Longer-term effects of early cholesterol intake

on cholesterol biosynthesis and plasma lipids

Théa A. Demmers

School of Dietetics and Human Nutrition, McGill University, Montréal, Québec

Submitted November 2004

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science in Human Nutrition.

TABLE OF CONTENTS

Acknowledgements.....3

Contribution of authors.....5

Abstract.....6

Introduction.....8

Literature review.....10

Background.....11

Pathophysiology of cardiovascular disease.....11
Cholesterol metabolism.....12
Measurement of cholesterol metabolism.....13
Cholesterol metabolism in the neonatal period.....14
Cholesterol metabolism and infant nutrition.....16

Preliminary data and previous work.....18

Experimental studies in animals.....18
Experimental studies in infants.....19
Observational studies.....21

Rationale and objective.....23

Manuscript – "Effects of early cholesterol intake on cholesterol biosynthesis and plasma lipids in infants until 18 months of age".....24

Abstract.....25
Introduction.....27
Materials and methods.....30
Results.....34
Discussion.....36

Summary and conclusions.....42

Tables and figures.....45

References.....55

Appendix 1 – Laboratory procedures.....63

Appendix 2 – Compiled data.....69

Appendix 3 – Institutional review board certificate.....77

Appendix 4 – Copyright waiver forms.....79

ACKNOWLEDGEMENTS

This study was supported by the National Institutes of Health DK54504, RR08084, Nestlé, American Heart Association and the Natural Sciences and Engineering Research Council of Canada.

The clinical trial for the present work took place at Cincinnati's Children Hospital Medical Center General Clinical Research Center (GCRC), under the direction of Dr. James E. Heubi. The infants were recruited and followed by study coordinators Vivian Creutzinger and Susan Krug. Much thanks are extended to the staff of the GCRC for their assistance; to Cindy Deeks, Shanthi Rajan and Suzanne Spang for dietary analysis, and to the children and families for their participation.

Many thanks to Mahmoud Raieni and Chris Vanstone for technical assistance with the 4 month fractional synthesis rate (FSR) samples. Great appreciation is also afforded to members of Dr. Jones' research team: Dr. Naoyuki Ebine, Dr. Yanwen Wang and Melanie Journoud for providing training and mentoring in laboratory techniques. Also to Dr. Isabelle Demonty, Vivian Lau, Xiaming (Nina) Jia, and Christine Gurekian for support and encouragement along the way, sharing ideas and lab space. To Dr. Peter Jones, thank you for your sense of vision and ability to empower those around you to be more than they thought they could be. It has been an honor to have worked under your supervision.

Thank you also to Dr. Roger Cue and Dr. Yanwen Wang for consultation with the statistical analysis.

To my committee members Dr. Grace Egeland and Dr. Louis Beaumier for your valuable insights throughout this project.

To all those who make up the School of Dietetics and Human Nutrition – the staff, particularly, Lise Grant, Francine Tardif and Jocelyn Begin. Also to Dr. Katherine Gray-Donald, for your thoughtful approach to research and learning, and Dr. Linda Starkey for your advice and coordinating my research experiences with those of professional practice in dietetics. Also to colleagues through this journey, Iwona Rudkowska, Yiyi Jing and Sylvia Santosa. Thank you for friendship, support and encouragement.

I am very grateful for this opportunity to explore working in research and for all the people, blessings, and lessons that have aided in bringing me to this place and to tread this path I tread. Thank you to my family for fostering a love of learning and life.

A debt of gratitude to my husband, Chad Polito, who has been a rock of support, encouragement and love. Thank you for all of your help, your perspective and your sense of humor. I am very blessed and honored to share life with you.

CONTRIBUTION OF AUTHORS

Théa A. Demmers, as first author, was responsible for writing the draft and incorporating the input of the other authors for the manuscript that appears in this thesis. She was also responsible for assembling the data from all time points, completing required calculations and conducting the statistical analysis. Finally, she completed the laboratory analysis necessary for determination of Ch fractional synthetic rate (FSR), at 18 months.

Dr. Peter J.H. Jones, provided direction for the project throughout the FSR determination, and had significant input into the manuscript.

Dr. Yanwen Wang, provided assistance and training of laboratory techniques and statistical analysis and had significant input into the manuscript.

Susan Krug and Vivian Creutzinger were involved in participant recruitment and arranging the follow-ups to administer the dose of deuterium oxide required for FSR determination and collection of all outcome measures: blood collection, growth measures, and 3-day diet diaries from mothers' of infants. They reviewed the manuscript and provided some comments.

Dr. James E. Heubi was the principal investigator for the study, obtaining necessary funds to carry it out and providing direction throughout. He also had significant input into the manuscript.

ABSTRACT

Endogenous cholesterol (Ch) fractional synthesis rate (FSR) is inversely related to infant dietary Ch at 4 months (mo) of age. The objective of the present study was to determine whether level of infant dietary Ch induced changes in FSR that persisted at 18 mo. Forty-seven infants received, human milk (HM) from birth until weaned (n=15), or were randomized to receive modified cow-milk formula (MCF) with added Ch (n=15), or cow-milk formula (CF, n=17), for 12 mo. Ch contents of HM, MCF, and CF were 120, 80, and 40 mg/L, respectively. At 4 mo, FSR in the HM group was lower than in CF, but at 18 mo, there were no differences between groups. Therefore, while Ch intake prior to weaning affects FSR, the differences do not persist after weaning to unrestricted diet. These data provide further evidence that there is no imprinting of FSR in infancy by differing dietary levels of Ch.

RÉSUMÉ

Le taux de synthèse fractionée (TSF) du cholestérol (Ch) endogène est inversement proportionnel à la prise alimentaire du Ch à 4 mois (mo). L'objectif de cette étude était de verifier si ces différences du TSF persistent à 18 mo. Quarante-sept enfants ont reçu, dès la naissance, soit lait maternel (HM), jusqu'au sevrage (n=15), ou ont reçu de façon aléatoire soit du lait maternisé (CF), à base lait de vache (n=17), ou une formule modifiée par l'addition de Ch (MCF, n=15) durant 12 mo. Les HM, CF, et MCF contenaient respectivement 120, 80, 40 mg/L de Ch. À 4 mois, le TSF dans HM était plus faible que chez CF, mais cette différence avait disparue à 18 mois avec le sevrage au régime sans restriction. Ces données confirment que le Ch alimentaire chez le nourrisson n'affecte pas le TSF de façon permanente.

INTRODUCTION

One of the primary combination of risk factors for cardiovascular disease (CVD) is an elevated level of serum low-density lipoprotein (LDL) cholesterol (Ch) and low serum level of high-density lipoprotein (HDL) Ch (Schaefer 2002). The epidemiological evidence linking elevated serum Ch and CVD is well established (Grundy 2002), as CVD is uncommon in societies with mean serum total Ch concentrations below 4.6 mmol/L (180mg/dl) (Schaefer 2002). Moreover, cardiovascular health is believed to be influenced by early dietary intake (Waterland et al.1999). It is postulated that atherogenesis could begin in the very early stages of life, as infants have been found to have small collections of foam cells in regions of coronary arteries that are prone to development of lesions (Stary 1987; Stary 2000).

The role of nutrition in the perinatal period has been shown to have permanent effects on the development of the central nervous system (Dobbing 1981; Rassin et al. 1983; Smart 1986; Farquharson et al. 1992; Uauy et al. 2003), and on the concentration of products from other metabolic pathways (Innis et al. 1993; Savino et al. 2002; Agostoni et al. 2003). Some evidence has been found that Ch metabolism may be influenced by early dietary intake. The Ch content of human milk is typically higher (90-150 mg/L) compared with regular cow milk-based commercial formulas (10 to 40 mg/L) and soy milk-based formulas contain no Ch. The higher Ch concentration in breast milk has been speculated to endow nursing infants with an enhanced ability to metabolize Ch later in life (Reiser et al. 1972). The hypothesis of "fetal programming" or "metabolic imprinting", as it relates to lipid metabolism has been put forth by epidemiological and

observational studies (Waterland et al. 1999). Studies in rats (Reiser et al. 1979) and baboons (Mott et al. 1990) evaluated the relationship between high or low Ch intake in infancy and subsequent plasma Ch levels. It is hypothesized that differences in plasma lipid concentrations in infancy and adulthood relate to changes in endogenous Ch fractional synthesis rates (FSR), affected by the amount of dietary Ch (McNamara et al. 1987; Miettinen et al. 1989; Jones et al. 1996). In infants, previous studies have shown that Ch intake relates inversely to the fractional synthesis rate (FSR) of endogenous Ch at four months of age (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998). The principal purpose of the present study was to determine whether a high or low dietary intake of Ch in early life is a determinant of FSR, CVD risk and lipid levels later in life.

LITERATURE REVIEW

BACKGROUND

The importance of Ch as it relates to the pathophysiology of cardiovascular disease, its various roles in the body and an overview of Ch metabolism provide a primary basis for understanding.

Pathophysiology of cardiovascular disease. Cardiovascular disease (CVD) is the process of thickening of the arterial walls, leading to occlusions and narrowing which reduce blood flow to peripheral organs. Coronary heart disease (CHD) results from the absence of blood flow to the network of vessels surrounding the heart, often secondary to CVD. The Consensus Panel on "Triglyceride, High-Density Lipoprotein and Coronary heart disease" of the National Institutes of Health (NIH) has stated that elevated levels of triglyceride-rich very-low-density lipoproteins (VLDL) and Ch-rich LDL lipoproteins and lowered HDL levels can increase risk for heart disease (Schaefer 2002). Elevation of VLDL and LDL contribute to plaque formation which stiffens arteries and can lead to myocardial infarction. The major cause of CVD is atherosclerosis, a slow progressive disease that begins in childhood and takes decades to advance. Atherosclerosis involves structural and compositional changes in the innermost layer of the large arteries, as a result of plaque formation. Plaque build-up develops mainly from the accumulation of lipid and Ch in the matrix around the cells, over time this becomes termed "lesion", with varying grades of severity in terms of risk for coronary events (Type I-IV). The presence of initial fatty streaks is often seen in people younger than 30 years of age. In a study of infants, the incidence of type I lesions in coronary arteries was greatest in the first six

months after birth, with 50% of the infants exhibiting type I lesions (Stary 2000). Since Ch is one of the major contributing components to atherosclerosis development and progression, its metabolism and factors that affect its concentration require examination.

Cholesterol metabolism. Established homeostatic mechanisms that affect Ch kinetics and subsequent serum levels of Ch involve the level of dietary Ch absorption (Grundy et al. 1969), LDL receptor activity (Brown et al. 1986), and the rate of endogenous Ch synthesis (Miettinen et al. 1989). It is the complex interplay between dietary intake and endogenous synthesis that is the subject of interest in the present study. Ch is synthesized mainly in the liver, with some contributions from the small intestine. Briefly, Ch is synthesized from 2-carbon precursors, acetate, which are converted to mevalonate, a 6-carbon isoprenoid precursor. Six mevalonate molecules are converted via activated 5-carbon intermediates into squalene, a 30-carbon molecule. The last process involves the cyclization of squalene and its transformation to Ch, a molecule with 27 carbons (Bloch 1965).

It has been shown in numerous adult studies that the major metabolic changes in response to increased Ch intake are suppression of whole body cholesterogenesis, of which hepatic FSR is a good surrogate measurement, and/or increased re-excretion of absorbed dietary Ch as fecal neutral steroids (Grundy et al. 1969; McNamara et al.1987). However, the large individual variability of responses in other studies suggests that the relationship between adult dietary intake and endogenous synthesis rate is complex and highly individual (Miettinen et al. 1989; Jones et al. 1994). A recently postulated effect on cellular Ch biosynthesis involves a supernatant protein factor, a lipid transporter,

complexed with oxidized vitamin E, which by diminishing uptake of oxidized LDL reduces cellular Ch uptake which then induces Ch synthesis by cellular feedback mechanisms (Stocker et al. 2003). Dietary intake of vitamin E may play a role in the rate of Ch synthesis at the cellular level by this newly proposed mechanism. Internal factors such as the genetic polymorphism of apolipoprotein (apo) E and LDL particle size influence circulating Ch levels by affecting the level of Ch absorption, and potential for Ch oxidation (Skoglund-Andersson et al. 2003). Although genetics account for some of the variability in circulatory response to changes in dietary Ch level, the exact mechanisms through which such effects occur remain to be established (Jones et al. 1993).

Measurement of cholesterol metabolism. Whole body cholesterogenesis can be studied using the stable isotope tracer, deuterium oxide (^2H_2O). The rate of incorporation of deuterium from body water into erythrocyte membrane Ch can be determined because 22 of 46 hydrogen atoms in Ch originate from body water. Ch synthesis, as defined by compartmental analysis, is comprised of that synthesized by the central pool, which includes the liver, plasma, and intestine. The FSR, represents the fraction of rapidly turning over free cholesterol, within the central pool, that is synthesized per day. The rate of deuterium incorporation into red blood cell membrane Ch over a 24 hour period serves as an index of Ch fractional synthetic rate (FSR). Refer to Appendix 1 for a more complete discussion of methodology.

Cholesterol metabolism in the neonatal period. There is still much to be learned about Ch kinetics in the early stages of life, as the majority of literature deals with adults. Some distinctives of infant Ch metabolism include the C27 hydroxylation pathway for bile acid synthesis, and the preferential role of the acyl-coenzyme A:Ch acyltransferase (ACAT) isoenzyme-2. The adult metabolic pathway of bile acid synthesis begins with oxidation at the C7 position of Ch to produce 7-α-hydroxycholesterol. The Ch 27-hydroxylation pathway is the only one expressed in fetal and neonatal life, while 7-α-hydroxycholesterol generated in the liver is the major source of bile acids in older adults, up-regulating sometime after birth (Javitt 2002). C27 hydroxylation of Ch occurs in many different tissues, such as the arterial endothelium and macrophages, in addition to being found in the liver. The rate of production of 27-hydroxycholesterol in arterial endothelial cell culture studies indicate that it is substrate driven, thus linking rates of synthesis to accumulation of Ch within the cell. The pathway thus serves to enhance reverse Ch transport by the direct conversion of Ch to 27-hydroxycholesterol. It has also been found that phenotypic expression of accelerated atherosclerosis is related to low or absent Ch 27-hydroxylase activity (Javitt 2002). As this pathway is the only one existent during the perinatal period, and as its rate of activity appears to be substrate driven, the higher Ch content of human milk might be associated with higher levels of Ch 27-hydroxylase activity and higher rates of bile acid synthesis. To date, no study has looked at the influence of infant diet on this enzyme. The enzyme 27-hydroxylase is a mitochondrial cytochrome P-450 enzyme (CYP27), in various species including humans, CYP27 is the same enzyme that catalyzes 25-hydroxylation of vitamin D_3 to yield 25-hydroxy-vitamin D_3. It was thought that Ch supplemented formulas could negatively

affect vitamin D metabolism and consequently calcium and bone metabolism. No deleterious effects on vitamin D metabolism have been observed as a result of Ch formula supplementation to levels similar to or higher than that of human milk (Picaud et al. 2002).

In human fetuses, hepatic LDL receptors are functional as early as 10 weeks of fetal age. Functionality of receptors increases with growth of the fetus. The total serum Ch during gestation decreases with increasing fetal age, due to the increase in LDL receptor activity with increasing gestational age (Cai et al. 1991). The effect of infant dietary Ch on LDL receptor activity after birth has not been studied.

Acyl-coenzyme A:Ch acyltransferase (ACAT) is a membrane protein located in the endoplasmic reticulum. It catalyzes the formation of Ch esters, which are important lipid components of VLDL and LDL. There are two forms in mammals:ACAT-1 and ACAT-2. ACAT-2 is more directly involved in lipoprotein synthesis and assembly, while ACAT-1 may play a role in cellular Ch homeostasis and lipid droplet formation (Chang et al. 2000). ACAT-1 is present in both neonatal and adult cells in small intestine enterocytes and hepatocytes. While ACAT-2 is evident in adult enterocytes, it is not found in adult hepatocytes and is believed to perform a more significant catalytic role in the fetal liver and in the intestinal enterocytes (Chang et al. 2000). Differences in the expression of ACAT-2 as influenced by infant diet have not been studied, but could represent a possible point of metabolic imprinting during maturity of cholesterol metabolism from infancy to adulthood.

