Conjugated Linoleic Acid Intake of
Exclusively Breastfed Infants

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Honors Thesis
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Abstract

Conjugated linoleic acids (CLA) are a group of octadecaenoic fatty acids present in high concentrations in beef and milk fat. Several isomers of CLA exist, but the $\text{cis} \ 9, \ \text{trans} \ 11$-18:2 conformation, given the trivial name “rumenic acid” (RA), is the most abundant in the human diet. Consumption of CLA during infancy has shown to inhibit cancer growth in laboratory animals later in life. It is hypothesized that a similar effect may be realized in human infants. Though human milk contains significant levels of CLA, studies show that infant formula does not contain CLA. Additionally, increases in milk CLA concentration have been demonstrated through maternal diets high in CLA. To date, no documentations of CLA intake for exclusively breastfed infants have been published. Because of the potential health benefit CLA poses to infants and the lack of CLA in infant formula, average CLA intake of exclusively breastfed infants ($n = 10$) was documented and found to be $114.03 \pm 0.04$ mg/d (mean ± SEM). Exclusively breastfeeding infants participated in a single 24 hr weighed feeding period in conjunction with a single 24 hr milk collection period used to determine average daily CLA intake. Average maternal CLA intake was estimated using 3 d weighed food records and found to be $154.7 \pm 21.9$ mg/d (mean ± SEM). No significant relationship between maternal CLA intake and milk CLA concentration was observed. Data indicate no effect of maternal dietary CLA on infant CLA intake.
Conjugated Linoleic Acid: An Introduction

Conjugated linoleic acids (CLA) are octadecadienoic fatty acids with conjugated double bonds. Experimental studies have shown CLA to inhibit an impressive number of cancers, including those of the breast, prostate, and skin (Ip et al., 1999; Cornell et al., 1997; Shultz et al., 1992). In addition, studies suggest that CLA might influence the risk of several other chronic degenerative diseases, including those involving bone mineralization (Li and Watkins, 1998), atherogenesis (Lee et al., 1994) and glucose homeostasis (Houseknecht et al., 1998).

There are several geometric and positional isomers of CLA, the most abundant in the human diet being the cis 9, trans 11-18:2 isomer, given the trivial name “rumenic acid” (RA) (Kramer et al., 1998). Rumenic acid derives its name from the fact that foods of ruminant origin (e.g., beef and milk), contain high concentrations of this isomer. Other isomers of CLA are present in human foods, but RA tends to be the predominate form, comprising over 80% of CLA present in milk fat and 75% in beef fat (Ritzenhaler et al., 1998).

The presence of CLA was first documented in butter in 1933 by Booth et al., (1933a) while studying seasonal variations in butter fat. It was not until some fifty years later that the importance of CLA to human health was recognized by Pariza and colleagues who demonstrated that these fatty acids could inhibit cancer (Pariza et al., 1985). Since that time, many studies have been done to document the presence and concentration of CLA in many different foods. Generally, these studies show that in the human diet, CLA is present in significant quantities in dairy products, beef, ruminant tissues and human milk. It is believed that the biohydrogenation pathways of the microflora in the ruminant gut account for higher levels of CLA in ruminant milk and tissues as compared to CLA levels in monogastriode milk and tissues (Griinari et al.,
In a study of the CLA milk concentrations of several species it was found that ruminant (cow, goat and ewe) milk contained more CLA on average than that of monogastrides (mare, sow and woman), while human milk, was found to be higher in concentration than both the mare and the sow (Jahreis et al., 1999).

The diversity of potential health benefits attributed to CLA, the anticarcinogenic properties of RA and especially the presence of CLA in high concentrations in human milk has spawned a great deal of interest in the public health importance of CLA as a dietary component. Animal studies have shown that CLA intake during infancy may afford lifelong protection against some cancers (Ip et al., 1999) and improve postnatal weight gain in the nursing infant (Chin et al., 1994). Many studies have since been performed to ascertain the specific physiological effects of CLA and any further benefits. The following will review some of the literature published to date on the health benefits attributed to CLA.

**Health Benefits of CLA**

Recent investigations into the health benefits of CLA have focused on the specific activities of different isomers composing CLA. Recent evidence demonstrates distinct differences in the physiological effects of the cis 9, trans 11 and trans 10, cis 12 isomers (Park et al., 1999; Baumgard et al., 2000). Animal studies suggest that RA is the primary isomer responsible for the anticarcinogenic properties observed for CLA (Ip et al., 1999), whereas trans 10, cis 12-18:2 is likely the isomer linked to alterations in lipid metabolism and body composition (Park et al., 1999). Additionally, the distribution of CLA isomers in commercially produced supplements may be quite different than those of naturally occurring isomers in foodstuffs. This is significant because commercially produced supplements have generally been used as the testing material for many studies. While it has been suggested that CLA may possess
a wide range of potential health benefits, these benefits are not all attributed to one isomer. It is important to remember that in the human diet the majority of CLA consumed is in the isomeric form of RA (McGuire et al., 1999). Therefore, benefits ascribed to other isomers, namely trans 10, cis 12, may not be as accessible, outside supplementation, as benefits attributed to the dominant dietary isomer, RA. This should be kept in mind throughout the remainder of this review.

CLA and Cancer Amongst the many health benefits attributed to CLA, one of the initial and perhaps the most thoroughly examined to date is the ability of CLA to inhibit cancer. The initial work determining the anticarcinogenic properties of CLA was directed by Dr. Michael Pariza of the University of Wisconsin. Pariza initially found a mutagenesis modulator and bacterial mutagens in methylene chloride extracts of fried ground beef (Pariza et al., 1979, 1983). Pariza and Hargraves (1985) later demonstrated that these extracts from beef inhibited the initiation of mouse epidermal tumors by 7, 12-dimethylbenzanthracene (DMBA) when applied to the skin 5 min before DMBA. Purification of these extracts led to the discovery of linoleic acid derivatives with conjugated double bonds—what we now know as CLA (Ha et al., 1987). Further studies then determined that topical application of CLA before DMBA and promotion by 12-0-tetradecanoylphorbol-13-acetate (TPA) reduced both the number of papillomas and the tumor incidence (Ha et al., 1987). Thus, the anticarcinogenic properties of CLA were discovered.

