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CALCIUM SUPPLEMENTATION IN PREMATURE INFANTS FED BREAST MILK: The Effect of Timing of Calcium Supplementation on Calcium, Phosphorus, Magnesium and Fat Balance and on the Absorption Coefficients of Individual Fatty Acids

BY

Yiping Wang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CALCIUM SUPPLEMENTATION IN PREMATURE INFANTS FED BREAST MILK: The Effect of Timing of Calcium Supplementation on Calcium, Phosphorus, Magnesium and Fat Balance and on the Absorption Coefficients of Individual Fatty Acids submitted by Yiping Wang in partial fulfillment of the requirements for the degree of Master of Science.

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TO MY MOTHER FOR HER LOVE AND SUPPORT

ABSTRACT

1. Calcium and fatty acid absorption in premature infants can be affected by calcium soap formation with fatty acids in the intestinal lumen following oral calcium supplementation. Three seventy-two-hour balance studies were conducted in sixteen pair-matched premature infants with a gestational age of 30.8 ± 1.4 weeks and a birthweight of 1329 ± 168 grams. Calcium, phosphorus, magnesium, fat balance and the absorption coefficients of individual fatty acids were measured in infants given calcium supplementation between feedings and in infants given calcium supplementation with feedings of expressed preterm human milk.
2. Calcium and phosphorus content of preterm human milk (PTM) decreased gradually in the first four weeks of life.
3. Calcium lactate (1.3 mmole/Kg/day) administration between feedings significantly decreased phosphorus absorption and retention, magnesium retention, and increased total magnesium loss compared to giving calcium with feedings. Calcium absorption tended to be impaired in infants receiving calcium between feedings, although this was not statistically significant.
4. Total lipid and fatty acid C14:0, C16:0, C18:0 and C18:1(9) absorption was also decreased by calcium supplementation between feedings when compared to supplementation with feedings. However, the differences were not statistically significant. Infants given calcium between feedings seem to have gained less weight compared to infants receiving calcium with feedings, especially in the second

postnatal week, although it was not statistically significant.

5. A significant correlation between calcium intake and calcium loss in stool was observed. In both feeding regimens, fecal lipid was positively correlated with calcium and phosphorus content in the stool. Calcium and phosphorus excretions positively correlated with magnesium in the stool in infants given calcium between feedings. In infants receiving calcium with feedings, fecal calcium and magnesium positively correlated with phosphorus concentration in the stool.

6. The percentages of neutral lipid, calcium salts, free fatty acids and compound lipid in stool were 54.4%, 2.5%, 21.5%, and 21.7% respectively. Neutral lipid excretion increased and free fatty acids decreased gradually during the first four weeks of life, although they were not statistically significant.

7. It is concluded that intraluminal hydrolysis of milk lipid might be less complete when supplementing calcium between feedings. Timing change of calcium supplementation from with feedings to between feedings may also complicate divalent ion interaction within the intestinal lumen and eventually the loss of calcium, phosphorus, magnesium, total fat and certain long-chain saturated fatty acids, which would in turn affect growth in premature infants.

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Chapter I

LITERATURE REVIEW AND RATIONALE

A: Introduction

Preterm human milk has been highly recommended to premature infants for its suitable macronutrient content and immunological protection. However, calcium and phosphorus inadequacy in their mother's own milk put these infants at high risk of developing various nutritional abnormalities and eventually growth retardation. The practice of calcium supplementation in premature infants fed breast milk has been proven to reduce this risk significantly. As increased calcium intake interferes with the absorption of fat and other divalent cations, it is postulated that the change in timing of calcium supplementation from with feedings to between feedings will reduce these interactions. This chapter will review the content of certain nutrients in preterm human milk as compared to either term human milk or a commercial formula, the evidence of calcium and phosphorus deficiency in the premature infant and the effect of calcium supplementation. Factors affecting intestinal fat and calcium absorption will also be discussed, as well as intestinal phosphorus and magnesium absorption. Finally, the rationale, hypothesis and objectives of this thesis will be presented.

B: Literature Review

1. Introduction

Calcium supplementation has been found to relate to increased fecal lipid loss in numerous studies with orally fed premature infants. Following supplementation of calcium lactate the amount of calcium absorbed is significantly increased, but fecal excretion of fat increases as well, resulting in a significantly lower percentage of ingested fat being absorbed in association with calcium supplementation (1). This complication and the more appropriate calcium supplementation require further study.

2. Preterm Human Milk vs Term Human Milk or Formulas

Nutrient Compositions of Preterm Human Milk

Mother's own breast milk has been increasingly recognized as the optimal food for premature infants as a source of nutrition in the first weeks of life. When premature infants are given a mixture of fresh human milk and an infant formula, fat absorption is greater than for infants receiving the formula alone (2). In addition, comparing to banked mature human milk or infant formula, the protein and amino acid content of preterm human milk offers about 75% more protein relative to energy, enabling growth of the premature infant to approach intrauterine nitrogen retention rates. Thus a greater net weight gain is achieved at least in

human milk can be easily digested and absorbed by neonates, and improves calcium absorption as well (6). Therefore, in order to meet the estimated macronutrient requirement for premature infants to reach an intrauterine growth rate, preterm human milk is more appropriate than mature human milk during the first weeks of life.

The Superior Fat Absorption of Preterm Human Milk

The composition of preterm milk enables superior fat absorption. First, preterm milk contains more medium-chain fatty acids and long-chain polyunsaturated fatty acids that have better absorption coefficients than full-term milk (7). Second, a small amount of bile salt-stimulated lipase is present in human milk, which, to a certain extent, may compensate for the shortage of pancreatic lipase and bile salts in the premature infant and help to hydrolyze fat in intestinal lumen (7). Because this enzyme is relatively heat-unstable, premature infants fed pooled pasteurized preterm milk tend to grow more slowly than those fed fresh preterm milk (8). Pancreatic lipase in the intestinal lumen preferably hydrolyzes triglycerides to beta-monoglyceride, while the bile salt-stimulated lipase in human milk does not have this positional specificity. Therefore, in infants fed human milk, beta-monoglyceride produced by pancreatic lipase is well hydrolyzed by this bile salt-stimulated lipase and thus the intestinal hydrolysis of milk lipid is relatively complete (9). Because of superior fat absorption, premature infants fed their mothers' own milk gain more weight when compared to either formula or pooled and heat-treated breast milk (3,10).

The Role of Fatty Acids in Growth and Development

Human milk contains long-chain polyunsaturated fatty acids, whereas most infant formulas lack this essential element (11). Because a significant amount of omega-3 fatty acids, especially docosahexaenoic acid (DHA), accumulates in the adipose tissue during the last trimester of pregnancy mainly through placental transfer, and preferential desaturation of long-chain omega-3 fatty acids in brain tissue occurs only after 30 weeks gestation, provision of premature infants with these fatty acids is critically important both for energy production and for synthesis of structural lipid (12,13,14).

Omega-3 long-chain polyunsaturated fatty acids are especially important in the growth and development of eyes and brain of neonates, as animal studies show that dietary omega-3 fatty acid deficiency during pregnancy and lactation affects normal vision, causes irreversible changes in electroretinograms and impairs learning ability in the offspring (15,16). It is suggested that omega-3 fatty acids in the membranes of photorecepter cells and synapses are important in biogenesis of these membranes during the perinatal period, in normal function of the tissues and in response of the nervous system to ischemia, convulsion and retinal stimulation (17). In rats, omega-3 fatty acid deficiency increases the brain's susceptibility to exogeneous toxins (18). An altered fatty acid profile of cerebroside in phospholipid in developing brain may alter myelination as well (19).

As the proportion of omega-3 fatty acids increases gradually only after 30 week gestation through the maturation of the chain elongation and

desaturation enzymatic system (14), nutrition during the first weeks of life in premature infants has a decisive effect on brain development. Numerous studies show that dietary fat modulation can change the fatty acid profile of membrane lipid in various neural cells, and therefore influence brain function and development. A diet rich in omega-3 fatty acids increases the level of omega-3 fatty acids in synaptosomal and microsomal membrane lipids in rat brain (20,21). As DHA is essential for the eye and brain function and development in premature infants, feeding premature infants a formula with a high ratio of linoleic acid (LA) to linolenic acid (LNA) (30:1) produced reduced electroretinogram responsiveness early in infancy as compared to those fed human milk or a formula with low ratio of LA to LNA (9:1). Addition of fish oil rich in long-chain omega-3 fatty acids improved some electroretinogram responses as well as visual-acuity development (22). Functional and developmental changes caused by dietary fat may be the result of changes in membrane phospholipid biosynthesis and turnover (21), receptors and transport sites located at membranes and membrane enzymes controlling metabolic processes in brain (23). These changes may also relate to the alteration in ion permeability and nerve transmission (20,21). For example, brain phospholipid fatty acid composition, phosphatidyl ethanolamine methyltransferase acitivity and rate of phosphatidylcholine biosynthesis via the CDP-choline pathway can be manipulated by omega-6 to omega-3 fatty acid ratio. Other studies showed that dietary modulation affects the de novo synthesis of phosphatidylcholine in the developing brain (21,24). Therefore, long-chain omega-3 fatty acids present in preterm human milk

per day by active transport across the placenta between 25 and 36 weeks gestation (34). Therefore approximately 60 mg calcium intake per kilogram body weight per day by premature infants (1) does not meet the calcium requirement needed to achieve intrauterine accretion rates. When calcium accumulation measured for infants fed different milks is compared with the estimated amount of calcium accumulated by the fetus of equivalent gestational age in utero, premature infants fed breast milk have an absolute dietary deficiency of calcium (1,36).

The Evidence of Calcium and Phosphorus Deficiency in Premature infants and Its Improvement after Dietary Supplementations

Calcium is an essential element for body functions. Ninety-nine percent calcium is present in body skeleton, and the other 1% is an important cofactor for neural transmission, enzyme activity, blood coagulation and other extracullular or intracellular functions. Bone mineralization can be affected by calcium and phosphorus metabolism as the endogenous store at birth and ability to absorb and excrete exogenous calcium and phosphorus in infants vary. Calcium and phosphorus deficiency, or rickets, and phosphate depletion syndrome may develop in infants receiving long term breast feeding without supplementation (29,31,38). A lower bone mineralization rate has also been recorded (39). Although calcium absorption increases with increasing postnatal age, intrauterine calcium retention rate is not achieved when infants were fed with breast milk alone (40).

These studies indicate a necessity for early calcium supplementation

in the premature infant fed breast milk (31,33,41). Improvement in bone mineral content and plasma and growth parameters have been observed after supplementation. Disorders such as rickets and phosphate depletion syndrome are readily corrected by calcium and phosphorus supplementation (42). Preterm infants receiving fortified preterm human milk show higher serum calcium and phosphorus status, lower alkaline phosphatase level, and faster weight gain, which may be partially due to better fat absorption of preterm milk as well (43-45). Formula enriched in calcium and phosphorus also enables premature infants to achieve the faster mean weight gain and increment of other growth parameters compared to exclusively pooled preterm milk feeding, which has resulted in a higher alkaline phosphatase levels in these infants (29,46). Bone densitometry studies have shown increased bone density in calcium-supplemented infants (47,48).