Cholesterol metabolism and infant nutrition. It has been demonstrated that early dietary intake impacts whole body cholesterogenesis, as measured by hepatic FSR of endogenous Ch, but whether or not this effect is sustained throughout life has not been definitively confirmed.

The three major sources of nutrition in Western societies during early infancy are human milk, cow milk-based formulas and soy milk-based formulas. The compositions of these types of diet differ in several factors that may affect Ch homeostasis. Among the major factors which may affect Ch synthesis and metabolism are the Ch content, the polyunsaturated/saturated fatty acid ratio (P/S ratio), the protein composition, the phytoestrogen content, and the presence of hormones in breast milk (Cruz et al. 1994; Uauy et al. 2000; Nelson et al. 1999). Although each of these factors may affect Ch metabolism, the focus of this study is the difference between cow milk-based formula and breast milk as a function of Ch content.

Ch is essential for normal cellular function in mammals; it is an integral part of membranes (Bloch 1983) and a precursor for many hormones. Requirements for Ch are met by endogenous biosynthesis and dietary supplementation. Infants fed human milk obtain much greater amounts of Ch than those fed by commercial formulas. Human milk (HM) contains between 90-150 mg/L of Ch (Lammi-Keefe et al. 1984), providing a daily Ch intake of approximately 75 mg per day for a breast-fed newborn weighing 4 kg. In contrast, cow milk-based formulas contain 10 to 40 mg/L of Ch, giving an average daily Ch intake of approximately 9 mg per day. Soy milk-based formulas contain no Ch. Several infant studies have looked at the effects of infant diet on Ch metabolism by comparing lipid profiles. The findings of these studies demonstrated that breast-fed

infants have a higher serum Ch concentration compared to formula-fed infants (Friedman et al. 1975; Uauy et al. 2000; Hayes et al. 1992; Van Biervliet et al. 1992; Agostoni et al. 2000; Jooste et al. 1991). The differences in serum Ch are thought to be a result of the differential in Ch content between human milk and commercial formulas. Whether or not the low Ch content in commercial formulas presents any physiological or pathophysiological effects other than the difference in serum Ch concentration remains to be understood. It is postulated that the differences in serum lipid concentrations can be explained by shifts in endogenous Ch synthesis rates that are quantitatively influenced by dietary Ch (Miettinen et al. 1989).

PRELIMINARY DATA AND PREVIOUS WORK

Experimental studies in animals. Reiser and Sidelman were the first to suggest that a possible function for Ch in milk was the establishment of serum Ch homeostasis (Reiser et al. 1972). Early exposure to dietary Ch appeared to 'protect' against diet-induced hypercholesterolemia in adulthood, as the adult male offspring exhibited an inverse relationship between serum Ch concentration and the Ch content of their mothers' milk. Further animal studies disputed Reiser's original hypothesis; results from other rat studies both support (Reiser et al. 1979) and refute (Kris-Etherton et al. 1979); studies in pigs (Jones et al. 1990) support the hypothesis, while studies in baboons (Mott et al. 1990; Mott et al. 1991) and guinea pigs do not (Li et al. 1980).

Breast-feeding is associated with lower Ch production rates in baboons (Mott et al. 1991), and in pigs (Jones et al. 1990) in the neonatal period. Increases of hepatic hydroxymethylglutaryl CoA (HMG-CoA) reductase activity in formula-fed neonatal pigs and rats (Jones et al. 1990, Reiser et al. 1977), confirm the presence of feedback inhibition between dietary Ch and endogenous Ch production. Breast-fed baboons as infants have higher levels of hepatic acyl CoA Ch acyltransferase (ACAT) activity, higher concentrations of hepatic Ch esters than those formula-fed, and lower plasma lecithin Ch acyltransferase activity (LCAT), as they metabolized exogenous Ch (Mott et al. 1993a), compared to formula-fed baboons who relied on de novo Ch synthesis as their principal source of Ch. Breast-feeding also led to increases of 44 to 99% in LDL receptor mRNA compared with formula-feeding, which persisted into adolescence in baboons (Mott et al. 1993b; 1995), suggesting that long-term Ch homeostasis could be

affected by the level of dietary Ch in the infant diet. However in the hamster, stimulation of Ch catabolism by bile acid removal, rather than Ch feeding or early weaning during the neonatal period, improved the ability to handle Ch challenge in adulthood (Li et al. 1980).

Experimental studies in infants. Clinical studies for measurement of cholesterogenesis by FSR have been largely limited to adults with various underlying disease states. One of the first studies performed to determine FSR in infancy found an inverse relationship between the quantity of dietary Ch and endogenous Ch production rates (Wong et al. 1993). A year later, FSR was measured in four month old infants using deuterium incorporation methodology (Cruz et al. 1994). Infants were divided into four groups fed either human milk (HM), which contained 90-150 mg/L of Ch, a cow milk-based formula (CF), which contained 11 mg/L of Ch, or a soy milk-based formula (SF), which contained no Ch. A fourth group of infants were fed modified soy formula (MSF) with similar amounts of Ch as that found in regular cow milk-based formula (11 mg/L). The FSR of the HM infants was significantly lower than that of formula-fed infants, reaffirming the inverse relationship found by Wong et al. 1993. In addition, serum total Ch concentrations were different between the HM-fed and the formula-fed infants. There was a significant negative correlation between serum total Ch concentrations and FSR ($r = -0.59$), and a negative correlation between serum concentrations of LDL-Ch and FSR ($r = -0.73$). These results demonstrate that the type of dietary milk intake affects both the serum Ch concentrations and the endogenous Ch synthesis rates.

Another investigation involved a cohort of subjects who received either breast milk or a cow milk-based formula (SMA, Wyeth-Ayerst Laboratories), or cow milk-based formula with added crystalline-free Ch to provide a concentration of 130 mg/L (Bayley et al. 2002). At 4 months, measurements of FSR and lipid profiles confirmed again that infants adapted the rate of endogenous Ch production based on the amount of Ch that they ingested. The endogenous Ch FSR was 3.8 fold higher in infants fed cow milk-based formula vs. human milk-fed infants at 4 months. However, the bioavailability of the Ch in the study formula was questioned, as the results seen at 4 months in those infants did not mirror those seen in the breast-fed infants. Another group of infants underwent a Ch challenge between 11 and 12 months of age, with measurement of plasma lipids and FSR done before and after. Plasma Ch concentrations for all 3 groups were similar at 11 and 12 months of age, and no differences in FSR before or after the Ch challenge were observed within the 3 feeding groups at 11 and 12 months of age. These results demonstrated that early dietary Ch had no persistent effect on Ch metabolism 6 months after the dietary exposure (Bayley et al. 2002). It was thought that the form and emulsion of dietary Ch in the formula may have been responsible for the differences between the supplemented formula and breast milk. Breast milk contains a small percentage of its Ch, approximately 16.5 to 24 %, in esterified form (Lammi-Keefe et al. 1984), which may be absorbed differently than the Ch of the formula provided as free non-esterified Ch (Bayley et al. 2002). Additional studies are required to assess absorption and bioavailability of supplemented Ch in formulas, as compared to Ch found in human milk.

Observational studies. Metabolic imprinting describes the potential relationship between early nutritional experiences and later metabolic mechanisms that may either protect or lead to disease. It comprises adaptive responses that meet the following criteria: i) a susceptibility limited to a critical window of time early in development, ii) a persistent effect lasting through adulthood, iii) the response has a specific and measurable outcome, and iv) there exists a specific causal relation between a specific exposure and outcome (Waterland et al. 1999). Therefore, the possibility of reducing long-term risk of disease by nutrition interventions early in life presents an effective complement to other prevention strategies undertaken in later life.

The idea of perinatal programming by way of metabolic imprinting arose from the epidemiological studies of Barker who reported a relationship between birth weight and CHD mortality in a cohort of 1586 men from the UK (Barker et al. 1989). CHD mortality was inversely related to birth weight (Ingelfinger et al. 2002; Barker et al. 1993). Studies arising from the Dutch famine examined the association further, by assessing the method of infant feeding in the first weeks after birth and subsequent adult plasma lipid profiles. It was found that fasting LDL-Ch and the LDL/HDL ratio were higher in those who were bottle-fed than those who were breast-fed, which is indicative of a more atherogenic lipid profile in those who received formula (Ravelli et al. 2000). Another study confirmed an association between method of infant feeding and adult serum LDL-Ch and mortality from ischaemic heart disease (Fall et al. 1992). Those exclusively bottle-fed had higher total and LDL-Ch levels. However, these findings demonstrated a higher mean serum concentration of total-Ch and LDL-Ch in breast-fed men who had not been weaned before one year, compared to men who had been weaned

before one year (Fall et al. 1992). These data suggest that age of weaning may affect adult Ch concentrations. However in another study of 465 subjects, total-Ch was significantly higher in adult males breast-fed for the shortest period of time, less than 3 months, in contrast to those who were breast-fed for more than 9 months (Kolaček et al. 1993).

Infant studies demonstrate increased mean total-Ch and LDL-Ch, associated with the Ch content of human milk and study formulas (Friedman et al. 1975; Rassin et al. 1983; Wagner et al. 1988; Kallio et al. 1992; Hayes et al. 1992; Van Biervliet et al. 1992; Wong et al. 1993; Cruz et al. 1994; Mize et al. 1995; Bayley et al. 1998; Agostini C et al. 2000; Bayley et al. 2002). After weaning, however, the differences in total-Ch and LDL-Ch between those either breast-fed or given formula in infancy moderate, with no consistent differences in serum lipids seen from 1 to 16 years of age (Glueck et al. 1972; Friedman et al. 1975; Hodgson et al. 1976; Huttunen et al. 1983; Fomon et al. 1984; Jooste et al. 1991; Kallio et al. 1992; Owen et al. 2002). In the majority of adult studies, both total-Ch and LDL-Ch levels are lower in adults who had been breast-fed rather than bottle-fed (Marmot et al. 1980; Kolacek et al. 1993; Fall et al. 1995; Ravelli et al. 2000; Leeson et al. 2001).

In summary, high Ch intakes in infancy inhibit de novo Ch synthesis, while low Ch intakes stimulate synthesis. Theoretically, these adaptations in synthesis rates might remain for a long period and be the definitive mechanism by which Ch metabolism is imprinted (Hahn et al. 1990). An adjustment in the Ch fractional synthesis rate (FSR), by either high dietary Ch, as in the breast-fed infant, or by a low intake of dietary Ch, typical of formula-fed infants could potentially alter the adult metabolic response to dietary Ch.

RATIONALE AND OBJECTIVE

Cholesterol metabolism in adult life and atherosclerosis risk may be linked to early infant feeding practices. Previous studies have demonstrated an inverse relationship between Ch intake and Ch synthesis in early infancy, but further work is needed to evaluate the possibility of metabolic imprinting in later cholesterol homeostasis. The objective of this study was to determine whether the level of dietary Ch in early life induced changes in Ch FSR and plasma lipids that persisted beyond weaning at 18 months.

MANUSCRIPT

Effects of early cholesterol intake on cholesterol biosynthesis and plasma lipids in infants until 18 months of age

Théa A. Demmers[1], Peter J.H. Jones[1], Yanwen Wang[1], Susan Krug[2], Vivian Creutzinger[2], James E. Heubi[2]

[1]School of Dietetics and Human Nutrition, McGill University, Montréal, Québec, Canada H9X 3V9

[2]Division of Pediatric Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, United States 45229

Accepted for print publication in Pediatrics, October 13, 2004

ABSTRACT

Endogenous cholesterol (Ch) fractional synthesis rate (FSR) is inversely related to infant dietary Ch at 4 months of age; however it remains to be established whether this effect is permanent, possibly contributing to later hypercholesterolemia. **Objective:** To determine whether level of dietary Ch in infancy induced changes in FSR and plasma lipids that persisted at 18 months. **Methods:** A prospective, clinical trial in 47 infants who received, from their first week of life until 18 months, human milk (HM) until weaned (n=15), or were randomized to receive modified cow-milk formula (MCF) with added Ch (n=15), or cow-milk formula (CF, n=17), for 12 months. Ch contents of HM, MCF, and CF were 120, 80, and 40 mg/L, respectively. FSR and plasma lipids were measured at 4 and 18 months. **Results:** At 4 months, total-Ch and LDL-Ch were higher ($p<0.03$) in infants fed HM and MCF, than CF. HDL-Ch levels were higher ($p=0.005$) in MCF group than in HM and CF groups. FSR in the HM group (0.034±0.005 pools/day) was lower ($p<0.02$) than in CF (0.052±0.005 pools/day). There was no difference between HM and MCF (0.047±0.005 pools/day), nor between MCF and CF. At 18 months, there were no differences in FSR or plasma lipids between groups. **Conclusion:** While Ch intake prior to weaning affects FSR and plasma lipids at 4 months, these differences do not persist after weaning to unrestricted diet at 18 months. This provides further evidence that there is no imprinting of FSR in infancy by differing dietary levels of Ch.

Key Words: cholesterol, breast-fed, formula-fed, fractional synthesis rate, deuterium.

Abbreviations: FSR, fractional synthesis rate

Ch, cholesterol

HM, human milk

MCF, modified cow-milk formula

CF, cow-milk formula

T-Ch, total cholesterol

DI, deuterium incorporation

GCRC, General Clinical Research Center

INTRODUCTION

Early intake of cholesterol (Ch) and its possible imprinting of later Ch metabolism have been studied to understand the influence of infant nutrition on adult Ch metabolism and subsequent cardiovascular disease risk (Fall et al. 1992; Kolaček et al. 1993; Ravelli et al. 2000). The Ch content of human milk is typically higher (90-150 mg/L) than regular cow milk-based formulas (10-40 mg/L), while soy milk-based formulas contain no Ch. Reiser and Sidelman first hypothesized that the function of Ch in milk was to establish a control of serum Ch homeostasis, as a result of their studies in the rat (Reiser et al. 1972). Early exposure to dietary Ch appeared to 'protect' against diet-induced hypercholesterolemia in adulthood, as the adult male offspring exhibited an inverse relationship between serum Ch concentration and the Ch content of their mothers' milk. Results from studies in a variety of species have produced conflicting results: other rat studies support (Reiser et al. 1979) and refute (Kris-Etherton et al. 1979); studies in pigs (Jones et al. 1990) support the hypothesis, while studies in baboons (Mott et al. 1990; Mott et al. 1991) and guinea pigs do not (Li et al. 1980).