Much of the later work on CLA and cancer focused on the prevention of mammary cancer. For the first time, Ip et al. (1991) fed CLA as a part of a rat’s diet and demonstrated the effect of dietary CLA on DMBA-induced mammary tumors. Ip et al. (1991) also reported a dose-dependant reduction in incidence and number of mammary tumors in rats fed CLA.

Further work (Ip et al., 1994) determined that even smaller concentrations of CLA were effective
in reducing mammary tumor incidence and number; as little as 0.1% of the diet as CLA reduced the total number of mammary tumors present at autopsy. In this study, Ip et al. (1994) moved to shorter-term feeding studies in which CLA was fed only from weaning until about 5 weeks, a period of rapid mammary development. Administration of carcinogen occurred at the end of the dietary intervention, at which time all rats received the same diet devoid of CLA. Further evaluations of the effects of timing and duration of CLA consumption on mammary cancer have demonstrated that feeding of CLA during a period of rapid mammary development clearly protects against mammary cancer; however, continuous intake of CLA is required to inhibit tumorigenesis when CLA feeding is started after carcinogen administration (Ip et al., 1995, 1997a).

Recent evidence has also demonstrated that CLA is effective in reducing the growth of both human breast (Visonneau et al., 1997) and prostate cancer cells (Cesano et al., 1998) implanted into immune-deficient animals. Evidence that dairy products can elicit prevention of cancer has been provided by recent investigations by Ip et al. (1999). Using methylnitrosourea (MNU), a carcinogenic direct alkylating agent, Ip et al. (1999) fed rats from weaning until 50 d age a diet without additional CLA or with CLA from various sources. Reduction in tumor incidence and number occurred regardless of whether the CLA was provided by commercial sources or through butter. The cis 9, trans 11 CLA possessed the ability to inhibit cancer when provided as either butter or the relatively pure isomer. In a study examining the effects of CLA supplementation timing on the suppression of tumorigenesis, it was found that rats fed CLA from weaning at 21d until administration of carcinogen at age 50d were afforded protection from mammary cancer for life, while rats fed CLA after administration of carcinogen required continuous supplementation to achieve equivalent results (Ip et al., 1995).
CLA and Body Composition  Recent evidence suggests that CLA may alter body composition. Mice fed diets containing 0.5% CLA exhibited nearly 60% less body fat with an almost 10% increase in body protein upon autopsy (Park et al., 1997). Similar studies have demonstrated improved lean growth in growing pigs fed CLA enriched diets (Ostrowska et al., 1999). West et al. (1998) have also demonstrated a dramatic reduction in adipose depot weight in mice fed CLA. They attributed the decrease in adipose partly to reduced energy intake and increased metabolic rate. However, in a double blind, placebo-controlled study in obese subjects, preliminary evidence of an alteration in body composition in humans due to CLA was not detected (Pariza, 1998). Further research is necessary to determine whether humans can alter their amount of lean or adipose tissue through dietary supplementation of CLA and, if so, which isomer is responsible for this effect.

Further research has shown distinct differences between the activity of the cis 9, trans 11 and trans 10, cis 12 isomers on lipid metabolism. Park et al. (1999b) demonstrated that changes in body composition of mice were attributed to the trans 10, cis 12 isomer but not to the cis 9, trans 11 isomer. In vitro, the trans 10, cis 12 isomer reduced lipoprotein lipase activity, intracellular triacylglycerol and glycerol, and enhanced glycerol release into the medium, but the cis 9, trans 11 and trans 9, trans 11 isomers failed to effect these components (Park et al., 1999b). Data thus far suggests that it is primarily the trans 10, cis 12 isomer that is responsible for changes in lean and fat body mass ratios in animals. Further research is needed in this area to determine if the effects of this isomer could possibly be realized in humans.

CLA, Growth and Diabetes  Another physiological effect observed in conjunction with dietary supplementation of CLA is an alteration in growth and weight gain. In a study done to examine the effects of CLA supplementation on the suckling pup it was found that pups nursed
by rats fed 50% CLA during gestation and lactation or lactation only had improved postnatal body weight gain and feed efficiency (Chin et al., 1994). Further, dams fed CLA had leaner pups. No consistent response in milk protein content was observed, but the CLA content of the rat milk was enriched in the dams fed CLA.

Interestingly, it has been demonstrated that CLA may improve glucose intolerance associated with diabetes. The feeding of CLA to rats prone to developing diabetes normalized glucose tolerance and improved hyperinsulinemia as effectively as currently used medications (Housknecht et al., 1998). The CLA fed was a mixture of isomers, mainly cis 9, trans 11 (~ 42%) and trans 10, cis 12 (~ 43.5%). This mixture was fed at 1.5% (by weight) of diet for two weeks. The study was short term and needs to be extended and repeated before the results can be applied to human health. Nevertheless, if CLA can help to improve insulin resistance and glucose tolerance in addition to reducing adipose tissue, it could be of great benefit to humans with and prone to diabetes.