. Factors Influencing Intestinal Fat Absorption

ntraluminal Factors

The bioavailability of bile acids and lipolytic enzymes and the aturity of the digestive system influence fat absorption (2,49-52). Bile cid concentration is inversely correlated with steatorrhea (53) and fecal ile acid excretion is inversely correlated with absorption coefficients f total fat and unsaturated fat (51). Infants with normal absorption oefficients for fatty acids (greater than 80%) usually had a bile acid oncentration greater than 4.0 mM, while infants with steatorrhea

the characteristics of individual fatty acids and triglycerides in milk contribute to the efficiency of fat absorption. The position of a fatty acid on the glycerol moiety in a triglyceride can limit fat absorption (2,51,88). Most palmitate in fresh human milk is esterified in the beta-position of glycerol molecules and remains unhydrolyzed by lipase. The beta-monoglyceride thus formed is absorbed directly instead of forming an insoluble palmitic acid-calcium soap in intestine (2). In rats, a linear correlation between fat absorption and the proportion of a fatty acid in the beta-position is observed for palmitic, and to a lesser extent, for myristic and oleic acid (89). When newborn infants were given similar formulas in which palmitic acid was either randomized on the triglyceride molecule or located mainly at the beta-position, fat absorption was markedly reduced when palmitic acid was distributed at random, indicating triglyceride configuration is an important factor in improving fat absorption (90).

The chain length and degree of saturation of individual fatty acids also determine the rate of fat absorption. Unsaturated fatty acids have higher absorption coefficients (51). The absorption of saturated fatty acids varies inversely with chain length (51,91) and medium-chain triglycerides are better absorbed than long-chain triglycerides (51,55,92). It has been estimated that an approximately 10% decrease in fatty acid absorption occurs for each two carbon addition in fatty acid chain length (51), as aqueous solubility of a fatty acid decreases with increasing chain length. In other words, because medium-chain saturated fatty acids are relatively water soluble, they may not require micellar

solubilization by bile acids. Therefore, its absorption coefficient shows no linear correlation with bile acid concentration, or it is efficiently absorbed irrespective of the intraduodenal bile acid concentration (51). For instance, premature infants fed a medium-chain triglyceride formula have a markedly decreased stool volume and frequency and increased fat absorption rate (55,91-93). Long-chain triglycerides are rehydrolyzed prior to absorption and have a relatively poor solubility (2). Thus, fat absorption is very dependent on the fatty acid profile of feedings (88,94-96). Compared to formula containing long-chain triglycerides, an isocaloric formula containing medium-chain triglycerides significantly promotes weight gain and nitrogen retention, in addition to improving fat absorption rate (97).

As discussed above, total fat absorption and calcium absorption are closely correlated (41,49,88,92,95). The mean fat excretion value in infants receiving calcium supplementation is compared with the value in infants not receiving calcium, and it has been found that the fat excretion is increased with calcium supplementation (1,41,48,49,94). Long-chain saturated fatty acids form calcium-soap and increase both fat and calcium excretion (52), or excessive calcium interferes with fat absorption and individual fatty acids selectively (48,94), as palmitic and stearic acids have been identified previously as the major fecal fatty acids in the adult while oleic acid is preferentially absorbed (98).

4. Factors Influencing Intestinal Calcium Absorption

Dietary Calcium and Phosphorus Intake and Their Ratio

The absolute amount of calcium and phosphorus intake and their ratio play a significant role in calcium secretion and absorption (36,41,48,99-103). In suckling rats, net transport of calcium is related in a linear manner to the concentration of calcium in the intestinal lumen (6,104,105). Calcium secretion occurs when intraluminal concentration of calcium is lower than plasma ionized calcium concentration (104). Phosphorus supplementation with calcium, in order to avoid hypercalciuria and phosphate depletion syndrome, improves calcium retention (41,106). Since some of the retained phosphorus is used for new body cell synthesis in premature infants, less phosphorus is available for bone mineralization. Calcium is thus accumulated in the plasma of these breast milk fed infants and results in hypercalcemia and hypercalciuria, which can be readily corrected by phosphorus supplementation (107). A positive correlation between serum calcium concentration and urinary calcium excretion and a negative correlation between serum calcium concentration and serum phosphorus concentration or urinary phosphorus excretion have also been observed (107). Therefore, a Ca/P ratio of 1.7-2.0:1 has been suggested to approximately achieve the intrauterine bone mineralization curve in premature infants (108).

Vitamin D and Other Regulating Factors

Homeostatic regulation of calcium depends on vitamin D, parathyroid

hormone, and calcitonin. These hormones act on the intestine, kidney, and bone to maintain serum calcium at a physiological level. Vitamin D, 1,25-$(OH)_2$ D_3 controls calcium metabolism through calcium intestinal absorption, bone mobilization and renal reabsorption. It increases serum calcium and phosphorus level for normal bone mineralization by improving intestinal calcium absorption, bone reabsorption and renal reabsorption at the distal tubule.

The saturable calcium transport, predominant in proximal intestine, is regulated by vitamin D. This saturable process consists of entry of calcium across the brush-border membrane, intracellular diffusion, and extrusion across the basolateral membrane (109). Calcium transport across cell membrane is carrier-mediated and inhibited competitively by strontium and noncompetitively by magnesium (110). As membrane lipid composition determines the function of a mobile carrier in a lipid bilayer, liposome treated brush-border membrane vesicles showed a change in phospholipid and cholesterol and, consequently, a decrease in Vmax which is correlated with brush-border membrane vesicle cholesterol content and fluidity (110). Vitamin D can affect phospholipid metabolism of intestine, kidney, bone, and possibly parathyroid cells (111). Vitamin D also stimulates active calcium transport by synthesis of a cytosolic protein, calcium-binding protein, and acceleration of intracellular diffusion of calcium. In adults, 1,25-$(OH)_2$ D_3 stimulates this energy-dependent cellular process in intestinal epithelium and converts regions of net calcium secretion in ileum and colon to net absorption (112). In weanling and post-weanling rats, vitamin D enhances active calcium uptake, significantly elevating

plasma calcium (6).

Vitamin D is activated in the liver and kidney. The transformation is stimulated by decreases in serum calcium and phosphorus and increase in serum parathyroid hormone. The active form of vitamin D, 1,25 $(OH)_2$ D_3, then binds to a receptor in the nuclear membrane. The complex further binds to specific portions of nuclear DNA, causing transcription of specific genes that code for calcium and phosphorus transport proteins. Calcium-binding protein has been identified in the cytoplasm, which facilitates calcium diffusion from the microvillar pole of the enterocyte to the basolateral membrane. The rate of intracellular calcium diffusion in the absence of calcium-binding protein is only about 1/70 of that found in the vitamin D-replete cell (109). In vitamin D-deficient or vitamin D-replete chicks, giving vitamin D at normal calcium and phosphorus levels results in an elevation of duodenal calcium-binding protein and its mRNA levels (113,114). The low level of duodenal vitamin D-dependent calcium-binding protein in hypophosphatemic mice is associated with low calcium intestinal absorption and low bone mineralization (115). Vitamin D deficiency can also cause reduction in body weight, intestinal mucosa weight, total protein and DNA and some hydrolytic enzyme activities both in intestine and pancreas (116).

In premature human neonates, calcium absorption and retention may be increased by adding vitamin D, especially after the first week of life (42,106,117,118). It seems that intestinal calcium absorption is not very responsive to vitamin D feeding in the early days of life although vitamin D can be well absorbed in premature infants (117). Indeed, premature

infants with hypocalcemia did not show vitamin D activation in the first week of life (119). It is hypothesized that in the early days of life, a nonsaturable, or passive calcium transport process is predominant, which may be vitamin D-independent (105). Therefore, the calcium status at this stage is maintained primarily by the relatively high calcium content present in milk, endocytosis of ingested calcium or simple passive diffusion down the calcium concentration gradient. The presence of lactose in milk increases passive absorption of calcium in intestine (6). It might be possible that the intestine is not sensitive to vitamin D in the early days of life because of the lack of vitamin D receptor in the nuclear membrane.

Parathyroid hormone is another regulating factor. The effect of vitamin D on bone mobilization and renal reabsorption of calcium requires the presence of this hormone. Its synthesis and secretion are stimulated by the reduction in serum calcium and 1,25 $(OH)_2D_3$ levels. Parathyoid hormone acts directly on bone and kidney to increase extracellular calcium concentration and indirectly on intestine to increase calcium absorption by stimulating vitamin D activation in kidney (120). As the secretion of parathyroid hormone may be mediated by the magnesium concentration in this manner, magnesium might affect calcium metabolism as well.

The elevation in serum calcium stimulates the production and release of calcitonin from the parafollicular cell of thyroid gland. Calcitonin decreases serum calcium level by reducing bone resorption and tubular reabsorption of calcium and inactivation of vitamin D by modulating some enzyme activities such as adenyl cyclase, and vitamin D 1-alpha-

hydroxylase. Calcitonin also indirectly inhibits calcium absorption in intestine by decreasing gastrin and gastric acid secretion (120).

Dietary Fat

Calcium absorption is also related to fat absorption. As calcium salts are formed with fat and excreted in increased amounts when calcium is supplemented to feeds (1,2,41,48,94,117), intestinal calcium absorption is favored by complete fat absorption by pancreatic enzymes. Long-chain saturated fatty acids are mainly excreted in the form of insoluble soaps. Infants fed a formula containing medium-chain triglycerides absorb more calcium than infants fed a standard cow's milk-based formula (95). The enhanced calcium absorption is proportional to the medium-chain triglyceride concentration in the formula and is positively correlated with fat absorption (95). In other words, in newborn infants, fat excreted as calcium-soap is related to fatty acid composition of a formula (121). A high level of stearate and palmitate in formula increases calcium losses, and retention of calcium is greatest in a formula low in stearate and palmitate and high in oleate (121). In chicks fed diets supplemented with palmitic acid, a significant reduction in bone ash and bone calcium content has been found (122). Increasing the calcium in the diet further decreases calcium retention and bone calcium content associated with addition of fat (122).

Other Factors Affecting Calcium Absorption

Some electrolytes affect calcium transport and absorption in the

intestinal lumen or mucosa and renal glomeruli. Sodium induces hypercalciuria and alters calcium absorption in intestine and reabsorption in kidney (112,123,124). In intestine, sodium affects calcium absorption at the brush border by altering the transmembrane electrical gradient and at the basolateral membrane by exchanging with intracellular calcium (112). Sodium intake in the hypocalcemic premature infant is positively correlated with urinary calcium, phosphorus, and magnesium excretions (124). Moreover, magnesium inhibits calcium absorption by depressing passive calcium absorption in the jejunum of adult human subjects (125).

Some drugs have also been shown to influence calcium metabolism. Furosemide induces hypercalciuria (123,126,127), and bone demineralization and renal calcification are found in premature infants receiving this diuretic treatment (126,127). Glucocorticoids suppress calcium absorption in intestine in the suckling rat (128). Calcium absorption also increases with gestational age and postnatal age (36,55,102). As vitamin D can be digested by bile salt, inadequacy of luminal bile salt may decrease calcium absorption as well. Acidity of digestate may enhance calcium absorption, therefore, some sugars increase intestinal calcium absorption by lowering luminal pH and thus increasing the solubility of calcium (129).

5. Introduction to Phosphorus Absorption

The low phosphorus content of human milk, especially preterm human milk, and gradual decline of phosphorus over the first few weeks of lactation are insufficient for growing low birthweight infants (28,29,31).

Premature infants excrete more phosphorus in urine in the first week of life compared to term infants (130). These infants exhibit decreased serum phosphorus and increased alkaline phosphase levels (29). Phosphorus or phosphorus and calcium supplementation can correct this situation, as phosphorus retention, bone content, and weight gain have been improved (41,108,131-133). As phosphate depletion is related to neonatal hypercalciuria in the premature infant fed breast milk (107), it is necessary to briefly review the intestinal phosphorus absorption and its interactions with calcium and magnesium.

Eighty five per cent of phosphorus deposits are found in skeleton tissue, the other 15% are present in soft tissues (120). Its metabolism is mainly regulated by vitamin D and parathyroid hormone (120). Parathyroid hormone decreases phosphorus reabsorption in kidney. Vitamin D increases renal tubular reabsorption and intestinal absorption of phosphorus. Tubular reabsorption and serum concentration of phosphorus can be also decreased by calcitonin.

