DIETARY CHOLINE INTAKE AND BIOMARKERS OF CHOLINE STATUS ACROSS THE LIFE CYCLE

by

Alejandra M Wiedeman Manriquez

B.Sc. Universidad de Valparaiso (Chile), 2006

M.Sc. Universidad de Chile (Chile), 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2017

Abstract

Choline, an essential dietary nutrient for humans, is involved in a broad range of critical physiological functions as a precursor for the synthesis of acetylcholine, phospholipids, and betaine. The current dietary recommendations for choline were set as Adequate Intakes (AIs), estimated based on limited data and expressed as total choline. However, dietary choline is present in different forms, which can be classified as water-soluble forms (free choline, phosphocholine, and glycerophosphocholine), and lipid-soluble forms (phosphatidylcholine and sphingomyelin). Despite its importance, there is still limited information available about choline intake and plasma and milk concentrations. Therefore, the overall goal of my research was to advance our knowledge of choline nutrition.

To address this goal, I conducted four studies on healthy participants of different age groups across the life cycle, in which dietary choline intake was assessed and/or milk and plasma concentrations of choline and metabolites were determined by stable isotope dilution liquid chromatography-tandem mass spectrometry. The first study validated a food frequency questionnaire to assess dietary total choline intake. The second study showed that the concentrations of water-soluble forms of choline in human milk samples did not differ between lactating women from a high- (Canada) and a low-income (Cambodia) countries; in both cases, the estimated means of milk total choline were below the AI for infants aged 0 – 6 mo. The third study showed that one fasted or fed blood sample was adequate to quantify plasma choline concentrations, but recent food intake increased its concentrations in healthy adults. The last study showed that plasma free choline was not associated with choline intake among toddlers, children, and adults.

This research has generated a considerable body of information about choline nutrition across the life cycle. In conclusion, these results suggest an overestimation of the current choline AI for infants and possibly for lactating women, thus emphasizing a need for reevaluating the AIs. The absence of an association between dietary choline intake and plasma free choline suggests the need for a better understanding of choline nutrition and metabolism is warranted.

Lay summary

Choline is an essential nutrient needed in the diet to maintain human health. Choline is used in varied ways in the body and has especially important roles in early development. In 1998, dietary recommendations for choline were set using limited information. Despite the awareness of its importance, there are very few studies to date describing choline intake and levels in plasma or milk. Therefore, the overall goal of my research was to increase our knowledge about human choline nutrition at different stages of the life cycle. My results suggest that the current dietary recommendation for choline might be overestimated for infants and likely for lactating women. Also, dietary choline intake was not associated with the plasma level. Therefore, more studies on choline metabolism are needed to update the current recommendations.

Preface

This dissertation was prepared according to the requirements for a PhD thesis by the University of British Columbia Faculty of Graduate and Postdoctoral Studies. This research was the result of a collaborative effort between the University of British Columbia, Helen Keller International, the Cambodian Ministry of Health, and the Dutch State Mines (DSM) company. All the sample analyses presented in this dissertation were conducted in the Innis Nutrition and Metabolism Laboratory at the Child and Family Research Institute, Vancouver, British Columbia, Canada.

The research presented in Chapter 2 was designed by Dr. Sheila Innis and me. Funding was obtained from the Canadian Institutes of Health Research (CIHR). Staff and graduate students in Dr. Innis' laboratory assisted with the enrollment of study subjects and collection of dietary information. I was primarily responsible for study coordination, all data analysis, and interpretation. Dr. Timothy Green and I wrote the first draft of this chapter. Dr. Susan Barr, Dr. David Kitts, and Dr. Zhaoming Xu contributed to data interpretation and chapter revisions.

A version of Chapter 3 is currently being prepared for manuscript submission. The original trials included in the research presented in Chapter 3 were designed by Dr. Sheila Innis and Dr. Timothy Green. Funding was obtained from CIHR and Grand Challenges Canada Stars. Staff and graduate students in Dr. Innis' and Dr. Green's laboratory administered enrollment of study subjects and collection of milk samples. Roger Dyer was the senior laboratory technician who trained me to perform the laboratory analyses. I was primarily responsible for all laboratory analysis, data analysis, and interpretation. Dr. Susan Barr and I wrote the first draft of this chapter. Dr. Timothy Green, Dr. David Kitts, and Dr. Zhaoming Xu contributed to data interpretation and chapter revisions.

A version of Chapter 4 is currently being prepared for manuscript submission. The research presented in Chapter 4 was designed by Dr. Sheila Innis and me. Funding was obtained from the CIHR. Staff and graduate students in Dr. Innis' laboratory assisted with the enrollment of the study subjects and collection of the blood samples. I was primarily responsible for study coordination, all laboratory analysis, data analysis, and interpretation. Dr. David Kitts and I wrote the first draft of this chapter. Dr. Susan Barr, Dr. Timothy Green, and Dr. Zhaoming Xu contributed to data interpretation and chapter revisions.

A version of Chapter 5 will be prepared for manuscript submission. The original studies included in the research presented in Chapter 5 were designed by Dr. Sheila Innis. Funding was obtained from CIHR and DSM. Staff and graduate students in Dr. Innis' laboratory administered the study procedures, including enrollment of study subjects and collection of dietary information and blood samples. I was primarily responsible for all laboratory analysis, data analysis, and interpretation. Dr. Zhaoming Xu and I wrote the first draft of this chapter. Dr. Susan Barr, Dr. Timothy Green, and Dr. David Kitts contributed to data interpretation and chapter revisions.

Ethics approval was obtained from the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia and the British Columbia Children's and Women's Hospital; certificate number H11-01205 for Chapter 2 and 4; H08-70242, H09-01261, and H14-01654 for Chapter 3; and H09-02028, H09-01633, H09-00188, and H07-01486 for Chapter 5. Ethics approval was obtained from the Cambodian National Ethics Committee for Health Research certificate 0245NECHR for Chapter 3.

Table of contents

List of tables

List of figures

List of abbreviations

24HR	24-hour recalls
AI	Adequate Intake
ANOVA	analysis of variance
BHMT	betaine homocysteine methyl transferase
BMI	body mass index
CDP	cytidine diphosphate
CFRI	Child and Family Research Institute
CI	confidence interval
CIHR	Canadian Institutes of Health Research
CV	coefficient of variation
CVD	cardiovascular disease
d	day(s)
dpp	days postpartum
DSM	Dutch State Mines
DRI	Dietary Reference Intakes
EAR	Estimated Average Requirement
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FC	free choline
FFQ	food frequency questionnaire
GC	gas chromatography
GPC	glycerophosphocholine
HPLC	high performance liquid chromatography
ICC	intra-class correlation
IQR	inter-quartile range
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOA	limits of agreement

min	minute(s)
mo	month(s)
MS	mass spectrometry
n	sample size
NHANES	National Health and Nutrition Examination Survey
NTD	neural tube defect
RDA	Recommended Dietary Allowance
PC	phosphatidylcholine
PCho	phosphocholine
PEMT	phosphatidylethanolamine N-methyltransferase
PM	postmenopausal
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	standard deviation
SM	sphingomyelin
T1	first trimester
T2	second trimester
T3	third trimester
TC	total choline
TMA	trimethylamine
TMAO	trimethylamine-N-oxide
UL	tolerable upper intake level
USDA	United Stated Department of Agriculture
VLDL	very low-density lipoprotein
WHO	World Health Organization
wk	week(s)
y	year(s)

Acknowledgements

Firstly, I would like first to thank my supervisor, late Dr. Sheila Innis (1953 - 2016) for giving me the opportunity to come to Vancouver Canada to pursue a Doctoral degree at the University of British Columbia. I am very grateful for her guidance and effort to help in my academic growth. I will always remember her as the great researcher that she was.

I would also like to thank the members of my supervisory committee, Dr. Susan Barr, Dr. Timothy Green, Dr. David Kitts for their support and guidance throughout my graduate studies, and to Dr. Zhaoming Xu who stepped into the role of supervisor after Dr. Sheila Innis passed away. None of this research work would have been possible without the continuous support, encouragement, and dedication of all the members of my supervisory committee. Words cannot express how grateful I am for the immense contribution to my personal and training time at the University of British Columbia.

I am very grateful for working with all of the students, research assistants, and technicians in the Innis Laboratory at the Child and Family Research Institute. I would like to extend special thanks to Roger Dyer for his limitless patience, guidance, and assistance with my studies during all these years. I would like to thank my fellow trainees Kelly, Brian, Lynda, Sara, Cyrielle, Jie Yang (Jessica), and Kyly. I am especially grateful to Betina, Theresa, and Maria Fernanda for their friendship and encouragement during these challenging years. Also, I would like to extend my gratitude to the wonderful volunteers for their invaluable assistance in this research.

I would like to extend my gratitude to the faculty members and staff in Food, Nutrition, and Health, the Faculty of Land and Food Systems, and the Child & Family Research Institute. Especially to Dr. Yvonne Lamers, Dr. Angela Devlin, Dr. Rajavel Elango, Dr. Phoebe Lu, Dr. Gail Hammond, Lia, Shelley, and Barbara. Also, I would like to thank, for their interest and time, all the participants involved in the different studies.

I would like to acknowledge the funding sources for my studies including the Doctoral Scholarship to study abroad from the Government of Chile, provided through Becas-Chile-CONICYT, and the trainee support from the Child & Family Research Institute and the Nutritional Research Fellowship from the University of British Columbia.

Lastly, special thanks are owed to my wonderful family including my husband Jose Miguel, parents Lidia and Roberto, and brother Jorge, who have never stopped believing in me and have always supported me during my years of education, both morally and financially (*Gracias por ser incondicionales*).

To MAXMANES

“A smooth sea never made a skillful sailor”

Franklin D Roosevelt

Chapter 1: Introduction, literature review, and objectives and hypotheses

1.1 Introduction

Choline is required for the synthesis of the neurotransmitter acetylcholine, phospholipids such as phosphatidylcholine and sphingomyelin, and the methyl group donor betaine, and therefore choline plays important roles across the life cycle (1, 2). Evidence from animal studies has shown that choline plays critical roles in brain development, consumption of choline-deficient diets during perinatal periods leads to impaired neurodevelopment of the offspring (3, 4). In humans, most studies of choline have focused on pregnancy (5-11). To date, there is limited information on dietary choline intake and the relationship between dietary choline intake and plasma free choline concentrations, an indicator of choline status, throughout the life cycle, especially during early childhood and in postmenopausal women.

In 1998, the Dietary Reference Intakes (DRIs) were established for choline by the Food and Nutrition Board of the Institute of Medicine (12). However, the recommendations for choline were set as Adequate Intakes (AIs) due to a lack of sufficient evidence to establish Estimated Average Requirements (EARs) and therefore Recommended Dietary Allowances (RDAs) (12). For infants, the AI for choline was set based on the choline concentration in mature human milk and the daily volume intake consumed by breastfed infants (12). For adults, the AI for choline was set based on one depletion-repletion study conducted only in healthy men, which was then extrapolated to women and other age groups (12).

Although the recommendation for choline intake was set as total choline, dietary choline is present in water-soluble forms, including free choline, phosphocholine, and glycerophosphocholine, and lipid-soluble forms, including phosphatidylcholine and sphingomyelin (13). Interestingly, an average of 84% of the total choline in mature human milk is in water-soluble forms (14-17). In rodent models, it has been shown that different forms of choline present in milk differ in their metabolic fate and that the development of the immune system in the offspring can be influenced by the forms of choline consumed (18, 19). Our current knowledge on human milk choline concentrations is restricted to a limited number of studies with small sample sizes (14-17, 20, 21). It has been reported that supplementing lactating women with choline increases the milk concentration of choline (17). Moreover, most of the data are from high-income countries where maternal consumption of animal-source food, the richest source of dietary choline (13), is probably higher than in low- and middle-income countries (22, 23). As such, it is possible that differences in maternal usual dietary choline intake would impact the milk choline concentration.

The AI for choline for adults was based on only a single choline-restricted diet study (24). In this study, the concentration of plasma free choline in participants who received a choline-deficient diet, for three weeks, was lower compared to those receiving control diet. Further, elevated hepatic alanine aminotransferase was detected in the serum of choline-deficient participants, indicating liver damage, in those on a choline-deficient diet. However, both lower concentration of plasma free choline and hepatic lesion were corrected when choline was added back to the diet, suggesting that the endogenous choline synthesis not be sufficient to support the body's needs for choline (24). This study provided evidence suggesting choline as an essential

nutrient; however, it had a small sample size and was conducted only in males. Recent studies have shown that men and postmenopausal women are more susceptible to develop hepatic and muscular lesions when consuming low choline diets as compared to premenopausal women, suggesting that requirements may be lower in premenopausal women (25, 26). Estrogen has been shown to play an important role enhancing endogenous choline synthesis by gene estrogen response elements (27). Evidence suggests that dietary choline requirement is influenced by many factors, including both gender and physiological stage of the life. Thus, the validity of the current AI for choline for adults established based on a single study involving male participants is limited.

Plasma free choline concentration was used as the biomarker of body choline status to set dietary choline recommendations, as it varies in response to restricted and supplemental choline intakes, and has usually been used to evaluate choline status (28, 29). However, it has been suggested that plasma free choline concentrations may not be sensitive to reflect small changes within the ranges of usual dietary choline intake (30). Moreover, plasma free choline concentration has been reported to vary within a wide range among participants (31) and that it can be influenced by many factors, including sex, age, and ethnicity (32, 33). Taken together, it appears that the relation between plasma free choline concentration and choline intake in subjects following their usual diet is poorly understood.

The overall objective of my dissertation research project was to advance knowledge on choline nutrition with a focus on dietary choline intake and the concentration of plasma free choline in healthy individuals of various life stages.

1.2 Literature review

This literature review begins by providing background information on choline, followed by an overview of choline metabolism and biological functions. Next, the Dietary Reference Intakes for choline are described, and information on dietary intakes and food sources is provided. Finally, the literature review presents a summary of the current knowledge on circulating choline concentrations.

1.2.1 Background on choline

Choline, 2-hydroxyethyl-trimethyl-ammonium salt, is a positively charged molecule containing three methyl groups covalently attached to nitrogen (molecular weight 104 g/mol) (Figure 1.1) (34, 35). Choline was first described as part of animal bile (36), and almost a century later, choline-deficient diets were shown to lead to the development of hepatic steatosis and alterations in methionine metabolism in animal models (37, 38). Choline has many critical, diverse functions across all stages of the life cycle, including roles in neurotransmission, membrane synthesis, lipid transport, and one-carbon metabolism (1, 2, 39). Signs of choline deficiency have been reported in subjects fed experimental choline-deficient diets and also in patients receiving total parenteral nutrition (24, 40). In 1998, the Food and Nutrition Board of the Institute of Medicine established dietary recommendations for choline (12).

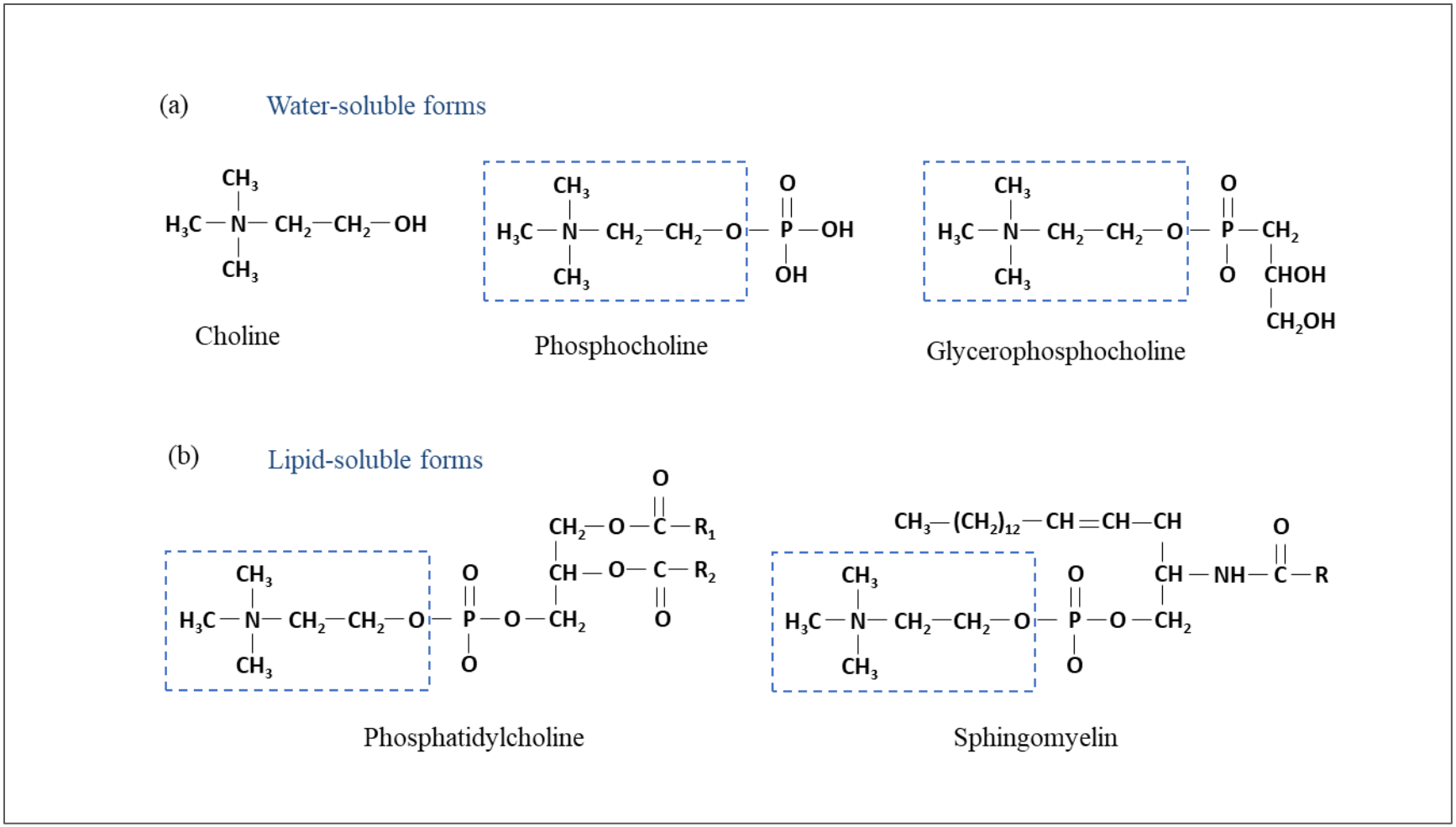

Figure 1.1 Structures of different forms of choline
(a) Water-soluble forms and (b) lipid-soluble forms of choline. The dashed blue box indicates the choline portion of the structure and R represents a fatty acid chain.

Choline can be obtained by endogenous synthesis or through dietary intake of choline-containing foods (12, 13). In foods, choline is found in both water-soluble (free choline, phosphocholine, and glycerophosphocholine) and lipid-soluble forms (phosphatidylcholine and sphingomyelin) (13). It is known that different forms of choline vary in absorption, with water-soluble reaching the liver via portal circulation and the lipid-soluble forms packaged into chylomicrons which are absorbed and transported through lymphatic circulation (41). As such, it has been suggested that the form in which choline is consumed may impact its metabolic fate and it should be considered (42). Therefore, the intake of different forms of choline may be relevant at specific stages of the life cycle.

1.2.2 Overview of choline metabolism and biological functions

1.2.2.1 Overview of choline metabolism

Choline metabolism can be divided into three main pathways which are involved in the synthesis of acetylcholine, betaine, and phospholipids (Figure 1.2). Choline is the precursor for the synthesis of the neurotransmitter acetylcholine, which reaction is catalyzed by choline acyltransferase in the cytosol of pre-synaptic cholinergic neurons (43). Acetylcholine is subsequently packaged into vesicles and released into the synaptic cleft, where it binds to receptors of the post-synaptic neuron in the central and peripheral nervous system (34, 44). Acetylcholine synthesis has also been reported in non-neural tissues including placenta, muscle, intestine, and lymphocytes (45-48).

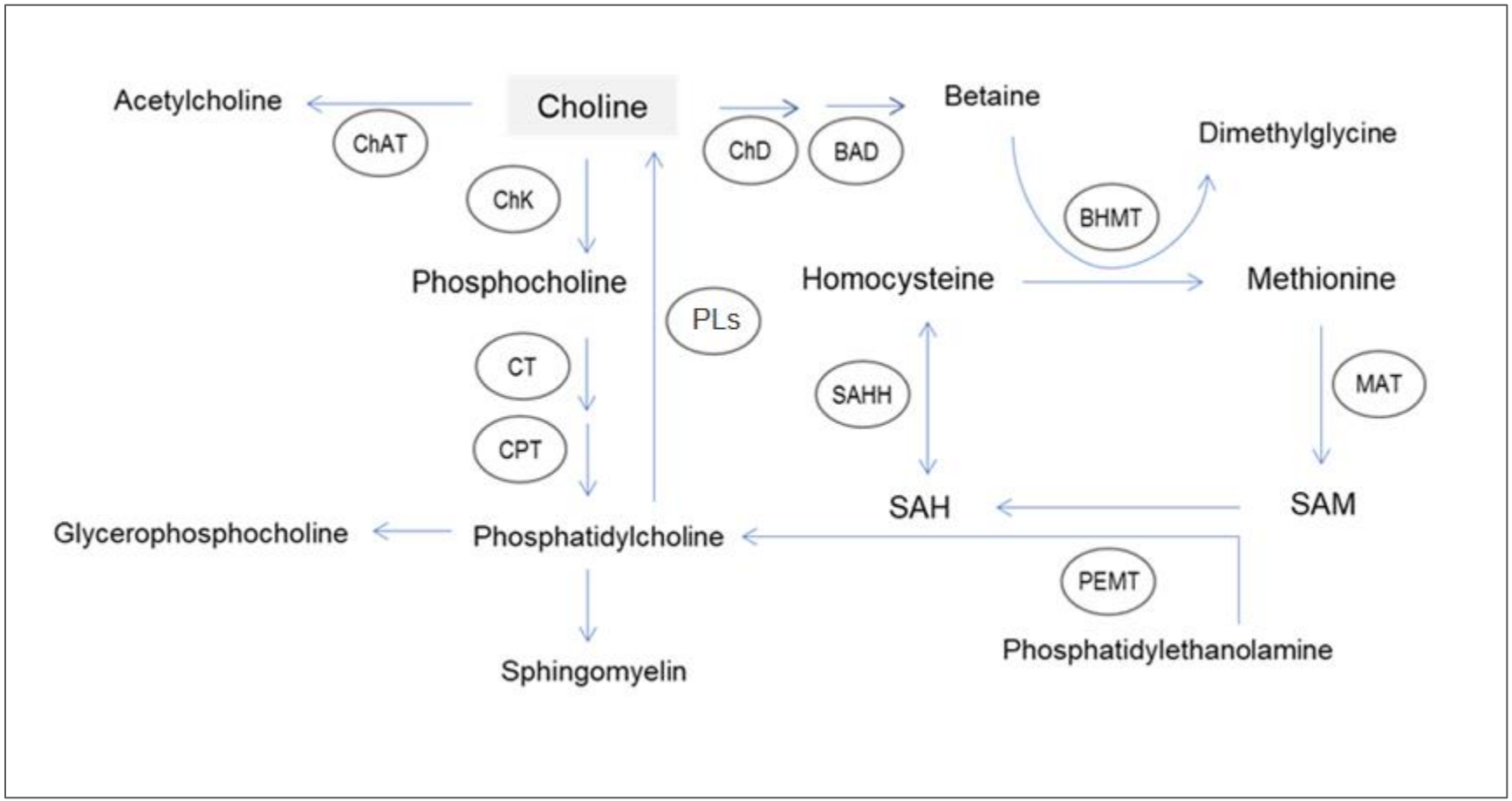

Figure 1.2 Simplified overview of choline metabolism
BAD: betaine aldehyde dehydrogenase, BHMT: betaine homocysteine methyltransferase, ChAT: choline acyltransferase, ChD: choline dehydrogenase, ChK: choline kinase, CPT: choline phosphotransferase, CT: CTP-phosphocholine cytidylyltransferase, MAT: methionine adenosyltransferase, PL: phospholipases, SAM: *S*-adenosylmethionine, SAH: *S*-adenosylhomocysteine, SAHH: SAH hydrolase, PEMT: phosphatidylethanolamine *N*-methyltransferase, PLs: phospholipases.

Choline can be irreversibly oxidized to yield betaine in a 2-step process reaction catalyzed by choline dehydrogenase (ChD) and betaine aldehyde dehydrogenase (BAD) predominantly in the liver and kidney (49, 50). Betaine is an important osmolyte and a methyl group donor (51, 52). As a methyl group donor, betaine participates in the re-methylation of homocysteine to methionine by betaine-homocysteine *S*-methyltransferase (BHMT), also producing dimethylglycine (53, 54). It has been reported that BHMT accounts for nearly half of the hepatic homocysteine re-methylation activity (55). This reaction is an alternative pathway, parallel to the ubiquitous vitamin B12-folate-dependent pathway for homocysteine re-methylation (51). Methionine is the precursor of the universal methyl donor *S*-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT). SAM is involved in several methylation reactions such as epigenetic regulation of DNA as well as the synthesis of phosphatidylcholine (56, 57). Dimethylglycine synthesis occurs primarily in the liver and kidney by BHMT, and further sequential demethylation of dimethylglycine produces sarcosine, which is metabolized to glycine, each time resulting in a carbon unit being transferred to the folate pool (58-61).

Finally, choline is a precursor for the synthesis of phosphatidylcholine, the most abundant phospholipid in the body. Phosphatidylcholine can be synthesized through the cytidine diphosphate (CDP)-choline pathway, which occurs in all nucleated cells (62). In this pathway, choline is first phosphorylated to phosphocholine by choline kinase (ChK), then phosphocholine is metabolized to CDP-choline by CTP: phosphocholine cytidylyltransferase (CT), and finally generating phosphatidylcholine by CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT) (62, 63). It has been estimated that 70% of total phosphatidylcholine in the liver is

synthesized by this pathway (64-67). Alternatively, phosphatidylcholine can be generated by the sequential methylation of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase (PEMT) (68-70). This reaction consumes three molecules of SAM, which in turn generates three molecules of *S*-adenosylhomocysteine (SAH), a precursor of homocysteine by *S*-adenosylhomocysteine hydrolase (SAHH) (71, 72). It has been estimated that up to 50% of homocysteine production may originate from PEMT activity, with the highest activity being detected in the liver, and also in other tissues such as the mammary gland (65, 68, 72-76). In humans, this is the only known *de novo* endogenous pathway for choline synthesis. Furthermore, sphingomyelin can be synthesized by the transfer of phosphocholine from phosphatidylcholine to ceramide; this reaction is catalyzed by sphingomyelin synthase (77). Further, the catabolism of phosphatidylcholine can generate lysophosphatidylcholine by phospholipases (78, 79), which can be further metabolized to generate glycerophosphocholine by lysophospholipase (80). Alternatively, glycerophosphocholine can be directly synthesized from phosphatidylcholine by phospholipase B (81).

In addition, before choline can be absorbed in the intestine, some can be metabolized by the intestinal microbiota to betaine and methylamines such as trimethylamine (TMA) (82, 83). After absorption, TMA can be metabolized to trimethylamine-*N*-oxide (TMAO) by flavin monooxygenases in the liver (84).

In summary, choline can be metabolized to the neurotransmitter acetylcholine and betaine, which is an important osmolyte and methyl group donor. Also, choline is the precursor of the phospholipid phosphatidylcholine and sphingomyelin.

1.2.2.2 Biological functions of choline

Choline has received considerable attention due to its inverse association with adverse health outcomes across the life cycle, including birth defects, neurodevelopment and cognition, hepatic steatosis, cardiovascular disease, and cancer (CVD) (2, 7, 25, 40, 85-98). Oxidation of choline to betaine and subsequent SAM synthesis involving methylation reactions is essential for epigenetic regulation of gene expression (99, 100). In rodents, maternal choline-deficient diets during the perinatal period altered DNA methylation in the offspring (101-103). In humans, maternal low choline intake during pregnancy alters DNA methylation in the placenta and cord blood (104). Notably, there is an inverse relationship between the risk of neural tube defect (NTD) and maternal choline intake (n = 424 cases and 440 controls, OR = 0.49) and plasma concentrations (n = 80 cases and 409 controls, OR = 0.40) have been reported during pregnancy (85, 87); to some extent, this is analogous to that reported for folate. In addition, other birth defects associated with choline inadequacy include cleft lip, hypospadias, and cardiac defects (105-108).

The role of choline in neurodevelopment and cognition involves not only the synthesis of acetylcholine and components of cellular membranes, but also gene expression. In rodents, maternal choline intake during the perinatal period impacts both anatomical and biochemical aspects of cognitive function, along with lifelong effects including memory decline in the offspring as they age (4, 109). The neuroprotective effect of maternal choline observed in rodent studies has also been studied in humans; however, with inconclusive results (6, 7, 88, 110, 111). In the only study published in children (n = 210, 5.6 y), no association between plasma free choline concentrations and child cognition was found, but plasma betaine was positively

associated with language ($\beta = 0.066$, $P = 0.04$) (89). In adults, a positive association between cognition and plasma free choline concentrations ($n = 2195$, $P < 0.01$), and also between dietary choline intake and better cognitive performance ($n = 1391$, $\beta = 0.60$, $P < 0.01$) have been described (112, 113). However, other studies that have examined choline supplementation have reported inconsistent results (114-119). Therefore, more research is still needed to determine the relationship between choline and cognitive function in different age groups.

In humans, liver damage (increase in serum alanine aminotransferase concentration) occurs in healthy men after three weeks of dietary choline restriction (< 13 mg/d, $n = 15$) (24). In the same study, a 30% decrease in plasma free choline concentration was observed in the choline-deficient group after three weeks. Similarly, muscular damage (increase serum creatine phosphokinase concentration) also has been reported to occur after three weeks of dietary choline restriction (< 50 mg/d, $n = 8$) (120). These tissue damages have been attributed to altered structural integrity and increased membrane permeability that arises due to a decreased phosphatidylcholine to phosphatidylethanolamine ratio (121-124). In addition, the production of very low-density lipoproteins involves phosphatidylcholine synthesis in the liver (125, 126). Without an adequate supply of choline for phosphatidylcholine synthesis, triacylglycerides will accumulate and result in fatty liver (127, 128). Similar alterations have been reported in patients receiving long-term total parenteral nutrition devoid of choline (129, 130).

The reported association between choline status and CVD risk is linked to homocysteine and TMAO concentrations. Elevated homocysteine concentrations have been positively associated with risk of CVD (131, 132). In prospective cohort studies, dietary choline intakes have been negatively associated with homocysteine concentrations ($n = 903$, $P < 0.05$) and

plasma betaine concentrations negatively associated with risk of CVD (n = 7074, $P < 0.05$) (133, 134). A recent meta-analysis reported no evidence of a positive association between dietary choline or betaine and CVD incidence (135). Intervention studies, with phosphatidylcholine or betaine supplementation, have reported a reduction in homocysteine concentrations (136-138). However, lowering homocysteine concentrations, with B-vitamins such as folate and B12, does not reduce CVD risk (139, 140). Furthermore, the concern about choline intake and CVD is related to a possible increase in TMAO concentration, which has been positively associated with CVD risk (141-143). However, it has been reported that only a low proportion (14%) of choline intake from eggs is converted to TMAO (144), which is then excreted and does not accumulate in the blood (145). In addition to choline intake, and metabolism by the intestine microbiota, TMAO concentrations are controlled by renal excretion (146). To date, the mechanisms by which TMAO increases CVD risk and the identification of the type of bacteria involved in TMA synthesis are just starting to be understood (83, 147).

In summary, choline and its metabolites participate in different metabolic pathways, and they have been related to diverse health outcomes. In animals, low choline intake during the perinatal period leads to impaired neural development with long-lasting effects throughout adulthood. In humans, higher choline intake during pregnancy has been associated with a lower risk of neural tube defects. In addition, studies conducted in healthy adults have shown that choline deficiency results in hepatic steatosis and muscular damage. However, the specific underlying mechanisms behind these associations between choline and its metabolites and health outcomes are complex and not completely understood.

1.2.3 Dietary reference intakes for choline

In 1998, the Food and Nutrition Board of the Institute of Medicine published the DRIs for choline, as part of a set of reference values for nutrient intakes for healthy populations in the United States and Canada (12). Due to the lack of sufficient evidence at the time, an EAR could not be calculated, and, instead, an AI was used as the dietary recommendation for total choline intake.

1.2.3.1 Choline deficiency

At the time when the DRIs for choline were being established, choline deficiency had been reported in only one experimental depletion-repletion study, which involved only eight and seven healthy men in the control and the deficient group, respectively (24). This study assigned participants to either a choline-deficient (< 13 mg/70 kg/d) or a control diet (500 mg/70 kg/d) for three weeks. After this period, hepatic damage indicated by a significant increase in plasma alanine aminotransferase activity (48%), along with a significant decrease in free choline concentrations compared to the baseline (30%, 10.3 vs. 7.3 µmol/L) were reported in the choline-deficient group. In contrast, no significant changes in the plasma alanine aminotransferase and plasma free choline concentration were reported in the control group (24). Similarly, elevated plasma alanine aminotransferase concentrations, hepatic steatosis, and decreased plasma free choline concentrations have been reported in patients receiving total parenteral nutrition devoid of choline (40, 148). As such, these available data supported the conclusion that the endogenous synthesis of choline was not sufficient to meet the body's requirements.

1.2.3.2 Adequate intakes of choline by stage of the life cycle

The AI for choline for infants from 0 – 6 mo old was set at 125 mg/d (Table 1.1), based on a milk volume intake of 0.78 L/d and milk choline concentration of 160 mg/L (12, 149). The mean milk volume intake was estimated from test weighing before and after each feeding by healthy, full-term birth infants who were exclusively breastfed (150, 151). It is important to mention that at the time that the AI for choline was set, the only one available study, measuring all five individual forms of choline present in mature human milk, reported a lower mean concentration of 134 mg/L (*n* = 16) (14). However, no rationale was provided for the 20% increase in total choline concentration in human milk (134 rounded to 160 mg/L) to establish the AI for early infancy. For infants from 7 – 12 months old, the AI was set at 150 mg/d using body weight ratio calculation to extrapolate the AI from early infancy.

Table 1.1 Dietary reference intakes for choline

		Adequate Intake (mg/d)		**Tolerable upper intake level (mg/d)**
		Males	**Females**	
Infants	0 – 6 mo	125	125	-
	7 – 12 mo	150	150	-
Children	1 – 3 y	200	200	1000
	4 – 8 y	250	250	1000
	9 – 13 y	375	375	2000
	14 – 18 y	550	400	3000
Adults	≥ 19 y	550	425	3500
Pregnancy		-	450	3000
Lactation		-	550	3500

For adults, the AI for choline was set at 550 mg/d for men and 425 mg/d for women. This value was based on the amount (7 mg/kg/d) that prevented hepatic alteration, defined as elevated alanine aminotransferase concentration in serum (24), and reference body weight of 76 kg and 59 kg for men and women, respectively (149). This value was obtained from a previously described small depletion-repletion study conducted only in men, which does not provide information if less choline would be effective as they only used one dose (24). Then, the AIs for children and adolescents were extrapolated using the following formula: AI = AI adult (weight child/weight adult) $^{0.75}$ (1 + growth factor). The growth factors for children are 0.30 between 7 mo – 3 y, 0.15 between 4 – 13 y, 0.15 for males between 14 – 18 y, and 0.00 for females between 14 – 18 y (149). For pregnant women, the AI for choline was set at 450 mg/d for all trimesters, which was calculated as AI for adult women plus fetal and placental choline accumulation, based on animal data (152-154). For lactating women, the AI for choline was set at 550 mg/d, calculated as the AI for adult women plus an increment to cover choline output in mature milk, using the AI set for early infancy.

1.2.3.3 Tolerable upper intake levels

The UL for choline was set as 3.5 g/d for adults based on the prevention of hypotension. One study reported a hypotensive effect after the oral administration of 10 g/d of choline chloride (equivalent to 7.5 g choline) (155). A lowest-observed-adverse-effect level of 7.5 g/d was divided by an uncertainty factor of 2 to obtain a UL of 3.75 g/d for adults, which was rounded down to 3.5 g/day. This value was also used to set UL for pregnant and lactating women, and the ULs for children and adolescents were extrapolated using body weight.

Studies conducted in the last decade, after the DRIs were published, have shown that the occurrence of fatty liver or muscle damage after choline-deficient diets differs between sex and age groups (25, 26). Specifically, men and postmenopausal women are more susceptible to organ dysfunction compared to premenopausal women, when consuming choline-deficient diets (< 50 mg/d, up to 42 d) (25, 26). This observation was related to higher estrogen concentrations in premenopausal women, which may enhance the endogenous synthesis of phosphatidylcholine via the PEMT pathway (16). Additional recent studies have identified several single nucleotide polymorphisms to impact PEMT and other enzymes involved in the one-carbon metabolism, thus influencing the susceptibility to organ dysfunction (28, 156).

In summary, due to the limited data published available on choline nutrition, the DRIs were set as AIs instead for all age groups in 1998. The recommendations were based on only two small studies: for infants, using an overestimated concentration of total choline in human milk; and for other age groups, the dietary total choline intake needed to prevent hepatic alterations from a depletion-repletion study conducted in men. In addition, in 1998, a database of choline content in foods was not available, and thus no data on dietary choline intake estimation were possible in both the United States and Canada at that time. These antecedents highlight the scarce data about choline available for establishing the DRIs, as the AIs for choline were set based on insufficient information.

1.2.4 Dietary intake of choline

1.2.4.1 Choline content in dietary food sources

The first database on the content of total choline, and its individual forms, in foods that are common in North American diets was made available in 2004 by the United States Department of Agriculture (USDA). The database listed 434 food items (157), which was updated and expanded in 2008 (13). These databases include values for the individual forms of choline, including free choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin, as well as values for total choline and betaine. Although betaine is a choline metabolite, it is generated by two successive irreversible reactions (158); it is not a choline-containing molecule, nor can it be used to resynthesize choline (159). Therefore, betaine is not included in the total choline value. However, dietary betaine may have a choline-sparing effect, particularly decreasing the use of choline needed to synthesize betaine (160).

Total choline content is higher in foods of animal origin compared to foods of vegetable origin on a per unit of weight basis (13). Foods that contain the highest content of choline include liver, eggs, beef, fish, pork, and chicken (Table 1.2) (13). In these foods, the majority of choline is present as phosphatidylcholine, a lipid-soluble form, as part of cell membrane. Milk is also a good food source for water-soluble forms of choline, which contribute to 87% of the total choline content (13). Information on choline for specific food groups continues to expand with recent data for choline content in pulses becoming available (161). However, the information on choline content of many foods from outside North America remains scarce, thus limiting the estimation of dietary choline intake worldwide.

Table 1.2 Choline and betaine content in different food sources

Food item	Betaine	Choline					
		FC	PCho	GPC	PC	SM	TC
		Content (mg per 100 g of weight)					
Beef liver, cooked	5.6	62.0	12.0	83.0	250.0	24.0	431.0[1]
Egg, hard boiled	0.6	0.7	0.5	0.5	210.0	14.0	225.7[1]
Beef steak, cooked	13.0	0.7	1.3	5.2	86.0	11.0	104.2[1]
Salmon, cooked	1.8	7.8	1.2	41.0	37.0	3.4	90.4[1]
Pork chops, cooked	2.8	1.1	0.6	12.0	57.0	7.5	78.2
Chicken breast, cooked	6.4	3.2	2.1	1.6	46.0	8.9	61.8
Nuts, almonds	0.5	9.4	1.9	1.2	40.0	0.0	52.5[1]
Broccoli, cooked	0.1	8.5	9.3	1.3	21.0	0.0	40.1
Beans, baked canned	0.1	17.0	0.8	1.3	12.0	0.0	31.1[1]
Milk, 2% fat	0.9	2.8	1.6	10.0	1.2	0.9	16.5[1]
Red potato, cooked	0.2	8.5	1.2	3.8	5.3	0.0	18.8
White rice, cooked	0.3	0.7	0.0	1.0	0.4	0.0	2.1

[1]TC is reported as the sum of all individual choline forms when a discrepancy was found with the value reported for TC in the database.
FC: free choline, GPC: glycerophosphocholine, PC: phosphatidylcholine, PCho: phosphocholine, SM: sphingomyelin, TC: total choline (sum FC + PCho + GPC + PC + SM). Source: USDA choline content database release two (13).

1.2.4.2 Choline concentration in human milk

Human milk is the only source of choline for exclusively breastfed infants during the first six months of life and is considered the optimal source of nutrition for infants by the World Health Organization (WHO) (162) and other agencies such as Health Canada (163). Neonates and infants require large amounts of choline to support a rapid growth rate and optimal development (164). Studies have reported a total choline in mature human milk ranging from 125 to 166 mg/L (1198 to 1600 µmol/L) (Table 1.3) (16, 165). In human milk, phosphocholine is the predominant form of choline, followed by glycerophosphocholine; thus, the water-soluble forms of choline account for approximately 84% of the total choline concentration in mature human milk (14, 15, 17, 166).

In contrast, the lipid-soluble forms of choline (phosphatidylcholine and sphingomyelin) are mainly found as part of the milk fat globule membrane, and make up a relatively small fraction of the total choline in human milk (167, 168). Milk choline is either transported from the maternal circulation or obtained through *de novo* synthesis via the PEMT pathway in the mammary gland (169, 170). The concentration of total choline in human milk almost double during the first week after birth and remains relatively constant after that in mature milk (15, 20, 166). Studies in rodents have suggested that the different forms of choline vary in bioavailability and impact on infant development, due to different absorption and tissue uptake (18, 19). However, in humans, the relationship between the different forms of choline in milk and infant development is not well understood.