Human infants have increased serum Ch proportionate to the Ch content of human milk and study formulas (Kallio et al. 1992; Hayes et al. 1992; Van Biervliet et al. 1992; Mize et al. 1995; Agostoni et al. 2000; Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002). After weaning, however, the differences in serum Ch moderate, with no consistent differences seen from 1 to 16 years of age (Kallio et al. 1992; Hodgson et al. 1976; Huttenen et al. 1983; Fomon et al. 1984; Jooste et al. 1991; Owen et al. 2002). In most adult studies, both total-Ch (T-Ch) and LDL-Ch levels are

lower in adults who had been breast-fed as infants (Kolaček et al. 1993; Ravelli et al. 2000; Marmot et al. 1980; Fall et al. 1995; Leeson et al. 2001). Arising out of the above observations are numerous speculations concerning the mechanisms by which neonatal dietary Ch may be responsible for long-lasting perturbations of Ch metabolism (Reiser et al. 1977; Mott et al. 1982; Mott et al. 1993; Mott et al. 1995, Mahaney et al. 1999). It has been hypothesized that differences in plasma lipid concentrations in infancy and adulthood may be partially accounted for by changes in endogenous Ch fractional synthesis rates (FSR), modulated by the quantity of dietary Ch (McNamara et al. 1987; Miettinen et al. 1989; Jones et al. 1996). Theoretically, adaptations in synthesis rates related to Ch exposure from infancy might persist and be the definitive mechanism by which Ch metabolism is imprinted (Hahn et al. 1990). An adjustment in the Ch FSR, by either high dietary Ch, as in the breast-fed infant, or by a low intake of dietary Ch, typical of formula-fed infants, could potentially alter the adult metabolic response to dietary Ch. Studies in infants have shown that Ch FSR is inversely related to Ch intake at age 4 months (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002). However, the formulas used boasted different levels of phytosterols, phytoestrogens, or hormones, compounded by different fatty acid profiles and questions regarding the bioavailability of supplemented Ch (Bayley et al. 2002). The present study attempted to address all the above issues by feeding identical formulas, different only in terms of Ch content, where the modified cow-milk formula (MCF) was supplemented with a more bioavailable form of Ch and to an intermediate level between regular cow-milk formula (CF) and human milk (HM) to clearly elucidate the relationship between dietary Ch, plasma lipids, and FSR. An understanding of the impact of dietary Ch on these variables

and metabolic parameters, taken with the plausibility of imprinting, could have significant ramifications on the production of infant formulas by pointing to a benefit from supplementation with Ch.

The purpose of the present study was to determine whether the level of dietary Ch in early life induced changes in Ch FSR and plasma lipids that persisted beyond weaning at 18 months, and to assess whether an intermediate level of Ch supplementation to cow-milk formula resulted in corresponding intermediate FSR. It was hypothesized that at age 4 months, infants fed HM would have a lower FSR compared to those fed MCF, who would have a lower FSR than those fed CF. The level of dietary Ch would vary inversely with endogenous Ch FSR. Furthermore, at 18 months, the pattern of FSR would remain similar to that of infants at age 4 months.

MATERIALS AND METHODS

Subjects, study design and protocol. A total of 68 full-term healthy infants who were appropriate for gestational age, and who had no parental history of hypercholesterolemia or hypertriglyceridemia were recruited during the first two weeks of life. This double-blind, partially randomized, prospective clinical trial took place at the Cincinnati Children's Hospital Medical Center (CCHMC) and other area hospitals, from January 1999 until June 2002. Fifty-two infants were followed until 4 months, of whom 47 were followed until 18 months of life on an unrestricted diet (Figure 1). The HM group included 18 infants exclusively breast-fed until 6 months, after which they received HM, supplemented with intake of MCF until 12 months of age; in this way the HM group served as a control group with continuous high Ch intake. The remaining infants were randomized by the study coordinator according to a computer-generated random numbers table to receive MCF (ready to serve Carnation Good Start + 40 mg/L Ch; Nestlé Laboratories, Eau Claire, WI) (n=16) or CF (ready to serve Carnation Good Start; Nestlé Laboratories, Eau Claire, WI) (n=18). HM infants could not be randomized, as breast-feeding involves an a priori decision and commitment on the part of the mother. The added Ch (G.W. Greeff and Co, Inc. as Chilesetrol National Formulary) was solubilized with a small quantity of ethanol. The ethanol was then evaporated and the Ch distributed evenly throughout the formula by the manufacturer. MCF and CF infants received only the assigned formula, with introduction of solid foods in all study groups after the age of 4 months by parent/physician. Sample sizes at 18 months were: HM (n=15), MCF

(n=15) and CF (n=17). The Ch contents of HM, MCF and CF were 120, 80, and 40 mg/L, respectively, as shown in Table 1.

The study was comprised of two test periods; the first from recruitment until 4 months of age and the second from 4 months of age until the end of the study at 18 months of age. Period one evaluated the effects of Ch supplementation of infant formulas on Ch FSR at 4 months of age, since at this age infants receive exclusively human milk or formula, thus eliminating the potential for interference from other diet components. At 18 months, infants have typically been introduced to solid foods, hence the purpose of period two was to evaluate the imprinting hypothesis. The outcome measures FSR, serum lipid profiles, and weight were obtained at 4 and 18 months (Figure 1).

The Ch content was the only difference in composition between MCF and CF. Stability and solubility of the Ch concentration in the formula was tested at different points in time to assess composition and assure bioavailability. All infants began receiving formula within the first 3-7 days of life. Formula was provided to the subjects at no cost for the entire duration of the study to improve compliance. Breast milk Ch content was analyzed from each mother of the breast-feeding infants at 4, 8 and 12 months. Mothers from the 3 groups were required to keep bimonthly 3-day diet diaries recording either the volume of formula intake/d (MCF or CF) or for the HM group, the frequency of breast-feeding, and the volume of supplemental MCF/d after the infant was 6 months old. During period 2, the 3-day diet diary recording was obtained every 3 months. The food records were analyzed using Food Processor (ESHA database, V. 7.4) by a registered dietitian to determine whether any significant deviations in food intake

occurred which might confound the outcome variable of FSR. Total energy intake, as well as fat and Ch levels were compared among the diet groups. The study protocol was reviewed and approved by the Institutional Review Board at the CCHMC, and informed consent was obtained from parents before enrollment of the infants.

Plasma lipid analysis. Plasma T-Ch, triglyceride (TG), HDL-Ch, and LDL-Ch levels were determined using enzymatic techniques validated by the National Institutes of Health Lipid Research Clinics, and used previously in infants (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002). (See Appendix 1).

Cholesterol biosynthesis measurement. Over the two day FSR study period, two blood specimens were obtained by the CCHMC GCRC nurses, placed on ice, and centrifuged within 30 minutes. On day 1, 8 ml blood was obtained to determine baseline body water and erythrocyte membrane Ch deuterium enrichment. Infants were then given orally 500 mg/kg body weight of deuterium oxide (D_2O, 99.96% deuterium; Isotec Inc., Miamisburg, OH) orally. On day 2, 8 ml blood for deuterium excess enrichment was obtained. All blood samples were taken between 09:00 – 12:00 h. For each day, the sample was fractionated and frozen at -80°C until analysis, such that the plasma was used for determination of lipid concentrations and the red blood cell fraction was used for Ch FSR determination.

Ch fractional synthesis rates were determined as the incorporation rate of the stable isotope, deuterium oxide (D_2O / ^2H_2O) from body water into red blood cell membrane Ch, which serves as an index of hepatic Ch synthesis rates. The analytical

procedure for FSR has been described previously (Bayley et al. 1998; Bayley et al. 2002). (See Appendix 1).

Statistical analysis. Variables were tested for normality, and in the case of non-normality, variables were natural log-transformed. Percent changes were calculated as the average of the differences between outcome variables at 18 months and 4 months, divided by the value at 4 months. One-way ANOVA was used to test the effect of infant diet on outcome measures and percent changes (SAS, v.8; Cary, NC), with 4 month outcome variables included as covariates for testing the effect of infant diet at 18 months. The Tukey-Kramer test was used to adjust for multiple comparisons and determine differences between pairs of groups. Using a standard deviation of 1.11 for FSR observed in cow milk-based formula-fed infants at 4 months from previous studies (Bayley et al. 1998), a sample size of 14 in each group was predicted to yield a power of 80% in detecting a 20% difference in FSR means between groups at an alpha value of 0.05 for a 2-sided test. Analysis at 18 months included all participants for whom data at 4 months existed. Statistical significance was considered for $p < 0.05$. Results are presented as mean ± SEM, and where applicable as geometric means (see Appendix 2 for compiled data presented as arithmetic means).

RESULTS

Body weights are summarized in Table 2. There were no significant differences in weight at any time during the study between the diet groups. Sixty-eight infants were recruited, of whom fifty-two infants participated in the first test period at four months, of whom 47 continued and completed the second test period at 18 months. During the first test period, reasons for dropping out were largely due to, (i) parent preference (n=12), and (ii) families lost to follow-up (n=2). In the HM group additional reasons for dropping out included, (iii) introduction of formula before 4 months (n=1), and (iv) unwillingness to participate in the blood draw (n=1). Reasons for dropping out during the second test period included, (i) introduction of formula and weaning from HM before 1 year of age in the HM group (n=3), and (ii) families lost to follow-up due to moving (n=2), in the MCF and CF groups. No adverse effects in response to formulas used were reported. There was insufficient blood drawn to determine FSR at 4 months in one infant from the MCF group, this was not a problem at 18 months. The average caloric and Ch intakes at 4 and 18 months of age are shown in Table 3. Results at 18 months are shown adjusted for the corresponding 4-month outcome variables, which were included as covariates in the statistical analysis. The infants' gender was included in the statistical model, with no significant effect on FSR or plasma lipids. Over the course of the study, CF infants had the largest percent change in dietary Ch from 4 to 18 months, and HM infants had the largest percent change in caloric intake.

At 4 months plasma T-Ch concentrations of infants fed HM (4.07±0.15 mmol/L) and MCF (3.85±0.16 mmol/L) did not differ statistically, but both were higher ($p<0.02$)

than those fed CF (3.28±0.15 mmol/L), as shown in Table 4. HDL-Ch levels were higher ($p=0.005$) in the MCF group (1.43±0.07 mmol/L) than in the HM (1.09±0.06 mmol/L) and CF (1.15±0.06 mmol/L) groups, see Figure 2. LDL-Ch levels were higher in the HM group (2.08±0.11 mmol/L) than MCF (1.56±0.12 mmol/L, $p<0.003$) and CF (1.20±0.11 mmol/L, $p<0.0001$); MCF mean LDL-Ch was also higher ($p=0.0321$) than that of the CF group (Figure 2). There were no statistically significant differences among groups in VLDL-Ch, and triglyceride levels (Figure 3). The ratio between T-Ch:HDL-Ch was lower in MCF (2.76±0.17, $p<0.0001$) and CF (2.96±0.16, $p=0.004$) groups than in HM group (3.81±0.16), as seen in Table 4. At 18 months, there was a non statistically significant trend toward lower plasma T-Ch, HDL-Ch, and LDL-Ch levels in the HM infants compared to the MCF and CF groups, as presented in Table 4.

FSR at 4 months was affected by infant feeding ($p<0.05$) among the three groups. FSR in the HM group (0.034±0.005 pools/day) was lower ($p<0.02$) than in the CF group (0.052±0.005 pools/day), however, there was no statistically significant difference between HM and MCF (0.047±0.005 pools/day), nor between MCF and CF, as shown in Table 5. At 18 months there was no significant effect of earlier infant feeding, nor any differences in FSR between the three dietary groups. The percent change between the FSR at 4 months and 18 months is presented in Table 5; the HM group exhibited the largest absolute percent change in FSR, while the formula groups' FSR showed more minimal differences over the course of the two test periods, see Figure 4.

DISCUSSION

This is the first study to evaluate the potential imprinting of FSR beyond one year of age. It was hypothesized that at age 4 months, infants fed HM would have a lower FSR compared to those fed MCF, who would have a lower FSR than those fed CF. The level of dietary Ch would vary inversely with endogenous Ch FSR. We demonstrated decreased cholesterogenesis and an increase in circulating plasma Ch concentrations as the level of dietary Ch increased between CF, MCF and HM-fed infants, at 4 months of age, thus confirming the original hypothesis. However, the differences seen as a result of infant feeding were not reflected at 18 months of age, suggesting that there is no imprinting of Ch biosynthesis or lasting differences in plasma lipids at early life cycle stages due to the level of Ch intake prior to weaning.

The positive relationship between dietary Ch intake and serum lipid levels in the neonatal period is well established in both animal (Reiser et al. 1972; Reiser et al. 1979; Kris-Etherton et al. 1979; Jones et al. 1990; Mott et al. 1990; Mott et al. 1991; Li et al. 1980) and infant studies (Kallio et al. 1992; Hayes et al. 1992; Van Biervliet et al. 1992; Mize et al. 1995; Agostoni et al. 2000; Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002). The higher HDL-Ch exhibited by the MCF infants (Figure 2) compared to HM and CF infants, might be due in part to differences in the form of Ch in HM and that in MCF. HM Ch contains both free Ch (85%) and esterified Ch (15%) as components of the total Ch content; while the MCF contained 100% free Ch. Other differences in formulas that could impact serum lipids include: a higher proportion of medium chain fatty acids and a lower proportion of longer-chain polyunsaturated fatty

acids, higher phytosterol concentrations, and galactose concentrations (Huisman et al. 1996). The present study confirms and reinforces the notion that dietary Ch in infancy elevates plasma total-Ch by a direct mechanism and that the effect persists only until weaning. At 18 months, plasma T-Ch, HDL-Ch and LDL-Ch levels tended to be lower in the HM compared to MCF and CF-fed infants; however, the differences were not significant. A larger sample size, with better control of dietary Ch may have resulted in significant differences; however, the study sample size was determined for differences in FSR and not plasma lipid levels. Previous human infant studies have shown a significant inverse relationship between dietary Ch and FSR at 4 months (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002). Using deuterium incorporation (DI) methodology, previous investigators have estimated that endogenous Ch FSR ranged from 0.02 pools/d in breast-fed infants to 0.11 pools/d in infants fed formulas with varying concentrations of Ch.

The present study demonstrated an effect of infant diet on FSR at 4 months, with CF infants having an up-regulated rate of endogenous Ch production, compared to HM infants. The infants receiving MCF had an FSR that was intermediate between the HM and CF infants, suggesting that the supplementation of Ch brought the MCF infants' FSR closer to the physiological range seen in breast-fed infants. These results demonstrate that adaptive regulatory mechanisms in early infancy enable human infants to respond to differences in Ch intakes. Theoretically, these homeostatic mechanisms would prevent excess Ch accumulation during high Ch intakes, or conversely, provide for an increase in Ch availability during instances of low or negligible intake. Assuming that HM is the "gold standard" for infant nutrition and values for FSR in HM-fed infants are considered

to be "normal", then these data would indicate that CF-fed infants have a 53% increase in FSR, as compared to HM-fed infants, during the first 4 months of life. This is indicative of the need for Ch in early infancy, a period of rapid growth. The results seen in MCF infants are in contrast those seen by Bayley et al. (1998), where the infants fed Ch supplemented formula had FSRs similar to infants receiving the regular formula at 4 months. Neither were any differences seen in FSR before or after a Ch challenge at 11 and 12 months of age (Bayley et al. 2002), however, the bioavailability of the Ch in the study formula was suspect. In the present study, at 18 months, there were no differences in FSR between dietary groups; the HM and MCF infants had an increase in endogenous Ch production, while the CF infants had a slight decrease in FSR. Small sample size and individual variability in FSR over time precluded the observation of a statistically significant difference in percent change across the groups from 4 to 18 months. At 18 months, it appears that FSR responds to the level of dietary Ch in the infant's diet, as the HM infants had a decrease by 4% in average Ch intake per day, the increase in FSR would compensate for this. Despite dramatic increases in Ch intake, the absence of change in 18 month FSRs in MCF and CF infants suggests a form of up-regulation; its permanency is a question that deserves further research.

Breast-feeding is associated with lower Ch production rates in baboons (Mott et al. 1991), and pigs (Jones et al. 1990) in the neonatal period. Increases of hepatic hydroxymethylglutaryl CoA (HMG-CoA) reductase activity in formula-fed neonatal pigs and rats (Jones et al. 1990; Reiser et al. 1977), confirm the presence of feedback inhibition between dietary Ch and endogenous Ch production. Breast-fed baboons as infants have higher levels of hepatic acyl CoA Ch acyltransferase (ACAT) activity,

higher concentrations of hepatic Ch esters than those formula fed, and lower plasma lecithin Ch acyltransferase activity (LCAT), as they metabolized exogenous Ch (Mott et al. 1993), compared to baboons formula-fed who relied on de novo Ch synthesis as their principal source of Ch. Breast-feeding also led to increases of 44 to 99% in LDL receptor mRNA compared with formula-feeding, which persisted into adolescence in baboons (Mott et al. 1993; Mott et al. 1995; Mahaney et al. 1999), suggesting that long-term Ch homeostasis could be affected by the level of dietary Ch in the infant diet. However, these effects were not seen in guinea pigs (Li et al. 1980), and in human fetuses increases of hepatic LDL-receptor activity were positively associated with gestational age and inversely correlated to serum T-Ch and LDL-Ch, (Cai et al. 1991). The current study confirms feedback inhibition of Ch synthesis in human infants dependent upon exposure to dietary Ch.