Several factors may affect phosphorus absorption and retention. Increasing amount of phosphorus in milk may increase phosphorus retention (102). Calcium supplementation increases not only calcium absorption but also causes a dose-dependent phosphorus retention in premature infants (41,102,134). Magnesium in diets may decrease phosphorus absorption by forming insoluble salt with magnesium in intestinal lumen. Phosphorus uptake by the intestinal mucosa decreases with aging. Although vitamin D and calcium intake can stimulate phosphorus uptake (135), the capacity is most intensified in young animals (136). Phosphorus absorption and

retention is also enhanced by a medium-chain fatty acid-enriched diet (50%) in adult rats receiving intestinal resection (137). Aluminum commonly contained in antacids interferes with intestinal phosphorus absorption, resulting in phosphate depletion (138). Urinary phosphorus excretion can be increased by ingestion of anions irrespective of their sodium or potassium salts (139).

6. Introduction to Magnesium Absorption

When compared to formulas, preterm human milk has a relatively low concentration of magnesium, which does not enable premature infants to achieve intrauterine magnesium retention rate (31).

More than half of total body magnesium is present in skeleton, whereas the rest is found in soft tissues. Magnesium is a very important constituent in bone mass. It is also involved in various enzymatic reactions in both energy metabolism and synthesis of proteins and nuclear acids.

Magnesium is absorbed throughout the intestine, mainly distal small intestine. The mechanism of the intestinal absorption includes passive diffusion, solvent drag, and active transport, with the passive diffusion being the major pathway and, possibly, a limited active transport in descending colon in some adult or aged animals (140). In fact, in suckling or adolescent rats, no active transport of magnesium is found. Extracellular magnesium is filtered at the glomerulus and most filtered magnesium is reabsorbed in the proximal tubule and thick ascending limb.

Various factors can influence magnesium absorption or retention.

There are also complex interactions between the divalent cations in intestinal lumen and complicated effect of vitamin D on magnesium (140). Increased magnesium intake is associated with increased absorption and bone accumulation (141). Duodenal magnesium absorption is suggested to be totally concentration-dependent (142). Pharmacological doses of vitamin D administration increases magnesium absorption and serum magnesium concentration (140,143), whereas a substantial amount of magnesium absorption is independent of vitamin D (140). As vitamin D also increases urinary magnesium excretion, the retention rate of magnesium may not be greatly affected by vitamin D (140). In patients with hypomagnesemia after intestinal resection, magnesium status is improved by vitamin D and magnesium supplementation (144).

Dietary calcium interferes with magnesium absorption, possibly by competition for a common carrier system, modulation of membrane permeability to magnesium or a specific magnesium carrier, direct competition in passive transport, or formation of a complex agent with each other. It is also possible that this interaction, to a certain extent, is conducted by hormonal feedback, as both vitamin D and parathyroid hormone are very responsive to the serum concentration of calcium and magnesium. Phosphorus in the diet also affects magnesium absorption by forming insoluble salt with magnesium, resulting in greater magnesium excretion in stool. In one study with premature infants fed formula with calcium and phosphorus supplementation, magnesium absorption and retention were severely impaired and showed a negative balance (145).

Some other dietary factors affect intestinal magnesium absorption.

Phytate in soybean products decreases magnesium absorption and bone content compared to casein-predominant formulas because of Mg-Ca-phytate complex formation in the intestinal lumen (141). Lactose present in milk decreases luminal pH through intestinal microbial fermentation and thus enhances magnesium solubility and passive magnesium absorption (141). Intraluminal potassium depresses magnesium absorption (146,147). A diet supplemented with anions increases urinary magnesium excretion and, therefore, the retention rate is reduced (139). In rats, short-chain fatty acids derived from fermentation of carbohydrate in the large intestine stimulate magnesium absorption by offering protons to Mg^{++}/H^{+} in the apical membrane of epithelium (147). Magnesium secretion in proximal intestine is greatly increased in rats with intestinal resection (148).

Magnesium deficiency in the diet causes hypocalcemia and hypomagnesemia. Vitamin D administration improves both calcium and magnesium status (143). It is suggested that magnesium deficiency impairs synthesis or target organ response to vitamin D (143). A protective effect of magnesium in renal calcinosis in furosemide-treated rats with magnesium deficiency has also been suggested (149).

7. Summary

Preterm human milk is recommended for the premature infant as it contains more medium-chain fatty acids, long-chain polyunsaturated fatty acids, and bile salt-stimulated lipase, therefore ensuring superior fat absorption. Long-chain omega-3 fatty acids in human milk are essential to the function and development of premature infants' eyes and brain.

However, the calcium and phosphorus content of breast milk is insufficient for normal bone mineralization in the premature infant. Therefore, calcium supplementation is necessary to obtain an intrauterine calcium accretion rate. Calcium supplementation increases lipid loss in stool as it forms calcium soap with long-chain saturated fatty acids in intestinal lumen. Factors influencing calcium absorption include absolute intake of calcium and phosphorus and their ratio, vitamin D status, dietary fat, some cations and drugs, and gestational and postnatal age. Fat absorption can be affected by the presence and concentration of bile salts, lipolytic enzymes, postnatal age, calcium intake, properties of milk lipids such as fatty acid saturation, chain length, and the position of fatty acids in glycerol molecules. Poor fat absorption in the premature infant due to lower intestinal concentration of bile salts and pancreatic lipase can be improved with breast milk feeding because of the compensation of intragastric lipolysis by lingual lipase and gastric lipase and intestinal hydrolysis by bile salt-stimulated lipase present in human milk. Lingual lipase does not only hydrolyze fat in stomach but also aids intestinal fat absorption. As well, bile salt-stimulated lipase makes intestinal lipolysis more complete. Intestinal phosphorus absorption may be enhanced by dietary intake of phosphorus, calcium, and medium-chain fatty acids and diminished by dietary magnesium, aluminum and some anions. Intestinal magnesium absorption can be improved by magnesium, short-chain fatty acids, and lactose in diets and impaired by dietary calcium, phosphorus, phytate, potassium and some anions. Vitamin D, parathyroid hormone and calcitonin regulate calcium, phosphorus and magnesium metabolism by acting

on the intestine, kidney and bone and thus maintain their serum concentrations at physiological levels.

C: Rationale

Because of the superior nutritional and immunological value of human milk, feeding premature infants' their own mother's milk has been recommended, especially in the first few weeks of life. However, calcium and phosphorus deficiency is quite evident in the premature infant fed exclusively human milk, as the calcium and phosphorus content in human milk is relatively low and mineral accumulation of the premature infant is very limited. Therefore, calcium or calcium and phosphorus supplementation is essential for the premature infant to reach the intrauterine mineral accretion rate. Unfortunately, calcium soap formation with fatty acids, especially long-chain saturated fatty acids complicates this practice, as the energy intake and, possibly, some essential fatty acids and consequently growth and development of the premature infant will be affected by the increased excretion of calcium-fatty acid soaps following calcium supplementation.

It is obvious that a more appropriate method of calcium supplementation requires investigation. In most related studies, calcium supplements have been given orally with feedings of either human milk or formula. In this study, an alternative way of calcium supplementation was compared with this practice, i.e. giving the supplement between two feedings to the premature infant fed expressed preterm human milk. The study will, to a certain extent, further clarify the interactions of

lipids and calcium and other divalent cations which may interfere with calcium metabolism.

1. Hypothesis

It is hypothesized that by changing the timing of calcium supplementation from with feedings to between feedings in the premature infant fed breast milk, insoluble calcium salt formation will be decreased. It is hypothesized that both fat and calcium losses in stool will decrease and the absorption coefficient of long chain saturated fatty acids will increase in the infant receiving calcium supplementation between feedings.

2. Objectives

a. To investigate the effect of timing of calcium supplementation on calcium and fat absorption, and absorption coefficients of selected fatty acids in the premature infant fed breast milk.

b. To investigate the interaction of intestinal minerals (Ca, P, Mg) and fatty acids in orally fed premature infants.

E: References

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Chapter II

CALCIUM SUPPLEMENTATION IN PREMATURE INFANTS FED BREAST MILK: The Effect of Timing of Calcium Supplementation on Calcium, Phosphorus, Magnesium and Fat Balance and on the Absorption Coefficients of Individual Fatty Acids

A: Introduction

Lipid and calcium soap formation has been extensively studied in newborn infants by giving various forms of calcium supplement with either human milk or formulas. However, giving the supplement other than together with feedings has not been studied. Therefore, in this study, sixteen medically stable premature newborns were pair-matched according to their gestational age and birthweight, divided into two groups at random and given calcium lactate either with or between expressed human milk feedings in the first four weeks of life. The measurement and observation focused on growth parameters, lipid, calcium (Ca), phosphorus (P) and magnesium (Mg) balance and the absorption coefficients of certain fatty acids. This chapter will present the experimental design, methodology, results of the study by tables and figures and discussion of these outcomes.

B: Study Subjects and Methodology

Infant nursing, milk and supplement feeding, sample collecting and anthropometric measuring were carried out by the research nurse in NICU. Calcium, phosphorus and magnesium in milk, stool, and urine and the total fat in milk and stool were measured in the Neonatal Research Laboratory of University of Alberta.

1. Study Design

Study Subject Description

Three-day metabolic balance studies were carried out during the first, second and fourth postnatal week in 16 infants born at 30.8±1.4 weeks gestation and weighing 1329±168 grams (Mean±SD) at birth at the Neonatal Intensive Care Unit (NICU) of the University of Alberta Hospital. All infants had normal renal function and acid-base balance, no congenital abnormalities, no steroid, diuretic or sodium medications and tolerated full feeds. Infants were nursed in incubators in a thermoneutral environment (1) (infants' core temperature ranged from 36.7 °C to 37.3 °C).

Feeding Regimen

Infants were pair-matched according to gestational age and birthweight and randomly divided into two groups. After expressed own mother's milk was collected (see Collection of Preterm Human Milk), infants in both groups were fed with calibrated syringes every two hours.

Calcium Supplementation

Once the infants tolerated 50% oral feeds, calcium lactate (1.3 mmole calcium /Kg /day) was administered every two hours with milk to 8 infants and exactly in between feedings to the other 8 infants. Other oral supplements, including vitamin C (100mg for first week), vitamin E (50IU for 60 days or until discontinuation of oxygen, whichever was longer), and vitamin D (1000IU), were given daily to each infant.

2. Collection of Preterm Human Milk

Expressed own mother's milk was delivered fresh or frozen to the NICU within 24 hours after collection in sterile glass containers. The milk was mixed and then divided into aliquots for feeding from the total 24-hour expressed volume. A 10 ml aliquot for each study day was frozen at -20 °C for analysis of calcium, phosphorus, magnesium, total fat and fatty acids. All expressed breast milk was fed within 48 hours of fresh collection or within 24 hours after thawing. Volume of milk intake was calculated from the fluid fed with calibrated syringes, minus gastric aspirates and vomitus which, if it occurred, was collected on ashless paper placed beneath the infant's mouth.

3. Collections of Urine and Stool Samples

Samples were collected, pooled and volumes measured in each study period. Urine was collected with an adaptive urine collector. All urine samples from each balance period were pooled and the total volume measured. An aliquot was removed and stored at -20 °C for later analysis.

Leakage was calculated from the weight of a pre-weighed diaper. Stools were collected on a large square sheet of polyethylene placed under the infant, frozen immediately at -20°C. They were later pooled and weighed for the 72-hour period, and then freeze dried and stored at -20°C for analysis.

4. Anthropometric Measurement

Anthropometric measurements included weekly head circumference using a fiberglass measuring tape, weekly body length using a plexiglass measuring board calibrated in milimeter increments, weekly skinfold at the triceps, subscapular and periumbilical regions with a Harpenden caliper stabilized for 30 seconds and daily body weight with an electronic scale to an accuracy of ± 1 gram.