Table 1.3 Studies reporting on choline concentrations in mature human milk

Country	n	Choline						Reference
		FC	PCho	GPC	PC	SM	TC[1]	
		Concentrations (µmol/L)[1]						
United States	10	85	-	-	180	206	-	(171)
United States	16	116	570	362	82	124	1254	(14)
United States	48	83	553	388	107	67	1198	(16)
United States	60	158	-	-	-	-	-	(172)
United States	28	84	500	403	63	175	1225	(17)
Turkey	12	286	438	465	155	97	1441[2]	(15)
Turkey[3]	54	93	351	958	-	-	1532	(173)
Japan	62	-	-	-	-	-	950	(21)
Korea	36	283	-	-	-	-	1600	(165)
Sweden	1	188	704	672	-	-	-	(174)

[1]Data are presented as mean concentrations unless otherwise noted.
[2]TC is reported as the sum of all individual choline forms when a discrepancy was found with the value reported for TC in each study.
[3]Median is presented.
FC: free choline, GPC: glycerophosphocholine, PC: phosphatidylcholine, PCho: phosphocholine, SM: sphingomyelin, TC: total choline (sum FC + PCho + GPC + PC + SM).

Some authors have suggested that maternal dietary choline intake may influence the milk choline concentration (22, 23, 171). Choline supplementation (> 550 mg/d) has been reported significantly increase (ranging between 20 to 38%) the concentrations of free choline, phosphocholine, glycerophosphocholine, and total choline in mature milk (16, 17). As mentioned before, the main dietary source of choline for lactating women are those foods of animal origin, including meats, eggs, and dairy (10). Studies of the choline concentration in human milk have mainly come from high-income countries, where consumption of food of animal origin, thus choline, is presumably higher than in low-income countries (175-181).

A small study comparing choline concentrations, including free choline, phosphatidylcholine, and sphingomyelin in milk from lactating women in the United States (*n* = 11) and Ecuador (*n* = 55) reported that women from Ecuador had a lower concentration of free choline than that in the United States, but lipid-soluble forms did not differ (22). This observation was attributed to a possible difference in dietary choline intake; however, the actual intake of choline was not assessed in the study. Furthermore, free choline represents only a small fraction (approximately 10%) of total choline in human milk; thus this difference may not be of biological relevance. More research in this area is required to substantiate the difference in choline concentrations in mature human milk samples of lactating women from different countries with different choline intake levels.

1.2.4.3 Dietary choline intakes in different age groups

The first report describing dietary choline intake (*n* = 32 adults) was published in 2005 in the United States (182). The 2007–2008 National Health and Nutrition Examination Survey (NHANES), which is a population representative sample survey of the United States, indicated that the estimated mean choline intake in adults was 396 mg/day for men and 260 mg/day for women using two 24-hour recalls (24HR) (Table 1.4) (183). This report also included information indicating that choline intakes differ by ethnic background (183). Comparable estimated mean choline intakes have been reported from 2009-2010, 2011-2012, and 2013-2014 NHANES (184, 185). In Canada, mean total choline intake in adults has been estimated at 372 mg/day for men and 292 mg/day for women in Newfoundland using a food frequency questionnaire (186). Of relevance, only a few dietary instruments have been validated for dietary choline intakes (187-190), which is a crucial step to obtain accurate dietary estimations.

A recent report describes the dietary choline intake and food sources from national surveys performed in nine European countries (191). These data showed that the highest dietary intake was collected from people in the Northern countries, whereas Mediterranean countries had the lowest intakes (191). Worldwide, total choline intake in adult ranges from 291 mg/d in Greece to 468 mg/d in Sweden in men, and from 279 mg/d in the United States to 374 mg/d in Sweden in women (191). Given that the AIs were set based on a single study conducted in men where only one choline dose was used, it is interesting to note that a common finding is that mean intakes are below the corresponding dietary choline recommendation.

Table 1.4 Studies reporting on dietary total choline intake in healthy adults

Country	Dietary total choline intake (mg/d)[1]				Reference
	Men		Women		
	n	Intake	n	Intake	
United States	-	-	39246	331 ± 80	(192)
United States	5419	325 ± 140	6043	288 ± 115	(193)
United States	428	369 ± 67	424	316 ± 57	(194)
United States[2]	2563	421 (408)	2704	279 (271)	(185)
Canada	822	372 ± 287	2232	292 ± 213	(186)
Greece	1514	291 ± 79	1528	285 ± 75	(91)
New Zealand	-	-	125	316 ± 65	(195)
China	18763	318 ± 92	37432	289 ± 85	(90)
Finland[2]	585	450 (425)	710	344 (327)	(191)
France[2]	936	370 (362)	1340	291 (283)	(191)
Ireland[2]	634	461 (443)	640	318 (314)	(191)
Italy[2]	1068	357 (341)	1245	293 (282)	(191)
The Netherlands[2]	1023	448 (425)	1034	334 (317)	(191)
Sweden[2]	623	468 (442)	807	374 (356)	(191)
United Kingdom[2]	560	407 (385)	706	294 (282)	(191)

[1]Data are presented as mean ± SD or mean (median).
[2]Data were obtained from a number of nationally representative surveys.

Only a small number of studies have reported on the individual choline forms in addition to total choline intake. In adults, lipid-soluble choline forms contribute between 45 to 60% of total choline intake, with phosphatidylcholine being the primary form (94, 192, 196-199). With respect to the intake of water-soluble choline forms, free choline and glycerophosphocholine contribute to approximately 25% and 15% of total choline, respectively (33, 187). The richest food groups identified contributing to dietary choline intake in the United States are the animal-food sources: meat, poultry, and fish (183). Major sources of dietary choline vary by country. For example, eggs, meat, and dairy are the major sources of total dietary choline in New Zealand (195). In contrast, eggs, seafood, meats, and soy products are the major sources of total dietary choline in Japan and China (90, 199).

In comparison to data obtained from adults in general, there is less information available on dietary choline intake levels and their major food group sources in specific life-stage groups in the life cycle, including infancy, childhood, lactation, and in the elderly (Table 1.5). In North America, the estimated mean choline intake during late pregnancy 353 – 363 mg/d, is similar to the choline intake estimated during lactation (346 – 369 mg/d) (6, 10). During pregnancy and lactation, phosphatidylcholine and glycerophosphocholine have been described as the main contributors to total choline intake (7, 11, 200). As for adults, the estimated mean total choline intakes in pregnant and lactating women are commonly reported to be below the current recommended AI (5-7, 10, 11, 176, 185, 191, 200). A similar situation exists for older adults (184, 185, 191). On the contrary, the mean intakes reported for young children in Germany and toddlers and young male children in the United States are above the corresponding AI for choline (184, 185, 191).

Table 1.5 Studies reporting on dietary choline intake in specific age groups in healthy subjects

Life stage	Country	Total choline intake (mg/d)[1]			Reference
		Specific group	n	Intake	
Toddlers (1-3 y)	Finland[2]	-	500	176 (172)	(191)
	United States[2]	-	1316	224 (217)	(185)
Children (4-10 y)	Germany[2]	Male	426	304 (291)	(191)
		Female	409	272 (260)	
	United States[2]	Male	1452	265 (258)	(185)
		Female	1322	218 (211)	
Pregnancy	United States[2]	-	593	319 (309)	(185)
		T3	50	363 ± 90	(6)
	Canada	T1	123	340 ± 148	
		T2	562	349 ± 154	(10)
		T3	493	353 ± 144	
	Latvia[2]	-	990	356 (330)	(191)
	Jamaica	T1	16	279 ± 116	(5)
	Bangladesh	T3	103	190 ± 98	(176)
Lactation	Canada	90 dpp	488	346 ± 151	(10)
	United States	45 dpp	50	369 ± 124	(6)
Older adults (>71 y)	Italy[2]	Male	69	335 (320)	(191)
		Female	159	269 (269)	
	United States[2]	Male	1099	363 (351)	(185)
		Female	1145	266 (259)	

[1]Data are presented as mean (median) or mean ± SD.
[2]Data were obtained from a number of national surveys.
dpp: days postpartum, T1: first trimester, T2: second trimester, T3: third trimester.

Interestingly, no differences have been reported in total choline intake during lactation compared to pregnancy (6, 10). This observation is relevant since the AI for choline was set higher for lactating women (550 mg/d) compared to pregnancy (450 mg/d). Additionally, it has been highlighted by several authors, in both animal and human studies, that a higher choline intake during the perinatal period may be a protective effect in the offspring (17, 29, 39, 85, 104, 129, 201-205). In humans, higher dietary choline intakes (> 480 mg/d compared to < 290 mg/d) are inversely associated with risk of neural tube defects (85). Choline supplementation studies during pregnancy and lactation suggest that maternal choline above the current AI intakes (980 vs. 430 mg/d) decreases preeclampsia risk marker (205) and increases milk choline concentrations (17). Currently, most of the commercially available prenatal supplements do not contain choline, and the estimated choline intake from supplements has been estimated to be low, ranging between 14 to 25 mg/d (185). Recently, the American Medical Association adopted the inclusion of choline in all prenatal supplements (206). However, more research is still needed to determine the specific choline requirement for both pregnant and lactating women.

In summary, our current knowledge of the concentration of choline in human milk is limited to relatively few studies with small sample sizes. Most studies are from high-income countries, where lactating women presumably consume larger amounts of animal products, thus choline, than in low-and-middle-income countries. In addition, information on the usual dietary choline intake across different age groups during the life cycle remains scarce, particularly for early childhood and postmenopausal women.

1.2.5 Circulating choline concentrations

1.2.5.1 Assessment of choline status

Plasma free choline concentration was the selected biomarker for choline status used to set dietary choline recommendations (12). Plasma free choline concentration varies in response to choline intake, decreasing between 30 to 40% when subjects are consuming choline-deficient diets (< 50 mg/d) (24, 25, 28). Also, plasma free choline increases between 63 to 400% after choline supplementation (i.e., 860 to 5000 mg) compared with baseline concentration (156, 207-210). However, plasma free choline concentration has been reported to not reflect small variations in dietary choline intake (i.e., change from 344 to 486 mg/d) in premenopausal women (30), and weak correlations have been reported in pregnant (211) and lactating women (16). Further, the relationship between usual dietary choline intake and plasma free choline in other age groups, such as children and postmenopausal women, has not been addressed.

Potential factors that can influence the measurement of free choline concentrations are the subject's fasting state at blood collection, analysis of choline from serum or plasma, and the different analytical methods used to quantify choline concentration (212). Relevant is the fact that the fasting state of the participants is not always reported, so it is not clear whether the participants were requested to fast overnight when sampled. Commonly, blood samples for specific subject groups that include infants, children, pregnant and lactating women are collected at random during the day, mostly due to ethics regulations of requesting sampling after an overnight fast. As such, this raises the question whether measuring plasma free choline concentrations are significantly increased after recent food intake.

1.2.5.2 Plasma free choline concentrations at different stages of the life cycle

Plasma free choline concentrations have previously been reported for different age groups across the life cycle (Table 1.6). Most of the data on plasma free choline concentration have been reported during pregnancy and lactation from different countries (5, 8, 15, 16, 213-216). During pregnancy, free choline concentrations increase throughout the gestational period (ranging from 38% to 64%), with similar relative increases being reported from independent studies conducted in different countries (8, 214-216). At birth, plasma free choline concentrations in newborns, measured in the umbilical cord, are several folds higher in comparison with their mothers (213, 216-218). During lactation, only a couple of studies have reported plasma free choline concentrations within the first three months postpartum, which are close to the early pregnancy concentrations (16, 214).

Less information is available about plasma free choline concentrations during other stages such as early childhood. It has been observed that circulating free choline concentrations decrease progressively until the second year of life, when they reach similar concentrations found in adulthood (15). A similar tendency in the difference in plasma free choline concentration has been described in animals (164, 219). Only two studies have reported on plasma free choline concentrations in toddlers and children (89, 217). Among adults, fasting plasma free choline concentrations have been reported to range from 6 to 13 µmol/L (31). Not all studies report sex-specific free choline concentrations (15, 31, 113, 220). This lack of detail makes it difficult to make comparisons between studies since free choline concentrations are described to be higher in men than women (32, 134).

Table 1.6 Studies reporting on plasma free choline concentrations in healthy subjects

Life stage	Country	Plasma free choline (µmol/L)			Reference
		Specific group	n	Concentration[1]	
Pregnancy	Curacao	T1	50	6.6 (4.5, 9.7)[2]	(214)
	Curacao	T3	50	10.8 (7.5, 15.6)[2]	(214)
	Canada	T2	264	7.2 ± 1.8[4]	(8)
	Canada	T3	220	9.9 ± 2.3[4]	(8)
	Jamaica	T1	16	8.4 ± 1.6[4]	(5)
Birth	Canada	Mothers	296	10.7 (10.4, 11.1)[2]	(216)
	Canada	Umbilical cord	266	34.5 (33.4, 35.7)[2]	(216)
	Ireland	Mothers	200	12.3 (11.9, 12.8)[2]	(213)
	Ireland	Umbilical cord	200	36.6 (34.9, 38.4)[2]	(213)
Lactation	Curacao	105 dpp	40	7.9 (5.4, 11.5)[2]	(214)
	United States	45 dpp	48	7.7 ± 2.1	(16)
Toddlers	United States		12	12.8 ± 2.0	(217)
Children	The Republic of Seychelles		210	9.2 ± 2.1	(89)
Adults	Norway	Men	1271	9.9 (9.8, 10.0)[2]	(32)
	Norway	Women	1686	9.1 (9.0, 9.2)[2]	(32)
	Canada	Women	88	7.7 ± 2.0	(8)
PM women	United States	-	835	9.4 ± 2.2[4]	(221)
	Ireland	-	23	6.9 (6.1 – 7.6)[3]	(222)
	Norway	-	1580	9.8 (9.7, 10.0)[2]	(32)

[1]Data are presented as mean ± SD, unless otherwise stated.
[2]Mean (95% CIs) is presented.
[3]Median (IQR) is presented.
[4]Blood samples were collected after an overnight fasting.
dpp: days postpartum, PM: postmenopausal, T1: first trimester, T3: third trimester.

It has been described that free choline concentrations in plasma are more stable compared to serum when stored at room temperature up to 72 hours, with free choline quantified in serum reporting a seven times fold increase (223). Different laboratory methodologies have been used to determine plasma free choline concentrations including chemical radio-enzymatic assays and analytical methods such as gas chromatography, liquid chromatography, and liquid chromatography-tandem mass spectrometry (154, 223-225). The higher accuracy and precision of liquid chromatography-tandem mass spectrometry analysis of choline and related metabolites makes it the current method of preference. Moreover, individual variability may also affect plasma free choline concentrations (226). For choline status, the information on the variability of plasma free choline is limited to fasting state (227, 228). This is relevant as in specific age groups, such as in infancy, childhood, and pregnancy, a collection of repeated fasting blood samples after an overnight fast might be not possible, or ethical.

In summary, it has been suggested that plasma free choline is impacted by both deficient and supplemental choline intake in adults. However, it has also been reported that plasma free choline concentrations do not reflect small changes in dietary choline intake in premenopausal women. As such, it is crucial to assess the individual variability of the biomarker, and also to expand the data available on the assessment of the relationship between plasma free choline concentration and the usual dietary choline intake in different age groups.

1.3 Summary of the rationale

Choline is an essential dietary nutrient required for diverse metabolic functions in the body (12). In humans, evidence suggests that low choline intakes or plasma concentrations are associated with adverse health outcomes including an increased risk for neural tube defects (85-87) and poor cognition (7, 88, 89). Also, hepatic and muscular alterations (25, 40, 90), cardiovascular disease (91), and cancer (92-98) have also been reported to be associated with choline during adulthood.

Valid dietary instruments for assessing choline intake and reliable biomarkers are critical to obtaining an estimation of dietary intakes and choline status, respectively. The richest sources of total choline are foods of animal origin (12, 24), which may not be consumed on a daily basis and only a few dietary instruments have been validated for dietary choline intakes (187-190). Also, choline is present in foods in water-soluble (free choline, phosphocholine, and glycerophosphocholine) and lipid-soluble (phosphatidylcholine and sphingomyelin) forms (159), which may differ in absorption and metabolic fate (18, 19). Thus, the dietary intake of individual forms of choline may be relevant for specific age groups (42).

Exclusive breastfeeding is recommended from 0 – 6 months of age (162), and the majority of the choline (mean 84%) present in human milk is found in the water-soluble forms (15-17, 20). Most of the information available on choline concentration in human milk are from high-income countries, where maternal animal-source food consumption, the richest sources for choline intake, is higher than in low- or middle-income countries (175, 179-181). As such, lactating women from countries with less availability of animal-source foods, as a proxy for choline intake, might produce milk with lower choline concentrations.

It is common that, in clinical studies, biomarker quantification is based on only one blood sample collection per subject to assess nutrient status, raising the concern of the significance of the inherent biological intra-individual variability (226). This consideration is important as in specific age groups, such as in infancy, childhood, and pregnancy, a collection of repeated blood samples after an overnight fast might be not possible, or ethical. For choline status, the information on the variability of plasma free choline is limited to fasting state (227, 228).

Plasma free choline concentration has been shown to be directly correlated to supplemental or restricted choline intake (16, 24, 222). However, no or weak correlations between dietary total choline intakes and plasma free choline concentrations have been reported in healthy premenopausal women (30), and also in pregnant (211) and lactating (16) women. As such, studies on choline nutrition are needed to better understand the relationship between dietary choline intake and plasma choline concentrations in other age groups.

The DRIs for choline were established in 1998, and the limited data available did not allow the calculation of EAR, and therefore AIs were set instead for choline (12). For infants, the AI was based on a 20% increase of total choline concentrations in mature milk from a single study with small sample size (14, 24). For men, the AI was set based on a single depletion-repletion study conducted to determine the amount necessary to prevent hepatic alterations (24). For the other sex and age groups, the AI for choline was then extrapolated from the AI for men by weight, growth factor, or output in milk according to the age group (12). At that time, no information on dietary choline intakes was available, and it was unknown how a population's intake related to the AIs established for choline, and even up to date information on dietary choline intake is still scarce.

1.4 Research objectives and hypotheses

The objectives of my dissertation were:

1. To validate a semi-quantitative food frequency questionnaire (FFQ) as an instrument for the assessment of choline and betaine intake, by comparing it with the intake estimated from multiple 24HR among healthy adults of both sexes from Vancouver, Canada.
    a. *Null hypothesis:* there will be no statistically significant associations between dietary choline and betaine intake estimated from the FFQ and intake estimated using the mean of multiple 24HR.
    b. *Research hypothesis:* there will be statistically significant positive associations ($r \geq 0.50$) between dietary choline and betaine intake estimated from the FFQ and intake estimation using the mean of multiple 24HR.

2. To determine whether the concentrations of choline in mature milk differ between lactating women from Canada and Cambodia, where animal-source food availability is thought to differ drastically.
    a. *Null hypothesis*: the concentrations of water-soluble forms of choline in milk will not differ between lactating women from Canada (a country with high availability of animal-source foods, and thus choline) and Cambodia (a country with low availability of animal-source foods, and thus choline).
    b. *Research hypothesis*: the concentration of water-soluble forms of choline in milk will be different ($P < 0.05$) in lactating women from Canada (a country with high

availability of animal-source foods, and thus choline) than from Cambodia a country with low availability of animal-source foods, and thus choline).

3. To determine the individual variability of plasma free choline concentrations and its associated metabolites in fasting and fed states in healthy adults from Vancouver, Canada.
    a. *Null hypothesis*: plasma choline and its associated metabolite concentrations will be not correlated when collected in the fasted state, and also will be not correlated when comparing fasted to the fed state.
    b. *Research hypothesis*: plasma choline and its metabolite concentrations will be at least moderately correlated (intra-class correlation (ICC) ≥ 0.50) between repeated fasted samples, and also when comparing fasted to fed state within the same participant.

4. To determine the association between dietary total and individual forms of choline and betaine intakes and the concentrations of plasma free choline and its metabolites in toddlers, children, men, and postmenopausal women from Vancouver, Canada.
    a. *Null hypothesis*: there will be no association between dietary total and individual forms of choline and betaine intakes and the concentrations of plasma free choline and its metabolites regardless of age.
    b. *Research hypothesis*: dietary total and individual forms of choline and betaine intakes will be positively associated ($r \geq 0.50$) with the concentrations of plasma free choline and its metabolites regardless of age.

Chapter 2: Validation of a food frequency questionnaire to assess usual dietary choline and betaine intakes among adults

2.1 Chapter synopsis

Choline is an essential nutrient that plays a number of important roles, including as the precursor for the synthesis of acetylcholine, phosphatidylcholine, and betaine. In humans, studies on choline or betaine intakes and the association with adverse health outcomes have been reported with a mixed result. Food frequency questionnaires (FFQs) are often selected as the dietary assessment instrument to estimate usual dietary intake in large epidemiological surveys. An adequate dietary intake assessment of usual choline and betaine intakes, reflecting long-term daily intake, is a prerequisite for the association with health and disease outcomes. Therefore, the purpose of this study was to validate a semi-quantitative FFQ to estimate daily choline and betaine intakes in apparently healthy adults (n = 40, both sexes, mean age 33 y) living in Vancouver, Canada. The estimation of usual intake of choline and betaine from the FFQ was compared to the mean of three non-consecutive days' 24-hour recalls (24HRs) as the reference instrument. The relative validity was assessed by evaluating the instruments' mean, estimated differences, correlation coefficients, limits of agreement, and cross-classification analysis. The results of this study indicated a significantly positive moderate to strong de-attenuated correlation coefficients (r = 0.47 – 0.70) and acceptable agreement between the estimation of dietary choline and betaine intakes obtained from FFQ compared to the mean of three 24HRs. These findings suggest that this FFQ is a valid instrument to assess dietary choline intakes in adults, but less accurate to estimate betaine intake.

2.2 Introduction

Choline is an essential dietary nutrient that plays a number of important roles in metabolism (12). Choline is required for the synthesis of the neurotransmitter acetylcholine involved in muscle contraction and cognition (34, 229), as well as phospholipids, phosphatidylcholine, and sphingomyelin, thus is essential for membrane synthesis and maintenance (24, 34). In addition, choline can be irreversibly oxidized to betaine, which is an important methyl group donor involved in the remethylation of homocysteine to methionine (52). Although it can be obtained by the endogenous synthesis of phosphatidylcholine (230), choline synthesized from endogenous source is not enough to meet the body requirements (24), and thus choline needs to be consumed in the diet (12). In addition to the endogenous synthesis from choline, betaine can also be directly obtained from the diet (13). Given the interrelation between choline and betaine, dietary choline and betaine intakes should be assessed together.

Recent interest in the assessment of dietary choline and betaine intakes in humans is related to the evidence from its well-established roles in health outcomes during development and adulthood in rodents (101, 109, 203, 231, 232). In humans, an association between low choline or betaine intakes and an increased risk of adverse health outcomes has been described in a number of studies (7, 85, 86, 88, 91-93, 112), particularly including neural tube defects (85, 86), poor cognition (7, 88, 112) cardiovascular disease (91), and cancer (92, 93). However, other studies have found no or only a weak association between choline or betaine intakes and these adverse health outcomes (111, 133, 233). The inconsistency of the results in the influence of dietary choline and betaine intakes in health outcomes raises the question of the validity of the instrument used to assess dietary choline and betaine intakes.

FFQs are widely used to estimate dietary intake in epidemiological studies (33, 234) because of their low participant burden and cost (235). The purpose of an FFQ is to obtain information on the usual frequency of food consumption and to rank individuals according to their intake level (236). In some cases, FFQs also include information on portion sizes, allowing the estimation of absolute daily intakes (237). However, absolute daily intakes from an FFQ are not usually as accurate as those derived from other instruments such as a 24HR or weighed food record, which allows the collection of more detailed information on the portion size of foods consumed (237-240). Therefore, the instrument selected to collect dietary intake data can influence the estimation of the nutrient of interest.

Although choline is commonly classified together with other water-soluble vitamins, choline is also present in lipid-soluble forms in the diet. Specifically, water-soluble forms of choline include free choline, phosphocholine, and glycerophosphocholine, and lipid-soluble forms of choline include phosphatidylcholine and sphingomyelin (159). The current dietary recommendations for choline are expressed for total choline, as the sum of all forms of choline (12), and no dietary intake recommendation has been set for betaine. The specific forms of choline consumed have been reported to differ between age groups, with infants consuming most of their choline in water-soluble forms naturally present in human milk (14). In contrast, adults consume the majority of choline as lipid-soluble forms, mainly as phosphatidylcholine, present in eggs and meats (10, 33, 161, 187). Notably, these different forms of choline differ in absorption and metabolic fate, which is related to the chemical structure of these compounds (18, 19). Thus, information on the dietary intake of individual forms of choline may be relevant for specific age groups (42).

Before using any dietary instrument, it should be validated in the population and for the nutrient of interest. However, only a small number of FFQs (n = 4) have been validated for the assessment of dietary choline and betaine intakes over the past three to 12-month period (187-190), and no studies have focused on the individual forms of choline. Given there is often no 'gold standard' methodology for dietary nutrient intakes, relative validity is often assessed where a selected instrument is compared to another reference instrument (235). The mean of multiple 24HRs has frequently been used as a reference instrument to assess the validity of FFQs (236). The administration of only one 24HR does not adequately represent the usual intake and the number of days required to estimate usual nutrient intakes at the individual level varies considerably, with a range of three to 41 days depending on the nutrient (241). The numbers of days necessary to assess dietary intake of choline or betaine are not known. However, for practical purposes, the number of days of 24HRs used to assess the validity of FFQs is frequently lower, and the mean of at least two 24HRs has been suggested for energy intake estimation (242).

The relative validity of each new FFQ requires testing (243), which is often assessed by the use of different statistical analyses ideally in the population that it is going to be used (244). Commonly, statistical analyses include correlation coefficients (245, 246), Bland-Altman plots (247, 248), cross-classification, and weighted Cohen's kappa coefficients (249, 250). The overall objective of the study presented in this chapter was to validate a semi-quantitative FFQ as an instrument for the assessment of dietary choline and betaine intakes, by comparing it with the mean intake estimated from three 24HRs among healthy adults (>19 y, 23-61 y) of both sexes from Vancouver, Canada.

2.3 Participants and methods

2.3.1 Study design and participants

This study used a repeated measures design in a convenience sample of 40 adults recruited from Vancouver, Canada. A power analysis calculation indicated a minimum of 38 participants was required to provide 90% power to detect an energy-adjusted de-attenuated correlation coefficient of $r \geq 0.5$ between the two dietary assessment instruments (236, 251), with significance at 5% (two-tailed). Inclusion criteria were apparently healthy adults (both sexes) between the ages of 19 – 65 y who were able to understand and speak English. Exclusion criteria included: history of chronic disease, taking prescribed medication, taking dietary supplements containing choline or betaine, and following a special diet (i.e., vegan). In addition, women were excluded if they were pregnant or breastfeeding. Potential participants were screened to check their eligibility for the study. Eligible participants were invited to attend three study visits at the Child and Family Research Institute at the Oak Street campus of the University of British Columbia in Vancouver, Canada.

On the first visit, sociodemographic data including age, sex, and ethnicity were self-reported and collected by questionnaire (Figure 2.1). Also, anthropometric measurements were taken using standard procedures, with subjects in light clothing and no shoes. Body weights were measured using a digital scale to the nearest 0.1 kg. Heights were measured using a wall-mounted stadiometer to the nearest 1 mm. Waist circumferences were measured in the standing

position, with measurements obtained midway between the lateral lower rib margin and the iliac crest. Measurements were repeated at least three times, and the average values were used.

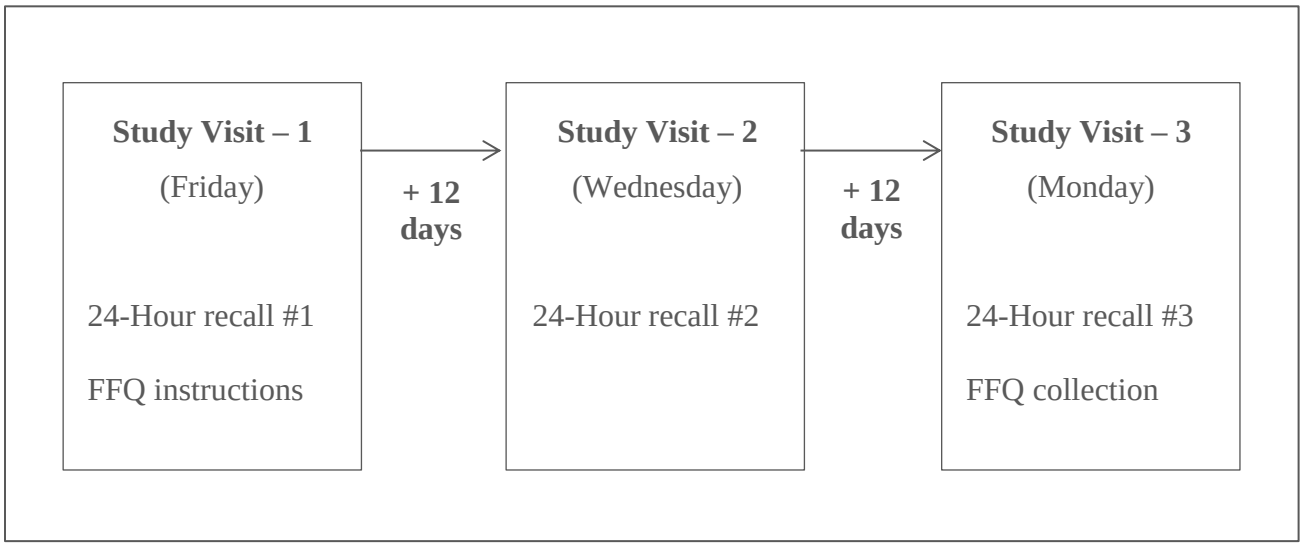

Figure 2.1 Diagram of the study design

All procedures included in the protocol of this study were reviewed and approved by the University of British Columbia – Children's & Women's Health Centre of British Columbia Research Ethics Board according to the guidelines of the Declaration of Helsinki (252, 253). All subjects provided written informed consent prior to participation.

2.3.2 Multiple 24-hour recalls

On each of the three study visits, a 24HR (reference instrument) was conducted by a trained interviewer using the multiple pass method (254). The participants were asked to report all consumed foods and drinks on the previous day. Food models and common kitchen measurement tools were utilized to aid in portion size estimates. The 24HRs were conducted on

three non-consecutive days, including the collection of information for two weekdays and one weekend day, as recommended for optimal energy intake estimation (242). The mean of three 24HRs was used as the reference instrument because it is reliable in measuring energy intake and it can capture within-subject variability in dietary patterns (235, 243). It also represented a feasible compromise between the number of days required to assess usual intake accurately (241) and participant burden.

2.3.3 Food frequency questionnaire

The FFQ, tested instrument, was a semi-quantitative questionnaire designed to assess usual intake during the previous month (Appendix A). The FFQ was adapted from our previously developed FFQ designed to estimate usual fatty acid intake (255, 256), by revising food items listed and clarifying food descriptions and the options for amount and frequency of consumption. The final FFQ included 170 food items divided into 13 groups: milk products; cereal, grain products, and baked goods; rice and pasta; combination foods and meals; meats, poultry, and alternatives; fish and seafood; potato and salads; cooking fat; beverages; alcohol; fruit; vegetables; and snacks. A reference portion size and unit were specified for each food item, with the space to modify as necessary to reflect actual food intake. The FFQ included eight questions asking about the consumption of any other foods not included in the FFQ at the end of some of the food item sections.

During the first study visit, participants received a detailed explanation of how to complete the self-administrated FFQ. For each food item, participants were instructed to select their answer from a list of portion size categories (i.e., volume consumed in oz, cup, or ml for milk; and the number of slices of bread) and frequencies (per day, week, or month). The FFQ

was completed on the participant's own time after the first study visit, and it was returned at the third study visit. Each returned FFQ was reviewed and checked for errors or omissions by trained study personnel, and errors were clarified with the participant. Nutrient intakes estimated by the FFQ were compared to nutrient intakes estimated by the mean of three 24HRs.

2.3.4 Daily dietary intake assessment

First, the frequency data from the FFQ were converted to servings per day. Then, both dietary data from FFQ and the three 24HRs were entered, and daily intake was estimated using a nutrient analysis software (ESHA Food Processor SQL, version 10.14.41; Salem, OR). Although the software for analyzing Canadian diets includes the nutritional composition for 163 nutritional components (257), it does not provide information on the individual forms of choline (free choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin), as only total choline is reported in the Canadian Nutrient File (258). However, the United States Department of Agriculture (USDA) database for the Choline Content of Common Foods (release 2) has the content of individual forms of choline in foods (13). Therefore, the nutrient analysis software database was manually updated for the individual forms of choline in the food items consumed by the participants using the USDA database. The updated nutrient analysis software was then used to assess dietary intake of total and individual forms of choline by the participants. Water-soluble forms of choline correspond to the sum of free choline, phosphocholine; lipid-soluble forms of choline correspond to the sum of phosphatidylcholine and sphingomyelin; and total choline corresponds to the sum of all the individual forms. Dietary intake of energy was expressed as kcal per day, and choline and betaine intakes were expressed as mg per day.

2.3.5 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 19; SPSS Inc. Chicago, US). The significance level was set at $P < 0.05$ for two-sided testing. Normality of the data was assessed using the Kolmogorov–Smirnov test. If distributions were skewed, data were then normalized using logarithmic transformations to improve normality before further analysis. If normality was not achieved after logarithmic transformation, non-parametric tests were used instead. All data are presented as mean ± SD, unless otherwise noted. Participant's weights, measured at study visit-1 and visit-3, were compared using paired samples Student's t-test. Daily dietary intake data are presented as both absolute and energy-density intakes (per 1000 kcal) from the FFQ and the mean of the three 24HRs.

Paired samples Student's t-test was used to compare the dietary intake assessed by the FFQ against the mean of the three 24HRs, using both absolute and energy-density intakes. Pair-wise correlation coefficients were calculated using Pearson's or Spearman's test on unadjusted and energy-adjusted nutrient intakes from the FFQ and the mean of the three 24HRs, and used to measure the direction and strength of the relationship between the instruments. Energy-adjusted nutrient intakes were computed as the residual for a participant from the regression model with the nutrient intake as the dependent variable and total caloric intake as the independent variable, and the mean nutrient intake of the study population was used to calculate the energy-adjusted intake for each nutrient of interest (250). Adjustment of nutrient intakes to the mean energy intake of the group mitigates the effects of measurement error in data collection using self-reported dietary assessments (259). This allows the comparison between the FFQ and the mean of three 24HRs without the influence of individual differences in estimates from the two

instruments of the total amounts eaten. In addition, to correct for the within-subject variation for nutrient intake in the three 24HRs, the observed correlation coefficient was multiplied by a de-attenuation factor, which was calculated as: $= \sqrt{(1 + \lambda/n)}$, where λ is the ratio of the within- to the between-subject variation, and *n* is the number of 24HRs used (245, 260). Interpretation for all correlation coefficients was defined as poor < 0.20, acceptable between 0.20 – 0.49, and good ≥ 0.50 (261).

Bland-Altman plots were constructed as the mean of the two instruments' measures against the difference between the two instruments' measures, and the limits of agreement (LOAs) were calculated as ± 1.96 SD of the mean difference. The LOAs allow the identification of the presence and direction of bias, as well as whether or not bias is consistent depending on the magnitude of the value (262, 263). Normal distribution of the differences was assessed by using the Kolmogorov–Smirnov test. Acceptable agreement between the two instruments was defined as the absence of constant bias. The agreement between the FFQ and the mean of three 24HRs was also determined by tertiles cross-classification. For each instrument, participants were divided into tertiles and the percentage of participants classified into the same, adjacent, and opposite tertiles of nutrient intake were calculated (251, 264). Interpretation for the classification of participants in the opposite tertile was defined as poor > 10% and good ≤ 10% (261). In addition, the agreement between both instruments was assessed using weighted Cohen's kappa coefficient (κ_w) (249, 250). Interpretation for κ_w was defined as poor < 0.20, acceptable between 0.20 – 0.60, and good ≥ 0.61 (261, 265).

2.4 Results

2.4.1 Sociodemographic characteristics of the participants

A total of 40 participants completed the study, and their characteristics are provided in Table 2.1. The age of the participants was 33 ± 12 y, with a BMI of 24.9 ± 4.9 kg/m^2. Participants' weight compared between study visit-1 and visit-3 (mean ± SD of 73.3 ± 18.4 and 73.3 ± 8.6 kg, respectively) did not differ (P = 0.987). A little over 50% of participants were male, and the majority of all participants (60%) was of European ethnicity.

Table 2.1 Sociodemographic characteristics of the participants

Characteristics	**Participants** (*n* = 40)
	Mean ± *SD*
Age (y)	33.4 ± 12.4
Weight (kg)	73.3 ± 18.4
Height (m)	1.7 ± 0.1
BMI (kg/m^2)	24.9 ± 4.9
Waist circumference (cm)	84.7 ± 14.4
Sex (*n;* %)	
Men	21; 52.5
Women	19; 47.5
Ethnicity (*n*; %)	
European	24; 60.0
Chinese	7; 17.5
Other Asian	7; 17.5
Latin-American	2; 5.0

2.4.2 Estimated daily dietary intakes

The estimated intakes of energy, total and individual forms of choline, and betaine intakes are presented as absolute unadjusted daily intakes in Table 2.2, and as energy-density daily intakes per 1000 kcal in Table 2.3. For the FFQ, energy, free choline, water-soluble forms of choline, phosphatidylcholine, and lipid-soluble forms of choline intakes were normally distributed, while betaine, phosphocholine, glycerophosphocholine, and sphingomyelin were skewed. For the mean of three 24HRs, the estimated intakes were normally distributed, except for betaine, free choline, glycerophosphocholine, and water-soluble forms of choline intakes. Unadjusted total energy intake was (mean ± SD) 2232 ± 873 kcal/d estimated from the FFQ and 1920 ± 565 kcal/d based on the mean of three 24HRs. Overall, the mean unadjusted intakes estimated by the FFQ were significantly higher compared to the estimated intake by the mean of three 24HRs, except for energy, phosphatidylcholine, and sphingomyelin, which did not differ.

The estimated intakes of energy, total and individual forms of choline, and betaine intakes are presented as energy-density daily intakes per 1000 kcal in Table 2.3. For the FFQ, most of the estimated intakes were normally distributed, but betaine was skewed. For the mean of three 24HRs, most of the estimated intakes were normally distributed, except for betaine, glycerophosphocholine, and water-soluble forms of choline intakes. When nutrients were compared as energy-density per 1000 kcal values, intakes did not differ except for significantly higher intakes of phosphocholine and betaine estimated from FFQ compared to those by the mean of three 24HRs.

Table 2.2 Daily energy and nutrient intakes as absolute values estimated from the FFQ and the mean of three 24HRs

	Absolute daily intake (*n* = 40)				
	FFQ		**24HRs**		***P*-value**[1]
Intakes	*Mean ± SD*	*Median (IQR)*	*Mean ± SD*	*Median (IQR)*	
Energy (kcal/d)	2232 ± 873	2111 (1695-2725)	1920 ± 565	1824 (1518-2341)	0.075
Total choline (mg/d)	395 ± 168	368 (282-499)	340 ± 133	318 (251-397)	0.025
Free choline (mg/d)	85 ± 34	81 (58-101)	71 ± 29	64 (53-90)	0.007
Phosphocholine (mg/d)	16 ± 9	15 (10-21)	12 ± 5	13 (9-15)	0.004
Glycerophosphocholine (mg/d)	72 ± 43	64 (46-84)	56 ± 35	50 (36-68)	0.002
Phosphatidylcholine (mg/d)	202 ± 98	209 (124-247)	184 ± 87	176 (135-228)	0.233
Sphingomyelin (mg/d)	21 ± 12	16 (12-31)	18 ± 9	16 (11-22)	0.379
Betaine (mg/d)	196 ± 148	152 (104-244)	128 ± 87	97 (75-165)	0.001

[1]Group differences were compared by paired samples Student t-test after log-transformation.
FFQ: food frequency questionnaire, 24HRs: 24-hour recalls.

Table 2.3 Daily energy and nutrient intakes as energy-density values estimated from the FFQ and the mean of three 24HRs

	Energy-density per 1000 kcal daily intake (*n* = 40)				
	FFQ		**24HRs**		***P*-value**[1]
Intakes	*Mean ± SD*	*Median (IQR)*	*Mean ± SD*	*Median (IQR)*	
Total choline (mg/d)	179.4 ± 41.9	172.5 (147.0-202.0)	178.1 ± 40.5	178.5 (150.2-205.2)	0.825
Free choline (mg/d)	39.0 ± 9.0	38.8 (32.8-45.3)	37.5 ± 10.9	36.6 (31.1-43.2)	0.367
Phosphocholine (mg/d)	7.4 ± 2.5	7.1 (5.8-9.2)	6.6 ± 2.3	6.5 (4.8-8.4)	0.049
Glycerophosphocholine (mg/d)	31.5 ± 10.8	28.4 (22.7-39.9)	29.0 ± 11.9	26.1 (21.3-32.9)	0.084
Phosphatidylcholine (mg/d)	92.2 ± 33.2	87.9 (66.5-114.8)	95.7 ± 33.9	96.9 (76.4-117.0)	0.447
Sphingomyelin (mg/d)	9.2 ± 4.8	8.4 (6.0-11.8)	9.2 ± 4.0	9.0 (6.1-11.0)	0.990
Betaine (mg/d)	90.5 ± 58.1	74.6 (49.1-110.1)	65.0 ± 30.6	60.6 (47.5-75.5)	0.008

[1]Group differences were compared by paired samples Student t-test after log-transformation.
FFQ: food frequency questionnaire, 24HRs: 24-hour recalls.

2.4.3 Correlations between instruments

The correlation coefficients between nutrient intakes estimated by the FFQ and the mean of three 24HRs are presented in Table 2.4. The correlation coefficients ranged from 0.42 for betaine to 0.72 for glycerophosphocholine for unadjusted intakes. For energy-adjusted values, the correlation coefficients ranged from 0.39 for betaine to 0.60 for phosphocholine, the correlation coefficients for all nutrients increased when corrected by de-attenuation. A good correlation was observed for total choline (r = 0.70), whereas the correlation coefficient for betaine intake was within the range of acceptable coefficients (r = 0.47).

