded duration from less than 1 month to 9 months were compared to 38 mixed formula-fed infants by the Teller acuity card procedure and Fagan recognition test (55). No significant differences were found between the groups in visual acuity or cognition; however, boys had significantly better visual acuity, and girls scored better on the Fagan infant test.

Results obtained by Neuringer et al. and Auestad et al. have been contradictory (21,56). Infants in a yet unpublished trial received (a) standard formula, (b) formula supplemented with 0.2% DHA from low-EPA marine oil, or (c) formula with 0.12% DHA and 0.43% arachidonic acid. No significant differences could be detected among the three diet groups by ERG at 4 months or by the Bayley Scales of Infant Development test at 12 months. Furthermore, a negative correlation was demonstrated between red cell DHA at 4 months and language development assessed by the MacArthur Communicative Development Inventory given at 14 months. Language development skills in these infants were negatively correlated with red cell DHA concentrations ($r = -0.20$ to $-0.37$, $p < 0.05$) (21). This finding could be associated with the source of DHA, but a clear interpretation is lacking, and it warrants further investigation.

Assessment and analysis of these divergent vision and cognitive function developmental test results are complex and confounded by differences in experimental design and other methodologic issues addressed previously. The most frequent confounders are the selection of test diets and the definition of outcomes measures of neurodevelopment.

Studies reviewed in this chapter provide evidence that dietary $n - 3$ fatty acid deficiency affects eye and brain function of preterm infants as measured by ERG, cortical VEP, and behavioral testing of visual acuity. Preterm infants require DHA in their diet because they are unable to form long-chain derivatives in sufficient quantity from LNA provided by soybean-oil-based formula products. Provision of dietary $n - 3$ and $n - 6$ LC-PUFA results in discernible differences in the fatty acid composition of plasma and red cell membrane lipids. Changes in membrane chemical structure are probably responsible for the observed functional effects. Preliminary evidence from term infants suggests that DHA supplementation is also required by this group.

**BIOLOGICAL SIGNIFICANCE OF THE EFFECTS**

The presence of an effect does not in itself make it of biological significance. As pointed out, diet-induced changes in triglyceride composition are of little consequence except to validate compliance with study formulas. Moreover, tissue fatty acid pools, particularly those required for retinal and CNS neural membrane formation, may not be reflected by plasma or even red blood cell fatty acid composition.
For example, we have found that in response to a low–n−3 diet, the DHA concentration in plasma and red cells falls steadily during the first 6 months postnataally; however, though the diet-induced alteration in rod b-wave threshold measured by ERG can be documented early (that is, at 36 weeks postconceptional age), after 4 months on the n−3–deficient diet, threshold values are similar to those of the control group receiving DHA or of a human-milk-fed reference group. The biochemical effects cannot be used as surrogates for functional measures; only in the presence of established correlations could one use the biochemical indices as predictive.

The selected outcome measure will ideally be clinically relevant, but the sensitivity of clinical responses is usually low; thus, functional responses are considered valid to define biologically significant outcomes. In the case of LC-PUFA supplementation, growth is affected only in extreme n−6 fatty acid deficiency and thus is not considered a sensitive measure of n−6 sufficiency. The effects of n−3 fatty acids on sensory maturation and cognitive development are the outcomes of greater interest in studies of n−3 supplementation.

The duration and reversibility of diet-induced effects is another important consideration. In evaluating diet-induced changes in developmental outcomes, there may be transient effects that reflect the acceleration or the slowing of a maturational process with a fully normal final outcome. This is of special relevance during the first few months of life, when visual maturation is progressing rapidly. Several studies have shown significant effects of the dietary LC-PUFA on visual maturation in the first 4 months of life, but in most cases the delayed response becomes normal at 6 months or at most by 1 year of age. Should we dismiss this phenomenon as being transitory and of limited significance or assume that we failed to detect a significant change at a year because our tools were not sensitive enough or that other related functions are indeed affected? In the same example, we failed to detect differences in visual acuity at 6 months, but space perception, assessed by stereovisual responses, was different at 3 years of age. These examples illustrate that unless sensitive outcome measures are used and sufficient follow-up time is provided, there may actually be long-term consequences of early developmental effects.