5. Calcium, Phosphorus, Magnesium, Total Fat and Fatty Acids Measurement

Calcium in milk and supplement, stool and urine was measured with atomic absorption flame spectrophotometry (2). Lyophilised stool sample was wet ashed, and urine was diluted before calcium analysis. Phosphorus in milk, stool and urine was measured with a modified method of Fiske and Subbarow (3). Magnesium in all milk, stool and urine samples was also measured by atomic absorption spectrophotometry (2). Total fat in milk was measured by a gravimetric method (4), and fatty acids in milk were extracted with a modified Folch extraction (5) (see Appendix I) and analyzed by capillary gas-liquid chromatography (6). Total fat in stool was measured by a modified Jeejeebhoy procedure with the addition of HCl

to release bound fatty acids (7). Fatty acids in stool were extracted by a modified method of Watkins and Bliss in triplicate (8) (see Appendix II) and then analyzed by capillary gas-liquid chromatography (6). Blood work included initial acid-base status, calcium and phosphorus levels, creatinine and blood urea nitrogen. Both calcium and phosphorus levels were measured at the end of the study as well.

6. Statistics Applied

Statistics include multiple analysis of variance and correlation analysis. In addition, Student-Newman-Keuls test was used to further compare the difference between three study weeks. Student t test was used to measure the difference between the two treatments within each study week.

C: Results

An overall significant variation among individual premature infants was observed. Results are presented in tables and figures. Data are expressed as means ± SD.

1. Composition of Preterm Human Milk

Table II-1 shows the composition of milk consumed by the study subjects. Infants from different groups ingested similar volumes of milk per kilogram body weight per day. The pooled intake volume was 162±17 ml/Kg/d. Solid weight and total lipid content of the milk were the same for the different groups and different study weeks (pooled value: 12.6±0.7

and 3.3±0.6 g/dl, respectively). There was no difference in Ca, P, and Mg content between the two groups. However, a progressive decline of Ca and P concentrations with postnatal age was observed. Preterm human milk had a significantly higher concentration of Ca in the first week after delivery which decreased gradually during the following study weeks (0.65±0.17, 0.58±0.14, 0.57±0.11 mmole/dl, respectively). The high P content in the milk lasted for the first two weeks of life (0.53±0.11, 0.49±0.09, 0.43±0.07 mmole/dl). However, the longitudinal decline in Mg concentration was not significant (0.13±0.04, 0.13±0.04, 0.12±0.04 mmole/dl).

2. Anthropometric Data

Table II-2 shows anthropometric data of the study subjects. The postnatal age and body weight at which the balance studies were conducted were not different between the two treatment groups. The growth parameters such as body weight, head circumference and length increased progressively with postnatal age and no difference was observed between the two treatments. Infants receiving calcium supplement between feedings showed less weight gain when compared to infants receiving calcium with feedings, especially in the second postnatal week (one-tailed t test: $P<0.05$), although it was not statistically significant. The pooled weight gain was 11.8±4.0 g/Kg/d. The mean weight gains in 'Ca between feedings' and 'Ca with feedings' were 11.4±4.6 and 12.2±4.1 g/Kg/d, respectively. The mean weight gains during three study periods were 9.7±3.9, 11.7±4.1, and 13.9±5.3 g/Kg/d, respectively. However, none of these was

statistically significant.

3. Balance Studies

In general, balance studies show increased fecal excretions of lipid, Ca, P, and Mg in the infants receiving Ca between feedings compared to the infants receiving Ca with feedings. The absorption and retention rate of these elements was correspondingly affected. The following summaries and tables present detailed outcomes of each balance study.

Lipid Balance Study

As milk intake and lipid content of the milk were relatively consistent, lipid intakes between groups during the three balance studies were quite similar. The pooled value was 5.40±1.11 g/Kg/d. The lipid loss in stools was higher in infants given Ca between feedings compared to infants given Ca with feedings, but the difference was not statistically significant (1.30±0.85 vs 0.95±0.80 g/Kg/d). However, the difference between lipid intake and lipid loss in stool was statistically significant in the first postnatal week (two-tailed t test: $P<0.05$). This may, in part, explain the difference in total lipid absorption rates between the two groups (75.4±16.8% vs 82.3±15.3%), which was not statistically significant (Table II-3).

Calcium Balance Study

There was no difference in Ca intake between groups. Infants from both groups ingested significantly more Ca in the first week of life,

Table II-4: Calcium Balance in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1 n=8	Week 2 n=7	Week 4 n=6
Ca intake from milk (mmole/Kg/d)			
Ca between	1.03±0.26	0.96±0.27	0.89±0.23
Ca with	1.04±0.30	0.92±0.27	1.03±0.14
Ca Lactate intake (mmole/Kg/d) *			
Ca between	1.36±0.23	1.18±0.08	1.08±0.18
Ca with	1.35±0.23	1.21±0.10	1.14±0.16
Total Ca intake (mmole/Kg/d) *			
Ca between	2.29±0.39	2.13±0.26	1.97±0.25
Ca with	2.31±0.31	2.13±0.32	2.17±0.23
Ca loss in stool (mmole/Kg/d)			
Ca between	0.87±0.52	0.71±0.46	0.65±0.25
Ca with	0.67±0.36	0.50±0.29	0.58±0.39
Ca absorption (%)			
Ca between	63.7±21.4	66.4±22.9	67.5±10.4
Ca with	71.5±12.4	77.4±10.3	73.9±17.0
Ca loss in urine (mmole/Kg/d) *			
Ca between	0.48±0.31	0.56±0.19	0.62±0.20
Ca with	0.38±0.24	0.54±0.23	0.61±0.25
Total Ca loss (mmole/Kg/d)			
Ca between	1.28±0.53	1.27±0.41	1.27±0.21
Ca with	1.05±0.32	1.06±0.16	1.19±0.35
Ca retention (%)			
Ca between	46.8±19.8	40.3±20.3	35.8±5.3
Ca with	55.1±10.7	51.1±5.2	45.3±14.7

1. Data are presented as Mean ± SD;
2. * significant difference among postnatal weeks ($P<0.05$).

Table II-5: Phosphorus Balance in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=7	n=6
P intake (mmole/Kg/d) *			
Ca between	0.82±0.14	0.80±0.18	0.66±0.09**
Ca with	0.83±0.17	0.78±0.16	0.81±0.13**
P loss in stool (mmole/Kg/d)			
Ca between	0.13±0.13	0.09±0.05	0.10±0.05
Ca with	0.06±0.03	0.06±0.04	0.06±0.04
P absorption (%) **			
Ca between	85.6±13.1	88.2±8.0	84.4±7.1**
Ca with	93.2±4.0	92.9±4.3	92.9±4.7**
P loss in urine (mmole/Kg/d)			
Ca between	0.003±0.003	0.002±0.002	0.002±0.002
Ca with	0.005±0.006	0.004±0.002	0.006±0.008
Total P loss (mmole/Kg/d)			
Ca between	0.13±0.13	0.09±0.05	0.11±0.05
Ca with	0.06±0.04	0.06±0.04	0.06±0.04
P retention (%) **			
Ca between	85.3±12.9	87.9±8.0	84.0±7.1**
Ca with	92.5±4.3	92.4±4.3	92.5±4.9**

1. Data are presented as Mean ± SD;

2. * significant difference among postnatal weeks ($P<0.05$);

3. ** significant difference between two treatments ($P<0.05$).

Table II-6: Magnesium Balance in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=7	n=6
Mg intake (mmole/Kg/d)			
Ca between	0.21±0.07	0.23±0.10	0.19±0.06
Ca with	0.21±0.03	0.19±0.04	0.20±0.07
Mg loss in stool (mmole/Kg/d)			
Ca between	0.12±0.09	0.09±0.05	0.08±0.04
Ca with	0.07±0.03	0.06±0.04	0.06±0.03
Mg absorption (%)			
Ca between	42.2±41.2	54.9±30.6	55.7±23.2
Ca with	68.3±13.4	69.6±15.3	68.2±9.1
Mg loss in urine (mmole/Kg/d) *			
Ca between	0.09±0.03	0.08±0.02	0.08±0.04
Ca with	0.07±0.02	0.07±0.02	0.05±0.03
Total Mg loss (mmole/Kg/d) *,**			
Ca between	0.21±0.08**	0.18±0.04**	0.15±0.03**
Ca with	0.13±0.02**	0.13±0.02**	0.12±0.03**
Mg retention (%) **			
Ca between	-3.0±36.6**	11.7±36.6	12.5±27.7
Ca with	35.0±9.4**	28.0±7.2	36.8±23.7

1. Data are presented as Mean ± SD;

2. * significant difference among postnatal weeks ($P<0.01$);

3. ** significant difference between two treatments ($P<0.05$).

respectively) (Table II-6).

4. Correlation of Calcium Parameters

Figure II-1 shows the correlation analysis of Ca intake and Ca loss in stool ($P<0.01$). Ca intake positively correlated with Ca loss in stools, this correlation being more obvious in infants receiving Ca with feeding ($r=0.70$ vs $r=0.33$).

5. Correlations of Fecal Lipid, Calcium, Phosphorus and Magnesium

Figures II-2 to II-6 illustrate the result of fecal total lipid, Ca, P, and Mg correlation analysis ($P<0.01$). Fecal lipid was significantly correlated with Ca and P content in stools. The correlations were greater in infants given the Ca supplement with milk (lipid and Ca: $r=0.91$ vs $r=0.63$; lipid and P: $r=0.92$ vs $r=0.23$). Fecal P and Mg were strongly correlated in both groups ($r=0.77$ and $r=0.78$). In the 'Ca with feeding' group the correlation between Ca and P was very obvious ($r=0.83$), while in the other group the correlation between Ca and Mg was more significant ($r=0.82$).

6. Absorption Coefficients of Individual Fatty Acids

Table II-7 shows the absorption coefficient of fatty acids C12:0, C14:0, C16:0, C18:0, C18:1(9) and C18:2(6). The absorption coefficients of C14:0, C16:0, C18:0, and C18:1(9) appear to decrease in 'Ca between feedings' group, especially in the first two weeks, although they were not statistically significant (C14:0-81.4±20.7% vs 91.3±8.1%, C16:0-72.1

Figure II-1: Correlation of Ca Intake and Ca Loss in Stool in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second and Fourth Postnatal Week