It has been postulated that approximately half the difference in FSR between the HM and formula-fed groups at 4 months can be explained by an expanded Ch central pool (Wong et al. 1993). The remainder is most likely due to down-regulation of HMG-CoA reductase and Ch synthesis. The expansion of the central pool may be due to the increased absorption of dietary Ch in the breast-fed infants, coupled with a modulation of LDL-receptor activity in the liver. The exact mechanisms and the physiological health implications deserve further research to determine the effects of early dietary Ch on absorption, LDL-receptor expression and activity, and whether such effects persist. However, such studies might be difficult to conduct in an infant population due to the extensive amount of blood needed and high costs associated with following a cohort long-term. To date, the present study has been unique in looking at the effects of early

Ch on FSR prospectively beyond one year of age. Study of toddlers at 18 months may be too early to see an effect, since differences in plasma lipids between those breast-fed or formula-fed as infants have not generally been seen until beyond 17 years of age (Kolaček et al. 1993; Ravelli et al. 2000; Owen et al. 2002; Marmot et al. 1980; Fall et al. 1995; Leeson et al. 2001).

The functionality of the higher Ch content in human milk has been the subject of debate for several decades, especially since in this regard, infant formula composition has stood in stark contrast. The advancement of knowledge in this area has been encumbered by the difficulty of separating the metabolic effects of dietary Ch from those of dietary fatty acids, since human milk differs from most formulas in this domain as well. Individual fatty acids can impact Ch metabolism, affecting concentrations of serum-lipoproteins, independent from intake of dietary Ch (Hayes et al. 1992; Van Biervliet et al. 1992; Mize et al. 1995; Grundy et al. 1990). As human milk fatty acid profile and content is modified by maternal diet (Agostoni et al. 2000, Koletzko et al. 1992, Jensen et al. 2000) and varies throughout a feeding period, the fatty acid composition cannot be mirrored by formulas. No direct inferences with regard to the effect of fatty acids on Ch metabolism can be made in the present study, and a potential confounding effect of fatty acid composition cannot be ruled out.

In the present study, as in previous infant studies (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002) limited blood sample size did not allow for a more comprehensive analysis of Ch metabolism. As FSR was the main outcome variable in the present study, fecal Ch excretion was not measured; complete collection of stools during infancy would have been difficult due to stool consistency.

Methods for determination of endogenous Ch synthesis based on deuterium incorporation (DI) have been well established (Wong et al. 1993; Jones et al. 1993; Jones et al. 1994), being advantageous because it is a direct, short-term, and non-invasive method, compared to intake balance methods (Huang et al. 1976; Potter et al. 1976; Nestel et al. 1979) or isotopic kinetic decay analyses (Ferezou et al. 1983; Dell et al. 1985; Schwartz et al. 1993). The efficacy of using erythrocyte Ch deuterium enrichment to study human lipid metabolism has been described previously (Wong et al. 1993; Cruz et al. 1994; Bayley et al. 1998; Bayley et al. 2002; Jones et al. 1994). Measurement of Ch synthesis by DI has been validated against sterol balance (Jones et al. 1998), mass isotopomer distribution analysis (Di Buono et al. 2000) and sterol precursors (Jones et al. 1992).

In conclusion, we have examined for the first time endogenous Ch synthesis rates in human infants beyond one year of age; determining that while early intake of Ch affects FSR and plasma lipids at 4 months, the differences observed do not persist at 18 months. This indicates that there is no imprinting of Ch biosynthesis at early life cycle stages by differing dietary levels of Ch in infancy.

SUMMARY AND CONCLUSIONS

The present study is the first to test the imprinting hypothesis of FSR beyond one year of age. A prospective, clinical trial was conducted in 47 infants who received, from their first week of life until 18 months, diets varying in Ch content for 12 months. FSR, plasma lipids and infant weight were measured at 4 and 18 months. Dietary intake of Ch, fat and energy was verified by 3-day diet diaries, kept by mothers at 4 months and 18 months. Decreased cholesterogenesis and increased circulating plasma Ch concentrations were found with increasing levels of dietary Ch between HM, MCF and CF infants, at 4 months of age. However, differences seen as a result of infant feeding were not reflected at 18 months. There were no significant differences in weight between the diet groups at any time during the study. These results suggest that there is no imprinting of Ch biosynthesis or lasting differences in plasma lipids at early life cycle stages due to the level of Ch intake prior to weaning. It is possible that the present study of toddlers at 18 months may have been too early to see an effect, since differences in plasma lipids between those breast-fed or formula-fed as infants have not generally been seen until beyond 17 years of age (Ravelli et al. 2000; Owen et al. 2002). Further research is needed to investigate if differences exist in adult Ch FSR, as a function of infant feeding.

Other potential points of imprinting that affect Ch kinetics include, i) levels of HMG-CoA reductase activity, the key enzyme regulating endogenous Ch synthesis, and levels of the enzymes ii) ACAT and iii) LCAT which esterify exogenous dietary Ch and endogenous Ch, respectively. Differences have been observed between breast-fed and formula-fed baboons in levels of these enzymes (Mott et al. 1993). In addition, further

study is needed concerning the developmental expression of LDL receptors. A lasting increase in LDL receptor mRNA was seen in breast-fed baboons compared to those formula-fed (Mott ct al. 1993; Mott et al. 1995), but the effect was not observed in guinea pigs (Li et al. 1980), and has yet to be studied in infants.

Strengths of the present work include the fact that the trial was prospective from birth until 18 months. The study formula provided was improved over that of previous studies; the cholesterol was more thouroughly matrixed into the formula by the manufacturer and provided in ready-to-feed cans. The formulas used also had identical polyunsaturated to saturated fatty acid ratios, which had not been the case in previous studies looking at infant FSR. However, the fatty acid composition of the formula cannot be made identical to the fatty acid composition of human milk, which means that no direct inferences as far as the effect of fatty acids on cholesterol metabolism can be made in the present study. The protein composition and contents of the formulas used also differ from human milk, specifically with respect to casein: whey ratios and actual concentrations of proteins. More research into the effects of proteins in formulas versus those in human milk on lipid metabolism is needed.

A rather permissive level of dietary control over the second year of life was part of the study design. An absence of regulations governing solid food intake allowed for more normal variation and a more "real life" test of the imprinting hypothesis, with 3-day diet records used to assess the variation. Differences in infant diet related to characteristics of mothers who choose to breast-feed versus those who formula-feed would also be detected by 3-day diet records, which have been an acceptable and reliable method used to estimate variation of dietary intake in infants (Ernst et al. 1990; Cruz et

al. 1994; Bayley et al. 1998; Bayley et al. 2002). However, further work is needed in order to truly test Reiser's hypothesis that level of early Ch intake may protect against later hypercholesterolemia. The current study design did not incorporate a cholesterol challenge; the possibility of following the cohort and incorporating a challenge, while controlling for other environmental factors needs to be considered. In conclusion, further research, encompassing the above mentioned points, is needed to examine the hypothesis of metabolic imprinting as it relates to cholesterol metabolism and cardiovascular disease risk.

In conclusion, the present study has examined for the first time endogenous Ch synthesis rates in human infants beyond one year of age; determining that while early intake of Ch affects FSR and plasma lipids at 4 months, the differences observed do not persist at 18 months. This suggests that there is no imprinting of Ch biosynthesis at early life cycle stages by differing dietary levels of Ch in infancy.

TABLES AND FIGURES

Table 1. Composition of types of milk and formulas used.

	HM*	MCF	CF
Calories (kcal)	680	667	667
Protein (g)	10.5	15.1	15.1
Carbohydrates (g)	72.0	74.7	74.7
Fat (g)	39.0	34.2	34.2
Polyunsaturated/ saturated ratio	0.3	0.46	0.46
Cholesterol (mg)	120.0	80.0	40.0
Linoleic acid (mg)	3,971	5,695	5,695

*Composition of human milk (HM) from a literature composite (Cruz et al. 1994; Bayley et al. 1998, Bayley et al. 2002). HM varies with stage of lactation and among mothers. Composition of formulas based on manufacturer's estimates. Values are per litre.

MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch).

CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch).

Table 2. Effect of infant diet on body weights.

Age (months)	HM	MCF	CF	*p value*
0	3.5±0.1	3.3±0.1	3.5±0.1	0.0649
4	6.8±0.2	6.6±0.2	7.0±0.2	0.3358
18	11.1±0.3	11.7±0.4	11.7±0.3	0.4025

Values are mean ± SEM, (kg).

HM = Human Milk, birth (n=18), 4 month (n=18), 18 month (n=15).

MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), birth (n=16), 4 month (n=16), 18 month (n=15).

CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch), birth (n=18), 4 month (n=18), 18 month (n=17).

Table 3. Average dietary cholesterol and caloric intakes.

	Average dietary cholesterol (mg)			
Age (months)	HM	MCF	CF	*p value*
4	124.2±2.2	126.0±5.5	59.6±3.0‖	<0.0001
18	119.1±14.3	182.8±24.0	156.5±16.2	0.0685
% change**	-4%	45%	163%*†	<0.0001
	Average calories (kcal)			
Age (months)	HM	MCF	CF	*p value*
4	619.4±11.1¶	867.4±38.2	894.9±44.4	<0.0001
18	1076.5±62.0	1245.3±60.9	1175.0±52.3	0.5474
% change**	74%‡§	44%	31%	0.0045

Values are mean ±SEM.

*CF vs. HM, p<0.0001

†CF vs. MCF, p=0.002

‡HM vs. MCF, p=0.0420

§HM vs. CF, p=0.0114

‖CF vs. HM, CF vs. MCF, p<0.0001

¶HM vs. MCF, HM vs. CF, p<0.0001

**presented as arithmetic means, calculated using difference between FSR group means, divided by 4 mo. FSR group mean, multiplied by 100.

HM = Human Milk, 4 month (n=18), 18 month (n=15).

MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), 4 month (n=16), 18 month (n=15).

CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch), 4 month (n=18), 18 month (n=17).

Table 4. Effect of infant diet on plasma lipid concentrations.

Total Ch (T-Ch)				
Age (months)	HM	MCF	CF	*p value*
4	4.07±0.15	3.85±0.16	3.28±0.15*	0.0013
18	3.58±0.16	3.80±0.15	4.12±0.16	0.0931
HDL-Ch				
Age (months)	HM	MCF	CF	*p value*
4	1.09±0.06	1.43±0.07†	1.15±0.06	0.0018
18	0.98±0.04	1.10±0.04	1.06±0.04	0.1496
LDL-Ch				
Age (months)	HM	MCF	CF	*p value*
4	2.08±0.11‡	1.56±0.12§	1.20±0.11	<0.0001
18	1.87±0.17	2.19±0.14	2.46±0.15	0.0736
T-Ch:HDL-Ch				
Age (months)	HM	MCF	CF	*p value*
4	3.81±0.16‖	2.76±0.17	2.96±0.16	<0.0001
18	3.67±0.20	3.69±0.18	4.02±0.16	0.2293

Values are mean ±SEM, (mmol/L). HM = Human Milk, 4 month (n=18), 18 month (n=15). MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), 4 month (n=16), 18 month (n=15). CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch), 4 month (n=18), 18 month (n=17).

* CF vs. MCF, p=0.0104, CF vs. HM, p=0.0004 at 4 months

† MCF vs. HM, p=0.0008, MCF vs. CF, p=0.0046 at 4 months

‡ HM vs. MCF, p=0.0027, HM vs. CF, p<0.0001 at 4 months

§ MCF vs. CF, p=0.0321 at 4 months

‖ HM vs. MCF, p<0.0001, HM vs. CF, p=0.004 at 4 months.

Table 5. Effect of infant diet on fractional synthesis rate (FSR) of endogenous cholesterol.

Age (months)	HM	MCF	CF	*p value*
4	0.034±0.005†	0.047±0.006	0.052±0.005	0.0479
18	0.052±0.007	0.053±0.007	0.047±0.006	0.8232
% change*	53%	13%	-10%	0.4790

Values are mean ± SEM, (pools/day).

HM = Human Milk, 4 month (n=18), 18 month (n=15).

MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), 4 month (n=16), 18 month (n=15). CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch), 4 month (n=18), 18 month (n=17).

*calculated using difference between FSR group means, divided by 4 mo. FSR group mean, multiplied by 100.

† HM vs. CF, p=0.0171

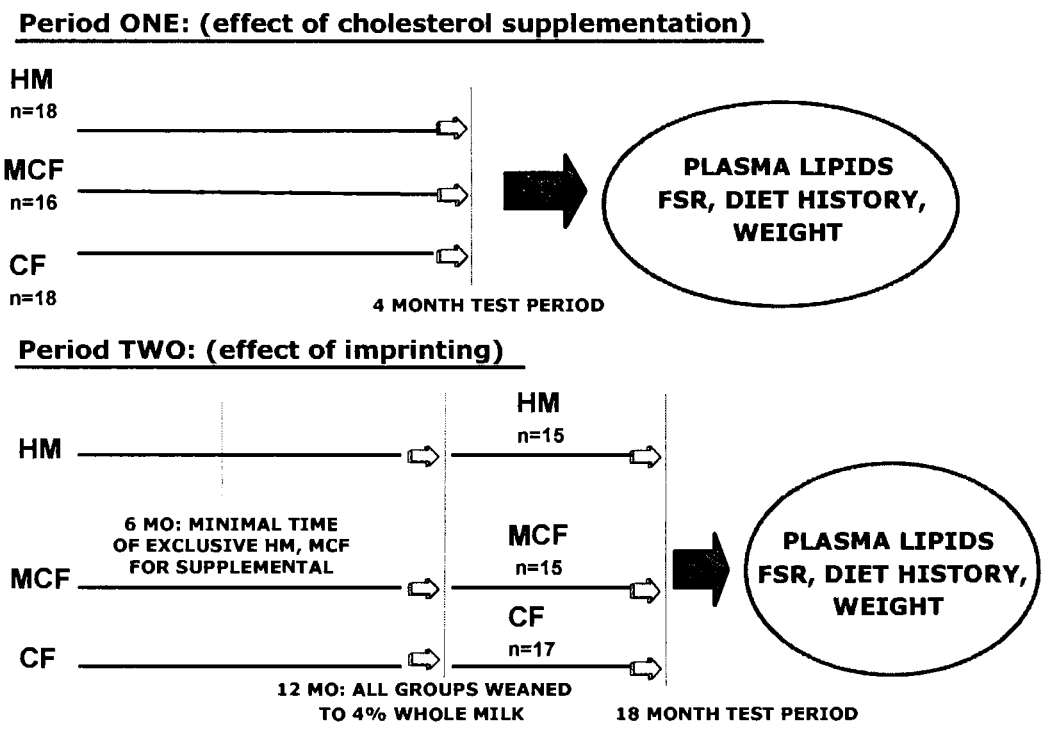

Figure 1. Study Design and Protocol.

HM = Human Milk, MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch). Plasma lipids include total-cholesterol (Ch), LDL-Ch, HDL-Ch, VLDL-Ch, and triglycerides.

FSR = fractional synthesis rate of endogenous cholesterol. Diet histories were analyzed for average kcal/day and average Ch/day.