CLA and Milk Fat Depression Infusions of commercial mixtures of CLA given to cows have been shown to dramatically reduce milk fat percentage and hence lipogenesis (Couinard et al., 1999). Baumgard et al. (2000) reported that the active isomer of CLA responsible for the depression of milk fat was the trans 10, cis 12-CLA; no effects on milk fat percentage were detected with infusion of RA. A similar effect of milk fat depression was demonstrated in a human study in which lactating women were given CLA supplements (Masters et al., 2002). The subjects showed increased milk concentrations of RA and trans 10, cis 12 CLA and significantly decreased milk fat percentages during the periods of CLA supplementation. The CLA supplements used were isomeric mixtures of CLA and provided each subject with approximately 560mg of the trans 10, cis 12 isomer and 547mg of RA daily. Conversely, Ritzenthaler et al.
(2002) found that increased CLA intake through consumption of a high-CLA cheddar cheese increased the RA concentrations of human milk but did not alter the milk fat percentage. This is an important change in milk composition, and further study is needed in this area. A possible explanation of the contradictory results of these studies is the presence of a significant percentage of \textit{trans} 10, \textit{cis} 12 CLA, the milk fat depressing isomer, in the Masters study (2002); whereas cheese, a natural product containing a majority of RA, the isomer not responsible for milk fat depression, was used in the Ritzenthaler study (2001).

**Documentation of CLA Intake in Humans**

In regard to all the potential public health benefits of CLA, accurate documentation of average human CLA consumption is of value. Conjugated linoleic acid intake in humans has been estimated using limited nutrient databases in conjunction with 3-d dietary records, semiquantitative food-frequency questionnaires and data from a German national food intake survey. In a study done to test the accuracy of written food records and nutrient databases, CLA intake estimated by written dietary assessments was shown lower than actual intake estimated by food duplicate methodology (Ritzenthaler et al., 2001). Results from this study showed estimates of CLA intake from dietary records to be 176 and 104 mg/d for men and women, respectively, while simultaneous evaluation of the diet by food duplicate methodology showed actual CLA intakes to be (on average) 212 and 151 mg/d for men and women, respectively. While earlier estimates suggested that humans may consume as much as 500 to 1000 mg/d CLA (Ip et al., 1994), more vigorously attained data suggests intake levels much lower. Other published estimates of human RA intake range from 430 and 350 mg/d for German males and females (Fritsche et al., 1998) to studies of American college students with intake levels estimated at 137 and 52 mg/d for males and females, respectively (Ritzenthaler et al., 1998).
Table 2 presents published estimates of human RA intakes. The wide variance in intake estimation is likely seen in part from varying methods of analysis and in part from the incompleteness of nutrient databases used in estimations of intake.

The National Research Council has stated that CLA unequivocally inhibits cancer in animal models in certain doses (NRC, 1996). Studying human CLA intake to determine if levels reach those shown to inhibit cancer in animal models seems warranted as a step in investigating the potential public health benefits of CLA consumption by humans.

Noteworthy is the fact that, although many studies have been done on the estimation of CLA intake in humans, all but one study has looked at limited populations. The one exception is the study published from the German national food intake survey, which surveyed the overall German population (Frischt et al., 1998). To date, no data have been published on estimations of dietary intake of CLA infants, school-aged children or adolescents.

**CLA in Infancy**

Studies suggesting that CLA consumption during infancy may afford long term health benefits and the presence of high concentrations of CLA in human milk raise the important possibility that CLA may be beneficial to infant health, and that these benefits can be passed from mother to child through human milk. Any possibility of infant health improvement is of public health importance and deserves investigation.

*Potential Benefits* Conjugated linoleic acid consumption has been shown in various study models to have diverse physiological health benefits. Of these studies, some have shown the effects of CLA to be dependant on timing of consumption. In studies using mice, Ip et al. (1995, 1997) demonstrated that CLA afforded life-long protection against mammary cancer if consumed during infancy. The implication for the human infant is that CLA consumption during
infancy may also provide protection from mammary cancer later in life. Further investigations of the fetal or early childhood hypothesis of disease suggest that chronic later life conditions such as obesity, diabetes, bone demineralization, atherosclerosis and cancer, all conditions that animal studies of CLA supplementation have shown to inhibit, may originate partly in the diet of expectant mothers, infants and young children. These studies suggest that early childhood diet may impact health later in life. It is possible that human milk contains CLA in high enough concentrations to impart levels of CLA to an infant equal to those used in animal studies that suggest potential health benefits. As demonstrated in part by the Ip studies (1991-1999), it is possible that CLA as a part of the diet at an early age may have positive effects on health at a later age.

CLA Concentrations in Human Milk  Several studies documenting the concentration of CLA in human milk have been published. The first documentation of CLA in human milk was published in a study of CLA concentrations in the lipids of foodstuffs, human plasma and milk (Fogerty et al., 1988). A single sample of mature milk from each of 18 healthy, lactating women consuming a conventional Australian diet and 8 women belonging to the Hare Krishna religious sect, was collected. In this study, neither time postpartum nor time of collection was controlled for. Utilizing thin-layer chromatography and column chromatography, the lipids of the human milk were separated into classes and methylated to produce fatty acid methyl esters (FAME), from which the individual fatty acids were quantified using gas chromatography. Mean CLA content was found to be 5.8 mg/g fat. In a study using similar methods, percent CLA present as RA in human milk was found to be 2.23 to 5.43 mg/g lipid, or 83 to 100% of total CLA (McGuire et al., 1997). While both the McGuire and Fogerty studies used gas-liquid chromatography with retention times of standards for identification of CLA in human milk,
Jensen et al. (1998), used gas chromatography in conjunction with mass spectroscopy to confirm the presence of CLA in human milk. The results of the Jensen study showed somewhat lower concentration of CLA than previous studies. It is thought that the use of human milk samples from a previous study of fish oil supplementation may have been responsible for the much lower concentrations than those found in other studies. Table 1 presents published findings on the CLA concentrations of human milk.