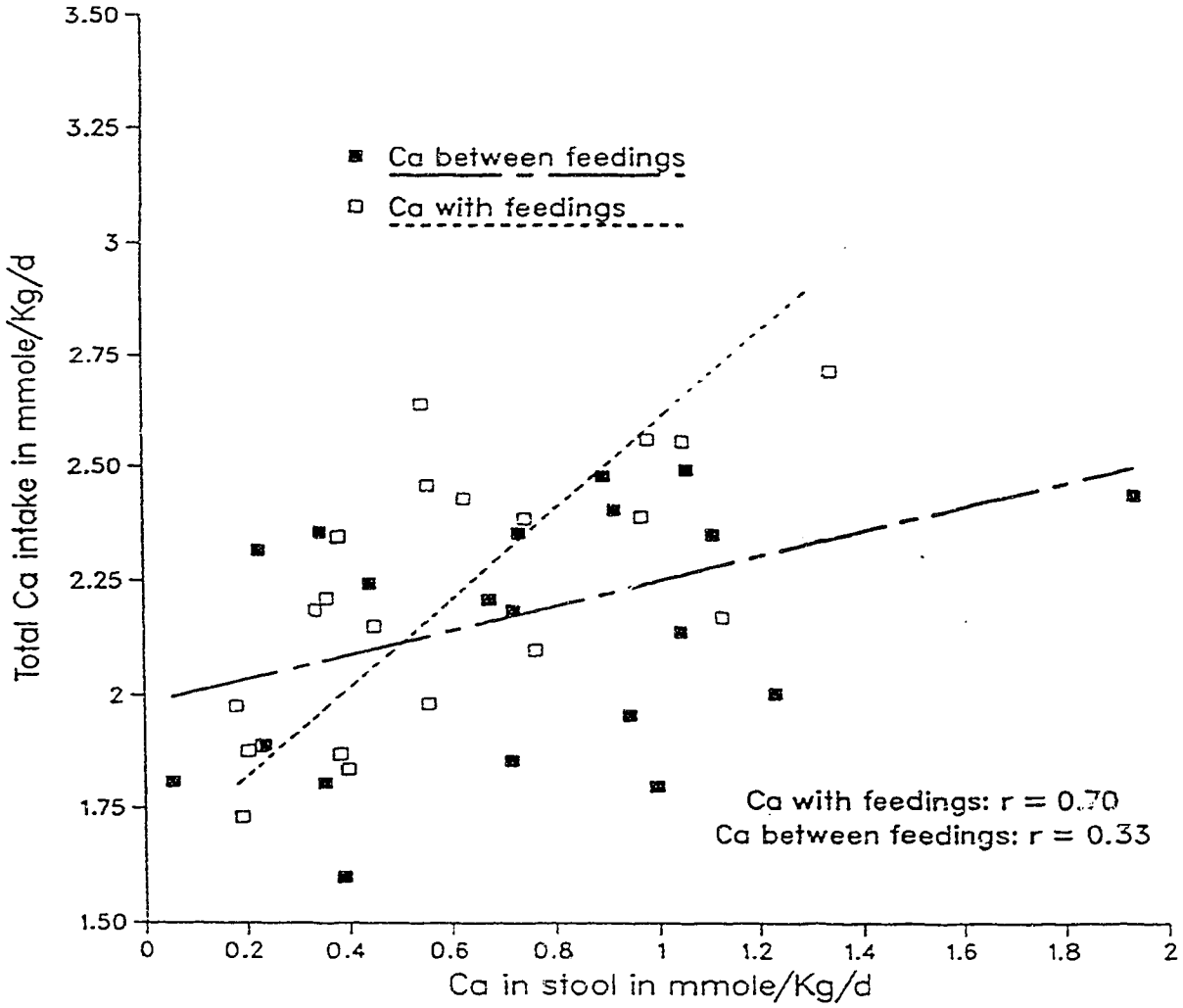

Figure II-2: Correlation of Fecal Lipid and Ca Losses in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second and Fourth Postnatal Week

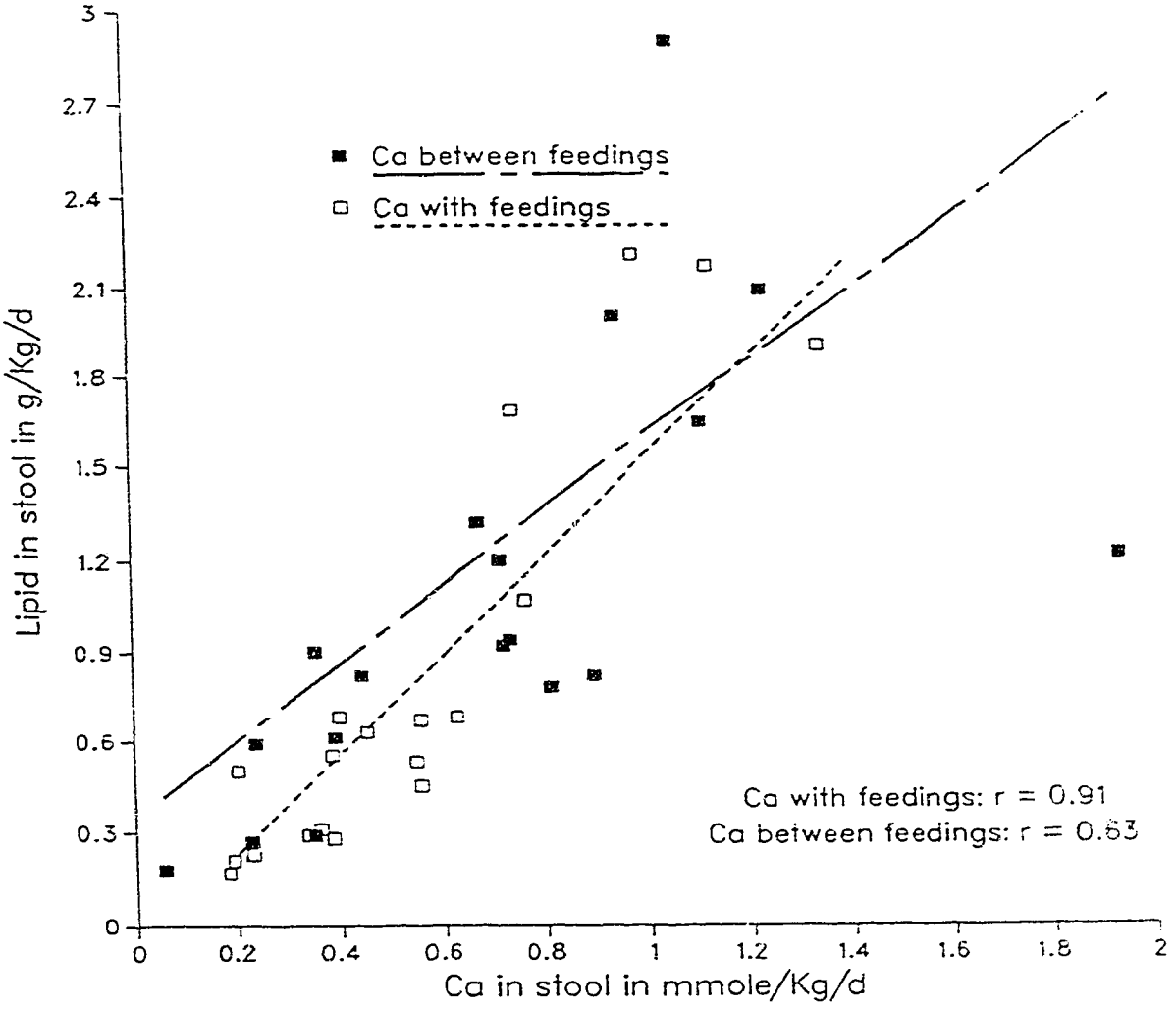

Figure II-3: Correlation of Fecal Lipid and P Losses in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second and Fourth Postnatal Week

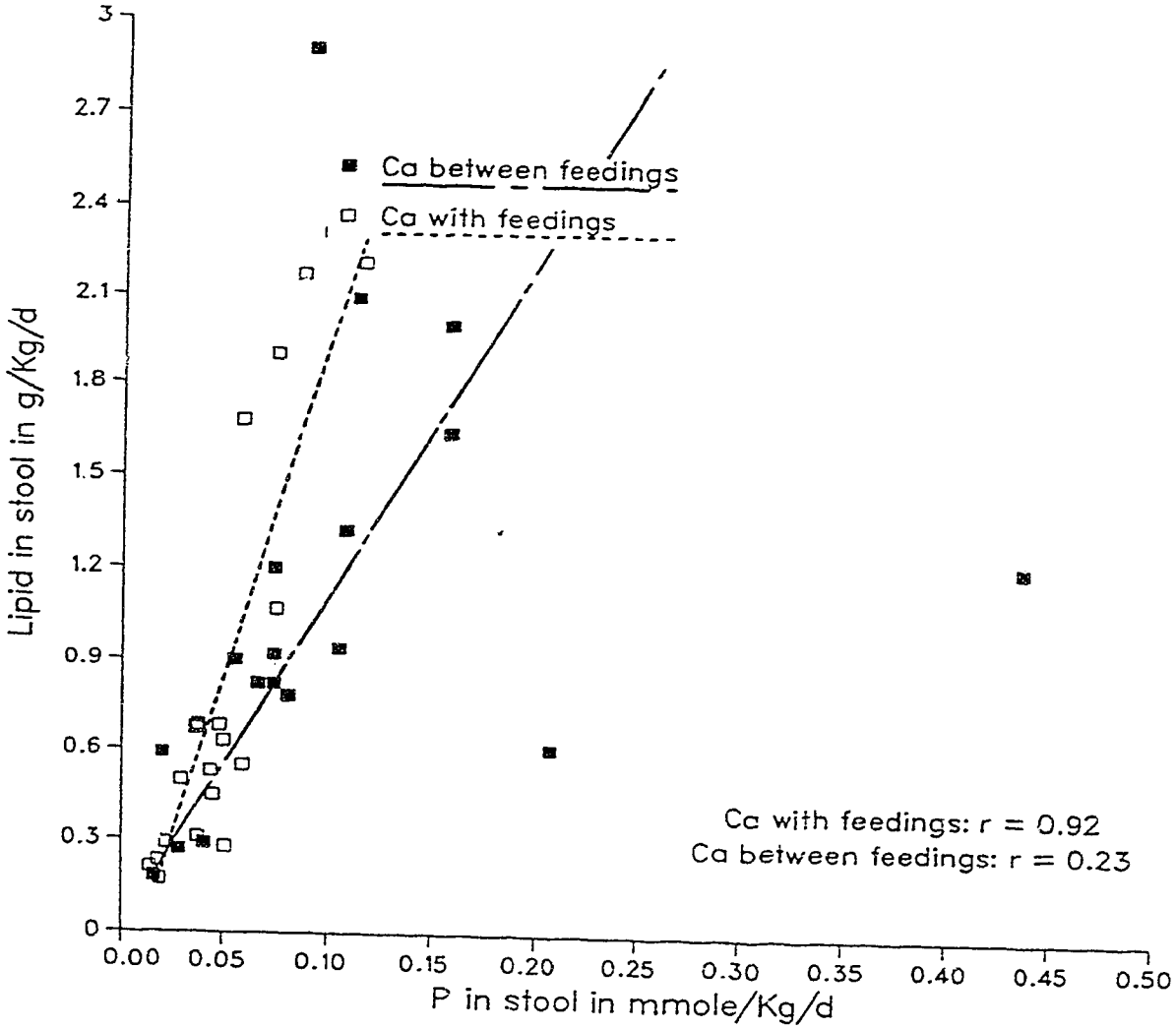

Figure II-4: Correlation of Fecal P and Mg losses in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second and Fourth Postnatal Week