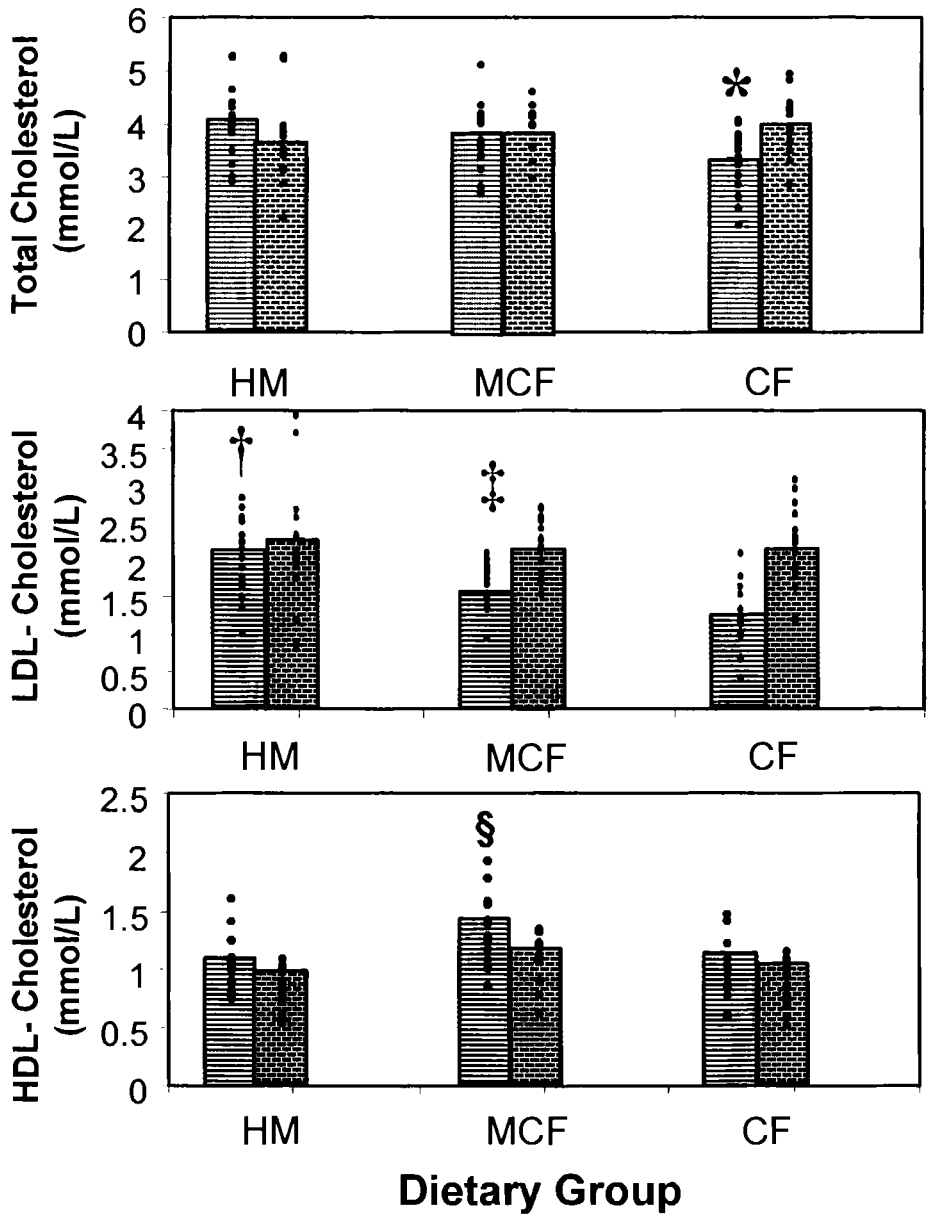

Figure 2. Effect of infant diet on Total-cholesterol (Ch), LDL-Ch and HDL-Ch. **4 months,** **18 months.** *CF vs. MCF, CF vs. HM, at 4 months, †HM vs. MCF, HM vs. CF at 4 months. ‡MCF vs. CF at 4 months. §MCF vs. HM, MCF vs. CF at 4 months, $p < 0.002$.

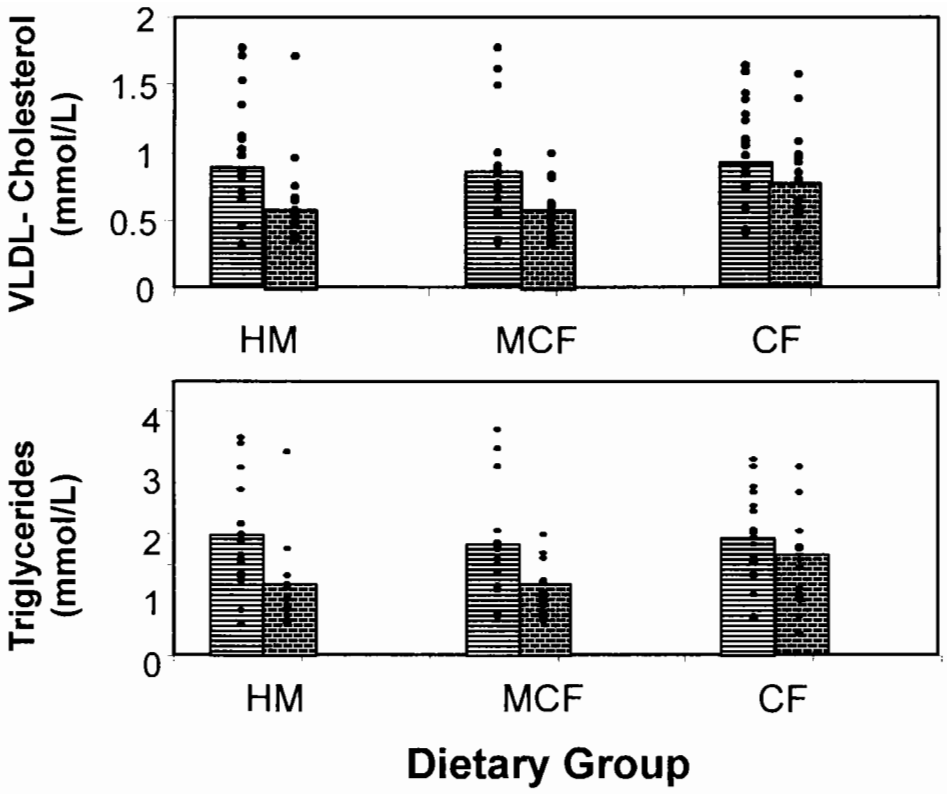

Figure 3. Effect of infant diet on VLDL-cholesterol and plasma triglycerides.

4 months, 18 months.

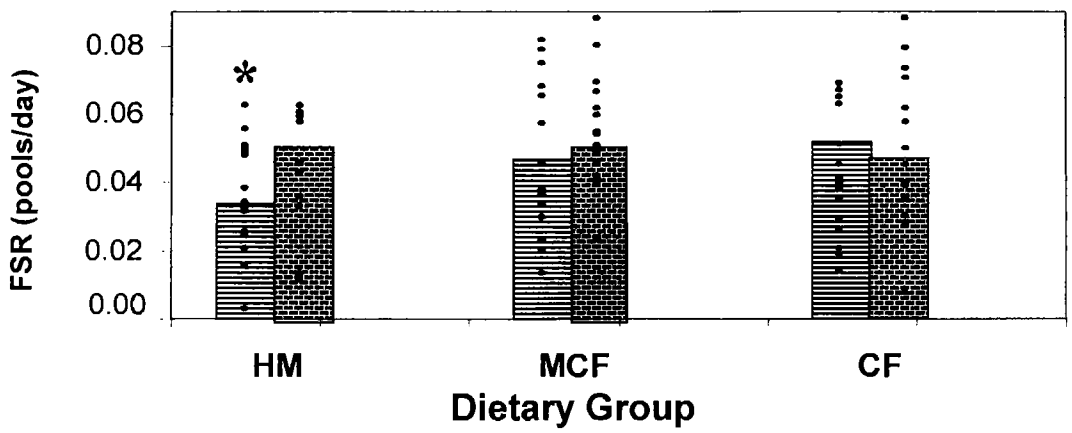

Figure 4. Effect of infant diet on fractional synthesis rate (FSR) of endogenous cholesterol. 4 months, 18 months. *FSR of HM was lower (p = 0.0171) at 4 months compared to CF. At 18 months, there were no significant differences between groups. The differences in percent change within each group were not statistically significant.

HM = Human Milk, 4 month (n=18), 18 month (n=15).

MCF = Modified Cow-milk formula (Carnation Good Start, 80 mg/L Ch), 4 month (n=16), 18 month (n=15).

CF = Cow-milk formula (Carnation Good Start, 40 mg/L Ch), 4 month (n=18), 18 month (n=17).

REFERENCES

Agostoni C, Riva E, Scaglioni S, Marangoni F, Radaelli G, Giovannini M 2000 Dietary fats and cholesterol in Italian infants and children. Am J Clin Nutr 72:S1384-91

Agostoni C, Haschke F 2003 Infant formulas. Recent developments and new issues. Minerva Pediatr 55:181-94

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ 1989 Weight in infancy and death from ischaemic heart disease. Lancet 2:577-80

Barker DJP, Martyn CN, Osmond C, Hales CN and Fall CHD 1993 Growth in utero and serum cholesterol concentrations in adult life. BMJ 307:1524-27

Bayley TM, Alasmi M, Thorkelson T, Krug-Wispe S, Jones PJH, Bulani JL, Tsang RC 1998 Influence of formula versus breast milk on synthesis rates in four-month-old infants. Pediatr Res 44:60-7

Bayley TM, Alasmi M, Thorkelson T, Jones PJH, Corcoran J, Krug-Wispe S, Tsang RC 2002 Longer term effects of early dietary cholesterol level on synthesis and circulating cholesterol concentrations in human infants. Metabolism 51:25-33

Bitman J, Wood DL, Mehta NR, Hamosh P, Hamosh M 1986 Comparison of the cholesteryl ester composition of human milk from preterm and term mothers. Pediatr Gastroenterol Nutr 5:780-6

Bloch KE 1965 The biological synthesis of cholesterol. Science 150:19-27

Bloch KE 1983 Sterol structure and membrane function. CRC Critical Reviews in Biochemistry 14:47-92

Brown MS, Goldstein JL 1986 A receptor-mediated pathway for cholesterol homeostasis. Science 232:34-47

Cai HJ, Xie CL, Chen Q, Chen XY, Chen YH 1991 The relationship between hepatic low-density lipoprotein receptor activity and serum cholesterol level in the human fetus. Hepatology 13:852-57

Chang CCY, Sakashita N, Ornvold K, Lee O, Chang ET, Dong R, et al. 2000 Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. J Biol Chem 275:28083-92

Cruz MLA, Wong WW, Mimouni F, Hachey DL, Setchell KDR, Klein PD, Tsang RC 1994 Effects of infant nutrition on cholesterol synthesis rates. Pediatr Res 35:135-40

Dell RB, Ramakrishnan R, Palmer RH, Goodman DS 1985 A convenient six-point

blood sampling schedule for determining whole body cholesterol kinetics in humans. J Lipid Res 26:575-82

Di Buono M, Jones PJH, Beaumier L, Wykes LJ 2000 Comparison of deuterium incorporation and mass isotopomer distribution analysis for measurement of human cholesterol biosynthesis. J Lipid Res 41:1516-23

Dietschy JM, Spady DK 1984 Measurement of rates of cholesterol synthesis using tritiated water. J. Lipid Res 25: 1469-76

Dobbing J 1981 Nutritional growth restriction and the nervous system, In: The Molecular Bases of Neuropathology, ed., T.R. Davidson AN, Edward Arnold: London, p. 221-33

Ernst JA, Brady MS, Rickard KA 1990 Food and nutrient intake of 6- to 12-month old infants fed formula or cow milk: a summary of four national surveys. J Pediatr 117:S86-100

Farquharson J, Cockburn F, Patrick WA, Jamieson EC, Logan RW 1992 Infant cerebral cortex phospholipids fatty-acid composition and diet. Lancet 340:810-13

Fall CH, Barker DJ, Osmond C, Winter PD, Clark PMS, Hales CN 1992 Relation of infant feeding to adult serum cholesterol concentration and death from ischaemic heart disease. BMJ 304:801-5

Fall CH, Osmond C, Barker DJP, Clark PMS, Hales CN, Stirling Y, Meade TW 1995 Fetal and infant growth and cardiovascular risk factors in women. BMJ 310:428-32

Ferezou J, Rautueau J, Coste T, Gouffier E, Chevalier F 1983 Cholesterol turnover in human plasma lipoproteins: studies with stable and radioactive isotopes. Am J Clin Nutr 36:235-44

Fomon SJ, Rodgers RR, Ziegler EE, Nelson SE, Thomas LN 1984 Indices of fatness and serum cholesterol at age eight years in relation to feeding and growth during early infancy. Pediatr Res 18:1233-38

Friedman G, Goldberg SJ 1975 Concurrent and subsequent serum cholesterols of breast- and formula-fed infants. Am J Clin Nutr 28:42-5

Gamble JL, Blackfan K 1920 Evidence indicating a synthesis of cholesterol by infants. J Biol Chem 42:401-9

Glueck CJ, Tsang R, Balistreri W, Fallat R 1972 Plasma and dietary cholesterol in infancy: effects of early low or moderate dietary cholesterol intake on subsequent response to increased dietary cholesterol. Metabolism 21:1181-92

Goodman DS, Smith FR, Seplowitz AH, Ramakrishnan R, Dell RB 1980 Prediction of

the parameters of whole body cholesterol metabolism in humans. J Lipid Res 21:699-713

Grundy SM, Ahrens EH, Davignon J 1969 The interaction of cholesterol absorption and cholesterol synthesis in man. J Lip Res 10:304-15

Grundy SM, Denke MA 1990 Dietary influences on serum lipids and lipoproteins. J Lip Res 31:1149-72

Grundy SM 2002 Low-density lipoprotein, non-high-density lipoprotein, and apolipoprotein B as targets of lipid-lowering therapy. Circulation 106:2526-29

Hahn P, Srubiski L 1990 Development of cholesterol metabolism: the effect of diet composition at weaning. Biol Neonate 58:1-7

Hayes KC, Pronczuk A, Wood RA, Guy DG 1992 Modulation of infant formula fat profile alters the low-density lipoprotein/high-density lipoprotein ratio and plasma fatty acid distribution relative to those breast-feeding. J Pediatr 120:S109-16

Huang CTL, Rodriguez JT, Woodward WE, Nichols B 1976 Comparison of patterns of fecal bile acid and neutral sterols between children and adults. Am J Clin Nutr 29:1196-203

Huisman M, van Beusekom CM, Lanting CI, Nijeboer HJ, Muskiet FA, Boersma ER 1996 Triglycerides, fatty acids, sterols, mono- and disaccharides and sugar alcohols in human milk and current types of infant formula milk. Eur J Clin Nutr 50:255-60

Hodgson PA, Ellefson RD, Elveback LR, Harris LE, Nelson RA, Weidman WH 1976 Comparison of serum cholesterol in children fed high, moderate, or low cholesterol milk diets during neonatal period. Metabolism 25:739-46

Huttenen JK, Saarinen UM, Kostiainen E, Siimes MA 1983 Fat composition of the infant diet does influence subsequent serum lipid levels in man. Atherosclerosis 46:87-94

Ingelfinger JR, Woods LL 2002 Perinatal programming, renal development, and adult renal function. Am J Hypertens 15:S46-9

Innis SM, Dyer R, Wadsworth L, Quinlan P, Diersen-Schade D 1993 Dietary saturated, monounsaturated, n-6 and n-3 fatty acids, and cholesterol influence platelet fatty acids in the exclusively formula-fed piglet. Lipids 28: 645-50

Javitt NB 2002 Cholesterol, hydroxycholesterols, and bile acids. Biochem Biophys Res Comm 292:1147-53

Jensen RG, Hagerty MM, McMahon KE 1978 Lipids of human milk and infant formulas: a review. Am J Clin Nutr 31:990-1016

Jensen CL, Maude M, Anderson RE, Heird WC 2000 Effect of docosahexaenoic acid supplementation of lactating women on the fatty acid composition of breast milk lipids and maternal and infant plasma. Am J Clin Nutr 71:S292-9