Table 2.4 Correlation coefficients between nutrient intakes estimated from FFQ and the mean of three 24HRs

	Correlation coefficients (n = 40) [1]				
	Unadjusted		**Energy-adjusted**		
Intakes	*r*	*P-value*	*r*	*P-value*	*De-attenuated r*
Energy	0.448	0.004	-	-	-
Total choline	0.526	<0.001	0.543	<0.001	0.696
Free choline	0.519	0.001	0.341	0.031	0.359
Phosphocholine	0.482	0.002	0.597	<0.001	0.621
Glycerophosphocholine	0.719	<0.001	0.358	0.023	0.409
Phosphatidylcholine	0.465	0.003	0.507[2]	<0.001	0.695
Sphingomyelin	0.660	<0.001	0.473[2]	0.002	0.610
Betaine	0.418	0.007	0.386[2]	0.014	0.467

[1]Data are presented as Pearson's correlation coefficients after log-transformation, unless otherwise noted.
[2]Data are Spearman's correlation coefficients.
FFQ: food frequency questionnaire, 24HR: 24-hour recalls.

2.4.4 Bland-Altman analysis

Details of mean differences and LOA based on unadjusted intakes are presented in Table 2.5. Similar results were obtained when using energy-adjusted values. Overall, the FFQ over-estimated nutrient intakes compared to the mean of three 24HRs. The Bland-Altman plots (Figure 2.2) showed that in most cases the bias did not differ systematically as a function of the magnitude of the difference, except for betaine, where it appears that the bias was more likely to be positive at higher intakes.

Table 2.5 Bias measured by Bland-Altman method

	Bland-Altman (*n* = 40)		
Intakes	**Mean difference**	**Lower LOA**	**Upper LOA**
Energy (kcal/d)	312	-1288	1913
Total choline (mg/d)	55	-110	220
Free choline (mg/d)	14	-31	59
Phosphocholine (mg/d)	4	-5	13
Glycerophosphocholine (mg/d)	16	-44	76
Phosphatidylcholine (mg/d)	18	-115	152
Sphingomyelin (mg/d)	3	-12	18
Betaine (mg/d)	68	-135	272

LOA: limits of agreement.

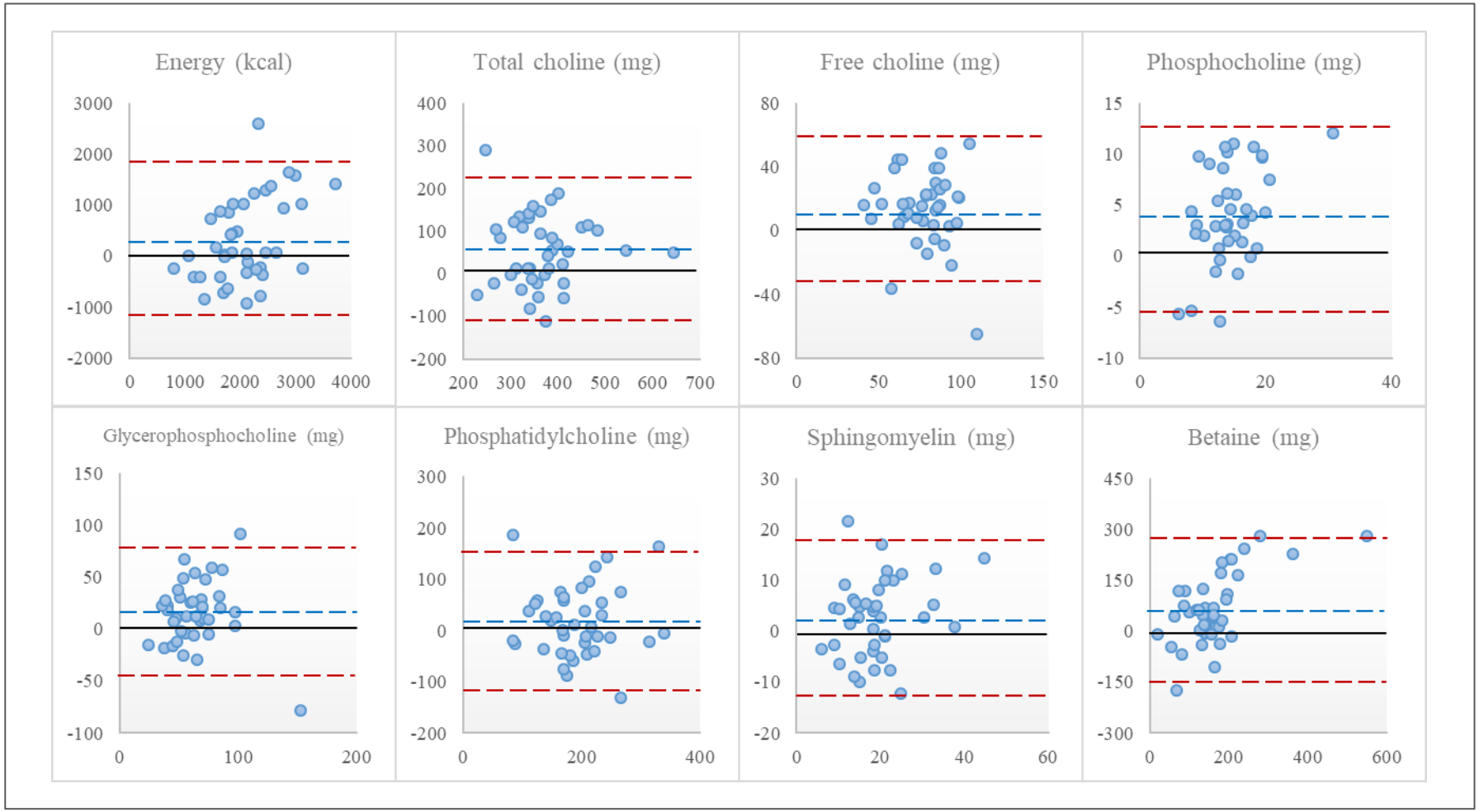

Figure 2.2 Bland-Altman plots
Plots showing the difference between the intake estimated by FFQ and the mean intake of three 24HRs in the Y-axis against the mean intake by the two instruments in the X-axis for each participant (n = 40). The solid black line represents the difference in intake of zero, the dotted blue line represents the mean difference, and the dotted red lines represent the upper and lower limits of agreement.

2.4.5 Cross-classification analysis

The results of the cross-classification analysis between the two instruments based on unadjusted intakes are presented in Table 2.6. Similar results were obtained when using energy-adjusted values. Overall, the proportion of participants categorized in the opposite dietary intake tertile was ≤ 10%, except for betaine, for which it was 15%. This result was consistent with the weighted Cohen's kappa coefficients, which were ≥ 0.2 for all nutrient intakes suggesting an acceptable agreement between instrument, apart from betaine ($\kappa_w = 0.192$) that did not reach statistical significance.

Table 2.6 Cross-classification between nutrient intakes estimated from FFQ and the mean of three 24HRs

	Cross-classification (*n* = 40)				
	Tertiles (%)			**Weighted Cohen's kappa**	
Intakes	*Same*	*Adjacent*	*Opposite*	κ_w	95% CI
Energy	42.5	50.0	7.5	0.259	0.028 – 0.490
Total choline	47.5	45.0	7.5	0.316	0.084 – 0.548
Free choline	47.5	45.0	7.5	0.316	0.084 – 0.548
Phosphocholine	50.0	40.0	10.0	0.316	0.077 – 0.556
Glycerophosphocholine	57.5	40.0	2.5	0.487	0.280 – 0.694
Phosphatidylcholine	50.0	45.0	5.0	0.373	0.150 – 0.596
Sphingomyelin	52.5	40.0	7.5	0.373	0.142 – 0.604
Betaine	43.6	41.0	15.4	0.192	-0.059 – 0.444

2.5 Discussion

The present study aimed to estimate the relative validity of a semi-quantitative FFQ to assess dietary choline and betaine intakes in the past month among apparently healthy adults (*n* = 40, both sexes, mean age 33 y) in Vancouver, Canada. These results showed: 1) acceptable to good correlations between the FFQ and the mean of three 24HRs for choline and betaine intake among adults; 2) in general, the Bland-Altman plots showed (except for betaine) that bias did not appear to vary as a function of the magnitude of the difference; and 3) cross-classification analysis indicated that miss-classification into opposite tertiles was ≤ 10%, apart from betaine intake. Likewise, weighted Cohen's kappa statistics were ≥ 0.20, indicating an acceptable agreement between instruments, with the exclusion of betaine intake.

FFQs are commonly used to assess dietary intake, as they reflect usual food intake of the participants, have a low respondent burden, and are feasible to use in large epidemiological studies (266). Before estimation of dietary intake, it is essential to validate the FFQ to use for the nutrients of interest. Only a few recent studies (*n* = 4) have reported on validation of instruments to assess choline and betaine intakes (187-190). This could be related to the fact that the first food composition database for these nutrients only became available for use less than 15 years ago (157). Moreover, no validation studies assessing the intake of the individual forms of choline have been published to date. In the study reported in this chapter, significantly higher intakes were found when comparing the FFQ to the mean of three 24HRs of total choline (395 vs. 340 mg/d, respectively; *P* = 0.025) and betaine (196 vs. 128 mg/d, respectively; *P* = 0.001). A similar tendency for higher intakes estimated from an FFQ compared to the mean intake from the reference method has been reported in the United States and Europe (188-190). In a previous

study from the United States (*n* = 42, pregnant women), with mean total choline intake of 391 vs. 376 mg/d, and betaine intake of 230 vs. 174 mg/d, estimated using an FFQ compared to the mean of two 24HRs, respectively (189). In Europe, dietary choline and betaine intakes appear to be lower compared to North America. In the United Kingdom (*n* = 64, premenopausal women), median total choline intake has been reported as 285 vs. 255 mg/d and betaine intake as 130 vs. 130 mg/d, estimated from FFQ and mean of three 24HRs, respectively (190). In Belgium (*n* = 80, premenopausal women), mean total choline intake has been reported of 287 vs. 284 mg/d and betaine intake of 179 vs. 147 mg/d, estimated from FFQ and mean of seven dietary records, respectively (188). As such, the present results in adults confirm previous findings from similar comparison studies in various populations, that FFQs commonly overestimate the absolute choline and betaine intakes when compared to 24HRs and dietary records. In addition, the estimated energy intake tended to be higher in the FFQ compared to 4HR, which supports the overestimation of intakes.

The discrepancy between FFQ and multiple 24HRs found in the present study has been commonly reported in other studies (188-190). A possible explanation could be the long list of food items included in FFQs, as participants may lose focus after a while, or the difficulty in assessing portion size or frequency of consumption. In contrast, it is also likely that under-reporting of energy from multiple 24HRs rather than an over-reporting of nutrient intake assessed by FFQ occurred, as has also been suggested by others (182). In the present study, total choline intake, when expressed as energy-densities mean intakes did not differ between instruments (179 vs. 178 mg/d/1000 kcal, respectively; $P = 0.825$). Another possibility is that the time frame, during which the reference instrument (the mean of three 24HRs) was administrated was not long enough to adequately quantify the usual choline and betaine intakes of the

participants. However, similar time frames have been used in other validation studies (267). Yet, for betaine the intake of rich food sources such as beets and quinoa (260 mg and 630 mg per 100 g of food, respectively) (13), that are not frequently consumed may be captured in the FFQ but not in the three 24HRs. Based on estimates of within-subject variability for other nutrients, it is probable that additional days of dietary recalls would have been required to improve the accuracy of the 24HRs at the individual level (241, 268). This limitation is inherent in most studies that use 24HRs as the reference instrument for validation, as reporting accuracy in 24HRs may diminish as the respondent burden increases. As such, there are multiple possibilities for higher intakes assessed by FFQs including underreporting energy and nutrient intakes by 24HRs.

It has been recommended to adjust by energy intake before assessing the association of estimated intake values between instruments (259). Additionally, as the within-subject variance in daily intake from 24HRs may affect the strength of the correlations, it has been suggested to correct this issue by using de-attenuation (250). It has been stipulated that reasonable correlation coefficients between instruments to assess dietary intake should be at least > 0.20 to be considered acceptable (261). In the present study, the energy-adjusted de-attenuated correlation coefficients were good (r = 0.70) for total choline intake and acceptable (r = 0.47) for betaine intake. This finding is consistent with the results from other studies, in which significant positive de-attenuated correlation coefficients were reported between 0.42 to 0.68 for total choline intake and 0.32 to 0.39 for betaine intake, among premenopausal women (188, 190). In contrast, another study reported a non-significant negative de-attenuated correlation coefficient of -0.40 for total choline in pregnant women (189). As such, our FFQ appears to have a better performance than previously validated FFQs for the assessment of dietary total choline and betaine intakes. However, it was noticed that betaine tended to differ systematically as a

function of the magnitude of the difference, with the bias being more likely to be positive at higher intakes which may limit the use of the FFQ.

In the present study, the cross-classification analysis showed that more than 85% of the participants' dietary intake estimations were classified in the same or adjacent tertiles, with a gross misclassification into the opposite tertile of 8% for total choline and 15% for betaine. A previous study, comparing an FFQ to seven days of dietary records in premenopausal women in Belgium, reported higher gross misclassification for total choline (20%) and similar for betaine (14%) intakes (188). A study including pregnant women, in the United States, reported higher gross misclassification rates, which was 38% for total choline and 31% for betaine when classified in quartiles (189). The small proportion of gross misclassification in the study presented in this chapter demonstrates a good ability of the FFQ to rank the participants, and thus a reasonable agreement with the mean of three 24HRs (251, 261). The weighted Cohen's kappa coefficients in the present study suggested acceptable agreement for total choline ($\kappa_w = 0.32$), but poor agreement for betaine ($\kappa_w = 0.19$) intake classification between instruments. Lower values for total choline ($\kappa_w = 0.10$) and similar for betaine ($\kappa_w = 0.21$) have been reported in only one previous study (188). As previously mentioned, the results of the present study indicate a better performance than previously validated FFQs for the assessment of dietary total choline intake, but the lower for betaine intake. However, the lower performance of the FFQ to estimate dietary betaine intake, as has been reported in previous studies, should be taken in account when using betaine intake to associate with another variable of interest, such as plasma concentrations or health outcomes.

The present study has a number of strengths. First, this is the first study performed to validate total and individual forms of choline and betaine intakes estimated from an FFQ among adults in Canada. Second, the reference instrument (the mean of three 24HRs) included two weekdays and one weekend day, which were assessed during a similar time frame to the FFQ. Third, a comprehensive examination was conducted using several statistical analyses, including an adjustment for within-subject variability that is known to attenuate correlations, which is strongly recommended in dietary intake validation studies when assessing correlations (246).

However, the present study has some limitations. First, a small sample size of participants was used (n = 40). Yet, it had enough power to detect correlation coefficients of 0.50, whereas a minimum of 0.20 has been recommended for acceptable validity (246, 261). Second, ideally, the reference instrument selected has different errors associated with it. For the FFQ, recall bias and incorrect estimation of the portion size are the major sources of error, whereas, portion size is commonly better estimated using 24HRs, however, recall bias is also an important source of error for 24HRs (269). As such, recall bias could not be eliminated as a common error in both, the instrument tested and the reference in the present study. Therefore, in this study, the 24HRs were conducted using in-person interviews with food models and the multiple-pass method in an effort to increase the accuracy of the information collected by the reference instrument (254). Third, self-reported energy intakes may not reflect actual energy intakes, and both instruments may have under-reported it (270). Thus, it is possible that choline and betaine intakes were also under-reported by both instruments. In addition, the betaine intake estimated from the mean of three 24HRs may not have been able to capture the consumption of rich food sources of betaine (i.e., beets and quinoa), as their intake may not be as frequent compared to other foods.

In summary, this is the first study to evaluate the relative validity of a semi-quantitative FFQ used to estimate dietary choline and betaine intakes among adults in Canada. Overall, the results suggest that the dietary intake estimated from the FFQ is comparable to the estimation of the mean of three 24HRs when energy-adjusted values were used. Acceptable correlation coefficients and agreement by cross-classification analyses were obtained for total choline and the individual forms. However, it may be less accurate for betaine intake, and thus estimated betaine intakes should be interpreted carefully. Overall, the findings suggest that this FFQ is a useful instrument for assessing the usual dietary intake of total and individual forms of choline among healthy adults.

Chapter 3: Concentrations of choline in milk from Canadian and Cambodian lactating women

3.1 Chapter synopsis

Human milk choline concentrations are thought to reflect maternal dietary choline intake. Since the main contributors to dietary choline intake are animal-source foods, lactating women from low-income countries with low intakes of these particular foods could produce milk lower in choline. In human milk, the majority (84%) of choline is present in the water-soluble forms. Moreover, limited data on mean concentrations in human milk were used to establish dietary choline recommendations for early infancy. To date, choline concentrations in mature human milk have been reported in studies with small numbers of participants. Even though most data on choline concentrations in mature human milk are from high-income countries, there was no information available from Canada. Further, there was only one study that reported on choline concentrations in human milk in a low-income country about three decades ago. Therefore, the principal aim of this study was to determine the concentrations of water-soluble forms of choline in mature human milk from a large number of lactating women living in Canada (n = 301) and Cambodia (n = 67, a low-income country). All human milk samples were collected using similar protocols and analyzed by stable isotope dilution liquid chromatography-tandem mass spectrometry. The key findings of this study indicate that the concentrations of water-soluble forms of choline in mature human milk did not differ between Canadian and Cambodian women ($P > 0.05$) and that the estimated infant's total choline intake was significantly below the current

intake recommendation for early infancy ($P > 0.001$). These results suggest that the current dietary recommendations for choline during early infancy should be revisited.

3.2 Introduction

Choline is an essential nutrient for humans and is critically important during periods of rapid growth and development, most notably in early infancy (12, 35). Although some choline is synthesized endogenously, the amounts are not sufficient to meet the needs of the body, and consequently, choline must be provided in the diet (24). Choline is a precursor for the synthesis of acetylcholine (52, 271), phospholipids (such as phosphatidylcholine and sphingomyelin), and betaine (1, 34, 51). Acetylcholine is a neurotransmitter that participates in muscle contraction and cognition (52). Phosphatidylcholine and sphingomyelin are essential for maintaining cell membrane structure and function, and for the synthesis of lung surfactant, bile, and lipoproteins (272), respectively. In contrast, betaine is an important osmolyte and methyl group donor for the remethylation of methionine and the one-carbon folate pool (51, 203). Such as, choline plays a crucial role in many biochemical and physiological processes.

During pregnancy, choline is transferred to the fetus through the placenta as free choline, while human milk provides choline as the sole dietary source for breastfed infants for the first six months postpartum (171, 273, 274). In human milk, choline is present in both water- and lipid-soluble forms. Water-soluble forms of choline, including free choline, phosphocholine, and glycerophosphocholine, contribute to an average of 84% (range 81 – 88%) of the total choline in human milk (14-16). The remaining portion of the total choline in human milk (mean 16%, range 12 – 19%) is present as lipid-soluble forms, including phosphatidylcholine and sphingomyelin as

part of the fat globule membrane (275). It has been reported that human milk choline concentrations are associated with serum free choline concentration in infants (15) and that elevated circulating free choline augments choline uptake across the blood-brain barrier (276, 277). A recent observational study suggested that a higher milk choline concentration is associated with better infant recognition memory ($n = 55$, $P < 0.05$) at six months postpartum (172).

Exclusive breastfeeding is recommended for 0 – 6 months old (162), and the average concentration of nutrients in mature human milk from healthy, well-nourished mothers is commonly used as the reference value for setting dietary recommendations for infants (12). Also, the amount of choline secreted in mature human milk is used to identify the increment of choline over the dietary recommendations of non-lactating women for lactation (12). For early infancy (0 – 6 months), the Adequate Intake (AI) for choline was set at 125 mg/d, based on a mean choline concentration of 1500 μmol/L in mature human milk, assuming a daily intake of 0.78 L of human milk (12).

In the view of the fundamental importance of adequate nutrient supply for infant growth and development, there remains limited knowledge about the concentrations of various nutrients in human milk, including choline. The current literature on choline concentration in human milk is scarce, with only 12 studies reported worldwide (14-17, 21, 22, 165, 171-174, 278). In these studies, the milk samples were collected from groups of lactating women after a full-term, pre-term, or mixed birth term pregnancy, with very small sample sizes ranging from one in Sweden (174) to 62 in Japan (21). Moreover, the report available from Ecuador, a low-middle-income country in 1982 (279), included the analysis of only free choline, phosphatidylcholine, and

sphingomyelin, which together contribute to 26% of total choline in human milk (22). Additionally, most of the limited data published on choline concentrations in human milk are from high-income countries, where maternal animal-source food consumption, the most important dietary source of choline, is higher than in low- or middle-income countries (175, 179-181). This information raises the question of whether lactating women consuming diets low in animal-source foods are more likely to produce milk that is lower in choline.

The primary objective of the study presented in this chapter was to determine the concentrations of choline in mature milk from lactating women in Canada and Cambodia, where the availability of animal-source foods is thought to differ drastically. According to the Food and Agriculture Organization's (FAO) food balance sheets, 9% (216 / 2411 kcal) of total energy available in the Cambodian diet is derived from animal products, compared to 27% (913 / 3419 kcal) in Canada (180). This study focused on the water-soluble forms of choline to estimate total choline, as they contribute an average of 84% of total choline in human milk (15-17, 20), and methodology to quantitate the lipid-soluble forms of choline was not available in our research group. A secondary objective was to explore the potential associations between maternal dietary choline intake during pregnancy and the concentrations of water-soluble forms of choline in human milk in a subset of Canadian participants.

3.3 Participants and methods

3.3.1 Study design and participants

The study presented in this chapter was a cross-sectional analysis of convenience samples of mature milk collected from apparently healthy lactating women (18 – 45 y) from Vancouver, Canada (n = 301) (280, 281), and Prey Veng province, Cambodia (n = 67) (282). A power analysis calculation indicated a minimum of 34 participants for each group was required to provide 90% power to detect a large effect size (d = 0.80) (283, 284) in group means when comparing two independent samples, with significance at the 5% (two-tailed). Only women who had low-risk pregnancies, uncomplicated deliveries, and gave birth to healthy, full-term (> 37 weeks of gestation) infants were included. Sociodemographic information including maternal age, education level, household income, and ethnicity, as well as infant age at sample collection, were obtained from the participants. For the secondary objective, a subset of the Canadian participants was used (n = 143). A power size calculation indicated a minimum of 38 participants was required to provide 90% power to detect a correlation of $r \geq 0.5$ (283, 284) between dietary intake and milk concentrations with significance at 5% (two-tailed).

In Canada, the participants were enrolled in two separate supplementation randomized controlled trials (neither of which contained choline) as described previously (280, 281), and therefore all participants, either assigned to treatments or control group, were included in the analysis presented in this chapter. In the first trial, women had consumed a daily supplement containing docosahexaenoic acid or placebo from 16 weeks of gestation through to the end of pregnancy (280). In the second trial, women had consumed a daily prenatal multivitamin and

mineral supplement and were assigned to one of three vitamin D supplement groups targeted between 13 – 24 weeks of gestation through eight weeks postpartum (281). In Cambodia, the participants had been consuming either a thiamin-fortified or placebo fish sauce *ad libitum* for six months prior to milk collection as part of a randomized controlled trial (282). Daily fish sauce consumption is estimated to provide only 2 mg of total choline per 16 ml (258, 285); thus, it did not represent a good source of choline, and therefore all participants, either assigned to treatments or control group, were included in the analysis presented in this chapter.

The Canadian trials were approved by the University of British Columbia – Children's & Women's Health Centre of British Columbia Research Ethics Board, while the Cambodian trial was approved by the Cambodian National Ethics Committee for Health Research, according to the guidelines of the Declaration of Helsinki (252, 253). All subjects provided written informed consent prior to participation.

3.3.2 Milk sample collection and handling

The Canadian participants, in both trials, provided a mature milk sample at eight weeks postpartum. In the first trial, hindmilk samples (n = 147) were collected at the participant's house, using a mechanical or electric pump, into pre-labeled tubes and stored in a home freezer for up to three days. The milk samples were then transported to the Child and Family Research Institute (CFRI) at the Oak Street campus of the University of British Columbia in Vancouver, where they were stored at -80°C. In the second trial, milk samples (n = 154) were collected during a visit at CFRI, using a mechanical or electric pump, as a full breast expression from the breast that had not been most recently emptied. The samples were immediately placed on ice,

and stored at -80°C within one hour until analysis. Previously, it has been reported that there is no difference among choline concentrations in fore, middle, and hindmilk (171).

In Cambodia, the participants provided a mature milk sample (*n* = 67) between 3 – 28 weeks postpartum. Previous studies have reported that choline concentration in human milk does not significantly change after two weeks postpartum (15, 21, 166) and remains constant throughout the first twelve months postpartum (21). Milk samples were collected, using a mechanical or electric pump as a full breast expression from the breast that had not been most recently emptied. Milk samples were provided by lactating women in their villages, placed on ice, and were subsequently transported to the National Institute of Public Health in Phnom Penh (< 5 hours) for storage at -80°C until they were shipped on dry ice to CFRI, Vancouver, Canada for analysis.

3.3.3 Water-soluble forms of choline analysis in milk

The concentrations of water-soluble forms of choline (free choline, phosphocholine, and glycerophosphocholine) in milk were quantified by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the method described previously (225), with modifications (172, 286). The concentration of betaine in the milk samples was also assessed, as betaine was included in method development. The LC-MS/MS was a Waters I-class ACQUITY UPLC system connected to a Quattro Micro tandem MS configured with an electrospray source (Waters Corporation, MA, USA). The LC was equipped with a Zorbax Rx-Sil pre-column (2.1 x 12.5 mm) and a column (2.1 x 150 mm), both with 5 µm particle size (Agilent Technologies, CA, USA).

Before analysis, frozen milk samples were thawed in a water bath (25°C) and homogenized to ensure constant quality of the samples. For analysis, aliquots of 20 µL of milk were transferred to an Eppendorf tube (1.5 mL) containing 10 µL of internal standards (choline-d9, phosphocholine-d9, glycerophosphocholine-d9, and betaine-d9; CDN Isotopes Inc, QC, Canada). Milk protein was precipitated by adding 30 µL of methanol containing 0.1% formic acid to the tube followed by vortexing and centrifugation (13,000 × *g*; 10 min; 4°C). Then, a 20 µL aliquot of the supernatant was transferred to a 150 µL glass insert placed inside a high-performance liquid chromatography vial, containing 100 µL of acetonitrile with 0.1% formic acid.

The chromatographic separation was carried out at a flow of 0.5 mL/min with a mobile phase consisting of A: acetonitrile with 0.1% formic acid and 0.1% trifluoroacetic acid, and B: 15 mmol/L ammonium formate with 0.1% formic acid and 0.2% trifluoroacetic acid in H_20. The gradient separation started at 95% A plus 5% B for 2 min followed by separation in a linear gradient to 55% A plus 45% B at 4 min and then held for 5.8 min after which the column was subsequently flushed with 100% B and re-equilibrated to starting conditions. The MS was operated in positive ion mode with multiple reaction monitoring, using the following paired precursor-product ion transitions: 103.9→59.9 choline and 113→68.9 choline-d9; 184.1→125.0 phosphocholine and 93.1→125.0 phosphocholine-d9; 258→124.9 glycerophosphocholine and 289.0→221.0 glycerophosphocholine-d9; and 117.9→58.9 betaine and 126.9→67.9 betaine-d9. The auto-sampler chamber and column were maintained at 5°C and 25°C, respectively. The injection volume used for the analysis was 4 µL, with a total analytical time of 9 min. Each milk sample was only analyzed one time.

A standard curve and an in-house prepared pooled human milk samples were analyzed every day for quality assurance. The standard curve, corresponding to 10 different concentrations of the choline metabolites of interest (Table 3.1), yielding a standard linear curve ($r > 0.99$; Figure 3.1). In addition, a reagent blank and the internal standard alone were run for quality control purposes. The unknown areas of identified peaks were compared with the area curve from the standard for quantification. The milk concentrations of free choline, phosphocholine, glycerophosphocholine, and betaine were expressed as µmol/L. Replicates of aliquots of pooled human milk were included for quality control and to determine the analytical variability. The inter- and intra-assay coefficients of variance, calculated based on ten replicates each, were: 4.8% and 1.4% for free choline, 5.4% and 0.2% for phosphocholine, 3.6% and 2.1% for glycerophosphocholine, and 5.9% and 2.3% for betaine, respectively.

Table 3.1 Concentrations of standard solutions for milk concentrations

Standards	Free Choline	Phosphocholine	Glycerophosphocholine	Betaine
		Concentrations (µmol/L)		
1	6.0	15.0	20.0	0.6
2	15.0	37.5	50.0	1.5
3	30.0	75.0	100.0	3.0
4	45.0	112.5	150.0	4.5
5	60.0	150.0	200.0	6.0
6	120.0	300.0	400.0	12.0
7	240.0	600.0	800.0	24.0
8	360.0	900.0	1200.0	36.0
9	480.0	1200.0	1600.0	48.0
10	600.0	1500.0	2000.0	60.0

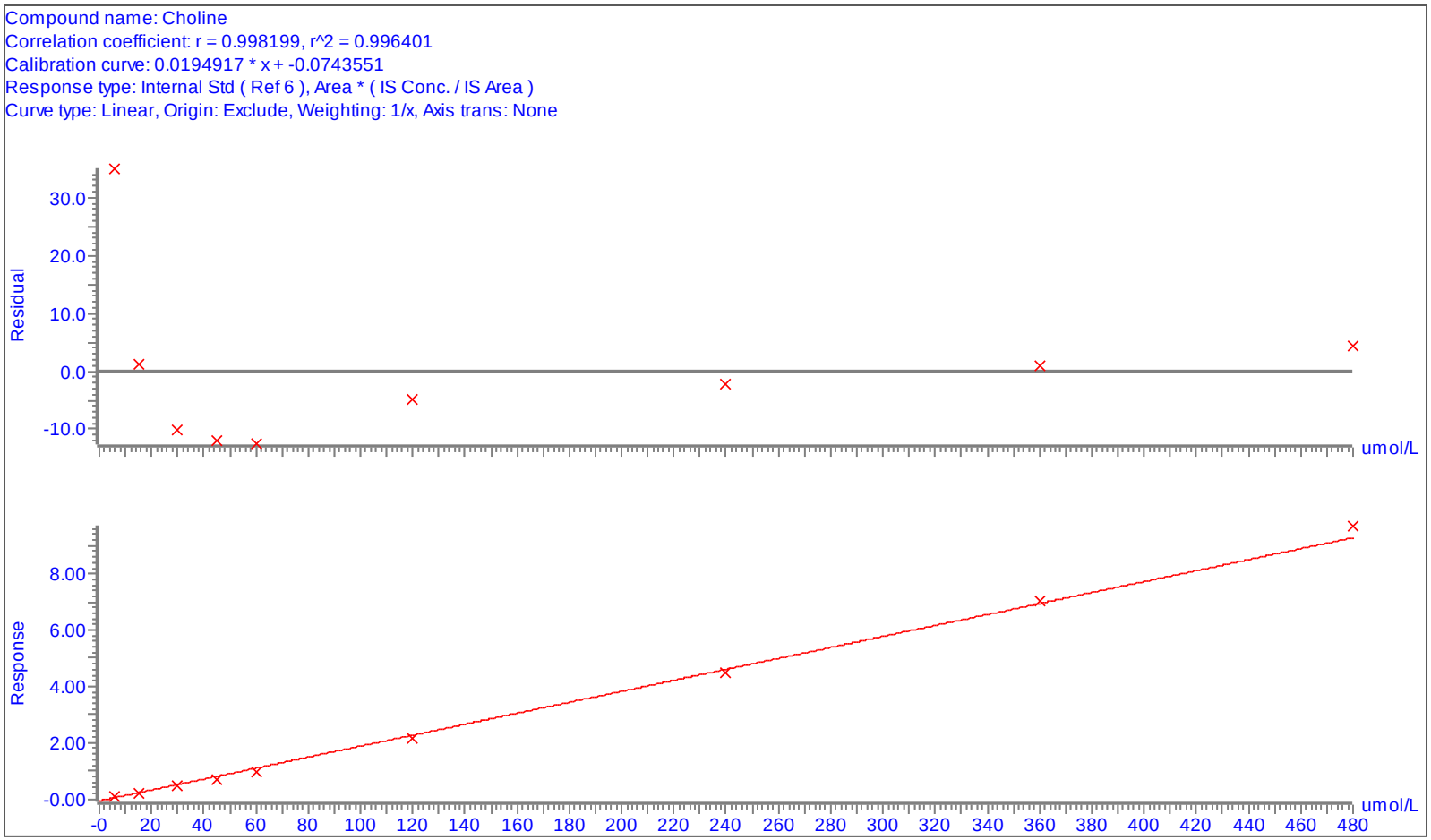

Figure 3.1 A representative plot of response factors and residuals from chromatograms of standard curve for free choline in milk

3.3.4 Calculation of total choline intake from milk in infants

The current AI recommendation for choline of 125 mg/d for infants aged 0 – 6 months was based on milk choline concentrations and volume intake (12). Specifically, the assumption that breastfed infants consumed an average of 0.78 L/d of human milk during the first six months of life was used (12, 149-151, 287, 288). The available data provide information that on average the water-soluble forms of choline make up 84% while the lipid-soluble forms contribute to 16% of total choline in human milk (14-16, 166). In this study, the concentration of total choline in the milk samples was calculated by multiplying the concentration of the sum of the water-soluble forms of choline of each individual participant, quantified as previously described, by a conversion factor of 1.19. This factor was calculated as the ratio between total choline and the contribution of the water-soluble forms of choline to total choline (1.19 = 1.00 / 0.84). The molar mass of choline (104 g/mol) was used to express the data as mg/d and is referred to as total choline estimation. This estimation allowed the values to be compared with the AI for choline.

3.3.5 Dietary intake assessment in a subset of the Canadian participants

Dietary intake information from participants was available from the first of the two Canadian trials (*n* = 143). In this trial, dietary intake was assessed using a food frequency questionnaire (FFQ) administered by face-to-face interviews, at 16 and 36 weeks of gestation (289). Previous studies have observed a minor and non-significant variation in the maternal diet when comparing total choline intakes during pregnancy and lactation (6, 10). The FFQ gathered information on the foods and beverages consumed, including frequency of intake and portion sizes, over the previous month. Dietary choline and betaine intakes were estimated using a nutrient analysis software (ESHA Food Processor SQL, version 10.14.41; Salem, OR) (257) and

the United States Department of Agriculture (USDA) database on the Choline Content of Common Foods (Version 2) (13). Detailed information is provided in Chapter 2 of this dissertation. The estimated dietary choline and betaine intakes were expressed as mg per day.

3.3.6 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 19; SPSS Inc. Chicago, US). The significance level was set at $P < 0.05$ for two-sided testing. Normality of the data was assessed using the Kolmogorov–Smirnov test. If distributions were skewed, data were then normalized using logarithmic transformations to improve normality before further analysis. If normality was not achieved after logarithmic transformation, non-parametric tests were used instead. All data are presented as mean ± SD, unless otherwise noted.

Prior to the hypothesis testing, the concentration of water-soluble forms of choline and betaine in milk were compared by randomization groups within each trial, by independent samples Student's t-test or one-way ANOVA, as appropriate. Next, the concentration of water-soluble forms of choline and betaine in milk were compared between the two Canadian trials, as the trials differed in the fraction of milk sample collected (hindmilk vs. full breast expression) by independent samples Student's t-test or Mann-Whitney U test, as appropriate. Since Cambodian milk samples were collected between 3 – 28 weeks postpartum (whereas all Canadian samples were collected at eight weeks), the association between the concentration of water-soluble forms of choline and betaine in milk and weeks postpartum was assessed by Pearson's correlation test in the Cambodian trial. None of these results were statistically significant ($P > 0.05$ for all comparisons; Appendix B). Therefore, participants from both Canadian trials were merged into one group, and there was no need to control for weeks postpartum among the Cambodian group.

Descriptive statistics were used to summarize the sociodemographic characteristics of the participants and were compared between Canadian and Cambodian participants, using Mann-Whitney U test for age and Fisher's exact test for categorical variables. Milk concentrations of water-soluble forms of choline and betaine were compared between Canadian and Cambodian participants by independent samples Student's t-test. The estimated total choline intake of infants was compared to the choline AI for early infancy (125 mg/d), in both Canadian and Cambodian participants separately and also together as one group, by one sample Student's t-test.

In one of the Canadian trials, dietary information from the participants (n = 143) was available at 16 and 36 weeks of gestation. A paired samples Student's t-test was used to determine the difference between dietary choline intakes collected at the two-time points and a pair-wise correlation coefficient was calculated using Pearson's correlation test between dietary total choline intake estimated at 16 and 36 weeks of gestation. As dietary choline intakes estimated at both time points did not differ ($P = 0.927$) and were strongly correlated ($r = 0.590$, $P < 0.001$; Appendix C), only the second-time point was used. The estimated total choline intake was compared to the choline AI, for both pregnancy and lactation (450 and 550 mg/d, respectively), by one sample Student's t-test. The relationships between maternal dietary choline and betaine intakes during pregnancy and the concentrations of water-soluble forms of choline and betaine in milk samples at eight weeks postpartum were explored using Pearson's or Spearman's correlation test, as appropriate. Interpretation for all correlation coefficients was defined as weak < 0.30, moderate between 0.30 – 0.49, and strong > 0.50 (284). Bonferroni correction was performed to adjust for multiple comparisons of dietary intakes on the same metabolite concentration in milk (0.05 / 9 = 0.0055, significance at $P < 0.006$).

3.4 Results

3.4.1 Sample analysis

In the first Canadian trial, 148 participants provided a milk sample, from which 147 milk samples were available for analysis (Figure 3.2). In the second Canadian trial, 154 participants provided a milk sample, and all were included in the analysis. Of the 68 participants that provided a milk sample in the Cambodian trial, 67 milk samples were available for analysis. For a subset (n = 143) of the Canadian participants in the first trial who provided milk samples, dietary intakes were available during pregnancy.

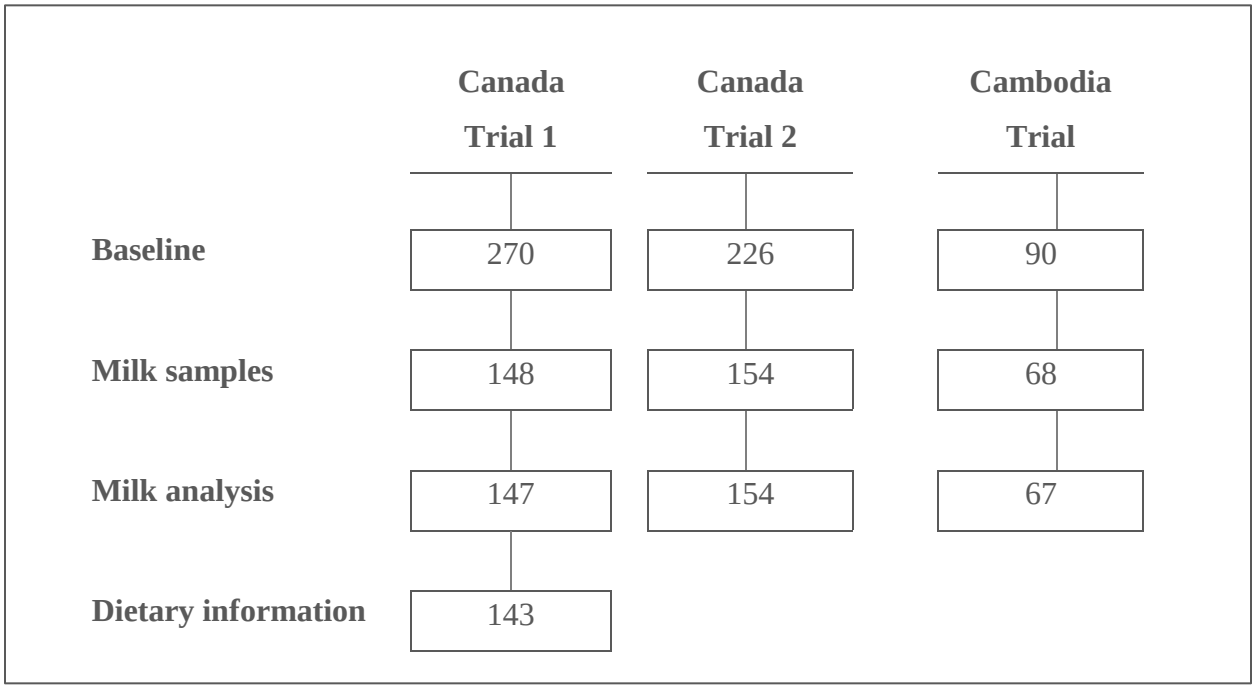

Figure 3.2 Diagram of milk samples and dietary information analyzed

3.4.2 Sociodemographic characteristics of the participants

The sociodemographic characteristics of the participants are shown in Table 3.2. In this study, Cambodian lactating women (mean ± SD, 26 ± 5 y) were significantly younger than Canadian women (33 ± 4 y) ($P < 0.001$). Canadian women were primarily of European descent (78%) followed by Asian descent (13%), and all Cambodian women were Khmer. Most lactating women were primiparous, giving birth to their first child in both Canadian (53%) and Cambodian (51%) participants. Canadian women were significantly better educated ($P < 0.001$) and had a higher household income ($P < 0.001$) compared to Cambodian women, reflecting a higher socio-economic status.

3.4.3 Concentrations of water-soluble forms of choline and betaine in milk

The concentrations of the water-soluble forms of choline and betaine are presented in Table 3.3. The milk concentrations of most metabolites were skewed, while phosphocholine in both groups and glycerophosphocholine in Cambodian women were normally distributed. The concentrations of the water-soluble forms of choline and betaine in milk were not significantly different between Canadian ($n = 301$) and Cambodian ($n = 67$) lactating women ($P > 0.05$). Therefore, as the concentrations did not differ between groups, the concentrations for all milk samples were combined. Among all samples ($n = 368$), phosphocholine and glycerophosphocholine were the predominant water-soluble forms of choline in mature milk, contributing 49% and 37% of the sum of the individual water-soluble forms of choline, respectively.