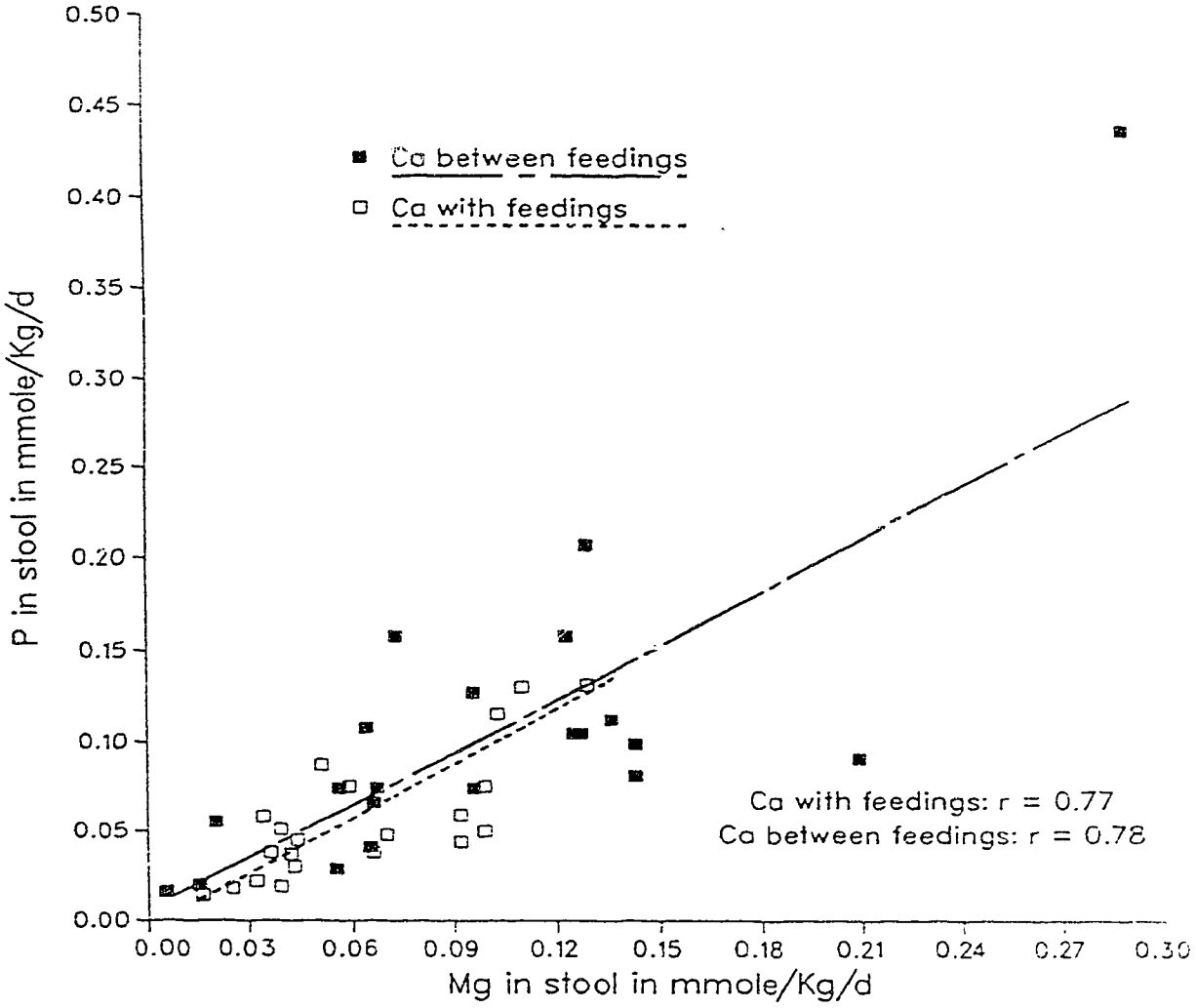

Figure II-5: Correlation of Fecal Ca and P Losses in Breast Fed Premature Infants Receiving Ca Lactate With Feedings in the First, Second and Fourth Postnatal Week

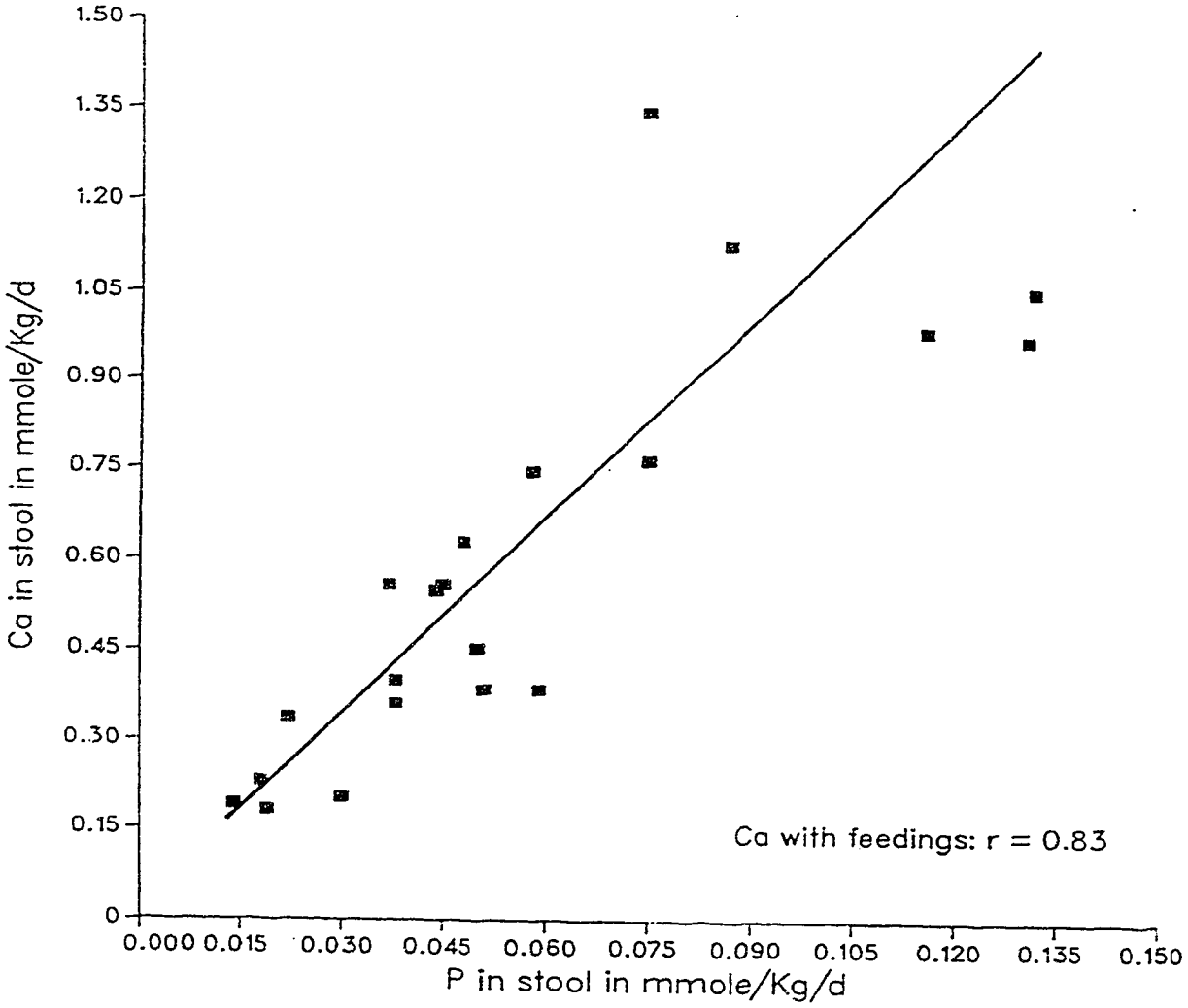

Figure II-6: Correlation of Fecal Ca and Mg Losses in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings in the First, Second and Fourth Postnatal Week

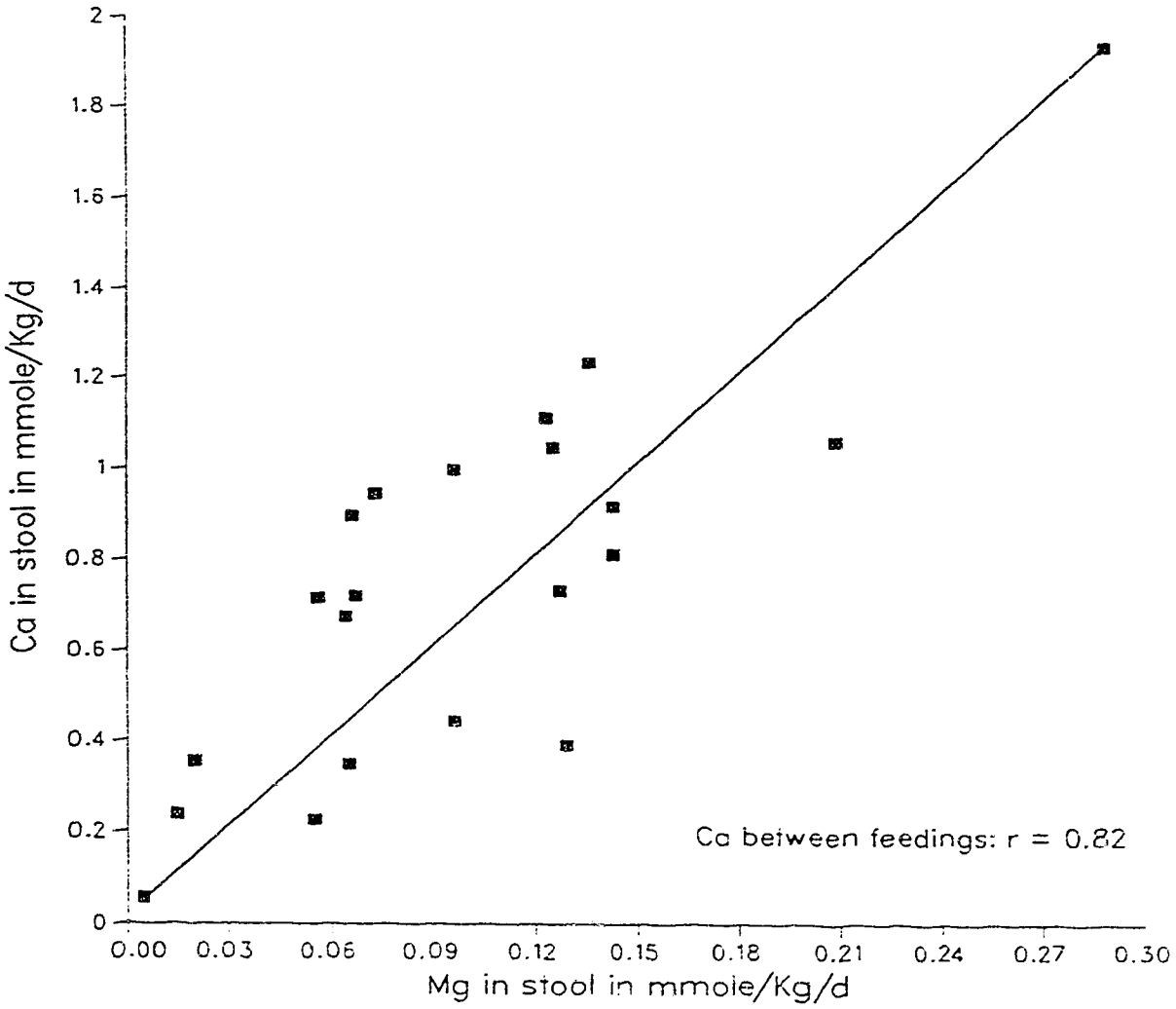

Table II-7: Absorption Coefficients for Selected Fatty Acids in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
C12:0 (%)			
Ca between	98.3±3.8	94.8±10.4	100.0±0.0
Ca with	99.5±0.9	99.3±1.3	98.9±2.7
C14:0 (%)			
Ca between	84.9±14.7	69.7±30.3	88.2±12.9
Ca with	91.2±8.1	93.2±6.0	89.7±10.8
C16:0 (%)			
Ca between	74.6±25.8	56.1±40.6	84.8±10.2
Ca with	85.9±11.8	87.5±10.4	84.8±17.8
C18:0 (%)			
Ca between	52.8±30.2	33.9±40.9 *	57.6±13.3
Ca with	71.2±16.6	75.6±17.0 *	71.5±23.9
C18:1(9) (%)			
Ca between	73.7±24.5	60.7±35.5	75.9±20.9
Ca with	76.5±27.1	87.5±13.5	77.3±26.8
C18:2(6) (%)			
Ca between	85.9±10.9	70.1±36.4	68.3±36.5
Ca with	69.6±38.7	78.0±38.8	72.1±39.3

1. Data are presented as Mean ± SD;

2. * significant difference between two treatments ($P<0.05$).