Jones PJH, Hrboticky N, Hahn P, Innis SM 1990 Comparison of breast-feeding and formula feeding on intestinal and hepatic cholesterol metabolism in neonatal pigs. Am J Clin Nutr 51:979-84

Jones PJH, Pappu AS, Illingworth DR, Leitch CA 1992 Correspondence between plasma mevalonic acid levels and deuterium uptake in measuring human cholesterol synthesis. Eur J Clin Invest 22:609-13

Jones PJH, Leitch CA, Li ZC, Connor WE 1993 Human cholesterol synthesis measurement using deuterated water: theoretical and procedural considerations. Arterioscler Thromb 13:247-53

Jones PJH, Main BF, Frohlich JJ 1993 Response of cholesterol synthesis to cholesterol feeding in men with different apolipoprotein E genotypes. Metabolism 42:1065-71

Jones PJH, Lichtenstein AH, Schaefer EJ. 1994 Interaction of dietary fat saturation and cholesterol level on cholesterol synthesis measured using deuterium incorporation. J Lip Res 35:1093-01

Jones PJH, Pappu AS, Hatcher L, Li ZC, Illingworth R, Connor WE 1996 Dietary cholesterol feeding suppresses human cholesterol synthesis measured by deuterium incorporation and urinary mevalonic acid levels. Arterioscler Thromb Vasc Biol 16:1222-28

Jones PJH, Ausman LM, Croll DH, Feng JY, Schaefer EA, Lichtenstein AH 1998 Validation of deuterium incorporation against sterol balance for measurement of human cholesterol biosynthesis. J Lipid Res 39: 1111-7

Jooste PL, Rossouw LJ, Steenkamp JE, Rossouw JE, Swanepoel ASP, Charlton DO 1991 Effect of breast feeding on the plasma cholesterol and growth of infants. J Pediatr Gastroenterol Nutr 13:139-42

Kallio MJT, Salmenperä L, Siimes MA, Perheentupa J, Miettinen TA 1992 Exclusive breast-feeding and weaning: effect on serum cholesterol and lipoprotein concentrations in infants during the first year of life. Pediatrics 89:663-6

Kolaček S, Kapetanović T, Zimolo A, Lužar V 1993 Early determinants of cardiovascular risk factors in adults. A. plasma lipids. Acta Pediatr 82:699-704

Koletzko B, Thiel I, Abiodun PO 1992 The fatty acid composition of human milk in Europe and Africa. J Pediatr 120:S62-70

Kris-Etherton PM, Layman DK, York PV, Frantz ID 1979 The influence of early nutrition on the serum cholesterol of the adult rat. J Nutr 109:1244-57

Lammi-Keefe CJ, Jensen RG 1984 Lipids in human milk: a review 2 - composition and fat-soluble vitamins. J Pedia Gastro and Nutr 3:172-198

Leeson CPM, Kattenhorn M, Deanfield JE, Lucas A 2001 Duration of breast feeding and arterial distensibility in early adult life: population based study. BMJ 322:643-7

Li JR, Bale LK, Kottke BA 1980 Effect of neonatal modulation of cholesterol homeostasis on subsequent response to cholesterol challenge in adult guinea pig. J Clin Invest 65:1060-8

London IM, Schwartz H 1953 Erythrocyte metabolism: the metabolic behavior of the cholesterol of human erythrocytes. J Clin Invest 32:1248-52

Mahaney MC, Blangero J, Rainwater DL, Mott GE, Comuzzie AG, MacCluer JW, et al. 1999 Pleiotropy and genotype by diet interaction in a baboon model for atherosclerosis – a multivariate quantitative genetic analysis of HDL subfractions in two dietary environments. Arterioscler Thromb Vasc Biol 19:1134-41

Marmot MG, Page CM, Atkins E, Douglas JW 1980 Effect of breast-feeding on plasma cholesterol and weight in young adults. J Epidemiol Community Health 34:164-7

McNamara DJ, Kolb R, Parker TS, Batwin H, Samuel P, Brown CD, Ahrens EH 1987 Heterogeneity of cholesterol homeostasis in man: response to changes in dietary fat quality and cholesterol quantity. J Clin Invest 79:1729-39

Miettinen TA, Kessaniemi YA 1989 cholesterol absorption: regulation of synthesis and elimination and within population variations of serum cholesterol levels. Am J Clin Nutr 49:629-35

Mize CE, Uauy R, Kramer R, Benser M, Allen S, Grundy SM 1995 Lipoprotein-cholesterol responses in healthy infants fed defined diets from ages 1 to 12 months: comparisons of diets predominant in oleic acid versus linoleic acid, with parallel observations in infants fed a human milk-based diet. J Lipid Res 36:1178-87

Mott GE, McMahan CA, Kelley JL, Mersinger Farley C, McGill HC 1982 Influence of infant and juvenile diets on serum cholesterol, lipoprotein cholesterol, and apolipoprotein concentrations in juvenile baboons. Atherosclerosis 45:191-202

Mott GE, Jackson EM, McMahan CA, McGill HC 1990 cholesterol metabolism in adult baboons is influenced by infant diet. J Nutr 120: 243-51

Mott GE, Lewis DS, McGill HC 1991 Programming of cholesterol metabolism by breast

or formula feeding. Ciba Foundation Symposium 156:56-76

Mott GE, Lewis DS, McMahan CA 1993 Infant diet affects serum lipoprotein concentrations and cholesterol esterifying enzymes in baboons. J Nutr 123:155-63

Mott GE, DeLallo L, Driscoll DM, McMahan CA, Lewis DS 1993 Influence of breast and formula feeding on hepatic concentrations of apolipoprotein and low-density lipoprotein receptor mRNAs. Biochim Biophys Acta 1169:59-65

Mott GE, Jackson EM, DeLallo L, Lewis DS, McMahan CA 1995 Differences in cholesterol metabolism in juvenile baboons are programmed by breast-versus formula-feeding. J Lipid Res 36:299-307

Nelson CM, Innis SM 1999 Plasma lipoprotein fatty acids are altered by the positional distribution of fatty acids in infant formula triacylglycerols and human milk. Am J Clin Nutr 70:62-9

Nestel PJH, Poser A, Bolton T 1979 Changes in cholesterol metabolism in infants in response to dietary cholesterol and fat. Am J Clin Nutr 32:2177-82

Owen CG, Whincup PJ, Odoki K, Gilg JA, Cook DG 2002 Infant feeding and blood cholesterol: a study in adolescents and a systematic review. Pediatrics 110:597-608

Picaud JC, Boucher P, Lapillonne A, Berthouze M, Delvin E, Boehm G, et al. 2002 Influence of dietary cholesterol on vitamin D metabolism in formula-fed preterm neonates. J Pediatr Gastroenterol Nutr 35:180-4

Potter JM, Nestel P 1976 Greater bile acid excretion with soy bean than with cow milk in infants. Am J Clin Nutr 29:546-51

Rassin DK, Gault GE, Järvenpää AL, Räihä NCR 1983 Feeding the low-birth-weight infant: II. Effects of taurine and cholesterol supplementation on amino acids and cholesterol. Pediatrics 71:179-86

Ravelli ACJ, van der Meulen JHP, Osmond C, Barker DJP, Bleker OP 2000 Infant feeding and adult glucose tolerance, lipid profile, blood pressure, and obesity. Arch Dis Child 82:248-52

Reiser R, Sidelman Z 1972 Control of serum cholesterol homeostasis by cholesterol in the milk of suckling rat. J Nutr 102:1009-16

Reiser F, Henderson GR, O'Brien BC, Moore RW 1977 Persistence of dietary suppression of 3-hydroxy-3-methylglutaryl coenzyme-A reductase during development in rats. J Nutr 107:1131-38

Reiser R, O'Brien BC, Henderson GR 1979 Studies on a possible function for

cholesterol in milk. Nutr Rep Int 19:835-49

Roseboom TJ, van der Meulen JHP, Osmond C, Barker DJP, Ravelli ACJ, Bleker OP 2000 Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. Am J Clin Nutr 72:1101-6

Savino F, Costamagna M, Prino A, Oggero R, Silvestro L 2002 Leptin levels in breast-fed and formula-fed infants. Acta Paediatr 91:897-902

Schaefer EJ 2002 Lipoproteins, nutrition and heart disease. Am J Clin Nutr 75:191-212

Skoglund-Andersson C, Ehrenborg E, Fisher RM, Olivecrona G, Hamsten A, Karpe F 2003 Influence of common variants in the CETP, LPL, HL and APO E genes on LDL heterogeneity in healthy, middle-aged men. Atherosclerosis 167:311-7

Smart J 1986 Undernutrition, learning and memory: a review of experimental studies, In: Proceedings of XII International Congress of Nutrition, ed. J.N. Taylor TG , John Libbey: London, p. 74-8

Stary HC 1987 Macrophages, macrophage foam cells, and eccentric intimal thickening in the coronary arteries of young children. Atherosclerosis 64:91-108

Stary HC 2000 Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. Am J Clin Nutr 72:S1297-306

Stocker A, Baumann U 2003 Supernatant protein factor in complex with RRR-α-tocopherylquinone: A link between oxidized vitamin E and cholesterol biosynthesis. J Mol Biol 332:759-65

Schwartz CC, Zech LA, VandenBroek JM, Cooper PS 1993 Cholesterol kinetics in subjects with bile fistula – positive relationship between size of the bile acid precursor pool and bile acid synthetic rate. J Clin Invest 91:923-38

Uauy R, Mena P, Wegher B, Nieto S, Salem N 2000 Long chain polyunsaturated fatty acid formation in neonates: effect of gestational age and intrauterine growth. Pediatr Res 47:127-35

Uauy R, Mize CE, Castillo-Duran C 2000 Fat intake during childhood: metabolic responses and effects on growth. Am J Clin Nutr 72:S1354-60

Uauy R, Castillo C 2003 Lipid requirements of infants: Implications for nutrient composition of fortified complementary foods. J Nutr 133:S2962-72

Van Biervliet JP, Vinaimont N, Vercaemst R, Rosseneu M 1992 Serum cholesterol, cholesteryl ester, and high-density lipoprotein development in newborn infants: response to formulas supplemented with cholesterol and γ-linolenic acid. J Pediatr 120:S101-8

Wagner V, Stockhausen HB 1988 The effect of feeding human milk and adapted milk formulae on serum lipid and lipoprotein levels in young infants. Eur J Pediatr 147:292-5

Waterland RA, Garza C 1999 Potential mechanisms of metabolic imprinting that lead to chronic disease. Am J Clin Nutr 69:179-97

Wong WW, Hachey DL, Insull W, Opekun AR, and Klein PD 1993 Effect of dietary cholesterol on cholesterol synthesis in breast-fed and formula-fed infants. J Lipid Res 34:1403-11

APPENDIX 1

LABORATORY PROCEDURES

Determination of plasma lipids. Plasma Ch, triglyceride (TG), HDL-Ch, and LDL-Ch levels were determined using enzymatic techniques validated by the National Institutes of Health Lipid Research Clinics. In the presence of Mn2+ and heparin, chylomicrons, very-low-density lipoprotein-Ch (VLDL-Ch), and LDL-Ch were selectively precipitated, leaving only HDL-Ch in solution. The precipitated lipoproteins were sedimented by centrifugation, leaving the clear supernate containing HDL-Ch that was then analyzed enzymatically. TG was also determined enzymatically. Plasma LDL-Ch concentrations were calculated from plasma total-Ch (TC) concentrations using the Friedwald equation (Friedwald et al. 1972), used previously with infants, (Kallio et al. 1992; Wong et al. 1993; Cruz et al. 1994; Mize et al. 1995; Bayley et al. 1998; Bayley et al. 2002).

Deuterium incorporation methodology. Methods for determination of endogenous Ch synthesis based on deuterium incorporations (DI) have been well established (Jones et al. 1993; Wong et al. 1993; Jones et al. 1994), being advantageous because it is a direct, short-term, and non-invasive method, compared to intake balance methods (Gamble et al. 1920; Huang et al. 1976; Potter et al. 1976; Nestel et al. 1979) or isotopic kinetic decay analyses (Goodman et al.1980; Ferezou et al. 1983; Dell et al. 1985; Schwartz et al. 1993). The efficacy of using erythrocyte Ch deuterium enrichment to study human lipid metabolism was first reported by London and Schwartz (London et al. 1953). Subsequently, use of deuterium incorporation into newly synthesized erythrocyte membrane free Ch, based on the tritiated water uptake methodology developed in animals

(Dietschy et al. 1984), was further refined and developed for measurement of short-term Ch synthesis in humans (Jones et al. 1990; Jones et al. 1993). The interpretation of endogenous Ch synthesis results, determined by DI, relies on three principal assumptions (Jones et al. 1990; Jones et al. 1993). The first is that a constant fraction of deuterium atoms in free Ch synthesized *de novo* originates from plasma water (Dietschy et al. 1984). Because water freely and rapidly diffuses across cell membranes, plasma water deuterium concentration provides a measure of precursor enrichment, and deuterium enrichment of the newly synthesized free Ch molecules (unesterified Ch) corresponds to enrichment of the central pool, which is comprised of the liver, plasma and intestine (Dell et al 1985; Schwartz et al. 1993). A second assumption is that within the central pool, rapid movement of newly synthesized Ch from the liver to plasma lipoproteins and then to cell membranes occurs, and therefore the deuterium content of erythrocyte membranes reflects newly synthesized Ch (London et al. 1953). The third assumption is that deuterium uptake by cell membranes represents synthesis from the central pool only, and it does not represent influx of newly formed Ch from other pools or synthesis by erythrocytes.

Measurement of Ch synthesis by DI has been validated against both sterol balance (Jones et al. 1998), mass isotopomer distribution analysis (Buono et al. 2000) and sterol precursors (Jones et al. 1992). Using sterol balance methodology, daily whole body Ch balance agreed with incorporation of deuterium oxide into newly synthesized Ch over a 24-h period (Jones et al. 1998). The correlation between the two methods was $r = 0.745$ ($p<0.001$). Subjects who received oral deuterium oxide coincident with constant infusion of sodium [1-^{13}C] acetate to measure both incorporation of deuterium into Ch and mass

isotopomer distribution pattern of newly synthesized Ch, also yielded similar measurements of FSR. The correlation between these two methods was r = 0.84 (p = 0.0007), (Buono et al. 2000). DI is a sensitive, direct and relatively short-term measurement, using a stable isotope that is safe, with minimal adverse affects, suitable for studies involving infants.

Determination of fractional synthesis rate. Over the two day FSR study period, two blood specimens were obtained by the CCHMC hospital phlebotomist, placed on ice, and centrifuged within 30 minutes. On day 1, 8 ml blood was obtained to determine baseline body water and erythrocyte membrane Ch deuterium enrichment. Infants were then orally given 500 mg/kg body weight of deuterium oxide (D_2O, 99.96% deuterium; Isotec Inc., Miamisburg, OH). On day 2, 8 ml blood for deuterium excess enrichment was obtained. All samples were taken between 09:00 – 12:00 h. For each day, the sample was fractionated such that the serum was used for determination of plasma lipid concentrations and the red blood cell fraction was used for Ch biosynthesis/FSR determination. The red blood cell fraction was frozen at -80°C in Cincinnati and sent to McGill University for FSR determination.

Ch FSR was determined as the incorporation rate of the stable isotope, deuterium oxide (D_2O / ^2H_2O) from body water into red blood cell membrane Ch, which serves as an index of hepatic Ch synthesis rates. The laboratory procedure for FSR begins with erythrocyte lipid extraction by organic solvent and drying under nitrogen. Duplicates of 1.5 ml red blood cells (RBC) samples were used, where there was a lack of RBC, the infants plasma was used as well, as free Ch associated with LDL-Ch in the plasma and in

cell membranes is in constant equilibrium (Brown et al. 1986, Schwartz et al. 1993). A total of 8 ml of methanol was added, and samples were heated in a 55° C water bath for 15 minutes. Hexane/chloroform (4:1 vol/vol, 24 ml) was added and the mixture shaken for 15 minutes, upon which 2 ml of doubly distilled water was added and shaken again for another 15 minutes. This was followed by centrifugation for 15 min. at 1,500g. The upper hexane-chloroform layer containing the lipid was removed. The lipid was re-extracted using hexane-chloroform, upon which the upper layer was again removed and combined with the first; this was repeated for a total of 3 extractions. The solvent was then evaporated by drying under nitrogen, leaving the lipid extract.