**Importance of Documenting Infant CLA Intake** If it is possible that CLA consumption during infancy affords benefits to the individual later in life, as animal studies suggest; then, in the interest of public health, this should be investigated. If infant CLA intake can be quantified, levels can then be accurately compared to supplementation doses used in animal studies that resulted in positive health effects. In addition, a study investigating the CLA concentrations of human milk and infant formula found CLA in trace amounts or lower than detection limits of the assay used in three of four brand name infant formulas analyzed. Interestingly, one brand which derived 30% of its lipids from beef tallow showed consistently higher levels of CLA (McGuire et al., 1997). The lack of CLA in infant formulas may be of concern because, by design, propriety infant formulas are intended to mirror human milk composition in all ways industrially feasible. If CLA does in fact impart health benefits to the breastfed infant, then the formula-fed infant should receive CLA through formula as well. Conjugated linoleic acid consumption by the breastfed infant needs to be quantified in order to make accurate recommendations for CLA fortification of infant formula.

Studies suggest that maternal intake of CLA can affect human milk concentrations of CLA, thus delivering more CLA to the breastfed infant. In the study first documenting the presence of CLA in human milk, milk samples from two different subpopulations of women
were collected and found to be quite different in their fatty acid profile. Milk from women of the Hare Krishna religious sect had CLA concentrations of almost double those of women on a conventional diet. The difference in the milk CLA concentrations was initially attributed to the fact that the Hare Krishna women, who exclude meat from their diet, consumed larger amounts of butter or ghee in their diet (foods rich in CLA) than women consuming a conventional diet. Higher dietary intake of CLA was hypothesized as the reason for higher CLA concentrations in the milk of the Hare Krishna women, but diet was not controlled for in either population (Fogerty et al., 1988). In a later study designed to test the hypothesis that maternal diet affects CLA concentrations of human milk, sixteen healthy breastfeeding women were recruited from the Palouse region of Washington and Idaho. Each subject underwent a one week depletion period during which the women were asked to consume diets low in RA and dairy products in an attempt to standardize initial milk RA concentrations. Women then consumed either a high dairy or low dairy diet for one week, during which a milk sample was collected on the third and seventh day. Following this period, women consumed the other diet. Results showed that milk collected during the high and low dairy periods had CLA concentrations of 13.5 ± 0.1 and 8.2 ± 0.4 µmol/g lipid, respectively (Park et al., 1999a). Results imply that maternal intake of CLA can alter human milk concentrations of CLA. If CLA is shown to have health benefits for humans similar to those found in animal studies, then accurate intake data would be valuable in setting intake recommendations for breastfeeding mothers to maximize the potential benefit to their infant.

Infant CLA intake data is also important for setting CLA intake and supplementation recommendations for breastfeeding mothers who may consume diets low in CLA (e.g., vegans); which would result in lower CLA concentrations of milk. It is possible that CLA supplements
could increase milk RA concentrations, benefiting the health of the breastfed infant whose mother consumes a diet low in CLA. Animal studies have demonstrated that certain long-term health benefits attributed to CLA are derived from consumption of CLA during infancy (Ip et al., 1994). In this regard, it is important that infants consume either human milk, which is a food source rich in CLA, or a formula that has been fortified with CLA. This knowledge, coupled with the fact that infant formula is unfortified with CLA and accurate data of breastfed infant CLA consumption remains undocumented, makes evident the need for documentation of CLA during infancy. The lack of accurate data on infant CLA intake has been the impetus for this study. Many important health benefits have been attributed to CLA and CLA consumption; in particular, the potential benefits CLA intake poses to the infant. For these reasons, this study was designed to document CLA consumption in exclusively breastfed infants.
General Objectives and Hypotheses

The following general objectives were proposed for this study.

1) To perform for each of ten subjects a single documentation of daily intake of milk by exclusively breastfed infants by 24hr weighed feedings.
2) To determine average human milk CLA concentrations by biochemical analysis of human milk samples.
3) To determine the average daily CLA intake of ten lactating women by 3d dietary records in conjunction with nutrient databases.

The following hypotheses were tested.

1) It is hypothesized using previous data on the CLA concentrations of human milk (McGuire et al., 1997) and information on average milk intakes (Nutrition During Lactation, 1991), that average infant CLA intakes would range from 15 to 240 mg/d.
2) It was hypothesized from previously published data on maternal CLA intake and human milk CLA concentrations (Fogerty et al., 1988; Park et al., 1999a; Ritzenthaler et al., 2002) that infants whose mothers consume diets high in CLA will have higher CLA intakes than infants whose mothers consume diets lower in CLA.
Introduction

Conjugated linoleic acids (CLA) are a group of octadecadienoic (18:2) fatty acids with conjugated double bonds. First documented in foods in 1933 (Booth et al., 1933a; Booth et al., 1933b), the importance of these fatty acids to human health was not recognized until Pariza and colleagues demonstrated that these fatty acids could inhibit cancer (Ha et al., 1987). Since that discovery, CLA has been shown to inhibit an impressive number of cancers, including those of the breast, prostate, and skin (Ip et al., 1999; Cornell et al., 1997; Shultz et al., 1992). In addition, experimental studies suggest that CLA might influence the risk of several other chronic degenerative diseases, including those involving bone mineralization (Li and Watkins, 1998), atherogenesis (Lee et al., 1994) and glucose homeostasis (Houseknecht et al., 1998). Although there are many isoforms of CLA, the form found most abundantly in the human diet is the c9, t11-18:2 isomer, recently given the trivial nomenclature of rumenic acid (RA; Kramer et al., 1998). This fatty acid is produced via the biohydrogenation pathways of bacteria residing in the ruminant gastrointestinal tract. Thus, foods obtained from ruminant tissues (e.g., beef and dairy) contain significant amounts of RA.