±29.0% vs 86.0±12.8%; C18:0-48.6±30.4% vs 72.6±18.2%; C18:1(9)-70.5±26.7% vs 80.1±23.1%). Only the absorption coefficient of fatty acid C18:0 in the infants receiving Ca between feedings in the second study week was significantly different from that in the infants receiving Ca with feedings (two-tailed t test: $P<0.05$).

Table II-8 illustrates the quantitative measurement of these six fecal fatty acids. Infants receiving Ca between feedings excreted more C14:0, C16:0, C18:0, C18:1(9) and C18:2(6) in the first two postnatal weeks compared to infants receiving Ca with feedings (C14:0-0.07±0.06 vs 0.05±0.06g/Kg/d; C16:0-0.30±0.23 vs 0.25±0.28g/Kg/d; C18:0-0.17±0.08 vs 0.13±0.12g/Kg/d; C18:1(9)-0.40±0.35 vs 0.32±0.34g/Kg/d; C18:2(6)-0.07±0.07 vs 0.04±0.05g/Kg/d). However, these differences were also not statistically significant.

7. Fecal Lipid Fractions

Table II-9 presents the percentage of four fecal lipid fractions: neutral lipid, Ca salts, free fatty acids, and compound lipid (the pooled values were approximately 54.4%, 2.5%, 21.5%, and 21.7%, respectively). Neutral lipid excretion increased gradually in the first four weeks of life (48.6±21.1%, 54.3±20.7%, and 62.1±17.7%, respectively), whereas free fatty acids decreased during the same period (25.8±13.5%, 19.7±10.9%, and 17.5±7.5%, respectively). In infants given Ca between feedings, the percentage of free fatty acids in stool was decreased (18.2±9.9% vs 24.8±12.3%) and the percentage of neutral lipid increased (57.1±23.7% vs 51.6±16.3%), although they were not statistically significant.

Table II-8: Quantitative Measurement of Selected Fecal Fatty Acids in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
C12:0 (mg/Kg/d)			
Ca between	5±11	13±25	0±0
Ca with	1±2	6±14	9±21
C14:0 (mg/Kg/d)			
Ca between	63±50	97±72	41±44
Ca with	48±50	49±54	64±81
C16:0 (mg/Kg/d)			
Ca between	302±211	419±290	180±128
Ca with	236±239	219±213	286±402
C18:0 (mg/Kg/d)			
Ca between	165±77	204±97	145±35
Ca with	125±79	120±93	156±179
C18:1(9) (mg/Kg/d)			
Ca between	382±352	493±402	312±348
Ca with	293±327	308±391	356±372
C18:2(6) (mg/Kg/d)			
Ca between	61±66	88±82	52±68
Ca with	37±46	40±57	52±67

Data are presented as Mean ± SD.

Table II-9: Percentages of Fecal Lipid Fractions in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (%) *			
Ca between	51.2±26.5	59.5±24.4	62.7±21.4
Ca with	46.2±15.4	49.2±16.8	61.4±15.2
Ca Salts (%)			
Ca between	2.9±2.5	1.6±0.6	3.2±3.2
Ca with	2.6±1.9	2.3±2.2	2.0±1.4
Free Fatty Acids (%) *			
Ca between	21.8±11.9	17.2±10.2	14.2±5.7
Ca with	29.8±14.6	22.2±12.0	20.8±8.1
Compound Lipid (%)			
Ca between	24.2±21.2	21.9±16.5	19.9±22.8
Ca with	21.5±11.8	26.1±15.9	15.7±9.8

1. Data are presented as Mean ± SD;

2. * significant difference among postnatal weeks ($P<0.05$).

Data of fecal lipid fractions from Table II-10 shows the quantitative change in these fractions. Table II-11a to Table II-11f show the fractional distribution of selected fatty acids in stool. In general, more neutral lipid that consisted of fatty acid C14:0, C16:0, and C18:0 had been hydrolyzed (65% of fecal fatty acid C14:0 was found in neutral lipid fraction, 47% of C16:0, and 33% of C18:0 vs 83% of C12:0, 86% of C18:1, and 91% of C18:2). Infants receiving Ca between feedings seem to have excreted more C16:0, C18:0, C18:1(9), and C18:2(6) in the form of neutral lipid (C16:0-156±187 vs 98±121 mg/Kg/d; C18:0-62±53 vs 38±38 mg/Kg/d; C18:1(9)-351±356 vs 246±271 mg/Kg/d; C18:2(6)-62±70 vs 34±44 mg/Kg/d). Longitudinal decline of free fatty acid formation also appears to exist in fatty acid C14:0 and C18:1(9) (C14:0-11.7±9.3, 9.6±6.4, 6.6±7.9 mg/Kg/d; C18:1(9)-33.1±42.1, 19.3±19.3, 15.2±11.1 mg/Kg/d). However, because of the great individual variations, none of these were statistically significant.

Table II-10: Quantitative Measurement of Fecal Lipid Fractions in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	776±850	1179±1055	693±649
Ca with	484±517	493±540	746±813
Ca Salts (mg/Kg/d)			
Ca between	29±21	22±10	31±31
Ca with	27±34	27±30	26±36
Free Fatty Acids (mg/Kg/d)			
Ca between	238±154	228±132	118±33
Ca with	245±250	143±112	216±292
Compound Lipid (mg/Kg/d)			
Ca between	202±135	271±202	162±162
Ca with	166±147	290±482	174±265

Data are presented as Mean ± SD.

Table II-11a: Quantitative Measurement of Fecal Fatty Acid C12:0 in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	4.39±9.75	11.89±24.05	0.00±0.00
Ca with	0.73±1.68	4.03±8.94	7.53±17.75
Ca Salts (mg/Kg/d)			
Ca between	0.03±0.05	0.08±0.12	0.00±0.00
Ca with	0.01±0.02	0.06±0.15	0.12±0.29
Free Fatty Acids (mg/Kg/d)			
Ca between	0.41±0.80	0.49±0.76	0.00±0.00
Ca with	0.34±0.69	0.58±1.19	0.54±1.22
Compound Lipid (mg/Kg/d)			
Ca between	0.11±0.24	0.63±1.24	0.00±0.00
Ca with	0.08±0.18	1.60±3.78	0.66±1.53

Data are presented as Mean ± SD.

Table II-11b: Quantitative Measurement of Fecal Fatty Acid C14:0 in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	39.1±48.9	70.9±70.3	28.5±41.7
Ca with	26.6±35.6	27.0±30.2	45.8±57.4
Ca Salts (mg/Kg/d)			
Ca between	1.38±0.89	1.20±0.59	1.29±1.99
Ca with	1.86±2.95	1.58±1.87	1.52±2.28
Free Fatty Acids (mg/Kg/d)			
Ca between	12.0±7.7	12.1±6.0	4.3±1.9
Ca with	11.4±11.3	7.1±6.4	8.8±11.0
Compound Lipid (mg/Kg/d)			
Ca between	10.6±6.8	12.2±6.8	6.5±7.4
Ca with	7.7±7.1	13.5±21.6	7.9±12.2

Data are presented as Mean ± SD.

Table II-11c: Quantitative Measurement of Fecal Fatty Acid C16:0 in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	141±183	237±248	95±106
Ca with	80±99	83±88	138±177
Ca Salts (mg/Kg/d)			
Ca between	11±9	8±5	9±13
Ca with	11±15	11±13	10±17
Free Fatty Acids (mg/Kg/d)			
Ca between	88±62	90±59	33±11
Ca with	89±102	52±41	88±142
Compound Lipid (mg/Kg/d)			
Ca between	61±52	84±53	43±47
Ca with	55±55	73±95	50±75

Data are presented as Mean ± SD.

Table II-11d: Quantitative Measurement of Fecal Fatty Acid C18:0 in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	51.9±57.4	82.4±69.5	55.1±21.5
Ca with	28.7±24.7	33.0±23.1	54.9±59.4
Ca Salts (mg/Kg/d)			
Ca between	7.6±6.5	4.9±2.4	8.2±7.6
Ca with	5.7±5.9	5.8±5.8	6.4±8.5
Free Fatty Acids (mg/Kg/d)			
Ca between	59.4±34.9	60.2±36.3	37.9±16.6
Ca with	56.8±44.0	37.2±23.2	57.7±69.0
Compound Lipid (mg/Kg/d)			
Ca between	46.1±30.7	56.2±30.8	43.6±43.7
Ca with	33.7±21.9	56.8±81.0	36.5±45.8

Data are presented as Mean ± SD.

Table II-11e: Quantitative Measurement of Fecal Fatty Acid C18:1(9) in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	332±352	449±406	278±354
Ca with	229±260	207±258	309±333
Ca Salts (mg/Kg/d)			
Ca between	3±2	4±2	4±5
Ca with	4±4	4±5	4±6
Free Fatty Acids (mg/Kg/d)			
Ca between	28±20	18±11	12±6
Ca with	39±58	20±26	18±15
Compound Lipid (mg/Kg/d)			
Ca between	20±18	22±16	18±21
Ca with	21±28	12±9	25±41

Data are presented as Mean ± SD.

Table II-11f: Quantitative Measurement of Fecal Fatty Acid C18:2(6) in Breast Fed Premature Infants Receiving Ca Lactate Between Feedings or With Feedings in the First, Second, and Fourth Postnatal Week

	Week 1	Week 2	Week 4
	n=8	n=6	n=6
Neutral Lipid (mg/Kg/d)			
Ca between	56.6±65.6	83.7±83.1	47.9±68.0
Ca with	30.3±35.7	22.7±28.2	50.6±64.8
Ca Salts (mg/Kg/d)			
Ca between	0.38±0.32	0.36±0.27	0.65±0.77
Ca with	0.49±0.73	0.54±1.10	0.32±0.30
Free Fatty Acids (mg/Kg/d)			
Ca between	2.53±2.25	1.46±0.96	1.47±0.92
Ca with	3.34±5.54	1.21±1.55	1.01±0.63
Compound Lipid (mg/Kg/d)			
Ca between	1.78±1.78	1.93±1.54	1.74±2.10
Ca with	3.04±6.17	4.22±8.81	1.55±2.07

Data are presented as Mean ± SD.

D: Discussion and Conclusions

1. Chronological Changes in Milk Composition

Calcium and phosphorus content of preterm milk decreased gradually in the first four weeks of life, which is consistent with previous reports (9,10,11,12). Mean calcium concentration of preterm milk during this period is also consistent with other studies (13,14). However, the decline in magnesium concentration was not as obvious as that in calcium and phosphorus. Mean milk volume consumed by infants from both groups in the first four postnatal weeks was 162 ± 17 ml/Kg/d which is within the range of 159-182 ml of other four similar studies (12,14,15,16). This volume may provide the infant with about 105 Kcal/Kg/d if it was well absorbed (16). The total lipid in preterm milk, 3.34 ± 0.63 g/dl, was a little less than the approximate amount of 4 g/dl in another study of preterm human milk (13).

2. Weight Gains in Anthropometric Study

Infants from both groups grew steadily as measured by body weight, body length and head circumference. However, weight gain of all infants in this study was less than other studies of non-supplemented preterm milk fed premature infants (14,16). Morever, infants receiving calcium between feedings showed even less weight gain compared to infants receiving calcium with feedings, especially during the second week of life, possibly because of the increased loss of ingested lipid from stool in relation to the decreased energy intake. Attendency to increase weight gain with

postnatal age in the first four weeks of life also seems to exist.

3. Effects of Calcium Supplementation on Intestinal Fat and Mineral Absorption

Mean lipid intake was about 5.4 ± 1.1 g/Kg/d which is in agreement with previous studies (12,15,16). Fecal lipid excretion in the infants given calcium between feedings was much higher when compared to the premature infants who were fed exclusively preterm human milk (15,16). The latter usually exhibits a fat absorption rate of more than 80% (15,17). However, the infants given calcium between feedings in the present study absorbed approximately 75% of milk lipid. Accordingly, all calcium, phosphorus, and magnesium absorption seem impaired when the calcium supplement was given between feedings with human milk (calcium 66% vs 74%, phosphorus 86% vs 93%, and magnesium 50% vs 72%).