Table 3.2 Sociodemographic characteristics of participants

Characteristics	**Canadian** (*n* = 301)	**Cambodian** (*n* = 67)	***P*-value**[1]
Age (y)[2]	33.3 ± 4.1	26.1 ± 4.7	< 0.001
Parity (*n;* %)			0.720
1	161; 53%	34; 51%	
2	112; 37%	24; 36%	
3 or more	28; 9%	9; 12%	
Education (*n;* %)			< 0.001
None	0; 0%	6; 9%	
Some primary	0; 0%	31; 46%	
Some secondary	11; 4%	30; 44%	
Some postsecondary	290; 96%	0; 0%	
Ethnicity (*n;* %)			< 0.001
European	234; 78%	-	
Asian	40; 13%	-	
Khmer	-	67; 100%	
Other	27; 9%	-	
Household total income (*n;* %)			< 0.001
< $20 000 CAD	10; 3%	67; 100%	
$20 000 – $50 000 CAD	47; 16%	0; 0%	
> $50 000 CAD	244; 81%	0; 0%	

[1]Continuous data were analyzed by Mann-Whitney U test, and categorical data analyzed by Fisher's exact test.
[2]Data are presented as mean ± SD.

Table 3.3 Concentration of water-soluble forms of choline and betaine in milk

Milk metabolites		**All women** (*n* = 368)	**Canadian** (*n* = 301)	**Cambodian** (*n* = 67)	***P*-value**[1]
			Concentrations (µmol/L)[2]		
Free choline	Mean ± SD	151 ± 90	155 ± 90	143 ± 90	0.071
	Median (IQR)	130 (87-191)	133 (92-193)	130 (72-164)	
Phosphocholine	Mean ± SD	540 ± 206	535 ± 207	562 ± 204	0.336
	Median (IQR)	533 (414-658)	519 (408-654)	567 (466-687)	
Glycerophosphocholine	Mean ± SD	411 ± 153	416 ± 156	390 ± 137	0.178
	Median (IQR)	383 (305-494)	383 (305-500)	380 (296-481)	
Water-soluble choline[3]	Mean ± SD	1102 ± 298	1106 ± 304	1095 ± 272	0.686
	Median (IQR)	1067 (922-1235)	1066 (922-1237)	1072 (925-1204)	
Betaine	Mean ± SD	4.9 ± 2.8	4.8 ± 2.4	5.1 ± 4.4	0.446
	Median (IQR)	4.2 (3.1-6.0)	4.2 (3.2-6.0)	3.6 (2.6-5.6)	

[1]Group differences were compared by independent samples Student t-test after log-transformation.
[2]Water-soluble forms of choline and betaine concentrations were quantified using liquid chromatography-tandem mass spectrometry.
[3]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

3.4.4 Comparison of estimated daily total choline intake from milk to the adequate intake for choline for infants aged 0 – 6 months

The estimated infant total choline intake from milk in Canada (n = 301), Cambodia (n = 67), and all milk samples (n = 368) was (mean ± SD) 105 ± 26 mg/d, 107 ± 29 mg/d, and 106 ± 29 mg/d, respectively. Total choline intake was calculated based on an estimated infant daily milk consumption of 0.78 L and the concentration of total choline (water-soluble forms of choline multiplied by the factor 1.19) at the individual level.

The estimated mean total choline intake from milk was significantly below the AI for choline for early infancy (0 – 6 months) of 125 mg/d in both Canadian and Cambodian participants separately and also as one group ($P < 0.001$). The estimated total choline intake compared with the AI for choline for early infancy (0 – 6 months) is illustrated in Figure 3.3 for all milk samples (n = 368). Based on these data, 19% of infants in both Canadian and Cambodian participants separately and also as one group would have estimated total choline intakes at or above the current AI for early infancy.

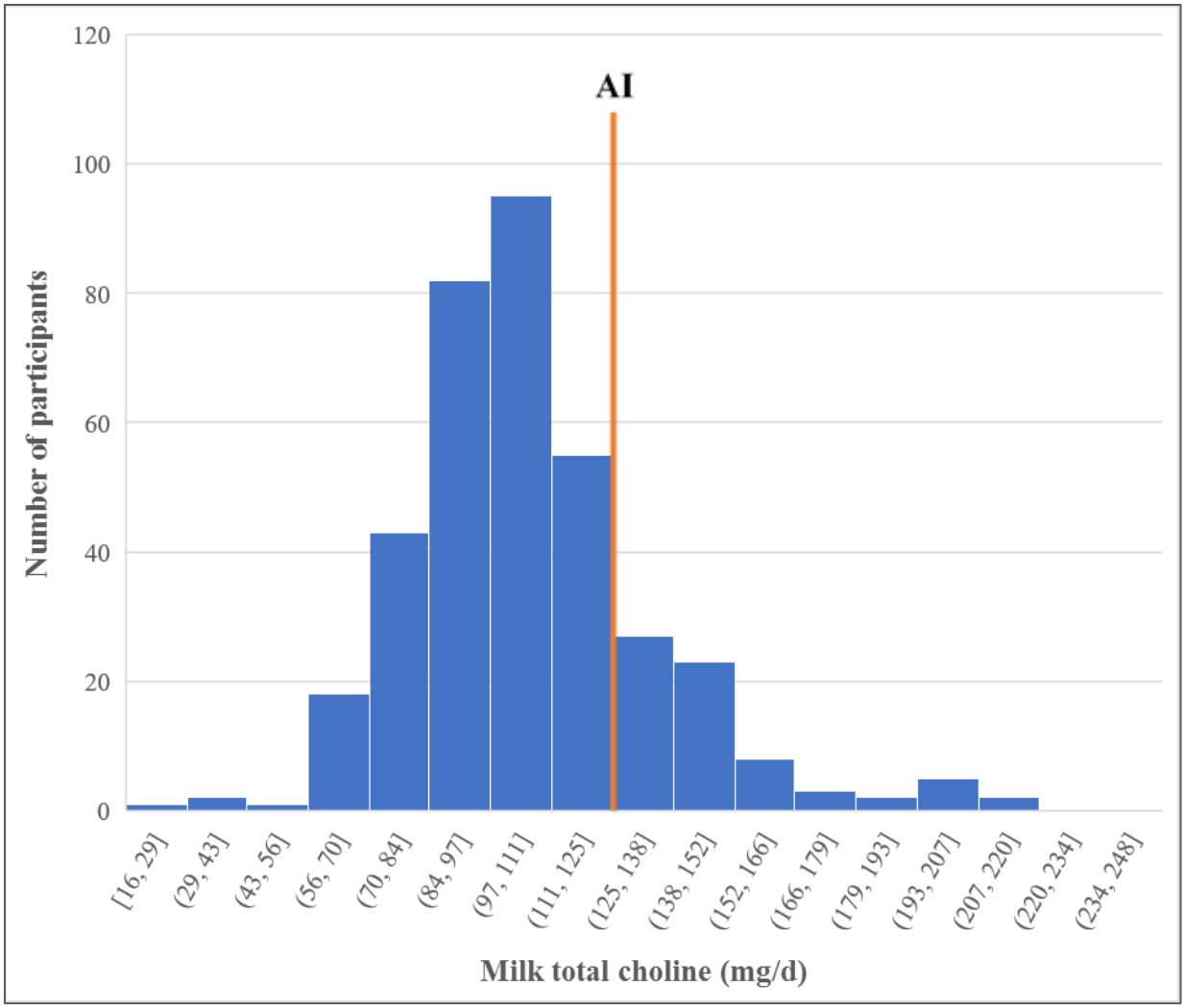

Figure 3.3 Distribution of estimated daily total choline intake from milk in infants

Data are presented for all milk samples analyzed (*n* = 368). Water-soluble forms of choline concentrations were quantified using liquid chromatography-tandem mass spectrometry. Total choline was calculated using a factor of 1.19 (based on the assumption that water-soluble forms contribute an average of 84% of total choline), and daily intake was calculated based on a consumption of 0.78 L/d and 104 g/mol. The vertical orange line corresponds to the Adequate Intake (AI) for choline for 0 – 6 months old infants of 125 mg/d.

3.4.5 Associations between estimated daily dietary choline and betaine intakes and water-soluble forms of choline in milk in a subset of the Canadian participants

The estimated dietary intakes of the total choline, individual forms of choline, the sum of water- and lipid-soluble forms of choline, and betaine at 36 weeks of gestation during pregnancy for a subset of the Canadian participants (n = 143) are presented in Table 3.4. Total choline, glycerophosphocholine, phosphatidylcholine, and lipid-soluble forms of choline intakes were normally distributed, while the distribution of free choline, phosphocholine, water-soluble forms of choline, sphingomyelin, and betaine intakes were skewed. The maternal dietary total choline intake was estimated at (mean ± SD) 408 ± 111 mg/d, which was significantly below the AI for choline for both pregnancy and lactation (450 and 550 mg/d, respectively; $P < 0.001$). At the individual level, only 32% and 10% of the participants had estimated total choline intakes above the AI for pregnancy and lactation, respectively.

The correlation coefficients between maternal dietary total choline, individual forms of choline, and betaine intakes and the concentrations of free choline, phosphocholine, glycerophosphocholine, and the sum of water-soluble forms of choline in milk are presented in Table 3.5. The sum of the water-soluble forms of choline in milk were positively, but weakly correlated with dietary total choline intake ($r = 0.166$, $P = 0.048$), dietary free choline intake ($r = 0.223$, $P = 0.007$), and dietary water-soluble forms of choline intake ($rho = 0.182$, $P = 0.029$). After adjusting for multiple comparisons (Bonferroni's correction, $P < 0.006$), the correlations were not significant anymore ($P \geq 0.006$, for all comparisons).

Table 3.4 Dietary choline and betaine intakes from a subset of Canadian participants

Dietary intakes	**Canadian participants** (*n* = 143)	
	Mean ± SD	Median (IQR)
	Dietary intakes (mg/d)[1]	
Total choline[2]	408 ± 111	409 (327 – 472)
Free choline	87 ± 24	84 (71 – 99)
Phosphocholine	24 ± 11	22 (18 – 28)
Glycerophosphocholine	92 ± 35	93 (71 – 109)
Water-soluble choline[3]	203 ± 58	196 (171 – 237)
Phosphatidylcholine	185 ± 66	183 (135 – 222)
Sphingomyelin	20 ± 6	20 (16 – 24)
Lipid-soluble choline[4]	206 ± 71	200 (149 – 246)
Betaine	142 ± 69	131 (97 – 174)

[1]Dietary intakes were estimated during pregnancy at 36 weeks of gestation using a Food Frequency Questionnaire and the United States Department of Agriculture database on choline content in common foods.
[2]Total choline corresponds to the sum of all individual forms of choline.
[3]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.
[4]Lipid-soluble choline corresponds to the sum of phosphatidylcholine and sphingomyelin.

Table 3.5 Correlation between estimated daily dietary choline and betaine intakes during pregnancy and water-soluble forms of choline in milk from a subset of Canadian participants

	Milk metabolites (n = 143)[1]				
Dietary intakes	**Free choline**	**Phospho-choline**	**Glycerophospho-choline**	**Water-soluble forms of choline**[2]	**Betaine**
Total choline[3]	0.043	0.150	0.017	0.166	0.119[5]
Free choline	0.054	0.125	0.129	0.223	0.150[5]
Phosphocholine	0.134[5]	0.063[5]	-0.007[5]	0.128[5]	0.097[5]
Glycerophosphocholine	0.105	0.034	0.101	0.130	0.050[5]
Water-soluble choline[2]	0.102[5]	0.094[5]	0.093[5]	0.182[5]	0.116[5]
Phosphatidylcholine	-0.016	0.145	-0.056	0.091	0.096[5]
Sphingomyelin	-0.031[5]	0.147[5]	-0.109[5]	0.036[5]	0.110[5]
Lipid-soluble choline[4]	-0.016	0.150	-0.058	0.093	0.102[5]
Betaine	-0.026[5]	0.091[5]	0.057[5]	0.142[5]	0.010[5]

[1]Data are presented as Pearson's correlation coefficients after log-transformation, unless otherwise noted. None of the observed associations was significant after Bonferroni's correction (0.05 / 9 = 0.0055; *P*-value obtained was > 0.006, for all comparisons).
[2]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine
[3]Total choline corresponds to the sum of all individual forms of choline.
[4]Lipid-soluble choline corresponds to the sum of phosphatidylcholine and sphingomyelin.
[5]Data are Spearman's correlation coefficients.

3.5 Discussion

This study is the first report presenting data on concentrations of the water-soluble forms of choline in mature milk samples from both Canadian (*n* = 301) and Cambodian (*n* = 67) lactating women. The main findings from this study are: 1) the concentrations of water-soluble forms of choline did not differ between milk samples collected from Canadian and Cambodian women; 2) the estimated intake of total choline from milk was lower than the current AI for choline for infants aged 0 – 6 mo; and 3) the concentrations of water-soluble forms of choline in milk were not associated with dietary choline intake during pregnancy in a subset of the Canadian participants.

It has been previously reported that the concentration of choline in human milk is influenced by maternal choline intake. Specifically, lactating women consuming a choline-supplemented diet close to 1000 mg/d (930 – 1088 mg/d) had significantly higher choline concentration in milk by 20% compared to a control group (364 – 480 mg/d) (16, 17). In addition, a study from 1982 reported that free choline concentration in milk was lower in lactating women from Ecuador (*n* = 55, 40% < 100 µmol/L) compared to the United States (*n* = 11, 55% > 300 µmol/L) (22). The authors hypothesized that the difference was a result of the low dietary choline intake; however dietary information from lactating women in Ecuador and United States was not collected. In Cambodia, it has been reported that food intake primarily consists of rice and is low in animal foods (181, 290). Even though food balance sheets from FAO cannot be used to quantitate dietary choline intake, they suggest that it is likely that choline intakes in Cambodia are lower compared to Canada given the low intake of energy from animal products (9% compared to 27%, respectively) (180). As such, the present hypothesis was that Cambodian

lactating women would produce milk with lower choline concentrations compared with Canadian lactating women. Surprisingly, concentrations of water-soluble forms of choline in mature milk did not differ between Canadian and Cambodian (median, 1066 and 1072 µmol/L, respectively, $P = 0.737$) lactating women. In the present study, usual dietary choline intake in Canada (408 mg/d) was closer to the amount of choline consumed in the control groups of previous studies (364 – 480 mg/d) than the supplemented groups (close to 1000 mg/d) (16, 17). Thus, the difference in the dietary choline intake between Canada and Cambodia may not reach the range in which a difference in milk choline concentration was observed.

Additionally, the mammary gland can obtain choline from either maternal circulation (169) as well as from endogenous synthesis through the phosphatidylethanolamine methyltransferase (PEMT) pathway (170). Although it has been described that PEMT is mostly active in liver and kidney (68, 291), its activity has also been identified in the epithelial cells of the mammary gland (170). The PEMT pathway may contribute to ensuring that sufficient choline is excreted in milk for the rapid growth and development that occurs during infancy. Taken together, we did not find a statistically significant difference in milk water-soluble forms of choline concentrations between Canadian and Cambodian lactating women. This may be explained by dietary choline intakes in both populations lower than the threshold at which differences are observed in previous studies, as well as a potential mediating effect by endogenous choline synthesis in the mammary gland.

The estimated mean concentration of total choline in mature milk reported in this study was 1312 µmol/L ($n = 368$), which is in agreement with previously described values in the literature. A previous study from the United States reported a milk concentration of total choline

of 1254 µmol/L (*n* = 16) (14). Later studies, from the same country, have reported similar concentrations of total choline of 1198 µmol/L (*n* = 48) (16) and 1225 µmol/L (*n* = 28) (17). In Turkey, higher concentrations of total choline in milk have been found, ranging between 1441 µmol/L (*n* = 12) (15) and 1588 µmol/L (*n* = 54) (173). It is important to note that different analytical methods have been used for choline quantification, such as LC-MS/MS in the present study and the ones from the United States, whereas enzymatic and LC-electrochemical detection for the previous studies in Turkey. The difference in the methods, including extraction steps and instrument sensitivity, might have impacted the higher choline concentrations reported in Turkey (15, 202, 292, 293).

The results of the present study indicate that the mean estimated total choline intake of 106 mg/d is significantly below the current AI for choline (125 mg/d) for infants aged 0 – 6 months (12). In 1998, the Institute of Medicine set dietary choline recommendations as an AI of 125 mg/d during early infancy. Typically, the AI for infants is based on the mean concentrations of nutrients in milk from well-nourished lactating women. However, it is important to note that the AI for choline for infants of 0 – 6 months reflected a milk total choline concentration of 1500 µmol/L. This concentration is 20% higher than the mean concentration of total choline in human milk published at that time (1254 µmol/L, *n* = 16) (14). No rationale was provided as to why a higher concentration was used to set the AI. Accordingly, this suggests that the current AI for choline for infants of 0 – 6 months as a cut-off should be interpreted with caution as the current recommendation may not accurately reflect the mean total choline concentration in human milk.

The milk concentrations reported in the present study are also relevant from a clinical perspective, as the nutrient concentrations in human milk may be used as a guideline to develop

human milk substitutes and enteral formulas for infants. Current guidelines for total choline content in infant formula recommend a minimum of 7 mg/100 kcal and a maximum between 30 to 50 mg/100 kcal for choline content (294, 295). This range is equivalent to a total choline intake between 37 to 265 mg/d, based on volume consumption of 0.78 L/d and energy content of 68 kcal/100 ml (294). Most of the commercially available infant formulas add choline as choline chloride (74% choline) and also include a small amount coming from soy lecithin (2% choline, and included as an ingredient in many formulas). Hence, total choline content ranges between 12 – 24 mg/100 kcal in infant formulas (estimated total choline intake 64 – 127 mg/d). Moreover, the database on choline provides information on the proportion of the different forms of choline present in infant formula: free choline contributes 57% of total choline (13) compared to only 10% in human milk, thus indicating that infant formulas do not mimic the profile of choline forms present in human milk. The reason why most choline in human milk is present in other forms, including phosphocholine and glycerophosphocholine, rather than as free choline, is not known presently. It is possible that these forms of choline may be physiologically meaningful, as they differ specifically in absorption and metabolic fate (18).

Furthermore, serum free choline concentrations in breastfed infants have been positively correlated with concentrations of total choline in human milk ($r = 0.306$, $P < 0.01$), and free choline concentrations in breastfed infants were two-fold higher compared to formula-fed infants (21.8 vs. 10.8 µmol/L, respectively) (15). In that study (15), choline in infant formulas was analyzed and ranged between 311 – 2270 µmol/L (estimated total choline intake 26 – 189 mg/d), however no specific identification on the actual formula consumed by the formula-fed infants was provided. Therefore, further work is needed to confirm this observation of a different serum free choline concentrations between breastfed and formula-fed infants, and the relationship

between infants' dietary choline intake and serum free choline concentrations. It is important to mention that choline transport through the blood-brain barrier has been described to be bidirectional and dependent on circulating free choline concentrations (296); this may suggest an elevated uptake of choline in breastfed infants compared to formula-fed infants.

In this study, dietary total choline intakes were estimated using an FFQ during pregnancy in a subset of the Canadian participants. It was found that total choline intake was not significantly associated with the concentrations of water-soluble forms of choline in milk, nor were dietary intakes of individual water-soluble forms associated with their concentrations in milk. In line with this finding, another study reported that habitual choline intake was not associated with any of the milk individual water-soluble forms of choline, and only total choline intake was weakly associated with milk phosphatidylcholine concentration (R^2 = 0.11, P = 0.007) (16). It, however, could be possible that, in the present study, maternal intake during pregnancy was not the same as when the milk samples were collected at 8 weeks postpartum. However, in the present study, it was found that total choline intakes at 16 and 36 weeks of gestation (410 and 408 mg/d, respectively) were positively associated (r = 0.590, P < 0.001), suggesting that choline intake was stable over time, at least during pregnancy. This result agrees with previous studies that have observed a minor and non-significant variation in the maternal diet when comparing intakes during pregnancy and lactation periods (6, 10).

Limited data are available on dietary choline intake during pregnancy and lactation. In the United States, choline intake in pregnancy has been estimated to range between 328 mg/d (FFQ, n = 813) (7) and 364 mg/d (3-day food record, n = 46) (16), and 369 mg/d (3-day food record, n = 50) during lactation (6). In Canada, choline intake during pregnancy has been

estimated to range between 302 mg/d (FFQ, *n* = 290) (11) to 353 mg/d (24-hour recall, *n* = 493) (10). In the present study, in a subset of Canadian women, the estimated dietary choline intake was 408 mg/d in late pregnancy (FFQ, *n* = 143). While the estimated dietary choline intake is comparable to the high end of the previous reports from Canada and United States, it was significantly lower than both AI for choline for pregnant and lactating women (450 and 550 mg/d, respectively; $P < 0.001$ for both comparisons). Outside of North America, dietary choline intakes during pregnancy appear to be lower: 208 mg/d in the Republic of Seychelles, a high-income country (4-d food record, *n* = 273) (297); 279 mg/d in Jamaica, a middle-income country (FFQ, *n* = 16) (5); and 190 mg/d in pregnant women in Bangladesh, a low-income country (24-hour recall, *n* = 103) (176).

This study reports, for the first time, choline concentrations measured in mature human milk samples collected from lactating women in Canada and Cambodia, in a study that could be considered the largest recruitment of lactating women obtained to date for choline assessment (*n* = 368). In addition, choline concentrations were quantified by LC-MS/MS, enabling results that had both high sensitivity and specificity. Here, the association between dietary choline intake and choline concentration in milk in Canada was explored. A potential limitation here was that milk samples were collected at different time points during lactation among the Cambodian participants (3 – 28 weeks postpartum). However, it has been described that choline concentration in milk increases from birth to 2 weeks postpartum, and then stays stable beyond 6 months after birth (15, 21, 165, 166). In addition, in the present study choline concentration was not associated with the week of lactation of the milk collection ($P = 0.915$). Another limitation was that only the water-soluble forms of choline were quantified, without including the lipid-soluble forms. However, the water-soluble forms of choline contribute 84% of the total choline

concentration in human milk (14-17, 173). Also, dietary information was collected only from a subset of the Cambodian participants, and thus the association between dietary choline intake and water-soluble forms of choline in milk could not be assessed.

In summary, the present study is the first report showing that the concentration of water-soluble forms of choline in mature human milk did not differ between Canadian and Cambodian lactating women, despite what appears to be substantial differences in the availability of animal-source foods (and thus choline) in national diets. The estimated choline intake during early infancy was significantly below the AI for infants aged 0 – 6 months. Taken together, these data are particularly relevant because they help to define the normal range for choline in human milk, regardless of mother's location or socioeconomic status. Finally, given the discrepancy between the milk choline concentrations reported here and the higher concentration used to set the AI, the current choline AI for infants aged 0 – 6 months should be revisited.

Chapter 4: Assessing individual variability of the concentrations of choline and its associated metabolites in plasma among adults

4.1 Chapter synopsis

The associations between health-related outcomes and nutrient status are usually assessed using a single blood sample for the biomarker. Although choline is found in different forms in plasma, including free choline, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, and its associated metabolites that include betaine and dimethylglycine, the assessment of choline status is usually performed using plasma free choline concentrations. Limited information exists on the intra-individual day-to-day variability and postprandial changes in repeated measures for biomarkers of choline and its metabolites in plasma. In the present study, the intra-individual variability of choline and its metabolites in plasma was determined in apparently healthy adults (n = 40, mean age 33 y), based on three blood samples collected 12 days apart in a fasted state. Variability of choline and its metabolites in plasma was also determined after a recent meal intake, using a subset of the participants (n = 19). Plasma samples were analyzed for free choline and water-soluble forms using stable isotope dilution liquid chromatography-tandem mass spectrometry, and the lipid-soluble forms of choline using high-performance liquid chromatography with evaporative light-scattering detection. The results from this study indicated a moderate to good correlations (ICC $\geq$ 0.6) between the concentrations of choline and its associated metabolites in a fasted state and also in the fed state (ICC $\geq$ 0.5). Although most of the concentrations were significantly higher in the fed compared to the fasted state ($P < 0.05$), they still fall within the normal reference interval determined. These findings

suggest that a single time point blood sample can be considered adequate to assess choline and its associated metabolites status, in adults that are either fasted or in the fed state.

4.2 Introduction

Circulating concentrations of biomarkers are often used to monitor nutrient status and assess the risk for adverse health outcomes in populations (298). Typically, in human clinical and epidemiological health studies, biomarker information is based on obtaining only one blood sample per subject, thus producing a single time point measurement to predict health status. As a result, it is often uncertain if a single measurement is truly reflective of an individual averaged over an extended time period. It is therefore critical to know the variability in biomarker concentrations over time and whether this is caused by sample handling, sample processing, the analysis process itself, or merely the extent of temporal variability in individuals (223, 226, 299).

Choline is an essential dietary nutrient with different metabolic functions that include being a precursor molecule for the neurotransmitter acetylcholine (3, 229). Choline also serves as a component of phospholipids, such as phosphatidylcholine and sphingomyelin, which are essential for cell membrane synthesis, integrity, and lipid transport (62, 63, 300). In addition, choline can be oxidized to betaine, an important methyl group donor for the remethylation of homocysteine and thus the synthesis of methionine and dimethylglycine in the liver and kidney (301-303). In humans, circulating choline and betaine concentrations have been associated with several clinical outcomes in a number of studies (34), including neural tube defects (87), cognitive function (7, 88), fatty liver disease (24, 304), cardiovascular disease (91, 305, 306),

and cancer (92, 93). As such, circulating choline and betaine concentrations have emerged as biomarkers of potential risk of pathological processes.

Commonly, choline status is assessed using a single blood sample to quantify plasma free choline concentrations (2), and several factors can influence plasma choline concentrations and status, measured in an individual at a single time point. First, while typically plasma free choline is quantified, choline is found in plasma in the free form as well as lipid-soluble bound forms, such as phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine as components of lipoproteins (24, 307). Further, an important consideration is the intra-individual variability of the biomarkers, related to the inherent biological variability, raising the question of whether the individuals might be misclassified by performing the assessment only at a single time point.

Another important factor that can influence plasma choline concentration is whether the blood sample was obtained in the fasting state or following the consumption of a choline-containing meal. Although choline requirements are partially met by endogenous synthesis following sequential methylation of phosphatidylethanolamine (230, 308), the diet indeed is a major source of choline (12, 24). In addition to choline, betaine is also obtained from the diet (13, 159). Hence differences in dietary patterns concerning choline and betaine intakes might explain differences in plasma concentrations when blood samples are collected in the postprandial state (309). It is common that most of the protocols used to analyze nutrition related biomarkers require fasting blood samples (overnight > eight-hour) for adults. However, other specific age groups, such as infants, children, and pregnant women, a collection of fasting blood samples after an overnight fast may be not be feasible, nor ethical.

There are only a few studies in the literature that report on the intra-individual variability of plasma free choline, while more have reported on betaine concentrations (210, 223, 224, 227, 228, 306, 309-313). These studies usually have used limited sampling protocols that relied on collecting at only two time points. In addition, most of these studies have reported on variability in participants having chronic health conditions, such as diabetes (227) or lipid disorders (306) in the fasting state, while few reported on the impact of food intake on change of plasma choline concentrations (210, 224, 313). Moreover, earlier studies in humans described that the intake of different forms of choline could affect serum free choline concentrations differently, with one showing higher concentrations following intake of phosphatidylcholine compared to choline chloride (207, 210).

The overall objective of the present study was to determine the intra-individual variability of the concentrations of choline and its associated metabolites in plasma within repeated fasting blood samples (defined as > eight-hour) across three time-points, each collected 12 days apart in apparently healthy adults. Earlier studies have described that plasma free choline concentrations increase after food intake (210, 224, 313). Therefore, a secondary objective of this study was to assess the intra-individual variability of the concentrations of choline and its associated metabolites in plasma in a subset of participants that were in both fasted and also fed (defined as four-hour after food intake) states.

4.3 Participants and methods

4.3.1 Study design and participants

This study used a repeated measures design, where each participant served as their own control for the concentrations of choline and its associated metabolites in plasma. The study included three clinical study visits, each scheduled 12 days apart (Figure 4.1). For the first part, fasting blood samples were drawn during early morning after an overnight fast (> eight-hour), with only water consumed before each of the three visits. The participants recruited for this study were a convenience sample of 40 apparently healthy adults (both sexes) between the ages of 19 – 65 y from Vancouver, Canada as described in Chapter 2 of this dissertation. A sample size calculation indicated a minimum of 32 participants were required to provide 90% power to detect an intra-class correlation coefficient of ICC ≥ 0.75 (314) using three repeated observations per subject with significance at the 5% (two-tailed). The second part involved a subset of the participants (n = 19), who agreed in providing a fed state blood sample in the postprandial state after consuming a self-selected breakfast. A power analysis calculation indicated a minimum of 19 participants was required to provide 90% power to detect a large effect size (d = 0.80) (283, 284) in group means when comparing two repeated samples, with significance at the 5% (two-tailed). This postprandial state was defined as four hours after food intake, and it was selected based on the time necessary to significantly affect both the free choline (210, 313) and the lipid-soluble forms of choline (315) compared to fasted baseline. No particular dietary indications or control of intake occurred, as the intent was to reproduce a real-life scenario where the dietary intake of the participants is not controlled.

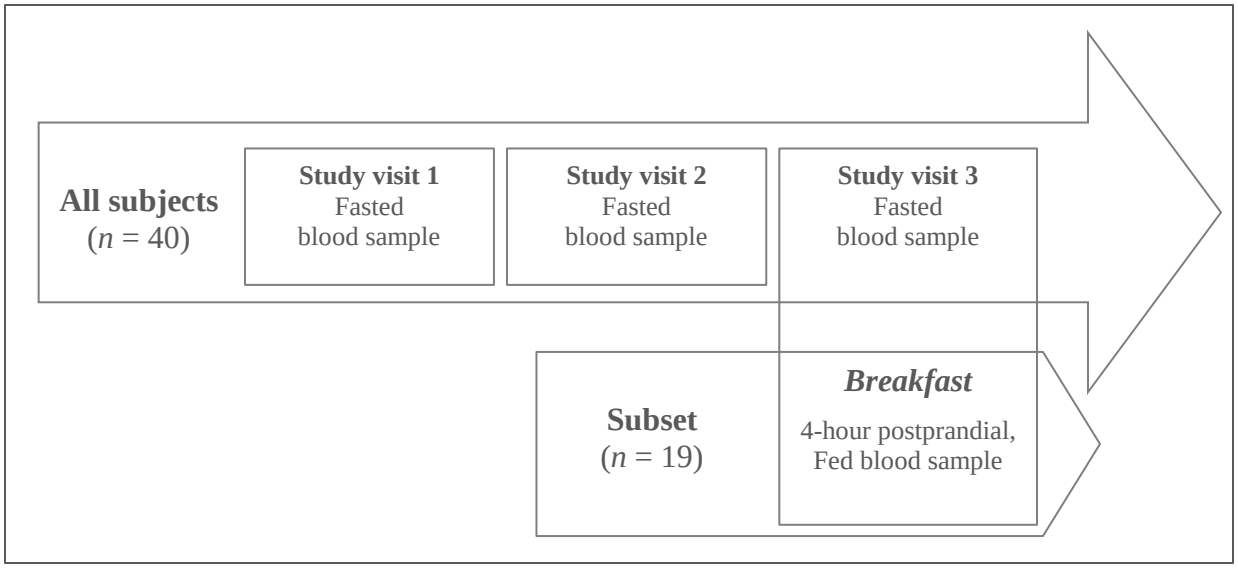

Figure 4.1 Diagram of study design

All protocols were reviewed and approved by the University of British Columbia – Children's & Women's Health Centre of British Columbia Research Ethics Board, according to the guidelines of the Declaration of Helsinki (252, 253). All subjects provided written informed consent prior to participation.

4.3.2 Blood sample collection and processing

At each clinical study visit, fasting venous blood was drawn by a trained phlebotomist and collected into ethylenediaminetetraacetic acid (EDTA) pre-coated vacutainers. Immediately after collection, the blood samples were placed on ice and transported to the laboratory to be processed. Subsequently, plasma was separated by centrifugation (2000 × *g;* 15 min; 4°C), then aliquots transferred into cryostat tubes, and frozen at -80°C until analyses. All blood samples were processed within 20 min from the time of blood collection.

4.3.3 Free choline, betaine, and dimethylglycine analysis in plasma

The concentrations of free choline, betaine, and dimethylglycine in plasma were quantified by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described previously (286, 316). The LC-MS/MS was a Waters I-class ACQUITY UPLC system connected to a Quattro Micro tandem MS configured with an electrospray source (Waters Corporation, MA, USA). The LC was equipped with a Zorbax Rx-Silicon pre-column (2.1 x 12.1 mm) and a column (2.1 x 150 mm), both with 5 µm particle size (Agilent Technologies, CA, USA).

For analysis, thawed aliquots of 50 µL of plasma were transferred to an Eppendorf tube (1.5 ml) containing 10 µL of internal standards (choline d-9, betaine d-9, and dimethylglycine d-6; CDN Isotopes Inc, QC, Canada). Plasma protein was precipitated by adding 100 µL of acetonitrile containing 0.1% formic acid to the tube followed by vortexing and centrifugation (13,000 × *g*; 10 min; 4°C). A 20 µL aliquot of the supernatant was transferred to a 150 µL glass insert placed inside a high-performance liquid chromatography vial, containing 100 µL of the mobile phase. The chromatographic separation was carried out using an isocratic mobile phase with a flow of 0.5 mL/min. The mobile phase consisted of 19% 15 mmol/L ammonium formate + 0.1% formic acid in H_2O, and 81% acetonitrile.

The MS was operated in positive ion multiple reaction monitoring mode, using the following paired precursor-product ion transitions: 103.9→59.9 choline and 113→68.9 choline-d9; 117.9→58.9 betaine and 126.9→67.9 betaine-d9; and 104.3→58.5 dimethylglycine and 110.4→64.2 dimethylglycine-d6. The auto-sampler chamber and column were maintained at 5°C

and 25°C, respectively. The injection volume used for the analysis was 4 µL, with a total sample run time of 7 min. Each plasma sample was only analyzed one time.

A standard curve and an in-house prepared pooled plasma samples were analyzed every day for quality assurance. The standard curve, corresponding to 7 different concentrations of the choline metabolites of interest (Table 4.1), yielding a standard linear curve ($r > 0.99$; Figure 4.2). In addition, the reagent blank and internal standard alone were run for quality control purposes. The unknown areas of identified peaks were compared with the area curve from the standard for quantification. The plasma concentrations of free choline, betaine, and dimethylglycine were expressed as µmol/L. Replicates of aliquots of pooled plasma were included for quality control and to determine the analytical variability.

Table 4.1 Concentrations of standard solutions for plasma concentrations

Standards	**Free Choline**	**Betaine**	**Dimethylglycine**
		Concentrations (µmol/L)	
1	1.0	5.0	0.5
2	2.0	10.0	1.0
3	3.0	15.0	1.5
4	5.0	25.0	2.5
5	10.0	50.0	5.0
6	15.0	75.0	7.5
7	20.0	100.0	10.0

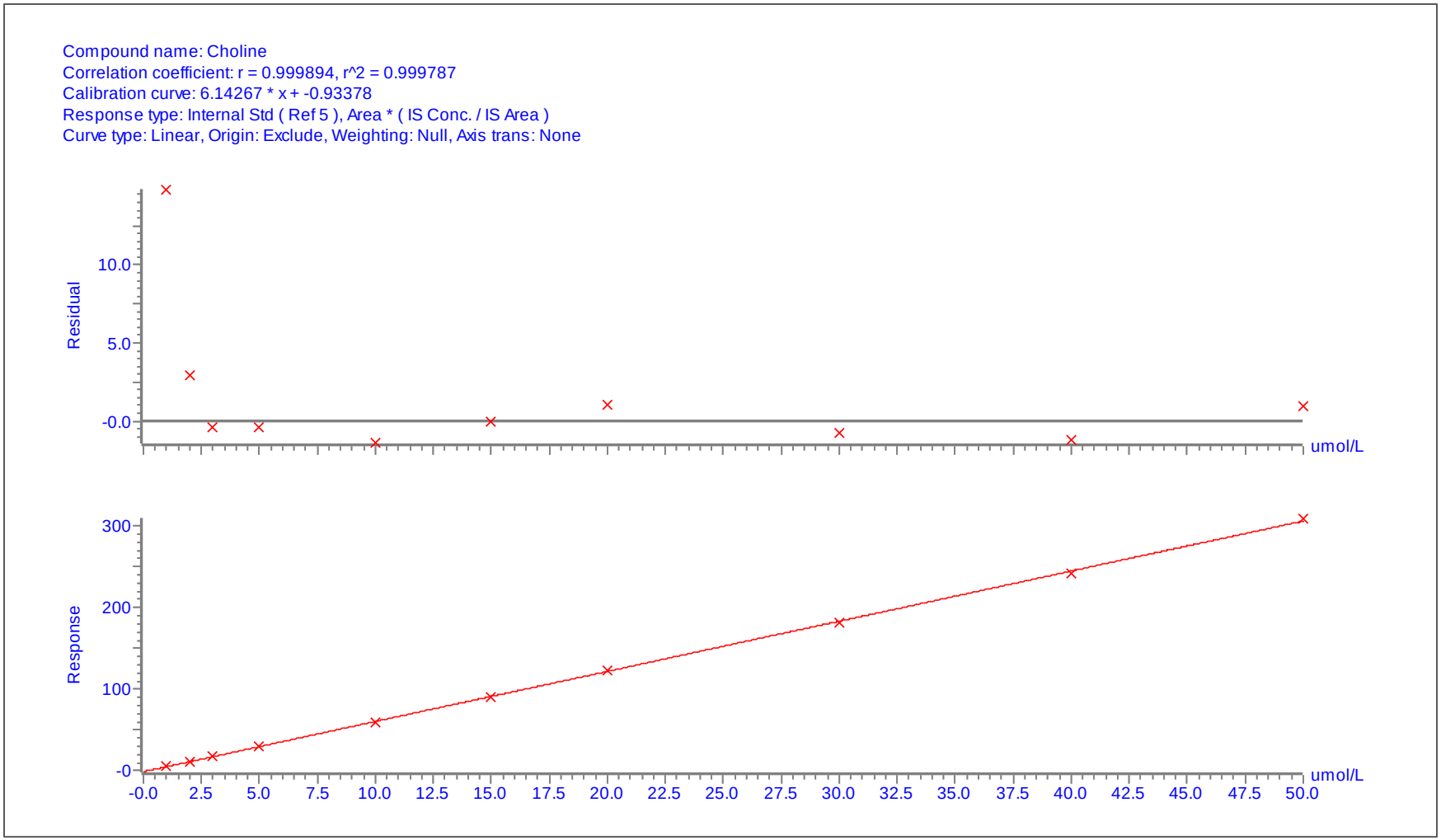

Figure 4.2 A representative plot of response factors and residuals from chromatograms of standard curve for free choline in plasma

4.3.4 Phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine analysis in plasma

Plasma phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine concentrations were quantified by high-performance liquid chromatography (HPLC), as described previously (317). The HPLC instrument (Waters 2690, Alliance Milford, MA) was equipped with an evaporative light scattering detector (Waters 2424, Alliance Milford, MA), an auto-sampler, and column heater. The column used for separating lipid classes was a YMC-Pack Diol120NP (25 cm × 4.6 mm, 5 µm).

Before analysis, total lipids were extracted from 250 µL of thawed plasma based on the Folch's method (318) with minor modifications (319); specifically, using relative proportions of chloroform:methanol:saline + plasma (6:3:2.25 v/v/v). The saline (0.9% sodium chloride) contained 15% EDTA in H_2O (w/v). Tubes were vortexed and centrifuged (2000 rpm; 5 minutes; 4ºC) to facilitate phase separation, and the organic layer was transferred to a new tube. The remaining aqueous layer was re-extracted twice with 6 mL chloroform, centrifuged, and transferred to the first two collections. The organic layer was dried under nitrogen, reconstituted in 160 µL of 2:1 chloroform:methanol (v/v), and transferred to HPLC autosampler vial. At this point, 30 µL of internal standard (1 mg/mL betulin) was added to allow for quantification, and the vial was dried down. Samples were prepared no more than one week in advance of being analyzed, and vials were stored at -80°C until analysis.

For analysis, the vials containing lipid extracts were re-suspended in 60 µL of HPLC solvent of methanol: chloroform:hexane:acetone (6:4:1:1 v/v). The separation of the different lipid classes used a quaternary linear gradient solvent system. Solvent A: hexane, B: methanol,

C: acetone-triethylamine 1000:17 (v/v), and D: isopropanol-acetic acid 1000:50 (v/v) with a flow rate of 2 mL/min. The injection volume used for the analysis was 20 µL, with a total analytical time of 60 min. Each plasma sample was only analyzed one time.

A standard curve was created for each lipid class of interest to allow identification and quantification of the individual peaks eluted and shown on the chromatogram. Area ratio of phosphatidylcholine/betulin, sphingomyelin/betulin, and lysophosphatidylcholine/betulin were calculated based in 8 different concentrations including 30 µL of internal standard (1 mg/mL betulin). The unknown areas of identified peaks were compared with the area curve from the standard for quantification. The plasma concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were expressed as mg/dL. Egg phospholipid extractions were included in each analytical run to check for consistency of the system each day.

4.3.5 Dietary intake assessment in a subset of the participants

Dietary choline and betaine intakes were estimated from a subset of the participants that provided a blood sample in a fed state (n = 19) after consuming a self-selected breakfast meal in the hospital cafeteria. The foods consumed at breakfast by each individual were specified on the receipt they received after purchasing the items. Dietary intakes of choline and betaine were estimated using a nutrient analysis software (ESHA Food Processor SQL, version 10.14.41; Salem, OR) (257) and the United States Department of Agriculture (USDA) database on the Choline Content of Common Foods (Version 2) (13). Detailed information is provided in Chapter 2 of this dissertation. The estimated dietary total and individual forms of choline and betaine by the participants were expressed as mg per meal.

4.3.6 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 19; SPSS Inc. Chicago, US) and MedCalc (version 17.9.2; MedCalc software bvba, Ostend, Belgium) was used to calculate reference intervals. The significance level was set at $P < 0.05$ for two-sided testing. Normality of the data was assessed using the Kolmogorov–Smirnov test. If distributions were skewed, data were then normalized using logarithmic transformations to improve normality before further analysis. If normality was not achieved after logarithmic transformation, non-parametric tests were used instead. All data are presented as mean ± SD, unless otherwise noted.