The extract was redissolved in a small amount of chloroform and streaked on to thin-layer silica chromatography (TLC) plates to isolate the free Ch. Plates were developed in petroleum ether/ethyl ether/acetic acid (135:15:1.5 vol/vol/vol) for approximately 1 hour and air dried. Lipid bands were visualized in iodine vapor against a Ch standard. The free Ch band appeared as the first band from the bottom of the TLC plate, and was then eluted from the TLC silica scrapings using: i) 8 ml of freshly prepared 50% methanolic KOH solution, ii) heating for 2 hrs in a dry bath, and iii) extraction with 8 ml of petroleum ether. The pooled solvent was dried under nitrogen, leaving the purified Ch which was quantitatively transferred to Pyrex combustion tubes containing CuO (0.6 mg) and a 2 to 2.5 cm piece of silver wire. Tubes were flame-sealed with an oxygen torch under vacuum, and Ch combusted completely for 4 hours at 520°C to produce CO_2 and water.

Combustion water was cryogenically separated from CO_2 by vacuum distillation into Pyrex tubes containing 60 mg of zinc. These were flame-sealed under vacuum with

an oxygen torch, and the water reduced to hydrogen-deuterium gas at 520°C for 30 min. Plasma-water enrichment was measured after dilution of 0 and 24 hour plasma samples with Montreal tap water of known isotopic abundance to bring the enrichment into the working range of the International Atomic Energy Agency (Vienna) mass spectrometer calibration standards. The plasma dilutions were placed under nitrogen into Pyrex tubes containing 60 mg of zinc. Tubes were sealed under vacuum, using an oxygen torch, and water was reduced in a 520°C oven for 30 min. to produce hydrogen-deuterium gas.

Deuterium enrichment of the resultant gas was measured by dual inlet isotope ratio mass spectrometry (VG Isogas 903D). Values were expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per million. Erythrocyte Ch deuterium enrichment values at 0 and 24 hours were expressed relative to the corresponding mean plasma water sample enrichment at each time point, to yield FSR (pools/day) for the free Ch pool. The FSR index represents that fraction of the free portion of the rapidly turning over central Ch pool that is synthesized in 24 hours. It is expressed as percent per day, and calculated using the formula:

$$\text{FSR(pools/day)} = \frac{24\text{hrs/day} \times \text{Slope(at \% excess/hour)}}{0.4783 \times \text{Body Water } {}^{2}\text{H(at \% excess)}}$$

The slope is calculated using the enrichment above the baseline versus time. The constant 0.4783 is the ratio of hydrogen atoms (n=22) in the Ch molecule derived from body water to the total number of hydrogen atoms in the Ch molecule (n=46).

APPENDIX 2

APPENDIX 2 - Compiled Results

4 MONTH DATA
HUMAN MILK (HM)

BABY # (SAS)	Subject	INITIALS	FSR	TRIG	TOTAL CHOL	HDL-C	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOL mg/DAY	CAL AVG/day	WEIGHT (kg)
1	1104	J-H	0.029	1.966	149	38	76	35	3.92	118.08	588.77	6.47
2	1117	HAR	0.049	3.684	162	36	61	65	4.5	119.72	596.96	6.56
3	1119	CMN	0.03	1.932	155	39	82	34	3.97	131.22	654.29	7.19
4	1120	DAH	0.013	3.164	207	42	109	56	4.93	104.94	523.25	5.75
5	1122	DSLS	0.032	2.791	208	45	114	49	4.62	128.85	642.46	7.06
6	1124	WSA	0.047	2.057	159	36	87	36	4.42	142.54	710.71	7.81
7	1127	ADEK	0.022	1.367	157	43	90	24	3.65	127.93	637.91	7.01
8	1128	MRK	0.023	1.333	146	51	71	24	2.86	124.83	622.44	6.84
9	1131	EPH	0.049	3.571	168	64	41	63	2.63	111.14	554.19	6.09
10	1133	EMS	0.03	2.215	154	33	82	39	4.67	117.53	586.04	6.44
11	1135	DJU	0.061	1.379	125	46	55	24	2.72	132.86	662.48	7.28
12	1137	JAP	0.036	2.237	169	35	94	40	4.83	130.49	650.65	7.15
13	1153	AMC	0.018	0.768	172	57	101	14	3.02	118.44	590.59	6.49
14	1154	CCD	0.048	1.639	159	39	91	29	4.08	139.43	695.24	7.64
15	1166	KCH	0.022	1.559	182	51	103	28	3.57	119.90	597.87	6.57
16	1167	XMN	0.054	1.706	135	39	66	30	3.46	127.39	635.18	6.98
17	1168	CDG	0	0.520	112	34	69	9	3.29	119.90	597.87	6.57
18	1169	CMS	0.046	1.266	116	34	60	22	3.41	120.82	602.42	6.62
		AVG	**0.034**	**1.953**	**157.500**	**42.333**	**80.667**	**34.500**	**3.808**	**124.223**	**619.407**	**6.807**
		STDEV	**0.016**	**0.883**	**26.185**	**8.636**	**19.932**	**15.565**	**0.743**	**9.404**	**46.885**	**0.515**
		SEM	**0.004**	**0.208**	**6.172**	**2.036**	**4.698**	**3.669**	**0.175**	**2.217**	**11.051**	**0.121**

- Note:1117, 1119, 1168 dropped out, not included in 18 mo. statistical analysis, nor in comparisons across time.

APPENDIX 2 - Compiled Results

4 MONTH DATA
STUDY FORMULA- MODIFIED COW FORMULA (MCF)

BABY # (SAS)	SUBJ #	INITIALS	FSR	TRIG	CHOL	HDL	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOLmg/DAY	CAL AVG/day	WEIGHT (kg)
1	1102	MAV	0.032	1.480	111	51	34	26	2.18	93.86	696	4.92
2	1103	TTR	0.035	1.605	140	56	56	28	2.5	.	.	.
3	1105	CJD	0.018	1.187	167	74	72	21	2.26	.	.	.
4	1110	TJS	0.044	1.989	173	79	59	35	2.19	129.56	961	7.18
5	1111	JMS	0.028	3.266	204	69	77	58	2.96	115.16	854	6.47
6	1114	GGB	0.078	3.560	160	47	50	63	3.4	119.77	888	6.42
7	1132	MQR	0.064	2.181	161	41	81	39	3.93	126.11	960	6.89
8	1134	JMS	0.011	0.735	135	55	67	13	2.45	101.35	752	6.20
9	1143	LVS	0.074	0.768	134	50	70	14	2.68	108.83	807	6.88
10	1144	ACW	0.021	1.898	160	62	64	34	2.58	92.13	683	6.04
11	1146	M-K	0.067	3.876	164	44	51	69	3.73	.	.	.
12	1148	TIN	0.064	1.232	106	49	35	22	2.16	90.98	675	7.54
13	1150	BKY	0.056	1.164	135	62	52	21	2.18	146.26	1085	7.18
14	1151	LAT	0.036	1.865	124	36	55	33	3.44	114.3	848	5.54
15	1155	MLR	.	1.243	147	51	74	22	2.88	119.77	888	6.75
16	1156	ARS	0.081	1.695	162	61	71	30	2.66	158.93	1179	7.52
		AVG	**0.047**	**1.859**	**148.938**	**55.438**	**60.500**	**33.000**	**2.761**	**126.048**	**867.385**	**6.579**
		STDEV	**0.023**	**0.947**	**24.933**	**11.832**	**14.000**	**16.805**	**0.581**	**20.458**	**152.902**	**0.767**
		SEM	**0.006**	**0.237**	**6.233**	**2.958**	**3.500**	**4.201**	**0.145**	**5.115**	**38.226**	**0.192**

- Note: 1132 was a drop out, included in 4 mo., but not included in 18 mo. statistical analysis, nor in comparisons across time. 1155 had no red bloc cells to calculate FSR at 4 mo., but FSR was calculated at 18 mo., and 1155 was included in 18 mo. statistical analysis.

APPENDIX 2 - Compiled Results

4 MONTH DATA
CONTROL FORMULA -COW FORMULA (CF)

BABY # (SAS)	SUBJ #	INITIALS	FSR	TRIG	CHOL	HDL	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOLmg/DAY	CAL AVG/day	WEIGHT (kg)
1	1101	SAH	0.033	0.701	123	46	65	12	2.67	61.78	927	7.99
2	1106	DMS	0.022	1.650	91	30	32	29	3.03	38.86	583	5.74
3	1109	NJG	0.049	3.266	130	31	41	58	4.19	54.66	820	7.25
4	1113	EJR	0.042	2.927	129	37	40	52	3.49	48.96	734	7.42
5	1115	EMF	0.024	0.746	140	62	65	13	2.26	54.37	822	6.34
6	1121	ODQ	0.055	2.147	158	43	77	38	3.67	.	.	.
7	1126	DJR	0.017	1.752	99	48	20	31	2.06	75.72	1136	6.85
8	1129	LJS	0.073	2.599	132	53	33	46	2.49	54.94	831	7.42
9	1136	JMD	0.069	2.068	146	62	47	37	2.35	44.69	670	5.75
10	1138	RCS	0.049	1.661	144	60	55	29	2.4	66.05	991	5.99
11	1140	CMB	0.043	2.836	125	40	35	50	3.13	52.95	794	5.80
12	1141	RPL	0.039	3.379	139	40	39	60	3.48	71.17	1068	7.29
13	1142	JAT	0.122	1.424	77	31	21	25	2.48	91.1	1366	7.53
14	1149	CSC	0.029	2.509	115	62	9	44	1.85	53.24	799	8.15
15	1159	C-C	0.091	1.944	107	30	43	34	3.57	57.22	858	7.01
16	1161	RAB	0.067	2.204	155	39	77	39	3.97	60.92	914	7.01
17	1162	AMM	0.071	1.096	136	40	77	19	3.4	54.94	824	6.60
18	1163	PJC	0.045	1.446	134	48	60	26	2.79	71.74	1076	8.36
		AVG	**0.052**	**2.020**	**126.667**	**44.556**	**46.444**	**35.667**	**2.960**	**59.606**	**894.882**	**6.971**
		STDEV	**0.027**	**0.796**	**21.717**	**11.356**	**20.515**	**14.204**	**0.685**	**12.581**	**188.411**	**0.835**
		SEM	**0.006**	**0.188**	**5.119**	**2.677**	**4.835**	**3.348**	**0.161**	**2.965**	**44.409**	**0.197**

- Note: 1162 dropped out, not included in 18 mo. statistical analysis, nor in comparisons across time, 1159 FSR was previously mistakenly reported did not match calculation and mass spec enrichment values.

- Normal Ranges: TRIG: 30-104mg/dL CHOL: 170-200mg/dL HDL: Not Estab. LDL: <110mg/dL VLDL: <20mg/dL

APPENDIX 2 - Compiled Results

18 MONTH DATA
HUMAN MILK (HM)

BABY # (SAS)	SUBJ #	INITIALS	FSR	TRIG	TOTAL CHOL	HDL	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOL mg/DAY	CAL AVG/day	WEIGHT/(kg)
1	1104	J-H	0.097	0.825	139	34	90	15	4	.	.	.
2	1117	HAR	.	.	.	.	.	.	.	.	.	.
3	1119	CMN	.	.	.	.	.	.	.	.	.	.
4	1120	DAH	0.111	0.983	87	26	44	17	3	36.91	761.18	9.335
5	1122	DSLS	0.034	1.232	210	33	155	22	6	199.6133	1727.66	14.5
6	1124	WSA	0.047	1.842	153	38	82	33	4	231.99	1101.94	11.32
7	1127	ADEK	0.044	0.588	157	43	104	10	4	95.3	902.94	10.22
8	1128	MRK	0.061	0.983	125	33	75	17	4	.	.	.
9	1131	EPH	0.106	3.480	136	44	30	62	3	192.9167	1233.43	11.09
10	1133	EMS	0.013	1.390	208	38	145	25	5	90.59333	895.68	9.56
11	1135	DJU	0.062	0.644	124	35	78	11	4	90.48667	982.23	10.7
12	1137	JAP	0.012	0.994	156	39	99	18	4	98.36	1400.22	14.405
13	1153	AMC	0.064	0.994	134	39	77	18	3	118.79	910.96	11.035
14	1154	CCD	0.013	0.814	149	46	89	14	3	86.33667	952.21	11.615
15	1166	KCH	0.037	1.164	158	38	99	21	4	175.215	1161.04	10.7
16	1167	XMN	0.014	0.791	123	35	74	14	4	79.155	1116.55	10.505
17	1168	CDG	.	.	.	.	.	.	.	.	.	.
18	1169	CMS	0.059	0.972	113	29	67	17	4	53.08	847.93	9.755
		AVG	**0.052**	**1.180**	**144.800**	**36.667**	**87.200**	**20.933**	**3.933**	**119.134**	**1076.459**	**11.134**
		STDEV	**0.033**	**0.708**	**32.358**	**5.407**	**32.287**	**12.714**	**0.799**	**60.715**	**262.846**	**1.621**
		SEM	**0.008**	**0.167**	**7.627**	**1.274**	**7.610**	**2.997**	**0.188**	**14.311**	**61.953**	**0.382**

APPENDIX 2 - Compiled Results

18 MONTH DATA
STUDY FORMULA -MODIFIED COW FORMULA (MCF)

BABY # (SAS)	SUBJ #	INITIALS	FSR	TRIG	CHOL	HDL	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOL mg/DAY	CAL AVG/day	WEIGHT (kg)
1	1102	MAV	0.065	1.074	116	40	57	19	3	.	.	13.2
2	1103	TTR	0.079	0.768	141	51	76	14	3	284.41	1356.09	11.43
3	1105	CJD	0.021	1.831	182	51	99	32	4	145.7	1826.72	12.7
4	1110	TJS	0.038	0.701	164	55	97	12	2.98	126.41	1287.74	11.55
5	1111	JMS	0.053	0.746	129	46	70	13	2.8	104.36	1307.08	10.52
6	1114	GGB	0.052	1.740	172	48	93	31	4	138.31	1118.1	11.7
7	1132	MQR	.	.	.	.	.	.	.	.	.	.
8	1134	JMS	0.087	1.356	138	30	84	24	5	129.72	885.19	10.3
9	1143	LVS	0.068	0.938	166	46	103	17	4	152.12	1341.15	11.83
10	1144	ACW	0.038	0.667	139	52	75	12	3	.	.	9.52
11	1146	M-K	0.058	0.983	158	36	105	17	4	192.06	1124.35	10.83
12	1148	TIN	0.04	1.153	130	46	64	20	3	.	.	13.2
13	1150	BKY	0.044	1.322	141	36	82	23	4	71.76	1087.77	11.03
14	1151	LAT	0.047	2.136	140	36	66	38	4	.	.	10
15	1155	MLR	0.06	1.175	156	47	88	21	3	393.5	1051.06	11.77
16	1156	ARS	0.049	0.836	158	56	87	15	3	271.94	1313.1	12.19
		AVG	**0.053**	**1.162**	**148.667**	**45.067**	**83.067**	**20.533**	**3.519**	**182.754**	**1245.305**	**11.451**
		STDEV	**0.017**	**0.445**	**18.169**	**7.796**	**14.849**	**7.873**	**0.655**	**95.670**	**243.466**	**1.106**
		SEM	**0.004**	**0.111**	**4.542**	**1.949**	**3.712**	**1.968**	**0.164**	**23.918**	**60.867**	**0.277**

- Note: 1132 was a drop out, included in 4 mo., but not included in 18 mo. statistical analysis, nor in comparisons across time. 1155 had no red bloo cells to calculate FSR at 4 mo., but FSR was calculated at 18 mo., and 1155 was included in 18 mo. statistical analysis.