In the original hypothesis, it is assumed that the less time that calcium and lipid interact in the intestinal lumen, the less chance they will have to form insoluble soap. However, the results of this experiment suggest that, as compared to giving calcium between feeding, giving calcium with the milk actually facilitates calcium and fat absorption, resulting in reduction of calcium-soap formation, and relatively less calcium and fat excretion in stool. As the interaction of minerals is also present in intestinal lumen, the change in fat absorption may alter phosphorus and magnesium absorption as well.

Possible Activation of Lipolytic Enzyme(s)

Some mechanisms may be involved in the enhancement of fat absorption when fat is ingested with calcium. Calcium may improve fat absorption by forming micelles, which incorporate or remove the lipolytic product of fat digestion, i.e. free fatty acids, in the stomach and intestine, thereby preventing a product inhibition effect. Another possibility is that calcium given with feedings activates the lipolytic enzymes, lipase(s), in the intestine. Calcium may have acted on pancreatic lipase or bile salt-stimulated lipase in the breast milk. The activated lipase digested milk fat more efficiently and, therefore, both fat and calcium absorption were enhanced. In vitro, calcium ion incubation decreases not only the lag phase before the accelerated hydrolysis of triglycerides reaches a high rate (18), but also decreases the rate of resynthesis of glyceride ester bonds (19). Calcium ions may have also acted on colipase synthesis and thus the binding strength and enzyme activity of lipase-colipase increases. In the presence of calcium ions, a smaller form of colipase, $colipase_{85}$, appears, whose specific activity is five times higher than that of regular $colipase_{101}$ (20). With intralipid as substrate, $colipase_{85}$ enables lipase to reach the triacylglycerol substrate more rapidly and shortens the lag phase for a given concentration (20). This type of interaction has further been confirmed recently by a study of triglyceride emulsions in vitro showing that the micelle-lipase-colipase complex exhibits decreased activity in the absence of calcium ions; whereas, in the presence of calcium, it becomes more active and binds more easily to interfaces (21).

Calcium Soap Formation

As expected, calcium soap formation occurred as shown from the positive correlation between fecal lipid and calcium content in the stool, especially in the infants receiving calcium with feedings (calcium with feedings: r=0.91 vs calcium between feedings: r=0.63). The long-chain saturated fatty acids were most involved in the soap formation and consequently excreted, as indicated by the increased loss of fatty acid C16:0 and C18:0 in stool and the decreased absorption coefficient of these two fatty acids.

Interactions of Divalent Cations

Complex interactions among cations are also apparent from the correlation analysis of calcium, phosphorus and magnesium excreted in stool, indicating calcium phosphate (fecal calcium and magnesium correlation: calcium with feedings: r=0.83) or magnesium phosphate (fecal phosphorus and magnesium correlation: calcium between feedings: r=0.78 and calcium with feedings: r=0.77) may be formed. Calcium-phosphorus-fat complex may also be formed as the correlations among fecal lipid, calcium, and phosphorus were quite significant.

Intraluminal calcium can modulate lipid dynamics of intestinal brush-border membrane in rat (22), and the change in membrane physiological processes may further complicate the mineral interactions. In vivo calcium gavage feeding results in a decrease in sphingomyelinase and an increase in sphingomyelin synthase in brush-border membrane, which enhance the synthesis of sphingomyelin in the membrane and, consequently,

decreases fluidity of brush-border membrane. The change in membrane lipid composition and fluidity may influence certain enzyme activities, receptor functions, permeability, and some ion channels within the membrane. It is possible that absorption of fatty acids, magnesium and phosphorus was affected in this manner.

4. Absorption Coefficients of Individual Fatty Acids

Fatty acids C12:0 and C14:0 were efficiently absorbed in the infants receiving calcium supplementation. However, fatty acids with sixteen and eighteen carbons seem to be affected by the calcium supplementation, among which C18:0 was most impaired (pooled absorption coefficient: 60.59%) because of the increased fecal loss of long-chain saturated fatty acids by forming insoluble calcium soaps in the intestinal lumen of the infants receiving calcium supplementation. This is in an agreement with other studies.

5. Fecal Lipid Fractions

The percentage distribution of fecal lipid in this study is contrary to that observed in other studies. It has been reported that the calcium salt fraction in stool of normal nonsupplemented newborn infants accounts for 25% of total fecal lipid; another 56% of fecal lipid is neutral lipid and the remainder is ionized fatty acids and bound lipid (8). Using the same methodology, the four fractions of fecal lipid in this study are distributed as follows: neutral lipid 54.4%, calcium salts 2.5%, free fatty acids 21.5% and compound lipid 21.7%. The difference in the calcium

salt fraction of this study cannot be readily explained. Perhaps, the influence of calcium supplementation on fat absorption is minimal and should not be done, or the mechanism of increased lipid excretion as suggested by the calcium-fat soap formation should be questioned.

Another study with either a lard-modified formula or an unmodified formula shows that greater than 90% of fecal lipid is free fatty acid (23) in newborn infants fed either formula. The high level of intestinal lipolysis was not seen in this study. Instead, free fatty acids in stool gradually decreased during the first four postnatal weeks while neutral lipid was increasing, indicating that the intestinal hydrolysis of milk lipid was progressively reduced at first weeks of life. A possible explanation is that the amount of lingual and gastric lipase is temporarily sufficient while its production and that of intestinal lipolytic enzymes are still quite limited at the early stage of life. The longitudinal change of these two fractions was more obvious in the infants receiving calcium between feedings, which might further indicate relatively less activity of lipase(s) at a low concentration of luminal calcium.

In conclusion, calcium and phosphorus content of preterm human milk decreased gradually in the first four weeks of life. Increased calcium intake resulted in the greater calcium loss from stool. Calcium supplementation between feedings further reduced the intestinal absorption of milk lipid, calcium, phosphorus, and magnesium compared to the supplementation with feedings. The absorption coefficients of C16:0 and C18:0 were most diminished in these infants, who also showed less weight

gain during the balance study. Therefore, it is not appropriate to feed calcium supplement between feedings to premature infants fed preterm human milk in the first four weeks of life. Yet intraluminal interactions of milk lipid, calcium, phosphorus, magnesium, and, possibly, other nutrients in human milk, as well as the role of calcium in the intestinal lipohydrolysis of milk, and fecal lipid fractionation need to be further elucidated.

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Appendix I

EXTRACTION OF MILK LIPID

(Modified Folch Extraction)

1. Weigh approximately 0.2g freeze dried milk in a 50ml glass test tube with a screw cap and record the weight.
2. Add 1ml distilled deionized water and vortex.
3. Add 17.25ml Chloroform:Methanol solution (2:1 with Butylated Hydroxyl Toluene 0.005%) and 7.5mg internal standard Triglyceride C19:0 (0.75ml, 100mg/10ml in Chloroform:Methanol solution) in Fume Hood.
4. Polytron for 30 seconds.
5. Add 5ml 0.9% NaCl, vortex, and centrifuge at 2,000RPM for 10 minutes.
6. Extract 8ml chloroform carefully to a 15ml preweighed screw tube.
7. Evaporate to dryness with nitrogen protection in 50°C heating block.
8. Reweigh the tube, record the weight, and calculate the total weight of milk lipid: reweight of the tube (with dried lipid) - preweight of the blank tube.
9. Saponify the lipid by adding 2ml 0.5N methanolic KOH and heating at 100-105°C for 1 hour.
10. Methylate the sample by adding 2ml hexane and 1.5ml 14% Boron Trifluoride Methanol and heating at 100-105°C for 1 hour.
11. Add 2ml water, vortex, and centrifuge at 2,000RPM for 10 minutes.
12. Transfer the hexane to a 1.8ml vial, evaporate to dryness, and store in freezer (-20°C) under nitrogen protection for future GC analysis.

Appendix II

EXTRACTION OF FECAL LIPID

(Modified Method of Watkins and Bliss)

1. Weigh approximately 0.2mg freeze dried stool in a 50ml screw tube.
2. Add 5ml water and vortex for 2 minutes.
3. Add 10ml ether, vortex for 1 minute, and centrifuge at a maximum speed of 2,000RPM for 30 seconds.
4. Transfer the ether to a 15ml capped COREX tube and evaporate to 10ml at 40°C.
5. Repeat steps #3 and #4 another two times.

Ether Phase:

6. Centrifuge the COREX tube at 11,200RPM (i.e. 15,000G) at -5 to -10°C for 15 minutes to separate neutral lipid and calcium salts in ether.

Neutral lipid separation:

7. Transfer all neutral lipid-containing ether to a 25ml screw tube.
8. Add 10mg internal standard TG C19:0 (4ml, 2.5mg/1ml in hexane) and evaporate to dryness.
9. Saponify the lipid by adding 2ml 0.5N methanolic KOH and heating at 100-105°C for 1.5 hour.
10. Methylate the sample by adding 2ml hexane and 3ml 14% BF_3 and heating at 100-105°C for 1 hour.
11. Add 5ml water, vortex, and centrifuge at a maximum speed of 2,000RPM

for 30 seconds.

12. Transfer the hexane to a 1.8ml vial and evaporate to dryness.

Calcium salts separation:

13. Add 2ml water, 7-12 drops of 6N HCl and 2ml ether to the COREX tube and scrub the wall with Ca salt deposition repeatedly using a Pasteur pipet.
14. Transfer the ether-water to a 50ml screw tube.
15. Repeat steps #13 and #14 another two times.
16. Vortex and transfer ether to a 25ml screw tube and evaporate to dryness.
17. Repeat step #16 another two times.
18. Add 1mg internal standard FA C19:0 (2ml, 0.5mg/1ml in hexane).
19. Methylate the sample by adding 1ml 14% BF_3 and heating at 100-105°C for 1 hour.
20. Add 5ml water, vortex, and centrifuge at a maximum speed of 2,000RPM for 30 seconds.
21. Transfer the hexane to a 1.8ml vial and evaporate to dryness.

Water Phase:

22. Acidify the water phase to pH 1 by adding 6N HCl.
23. Add 20ml Chloroform:Methanol (2:1, 0.01% BHT) and 4ml saturated NaCl and vortex for 30 seconds.
24. Centrifuge at 2,000RPM for 5 minutes.

Free fatty acid separation:

25. Extract all chloroform to a 25ml screw tube and evaporate to dryness.
26. Add internal standard FA C19:0 4mg (2ml, 2mg/1ml in hexane) and 2ml 14% BF_3 and methylate at 100-105°C for 1 hour.
27. Add 5ml water, vortex, and centrifuge at a maximum speed of 2,000RPM for 30 seconds.
28. Transfer the hexane to a 1.8ml vial and evaporate to dryness.

Compound lipid separation:

29. Alkalize the remaining water phase to pH 10 by adding 10N NaOH.
30. Add internal standard TG C19:0 2mg (2ml, 1mg/1ml in hexane) and saponify it at 100-105°C overnight.
31. Reacidify to pH 1 by adding 6N HCl.
32. Add 20ml Chloroform:Methanol (2:1, 0.01% BHT) and 4ml saturated NaCl and vortex.
33. Centrifuge at 2,000RPM for 5 minutes.
34. Extract chloroform to a 25ml screw tube and evaporate to dryness.
35. Methylate the sample by adding 2ml hexane and 2ml 14% BF_3 and heating at 100-105°C for 1 hour.
36. Add 5ml water, vortex and centrifuge at a maximun speed of 2,000RPM for 30 seconds.
37. Transfer the hexane to a 1.8ml vial and evaporate to dryness.