Despite the knowledge of the potential health benefits of CLA, little accurate documentation of CLA intake has yet been performed, especially among children and infants (McGuire et al., 1999). Rumenic acid consumption was documented in adults living in the United States and daily intake was found to be 193 ±13 and 140 ±14 mg/d for males and females, respectively (Ritzenthaler et al., 2001). Dairy products contributed the majority (69%) of the RA consumed by the subjects.

Although it was known for over 50 years that CLA was found in cow’s milk, it was not until 1988 that the presence of CLA was documented in human milk (Fogerty et al., 1988).
Interestingly, negligible amounts of CLA have been found in infant formulas (McGuire et al., 1997). Conjugated linoleic acid intake has been estimated in breastfed infants, however the methodology used was not adequate to accurately document intake (Masters et al., 1999). Currently, there are no estimates of RA intake in any other populations (e.g., preschoolers and school-aged children). This information might prove important in future long-term studies, because it is thought that the genesis of several chronic diseases that may be influenced by CLA (e.g., cancer, diabetes, atherosclerosis and obesity) occur during these periods of time. Also documented is the increase of milk RA in lactating women consuming diets rich in high fat dairy products (Park et al., 1999a). Hence, the documentation of infant CLA intake will aid in the understanding of infant nutrition and may help provide more accurate recommendations for maternal diet during pregnancy and lactation. Additionally, documentation of CLA intake by breastfed infants could provide infant formula producers information on how to better fortify their formulas. Furthermore, animal studies suggest that CLA consumption early in life provides long-term protection from mammary tumors (Ip et al., 1999). If these results hold true for humans, then further documentation needs to be done on CLA intake in children so that effective recommendations can be set for intake.

The dramatic results of animal studies on the effects of CLA in the diet make evident the need for further studies on humans and CLA intake; but, before we can make further hypotheses concerning the effects of dietary CLA, we must first document average intakes in diverse populations. Documentation of intake in all populations is an essential first step in producing effective research in the future, as it provides a baseline for experiments on supplementation or deficiency.
In summary, CLA has many potential health benefits, though most need continued research to be verified. Documentation of CLA intake in infants is an important first step in continuing research on the effects of dietary CLA from infancy through childhood and into adult years. The lack of accurate data on infant CLA intake has been the impetus for this study. Many important health benefits have been attributed to CLA and CLA consumption; and in particular, the potential benefits CLA intake poses to the infant. For these reasons, this study was designed to accurately document CLA consumption in exclusively breastfed infants.

**Materials and Methods**

**Subjects** Eleven healthy breastfeeding mothers between 3 and 26 wks postpartum and their infants were recruited from the Palouse region of Washington and Idaho. Only those infants exclusively breastfed were eligible to participate, with exclusivity defined as consuming less than 1 Tbsp supplementary food per day. The general hypotheses of the study were explained to each subject, and written informed consent was obtained. All subjects were self reported healthy and free of chronic diseases. Maternal health, dietary inclination (vegetarian, vegan, etc.), activity level, parity and age were documented upon enrollment utilizing a self-administered questionnaire. All procedures described here were approved by Washington State University Institutional Review Board.

**Anthropometric Assessments** Maternal and infant height and length were measured using a wooden height board (Seca® Measure-All™, Hamburg, Germany) upon enrollment. Maternal body weight was measured using an electronic scale (Seca® Alpha, Model 770, Hamburg, Germany). Infant body weight was recorded upon enrollment utilizing an electronic infant scale (Seca® Model 727, Hamburg, Germany). Maternal body mass index (BMI) was calculated as measured weight (kg) divided by height squared (m²).
**Milk Sample Collection**  Human milk samples were collected at each feeding during a 24hr period. Milk was collected from alternate breasts by complete breast expression using an electric breast pump (Model SMR-B-R; Ameda-Egnell, Inc., Cary, IL or Model 01511 Medela®-Classic; Medela, Inc., McHenry, IL). At each feeding the subject pumped one breast while simultaneously feeding on the other breast. A 5% (by weight) sample of the collected milk was transferred to a collection vial and immediately frozen in the mother’s freezer.

**Milk Lipid Extraction**  Milk lipid was extracted using a modified procedure by Ingalls et al. (1995). Briefly, milk samples from each subject were pooled and three sets of milk (2 mL each) were extracted using 6 mL chloroform:methanol (2: 1, v/v; J.T. Baker, Phillipsburg, NJ). The chloroform:methanol layer was filtered through a granular anhydrous Na$_2$SO$_4$ Pasteur pipette column. For quantitative analysis of total milk lipid, the extraction procedure was repeated and completed with a final column rinse of 1mL chloroform:methanol. At this point one set of samples was stored at 4°C in an explosion-proof refrigerator for later fatty acid analyses. The remaining two sample sets, for total milk lipid quantification, were taken to dryness under nitrogen, and dried at 100°C overnight before the final weight was determined.