The analytical coefficient of variance ($CV_{analytical}$), also referred to as precision, for each metabolite was calculated from the same quality control pool using the equation: $CV_{analytical} = (SD/mean) \times 100$. To estimate the day-to-day intra-individual variability for the concentrations of choline and its associated metabolites in plasma, the total coefficient of variance (CV_{total}) was calculated for the three samples collected in fasting from each participant using the equation: $CV_{total} = (SD/mean) \times 100$. The intra-individual coefficient of variance ($CV_{intra-individual}$) was calculated using the formula: $CV_{intra-individual} = \sqrt{(CV_{total}^2 - CV_{analytical}^2)}$ (320). In addition, intra-individual variability for each metabolite was assessed by determination of intra-class correlation coefficient (ICC), sometimes referred to as the reliability coefficient. For the calculation of ICC from the repeated participant's samples, participants were considered as the random variable in the random-effects model. Interpretation for ICC was defined as poor < 0.50, as moderate between 0.50 – 0.74, good between 0.75 – 0.90, and excellent > 0.90 (314).

The effect of consuming a recent meal on the concentrations of choline and its associated metabolites in plasma was assessed by comparing fasted versus fed states using a paired samples Student's t-test in a subset of the participants (n = 19). Also, to evaluate the association of the concentrations of choline and its associated metabolites in plasma between fasted and fed state for each metabolite was assessed by determination of ICC and interpreted as previously defined. A reference interval (95% CI) was calculated for choline and its associated metabolites in plasma using all the fasting samples (n = 120) according to the current standards (2.5^{th} to 97.5^{th} percentile) (321). This reference interval was used to determine whether the concentrations of choline and its associated metabolites in plasma assessed in fed states fall within the limits of the reference interval.

In addition, the association between dietary choline and betaine intakes from breakfast and the difference in plasma concentration for each metabolite was assessed by using a Pearson's or Spearman's coefficient of correlation, as appropriate. Interpretation for all correlation coefficients was defined as weak < 0.30, moderate between 0.30 – 0.49, and strong > 0.50 (284). Bonferroni's correction was performed to adjust for multiple comparisons of dietary intakes on the same metabolite concentration in milk (0.05 / 9 = 0.0055, significance at $P < 0.006$).

4.4 Results

4.4.1 Sociodemographic characteristics of the participants

A total of 40 participants completed the protocol for repeated fasted blood samples collection, and their characteristics are provided in Table 4.2. The age of all the participants was (mean ± SD) 33 ± 12 y, with a BMI of 24.9 ± 4.9 kg/m^2. A subset of the participants (*n* = 19) completed the protocol for fasted versus fed blood samples collection and their characteristics are also presented in Table 4.2.

Table 4.2 Sociodemographic characteristics of the participants

	Participants	
Characteristics	**Repeated fasted** (*n* = 40)	**Fasted vs. fed** (*n* = 19)
	Mean ± SD	
Age (y)	33.4 ± 12.4	34.3 ± 12.4
Weight (kg)	73.3 ± 18.4	74.6 ± 20.1
Height (m)	1.7 ± 0.1	1.7 ± 0.1
BMI (kg/m^2)	24.9 ± 4.9	25.5 ± 5.0
Waist circumference (cm)	84.7 ± 14.4	85.9 ± 15.1
Sex (*n*; %)		
Men	21; 52.5	8; 42.1
Women	19; 47.5	11; 57.9
Ethnicity (*n*; %)		
European	24; 60.0	11; 57.9
Chinese	7; 17.5	4; 21.1
Other Asian	7; 17.5	2; 10.5
Latin-American	2; 5.0	2; 10.5

4.4.2 Concentrations and variability of choline and its associated metabolites in plasma from repeated samples collected in fasted state

The concentrations of free choline, betaine, dimethylglycine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine in plasma from three repeated samples per participant collected in the fasted state are presented in Table 4.3. Most of the plasma metabolites concentrations were normally distributed at all visits, except for free choline at visit-1, and phosphatidylcholine and sphingomyelin at visit-2.

The analytical, total, and intra-individual coefficients of variations calculated for plasma free choline, betaine, glycerophosphocholine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine from the three repeated fasted samples are presented in Table 4.4. For all metabolites, CVs were ≤ 5% for analytical variation. For total and intra-individual variability, the lowest CV found was for sphingomyelin concentrations (8.8% and 6.8%, respectively), while the highest was for dimethylglycine concentrations (13.5% and 13.1%, respectively). The ICCs were considered moderate for all metabolites, except for betaine and sphingomyelin, which were classified as good.

Table 4.3 Concentrations of choline and its associated metabolites in plasma from repeated samples collected in fasted state

Plasma metabolites		Concentrations in fasted state (n = 40)[1]		
		Visit 1	Visit 2	Visit 3
		Concentrations (µmol/L)		
Free choline	Mean ± SD	7.0 ± 1.8	7.3 ± 1.5	7.1 ± 2.1
	Median (IQR)	6.8 (6.0-7.5)	7.1 (6.4-8.1)	6.7 (6.0-8.5)
Betaine	Mean ± SD	37.5 ± 12.3	38.9 ± 11.4	37.2 ± 10.0
	Median (IQR)	34.3 (29.9-43.4)	39.1 (34.3-44.7)	36.5 (32.8-43.3)
Dimethylglycine	Mean ± SD	2.5 ± 0.8	2.5 ± 0.6	2.4 ± 0.6
	Median (IQR)	2.5 (1.9-2.8)	2.6 (2.1-3.0)	2.4 (2.0-2.8)
		Concentrations (mg/dL)		
Phosphatidylcholine	Mean ± SD	125.4 ± 25.9	138.7 ± 39.0	127.9 ± 25.7
	Median (IQR)	123.1 (107.5-146.4)	124.7 (118.1-158.5)	123.6 (108.6-147.0)
Sphingomyelin	Mean ± SD	23.6 ± 4.7	25.0 ± 6.2	23.4 ± 4.6
	Median (IQR)	24.0 (20.7-26.2)	24.2 (21.7-26.4)	23.0 (20.2-27.4)
Lysophosphatidylcholine	Mean ± SD	15.5 ± 4.1	16.5 ± 5.1	16.0 ± 3.9
	Median (IQR)	15.1 (13.1-17.5)	15.9 (13.0-18.8)	15.9 (14.1-17.3)

[1]Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography.

Table 4.4 Variability of the concentrations of choline and its associated metabolites in plasma from repeated samples collected in fasted states

Plasma metabolites	**Variability in fasted state** (n = 40)[1]			
	$CV_{analytical}$ (%)	**CV_{total}** (mean ± SD, %)	**$CV_{intra\text{-}individual}$** (mean ± SD, %)	**ICC**
Free choline	2.5	13.3 ± 7.4	12.9 ± 7.6	0.636
Betaine	2.2	12.1 ± 6.9	11.8 ± 7.1	0.770
Dimethylglycine	2.4	13.5 ± 9.4	13.1 ± 9.6	0.592
Phosphatidylcholine	5.0	11.2 ± 9.3	9.2 ± 10.2	0.629
Sphingomyelin	5.0	8.8 ± 8.4	6.8 ± 9.1	0.709
Lysophosphatidylcholine	5.0	12.8 ± 9.6	11.1 ± 10.5	0.687

[1]$CV_{analytical}$ was calculated from intra-assay samples, $CV_{intra\text{-}individual}$ was calculated = $\sqrt{(CV_{total}^2 - CV_{analytical}^2)}$.
ICC: intra-class correlation coefficient.

4.4.3 Concentrations and variability of choline and its associated metabolites in plasma from samples collected in fasted and fed state in a subset of the participants

The concentrations of free choline, betaine, dimethylglycine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine in plasma from a subset of participants that provided a blood sample in the fed state (n = 19) are presented in Table 4.5. Most of the plasma metabolites concentrations were normally distributed at both states, except for the concentration of phosphatidylcholine in fasted state and lysophosphatidylcholine in fed state. Overall, the plasma metabolites concentrations in the fed state were significantly higher compared to fasted state, except for sphingomyelin and lysophosphatidylcholine.

The highest significant increase in concentrations was observed for free choline (20%) followed by betaine (19%), while the lowest increases occurred for sphingomyelin and lysophosphatidylcholine (each 7%), which were not significantly different from the concentrations in a fasted state. For all metabolites, the ICCs were classified as moderate.

The reference interval for choline and its associated metabolites was calculated using the samples collected in a fasted state (n = 120) and is presented in Table 4.6. For choline and its associated metabolites in the fed state, none of the concentrations were outside the 95% CI of the lower and upper limits of corresponding reference interval calculated.

Table 4.5 Concentrations and variability of choline and its associated metabolites in plasma from samples collected in fasted compared to fed states in a subset of the participants

	Concentrations and variability in fasted and fed states (n = 19)[1]			
Plasma metabolites	**Fasted** Mean ± SD	**Fed** Mean ± SD	***P*-value**[2]	**ICC**
	Concentrations (µmol/L)			
Free choline	6.9 ± 1.7	8.3 ± 2.4	0.001	0.610
Betaine	35.7 ± 7.9	42.6 ± 11.4	0.001	0.567
Dimethylglycine	2.4 ± 0.6	2.8 ± 0.9	0.003	0.655
	Concentrations (mg/dL)			
Phosphatidylcholine	129.1 ± 24.4	143.4 ± 37.9	0.046	0.536
Sphingomyelin	24.0 ± 4.4	25.6 ± 6.4	0.125	0.679
Lysophosphatidylcholine	16.8 ± 4.3	18.0 ± 4.0	0.061	0.694

[1]Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography.
[2]Group differences compared by paired samples Student's t-test after log-transformation.
ICC: intra-class correlation.

Table 4.6 Reference intervals for choline and its associated metabolites in plasma from samples collected in fasted state

	Reference intervals for fasted state (n = 120)[1]			
	Lower limit		**Upper limit**	
Plasma metabolites	Mean	95% CI	Mean	95% CI
	Concentrations (µmol/L)			
Free choline	3.5	3.3 – 4.5	12.0	10.1 – 14.5
Betaine	13.6	10.7 – 19.0	63.2	57.0 – 73.7
Dimethylglycine	1.1	1.1 – 1.4	4.2	3.6 – 4.5
	Concentrations (mg/dL)			
Phosphatidylcholine	81.8	11.9 – 85.8	212.3	178.6 – 269.6
Sphingomyelin	15.8	13.1 – 16.2	37.8	31.9 – 42.8
Lysophosphatidylcholine	8.6	7.4 – 9.4	27.1	24.2 – 31.0

[1]Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography.
[2]Reference intervals were calculated using MedCalc according to current standards (321). The lower limit corresponds to 2.5th percentile, and the upper limit corresponds to 97.5th percentile of the concentrations in a fasted state.

4.4.4 Associations between estimated dietary choline and betaine intakes and the difference of the concentrations of choline and its associated metabolites in plasma from samples collected in fed and fasted states in a subset of the participants

The estimated intakes of choline and betaine that occurred at the breakfast meal are presented in Table 4.7. Most of the estimated intakes were normally distributed, while the distribution of the estimated phosphocholine, sphingomyelin, and betaine intakes were skewed.

The correlation coefficients between dietary choline and betaine intakes and the difference in the concentrations in plasma comparing fed and fasted states are presented in Table 4.8. Most of the difference in the concentrations in plasma comparing fed and fasted states were normally distributed, except for sphingomyelin, and betaine concentrations that were skewed.

The difference in free choline concentration was positively associated with dietary free choline (r = 0.481, P = 0.037), phosphocholine (*rho* = 0.553, P = 0.014), and betaine (*rho* = 0.611, P = 0.005) intakes. Similarly, the difference in betaine concentration was positively associated with dietary phosphocholine (*rho* = 0.580, P = 0.009) and betaine (*rho* = 0.526, P = 0.021) intakes. A significant negative correlation was also observed between the difference in lysophosphatidylcholine concentration and glycerophosphocholine intake (r = -0.503, P = 0.028). Total choline intake was not significantly correlated with any of the differences in plasma concentrations of choline and its associated metabolites. After adjusting for multiple comparisons (Bonferroni's correction, $P < 0.006$), only the positive, strong correlation between betaine intake and the difference in plasma free choline concentration remained significant.

Table 4.7 Estimated dietary choline and betaine intakes from a subset of participants

Nutrients	**Dietary intake** (n = 19)[1]	
	Mean ± SD	Median (IQR)
	Intakes (mg/breakfast meal)	
Total choline[2]	177.7 ± 119.4	176.0 (53.5 – 261.9)
Free choline	14.4 ± 7.3	15.0 (8.5 – 21.4)
Phosphocholine	1.9 ± 1.2	1.6 (1.2 – 2.2)
Glycerophosphocholine	8.8 ± 5.5	7.9 (5.2 – 11.0)
Water-soluble choline[3]	25.1 ± 11.8	28.2 (13.9 – 33.5)
Phosphatidylcholine	143.3 ± 113.4	156.2 (11.2 – 225.4)
Sphingomyelin	9.3 ± 7.8	8.7 (1.5 – 13.1)
Lipid-soluble choline[4]	152.6 ± 121.0	165.3 (12.3 – 240.5)
Betaine	22.5 ± 19.2	16.6 (15.0 – 22.1)

[1]Dietary intakes were estimated using the USDA database on choline content in common foods.
[2]Total choline corresponds to the sum of all individual forms of choline.
[3]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.
[4]Lipid-soluble choline corresponds to the sum of phosphatidylcholine and sphingomyelin.

Table 4.8 Correlation between estimated dietary choline and betaine intakes and the difference of the concentrations of choline and its associated metabolites in plasma from samples collected in fed and fasted states in a subset of the participants

	Difference in fed and fasted plasma (n = 19)[1]					
Dietary intakes	**Free choline**	**Betaine**	**Dimethyl-glycine**	**Phosphatidyl-choline**	**Sphingo-myelin**	**Lysophospha-tidylcholine**
Total choline[2]	0.020	0.073[5]	0.293	0.157	-0.074[5]	0.308
Free choline	0.481*	0.343[5]	0.268	0.089	0.001[5]	-0.050
Phosphocholine	0.553*[5]	0.580**[5]	0.450[5]	-0.070[5]	-0.229[5]	-0.341[5]
Glycerophosphocholine	0.201	0.165[5]	0.268	-0.242	-0.052[5]	-0.503*
Water-soluble choline[3]	0.439	0.372[5]	0.331	-0.070	-0.022[5]	-0.300
Phosphatidylcholine	-0.025	0.022[5]	0.255	0.159	-0.084[5]	0.331
Sphingomyelin	0.022[5]	0.097[5]	0.285[5]	0.184[5]	-0.049[5]	0.273[5]
Lipid-soluble choline[4]	-0.023	0.071[5]	0.162	0.162	-0.041[5]	0.334
Betaine	0.611***[5]	0.526*[5]	0.432[5]	0.146[5]	-0.006[5]	-0.272[5]

[1]Data are presented as Pearson's correlation coefficients, unless otherwise noted. Correlation is significant at * 0.05, ** 0.01, and *** 0.006. (Bonferroni's correction was used to adjust the significance at 0.006).
[2]Total choline corresponds to the sum of all individual forms of choline.
[3]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.
[4]Lipid-soluble choline corresponds to the sum of phosphatidylcholine and sphingomyelin.
[5]Data are Spearman's correlation coefficients.

4.5 Discussion

The objective of the present study was to estimate the variability of the concentrations of choline and its associated metabolites in plasma among apparently healthy adults (*n* = 40, both sexes, mean age 33 y) in Vancouver, Canada. The main findings of this study are: 1) in general, moderate ICCs were found for biomarker concentrations when three blood samples were collected in fasted state; 2) although most of the biomarker concentrations were higher in samples collected in fed compared to fasted state collected on the same day, all concentrations fell within the 95% CI of the reference interval calculated for the fasted state, and moderate ICCs were found between fasted and fed states for all biomarkers; and 3) the postprandial change in plasma free choline concentration was positively correlated with the prior meal's dietary intake of betaine, but no other association was found.

Previous reports have shown that variability in biomarker concentrations can arise from different factors related to pre-analytical, analytical, and intra-individual variabilities (322, 323). The use of standardized protocols for blood collection which include the time of collection, handling and storage procedures are essential to ensure that pre-analytical variability for assessing choline status is kept to a minimum. In this study, the low $CV_{analytical}$ values ($\leq$ 5%) found were accomplished by analyzing all samples from the same participant compiled in the same analytical run performed by one analyst to minimize the inter-assay variation (212). As such, in the present study, it was shown that the analytical variability reflects sound methodology and technical skills.

Intra-individual variability, also referred to as biological variability, is defined as random fluctuation around the homeostatic set points of individual sample analyses, which indicates the potential usefulness of the biomarker (212). The $CV_{intra\text{-}individual}$ obtained in this study (*n* = 40, apparently healthy adults, both sexes) based on three samples collected in fasted states over a 25-day period for plasma free choline (13%), betaine (12%), and dimethylglycine (13%) were similar to those reported from previous studies. The $CV_{intra\text{-}individual}$ has been reported to range between 14% (227) to 16% (228) for free choline, between 15% (227, 228) to 35% (310) for betaine, and 17% for dimethylglycine (227, 228) including healthy and diabetic participants. Taken together, it appears that plasma free choline, betaine, and dimethylglycine concentrations vary close to 17% between days in adults.

The ICCs, or coefficients of reliability, obtained in the present study ranged from moderate to good for plasma free choline (0.64), betaine (0.77), and dimethylglycine (0.59). For free choline, a similar ICC of 0.67 was found among diabetic patients (*n* = 243, fasting at four-time points at six-month intervals over a two-year period) (227), whereas a much lower ICC of 0.29 was found in healthy postmenopausal women (228). In general, moderate to excellent ICCs have been reported for plasma betaine and dimethylglycine, ranging from 0.63 (92) to 0.89 (324, 325) for betaine; and from 0.55 (317) to 0.93 (326) for dimethylglycine. The inconsistency observed between the ICC for free choline in the present study, and the postmenopausal women study (228) may be due to the fact that the samples were collected at different time intervals. Although samples were collected in the fasted state in both studies, in our study blood samples were collected at three time points at 12-day intervals over a 25-day period, while in the postmenopausal women study was at two-time points between 1 – 2 years apart (228). As such, the more extended time interval may reflect a longer-term variation. Alternatively, it may be

possible that in the postmenopausal women a decrease in the estrogen-enhanced endogenous synthesis of choline, mediated via phosphatidylethanolamine N-methyltransferase (25, 72), and thus impacting the variability in plasma concentrations. Taken together, it appears that the variability in plasma free choline concentrations may depend on the stage of the life cycle and the time interval that blood samples are collected.

Circulating betaine concentrations reflect both dietary intake (159) and choline oxidation (327), and it has been previously suggested that circulating betaine concentrations are homeostatically controlled (310, 328). It is unknown, however, if this is related to its role as an osmolyte or as a methyl group donor. There is no previous information available on the intra-individual variability for plasma phosphatidylcholine, sphingomyelin, or lysophosphatidylcholine, but the values obtained for these were in agreement with the values obtained for the other biomarkers analyzed in the present study. Taken together, the low mean intra-individual variability and moderate to good ICCs for the biomarkers analyzed found in this group of apparently healthy adults suggest that concentrations measured in the fasted state provide information of the status based on a single measurement.

In the present study, plasma concentrations collected (n = 19, healthy adults) under fed states (four-hour after a self-selected breakfast) were relatively higher (7 – 20%) compared to fasted states (≥ eight-hour overnight fasting). Although the increase was statistically significant, all fed concentrations fell within the reference interval calculated for the fasted state, and therefore it suggests that the increase may not be biologically relevant. Few data are available on the effect of recent food intake affecting the concentrations of choline and its associated metabolites in plasma. An early study reported a small (13%), but significant, increase in plasma

free choline one-hour after lunch which suggested that recent food intake influences plasma free choline concentrations (224). Later studies have shown an increase ranging from 25 – 80% in plasma free choline, betaine, and dimethylglycine concentrations between two to four hours postprandial (223, 309, 329). However, in a previous study reported intakes of 846 mg for choline and 846 mg for betaine (309), whereas in the present study dietary intakes were relatively lower at 178 and 23 mg, respectively. Accordingly, a potential explanation for the considerably higher increase in plasma concentrations may be due to the higher choline or betaine intakes consumed by the participants in the previous study.

The findings from the present study are in agreement with the limited data available on comparing the concentration of choline and its associated metabolites in fasted and fed states. For betaine concentration, a strong positive correlation (r = 0.92) has been found among samples collected two to six hours after breakfast compared to fasted state (310). Similarly, a positive correlation coefficient 0.65 has been reported for dimethylglycine concentrations (311). Overall, the previously reported reliability coefficients are similar to the ones obtained in the present study, which were 0.70 for betaine and 0.79 for dimethylglycine. Although the statistical difference in the concentrations of free choline, betaine, dimethylglycine, and phosphatidylcholine in fed state obtained in the present study should be recognized, there was a low variability between fasted and fed states as indicated by ICCs obtained. As such, the samples collected in fed states may still be useful indicators to suggest a relationship between plasma concentrations and health outcome risks.

This study had several strengths. First, standardized protocols were in place to minimize pre-analytical sources of variability such as constant blood collection time, single phlebotomist, handling and storage of the samples, as these have been shown to impact plasma concentrations (299). Second, the analytical variability was low, as a single analyst quantified plasma concentrations, also including calibration and quality controls for the instruments. Third, this study is the first study exploring the impact of recent choline and betaine intakes from a self-selected breakfast on the concentrations of choline and its associated metabolites in plasma. This scenario allowed the determination of intra-individual variability of plasma biomarker concentrations in a situation that resembles real-life of free-living subjects without controlling the choline amount consumed in the meal.

In addition, the present study also has some limitations. The blood samples were collected from a small number of individuals, in contrast to larger sample sizes from studies using patients from routine health-care monitoring examinations, including lipid and diabetic patient clinics. However, a sample size of six to 12 participants has been recommended for variability studies (330), and the sample size calculation showed adequate power to detect ICC > 0.75 (314). Also, the convenience sample was from a homogeneous set of apparently healthy individuals, and it has been suggested for some nutrients that intra-individual variability may differ between ethnic groups (331). Furthermore, in the present study, the assessment of the postprandial concentrations was performed at only one point in time (four-hour after a meal); this might not have captured the maximum difference between fasted and fed biomarker concentrations, that may potentially have impacted the value of the ICCs. Nevertheless, based on a previous study, concentrations in plasma samples collected four hours after a meal significantly differed in comparison to the fasted baseline (315).

In summary, the present study found low mean intra-individual variability based on three-time point blood samples collected in fasted states for choline and its associated metabolites. Plasma concentrations in the fed state were within the reference interval limits for the fasted state. Moreover, using a single time point sample from participants in the fed state still, would generally perform satisfactorily in ranking participants, based on the moderate to good correlations obtained. Therefore, the results presented herein suggest that status of plasma choline and its associated metabolites may well be assessed using a single blood sample in fasted or fed (four hours after a meal). These data are relevant for the assessment of individual subjects and study design, as undoubtedly, repeated sampling in research is impractical and not always possible. Given the important roles of choline in metabolism more research is warranted to determine choline status in the general population.

Chapter 5: Dietary choline and betaine intakes and the concentrations of free choline and its metabolites in plasma in various age groups in Canada

5.1 Chapter synopsis

Choline is an essential dietary nutrient that plays essential roles in the life cycle. Higher intakes of choline have been associated with a lower risk of neural tube defects and cancer, improved neurodevelopment and cognition, and prevention of fatty liver. However, there were limited data available on choline at the time that dietary choline recommendations were set in 1998. Since then, most of the studies have been focused on choline nutrition during pregnancy and lactation or the association between choline and the risk of adverse health outcomes in adults. Only a few studies have characterized choline nutrition, including dietary choline intake and plasma free choline concentrations. Data from healthy populations during childhood are also lacking. This cross-sectional study was conducted to characterize the dietary choline intake and plasma free choline concentrations across the life cycle including apparently healthy toddlers, children, men, and postmenopausal women from Vancouver, Canada. In this study, daily choline intakes were estimated using a semi-quantitative food frequency questionnaire and plasma free choline concentration was quantified using stable isotope dilution liquid chromatography-tandem mass spectrometry. The principal findings from this study included that the dietary intake of the individual forms of choline changed from primarily water-soluble to lipid-soluble from early life to adulthood and that dietary total choline intake was not significantly associated with plasma free choline concentrations. Overall, these findings contribute towards the information available about dietary total and individual forms of choline intakes and plasma free choline

concentrations in healthy populations. However, more research is needed for a better understanding of other factors influencing the relationship between dietary choline intake and plasma free choline across the life cycle.

5.2 Introduction

Increasing evidence suggests that both low dietary total choline intakes and low plasma free choline concentrations are associated with adverse health outcomes at different life stages in humans. During the periconceptional period, women consuming the highest quartile of dietary total choline intake (> 498 mg/d) had a reduced risk for neural tube defect (OR = 0.49), compared with the lowest quartile of choline intake (< 290 mg/d) (85, 87). In early childhood, dietary total choline intake and the concentrations of free choline and betaine in plasma have been positively correlated with cognitive test scores (88, 89). In addition to hepatic lesions (40, 90), choline-devoid diets (< 50 mg/d) have also been linked to muscular damage in both adult men and women (25). Moreover, choline has been linked to cardiovascular diseases (91) and various types of cancers (94-98), such as breast (94, 96), hepatic (98), and lung (95) cancer. Therefore, this evidence clearly demonstrates that choline is important for health throughout the life cycle (12, 24, 332).

Although the AIs for choline were set as total choline, dietary choline comprises both water-soluble (free choline, phosphocholine, and glycerophosphocholine) and lipid-soluble (phosphatidylcholine and sphingomyelin) forms of choline (159). Human milk is the principal dietary source of choline for breastfed infants, with the majority of choline in milk (84%) present as the water-soluble forms (14-16). In contrast, the predominant dietary form of choline for

adults has been reported as phosphatidylcholine (332), with liver, meats, and egg as the richest food sources (13). Studies conducted in rodents have shown that the different forms of dietary choline differ in bioavailability and metabolic fate (18), and differently impact the development of the immune system of the offspring (19). Thus, it may be relevant to consider both water- and lipid-soluble forms of choline, as well as total choline, in studies focusing on dietary choline intake assessment.

Plasma free choline concentration has been commonly used to assess choline status across the life cycle (89, 216, 222, 333). It has been shown that plasma free choline concentrations are significantly impacted when total dietary choline is modified. Specifically, plasma free choline concentrations decreased (-30%) after three weeks of low intake of choline (< 50 mg/d) in healthy men (24). Moreover, plasma free choline concentrations significantly increased (78%) after supplement choline intakes (1088 mg/d) (16). However, plasma free choline concentration cut-off points for determining nutrition status of choline in the body remain to be determined.

Presently, despite choline importance across the life cycle, the data available on dietary choline and betaine intakes and the concentrations of free choline and its associated metabolites in plasma are scarce (2). Information is particularly limited in early childhood and the later stages of life (15, 191, 217, 218). The few studies published have been mostly focused on choline nutrition during pregnancy and lactation (5, 7, 8, 10, 11, 218, 334) or adulthood (184, 186, 191, 233, 335). Therefore, the overall objective of the study presented in this chapter was to determine dietary choline and betaine intakes and the concentrations of free choline and its related metabolites in plasma in apparently healthy age groups including toddlers, children, men,

and postmenopausal women in Vancouver, Canada. A secondary objective of the present study was to explore the association between dietary choline and betaine intakes and the concentrations of free choline and its associated metabolites in plasma within each age group.

5.3 Participants and methods

5.3.1 Study design and participants

The study presented in this chapter was a cross-sectional analysis of convenience samples of apparently healthy subjects from different age groups of the life cycle. The participants were recruited from the Vancouver area between 2008 and 2015, using poster advertisements and through direct contact. Specifically, the participants were enrolled by our research group under different protocols including: toddlers (n = 133) (336), children (n = 285) (337), men (n = 77), and postmenopausal women (n = 77). A power analysis calculation indicated a minimum of 24 participants for each group was required to provide 90% power to detect a medium effect size difference (d = 0.40) (283, 284) in group means when comparing four independent samples, with significance at 5% (two-tailed). For the secondary objective, a power size calculation indicated a minimum of 38 participants was required to provide 90% power to detect a large effect size in correlation ($r \geq 0.5$) (283, 284) between dietary intake and plasma concentrations with significance at 5% (two-tailed). The clinical visits were conducted at the Child and Family Research Institute at the Oak Street campus of the University of British Columbia in Vancouver, Canada.

For toddlers and children, the inclusion criteria were age between 12 – 14 months (336) and 5.7 – 5.9 years (337), respectively, born at term gestation (> 37 weeks of gestation), birth weight ≥ 2500 g, singleton birth, maternal age at birth between 20 – 40 years, and caregiver able to understand and speak English. For men, the inclusion criteria were age 19 – 50 years, able to understand and speak English. For postmenopausal women, inclusion criteria were age 50 – 65 years, at least one year after menopause and not taking hormone replacement therapy, and able to understand and speak English. Exclusion criteria for all of the age groups were having any chronic health condition, taking supplements or prescriptions, and following a special diet (i.e., vegan).

At the clinical visit, sociodemographic information including age, sex, and household income (categorical: < $30 000, $30 000 – $50 000, and > $50 000) were collected by questionnaire. For adults, education level (categorical: high school or less, college or diploma, and university degree) and ethnicity were collected; for toddlers and children, maternal education and ethnicity information was collected. Anthropometric measurements were also taken using standard procedures, with subjects in light clothing and no shoes. Body weights were measured using a digital scale to the nearest 0.1kg. Heights were measured by a wall-mounted stadiometer to the nearest mm. Anthropometric measurements were repeated at least three times, and the average values were used.

All protocols were reviewed and approved by the University of British Columbia – Children's & Women's Health Centre of British Columbia Research Ethics Board, according to the guidelines of the Declaration of Helsinki (252, 253). All subjects (or their legal guardians) provided written informed consent prior to participation.

5.3.2 Dietary intake assessment

Daily dietary intakes of choline and betaine from the participants were estimated using a semi-quantitative food frequency questionnaire (FFQ). For toddlers and children, the primary caregiver completed the FFQ. The FFQ gathered information on the foods and beverages consumed, including frequency of food consumed and portion sizes, to capture the habitual diet over the previous month. Dietary intakes of choline and betaine were estimated using a nutrient analysis software (ESHA Food Processor SQL, version 10.14.41; Salem, OR) (257) and the United States Department of Agriculture (USDA) database on the Choline Content of Common Foods (Version 2) (13). Detailed information is provided in Chapter 2 of this dissertation. The estimated dietary total and individual forms of choline and betaine by the participants were expressed as mg per day.

Food group classifications were based on the USDA database (13), with some modifications. Food groups were divided as: eggs, dairy (milk, yogurt, cheese, and butter), meats (beef, pork, lamb, poultry, and seafood), grains (bread, crackers, rice, quinoa, and pasta), vegetables, fruits, legumes, mixed dishes (e.g., pizza, stew, and lasagna), pastry (e.g., muffins and cake), beverages (e.g., tea, coffee, beer, and wine), and others (e.g., oils and condiments). The contribution of each food group to the daily dietary total choline intake was expressed as a percentage.

5.3.3 Blood sample collection and processing

During the clinical visit, a venous blood sample was drawn by a trained phlebotomist and collected into ethylenediaminetetraacetic acid pre-coated vacutainers. For toddlers, children, and men, non-fasting blood samples were obtained. For postmenopausal women, fasting blood

samples (≥ eight hours) were collected during the early morning. Immediately after collection, the blood samples were placed on ice and transported to the laboratory to be processed as is described in Chapter 4 of this dissertation.

5.3.4 Free choline, betaine, and dimethylglycine analysis in plasma

Free choline, betaine, and dimethylglycine in plasma were quantified by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS), using a Waters ACQUITY UPLC system connected to a Quattro Micro tandem MS configured with an electrospray source (Waters Corporation, MA, USA). The method has been described previously (286, 316), and it was provided in detail in Chapter 4 of this dissertation. The concentrations of free choline, betaine, and dimethylglycine in plasma were expressed as µmol/L. Non-fasting blood samples were also obtained from mothers of children; results are presented in Appendix D.

5.3.5 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 19; SPSS Inc. Chicago, US). The significance level was set at $P < 0.05$ for two-sided testing. Normality of the data was assessed using the Kolmogorov–Smirnov test. If distributions were skewed, data were then normalized using logarithmic transformations to improve normality before further analysis. If normality was not achieved after logarithmic transformation, non-parametric tests were used instead. All data are presented as mean ± SD, unless otherwise noted.

The estimated total choline intake from each age group was compared with the corresponding AI for choline (200, 250, 550, and 425 mg/d for toddlers, children, men, and postmenopausal women, respectively), by one sample Student's t-test. The difference in the

contribution of the individual forms of choline to the daily dietary total choline intake between the age groups was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate. If the difference among group means was significant, it was followed by multiple comparisons analysis by Dunnett's T3 for parametric data or Dunn-Bonferroni test for non-parametric data. The difference in the concentrations of free choline and its associated metabolites in plasma between the age groups was analyzed by one-way ANOVA or the Kruskal-Wallis test, as appropriate. If the difference among group means was significant, it was followed by multiple comparisons analysis by Dunnett's T3 for parametric data or Dunn-Bonferroni test for non-parametric data.

The direction and strength of the relationship between dietary choline and betaine intakes and the concentrations of free choline and its associated metabolites in plasma, within each age group, was explored using pair-wise correlation coefficient using Pearson's or Spearman's correlation test, as appropriate. Interpretation for all correlation coefficients was defined as weak < 0.30, moderate between 0.30 – 0.49, and strong > 0.50 (284). Bonferroni correction was performed to adjust the original significance level ($P < 0.05$) for multiple comparisons of dietary intakes on the same concentration of free choline and its associated metabolites in plasma ($0.05 / 9 = 0.0055$, significance at $P < 0.006$).

5.4 Results

5.4.1 Sample analysis

Between 77 to 285 subjects were enrolled per age group as presented in Figure 5.1. Dietary information and blood samples were available for at least 62% of the subjects per age group.

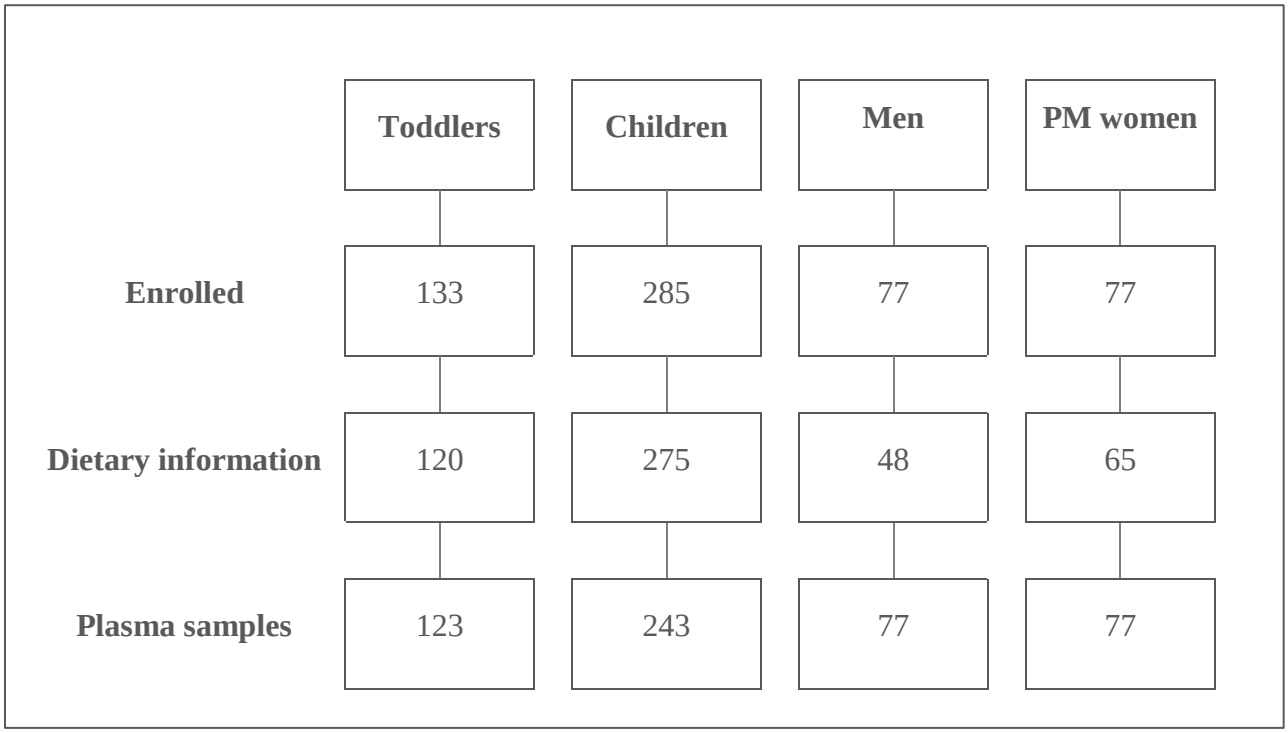

Figure 5.1 Diagram of dietary information and plasma samples analyzed
PM: postmenopausal.

5.4.2 Sociodemographic characteristics of the participants

The age of the participants per group was (mean ± SD) 1.1 ± 0.1, 5.7 ± 0.1, 33.1 ± 11.8, and 55.2 ± 3.6 y for toddlers, children, men, and postmenopausal women, respectively. For the toddlers and children groups, 61% and 51% of the participants were boys, respectively. The anthropometric characteristics of the participants are presented in Table 5.1.

Table 5.1 Anthropometric characteristics of the participants

	Toddlers	**Children**	**Men**	**PM women**
Characteristics	(*n* = 133)	(*n* = 285)	(*n* = 77)	(*n* = 77)
		Mean ± SD		
Weight (kg)	10.3 ± 1.3	20.8 ± 2.9	82.4 ± 14.9	65.5 ± 9.9
Height (m)	0.8 ± 0.0	1.1 ± 0.1	1.8 ± 0.1	1.6 ± 0.1
BMI (kg/m^2)	0.6 ± 0.9[1]	0.1 ± 1.0[1]	25.9 ± 4.6	24.5 ± 3.7

[1]For toddlers and children, BMI-for-age as z-score is presented.
PM: postmenopausal, BMI: body mass index.

Sociodemographic characteristics of the participants are presented in Table 5.2. Most of the mothers of the toddlers and children, and adult participants were university graduates (≥ 69%) and mainly from European background (≥ 64%). Most of the participants had a household income of > $50,000 CAD (≥ 75%).

Table 5.2 Sociodemographic characteristics of the participants

	Toddlers[1,2]	**Children[1]**	**Men**	**PM women**
Characteristics	(*n* = 133)	(*n* = 285)	(*n* = 77)	(*n* = 77)
		n; %		
Education				
High school or less	8; 7%	14; 5%	3; 4%	5; 6%
College or diploma	18; 15%	72; 25%	18; 23%	19; 25%
University degree	91; 78%	199; 70%	56; 73%	53; 69%
Ethnicity				
European	85; 71%	190; 67%	49; 64%	58; 75%
Asian	33; 28%	74; 26%	23; 30%	17; 22%
Other	2; 2%	21; 7%	5; 6%	2; 3%
Annual household income				
< $30 000 CAD	15; 13%	17; 6%	6; 8%	9; 12%
$30 000 – $50 000 CAD	13; 11%	37; 13%	13; 17%	10; 13%
> $50 000 CAD	87; 76%	231; 81%	58; 75%	58; 75%

[1]For toddlers and children, maternal education and ethnicity are presented.
[2]For toddlers, information on maternal education *n* = 16, maternal ethnicity *n* = 13, and annual household income *n* = 18 was missing. Percentages are thus expressed based on those who provided data.
PM: postmenopausal.

5.4.3 Estimated daily dietary choline and betaine intakes

The estimated daily dietary intakes of total choline and betaine across the different age groups are presented in Table 5.3. For all age groups, total choline intakes were normally distributed, while the distribution of betaine intakes were skewed.

Figure 5.2 illustrates the comparison between the estimated mean daily dietary total choline intake and the corresponding AI for choline per age group. For toddlers, men, and postmenopausal women the estimated total choline intakes were not significantly different from the corresponding AI ($P = 0.29 – 0.52$). In children, the estimated mean total choline intake was significantly above the corresponding AI for choline ($P < 0.001$). At the individual intake level per age group, 54%, 67%, 46%, and 49% of toddlers, children, men, and postmenopausal women, respectively, met their corresponding AI for choline.

Overall, across all age groups, the three food groups that made the largest contribution to the estimated daily dietary total choline intake were all the animal-source foods (dairy, eggs, and meats), which in sum ranged between 54% to 68% of total choline intake (Table 5.4).

Table 5.3 Estimated daily dietary total choline and betaine intakes

Intakes		**Toddlers** (*n* = 120)	**Children** (*n* = 275)	**Men** (*n* = 48)	**PM women** (*n* = 65)
			Daily dietary intake (mg/d)[1]		
Total choline[2]	Mean ± SD	209 ± 79	306 ± 106	531 ± 197	414 ± 107
	Median (IQR)	205 (149-258)	286 (224-365)	539 (377-650)	424 (337-472)
Betaine	Mean ± SD	55 ± 32	102 ± 44	187 ± 106	138 ± 69
	Median (IQR)	49 (31-68)	93 (71-119)	155 (127-224)	121 (88-175)

[1]Data were collected using a food frequency questionnaire and estimated using a food frequency questionnaire and choline content in common foods database (version 2) from the United States Department of Agriculture.
[2]Total choline corresponds to the sum of free choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin.
PM: postmenopausal.