Evidence of potential beneficial long-term effects of DHA supplementation on brain development of term infants is suggestive; however, proof is lacking. The resolution of this issue should be forthcoming because controlled clinical trials of DHA and DHA–arachidonic acid supplementation in term infants are now being completed. The follow-up of these infants beyond infancy should help to address the question of long-term effects.

CONCLUSIONS

The LC-PUFA have demonstrable benefits during development. The effects on neural development are of particular interest. Human milk is the best and only time-proven source of fat and EFA in the infant diet. Technological procedures based on chemical and physical separation of the unsaturated fatty acids have permitted the
elaboration of concentrated DHA and arachidonic acid for clinical use. The development of single-cell oil sources has allowed the provision of novel forms of LC-PUFA delivery. Before the 1990s, low LNA was found in most infant formulas; by now virtually all infant formulas in developed countries are supplemented with LNA, and several manufacturers in Europe and in Japan have added DHA or DHA plus arachidonic acid, and some have included GLA in preterm and term formulas. Efficacy seems less well established. The need for comprehensive safety evaluation is underscored before the practice of LC-PUFA supplementation can be advocated. Safety issues have been addressed in small to medium-size studies; larger-sample-size trials are required to identify potential side effects that are of low prevalence. Moreover, the public health implications of the beneficial effects need to be fully evaluated in order to support the practice of supplementing infant formula and possibly maternal diets on a global scale.

REFERENCES


DISCUSSION

Dr. Aggett: In what way would you regard the development of research protocols for looking at LC-PUFAs as a paradigm for many of the issues we have been addressing? For example, Dr. Lozoff has told me that if we had totally accepted some of the first studies on iron intervention, we would not necessarily have followed it up and pursued it more closely.

Dr. Uauy: Our knowledge is at various degrees of advancement; in fact, it is only this year that we have been able to say with certainty that DHA can be formed from ω-linolenic acid. Thus, even the biology of today is different from what we were learning 5 years ago, so study design has to reflect these rapid changes. We now have stable isotopes to see if we can enhance DHA formation from ω-linolenic. That question has been partly answered by Bill Heird and his group; they showed that no matter how much ω-linolenic they gave, they were unable to provide sufficient DHA for biochemical normality. So that is the first step, but there still needs to be more work before we do a definitive clinical trial. Now as to outcome, I would use state-of-the-art electrophysiological measurements to look at visual
acuity. I would include state-of-the-art noninvasive methods to look at electroretinography. After I have those outcomes properly established, I would then look at the long-term effects. I think we are still not ready to do the definitive clinical trials.

Dr. Klish: In Houston, Heird and Jensen have shown that the elongation of the $n-3$ and the $n-6$ series shares the same enzyme system and, as a result, there is competition between those two cascades (1). That brings up the question of the potential for toxicity if the ratios of those two groups of fatty acids are not correct. There are also data that suggest there is a potential for growth retardation in term infants (2). So, knowing this new biology, how comfortable do you feel at this point about using long-chain polyunsaturated fatty acids in clinical trials before we actually understand the relationship between these ratios?

Dr. Uaay: The evidence we have from preterm infants indicates that if we provide a DHA source with low EPA, we do not have any adverse effect on growth. There are at least three studies that support what I am saying (3–5). If you give a very high linolenic acid intake, which is the model that Bill Heird uses, then you may compromise growth, or if you use high-EPA marine oil, then you also have a drop in arachidonic acid, which is the most likely mechanism. It is not a new finding. If you go back to Hansen’s original data, low arachidonic acid compromised growth in infants in 1954, when he studied them.

Dr. Klish: What you are really saying is that there is a pharmacologic effect, and what we don’t know is the window of safety. We have some studies that are in the normal range and some that appear outside that range, and there are different ways of supplementing these fatty acids, none of which totally controls the ratios.