APPENDIX 2 - Compiled Results

18 MONTH DATA
CONTROL FORMULA -COW FORMULA (CF)

BABY # (SAS)	SUBJ #	INITIALS	FSR	TRIG	CHOL	HDL	LDL	VLDL	CHOL/HDL Ratio	DIET AVG CHOL mg/DAY	CAL AVG/day	WEIGHT (kg)
1	1101	SAH	0.072	1.921	171	45	92	34	4	261.48	1119.2	13.93
2	1106	DMS	0.056	0.463	127	36	83	8	4	129.33	904.47	10.43
3	1109	NJG	0.078	1.107	149	39	90	20	4	108.75	1120.38	11.19
4	1113	EJR	0.028	1.220	152	35	95	22	4	200.63	1356.79	12.25
5	1115	EMF	0.069	1.718	153	47	76	30	3	107.71	965.4	10.94
6	1121	ODQ	0.048	1.130	188	46	122	20	4	128.22	1144.27	12.33
7	1126	DJR	0.033	1.571	140	48	64	28	3	189.15	1616.38	11.27
8	1129	LJS	0.056	1.921	154	50	70	34	3	.	.	13.11
9	1136	JMD	0.06	1.028	167	46	103	18	4	92.08	899.89	9.95
10	1138	RCS	0.005	2.192	166	39	88	39	4	129.47	1589.84	9.73
11	1140	CMB	0.026	3.277	134	30	46	58	4	98.22	1262.04	9.87
12	1141	RPL	0.038	1.921	193	42	117	34	5	149.03	1086.24	13.08
13	1142	JAT	0.087	2.859	148	33	64	51	5	.	.	13.26
14	1149	CSC	0.043	1.062	109	42	48	19	3	83.1	978.62	12.28
15	1159	C-C	0.028	1.887	136	27	76	33	5	203.81	1164.83	11.59
16	1161	RAB	0.037	0.768	163	39	110	14	4	137.19	1080.31	13.54
17	1162	AMM	.	.	.	.	.	.	.	.	.	.
18	1163	PJC	0.043	1.955	140	33	72	35	4	329.38	1336	12.98
		AVG	**0.047**	**1.647**	**152.353**	**39.824**	**83.294**	**29.235**	**3.941**	**156.503**	**1174.977**	**11.866**
		STDEV	**0.021**	**0.727**	**21.287**	**6.748**	**22.090**	**12.872**	**0.659**	**68.895**	**221.813**	**1.365**
		SEM	**0.005**	**0.171**	**5.017**	**1.590**	**5.207**	**3.034**	**0.155**	**16.239**	**52.282**	**0.322**

- Normal Ranges: TRIG: 30-104mg/dL CHOL: 170-200mg/dL HDL: Not Estab. LDL: <110mg/dL VLDL: <20mg/dL

APPENDIX 2 - Compiled Results

Final results for main outcome measures

Variables	4 months				18 months			
	HM	MCF	CF	P value	HM	MCF	CF	P value
FSR	0.034± 0.005*	0.047±0.006	0.052±0.005	0.0479	0.052±0.007	0.053±0.007	0.047±0.006	0.8232
TG	1.99±0.2	1.83±0.2	1.94±0.2	0.8665	1.81±0.2	1.18±0.2	1.63±0.2	0.0679
TC	4.07±0.15	3.85±0.16	3.28±0.15†	0.0013	3.58±0.16	3.80±0.15	4.12±0.16	0.0931
HDL-C	1.09±0.06	1.43±0.07‡	1.15±0.06	0.0018	0.98±0.04	1.10±0.04	1.06±0.04	0.1496
LDL-C	2.08±0.11§	1.56±0.12‖	1.20±0.11	<0.0001	1.87±0.17	2.19±0.14	2.46±0.15	0.0736
VLDL-C	0.89±0.10	0.85±0.10	0.92±0.10	0.8359	0.54±0.08	0.54±0.08	0.75±0.07	0.0658
TC:HDL-C	3.81±0.16¶	2.76±0.17	2.96±0.16	<0.0001	3.67±0.20	3.69±0.18	4.02±0.16	0.2293

* HM vs. CF, p=0.0171
† CF vs. MCF, p=0.0104, CF vs. HM, p=0.0004
‡ MCF vs. HM, p=0.0008, MCF vs. CF, p=0.0046
§ HM vs. MCF, p=0.0027, HM vs. CF, p<0.0001
‖ MCF vs. CF, p=0.0321
¶ HM vs. MCF, p<0.0001, HM vs. CF, p=0.004

APPENDIX 3

INSTITUTIONAL REVIEW BOARD

NOTIFICATION OF FINAL APPROVAL

PRINCIPAL INVESTIGATOR Mahmood Alaani, M.D. CHMC PROTOCOL # 96-9-14

TITLE OF STUDY: Cholesterol supplementation and cholesterol synthesis rates in infants at one year of age

APPROVED ON: 9/26/96

☒ FULL REVIEW

☐ EXPEDITED REVIEW

PLEASE NOTE: The Review Board has determined that this is a minimal risk study with/without direct benefit to subjects, and that

☐ assent must be obtained from all participating minors. This must be documented on the consent form either by the child's signature or by the investigator checking a box to indicate that assent was obtained. Assent requires affirmative agreement by the subject not merely failure to object.

☐ assent must be obtained from participating minors age _____, unless they are _____.

☒ assent need not be obtained from participants because of age of participants

for this study, one (1)/~~two(2)~~ parents must give permission for inclusion of the child (unless one parent is dead, incompetent, unknown, not reasonably available, or if only one has legal responsibility).

COMMENTS:

1. Any adverse reactions or complications that occur as a result of this study, must be reported
   a) immediately to the Review Board Chairman (559-8039) if life threatening or severe or
   b) within 5 days if relatively minor.
2. A progress report must be filed at least every 12 months (more frequently if requested) with the Review Board as long as the study is active.
3. There may be no change or additions to the protocol or consent form, without prior approval of the Review Board.
4. If this protocol has not been initiated within two years of this date, you will be required to submit an updated protocol for reconsideration by the Review Board.
5. If the study remains active 5 years from this date, an updated protocol must be submitted for full review.
6. Approval by the Review Board does not indicate approval by other committees of the Medical Center, e.g., CRC Scientific Advisory Committee, Radiation Safety Committee, College of Medicine Institutional Review Board, etc.
7. It is the responsibility of the investigator to keep copies of the approved protocol, consent form and all correspondence and all changes pertaining to the study or consent form.

DHHS Assurance #M1170
IRB No. 01

Chairman, Institutional Review Board
Children's Hospital Medical Center
Cincinnati, Ohio

APPENDIX 4

COPYRIGHT ASSIGNMENT

MS # 2004-0997.R1

Conditions for Publication in *Pediatrics*

This form must be completed, signed by ALL authors, and BOTH PAGES returned to the Editorial Office. Until this completed form is received, we will not be able to publish your article.

Authors: Demmers, Théa; Jones, Peter J.H.; Wang, Yanwen; Krug, Susan; Creutzinger, Vivian; Heubi, James E.

Title: Effects of Early Cholesterol Intake on Cholesterol Biosynthesis and Plasma Lipids in Infants until 18 months of age

I agree to the conditions listed below:

Scientific Responsibility Statements: I certify that I have participated sufficiently in the work to take responsibility for a meaningful share of the content of this manuscript. This participation included:

(a) conception and/or design of the research/experiment(s); collection, analysis, and interpretation of data; and/or
(b) drafting the manuscript and/or revising its intellectual content; and
(c) approval of the manuscript as submitted and approval of the final version of the manuscript to be published; and
(d) approval of the abstract of the manuscript and attestation that the abstract accurately reflects the substance of the article.

Exclusive Publication Statement: I certify that none of the material in this manuscript has been published previously, and that none of this material is currently under consideration for publication elsewhere. This includes symposia, books, articles published by invitation, and preliminary publications of any kind except in abstract form.

Conflict of Interest: I affirm that I have no financial affiliation (e.g., employment, direct/indirect payments, stock holdings, retainers, consultantships, patent-licensing arrangements, or honoraria) or involvement within the last 3 years with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, except as disclosed in the attachment, which lists all financial disclosures and conflicts of interest. Any other potential conflict of interest is also disclosed. If no conflict of interest is declared, a statement to that effect will be published with the article. Any disclosed conflict of interest may also be published with the article. Attach additional pages if necessary. All information must be typed.

Copyright Transfer Agreement: Effective upon acceptance for publication, copyright (including the right to obtain copyright registration, whether separately or as part of a journal issue or otherwise) in and to the above article is hereby transferred throughout the world and for the full term and all extensions and renewals thereof, to:

THE AMERICAN ACADEMY OF PEDIATRICS

This transfer includes the right to adapt the article for use in conjunction with computer systems and programs, including reproductions or publication in machine-readable form and incorporation in retrieval systems.

Rights of Authors: Effective upon acceptance for publication, the American Academy of Pediatrics hereby licenses the following rights back to the author(s):

(a) Patent and trademark rights and rights to any process or procedure described in the article.
(b) The right to photocopy or make single electronic copies of the article for their own personal use, including for their own classroom use, or for the personal use of colleagues, provided the copies are not offered for sale and are not distributed in a systematic way outside of their employing institution (e.g. via an e-mail list or public file server). Posting of the article on a secure network (not accessible to the public) within the author's institution is permitted.
(c) The right, subsequent to publication, to use the article or any part thereof free of charge in a printed compilation of works of their own, such as collected writings, theses or lecture notes.

Note: All copies, paper or electronic, or other use of the information must include an indication of the American Academy of Pediatrics copyright and a full citation of the journal source. Please refer requests for all uses not included above, including the authorization of third parties to reproduce or otherwise use all or part of the article (including figures and tables), to the American Academy of Pediatrics, Permissions Department, 141 Northwest Point Blvd., Elk Grove Village, Illinois 60007; phone (847) 434-4000, fax (847) 434-8000.

Authorship: If copyright is held by the employer, the employer or an authorized representative of the employer must sign in addition to the author(s). If the author is a US Government employee, whose work falls within the public domain, and this work was created in that capacity, the copyright transfer applies only to the extent allowed by US law and the author should sign this form. Please confirm by checking the box following the signature line. Any other author who is not a US Government employee must also sign the copyright transfer form.

MS # 2004-0997.R1

Warranties: The author(s) warrant(s) that the article is the author's original work and has not been published before. The author(s) warrant(s) that the article contains no libelous or other unlawful statements, and does not infringe on the rights of others. If excerpts from copyrighted works are included, the author(s) has (have) obtained written permission from the copyright owners and will credit the sources in the article.

Person who is to receive page proofs:

Name: Théa Demmers

Address: 55 Montée Ste. Marie, Ste. Anne de Bellevue, QC H9X 2B7

- **Each author (and employer representative, if applicable) must sign his or her name and provide the date of signature.**
- **Check box if you are an U.S. government employee whose work falls within the public domain.**
- **If any author has declared a conflict of interest, the declaration must be attached to this form.**

I agree to the Conditions of Publication explained on this form.

Print Name	*Signature*	Date	
Théa Demmers	Théa Demmers	Oct 18, 2004	Government Employee Conflict of Interest Declared: Yes __ No ✓
Peter J.H. Jones		Oct 24' 04	Government Employee Conflict of Interest Declared: Yes __ No ✓
Yanwen Wang			Government Employee Conflict of Interest Declared: Yes __ No ✓
Susan Krug			Government Employee Conflict of Interest Declared: Yes __ No ✓
Vivian Creutzinger			Government Employee Conflict of Interest Declared: Yes __ No ✓
James E. Heubi			Government Employee Conflict of Interest Declared: Yes __ No ✓
			Government Employee Conflict of Interest Declared: Yes __ No __

AUTHORIZED EMPLOYER-REPRESENTATIVE SECTION: (if applicable)

Name of Employer: _____

Employer Representative's Name and Title (Print)

Signature Date

MS # 2004-0997.R1

Warranties: The author(s) warrant(s) that the article is the author's original work and has not been published before. The author(s) warrant(s) that the article contains no libelous or other unlawful statements, and does not infringe on the rights of others. If excerpts from copyrighted works are included, the author(s) has (have) obtained written permission from the copyright owners and will credit the sources in the article.

Person who is to receive page proofs:

Name: Ms. Thea Demmers

Address: 55-1 Montée Ste. Marie, Ste. Anne de Bellevue, QC H9X 2B7

- Each author (and employer representative, if applicable) must sign his or her name and provide the date of signature.
- Check box if you are an U.S. government employee whose work falls within the public domain.
- If any author has declared a conflict of interest, the declaration must be attached to this form.

I agree to the Conditions of Publication explained on this form.

Print Name	Signature	Date	Conflict of Interest Declared: Yes / No	
James Heubi, M.D.		10/14/04		☐ Government Employee
Susan Krug				☐ Government Employee
Vivian Creutzinger		10/15/04		☐ Government Employee
				☐ Government Employee
				☐ Government Employee
				☐ Government Employee
				☐ Government Employee

AUTHORIZED EMPLOYER-REPRESENTATIVE SECTION: (if applicable)

Name of Employer: _____

Employer Representative's Name and Title (Print)

Signature Date

AMERICAN ACADEMY OF PEDIATRICS, ELK GROVE VILLAGE, ILLINOIS, 60007

MS # 2004-0997.R1

Warranties: The author(s) warrant(s) that the article is the author's original work and has not been published before. The author(s) warrant(s) that the article contains no libelous or other unlawful statements, and does not infringe on the rights of others. If excerpts from copyrighted works are included, the author(s) has (have) obtained written permission from the copyright owners and will credit the sources in the article.

Person who is to receive page proofs:

Name: Ms. Thea Demmers

Address: 55-1 Montée Ste. Marie, Ste. Anne de Bellevue, QC H9X 2B7

- Each author (and employer representative, if applicable) must sign his or her name and provide the date of signature.
- Check box if you are an U.S. government employee whose work falls within the public domain.
- If any author has declared a conflict of interest, the declaration must be attached to this form.

I agree to the Conditions of Publication explained on this form.

Print Name	Signature	Date	Conflict of Interest Declared: Yes __ No __	
James Heubi, M.D.				☐ Government Employee
Susan Krug	Susan J. Krug	10/18/2004	Yes __ No ✓	☐ Government Employee
Vivian Creutzinger				☐ Government Employee
				☐ Government Employee
				☐ Government Employee
				☐ Government Employee
				☐ Government Employee

AUTHORIZED EMPLOYER-REPRESENTATIVE SECTION: (if applicable)

Name of Employer: _____

Employer Representative's Name and Title (Print)

Signature Date

AMERICAN ACADEMY OF PEDIATRICS, ELK GROVE VILLAGE, ILLINOIS, 60007

MS # 2004-0997

Warranties: The author(s) warrant(s) that the article is the author's original work and has not been published before. The author(s) warrant(s) that the article contains no libelous or other unlawful statements, and does not infringe on the rights of others. If excerpts from copyrighted works are included, the author(s) has (have) obtained written permission from the copyright owners and will credit the sources in the article.

Person who is to receive page proofs:

Name: Peter JH Jones

Address: Dietetics & Human Nutri, McGill Uni., 21,111 Lakeshore Rd, Montreal, QC, Canada H9X 3V9

- **Each author (and employer representative, if applicable) must sign his or her name and provide the date of signature.**
- **Check box if you are an U.S. government employee whose work falls within the public domain.**
- **If any author has declared a conflict of interest, the declaration must be attached to this form.**

I agree to the Conditions of Publication explained on this form.

YANWEN WANG Yanwen Wang Oct 29/04 ☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

☐ Government Employee
Print Name *Signature* Date Conflict of Interest Declared: Yes __ No __

AUTHORIZED EMPLOYER-REPRESENTATIVE SECTION: (if applicable)

Name of Employer: _____

Employer Representative's Name and Title (Print)

Signature Date