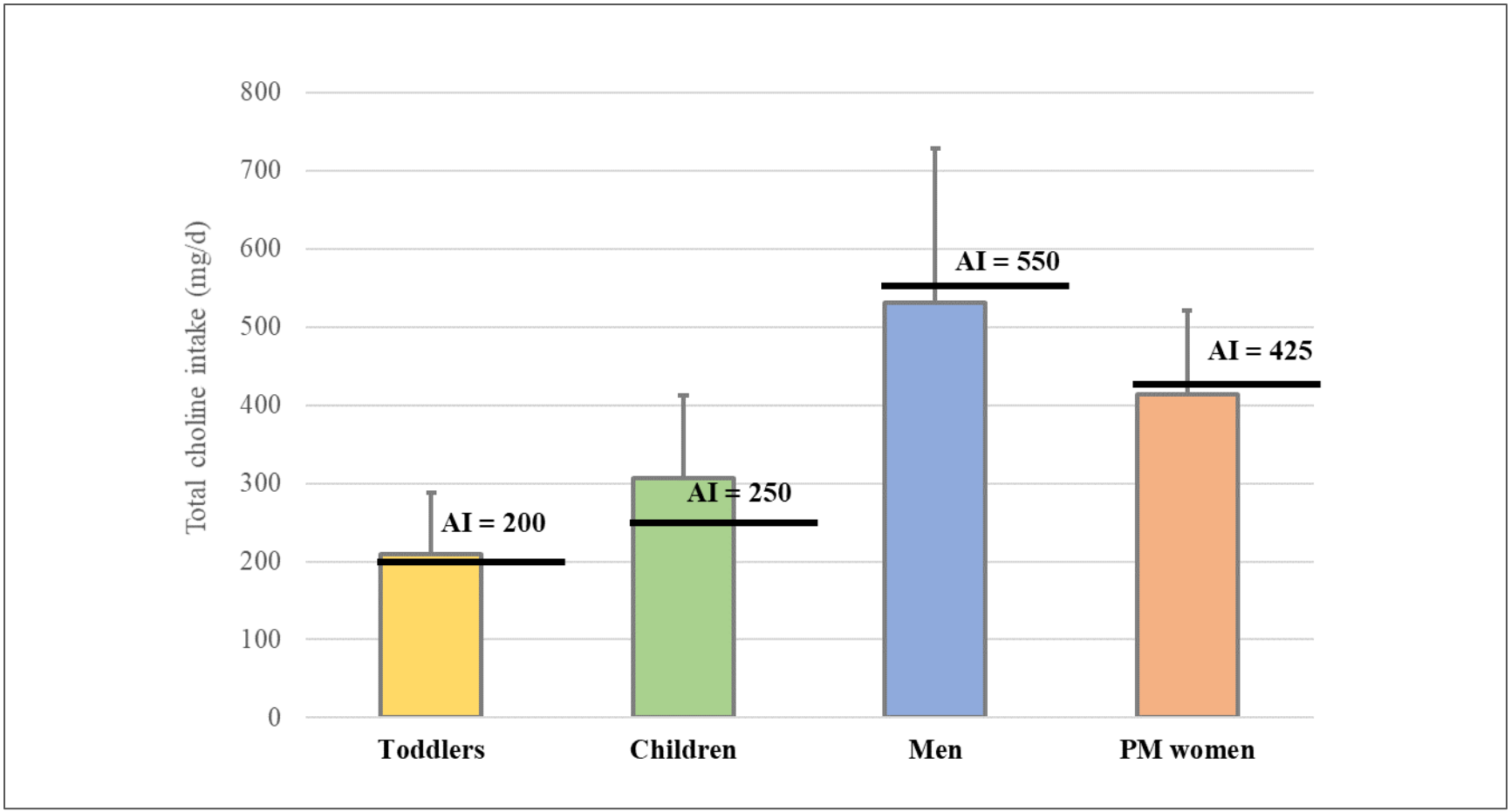

Figure 5.2 Comparison of estimated daily dietary total choline intake with the corresponding adequate AI for choline per age group. The means and SD for the estimated daily total choline intakes are presented in columns for toddlers, children, men, and PM women. Total choline intakes were estimated using a food frequency questionnaire and choline content in common foods database (version 2) from the United States Department of Agriculture. The AI recommendations for total choline per age group are presented as a solid black line per age group. AI: Adequate Intake, PM: postmenopausal.

Table 5.4 Contribution of different food groups to the estimated daily total choline intake

Food groups	Toddlers (*n* = 120)	Children (*n* = 275)	Men (*n* = 48)	PM women (*n* = 65)
	Contribution to estimated daily total choline intake (%)[1]			
Dairy	37.6	28.1	17.3	18.3
Eggs	17.8	12.8	17.3	12.8
Meats	12.9	17.8	25.9	22.5
Grains	6.6	9.1	6.4	6.1
Vegetables	8.5	8.2	7.5	16.0
Fruits	7.4	8.1	5.2	4.8
Legumes	4.5	4.1	5.0	7.7
Mixed dishes	3.5	5.6	6.2	3.5
Pastry	0.6	4.5	2.0	1.4
Beverages	0.0	0.0	5.1	4.4
Others	0.6	1.7	2.1	2.5

[1]Food group intakes were estimated using a food frequency questionnaire and choline content in common foods database (version 2) from the United States Department of Agriculture.
PM: postmenopausal.

5.4.4 Individual forms of choline contributing to the estimated daily dietary total choline intake

The contribution of the individual forms of choline to the estimated total choline intake is presented by age group in Figure 5.3. The intakes of phosphocholine and phosphatidylcholine were normally distributed in all age groups, while the distribution of free choline in children, glycerophosphocholine in toddlers, children, and men, and sphingomyelin in toddlers and children were skewed.

Among all participants, phosphatidylcholine was the major individual form of choline consumed in the diet, contributing between 39 – 50% of total choline intake. Among toddlers, the contributions of phosphocholine (7%, $P \leq 0.01$) and glycerophosphocholine (26%, $P \leq 0.02$) to the estimated daily dietary total choline intake were significantly higher compared to each of the other age groups. On the contrary, the contribution of phosphatidylcholine (39%, $P \leq 0.01$) to the estimated daily dietary total choline in toddlers was significantly lower compared to each of the other age groups.

The contribution of the water- and lipid-soluble forms of choline to the estimated daily dietary total choline intake were normally distributed and the results are presented in Table 5.5. The proportion of the water-soluble forms of choline contributing to the estimated daily dietary total choline intake was significantly higher in toddlers (56%, $P \leq 0.01$) compared to each of the other age groups. In children, the water-soluble forms of choline contributed to 50% of the estimated daily dietary total choline intake. For adults, the lipid-soluble forms of choline were the main contributor to the estimated daily dietary total choline intake (range 52% to 55%).

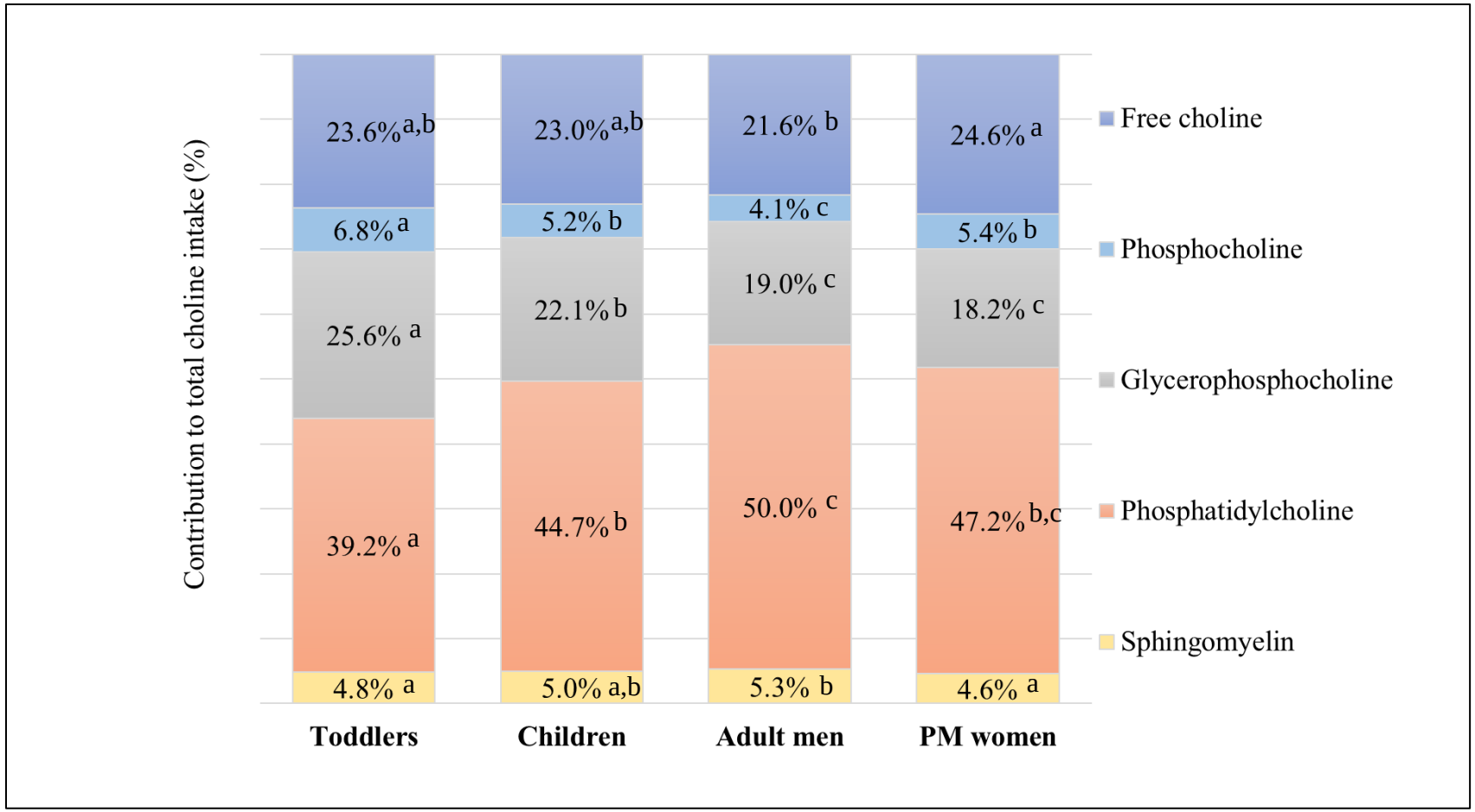

Figure 5.3 Relative contribution of the individual forms of choline to total choline intake.
Individual forms of choline intakes were estimated using a food frequency questionnaire and choline content in common foods database (version 2) from the United States Department of Agriculture. Values that not share a common superscript letter are significantly different based on multiple comparisons analysis by Dunnett's T3 test for phosphocholine and phosphatidylcholine, or Dunn-Bonferroni test for free choline, glycerophosphocholine, and sphingomyelin ($P \leq 0.05$). PM: postmenopausal.

Table 5.5 Contribution of the water-soluble and lipid-soluble forms of choline to the estimated daily dietary total choline intake

Intakes		**Toddlers** (*n* = 120)	**Children** (*n* = 275)	**Men** (*n* = 48)	**PM women** (*n* = 65)
		Contribution to daily total choline intake (%)[1,2]			
Water-soluble choline[3]	Mean ± SD	56 ± 13 [a]	50 ± 10 [b]	45 ± 10 [c]	48 ± 7 [b,c]
	Median (IQR)	56 (48-65)	51 (44-57)	43 (38-50)	48 (43-53)
Lipid-soluble choline[4]	Mean ± SD	44 ± 13 [a]	50 ± 10 [b]	55 ± 10 [c]	52 ± 7 [b,c]
	Median (IQR)	44 (35-52)	49 (43-56)	56 (50-62)	52 (47-57)

[1]Data were collected using a food frequency questionnaire and estimated using a food frequency questionnaire and choline content in common foods database (version 2) from the United States Department of Agriculture.
[2]Values that not share a common superscript letter are significantly different based on multiple comparisons analysis by Dunnett's T3 test ($P \leq 0.05$).
[3]Water- soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.
[4]Lipid- soluble choline corresponds to the sum of phosphatidylcholine and sphingomyelin.
PM: postmenopausal.

5.4.5 Associations between the estimated daily dietary choline and betaine intakes and concentrations of free choline and its associated metabolites in plasma

The distributions of plasma free choline, betaine, and dimethylglycine were skewed, and the concentrations are shown in Table 5.6. Plasma free choline concentrations were the highest in toddlers, similar in children and men. The lowest plasma free choline concentrations were from postmenopausal women, who provided fasting blood samples. Toddlers and postmenopausal women had lower plasma betaine concentrations than children and men. In addition, significant differences were found in plasma dimethylglycine concentrations between all age groups, with toddlers having the highest concentrations and postmenopausal women the lowest.

The correlation coefficients between the estimated dietary total choline and betaine intakes and the concentrations of free choline, betaine, and dimethylglycine in plasma are presented in Table 5.6. Total choline intake was positively but weakly correlated with plasma free choline among toddlers ($P = 0.046$). Similarly, total choline intake was positively associated with both plasma concentration of free choline ($P = 0.025$) and dimethylglycine ($P = 0.020$) in children. After adjusting for multiple comparisons, the correlations were no longer significant (Bonferroni's correction significant at $P < 0.006$). In the adult groups, no significant correlations were found between total choline intake and any of the plasma concentrations. After adjusting for multiple comparisons (Bonferroni's correction significant at $P < 0.006$), few correlations remained significant only among children. Specifically, the correlations between the estimated daily dietary phosphatidylcholine intake and plasma dimethylglycine concentration ($P = 0.003$), and between the sum of the lipid-soluble forms of choline intake and plasma dimethylglycine concentration ($P = 0.003$) remained significantly positively but weakly associated.

Table 5.6 Concentrations of free choline, betaine, and dimethylglycine in plasma

Plasma metabolites		**Toddlers** (*n* = 123)	**Children** (*n* = 243)	**Men** (*n* = 77)	**PM women** (*n* = 77)
			Concentration (µmol/L)[1,2]		
Free choline	Mean ± SD	10.4 ± 3.3	8.6 ± 2.1	8.9 ± 2.5	7.7 ± 2.1
	Median (IQR)	9.8 (8.2-12.1) [a]	8.3 (7.2-9.7) [b]	8.1 (6.9-10.6) [b]	7.3 (6.1-8.8) [c]
Betaine	Mean ± SD	41.1 ± 15.4	45.4 ± 13.7	45.7 ± 13.4	36.8 ± 9.8
	Median (IQR)	36.2 (27.8-46.7) [a]	43.4 (36.4-52.4) [b]	43.0 (36.4-54.7) [b]	36.1 (31.4-41.5) [a]
Dimethylglycine	Mean ± SD	4.1 ± 1.9	3.3 ± 1.0	2.7 ± 0.9	2.2 ± 0.6
	Median (IQR)	3.7 (3.4-4.8) [a]	3.2 (2.6-3.9) [b]	2.6 (2.2-3.0) [c]	2.1 (1.7-2.5) [d]

[1]Plasma concentrations were quantified by liquid chromatography-tandem mass spectrometry.
[2]Values that do not share a common superscript letter are significantly different based on multiple comparisons analysis by Dunn-Bonferroni test ($P < 0.05$).
PM: postmenopausal.

Table 5.7 Associations between dietary choline and betaine intakes and plasma free choline, betaine, and dimethylglycine

	Dietary intake[1]								
Plasma metabolites	**TC**	**FC**	**PCho**	**GPC**	**WSC**	**PC**	**SM**	**LSC**	**Betaine**
Free choline									
Toddlers	0.190*	-0.001	-0.013	0.086	0.041	0.126	0.069	0.124	-0.011
Children	0.145*	0.106	0.063	-0.016	0.054	0.176**	0.106	0.174**	0.091
Men	0.126	0.224	0.100	-0.043	0.089	0.132	-0.033	0.120	-0.148
PM women	-0.044	-0.048	-0.029	-0.103	-0.077	0.003	-0.079	-0.005	-0.111
Betaine									
Toddlers	-0.095	-0.196*	-0.228*	-0.111	-0.150	-0.010	-0.068	-0.014	-0.022
Children	0.013	0.028	-0.115	-0.170**	-0.098	0.124	-0.046	0.110	0.024
Men	0.237	0.230	0.161	0.031	0.131	0.262	0.072	0.245	0.061
PM women	-0.045	0.001	-0.007	-0.306*	-0.154	0.151	-0.127	0.130	-0.038
Dimethylglycine									
Toddlers	-0.061	-0.121	-0.107	-0.010	-0.077	-0.050	-0.093	-0.054	-0.101
Children	0.151*	0.050	0.096	0.023	0.052	0.191***	0.090	0.190***	0.062
Men	0.182	0.063	0.216	0.028	0.073	0.220	0.158	0.210	0.155
PM women	-0.117	-0.147	-0.106	-0.204	-0.179	0.104	-0.063	0.088	0.002

[1]Data are presented as Spearman's correlation coefficients. Correlation is significant at * 0.05, ** 0.01, and *** 0.006. (Bonferroni's correction was used to adjust the significance at 0.006).
FC: free choline, GPC: glycerophosphocholine, LSC: lipid soluble forms of choline (sum PC + SM), PC: phosphatidylcholine, PCho: phosphocholine, PM: postmenopausal, SM: sphingomyelin, TC: total choline (sum individual forms of choline), WSC: water-soluble forms of choline (sum FC + PCho + GPC).

5.5 Discussion

The present study is the first reporting on dietary choline and betaine intakes and the concentrations of free choline and its associated metabolites in plasma among apparently healthy toddlers, children, men, and postmenopausal women in Canada. The principal findings from the present study are: 1) the mean total choline intake was not significantly lower than the current AI for toddlers, children, men, and postmenopausal women; 2) the individual forms of choline significantly changed from primarily water-soluble forms in early stage of life to mostly lipid-soluble forms in adulthood, and animal-source foods were the main contributors to total choline intake across all age groups; and 3) dietary choline intakes were not significantly associated with plasma free choline concentrations.

The mean total choline intakes in toddlers (208 mg/d) observed in the present study is similar to the ranges reported in previous studies including toddlers in Europe (157 to 205 mg/d) (191) and the United States (229 mg/d) (184). In children, the mean total choline intake (306 mg/d) is similar to children in Europe (185 to 294 mg/d) (191). In men, reports on dietary total choline intakes range from 291 mg/d in Greece (91) to 468 mg/d in Sweden (191), which are relatively lower than the intakes estimated in the present study (531 mg/d). Similarly, the present study estimated a mean intake of 414 mg/d for total choline in postmenopausal women, which is higher than the range from previous studies in women (269 to 404 mg/d) (133, 184, 191, 338-340). Some of the differences in comparing with other studies, as in men and postmenopausal women, may be due to the instruments used to estimate dietary choline intake. Specifically because the richest food sources of total choline are liver and eggs (290 to 430 mg and 230 mg per 100 g of food, respectively) (13, 159). The use of 24-hour recalls or food records (191)

collecting dietary information from only a few days might not be adequate to capture the richest food sources of choline, which are likely not being consumed on a daily basis. Taken together, these data suggest that choline intakes are similar across Western countries in toddlers and children, but higher in men and postmenopausal women in North America compared to Europe.

It is important to mention that due to the limited data available on choline, an Estimated Average Requirement (EAR) could not be calculated and AIs were established instead (12). Therefore, the evaluation of choline intake must be done with caution, as intake levels above the AI imply a low probability of inadequate intake, but intake below the AI does not necessarily indicate inadequacy (341). Moreover, the current AIs were set based on findings obtained from a single study that was designed to study total choline intake and prevention of hepatic alterations as a functional outcome of choline-deficient intake in adult men (24). Further, these results were then extrapolated to women and other age groups, including toddlers and children (12). The selection of this functional outcome for choline was based on data available at that time, and although it has also been observed in children (342), it develops after a severe dietary choline restriction (< 50 mg/d). Therefore, to develop sound dietary intake recommendations, it would be helpful to identify other indicators to assess function at earlier stages of deficiency.

It is known that in breastfed infants, the water-soluble forms of choline represent an average of 84% of the total choline intake (14-16), whereas the lipid-soluble forms are the major contributors in adults (range between 52 to 61%) (33, 192, 195, 343). However, the physiological reason for this transition is unknown, and the change has not been well characterized in toddlers and children. In the present study, the proportion of the water-soluble forms contributing to total choline intake was 55% in toddlers and decreased to 50% in children,

which is closer to the range found in adults (43 – 48%). In addition, in the present study, at least half of total choline (50 – 68%) in the diet was provided by animal-source foods such as dairy, eggs, and meats. Similar food sources have been reported by other studies (10, 33, 187, 191, 192, 195, 199, 233, 343, 344). This is consistent with the higher intake of water-soluble forms of choline in toddlers, who consumed 38% of total choline from dairy (13). The water-soluble forms of choline are absorbed via the portal circulation to the liver (345), whereas the lipid-soluble forms are absorbed via lymphatic circulation, and thus bypassing the liver and reaching general circulation at the thoracic duct (41). A recent study in rodents provided evidence that maternal choline intake as phosphatidylcholine, compared to free choline, increased milk glycerophosphocholine concentrations and was related to a better immune development in the offspring (19). In humans, it has been reported that milk glycerophosphocholine concentrations are associated with serum concentrations in breastfed infants (15). Overall, this information suggests that the different forms of choline in the diet might be metabolized differently and thus potentially impact the metabolic fate of choline to serve as a precursor for methyl groups, acetylcholine, or phospholipids.

In the present study, plasma free choline concentration in toddlers was significantly higher than in all other age groups, including in children (10.4 and 8.6 µmol/L, respectively). Given the finding of significantly higher choline concentrations in fed versus fasted samples (Chapter 4), this might confound the comparisons of postmenopausal women to the other age groups. These results are consistent with the limited information available, with mean plasma free choline concentrations reported as 12.8 µmol/L in toddlers from the United States (217) and 9.2 µmol/L in children from the Republic of Seychelles (89). In addition, a similar decrease in plasma free choline concentrations in children, compared to toddlers, in the present study (17%)

has also been observed in serum free choline concentrations (20%) in Turkey (15). Other authors have suggested that the increased concentrations of circulating free choline during the early stage of life might be related to a higher choline supply needed to support organ growth and development (1, 39, 203, 332). This may be particularly important for choline uptake in the brain, as it has been described that choline is transported at a rate proportional to plasma concentration (296, 346, 347). This suggests a higher uptake at early ages, which coincides with a high rate of brain development during the first years of life (348-351). However, the uncertainty over the normal range of plasma free choline concentrations remains, with no reference cut-off points defined yet.

The present study found no evidence of a significant association between choline intake (total and individual forms of choline) and plasma free choline concentrations in any of the age groups. This result suggests that plasma free choline concentrations do not directly reflect usual dietary choline intake within the range consumed in the present study. Other authors have proposed that in women plasma free choline concentration is homeostatically regulated (30), and thus does not reflect small changes in choline intake. Furthermore, the specific origin of free choline in plasma is unknown, and in addition to dietary intake, it could reflect hepatic or plasma lipoprotein metabolism. It is possible that other factors might influence the relationship between dietary choline intake and plasma free choline concentrations, as emerging data on genetic polymorphisms have been reported to have an impact choline metabolism (16, 26, 28, 93, 352-358).

Dietary betaine intakes of 187 mg/d in men and 138 mg/d in postmenopausal women estimated in the present study are in agreement with the intake range of intake reported in

previous studies (97 to 189 mg/d) (33, 186, 192, 196, 233, 339, 344). No information on dietary betaine intake among toddlers or children has been published, and there is no dietary recommendation on the intake level for betaine at present. In the present study, mean plasma betaine concentrations were lower in toddlers and postmenopausal women (36 µmol/L) compared to children and men (43 µmol/L), which agrees with the concentration reported from the previous studies (89, 134, 228). Like choline, the results of the present study indicated that dietary betaine intakes were not significantly associated with plasma betaine concentrations in any age group. In contrast, dietary intakes of free choline, phosphocholine, and glycerophosphocholine were negatively associated with plasma betaine concentrations (*rho* = -0.170 to -0.306) in toddlers, children, and postmenopausal women. It is possible that the plasma betaine concentrations are influenced by several factors including betaine intake (13, 159), choline oxidation (61, 327), and hepatic or renal metabolism (303). In addition, the inverse association between the specific forms of choline and plasma betaine concentrations deserves further study.

The present study has several limitations that need to be mentioned. First, this study presents cross-sectional data on participants from a number of convenience samples, most of whom had high education levels and are from European ethnicity, and therefore, the results from this study may not be generalized to other sociodemographic groups. Second, the sample sizes for men and postmenopausal women groups were relatively small compared to toddlers and children. Third, the estimation of the dietary choline intake was calculated using the USDA database, which includes a limited number of food items; thus, the possibility of error in the daily dietary intake estimations due to differences in the food content in Canada may occur. Fourth, random blood samples were collected for practical and ethical reasons for most of the age groups

including toddlers, children, and men and recent food intake may have affected postprandial plasma concentrations. Moreover, blood samples from the postmenopausal women were collected in the fasted state, and this may also contribute to the apparently lower values in postmenopausal women when comparing to the other age groups.

In summary, this study was conducted in apparently healthy participants in Vancouver, Canada, with the goal to increase the information available on usual dietary choline intake and plasma free choline concentrations among different age groups. This is the first study reporting on choline nutrition during early childhood in Canada. Specifically, the results of this study provide novel information on dietary ranges for total choline, individual forms of choline, and betaine intakes, and also plasma concentrations of free choline, betaine, and dimethylglycine in apparently healthy Canadians. Specifically, this study highlights that total choline intakes were not significantly below the current AI of choline recommendations. Given the definition of AI, no conclusion on the prevalence of deficiency of choline intake can be made. Furthermore, the results from the present study also suggest that plasma free choline concentrations do not reflect dietary choline intake. Taken together, these findings expand our current knowledge describing the usual dietary choline intake and may contribute towards a future update on the current AIs for choline in different age groups. More work is needed to better understand the dietary requirements for choline in different age groups to establish EARs, bearing in mind the potential physiological relevance of the different forms of choline in the diet.

Chapter 6: General discussion, conclusion, and future directions

Chapter six presents the discussion of the key findings which are presented in context with the comparable published literature on choline. This is followed by a summary of the significance and overall contribution of this dissertation to the current literature on choline. In addition, the overall limitations of the research conducted are presented. Finally, this chapter includes potential future research directions.

6.1 General discussion of key findings

6.1.1 The concentration of the water-soluble forms of choline in mature milk did not differ between Canadian and Cambodian lactating women

One of the main findings from this research was that the concentrations of the water-soluble forms of choline in mature milk samples collected from healthy lactating women in Canada were similar to the lactating women in Cambodia (Chapter 3). This result was unexpected, as the Cambodian diet has been described to be low in animal-source foods (181, 290), which are the richest sources of choline (13, 159). The concentrations of the water-soluble forms of choline in mature milk samples in the present study are within the range the previous reports in the United States (14, 16, 17) and Turkey (15, 173), and no other studies quantifying the water-soluble forms of choline in human mature milk from a developing or low- and middle-income countries have been published to date. Little is known regarding the origin of choline in human milk and the factors that could influence milk choline concentrations. It has been reported

that supplemental total choline intakes at twice the corresponding Adequate Intake (AI) in lactating women have resulted in increased milk total choline concentrations (20%) compared to a control group (16, 17). Nevertheless, the proportion of the water-soluble forms of choline reported in the mentioned supplementation studies was similar between the supplementation groups and the control groups, ranging from 79 to 87% (16, 17). In the present research, no statistically significant association was found between dietary total choline intake and the concentration of water-soluble forms of choline in mature human milk. However, the estimated dietary total choline intakes in the present study were similar to the control groups in the supplemental studies. Thus, this observation suggests that the majority of choline in milk may originate from maternal metabolism (72, 170) rather than directly from dietary intake and that dietary choline intake within the usual range estimated in these lactating women has no significant impact on milk choline concentrations.

6.1.2 The estimated daily dietary total choline intake meets the corresponding adequate intake among most of the age groups, except for infants and lactating women

Another key finding from this research was that the mean dietary total choline intakes were not significantly lower than the corresponding AIs among healthy subjects (Chapter 5), thus meeting the recommendation, except for infants and lactating women (Chapter 3). In the present study, the mean intake of total dietary choline for 0 – 6 months old infants was estimated at 106 mg/d, which was significantly lower than the corresponding AI of 125 mg/d (12). Generally, the dietary intake recommendations for early infancy are established based on the mean concentration and volume intake of human milk of healthy breastfed infants, born full-term to

well-nourished mothers (149). However, the AI of choline for 0 – 6 months old infants was established based on a 20% increase in the mean total choline concentration from a single small study in mature human milk (14), and no rationale was provided for this increase over the original value. Interestingly, no difference was found when the estimated daily total choline for infants in the current study was compared with the value of the original study used to set the AI (106 vs. 102 mg/d, $P < 0.05$) (14). As such, this finding suggests that the milk choline concentration used when developing the current Dietary Reference Intakes (DRIs) for choline as AI for infancy may be overestimated, and revisions should be considered accordingly.

For all other age groups, apart from infants, daily dietary total choline intakes were estimated based on a semi-quantitative food frequency questionnaire (FFQ) and the United States Department of Agriculture choline database (13). For this research, the validity of the FFQ in estimating total choline intake was evaluated compared to the mean three of 24-hour recalls in adults, of both sexes (Chapter 2). A significantly strong positive correlation coefficient for total choline intake was found between these dietary assessment instruments in the present study, which is consistent with results from previous studies (188, 190). In the present research, the estimated daily dietary total choline intakes among toddlers, children, men, and postmenopausal women met the corresponding AIs (12) and were in agreement to the intake ranges reported worldwide (33, 91, 133, 184, 186, 191, 338-340, 344).

In the present research, the estimated mean daily dietary total choline intake during late pregnancy was used as a proxy for intake during lactation, which was significantly lower than the AI for choline for lactating women (12). Notably, the AI for choline is 22% higher in lactation than pregnancy (12), while data from previous studies have shown that the dietary

intake of total choline does not differ between these two periods (10, 11). Both AIs were based on the AI for adult women, and an increase in choline demand to support choline tissue accumulation and choline output in milk were added to the AI for pregnant and lactating women, respectively (12). Although the previously mentioned overestimation of milk choline would consequently affect the AI of choline for lactating women, the impact would be small (-4% change in the AI). Yet, the current choline AI for lactation may require revision.

6.1.3 Plasma free choline concentrations did not reflect the estimated daily dietary total choline intake and differed between age groups

A key finding of this research was that plasma free choline was not significantly associated with dietary total choline intake (Chapter 5), which is similar to the result of a previous study conducted in premenopausal women (30). This finding suggests that plasma free choline concentrations may not be an adequate biomarker for dietary choline intake. It is possible that the participants enrolled in the current research were consuming adequate dietary total choline. Thus, the estimated dietary choline intake of the participants presented in this dissertation would be below or above the threshold needed to affect plasma free choline concentrations as shown in previous depletion (24, 25, 28) or supplementation studies (156, 207-210). The exact origin of plasma free choline is not entirely understood, and it may be the net result of choline body status after choline intake, along with choline metabolism that is contributed by small intestinal microbiota (82, 83), and hepatic and lipoproteins (72). Nevertheless, since dietary choline intake and plasma choline concentrations have independently

been associated with clinical outcomes in different age groups (2), both assessments could provide useful information to help to determine choline requirements.

In the present research, plasma free choline concentrations significantly differed between age and sex groups, with the highest concentration found in toddlers, followed by similar concentrations in children and men, and the lowest concentration found in postmenopausal women (Chapter 5). These plasma free choline concentrations are consistent with those previously reported within the same age and sex group (89, 134, 217, 222, 359). In the present research, one non-fasted blood sample was collected per participant, except for postmenopausal women, from whom one blood sample was obtained after an overnight fast. It is important to mention that the effect of recent food intake on the plasma free choline concentrations was assessed in the present research, as the intra-individual variability comparing fasted (> eight hours) to non-fasted (four hours postprandial) state in adults (Chapter 4). The fact that all fed concentrations fell within the 95% CI of the reference interval calculated and the positive correlation between both states, obtained in the present research, suggests that one blood sample in fasted or fed state is adequate to assess plasma free choline concentrations, within the range of total choline intakes of these participants. However, plasma free choline concentrations increase after food intake, as has been similarly reported in earlier studies (223, 224), which may have affected the comparison of the plasma free choline concentrations in postmenopausal women compared to the other age groups. It is possible that in a different situation, in which individuals consume a deficient diet with lower dietary choline intakes compared to those obtained here, a blood collection in the fed state may produce a false positive assessment of choline status.

6.2 Significance and contribution of the research

This research generated three overall key findings regarding choline nutrition. First, the concentrations of the water-soluble forms of choline did not differ between mature milk samples collected from lactating women in Canada and Cambodia. As such, the results of this research suggest that the differences in choline intake, assumed from the differences in availability of animal-source foods between countries, may not significantly affect the concentrations of the water-soluble forms of choline in mature human milk. These data are particularly relevant because they may help to define the normal range for choline in human milk, regardless of mother's location or socioeconomic status.

Second, the estimated dietary total choline intake met the corresponding choline AIs recommendations in toddlers, children, men, and postmenopausal women, with the exception of infants and lactating women. The results indicated that choline intake of infants, estimated from milk samples of both Canadian and Cambodian lactating women, were lower than the current AI for choline. This can have implications for infants, as this value may be used in the formulation of infant formulas and enteral nutrition products. In addition, apparently healthy well-nourished women did not meet the choline AI for lactation based on data obtained using a validated FFQ for total choline intake. This suggests that it may not be appropriate to establish the recommendation based on the extrapolation of the result from a single small study conducted in men and an overestimated choline output in milk. As such, based on the current research it appears the AIs of choline for infancy and lactation should be reviewed.

Third, the fact that no significant association was found between dietary total choline intake and plasma free choline concentration indicates that dietary choline within the range of intake in the age groups studied is not reflected in plasma free choline concentrations. This finding suggests that plasma free choline concentrations were not affected by dietary choline as participants had adequate choline intakes. Furthermore, no reference intervals for normal plasma free choline concentrations have been defined for any age group so far. Thus, the interpretation of plasma free choline concentrations is challenging.

Taken together, the results generated by this work contribute to the information about choline nutrition from healthy populations across different age groups. In 1998, the current DRIs for choline were established as AIs for total choline for all age groups based on limited data, as the estimated choline intake from the population was not available due to the lack of information on choline content in foods. To date, 19 years after, there are still limited data available on dietary choline intake worldwide. Overall, this dissertation contributes to expanding the current data on choline nutrition in different age groups, including information on dietary choline intake and milk and plasma choline concentrations. In addition, this dissertation provides information on the validation of an FFQ for dietary choline intake assessment and individual variability of plasma choline concentrations, which may assist in delineating future research. Finally, these findings suggest that revisiting the current AI values for choline recommendations, especially during infancy and lactation might be warranted.

6.3 Overall limitations

The participants were recruited using convenience sampling, which may reduce the generalizability of the results because the subjects participating in the present research might differ from a national representative sample. In fact, the sociodemographic characteristics, including educational level and household income of the participants enrolled in the studies conducted in Vancouver, are higher than the reference data available from the National Household Survey in the Vancouver Metropolitan area (360). In addition, the participants in this research were enrolled on a volunteer basis. Thus, it is possible that the subjects were more interested in dietary intake and nutrition than the general population and therefore differ in dietary patterns.

Further, the dietary intake assessment was self-reported, and thus susceptible to memory bias, along with under-reporting or over-reporting of food intake, which may affect the accuracy of the estimation (270). However, these challenges are inherent and present in most of the dietary assessment strategies. In addition, dietary information was not collected from the Cambodian lactating women, and the assumption of the different choline intake between Canada and Cambodia was based on the energy available for consumption from animal source foods using the food balance sheets. Therefore, it may be possible that this assumption does not necessarily translate to dietary intake, as this does not provide information on actual consumption (178, 361). Moreover, the United States Department of Agriculture is the only available database to estimate choline content in foods and thus intakes (13). At present, all the studies published about choline intake have based their estimations on the same database. This database has limited information on ethnic-specific food items, and in addition, it is unknown whether the

choline content in foods varies in different countries. As such, self-report bias and an incomplete nutrient file database are recognized as limitations.

Blood samples were collected randomly during the day for most participants of the different age groups, without requesting an overnight fast, except for postmenopausal women. This may potentially have had an impact on plasma free choline concentrations. Although an assessment of the intra-individual variability after food intake was conducted among healthy adults, this study only included one overnight fasted sample and one fed sample four hours after a self-selected breakfast. Yet, a different study design including the collection of samples at several time points after the consumption of different test meals, varying in choline content (i.e., low, medium, and high choline content), might offer a more comprehensive insight into the postprandial effect on plasma free choline concentrations.

6.4 Future research directions

Although the estimations of dietary choline intakes in different studies worldwide (5, 10, 32, 128, 133, 187, 195, 196, 211, 233) are based on the same single database (13), these data have some limitations. This database contains information on a limited number of samples per food item analyzed, and the samples were obtained only from the United States (13). Therefore, the database may not be representative of certain foods consumed by a particular population, and it is uncertain whether the choline content of foods differs between countries. Some work has been done expanding the information available on choline content in legumes (161) and meats (362) in Canada. More work is still necessary to expand the current database, focusing particularly on the choline content in dairy products, which is

another important dietary source of choline, and dairy alternative foods, such as soy or almond milk, to allow a better estimation of dietary choline intake.

Studies with large sample sizes, of randomly selected healthy lactating participants, describing both total and individual milk choline concentration from different countries are needed. In addition, these sorts of studies are relevant as they could provide additional information during lactation, such as dietary choline intake, plasma choline concentrations, and polymorphisms in genes related to choline metabolism. The data collected may help to generate a potentially appropriate standard reference interval for choline concentration in human milk. This approach may also be applied to plasma free choline concentration, since cut-off points to define choline deficiency have not been determined yet. Efforts on collecting descriptive information from healthy participants worldwide started with the World Health Organization (WHO) growth chart standard (363). In Canada, blood samples have been collected from a representative sample from three to 79 years in the Canadian Health Measures Survey (CHMS) (364-366), from birth to 18 years in the Canadian Laboratory Initiative on Paediatric Reference Intervals (CALIPER) (367, 368), and from younger children in the Canadian Healthy Infant Longitudinal Development Study (CHILD) (369, 370). These biobanks appear as worthwhile opportunities to analyze the concentration of choline in plasma to be able to calculate reference interval values.

Finally, another area that requires further investigation is the relationship between choline and functional outcomes. The selection of liver damage as the criterion of functional outcome for choline-deficient intake was based on data available at that time from a depletion-repletion study conducted in healthy men (24). Although it has also been observed in children

receiving total parenteral nutrition (342), liver damage develops after a severe dietary choline restriction, a time point at which other adverse health outcomes may already be present. Since choline is involved in a broad range of body functions across the life cycle, the selection of an endpoint might be challenging. A recently published review provides a summary of the diverse health-related outcomes that have been previously reported in humans, including neurological and metabolic outcomes (2). It would be relevant to consider the rationale when selecting an appropriate endpoint at different age stages (371). For example, during development, the assessment of dietary choline intake or plasma choline concentration have been associated with neurodevelopmental tests scores as functional outcomes at different ages, which have provided mixed results (7, 88, 89, 110, 111). More work is needed to identify other indicators to assess choline function at earlier stages of deficiency. In addition, this information would be helpful to develop sound DRIs for choline based on functional health outcomes to establish Estimated Average Requirements.

Bibliography

1. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr 2006;26:229-50.

2. Leermakers ET, Moreira EM, Kiefte-de Jong JC, Darweesh SK, Visser T, Voortman T, Bautista PK, Chowdhury R, Gorman D, Bramer WM, et al. Effects of choline on health across the life course: a systematic review. Nutr Rev 2015;73(8):500-22.

3. Blusztajn JK, Wurtman RJ. Choline and cholinergic neurons. Science 1983;221(4611):614-20.

4. Meck WH, Williams CL. Metabolic imprinting of choline by its availability during gestation: implications for memory and attentional processing across the lifespan. Neurosci Biobehav Rev 2003;27(4):385-99.

5. Gossell-Williams M, Fletcher H, McFarlane-Anderson N, Jacob A, Patel J, Zeisel S. Dietary intake of choline and plasma choline concentrations in pregnant women in Jamaica. West Indian Med J 2005;54(6):355-9.

6. Cheatham CL, Goldman BD, Fischer LM, da Costa KA, Reznick JS, Zeisel SH. Phosphatidylcholine supplementation in pregnant women consuming moderate-choline diets does not enhance infant cognitive function: a randomized, double-blind, placebo-controlled trial. Am J Clin Nutr 2012;96(6):1465-72.

7. Boeke CE, Gillman MW, Hughes MD, Rifas-Shiman SL, Villamor E, Oken E. Choline intake during pregnancy and child cognition at age 7 years. Am J Epidemiol 2013;177(12):1338-47.

8. Wu BT, Innis SM, Mulder KA, Dyer RA, King DJ. Low plasma vitamin B-12 is associated with a lower pregnancy-associated rise in plasma free choline in Canadian pregnant women and lower postnatal growth rates in their male infants. Am J Clin Nutr 2013;98(5):1209-17.

9. Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Brenna JT, Stabler SP, Allen RH, Gregory JF, 3rd, Caudill MA. Pregnancy alters choline dynamics: results of a randomized trial using stable isotope methodology in pregnant and nonpregnant women. Am J Clin Nutr 2013;98(6):1459-67.

10. Lewis ED, Subhan FB, Bell RC, McCargar LJ, Curtis JM, Jacobs RL, Field CJ, APrON team. Estimation of choline intake from 24 h dietary intake recalls and contribution of

egg and milk consumption to intake among pregnant and lactating women in Alberta. Br J Nutr 2014;112(1):112-21.

11. Masih SP, Plumptre L, Ly A, Berger H, Lausman AY, Croxford R, Kim YI, O'Connor DL. Pregnant Canadian women achieve recommended intakes of one-carbon nutrients through prenatal supplementation but the supplement composition, including choline, requires reconsideration. J Nutr 2015;145(8):1824-34.

12. Institute of Medicine. Choline; in Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. National Academy Press, Washington (DC), 1998:390-422.

13. United States Department of Agriculture. Database for the choline content of common foods, release 2. Agricultural Research Service, Washington (DC), 2008.

14. Holmes-McNary MQ, Cheng WL, Mar MH, Fussell S, Zeisel SH. Choline and choline esters in human and rat milk and in infant formulas. Am J Clin Nutr 1996;64(4):572-6.

15. Ilcol YO, Ozbek R, Hamurtekin E, Ulus IH. Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk. J Nutr Biochem 2005;16(8):489-99.

16. Fischer LM, da Costa KA, Galanko J, Sha W, Stephenson B, Vick J, Zeisel SH. Choline intake and genetic polymorphisms influence choline metabolite concentrations in human breast milk and plasma. Am J Clin Nutr 2010;92(2):336-46.

17. Davenport C, Yan J, Taesuwan S, Shields K, West AA, Jiang X, Perry CA, Malysheva OV, Stabler SP, Allen RH, et al. Choline intakes exceeding recommendations during human lactation improve breast milk choline content by increasing PEMT pathway metabolites. J Nutr Biochem 2015;26(9):903-11.