Dr. Whitehead: I wonder if I could just ask you about the term “pharmacologic effect.” Are you looking on this as pharmacology, or are you looking on it as nutrition?

Dr. Uaay: I didn’t use the term pharmacologic effect. I think Dr. Klish meant outside the range found in human milk, but I had problems convincing the NIH reviewers that the safety components of the study needed to be included because, in fact, we were using amounts that are present in human milk.

Dr. Guesry: Your paper showed very clearly that there is no need to add arachidonic acid, even in premature babies, provided that you don’t give too much DHA and that you give as little EPA as possible, but then in your comment, you said, “You can add arachidonic acid if you like.” I realize you want to be nice, but why?

Dr. Uaay: This is a very important issue. Until we have data on DHA alone, low-EPA DHA, and DHA plus AA (arachidonic acid), I cannot provide you with the definitive answer, because there are other people saying that if arachidonic acid is in breast milk, then it should be added. I think the stable isotope work that I showed you will probably give us the answer, because accretion of arachidonic acid by the infant is double what is provided by human milk; the human milk-fed infant is synthesizing at least 50% of the arachidonic acid from linoleic acid. The functional data that we have, using carefully defined groups, show that the drop in arachidonic acid that we get, which is about 20%, has no influence on growth. But I have to accept that, in Carlson’s study, in which infants also had low zinc and low vitamin A and were as small as 700 g, the adverse effect on growth was apparently associated with the low arachidonic acid rather than the DHA supplementation.

Dr. Hamburger: Are you not implying by what you just said that you would have to have the exact proportions of all the lipids that are found in breast milk in order to not deviate from what the baby may be doing in the way of processing?

Dr. Uaay: The breast milk model offers you a wide range: you can go as low as 0.1 or as high as 1.2 with DHA, and as low as 0.2 or as high as 2.5 with AA. So human milk does not offer you exact numbers, but it provides a range, especially if you take the omnivorous
woman as a model, against which you can test your biochemistry and your function and hope to get a good answer.

Dr. Rey: I don’t understand why you continue to study the effect of corn oil or soybean oil or marine oil in preterm and term infants. It is known that the best ratio of \( \frac{\omega-6}{\omega-3} \) is around 6:1 to 10:1. Why don’t you only compare human milk with formulas with that ratio? Why are people working in this field continuously trying to prove that there is an advantage in a formula that differs so much from human milk? My second question is, what are the long-term effects you suggest?

Dr. Uauy: The studies we are doing at present are exactly as you have suggested; the only difference is that we are now using a pure DHA source or DHA plus AA to try to get the answer. There is no purpose in just adding AA. So our present studies include DHA only, with appropriate \( \alpha \)-linolenic and appropriate linoleic acids, a DHA plus AA, and a formula that has no LC-PUFAs and has the right balance. The preliminary data show that we do not have an effect of the addition of AA, we have functional effects of DHA, and we have no additional beneficial effects of DHA plus AA. Now, to your other question regarding long-term effects. The infants we studied in Dallas are now about 8 years of age. Out of the 80 infants we studied, we have been able to trace only ten by conventional methods. We are paying a private detective to try to find the subjects. In the urban United States, in a county hospital, getting a 7-year follow-up is not an easy job; I would say it is an impossible job. We need those long-term studies, and it is only the people who already have systems that will allow them to follow up for 7 or 8 years who will provide us with the answers.

Dr. Crozier: I find it difficult to reconcile the evidence that the baby is capable of synthesizing his fatty acids with the evidence that the baby needs a dietary source. You have mentioned that the relative contribution of synthetic and dietary arachidonic acid was roughly 50:50. Do you have any kind of estimate of what these numbers would be for DHA? And how does the high conservation of DHA in the retina and other neural tissues factor into this equation.