**Milk Fatty Acid Determination**  Following extraction of milk lipid, 300μL of each sample was aliquoted into duplicate tubes then dried under nitrogen at 47°C. Subsequently, 2 mL 0.5 N sodium methoxide in methanol (Aldrich Co., Milwaukee, WI) was added to dried samples and heated for 4 hr at 56°C (Blau and Halket, 1993). Following transesterification, samples were added to 5.5 mL of 0.2 N HCL and 3 mL methyl tert-butyl ether (MTBE):hexane (1:3; J.T. Baker, Phillipsburg, NJ) and mixed. The MTBE:hexane layer was removed and passed through a Pasteur pipette column containing a glass wool plug, 1 mm filter bed of silica gel and 3 cm anhydrous Mg SO$_4$/activated charcoal (Norite®; Sigma-Aldrich Chemical Co., St. Louis, WI)
mixture (1:10 w/w). A second extraction using 1 mL of MTBE:hexane followed and was
completed by a 1 mL final column rinse. Under nitrogen, samples were concentrated to
approximately 2 mL, 200 μL of methanol added, homogeneity checked and 15 μL of
trimethylsilyldiazomethane (TMSDAM, Aldrich Co, Milwaukee, WI) added (Hashimoto et al.,
1981). The reaction mixture was allowed to stand overnight at room temperature. The
TMSDAM was decomposed dropwise with 2% acetic acid in hexane until colorless and 1.5 mL
5% NaHCO₃ (w/v) added. The organic layer was removed and passed through a Pasteur pipette
column containing a glass wool plug and 4 cm silica gel overlaid with anhydrous Mg SO₄. A
second extraction using 1 mL of MTBE:hexane was followed by a 1 mL final column rinse.
Plasma samples were concentrated under nitrogen to approximately 200 μL.

Samples were analyzed on a gas chromatograph (Hewlett Packard 6890, Agilent
Technologies, Willington, DE) equipped with a capillary column (Restek #10641; 60m, 0.25 mm
ID with 0.5 μm film thickness; Stablewax®; Restek, Bellefonte, PA). Helium was used as
carrier gas at a constant flow mode with linear velocity set at 20 cm/sec. The flame ionization
detector was heated to 260° C, and detector gas flows were set at 40, 450, 49 mL/min for
hydrogen, compressed air and nitrogen, respectively. One μL samples were injected in the
splitless mode (injection temperature 260° C, on at purge at 0.75 min) with an initial oven temp
of 50° C with 4 min hold time, increased at 10° C/min to 200° C and at 2° C/min to the final
temperature of 240° C. The identities of fatty acid peaks were established by comparing
retention times to a milk fat reference standard obtained from the Commission of the European
Communities (CRM 164; European Community Bureau of Reference, Brussels, Belgium).

Determination of Maternal and Infant CLA Intakes Maternal dietary intake was
estimated using three-day weighed food records. Subjects weighed and recorded all food and
drink consumed for three 24hr periods, two weekdays and one weekend day (Electronic food scales were provided. Ohaus®, Florham Park, NJ). Data were evaluated using a computerized nutrient database (ESHA Research, Salem OR (version 7.50)) modified by us to contain quantities of total CLA and RA (mg/g fat) (Ritzenthaler et al., 2001).

Infant milk consumption was measured for a single 24hr period. Mothers were provided with electronic infant scales and asked to record their infants’ weights before and after each feeding. CLA intake by infants was calculated using the following equation:

\[
\text{CLA Intake (mg/d) = Avg. Milk Intake (g/d) x Avg. [CLA] of Milk (mg/g)}
\]

**Statistical Analyses** Data were analyzed using Excel (Microsoft® 1997; descriptive statistics) and Minitab (release 13.3; correlation statistics). For correlations, \( p < 0.05 \) was considered statistically significant. Values in this manuscript represent mean \( \pm \) SEM.

**Results**

**Description of Subjects** Descriptive statistics of the subjects are shown in Table 3. Data from one subject were excluded from the analysis due to the subject’s incompletion of the study; thus, data presented here represent those of the remaining women and infants \( (n = 10) \). In summary, women were young, of normal body weight and height and consuming standard diets. Infants were under the age of six months, healthy and of normal body proportions for their age.

**Maternal and Infant Dietary Intakes** Information concerning current maternal dietary intake estimated by 3 d weighed food records is shown in Table 4. Average maternal CLA and RA consumption were estimated as 154.7 ± 21.9 and 122.1 ± 18.5 mg/d, respectively. Average milk RA concentration was found to be 3.16 ± 0.31 mg/g lipid. Percent total CLA present in human milk as RA has been reported as 83 -100% (McGuire et al., 1997); for this reason, only the concentration of the RA isomer of CLA was quantified in milk fatty acid analysis. Likewise,
only the consumption of RA was determined for infant CLA intake. Overall, infant milk consumption ranged from 590 to 1053 g/d, with average milk consumption found to be $790.4 \pm 41.4$ g/d. Average infant RA intake was found to be $114.0 \pm 10.5$ mg/d.

**Correlation Analyses**  Correlations analyses were run on the following study variables: maternal parity, maternal age, maternal BMI, maternal CLA intake, maternal RA intake, time postpartum, average milk fat, percent RA fatty acid methyl esters (FAMES), RA concentration of total lipid, infant fat intake, RA concentration of total milk, infant milk intake, infant RA intake, infant weight and infant length. Significance values ($p$) from significant correlations are listed in Table 4. Analyses revealed significant relationships between (1) maternal age and maternal CLA and RA intake, (2) maternal BMI and RA intake, (3) milk concentrations of RA and infant RA intake, and (4) infant fat intake and infant milk intake. Graphical illustrations of these relationships are presented in Figures 1 through 5. The amount of RA infants consumed was affected only by the quantity of milk they consumed and the RA concentration of the milk they consumed. Analyses revealed no significant relationship between maternal RA intake and milk RA concentration. Women consuming diets high in RA did not produce milk of higher RA concentration. Hence, no significant relationship between maternal RA intake and infant RA intake was observed. Infants whose mothers consumed diets high in CLA did not have higher RA intakes than infants whose mothers consumed diets lower in CLA. Other intuitively significant relationships were observed between the following variables: time postpartum and infant weight and length, maternal CLA intake and maternal RA intake, infant fat intake and infant RA intake, and infant weight and infant length. These relationships and their $p$ values are presented in Table 4. Trends were also seen between parity and milk RA concentration ($p = 0.061$) and maternal RA intake and infant fat intake ($p = 0.073$).
Discussion

The results of this study show average infant RA intake to be $114.0 \pm 10.5$ mg/d. The significance of this finding lies in the more complete knowledge of human milk composition. Public health benefit might be derived from this finding through the production of infant formulas more accurately imitating human milk. Further benefit may be derived from this finding through comparison of average infant RA intake to RA supplementation in previous and future animal and human studies.