18. Cheng W-L, Holmes-McNary MQ, Mar M-H, Lien EL, Zeisel SH. Bioavailability of choline and choline esters from milk in rat pups. J Nutr Biochem 1996;7(8):457-64.

19. Lewis ED, Richard C, Goruk S, Dellschaft NS, Curtis JM, Jacobs RL, Field CJ. The form of choline in the maternal diet affects immune development in suckled rat offspring. J Nutr 2016;146(4):823-30.

20. Holmes HC, Snodgrass GJ, Iles RA. The choline content of human breast milk expressed during the first few weeks of lactation. Biochem Soc Trans 1996;24(3):350S.

21. Sakurai T, Furukawa M, Asoh M, Kanno T, Kojima T, Yonekubo A. Fat-soluble and water-soluble vitamin contents of breast milk from Japanese women. J Nutr Sci Vitaminol (Tokyo) 2005;51(4):239-47.

22. Zeisel SH, Stanbury JB, Wurtman RJ, Brigida M, Fierro-Benitez R. Choline content of mothers' milk in Ecuador and Boston. N Engl J Med 1982;306(3):175-6.

23. Allen LH. B vitamins in breast milk: relative importance of maternal status and intake, and effects on infant status and function. Adv Nutr 2012;3(3):362-9.

24. Zeisel SH, Da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, Beiser A. Choline, an essential nutrient for humans. FASEB J 1991;5(7):2093-8.

25. Fischer LM, daCosta KA, Kwock L, Stewart PW, Lu TS, Stabler SP, Allen RH, Zeisel SH. Sex and menopausal status influence human dietary requirements for the nutrient choline. Am J Clin Nutr 2007;85(5):1275-85.

26. Fischer LM, da Costa KA, Kwock L, Galanko J, Zeisel SH. Dietary choline requirements of women: effects of estrogen and genetic variation. Am J Clin Nutr 2010;92(5):1113-9.

27. Resseguie M, Song J, Niculescu MD, da Costa KA, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. FASEB J 2007;21(10):2622-32.

28. da Costa KA, Kozyreva OG, Song J, Galanko JA, Fischer LM, Zeisel SH. Common genetic polymorphisms affect the human requirement for the nutrient choline. FASEB J 2006;20(9):1336-44.

29. Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Stabler SP, Allen RH, et al. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am J Clin Nutr 2012;95(5):1060-71.

30. Abratte CM, Wang W, Li R, Axume J, Moriarty DJ, Caudill MA. Choline status is not a reliable indicator of moderate changes in dietary choline consumption in premenopausal women. J Nutr Biochem 2009;20(1):62-9.

31. Holm PI, Hustad S, Ueland PM, Vollset SE, Grotmol T, Schneede J. Modulation of the homocysteine-betaine relationship by methylenetetrahydrofolate reductase 677 C->t genotypes and B-vitamin status in a large-scale epidemiological study. J Clin Endocrinol Metab 2007;92(4):1535-41.

32. Konstantinova SV, Tell GS, Vollset SE, Ulvik A, Drevon CA, Ueland PM. Dietary patterns, food groups, and nutrients as predictors of plasma choline and betaine in middle-aged and elderly men and women. Am J Clin Nutr 2008;88(6):1663-9.

33. Yonemori KM, Lim U, Koga KR, Wilkens LR, Au D, Boushey CJ, Le Marchand L, Kolonel LN, Murphy SP. Dietary choline and betaine intakes vary in an adult multiethnic population. J Nutr 2013;143(6):894-9.

34. Zeisel SH, Blusztajn JK. Choline and human nutrition. Annu Rev Nutr 1994;14:269-96.

35. Zeisel SH. Choline: an essential nutrient for humans. Nutrition 2000;16(7-8):669-71.

36. Strecker A. Notiz über die Zusammensetzung des Leucins. Liebigs Annalen der Chemie 1842;72:89-91.

37. Best CH, Huntsman ME. The effects of the components of lecithine upon deposition of fat in the liver. J Physiol 1932;75(4):405-12.

38. Mackenzie CG, Du Vigneaud V. Effect of choline and cystine on the oxidation of the methyl group of methionine. J Biol Chem 1952;195(2):487-91.

39. Zeisel SH. The fetal origins of memory: the role of dietary choline in optimal brain development. J Pediatr 2006;149(5 Suppl):S131-6.

40. Buchman AL, Moukarzel A, Jenden DJ, Roch M, Rice K, Ament ME. Low plasma free choline is prevalent in patients receiving long term parenteral nutrition and is associated with hepatic aminotransferase abnormalities. Clin Nutr 1993;12(1):33-7.

41. Zeisel SH. Dietary choline: biochemistry, physiology, and pharmacology. Annu Rev Nutr 1981;1:95-121.

42. Lewis ED, Field CJ, Jacobs RL. Should the forms of dietary choline also be considered when estimating dietary intake and the implications for health?. Lipid Technology 2015;27(10):227-30.

43. Sarter M, Parikh V. Choline transporters, cholinergic transmission and cognition. Nat Rev Neurosci 2005;6(1):48-56.

44. Varoqui H, Erickson JD. The cytoplasmic tail of the vesicular acetylcholine transporter contains a synaptic vesicle targeting signal. J Biol Chem 1998;273(15):9094-8.

45. Everitt BJ, Robbins TW. Central cholinergic systems and cognition. Annu Rev Psychol 1997;48:649-84.

46. Fujii T, Takada-Takatori Y, Kawashima K. Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. J Pharmacol Sci 2008;106(2):186-92.

47. Kawashima K, Fujii T. Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. J Pharmacol Sci 2008;106(2):167-73.

48. Wessler I, Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 2008;154(8):1558-71.

49. Bianchi G, Azzone GF. Oxidation of choline in rat liver mitochondria. j biol chem 1964;239:3947-55.

50. Munoz-Clares RA, Diaz-Sanchez AG, Gonzalez-Segura L, Montiel C. Kinetic and structural features of betaine aldehyde dehydrogenases: mechanistic and regulatory implications. Arch Biochem Biophys 2010;493(1):71-81.

51. Lever M, Slow S. The clinical significance of betaine, an osmolyte with a key role in methyl group metabolism. Clin Biochem 2010;43(9):732-44.

52. Ueland PM. Choline and betaine in health and disease. J Inherit Metab Dis 2011;34(1):3-15.

53. Garrow TA. Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. J Biol Chem 1996;271(37):22831-8.

54. Delgado-Reyes CV, Wallig MA, Garrow TA. Immunohistochemical detection of betaine-homocysteine S-methyltransferase in human, pig, and rat liver and kidney. Arch Biochem Biophys 2001;393(1):184-6.

55. Feng Q, Kalari K, Fridley BL, Jenkins G, Ji Y, Abo R, Hebbring S, Zhang J, Nye MD, Leeder JS, et al. Betaine-homocysteine methyltransferase: human liver genotype-phenotype correlation. Mol Genet Metab 2011;102(2):126-33.

56. Pajares MA, Perez-Sala D. Betaine homocysteine S-methyltransferase: just a regulator of homocysteine metabolism?. Cell Mol Life Sci 2006;63(23):2792-803.

57. Zeisel SH. Dietary choline deficiency causes DNA strand breaks and alters epigenetic marks on DNA and histones. Mutat Res 2012;733(1-2):34-8.

58. Soloway S, Stetten D Jr. The metabolism of choline and its conversion to glycine in the rat. J Biol Chem 1953;204(1):207-14.

59. Haubrich DR, Gerber NH. Choline dehydrogenase. Assay, properties and inhibitors. Biochem Pharmacol 1981;30(21):2993-3000.

60. Grossman EB, Hebert SC. Renal inner medullary choline dehydrogenase activity: characterization and modulation. Am J Physiol 1989;256(1 Pt 2):F107-12.

61. McKeever MP, Weir DG, Molloy A, Scott JM. Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat. Clin Sci (Lond) 1991;81(4):551-6.

62. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res 2008;49(6):1187-94.

63. Pritchard PH, Vance DE. Choline metabolism and phosphatidylcholine biosynthesis in cultured rat hepatocytes. Biochem J 1981;196(1):261-7.

64. Kennedy EP. The synthesis of cytidine diphosphate choline, cytidine diphosphate ethanolamine, and related compounds. J Biol Chem 1956;222(1):185-91.

65. Sundler R, Akesson B. Biosynthesis of phosphatidylethanolamines and phosphatidylcholines from ethanolamine and choline in rat liver. Biochem J 1975;146(2):309-15.

66. DeLong CJ, Shen YJ, Thomas MJ, Cui Z. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. J Biol Chem 1999;274(42):29683-8.

67. Kent C. Regulatory enzymes of phosphatidylcholine biosynthesis: a personal perspective. Biochim Biophys Acta 2005;1733(1):53-66.

68. Bremer J, Greenberg DM. Biosynthesis of choline in vitro. Biochim Biophys Acta 1960;37:173-5.

69. Vance JE. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. J Lipid Res 2008;49(7):1377-87.

70. Ridgway ND, Yao Z, Vance DE. Phosphatidylethanolamine levels and regulation of phosphatidylethanolamine N-methyltransferase. J Biol Chem 1989;264(2):1203-7.

71. Cantoni GL. Biological methylation: selected aspects. Annu Rev Biochem 1975;44:435-51.

72. Vance DE, Ridgway ND. The methylation of phosphatidylethanolamine. Prog Lipid Res 1988;27(1):61-79.

73. Infante JP, Kinsella JE. Phospholipid synthesis in mammary tissue. Choline and ethanolamine kinases: kinetic evidence for two discrete active sites. Lipids 1976;11(10):727-35.

74. Bjornstad P. Phospholipase activity in rat liver mitochondria studied by the use of endogenous substrates. J Lipid Res 1966;7(5):612-20.

75. Noga AA, Vance DE. Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. J Lipid Res 2003;44(10):1998-2005.

76. Horl G, Wagner A, Cole LK, Malli R, Reicher H, Kotzbeck P, Kofeler H, Hofler G, Frank S, Bogner-Strauss JG, et al. Sequential synthesis and methylation of phosphatidylethanolamine promote lipid droplet biosynthesis and stability in tissue culture and in vivo. J Biol Chem 2011;286(19):17338-50.

77. Vance JE, Vance DE. Phospholipid biosynthesis in mammalian cells. Biochem Cell Biol 2004;82(1):113-28.

78. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 2009;50 Suppl:S237-42.

79. Billah MM, Anthes JC. The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J 1990;269(2):281-91.

80. Parthasarathy S, Subbaiah PV, Ganguly J. The mechanism of intestinal absorption of phosphatidylcholine in rats. Biochem J 1974;140(3):503-8.

81. Morgan CP, Insall R, Haynes L, Cockcroft S. Identification of phospholipase B from Dictyostelium discoideum reveals a new lipase family present in mammals, flies and nematodes, but not yeast. Biochem J 2004;382(Pt 2):441-9.

82. Zeisel SH, Wishnok JS, Blusztajn JK. Formation of methylamines from ingested choline and lecithin. J Pharmacol Exp Ther 1983;225(2):320-4.

83. Romano KA, Vivas EI, Amador-Noguez D, Rey FE. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. MBio 2015;6(2):e02481.

84. Baker JR, Chaykin S. The biosynthesis of trimethylamine-N-oxide. J Biol Chem 1962;237:1309-13.

85. Shaw GM, Carmichael SL, Yang W, Selvin S, Schaffer DM. Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. Am J Epidemiol 2004;160(2):102-9.

86. Lavery AM, Brender JD, Zhao H, Sweeney A, Felkner M, Suarez L, Canfield MA. Dietary intake of choline and neural tube defects in Mexican Americans. Birth Defects Res A Clin Mol Teratol 2014;100(6):463-71.

87. Shaw GM, Finnell RH, Blom HJ, Carmichael SL, Vollset SE, Yang W, Ueland PM. Choline and risk of neural tube defects in a folate-fortified population. Epidemiology 2009;20(5):714-9.

88. Wu BT, Dyer RA, King DJ, Richardson KJ, Innis SM. Early second trimester maternal plasma choline and betaine are related to measures of early cognitive development in term infants. PLoS One 2012;7(8):e43448.

89. Strain JJ, McSorley EM, van Wijngaarden E, Kobrosly RW, Bonham MP, Mulhern MS, McAfee AJ, Davidson PW, Shamlaye CF, Henderson J, et al. Choline status and neurodevelopmental outcomes at 5 years of age in the Seychelles Child Development Nutrition Study. Br J Nutr 2013;110(2):330-6.

90. Yu D, Shu XO, Xiang YB, Li H, Yang G, Gao YT, Zheng W, Zhang X. Higher dietary choline intake is associated with lower risk of nonalcoholic fatty liver in normal-weight Chinese women. J Nutr 2014;144(12):2034-40.

91. Detopoulou P, Panagiotakos DB, Antonopoulou S, Pitsavos C, Stefanadis C. Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study. Am J Clin Nutr 2008;87(2):424-30.

92. Xu X, Gammon MD, Zeisel SH, Lee YL, Wetmur JG, Teitelbaum SL, Bradshaw PT, Neugut AI, Santella RM, Chen J. Choline metabolism and risk of breast cancer in a population-based study. FASEB J 2008;22(6):2045-52.

93. Du YF, Luo WP, Lin FY, Lian ZQ, Mo XF, Yan B, Xu M, Huang WQ, Huang J, Zhang CX. Dietary choline and betaine intake, choline-metabolising genetic polymorphisms and breast cancer risk: a case-control study in China. Br J Nutr 2016;116(6):961-8.

94. Xu X, Gammon MD, Zeisel SH, Bradshaw PT, Wetmur JG, Teitelbaum SL, Neugut AI, Santella RM, Chen J. High intakes of choline and betaine reduce breast cancer mortality in a population-based study. FASEB J 2009;23(11):4022-8.

95. Ying J, Rahbar MH, Hallman DM, Hernandez LM, Spitz MR, Forman MR, Gorlova OY. Associations between dietary intake of choline and betaine and lung cancer risk. PLoS One 2013;8(2):e54561.

96. Zhang CX, Pan MX, Li B, Wang L, Mo XF, Chen YM, Lin FY, Ho SC. Choline and betaine intake is inversely associated with breast cancer risk: a two-stage case-control study in China. Cancer Sci 2013;104(2):250-8.

97. Sun S, Li X, Ren A, Du M, Du H, Shu Y, Zhu L, Wang W. Choline and betaine consumption lowers cancer risk: a meta-analysis of epidemiologic studies. Sci Rep 2016;6:35547.

98. Zhou RF, Chen XL, Zhou ZG, Zhang YJ, Lan QY, Liao GC, Chen YM, Zhu HL. Higher dietary intakes of choline and betaine are associated with a lower risk of primary liver cancer: a case-control study. Sci Rep 2017;7(1):679.

99. Rees WD, Hay SM, Cruickshank M. An imbalance in the methionine content of the maternal diet reduces postnatal growth in the rat. Metabolism 2006;55(6):763-70.

100. Luka Z, Mudd SH, Wagner C. Glycine N-methyltransferase and regulation of S-adenosylmethionine levels. J Biol Chem 2009;284(34):22507-11.

101. Niculescu MD, Craciunescu CN, Zeisel SH. Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. FASEB J 2006;20(1):43-9.

102. Kovacheva VP, Mellott TJ, Davison JM, Wagner N, Lopez-Coviella I, Schnitzler AC, Blusztajn JK. Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. J Biol Chem 2007;282(43):31777-88.

103. Davison JM, Mellott TJ, Kovacheva VP, Blusztajn JK. Gestational choline supply regulates methylation of histone H3, expression of histone methyltransferases G9a

(Kmt1c) and Suv39h1 (Kmt1a), and DNA methylation of their genes in rat fetal liver and brain. J Biol Chem 2009;284(4):1982-9.

104. Jiang X, Yan J, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Caudill MA. Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. FASEB J 2012;26(8):3563-74.

105. Shaw GM, Carmichael SL, Laurent C, Rasmussen SA. Maternal nutrient intakes and risk of orofacial clefts. Epidemiology 2006;17(3):285-91.

106. Yang W, Shaw GM, Carmichael SL, Rasmussen SA, Waller DK, Pober BR, Anderka M, National Birth Defects Prevention Study. Nutrient intakes in women and congenital diaphragmatic hernia in their offspring. Birth Defects Res A Clin Mol Teratol 2008;82(3):131-8.

107. Carmichael SL, Yang W, Correa A, Olney RS, Shaw GM, National Birth Defects Prevention Study. Hypospadias and intake of nutrients related to one-carbon metabolism. J Urol 2009;181(1):315-21; discussion 21.

108. Chan J, Deng L, Mikael LG, Yan J, Pickell L, Wu Q, Caudill MA, Rozen R. Low dietary choline and low dietary riboflavin during pregnancy influence reproductive outcomes and heart development in mice. Am J Clin Nutr 2010;91(4):1035-43.

109. Blusztajn JK, Slack BE, Mellott TJ. Neuroprotective actions of dietary choline. Nutrients 2017;9(8).

110. Signore C, Ueland PM, Troendle J, Mills JL. Choline concentrations in human maternal and cord blood and intelligence at 5 y of age. Am J Clin Nutr 2008;87(4):896-902.

111. Villamor E, Rifas-Shiman SL, Gillman MW, Oken E. Maternal intake of methyl-donor nutrients and child cognition at 3 years of age. Paediatr Perinat Epidemiol 2012;26(4):328-35.

112. Poly C, Massaro JM, Seshadri S, Wolf PA, Cho E, Krall E, Jacques PF, Au R. The relation of dietary choline to cognitive performance and white-matter hyperintensity in the Framingham Offspring Cohort. Am J Clin Nutr 2011;94(6):1584-91.

113. Nurk E, Refsum H, Bjelland I, Drevon CA, Tell GS, Ueland PM, Vollset SE, Engedal K, Nygaard HA, Smith DA. Plasma free choline, betaine and cognitive performance: the Hordaland Health Study. Br J Nutr 2013;109(3):511-9.

114. Ladd SL, Sommer SA, LaBerge S, Toscano W. Effect of phosphatidylcholine on explicit memory. Clin Neuropharmacol 1993;16(6):540-9.

115. Spiers PA, Myers D, Hochanadel GS, Lieberman HR, Wurtman RJ. Citicoline improves verbal memory in aging. Arch Neurol 1996;53(5):441-8.

116. Benton D, Donohoe RT. The influence on cognition of the interactions between lecithin, carnitine and carbohydrate. Psychopharmacology (Berl) 2004;175(1):84-91.

117. Knott V, de la Salle S, Choueiry J, Impey D, Smith D, Smith M, Beaudry E, Saghir S, Ilivitsky V, Labelle A. Neurocognitive effects of acute choline supplementation in low, medium and high performer healthy volunteers. Pharmacol Biochem Behav 2015;131:119-29.

118. Naber M, Hommel B, Colzato LS. Improved human visuomotor performance and pupil constriction after choline supplementation in a placebo-controlled double-blind study. Sci Rep 2015;5:13188.

119. Lippelt DP, van der Kint S, van Herk K, Naber M. No acute effects of choline bitartrate food supplements on memory in healthy, young, human adults. PLoS One 2016;11(6):e0157714.

120. da Costa KA, Badea M, Fischer LM, Zeisel SH. Elevated serum creatine phosphokinase in choline-deficient humans: mechanistic studies in C2C12 mouse myoblasts. Am J Clin Nutr 2004;80(1):163-70.

121. Wirtz KW, Zilversmit DB. Exchange of phospholipids between liver mitochondria and microsomes in vitro. J Biol Chem 1968;243(13):3596-602.

122. Devaux PF. Static and dynamic lipid asymmetry in cell membranes. Biochemistry 1991;30(5):1163-73.

123. Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A, Vance DE. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell Metab 2006;3(5):321-31.

124. Ling J, Chaba T, Zhu LF, Jacobs RL, Vance DE. Hepatic ratio of phosphatidylcholine to phosphatidylethanolamine predicts survival after partial hepatectomy in mice. Hepatology 2012;55(4):1094-102.

125. Higgins JA, Fieldsend JK. Phosphatidylcholine synthesis for incorporation into membranes or for secretion as plasma lipoproteins by Golgi membranes of rat liver. J Lipid Res 1987;28(3):268-78.

126. Vance DE. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. Curr Opin Lipidol 2008;19(3):229-34.

127. Corbin KD, Zeisel SH. Choline metabolism provides novel insights into nonalcoholic fatty liver disease and its progression. Curr Opin Gastroenterol 2012;28(2):159-65.

128. Guerrerio AL, Colvin RM, Schwartz AK, Molleston JP, Murray KF, Diehl A, Mohan P, Schwimmer JB, Lavine JE, Torbenson MS, et al. Choline intake in a large cohort of patients with nonalcoholic fatty liver disease. Am J Clin Nutr 2012;95(4):892-900.

129. Zeisel SH. Choline: an important nutrient in brain development, liver function and carcinogenesis. J Am Coll Nutr 1992;11(5):473-81.

130. Buchman AL, Ament ME, Sohel M, Dubin M, Jenden DJ, Roch M, Pownall H, Farley W, Awal M, Ahn C. Choline deficiency causes reversible hepatic abnormalities in patients receiving parenteral nutrition: proof of a human choline requirement: a placebo-controlled trial. JPEN J Parenter Enteral Nutr 2001;25(5):260-8.

131. Gerhard GT, Duell PB. Homocysteine and atherosclerosis. Curr Opin Lipidol 1999;10(5):417-28.

132. Leach NV, Dronca E, Vesa SC, Sampelean DP, Craciun EC, Lupsor M, Crisan D, Tarau R, Rusu R, Para I, et al. Serum homocysteine levels, oxidative stress and cardiovascular risk in non-alcoholic steatohepatitis. Eur J Intern Med 2014;25(8):762-7.

133. Dalmeijer GW, Olthof MR, Verhoef P, Bots ML, van der Schouw YT. Prospective study on dietary intakes of folate, betaine, and choline and cardiovascular disease risk in women. Eur J Clin Nutr 2008;62(3):386-94.

134. Konstantinova SV, Tell GS, Vollset SE, Nygard O, Bleie O, Ueland PM. Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr 2008;138(5):914-20.

135. Meyer KA, Shea JW. Dietary choline and betaine and risk of CVD: a systematic review and meta-analysis of prospective studies. Nutrients 2017;9(7).

136. Schwab U, Torronen A, Toppinen L, Alfthan G, Saarinen M, Aro A, Uusitupa M. Betaine supplementation decreases plasma homocysteine concentrations but does not

affect body weight, body composition, or resting energy expenditure in human subjects. Am J Clin Nutr 2002;76(5):961-7.

137. Steenge GR, Verhoef P, Katan MB. Betaine supplementation lowers plasma homocysteine in healthy men and women. J Nutr 2003;133(5):1291-5.

138. Olthof MR, Brink EJ, Katan MB, Verhoef P. Choline supplemented as phosphatidylcholine decreases fasting and postmethionine-loading plasma homocysteine concentrations in healthy men. Am J Clin Nutr 2005;82(1):111-7.

139. Huang T, Chen Y, Yang B, Yang J, Wahlqvist ML, Li D. Meta-analysis of B vitamin supplementation on plasma homocysteine, cardiovascular and all-cause mortality. Clin Nutr 2012;31(4):448-54.

140. Pan Y, Guo LL, Cai LL, Zhu XJ, Shu JL, Liu XL, Jin HM. Homocysteine-lowering therapy does not lead to reduction in cardiovascular outcomes in chronic kidney disease patients: a meta-analysis of randomised, controlled trials. Br J Nutr 2012;108(3):400-7.

141. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368(17):1575-84.

142. Mente A, Chalcraft K, Ak H, Davis AD, Lonn E, Miller R, Potter MA, Yusuf S, Anand SS, McQueen MJ. The relationship between trimethylamine-N-oxide and prevalent cardiovascular disease in a multiethnic population living in Canada. Can J Cardiol 2015;31(9):1189-94.

143. Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, Li L, Fu X, Wu Y, Mehrabian M, et al. Gut microbial metabolite TMAO Enhances platelet hyperreactivity and thrombosis risk. Cell 2016;165(1):111-24.

144. Miller CA, Corbin KD, da Costa KA, Zhang S, Zhao X, Galanko JA, Blevins T, Bennett BJ, O'Connor A, Zeisel SH. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. Am J Clin Nutr 2014;100(3):778-86.

145. DiMarco DM, Missimer A, Murillo AG, Lemos BS, Malysheva OV, Caudill MA, Blesso CN, Fernandez ML. Intake of up to 3 eggs/day increases HDL cholesterol and plasma choline while plasma trimethylamine-N-oxide is unchanged in a healthy population. Lipids 2017;52(3):255-63.

146. Mueller DM, Allenspach M, Othman A, Saely CH, Muendlein A, Vonbank A, Drexel H, von Eckardstein A. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. Atherosclerosis 2015;243(2):638-44.

147. Romano KA, Martinez-Del Campo A, Kasahara K, Chittim CL, Vivas EI, Amador-Noguez D, Balskus EP, Rey FE. Metabolic, epigenetic, and transgenerational effects of gut bacterial choline consumption. Cell Host Microbe 2017;22(3):279-90 e7.

148. Chawla RK, Wolf DC, Kutner MH, Bonkovsky HL. Choline may be an essential nutrient in malnourished patients with cirrhosis. Gastroenterology 1989;97(6):1514-20.

149. Institute of Medicine. Overview and Methods; in Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. National Academy Press, Washington (DC), 1998:27-38.

150. Hofvander Y, Hagman U, Hillervik C, Sjolin S. The amount of milk consumed by 1-3 months old breast- or bottle-fed infants. Acta Paediatr Scand 1982;71(6):953-8.

151. Butte NF, Garza C, Smith EO, Nichols BL. Human milk intake and growth in exclusively breast-fed infants. J Pediatr 1984;104(2):187-95.

152. Widdowson EM, McCance RA. The effect of finite periods of undernutrition at different ages on the composition and subsequent development of the rat. Proc R Soc Lond B Biol Sci 1963;158:329-42.

153. Welsch F. Studies on accumulation and metabolic fate of (N-Me3h)choline in human term placenta fragments. Biochem Pharmacol 1976;25(9):1021-30.

154. Pomfret EA, daCosta KA, Schurman LL, Zeisel SH. Measurement of choline and choline metabolite concentrations using high-pressure liquid chromatography and gas chromatography-mass spectrometry. Anal Biochem 1989;180(1):85-90.

155. Boyd WD, Graham-White J, Blackwood G, Glen I, McQueen J. Clinical effects of choline in Alzheimer senile dementia. Lancet 1977;2(8040):711.

156. Veenema K, Solis C, Li R, Wang W, Maletz CV, Abratte CM, Caudill MA. Adequate Intake levels of choline are sufficient for preventing elevations in serum markers of liver dysfunction in Mexican American men but are not optimal for minimizing plasma total homocysteine increases after a methionine load. Am J Clin Nutr 2008;88(3):685-92.

157. United States Department of Agriculture. Database for the choline content of common foods, release 1. Agricultural Research Service, Washington (DC), 2004.

158. Chern MK, Gage DA, Pietruszko R. Betaine aldehyde, betaine, and choline levels in rat livers during ethanol metabolism. Biochem Pharmacol 2000;60(11):1629-37.

159. Zeisel SH, Mar MH, Howe JC, Holden JM. Concentrations of choline-containing compounds and betaine in common foods. J Nutr 2003;133(5):1302-7.

160. Dilger RN, Garrow TA, Baker DH. Betaine can partially spare choline in chicks but only when added to diets containing a minimal level of choline. J Nutr 2007;137(10):2224-8.

161. Lewis ED, Kosik SJ, Zhao YY, Jacobs RL, Curtis JM, Field CJ. Total choline and choline-containing moieties of commercially available pulses. Plant Foods Hum Nutr 2014;69(2):115-21.

162. World Health Organization. The World Health Organization's infant feeding recommendation. World Health Organization, Geneva, 2001.

163. Health Canada, Canadian Pediatric Society , Dietitians of Canada, & Breastfeeding Committee for Canada. Nutrition for healthy term infants: Recommendations from six to 24 months. 2014. Accessed on September 18th 2017, from https://www.canada.ca/en/health-canada/services/food-nutrition/healthy-eating/infant-feeding/nutrition-healthy-term-infants-recommendations-birth-six-months/6-24-months.html.

164. Zeisel SH, Wurtman RJ. Developmental changes in rat blood choline concentration. Biochem J 1981;198(3):565-70.

165. Hanok Jeong YS, Young-Jin Chung. Choline and betaine concentrations in breast milk of korean lactating women and the choline and betaine intakes of their infants. Korean J Nutr 2010;43(6):588-96.

166. Holmes HC, Snodgrass GJ, Iles RA. Changes in the choline content of human breast milk in the first 3 weeks after birth. Eur J Pediatr 2000;159(3):198-204.

167. Bitman J, Wood DL, Mehta NR, Hamosh P, Hamosh M. Comparison of the phospholipid composition of breast milk from mothers of term and preterm infants during lactation. Am J Clin Nutr 1984;40(5):1103-19.

168. Patton S, Keenan TW. The milk fat globule membrane. Biochim Biophys Acta 1975;415(3):273-309.

169. Chao CK, Pomfret EA, Zeisel SH. Uptake of choline by rat mammary-gland epithelial cells. Biochem J 1988;254(1):33-8.

170. Yang EK, Blusztajn JK, Pomfret EA, Zeisel SH. Rat and human mammary tissue can synthesize choline moiety via the methylation of phosphatidylethanolamine. Biochem J 1988;256(3):821-8.

171. Zeisel SH, Char D, Sheard NF. Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. J Nutr 1986;116(1):50-8.

172. Cheatham CL, Sheppard KW. Synergistic effects of human milk nutrients in the support of infant recognition memory: an observational study. Nutrients 2015;7(11):9079-95.

173. Ozarda Y, Cansev M, Ulus IH. Breast milk choline contents are associated with inflammatory status of breastfeeding women. J Hum Lact 2014;30(2):161-6.

174. Wu J, Domellof M, Zivkovic AM, Larsson G, Ohman A, Nording ML. NMR-based metabolite profiling of human milk: a pilot study of methods for investigating compositional changes during lactation. Biochem Biophys Res Commun 2016;469(3):626-32.

175. Darapheak C, Takano T, Kizuki M, Nakamura K, Seino K. Consumption of animal source foods and dietary diversity reduce stunting in children in Cambodia. Int Arch Med 2013;6:29.

176. Goon S, Dey SR. A 24-hour dietary recall for assessing the intake pattern of choline among Bangladeshi pregnant women at their third trimester of pregnancy. Cent Asian J Glob Health 2014;3(1).

177. McDonald CM, McLean J, Kroeun H, Talukder A, Lynd LD, Green TJ. Household food insecurity and dietary diversity as correlates of maternal and child undernutrition in rural Cambodia. Eur J Clin Nutr 2015;69(2):242-6.

178. Mark HE, Houghton LA, Gibson RS, Monterrosa E, Kraemer K. Estimating dietary micronutrient supply and the prevalence of inadequate intakes from national Food Balance Sheets in the South Asia regiona. Asia Pac J Clin Nutr 2016;25(2):368-76.

179. Allen LH. Multiple micronutrients in pregnancy and lactation: an overview. Am J Clin Nutr 2005;81(5):1206S-12S.

180. Food and Agriculture Organization of the United Nations, Statistical Division. FAOSTAT Statistics database, 2013. Accessed on March 15th 2017, from www.fao.org/faostat/en/#compare.

181. Wallace LJ, Summerlee AJ, Dewey CE, Hak C, Hall A, Charles CV. Women's nutrient intakes and food-related knowledge in rural Kandal province, Cambodia. Asia Pac J Clin Nutr 2014;23(2):263-71.

182. Fischer LM, Scearce JA, Mar MH, Patel JR, Blanchard RT, Macintosh BA, Busby MG, Zeisel SH. Ad libitum choline intake in healthy individuals meets or exceeds the proposed adequate intake level. J Nutr 2005;135(4):826-9.

183. Chester DN, Goldman JD, Ahuja JK, Moshfegh A. Dietary intakes of choline: what we eat in America, NHANES 2007–2008. Food Surveys Research Group Dietary Data Brief No 9 2011.

184. Wallace TC, Fulgoni VL 3rd. Assessment of total choline intakes in the United States. J Am Coll Nutr 2016;35(2):108-12.

185. Wallace TC, Fulgoni VL. Usual choline intakes are associated with egg and protein food consumption in the United States. Nutrients 2017;9(8).

186. Gao X, Wang Y, Randell E, Pedram P, Yi Y, Gulliver W, Sun G. Higher dietary choline and betaine intakes are associated with better body composition in the adult population of Newfoundland, Canada. PLoS One 2016;11(5):e0155403.

187. Cho E, Zeisel SH, Jacques P, Selhub J, Dougherty L, Colditz GA, Willett WC. Dietary choline and betaine assessed by food-frequency questionnaire in relation to plasma total homocysteine concentration in the Framingham Offspring Study. Am J Clin Nutr 2006;83(4):905-11.

188. Pauwels S, Dopere I, Huybrechts I, Godderis L, Koppen G, Vansant G. Reproducibility and validity of an FFQ to assess usual intake of methyl-group donors. Public Health Nutr 2015;18(14):2530-9.

189. Brunst KJ, Kannan S, Ni YM, Gennings C, Ganguri HB, Wright RJ. Validation of a food frequency questionnaire for estimating micronutrient intakes in an urban US sample of multi-ethnic pregnant women. Matern Child Health J 2016;20(2):250-60.

190. Coathup V, Wheeler S, Smith L. A method comparison of a food frequency questionnaire to measure folate, choline, betaine, vitamin C and carotenoids with 24-h dietary recalls in women of reproductive age. Eur J Clin Nutr 2016;70(3):346-51.

191. Vennemann FB, Ioannidou S, Valsta LM, Dumas C, Ocke MC, Mensink GB, Lindtner O, Virtanen SM, Tlustos C, D'Addezio L, et al. Dietary intake and food sources of choline in European populations. Br J Nutr 2015;114(12):2046-55.

192. Cho E, Willett WC, Colditz GA, Fuchs CS, Wu K, Chan AT, Zeisel SH, Giovannucci EL. Dietary choline and betaine and the risk of distal colorectal adenoma in women. J Natl Cancer Inst 2007;99(16):1224-31.

193. Bidulescu A, Chambless LE, Siega-Riz AM, Zeisel SH, Heiss G. Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) study. Nutr J 2009;8:14.

194. Bertoia ML, Pai JK, Cooke JP, Joosten MM, Mittleman MA, Rimm EB, Mukamal KJ. Plasma homocysteine, dietary B vitamins, betaine, and choline and risk of peripheral artery disease. Atherosclerosis 2014;235(1):94-101.

195. Mygind VL, Evans SE, Peddie MC, Miller JC, Houghton LA. Estimation of usual intake and food sources of choline and betaine in New Zealand reproductive age women. Asia Pac J Clin Nutr 2013;22(2):319-24.

196. Chiuve SE, Giovannucci EL, Hankinson SE, Zeisel SH, Dougherty LW, Willett WC, Rimm EB. The association between betaine and choline intakes and the plasma concentrations of homocysteine in women. Am J Clin Nutr 2007;86(4):1073-81.

197. Lee JE, Giovannucci E, Fuchs CS, Willett WC, Zeisel SH, Cho E. Choline and betaine intake and the risk of colorectal cancer in men. Cancer Epidemiol Biomarkers Prev 2010;19(3):884-7.

198. Richman EL, Kenfield SA, Stampfer MJ, Giovannucci EL, Zeisel SH, Willett WC, Chan JM. Choline intake and risk of lethal prostate cancer: incidence and survival. Am J Clin Nutr 2012;96(4):855-63.

199. Nagata C, Wada K, Tamura T, Konishi K, Kawachi T, Tsuji M, Nakamura K. Choline and betaine intakes are not associated with cardiovascular disease mortality risk in Japanese men and women. J Nutr 2015;145(8):1787-92.

200. Brunst KJ, Wright RO, DiGioia K, Enlow MB, Fernandez H, Wright RJ, Kannan S. Racial/ethnic and sociodemographic factors associated with micronutrient intakes and inadequacies among pregnant women in an urban US population. Public Health Nutr 2014;17(9):1960-70.

201. Cohen EL, Wurtman RJ. Brain acetylcholine: control by dietary choline. Science 1976;191(4227):561-2.

202. Klein J, Gonzalez R, Koppen A, Loffelholz K. Free choline and choline metabolites in rat brain and body fluids: sensitive determination and implications for choline supply to the brain. Neurochem Int 1993;22(3):293-300.

203. Zeisel SH, Niculescu MD. Perinatal choline influences brain structure and function. Nutr Rev 2006;64(4):197-203.

204. Strupp BJ, Powers BE, Velazquez R, Ash JA, Kelley CM, Alldred MJ, Strawderman M, Caudill MA, Mufson EJ, Ginsberg SD. Maternal choline supplementation: a potential prenatal treatment for Down Syndrome and Alzheimer's disease. Curr Alzheimer Res 2016;13(1):97-106.

205. Jiang X, Bar HY, Yan J, Jones S, Brannon PM, West AA, Perry CA, Ganti A, Pressman E, Devapatla S, et al. A higher maternal choline intake among third-trimester pregnant women lowers placental and circulating concentrations of the antiangiogenic factor fms-like tyrosine kinase-1 (sFLT1). FASEB J 2013;27(3):1245-53.

206. American Medical Association. Business of the AMA House of Delegates 2017 Annual Meeting. 2017. Accessed on September 18th 2017, from https://www.ama-assn.org/about-us/business-ama-house-delegates-2017-annual-meeting#ANNOTATED%20Reference%20Committee%20Reports.

207. Wurtman RJ, Hirsch MJ, Growdon JH. Lecithin consumption raises serum-free-choline levels. Lancet 1977;2(8028):68-9.

208. Jope RS, Domino EF, Mathews BN, Sitaram N, Jenden DJ, Ortez A. Free and bound choline blood levels after phosphatidylcholine. Clin Pharmacol Ther 1982;31(4):483-7.

209. Wurtman RJ, Regan M, Ulus I, Yu L. Effect of oral CDP-choline on plasma choline and uridine levels in humans. Biochem Pharmacol 2000;60(7):989-92.

210. Hirsch MJ, Growdon JH, Wurtman RJ. Relations between dietary choline or lecithin intake, serum choline levels, and various metabolic indices. Metabolism 1978;27(8):953-60.

211. Wu BT, Dyer RA, King DJ, Innis SM. Low fish intake is associated with low blood concentrations of vitamin D, choline and n-3 DHA in pregnant women. Br J Nutr 2013;109(5):936-43.

212. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989;27(5):409-37.

213. Molloy AM, Mills JL, Cox C, Daly SF, Conley M, Brody LC, Kirke PN, Scott JM, Ueland PM. Choline and homocysteine interrelations in umbilical cord and maternal plasma at delivery. Am J Clin Nutr 2005;82(4):836-42.

214. Velzing-Aarts FV, Holm PI, Fokkema MR, van der Dijs FP, Ueland PM, Muskiet FA. Plasma choline and betaine and their relation to plasma homocysteine in normal pregnancy. Am J Clin Nutr 2005;81(6):1383-9.

215. Fernandez-Roig S, Cavalle-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Salat-Batlle J, Ueland PM, Murphy MM. Low folate status enhances pregnancy changes in plasma betaine and dimethylglycine concentrations and the association between betaine and homocysteine. Am J Clin Nutr 2013;97(6):1252-9.

216. Visentin CE, Masih S, Plumptre L, Malysheva O, Nielsen DE, Sohn KJ, Ly A, Lausman AY, Berger H, Croxford R, et al. Maternal choline status, but not fetal genotype, influences cord plasma choline metabolite concentrations. J Nutr 2015;145(7):1491-7.

217. Buchman AL, Sohel M, Moukarzel A, Bryant D, Schanler R, Awal M, Burns P, Dorman K, Belfort M, Jenden DJ, et al. Plasma choline in normal newborns, infants, toddlers, and in very-low-birth-weight neonates requiring total parenteral nutrition. Nutrition 2001;17(1):18-21.

218. Ozarda Ilcol Y, Uncu G, Ulus IH. Free and phospholipid-bound choline concentrations in serum during pregnancy, after delivery and in newborns. Arch Physiol Biochem 2002;110(5):393-9.

219. Zeisel SH, Epstein MF, Wurtman RJ. Elevated choline concentration in neonatal plasma. Life Sci 1980;26(21):1827-31.

220. Nitter M, Norgard B, de Vogel S, Eussen SJ, Meyer K, Ulvik A, Ueland PM, Nygard O, Vollset SE, Bjorge T, et al. Plasma methionine, choline, betaine, and dimethylglycine in relation to colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC). Ann Oncol 2014;25(8):1609-15.

221. Bae S, Ulrich CM, Neuhouser ML, Malysheva O, Bailey LB, Xiao L, Brown EC, Cushing-Haugen KL, Zheng Y, Cheng TY, et al. Plasma choline metabolites and colorectal cancer risk in the Women's Health Initiative Observational Study. Cancer Res 2014;74(24):7442-52.

222. Wallace JM, McCormack JM, McNulty H, Walsh PM, Robson PJ, Bonham MP, Duffy ME, Ward M, Molloy AM, Scott JM, et al. Choline supplementation and measures of

choline and betaine status: a randomised, controlled trial in postmenopausal women. Br J Nutr 2012;108(7):1264-71.

223. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 2003;49(2):286-94.

224. Eckernas SA, Aquilonius SM. Free choline in human plasma analysed by simple radio-enzymatic procedure: age distribution and effect of a meal. Scand J Clin Lab Invest 1977;37(2):183-7.

225. Koc H, Mar MH, Ranasinghe A, Swenberg JA, Zeisel SH. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. Anal Chem 2002;74(18):4734-40.

226. Fraser CG, Cummings ST, Wilkinson SP, Neville RG, Knox JD, Ho O, MacWalter RS. Biological variability of 26 clinical chemistry analytes in elderly people. Clin Chem 1989;35(5):783-6.

227. McEntyre CJ, Lever M, Chambers ST, George PM, Slow S, Elmslie JL, Florkowski CM, Lunt H, Krebs JD. Variation of betaine, N,N-dimethylglycine, choline, glycerophosphorylcholine, taurine and trimethylamine-N-oxide in the plasma and urine of overweight people with type 2 diabetes over a two-year period. Ann Clin Biochem 2015;52(Pt 3):352-60.