Dr. Uauy: The stable isotope work is very limited. With the amino acid, we know the precursor enrichment, but here, we really have no tool to evaluate precursor enrichment, especially at the tissue level, so we have to make a lot of assumptions. We are assuming that what we measure in the plasma reflects tissue equilibrium. We are assuming that we have steady-state conditions so that the kinetic models can be applied. And third, we have no measure of oxidation rates because we have no tool at the present time to measure how much of what we give is oxidized. So any figures that I could give you are really the minimum conversion, but for DHA, only 3% of the label appears as long chain over a 6-day period, as opposed to 60% of the label when linoleic acid is fed. The other source of data is the accretion data. These data show that it is impossible for a baby who is fed human milk to get all the arachidonic acid he or she needs from human milk. So that is a different set of observations. My view is that the amount of DHA in human milk is enough to satisfy 100% of the accretion need. From the accretion data and the stable isotope work, it looks as though the \( \omega-3 \) component is the most limiting. Your other question is crucial, because our data suggest that even when there are very small amounts of DHA in the plasma, the retina seems to manage perfectly well. We know from studies of turnover that if you inject labeled DHA in an eye, 99.5% of the label remains in the eye and does not enter the circulation. So the eye definitely traps it. We know there is recycling of segment and pigment epithelium, so that the cell turnover takes up all the DHA that is released with the segment renewal, which happens on a daily cycle. So the brain, and especially the retina, is very well protected; once it loads up with DHA, it is hard to lose it, that is why you don’t find DHA deficiency in mature animals or mature humans, no matter for how long they are given a low-DHA diet.
Dr. Lucas: I have a comment about the mimicability of human milk. The first thing to say is that the presence of something in breast milk doesn’t imply that it is important; the most prevalent component of human milk is lactose, present at 70 g per 1000 ml, and not considered to be an essential nutrient. But as far as human milk goes, there are 160 fatty acids, over 100 triglycerides, a unique stereo isometric structure, fat globules with a complex glycochalin over them, and so on; human milk is completely unimicible, and the fats that we have available to add LC-PUFAs are unphysiological ones that are not present in milk, for instance containing three arachidonates or three DHAs on the triglyceride molecule, which you will never have in human milk. So that does pose a problem. If you want to hypothesize that adding DHA to humans would improve visual development, what you would do is to look at the comparison of breast-fed and bottle-fed babies. There have been about 12 studies of this type, and as far as I can see, it is only in the studies where there has been a low linolenic acid content in the formula that there has been a difference between the breast-fed and the formula-fed group. When there has been a linolenic acid content greater than 0.7% of energy, then differences have not been observed. So on the basis of published data, would you in fact hypothesize that there would be a difference from adding DHA as opposed to the precursor?

Dr. Uauy: Our data using visual evoked potentials do show an effect, so with more subtle tools, you can pick up differences between formulas relating to α-linolenic acid content. But there is really no long-term study; you are correct. I fully agree that trying to mimic human milk is impossible, though you can vary the degree of closeness of matching.

Dr. Crozier: From the industry point of view, I feel I have to ask some questions about feasibility, and particularly in relation to the sourcing of these fatty acids. Professor Klish mentioned synthetic fatty acids, and I think he was referring to the single-cell oils that are enriched sources of fatty acids but, of course, are not pure sources—they are accompanied by a number of other compounds, for example, phytosterols and other fatty acids, which may interfere with the metabolism of these particular fatty acids. Do you have any comments about the safety of these sources?

Dr. Uauy: The sources need to be tested in all aspects: biological activity, toxicity, animal work, adult human work, and eventually infant work. We need more safety data, and the safety data will have to be obtained from sources that are economically feasible and industrially available. I agree with you: you first have to define a source and then test it and do your clinical trials.

Dr. Rey: The Scientific Committee for Food (SCF) of the European Union has prepared guidelines on the safety of novel foods. It is clear that DHA and AA prepared from single cells should be considered as novel ingredients according to these guidelines. This DHA was accepted in Holland, but I would not say what the opinion of the SCF would be regarding the acceptability of such fatty acid sources in the European Union. The use of novel ingredients in infant nutrition cannot be accepted without all the guarantees of safety.

REFERENCES