Further analyses indicate infant RA intake was influenced only by milk RA concentration. Data suggest that neither maternal CLA intake, RA intake nor BMI affected the RA concentration of milk, the milk fat percentage or the RA intake of the infant. To increase RA intake by exclusively breastfed infants, milk RA concentrations must increase. It was hypothesized based upon published work that maternal diet would influence milk RA concentration. Mothers consuming diets higher in RA were hypothesized to have higher milk RA concentrations, but this was not observed. These results are in contradiction with two published studies demonstrating an increase in milk RA through diets high in RA and through CLA supplementation of lactating women (Park et al., 1999; Masters et al., 2002). Several factors may contribute to the observed contradiction between results. The sample size of this study was too small to draw accurate conclusions on the effects of free-living maternal diets on milk RA applying to the general populace. Additionally, the accuracy of the nutrient database used to determine maternal CLA and RA intake was limited. The CLA and RA concentrations of many foods remain unknown, resulting in maternal CLA and RA intakes reported as lower than they may be in actuality.
While both studies demonstrating increases in milk RA as a consequence of maternal RA intake had relatively small sample sizes ($n = 16$; Park et al., 1999; $n = 10$; Masters et al., 2002) as well as having used a similarly limited nutrient database, both studies were interventions, either of high dairy diets (Park et al., 1999) or commercial CLA supplements (Masters et al., 2002). Recent evidence suggests that CLA supplementation may decrease milk fat, a very important change in human milk. Increases in milk RA concentration observed alongside decreases in milk fat (Masters et al., 2002) reported in studies using commercially produced CLA supplements may be observed due to an increase in proportion of RA present as a consequence of less total lipid present. This result may be hypothesized for studies using commercially synthesized CLA supplements, because these supplements contain a higher proportion of the \textit{trans} 10, \textit{cis} 12-18:2 isomer than do most foods normally found in the typical Western diet (Yurawecz et al., 1999; Ritzenthaler et al., 2001). The other study demonstrating an increase in milk RA through maternal dietary intervention used high fat dairy products as the intervention (Park et al., 1999). This is important because the majority of CLA in dairy products is present as RA, the isoform not responsible for milk fat depression. Indeed, in this study, an increase in milk fat was seen alongside an increase in milk RA. What then was the cause of the lack of a significant relationship between maternal RA intake and milk RA observed in the present study? Given the small sample size of the present study, it is likely that relationships observed between study variables are not indicative of the greater population. With such a small study size it is possible that milk RA is effected by maternal diet, it was just not observed in the subjects studied. The disconnect between the results of the present study and published work indicates the need for further research investigating the mechanisms by which milk fatty acid composition is regulated. It is possible that a physiological set point for milk RA concentration exists, but it could be
overridden by intervention. Longer trials and interventions between maternal dietary intake and milk RA concentrations are suggested for further research.

In addition, it is known that human milk composition is highly conserved, remaining constant almost regardless of maternal diet. Fatty acid composition of human milk has similar conservation. Roughly 30% of the fatty acids in human milk are derived from maternal diet (Hachey et al., 1987). Even with intervention, a great degree of change in fatty acid profile of human milk will not be seen through alteration of diet. Before attempting to alter milk composition through maternal diet, investigation should be directed toward determining if infants are consuming RA in quantities equal to those shown to have health benefits (e.g., anticarcinogenic effects) in animal studies. If average infant intake does meet these levels, there is yet another reason to endorse breastfeeding. If they do not, then finding some way to increase infant and RA intake would be advisable. If further research demonstrates a health benefit attributed to infant RA intake above and beyond that normally consumed, then continued evaluation of methods of increasing milk RA is suggested.

The results of this study do not indicate that maternal diet has any effect on milk RA concentration. In lieu of these results, suggestions for further research are directed towards determining if infant RA intakes meet those of supplemental intakes in animal studies. If further research demonstrates a health benefit attributed to infant RA intake above and beyond that normally consumed, then continued evaluation of methods of increasing milk RA is suggested.

Additional research is needed to determine the biological significance of the relationship between maternal age and maternal CLA and RA intakes, and maternal BMI and maternal RA intakes. The biological significance of these results and other trends observed in this data are unknown at this time.
Data suggest maternal CLA intake, RA intake or BMI have no affect on the RA concentration of milk, the milk fat percentage or the RA intake of the infant. The implication of these results is that maternal diet does not alter milk RA concentration, and therefore does not influence infant RA intake. Further research is needed to determine if CLA concentration in milk is controlled, and if so, by what mechanism.
Tables
Table 1. Concentrations of Rumenic Acid (RA) in Human Milk (means ± SEM)

<table>
<thead>
<tr>
<th>Publication</th>
<th>Description of Samples</th>
<th>Mean RA concentration of milk (mg/g fat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fogerty et al., 1988</td>
<td>Australian</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>(n=18; conventional diet)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=8; Hare Krishna diet)</td>
<td>11.2</td>
</tr>
<tr>
<td>Park et al., 1999</td>
<td>U.S.</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>(n=16; low dairy diet)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=16; high dairy diet)</td>
<td>3.8</td>
</tr>
<tr>
<td>McGuire et al., 1997</td>
<td>U.S. (n=14)</td>
<td>3.6</td>
</tr>
<tr>
<td>Masters et al., 1999</td>
<td>U.S.</td>
<td>30.0 ± 2.1^a</td>
</tr>
<tr>
<td></td>
<td>(n = 9; CLA supplement)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 9; placebo)</td>
<td>15.3 ± 1.8^a</td>
</tr>
<tr>
<td>Jahreis et al., 1999</td>
<td>German (n=29)</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>Precht and Molkentin, 1999</td>
<td>German (n=40)</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Jensen et al., 1998</td>
<td>U.S. (n=20)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

^aValues represent μmol RA/g lipid.
Table 2. Published Estimates of RA Intakes in Humans (means ± SEM)

<table>
<thead>
<tr>
<th>Publication</th>
<th>Description of Samples</th>
<th>RA Intake (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ip et al., 1994</td>
<td>U.S.</td>
<td>1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Parodi et al., 1994</td>
<td>Australia</td>
<td>500-1000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Herbal et al., 1998</td>
<td>Washington/Idaho, U.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Men and women combined</td>
<td>127&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fritsche et al., 1998</td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>350</td>
</tr>
<tr>
<td>Salminen et al., 1998</td>
<td>Finland</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Men and women combined</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High dairy foods diet (n = 80)</td>
<td>310 ± 41</td>
</tr>
<tr>
<td></td>
<td>High trans fatty acid diet (n = 40)</td>
<td>40 ± 6</td>
</tr>
<tr>
<td></td>
<td>High stearic acid diet (n = 40)</td>
<td>90 ± 16</td>
</tr>
<tr>
<td>Ritzenthaler et al., 1998</td>
<td>Washington/Idaho</td>
<td></td>
</tr>
<tr>
<td></td>
<td>College-aged subjects</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Males (n = 19)</td>
<td>137 ± 84</td>
</tr>
<tr>
<td></td>
<td>Females (n = 18)</td>
<td>52 ± 44</td>
</tr>
<tr>
<td>Park et al., 1997</td>
<td>Washington/Idaho, U.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactating Women (n = 16)</td>
<td>227 ± 180</td>
</tr>
<tr>
<td></td>
<td>Low dairy foods diet</td>
<td>15 ± 24</td>
</tr>
<tr>
<td></td>
<td>High dairy foods diet</td>
<td>291 ± 75</td>
</tr>
</tbody>
</table>

<sup>a</sup>As adapted from “Dietary Sources and Intakes of Conjugated Linoleic Acid Intake in Humans” (McGuire et al., 1999).

<sup>b</sup>Values represent intakes of all isomers of conjugated linoleic acid; other values represent intakes of RA only.
Table 3. Demographic Variables and Anthropometric Measurements of Women and Infant Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Maternal (n = 10)</th>
<th>Infant (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age (yr)</td>
<td>26.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td>27.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Time postpartum (wk)</td>
<td>11.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Parity (# children)</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>5882.5 ± 557.5</td>
<td></td>
</tr>
<tr>
<td>Length (cm)</td>
<td>59.6 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Maternal Daily Intakes of Macronutrients, Selected Fatty Acids, CLA, and Rumenic Acid (RA) as Estimated by 3-d weighed Food Records

<table>
<thead>
<tr>
<th>Dietary Component</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2277.7 ± 160.3</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>81.7 ± 7.3</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>318.4 ± 20.2</td>
</tr>
<tr>
<td>Lipid (g)</td>
<td>79.2 ± 8.5</td>
</tr>
<tr>
<td>Saturated FA (g)</td>
<td>30.4 ± 3.4</td>
</tr>
<tr>
<td>Monounsaturated FA (g)</td>
<td>23.1 ± 3.0</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>8.2 ± 1.5</td>
</tr>
<tr>
<td>CLA (mg)</td>
<td>154.7 ± 21.9</td>
</tr>
<tr>
<td>cis 9, trans 11-18:2 (RA; mg)</td>
<td>122.1 ± 18.5</td>
</tr>
<tr>
<td>Correlation Variable</td>
<td>Parity</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Time Postpartum (wks)</td>
<td></td>
</tr>
<tr>
<td>Maternal Age (yr)</td>
<td></td>
</tr>
<tr>
<td>Maternal BMI (kg/m²)</td>
<td></td>
</tr>
<tr>
<td>Maternal CLA Intake</td>
<td></td>
</tr>
<tr>
<td>Maternal RA Intake</td>
<td></td>
</tr>
<tr>
<td>% Milk Fat</td>
<td></td>
</tr>
<tr>
<td>mg RA/g Fat</td>
<td></td>
</tr>
<tr>
<td>mg RA/g milk</td>
<td></td>
</tr>
<tr>
<td>Infant Milk Intake</td>
<td></td>
</tr>
<tr>
<td>Infant Fat Intake</td>
<td></td>
</tr>
<tr>
<td>Infant RA Intake</td>
<td></td>
</tr>
<tr>
<td>Infant Weight (g)</td>
<td></td>
</tr>
<tr>
<td>Infant Length (cm)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Significance Values (p) for Data Correlations Between Maternal, Infant and Milk Composition Variables
Figures
Figure 1. Graphical illustration of the relationship between maternal age (yr) and maternal CLA intake (mg/g/d); $R^2 = 0.4837; p = 0.026$. 
Figure 2. Graphical illustration of the relationship between maternal age (yr) and maternal CLA intake (mg/g/d); $R^2 = 0.4473; p= 0.026$
Figure 3. Graphical illustration of the relationship between maternal BMI (kg/m²) and maternal RA intake (mg/d); \( R^2 = 0.4055; p = 0.026 \).
Figure 4. Graphical illustration of the relationship between milk RA (mg/g fat) and infant RA intake (mg/d); $R^2 = 0.6707; p = 0.004$. 
Figure 5. Graphical Illustration of the relationship between infant milk intake (g/d) and infant fat intake (g/d); $R^2 = 0.4553; p = 0.032$. 
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*Chem Ind* 52: 270.


*FASEB J* 11: (Abst. 3347).


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