228. Midttun O, Townsend MK, Nygard O, Tworoger SS, Brennan P, Johansson M, Ueland PM. Most blood biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway show adequate preanalytical stability and within-person reproducibility to allow assessment of exposure or nutritional status in healthy women and cardiovascular patients. J Nutr 2014;144(5):784-90.

229. Blusztajn JK, Liscovitch M, Richardson UI. Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. Proc Natl Acad Sci U S A 1987;84(15):5474-7.

230. Vance DE, Walkey CJ, Cui Z. Phosphatidylethanolamine N-methyltransferase from liver. Biochim Biophys Acta 1997;1348(1-2):142-50.

231. Meck WH, Williams CL. Perinatal choline supplementation increases the threshold for chunking in spatial memory. Neuroreport 1997;8(14):3053-9.

232. Jadavji NM, Emmerson JT, MacFarlane AJ, Willmore WG, Smith PD. B-vitamin and choline supplementation increases neuroplasticity and recovery after stroke. Neurobiol Dis 2017;103:89-100.

233. Bidulescu A, Chambless LE, Siega-Riz AM, Zeisel SH, Heiss G. Usual choline and betaine dietary intake and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. BMC Cardiovasc Disord 2007;7:20.

234. Kroke A, Klipstein-Grobusch K, Voss S, Moseneder J, Thielecke F, Noack R, Boeing H. Validation of a self-administered food-frequency questionnaire administered in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study: comparison of energy, protein, and macronutrient intakes estimated with the doubly labeled water, urinary nitrogen, and repeated 24-h dietary recall methods. Am J Clin Nutr 1999;70(4):439-47.

235. Thompson FE, Byers T. Dietary assessment resource manual. J Nutr 1994;124(11 Suppl):2245S-317S.

236. Cade J, Thompson R, Burley V, Warm D. Development, validation and utilisation of food-frequency questionnaires - a review. Public Health Nutr 2002;5(4):567-87.

237. Bingham SA, Gill C, Welch A, Day K, Cassidy A, Khaw KT, Sneyd MJ, Key TJ, Roe L, Day NE. Comparison of dietary assessment methods in nutritional epidemiology: weighed records v. 24 h recalls, food-frequency questionnaires and estimated-diet records. Br J Nutr 1994;72(4):619-43.

238. Block G. A review of validations of dietary assessment methods. Am J Epidemiol 1982;115(4):492-505.

239. Thompson RL, Margetts BM. Comparison of a food frequency questionnaire with a 10-day weighed record in cigarette smokers. Int J Epidemiol 1993;22(5):824-33.

240. Schatzkin A, Kipnis V, Carroll RJ, Midthune D, Subar AF, Bingham S, Schoeller DA, Troiano RP, Freedman LS. A comparison of a food frequency questionnaire with a 24-hour recall for use in an epidemiological cohort study: results from the biomarker-based Observing Protein and Energy Nutrition (OPEN) study. Int J Epidemiol 2003;32(6):1054-62.

241. Basiotis PP, Welsh SO, Cronin FJ, Kelsay JL, Mertz W. Number of days of food intake records required to estimate individual and group nutrient intakes with defined confidence. J Nutr 1987;117(9):1638-41.

242. Ma Y, Olendzki BC, Pagoto SL, Hurley TG, Magner RP, Ockene IS, Schneider KL, Merriam PA, Hebert JR. Number of 24-hour diet recalls needed to estimate energy intake. Ann Epidemiol 2009;19(8):553-9.

243. Gibson RS. Principles of Nutritional Assessment, 2nd ed. Oxford: Oxford University Press, 2005.

244. Cade JE, Burley VJ, Warm DL, Thompson RL, Margetts BM. Food-frequency questionnaires: a review of their design, validation and utilisation. Nutr Res Rev 2004;17(1):5-22.

245. Beaton GH, Milner J, Corey P, McGuire V, Cousins M, Stewart E, de Ramos M, Hewitt D, Grambsch PV, Kassim N, et al. Sources of variance in 24-hour dietary recall data: implications for nutrition study design and interpretation. Am J Clin Nutr 1979;32(12):2546-59.

246. Willett W. Nutritional Epidemiology, 3rd ed. Oxford: Oxford University Press, 2013.

247. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1(8476):307-10.

248. Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8(2):135-60.

249. Cohen J. Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit. Psychol Bull 1968;70(4):213-20.

250. Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH, Speizer FE. Reproducibility and validity of a semiquantitative food frequency questionnaire. Am J Epidemiol 1985;122(1):51-65.

251. Masson LF, McNeill G, Tomany JO, Simpson JA, Peace HS, Wei L, Grubb DA, Bolton-Smith C. Statistical approaches for assessing the relative validity of a food-frequency questionnaire: use of correlation coefficients and the kappa statistic. Public Health Nutr 2003;6(3):313-21.

252. World Medical Association. 18th General Assembly, Helsinki, Finland, June 1964.

254. Conway JM, Ingwersen LA, Vinyard BT, Moshfegh AJ. Effectiveness of the US Department of Agriculture 5-step multiple-pass method in assessing food intake in obese and nonobese women. Am J Clin Nutr 2003;77(5):1171-8.

255. Friesen RW, Innis SM. Dietary arachidonic acid to EPA and DHA balance is increased among Canadian pregnant women with low fish intake. J Nutr 2009;139(12):2344-50.

256. Stephens TV, Woo H, Innis SM, Elango R. Healthy pregnant women in Canada are consuming more dietary protein at 16- and 36-week gestation than currently recommended by the Dietary Reference Intakes, primarily from dairy food sources. Nutr Res 2014;34(7):569-76.

257. ESHA Research. Food Processor Nutrition Analysis Software. 2015.

258. Health Canada. Canadian Nutrient File. 2010.

259. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. Am J Clin Nutr 1997;65(4 Suppl):1220S-8S; discussion 9S-31S.

260. Beaton GH, Milner J, McGuire V, Feather TE, Little JA. Source of variance in 24-hour dietary recall data: implications for nutrition study design and interpretation. Carbohydrate sources, vitamins, and minerals. Am J Clin Nutr 1983;37(6):986-95.

261. Lombard MJ, Steyn NP, Charlton KE, Senekal M. Application and interpretation of multiple statistical tests to evaluate validity of dietary intake assessment methods. Nutr J 2015;14:40.

262. Ludbrook J. Linear regression analysis for comparing two measurers or methods of measurement: but which regression? Clin Exp Pharmacol Physiol 2010;37(7):692-9.

263. Giavarina D. Understanding Bland Altman analysis. Biochem Med (Zagreb) 2015;25(2):141-51.

264. Molag ML, de Vries JH, Ocke MC, Dagnelie PC, van den Brandt PA, Jansen MC, van Staveren WA, van't Veer P. Design characteristics of food frequency questionnaires in relation to their validity. Am J Epidemiol 2007;166(12):1468-78.

265. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33(1):159-74.

266. Willett WC. Future directions in the development of food-frequency questionnaires. Am J Clin Nutr 1994;59(1 Suppl):171S-4S.

267. Fayet F, Flood V, Petocz P, Samman S. Relative and biomarker-based validity of a food frequency questionnaire that measures the intakes of vitamin B(12), folate, iron, and zinc in young women. Nutr Res 2011;31(1):14-20.

268. Jackson KA, Byrne NM, Magarey AM, Hills AP. Minimizing random error in dietary intakes assessed by 24-h recall, in overweight and obese adults. Eur J Clin Nutr 2008;62(4):537-43.

269. Briefel RR, Flegal KM, Winn DM, Loria CM, Johnson CL, Sempos CT. Assessing the nation's diet: limitations of the food frequency questionnaire. J Am Diet Assoc 1992;92(8):959-62.

270. Garriguet D. Under-reporting of energy intake in the Canadian Community Health Survey. Health Rep 2008;19(4):37-45.

271. Zeisel SH. Choline: needed for normal development of memory. J Am Coll Nutr 2000;19(5 Suppl):528S-31S.

272. Zeisel SH. Choline phospholipids: signal transduction and carcinogenesis. FASEB J 1993;7(6):551-7.

273. Sweiry JH, Page KR, Dacke CG, Abramovich DR, Yudilevich DL. Evidence of saturable uptake mechanisms at maternal and fetal sides of the perfused human placenta by rapid paired-tracer dilution: studies with calcium and choline. J Dev Physiol 1986;8(6):435-45.

274. Grassl SM. Choline transport in human placental brush-border membrane vesicles. Biochim Biophys Acta 1994;1194(1):203-13.

275. Lopez C, Menard O. Human milk fat globules: polar lipid composition and in situ structural investigations revealing the heterogeneous distribution of proteins and the lateral segregation of sphingomyelin in the biological membrane. Colloids Surf B Biointerfaces 2011;83(1):29-41.

276. Stoll AL, Renshaw PF, De Micheli E, Wurtman R, Pillay SS, Cohen BM. Choline ingestion increases the resonance of choline-containing compounds in human brain: an in vivo proton magnetic resonance study. Biol Psychiatry 1995;37(3):170-4.

277. Babb SM, Ke Y, Lange N, Kaufman MJ, Renshaw PF, Cohen BM. Oral choline increases choline metabolites in human brain. Psychiatry Res 2004;130(1):1-9.

278. Moukarzel S, Soberanes L, Dyer RA, Albersheim S, Elango R, Innis SM. Relationships among different water-soluble choline compounds differ between human preterm and donor milk. Nutrients 2017;9(4).

279. World Bank. DataBank, 2013. Accessed on March 15th 2017, from http://data.worldbank.org/country/ecuador.

280. Mulder KA, King DJ, Innis SM. Omega-3 fatty acid deficiency in infants before birth identified using a randomized trial of maternal DHA supplementation in pregnancy. PLoS One 2014;9(1):e83764.

281. March KM, Chen NN, Karakochuk CD, Shand AW, Innis SM, von Dadelszen P, Barr SI, Lyon MR, Whiting SJ, Weiler HA, et al. Maternal vitamin D(3) supplementation at 50 mug/d protects against low serum 25-hydroxyvitamin D in infants at 8 wk of age: a randomized controlled trial of 3 doses of vitamin D beginning in gestation and continued in lactation. Am J Clin Nutr 2015;102(2):402-10.

282. Whitfield KC, Karakochuk CD, Kroeun H, Hampel D, Sokhoing L, Chan BB, Borath M, Sophonneary P, McLean J, Talukder A, et al. Perinatal Consumption of thiamine-fortified fish sauce in rural Cambodia: a randomized clinical trial. JAMA Pediatr 2016:e162065.

283. Cohen J. A power primer. Psychol Bull 1992;112(1):155-9.

284. Cohen J. Statistical Power Analysis for the Behavioral Sciences: Taylor & Francis, 2013.

285. Whitfield KC, Karakochuk CD, Kroeun H, Sokhoing L, Chan BB, Borath M, Sophonneary P, Moore K, Tong JK, McLean J, et al. Household consumption of thiamin-fortified fish sauce increases erythrocyte thiamin concentrations among rural Cambodian women and their children younger than 5 years of age: a randomized controlled efficacy trial. J Pediatr 2017;181:242-7 e2.

286. Innis SM, Hasman D. Evidence of choline depletion and reduced betaine and dimethylglycine with increased homocysteine in plasma of children with cystic fibrosis. J Nutr 2006;136(8):2226-31.

287. Neville MC, Keller R, Seacat J, Lutes V, Neifert M, Casey C, Allen J, Archer P. Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation. Am J Clin Nutr 1988;48(6):1375-86.

288. Allen JC, Keller RP, Archer P, Neville MC. Studies in human lactation: milk composition and daily secretion rates of macronutrients in the first year of lactation. Am J Clin Nutr 1991;54(1):69-80.

289. Elias SL, Innis SM. Bakery foods are the major dietary source of trans-fatty acids among pregnant women with diets providing 30 percent energy from fat. J Am Diet Assoc 2002;102(1):46-51.

290. Gibson RS, Cavalli-Sforza T. Using reference nutrient density goals with food balance sheet data to identify likely micronutrient deficits for fortification planning in countries in the Western Pacific region. Food Nutr Bull 2012;33(3 Suppl):S214-20.

291. Zeisel SH. Choline, homocysteine, and pregnancy. Am J Clin Nutr 2005;82(4):719-20.

292. Buyukuysal RL, Ulus IH, Aydin S, Kiran BK. 3,4-Diaminopyridine and choline increase in vivo acetylcholine release in rat striatum. Eur J Pharmacol 1995;281(2):179-85.

293. Hampel D, Allen LH. Analyzing B-vitamins in human milk: methodological approaches. Crit Rev Food Sci Nutr 2016;56(3):494-511.

294. Koletzko B, Baker S, Cleghorn G, Neto UF, Gopalan S, Hernell O, Hock QS, Jirapinyo P, Lonnerdal B, Pencharz P, et al. Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. J Pediatr Gastroenterol Nutr 2005;41(5):584-99.

295. Codex Alimentarius Commision. Codex Standard 72 for Infant Formula. Codex Alimentarius, 2007.

296. Klein J, Koppen A, Loffelholz K. Regulation of free choline in rat brain: dietary and pharmacological manipulations. Neurochem Int 1998;32(5-6):479-85.

297. Bonham MP, Duffy EM, Robson PJ, Wallace JM, Myers GJ, Davidson PW, Clarkson TW, Shamlaye CF, Strain JJ, Livingstone MB. Contribution of fish to intakes of micronutrients important for fetal development: a dietary survey of pregnant women in the Republic of Seychelles. Public Health Nutr 2009;12(9):1312-20.

298. Prentice AM. Applications of nutritional biomarkers in global health settings. Nestle Nutr Inst Workshop Ser 2016;84:15-23.

299. Yue B, Pattison E, Roberts WL, Rockwood AL, Danne O, Lueders C, Mockel M. Choline in whole blood and plasma: sample preparation and stability. Clin Chem 2008;54(3):590-3.

300. Yao ZM, Vance DE. Reduction in VLDL, but not HDL, in plasma of rats deficient in choline. Biochem Cell Biol 1990;68(2):552-8.

301. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem 1990;1(5):228-37.

302. Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr 2002;132(8 Suppl):2333S-5S.

303. Craig SA. Betaine in human nutrition. Am J Clin Nutr 2004;80(3):539-49.

304. Buchman AL, Dubin MD, Moukarzel AA, Jenden DJ, Roch M, Rice KM, Gornbein J, Ament ME. Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. Hepatology 1995;22(5):1399-403.

305. Guba SC, Fink LM, Fonseca V. Hyperhomocysteinemia. An emerging and important risk factor for thromboembolic and cardiovascular disease. Am J Clin Pathol 1996;106(6):709-22.

306. Lever M, George PM, Dellow WJ, Scott RS, Chambers ST. Homocysteine, glycine betaine, and N,N-dimethylglycine in patients attending a lipid clinic. Metabolism 2005;54(1):1-14.

307. Waite KA, Vance DE. Dimethylethanolamine does not prevent liver failure in phosphatidylethanolamine N-methyltransferase-deficient mice fed a choline-deficient diet. Biochim Biophys Acta 2004;1636(2-3):175-82.

308. Blusztajn JK, Zeisel SH, Wurtman RJ. Developmental changes in the activity of phosphatidylethanolamine N-methyltransferases in rat brain. Biochem J 1985;232(2):505-11.

309. Atkinson W, Elmslie J, Lever M, Chambers ST, George PM. Dietary and supplementary betaine: acute effects on plasma betaine and homocysteine concentrations under standard and postmethionine load conditions in healthy male subjects. Am J Clin Nutr 2008;87(3):577-85.

310. Lever M, Sizeland PC, Frampton CM, Chambers ST. Short and long-term variation of plasma glycine betaine concentrations in humans. Clin Biochem 2004;37(3):184-90.

311. Lever M, Atkinson W, Slow S, Chambers ST, George PM. Plasma and urine betaine and dimethylglycine variation in healthy young male subjects. Clin Biochem 2009;42(7-8):706-12.

312. Lever M, Atkinson W, George PM, Chambers ST. Sex differences in the control of plasma concentrations and urinary excretion of glycine betaine in patients attending a lipid disorders clinic. Clin Biochem 2007;40(16-17):1225-31.

313. Kirsch SH, Herrmann W, Rabagny Y, Obeid R. Quantification of acetylcholine, choline, betaine, and dimethylglycine in human plasma and urine using stable-isotope dilution ultra performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878(32):3338-44.

314. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. J Chiropr Med 2016;15(2):155-63.

315. Meikle PJ, Barlow CK, Mellett NA, Mundra PA, Bonham MP, Larsen A, Cameron-Smith D, Sinclair A, Nestel PJ, Wong G. Postprandial plasma phospholipids in men are influenced by the source of dietary fat. J Nutr 2015;145(9):2012-8.

316. Friesen RW, Novak EM, Hasman D, Innis SM. Relationship of dimethylglycine, choline, and betaine with oxoproline in plasma of pregnant women and their newborn infants. J Nutr 2007;137(12):2641-6.

317. Innis SM, Dyer RA. Brain astrocyte synthesis of docosahexaenoic acid from n-3 fatty acids is limited at the elongation of docosapentaenoic acid. J Lipid Res 2002;43(9):1529-36.

318. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226(1):497-509.

319. Innis SM, Nelson CM, Rioux MF, King DJ. Development of visual acuity in relation to plasma and erythrocyte omega-6 and omega-3 fatty acids in healthy term gestation infants. Am J Clin Nutr 1994;60(3):347-52.

320. Costongs GM, Janson PC, Bas BM, Hermans J, van Wersch JW, Brombacher PJ. Short-term and long-term intra-individual variations and critical differences of clinical chemical laboratory parameters. J Clin Chem Clin Biochem 1985;23(1):7-16.

321. Clinical & Laboratory Standards Institute (CLSI). Defining, establishing, and verifying reference intervals in the clinical laboratory. Approved Guideline 3rd ed. Wayne, PA 2008.

322. Blanck HM, Bowman BA, Cooper GR, Myers GL, Miller DT. Laboratory issues: use of nutritional biomarkers. J Nutr 2003;133(Suppl 3):888S-94S.

323. Potischman N. Biologic and methodologic issues for nutritional biomarkers. J Nutr 2003;133(Suppl 3):875S-80S.

324. Obeid R, Awwad HM, Keller M, Geisel J. Trimethylamine-N-oxide and its biological variations in vegetarians. Eur J Nutr 2016.

325. Kuhn T, Rohrmann S, Sookthai D, Johnson T, Katzke V, Kaaks R, von Eckardstein A, Muller D. Intra-individual variation of plasma trimethylamine-N-oxide (TMAO), betaine and choline over 1 year. Clin Chem Lab Med 2017;55(2):261-8.

326. Svingen GF, Ueland PM, Pedersen EK, Schartum-Hansen H, Seifert R, Ebbing M, Loland KH, Tell GS, Nygard O. Plasma dimethylglycine and risk of incident acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol 2013;33(8):2041-8.

327. Zhang J, Blusztajn JK, Zeisel SH. Measurement of the formation of betaine aldehyde and betaine in rat liver mitochondria by a high pressure liquid chromatography-radioenzymatic assay. Biochim Biophys Acta 1992;1117(3):333-9.

328. Slow S, Lever M, Chambers ST, George PM. Plasma dependent and independent accumulation of betaine in male and female rat tissues. Physiol Res 2009;58(3):403-10.

329. Atkinson W, Slow S, Elmslie J, Lever M, Chambers ST, George PM. Dietary and supplementary betaine: effects on betaine and homocysteine concentrations in males. Nutr Metab Cardiovasc Dis 2009;19(11):767-73.

330. Dunn G. Design and analysis of reliability studies. Stat Methods Med Res 1992;1(2):123-57.

331. Gillespie C, Ballew C, Bowman BA, Donehoo R, Serdula MK. Intraindividual variation in serum retinol concentrations among participants in the third National Health and Nutrition Examination Survey, 1988-1994. Am J Clin Nutr 2004;79(4):625-32.

332. Zeisel SH, da Costa KA. Choline: an essential nutrient for public health. Nutr Rev 2009;67(11):615-23.

333. Innis SM, Davidson AG, Melynk S, James SJ. Choline-related supplements improve abnormal plasma methionine-homocysteine metabolites and glutathione status in children with cystic fibrosis. Am J Clin Nutr 2007;85(3):702-8.

334. Pauwels S, Duca RC, Devlieger R, Freson K, Straetmans D, Van Herck E, Huybrechts I, Koppen G, Godderis L. Maternal methyl-group donor intake and global DNA (hydroxy)methylation before and during pregnancy. Nutrients 2016;8(8).

335. Huang JY, Butler LM, Wang R, Jin A, Koh WP, Yuan JM. Dietary intake of one-carbon metabolism-related nutrients and pancreatic cancer risk: The Singapore Chinese Health Study. Cancer Epidemiol Biomarkers Prev 2016;25(2):417-24.

336. Devlin AM, Chau CMY, Dyer R, Matheson J, McCarthy D, Yurko-Mauro K, Innis SM, Grunau RE. Developmental outcomes at 24 months of age in toddlers supplemented with arachidonic acid and docosahexaenoic acid: results of a double blind randomized, controlled trial. Nutrients 2017;9(9).

337. Mulder KA, Innis SM, Rasmussen BF, Wu BT, Richardson KJ, Hasman D. Plasma lutein concentrations are related to dietary intake, but unrelated to dietary saturated fat or cognition in young children. J Nutr Sci 2014;3:e11.

338. Malinowska AM, Szwengiel A, Chmurzynska A. Dietary, anthropometric, and biochemical factors influencing plasma choline, carnitine, trimethylamine, and trimethylamine-N-oxide concentrations. Int J Food Sci Nutr 2017;68(4):488-95.

339. Cho E, Holmes MD, Hankinson SE, Willett WC. Choline and betaine intake and risk of breast cancer among post-menopausal women. Br J Cancer 2010;102(3):489-94.

340. Marriott BP, Olsho L, Hadden L, Connor P. Intake of added sugars and selected nutrients in the United States, National Health and Nutrition Examination Survey (NHANES) 2003-2006. Crit Rev Food Sci Nutr 2010;50(3):228-58.

341. Institute of Medicine. Dietary Reference Intakes: Applications in Dietary Assessment. Washington, DC: The National Academies Press, 2000.

342. Misra S, Ahn C, Ament ME, Choi HJ, Jenden DJ, Roch M, Buchman AL. Plasma choline concentrations in children requiring long-term home parenteral nutrition: a case control study. JPEN J Parenter Enteral Nutr 1999;23(5):305-8.

343. Lu MS, Fang YJ, Pan ZZ, Zhong X, Zheng MC, Chen YM, Zhang CX. Choline and betaine intake and colorectal cancer risk in Chinese population: a case-control study. PLoS One 2015;10(3):e0118661.

344. Chu DM, Wahlqvist ML, Chang HY, Yeh NH, Lee MS. Choline and betaine food sources and intakes in Taiwanese. Asia Pac J Clin Nutr 2012;21(4):547-57.

345. Sheard NF, Zeisel SH. An in vitro study of choline uptake by intestine from neonatal and adult rats. Pediatr Res 1986;20(8):768-72.

346. Cornford EM, Braun LD, Oldendorf WH. Carrier mediated blood-brain barrier transport of choline and certain choline analogs. J Neurochem 1978;30(2):299-308.

347. Ehrlich BE, Diamond JM, Braun LD, Cornford EM, Oldendorf WH. Effects of lithium on blood-brain barrier transport of the neurotransmitter precursors choline, tyrosine and tryptophan. Brain Res 1980;193(2):604-7.

348. Grantham-McGregor S, Cheung YB, Cueto S, Glewwe P, Richter L, Strupp B, International Child Development Steering Group. Developmental potential in the first 5 years for children in developing countries. Lancet 2007;369(9555):60-70.

349. Grantham-McGregor S, International Child Development Committee. Early child development in developing countries. Lancet 2007;369(9564):824.

350. Rosales FJ, Reznick JS, Zeisel SH. Understanding the role of nutrition in the brain and behavioral development of toddlers and preschool children: identifying and addressing methodological barriers. Nutr Neurosci 2009;12(5):190-202.

351. Shen EY, Wu KH, Lin MF, Chen CY. Study of brain growth in children--a new approach to volume measurements using MRI-reconstructed 3D neuroimaging. Childs Nerv Syst 2010;26(11):1619-23.

352. Ganz AB, Cohen VV, Swersky CC, Stover J, Vitiello GA, Lovesky J, Chuang JC, Shields K, Fomin VG, Lopez YS, et al. Genetic variation in choline-metabolizing enzymes alters choline metabolism in young women consuming choline intakes meeting current recommendations. Int J Mol Sci 2017;18(2).

353. Song J, da Costa KA, Fischer LM, Kohlmeier M, Kwock L, Wang S, Zeisel SH. Polymorphism of the PEMT gene and susceptibility to nonalcoholic fatty liver disease (NAFLD). FASEB J 2005;19(10):1266-71.

354. da Costa KA, Corbin KD, Niculescu MD, Galanko JA, Zeisel SH. Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. FASEB J 2014;28(7):2970-8.

355. Schwahn BC, Chen Z, Laryea MD, Wendel U, Lussier-Cacan S, Genest J Jr., Mar MH, Zeisel SH, Castro C, Garrow T, et al. Homocysteine-betaine interactions in a murine model of 5,10-methylenetetrahydrofolate reductase deficiency. FASEB J 2003;17(3):512-4.

356. Kohlmeier M, da Costa KA, Fischer LM, Zeisel SH. Genetic variation of folate-mediated one-carbon transfer pathway predicts susceptibility to choline deficiency in humans. Proc Natl Acad Sci U S A 2005;102(44):16025-30.

357. Jacobs RL, Zhao Y, Koonen DP, Sletten T, Su B, Lingrell S, Cao G, Peake DA, Kuo MS, Proctor SD, et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. J Biol Chem 2010;285(29):22403-13.

358. Resseguie ME, da Costa KA, Galanko JA, Patel M, Davis IJ, Zeisel SH. Aberrant estrogen regulation of PEMT results in choline deficiency-associated liver dysfunction. J Biol Chem 2011;286(2):1649-58.

359. Oyen J, Nygard OK, Gjesdal CG, Ueland PM, Apalset EM, Schartum-Hansen H, Vollset SE, Meyer K, Tell GS. Plasma choline, nicotine exposure, and risk of low bone mineral density and hip fracture: the Hordaland health study. J Bone Miner Res 2014;29(1):242-50.

360. Statistics Canada. National Household Survey (NHS) Profile. 2011 National Household Survey. Catalogue no. 99-004-XWE. Ottawa. Released September 11, 2013.

361. Sasaki S, Kesteloot H. Value of Food and Agriculture Organization data on food-balance sheets as a data source for dietary fat intake in epidemiologic studies. Am J Clin Nutr 1992;56(4):716-23.

362. Lewis ED, Zhao YY, Richard C, Bruce HL, Jacobs RL, Field CJ, Curtis JM. Measurement of the abundance of choline and the distribution of choline-containing moieties in meat. Int J Food Sci Nutr 2015;66(7):743-8.

363. Garza C, de Onis M. Rationale for developing a new international growth reference. Food Nutr Bull 2004;25(Suppl 1):5S-14S.

364. Tremblay M, Wolfson M, Connor Gorber S. Canadian Health Measures Survey: rationale, background and overview. Health Rep 2007;18(Suppl):7S-20S.

365. Tremblay MS, Connor Gorber S. Canadian Health Measures Survey: brief overview. Can J Public Health 2007;98(6):453-6.

366. Adeli K, Higgins V, Nieuwesteeg M, Raizman JE, Chen Y, Wong SL, Blais D. Biochemical marker reference values across pediatric, adult, and geriatric ages: establishment of robust pediatric and adult reference intervals on the basis of the Canadian Health Measures Survey. Clin Chem 2015;61(8):1049-62.

367. Colantonio DA, Kyriakopoulou L, Chan MK, Daly CH, Brinc D, Venner AA, Pasic MD, Armbruster D, Adeli K. Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem 2012;58(5):854-68.

368. Teodoro-Morrison T, Kyriakopoulou L, Chen YK, Raizman JE, Bevilacqua V, Chan MK, Wan B, Yazdanpanah M, Schulze A, Adeli K. Dynamic biological changes in metabolic disease biomarkers in childhood and adolescence: A CALIPER study of healthy community children. Clin Biochem 2015;48(13-14):828-36.

369. Moraes TJ, Lefebvre DL, Chooniedass R, Becker AB, Brook JR, Denburg J, HayGlass KT, Hegele RG, Kollmann TR, Macri J, et al. The Canadian healthy infant longitudinal development birth cohort study: biological samples and biobanking. Paediatr Perinat Epidemiol 2015;29(1):84-92.

370. Subbarao P, Anand SS, Becker AB, Befus AD, Brauer M, Brook JR, Denburg JA, HayGlass KT, Kobor MS, Kollmann TR, et al. The Canadian Healthy Infant Longitudinal Development (CHILD) Study: examining developmental origins of allergy and asthma. Thorax 2015;70(10):998-1000.

371. Yetley EA, MacFarlane AJ, Greene-Finestone LS, Garza C, Ard JD, Atkinson SA, Bier DM, Carriquiry AL, Harlan WR, Hattis D, et al. Options for basing Dietary Reference Intakes (DRIs) on chronic disease endpoints: report from a joint US-/Canadian-sponsored working group. Am J Clin Nutr 2017;105(1):249S-85S.

Appendices

Appendix A: Food frequency questionnaire

Food Frequency Questionnaire
Nutrition Research and Metabolism Program, University of British Columbia

Subject Number: _____

MILK PRODUCTS:		Specify most common type:		
Milk – drinking -cow (homo, 2%, 1%, skim) -goat, rice, soy, almond -chocolate	◻ yes ◻ no		___ ◻ oz ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Hard cheese -cheddar, mozzarella, gouda, swiss, havarti -regular/low fat	◻ yes ◻ no		___ total all types ◻ oz/g ◻ tbsp ◻ 1 inch cube	___ ◻ Day ◻ Week ◻ Month
Soft cheese -brie, camembert, feta, goat	◻ yes ◻ no		___ total all types ◻ oz/g ◻ tbsp ◻ 1 inch cube	___ ◻ Day ◻ Week ◻ Month
Cottage cheese -regular/skim	◻ yes ◻ no		___ ◻ oz/g ◻ tbsp ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Other cheese: soy cheese, paneer	◻ yes ◻ no		___ ◻ oz/g ◻ tbsp ◻ 1 inch cube	___ ◻ Day ◻ Week ◻ Month
Yogurt -regular/full fat -plain/flavored - DHA/omega-3	◻ yes ◻ no		___ ◻ ¼ C./ 60g ◻ 125g ◻ 175g ◻ each	___ ◻ Day ◻ Week ◻ Month
Ice cream/Frozen yogurt	◻ yes ◻ no		___ ◻ oz/ ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Pudding or Custard	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
CEREALS/GRAIN PRODUCTS/BAKED GOODS:				
Breakfast cereal	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Milk added to cold cereal? - homo/ 2% /1% /skim	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month

Cooked cereal/oatmeal - Oatmeal, cream of wheat	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ Pkg ea	___ ◻ Day ◻ Week ◻ Month
Milk added to cooked cereal/oatmeal? - homo/ 2% /1% /skim	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ tsp ◻ tbsp	___ ◻ Day ◻ Week ◻ Month
Pancakes/Waffles -homemade, mix with eggs/milk added, mix with water added, restaurant	◻ yes ◻ no		___ ◻ pieces	___ ◻ Day ◻ Week ◻ Month
French toast	◻ yes ◻ no		___ ◻ pieces	___ ◻ Day ◻ Week ◻ Month
Added to pancakes, waffles, french toast? Whipped cream, sugar, syrup, butter, margarine	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ tsp ◻ tbsp	___ ◻ Day ◻ Week ◻ Month
Breakfast sandwiches -McMuffin, Tim Hortons	◻ yes ◻ no		___ ◻ piece ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Bread	◻ yes ◻ no		___ ◻ slices	___ ◻ Day ◻ Week ◻ Month
Dinner rolls/buns	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Bagels:	◻ yes ◻ no		___ ◻ small ◻ med ◻ large	___ ◻ Day ◻ Week ◻ Month
English muffin, crumpet	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Muffins *If homemade, made with butter/marg*	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Scones			___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Quick breads Banana Bread, Lemon loaf Homemade / store bought			___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Added to breads/ bagels/ crumpets/ muffins/ scones?				
Jam/jelly	◻ yes ◻ no		___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month

Nut/seed butters	◻ yes ◻ no		___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Butter/margarine	◻ yes ◻ no		___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Mayo (not incl. added to canned fish) -regular, low fat	◻ yes ◻ no		___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Cream cheese -light/regular	◻ yes ◻ no		___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Donuts, Pastries	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Croissants	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Cake, Cupcakes - yellow/choc cake, cupcakes with icing -purchased/homemade -made with butter/marg	◻ yes ◻ no	◻ with icing ◻ no icing	___ ◻ oz/g ◻ piece	___ ◻ Day ◻ Week ◻ Month
Pies/ Fruit crisps/ **Fruit crumbles:** fruit, pumpkin	◻ yes ◻ no	◻ added whip cream	___ ◻ oz/ ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Cookies -purchased/homemade -made with butter/marg	◻ yes ◻ no		___ ◻ piece(s)	___ ◻ Day ◻ Week ◻ Month
Crackers, plain -soda crackers	◻ yes ◻ no		___ ◻ pieces	___ ◻ Day ◻ Week ◻ Month
Crackers, savory - ritz, gold fish, etc	◻ yes ◻ no		___ ◻ pieces	___ ◻ Day ◻ Week ◻ Month
Chappati/Roti/Pita	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Steamed buns	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Tortillas (not in tacos/burritos) Corn, whole wheat, white -Specify size: 6-8", 10-12"	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month
Rice cakes	◻ yes ◻ no		___ ◻ each	___ ◻ Day ◻ Week ◻ Month

RICE/PASTA				
Rice, cooked	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Pastas and noodles: -spaghetti, linguini, fettuccini, macaroni, orzo	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Stuffed Pastas -tortellini, ravioli	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Tomato-based sauces with meat	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Tomato-based sauces no meat (vegetarian)	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Other Sauces added to pasta -Alfredo, Pesto	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Canned/frozen Pasta dishes - Alphagetti, Chef Boyardee, Lean Cuisine, Michaelinas	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Pasta, rice, potato, or noodle dishes prepared from package/box mix: -Sidekicks, Kraft dinner	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Lasagna - meat - vegetarian	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Instant Noodles - Mr. Noodles, Ichiban	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ serving	___ ◻ Day ◻ Week ◻ Month
Other cereal grain products -couscous, bulgar, quinoa	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
COMBINATION FOOD/MEALS				
Meat Pies -beef, chicken, other	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Shepherd's Pie	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Sausage rolls	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Quiche -meat, vegetable, seafood	◻ yes ◻ no		___ ◻ piece	___ ◻ Day ◻ Week ◻ Month

Potstickers/gyoza -pork, beef, shrimp, other	◻ yes ◻ no		___ ◻ piece	___ ◻ Day ◻ Week ◻ Month
Perogies -potato, potato w/cheese, other **Anything Added to Perogies?** -sour cream, bacon, butter/marg	◻ yes ◻ no		___ ◻ piece ___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Tacos/Burritos/Enchildas -beef/chicken/bean **Anything Added?** -sourcream, guacamole	◻ yes ◻ no		___ ◻ piece ___ ◻ tsp ◻ Tbsp	___ ◻ Day ◻ Week ◻ Month
Pizza	◻ yes ◻ no	Most common type:	___ ◻ piece	___ ◻ Day ◻ Week ◻ Month
Chili -meat/no meat	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Any other mixed dishes (e.g Cabbage Rolls, Casseroles)	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Soups, broth types - Chicken Noodle - Beef vegetable - Tomato	◻ yes ◻ no		___ all types ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Soups, cream types - Cream of mushroom, cream of broccoli, etc	◻ yes ◻ no		___ all types ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
MEATS, POULTRY & ALTERNATES				
Eggs - regular/omega-3	◻ yes ◻ no	*Usual Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ each	___ ◻ Day ◻ Week ◻ Month
Chicken/Turkey – Not fried - white/dark	◻ yes ◻ no	*Cooking Method?* _____ _	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month

Chicken nuggets, chicken fingers, or fried chicken	◻ yes ◻ no		___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month
Beef- roast beef or steak -roast, steak, ground (not including mixed dishes)	◻ yes ◻ no	*Cooking Method?* _____ _	___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month
Hamburgers/Veggie burgers	◻ yes ◻ no		___	◻ piece	___	◻ Day ◻ Week ◻ Month
Hamburger buns	◻ yes ◻ no		___	◻ piece	___	◻ Day ◻ Week ◻ Month
Hamburger/Veggie burger condiments - mayo/miracle whip, butter, margarine, ketchup, mustard	◻ yes ◻ no		___	◻ tsp ◻ tbsp	___	◻ Day ◻ Week ◻ Month
Hot dogs/Veggie dogs	◻ yes ◻ no		___	◻ piece	___	◻ Day ◻ Week ◻ Month
Hot dog buns	◻ yes ◻ no		___	◻ piece	___	◻ Day ◻ Week ◻ Month
Hot dog/Veggie dog condiments - mayo/miracle whip, butter, margarine, ketchup, mustard	◻ yes ◻ no		___	◻ tsp ◻ tbsp	___	◻ Day ◻ Week ◻ Month
Pork- roast or chops - roast, steak, chops	◻ yes ◻ no	*Cooking Method?* _____ _	___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month
Lamb - roast or chops	◻ yes ◻ no	*Cooking Method?* _____ _	___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month
Deli meats/ Processed meats -bologna, canned meats, salami, ham, turkey, liverwurst, other	◻ yes ◻ no		___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month
Sausages -beef, pork	◻ yes ◻ no		___	◻ oz/g ◻ cup/ml ◻ piece	___	◻ Day ◻ Week ◻ Month

Bacon	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Tofu -medium/firm/soft -dessert	◻ yes ◻ no	*Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Other meats (e.g. duck, deer, buffalo)	◻ yes ◻ no	*Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
FISH & SEAFOOD				
Salmon – fresh or frozen	◻ yes ◻ no	*Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
White Fish – fresh or frozen - sole, halibut, cod, snapper, tilapia	◻ yes ◻ no	*Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Canned salmon -water packed/oil packed	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Canned tuna -water packed/oil packed	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Mayo added to canned fish -regular, low fat, miracle whip			___ ◻ tsp ◻ tbsp ◻ cup/ml	
Battered or breaded fish (e.g. fish sticks, fish fillets)	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Shellfish -prawns, crab, mussels, oysters, shrimp, lobster, clams	◻ yes ◻ no	*Cooking Method?* _____	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month

Clam/seafood chowder	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Sushi/Sashimi	◻ yes ◻ no	Most common type:	___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
VEGGIES/SALAD				
Potatoes -boiled/mashed/baked -added milk/butter/oil	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
French Fries	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Other Fried Potatoes - hashbrowns, potato pancakes, potato nuggets)	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml ◻ piece	___ ◻ Day ◻ Week ◻ Month
Green/Spinach Salads	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Other Salads -potato salad, coleslaw, greek	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Salad Dressings/Dips - vinaigrette, Italian, ranch, -Fat-free, Low-Cal	◻ yes ◻ no	Most common type:	___ ◻ tsp ◻ tbsp	___ ◻ Day ◻ Week ◻ Month
COOKING FAT:				
Margarine/butter used in cooking (not including baking) - specify type		In what: _____ _____	___ ◻ tsp ◻ tbsp ◻ cup/ml ◻ oz/g	___ ◻ Day ◻ Week ◻ Month
Cooking oil -specify type		In what: _____ _____	___ ◻ tsp ◻ tbsp ◻ cup/ml ◻ oz/g	___ ◻ Day ◻ Week ◻ Month

BEVERAGES				
Orange juice and other citrus juices -omega-3 added?	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Apple juice, pure	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Other fruit juices	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Sweetened beverages, non pop, not diet	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Smoothies/milkshakes/ blended drinks	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Soda – Not Diet	◻ yes ◻ no	Most Common Type: _____	___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Soda –Diet	◻ yes ◻ no	Most Common Type: _____ _	___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Other Beverages -coffee, tea, hot chocolate	◻ yes ◻ no		___ ◻ oz/g ◻ cup/ml	___ ◻ Day ◻ Week ◻ Month
Sugar added to beverages	◻ yes ◻ no		___ ◻ tsp ◻ tbsp	___ ◻ Day ◻ Week ◻ Month

FRUIT

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Apple (each)	○	○	○	○	○	○	○	○	○	○	○
Orange (each)	○	○	○	○	○	○	○	○	○	○	○
Banana (each)	○	○	○	○	○	○	○	○	○	○	○
Grapes (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Grapefruit (1/2 Grapefruit)	○	○	○	○	○	○	○	○	○	○	○
Melon: watermelon (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Melon: cantaloupe (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Lychee (each)	○	○	○	○	○	○	○	○	○	○	○
Kiwi (each)	○	○	○	○	○	○	○	○	○	○	○
Pear (each)	○	○	○	○	○	○	○	○	○	○	○
Peach/ Nectarine (each)	○	○	○	○	○	○	○	○	○	○	○
Plum (each)	○	○	○	○	○	○	○	○	○	○	○
Apricot (each)	○	○	○	○	○	○	○	○	○	○	○
Pineapple (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Dried fruit/ raisins (1/4 Cup)	○	○	○	○	○	○	○	○	○	○	○
Fruit Cocktail (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Applesauce (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Strawberries (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Blueberries (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Raspberries (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Blackberries (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○
Mango (1/2 Cup)	○	○	○	○	○	○	○	○	○	○	○

Any other fruits? (papaya, etc.)

VEGETABLES

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Avocado (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Broccoli (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Carrots (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Celery (1 stalk)	O	O	O	O	O	O	O	O	O	O	O
Radish (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Cabbage (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Cucumber (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Pepper (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Tomato (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Green Beans (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Yellow Beans (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Spinach (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Brussel Sprouts (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Green Peas (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Cauliflower (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Corn (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Squash (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Sweet Potato (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Lentils (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Dried peas (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Canned beans (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Baked Beans (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O

Hummus? (store bought/homemade(oil?))

Any other Vegetable? (asparagus, kale, mushrooms, bok choy?)

Any butter/sauce added to vegetables?

SNACKS

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Potato Chips (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Cheezies (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Corn Chips (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Tortilla Chips/ Nacho Chips (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Pretzels (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O
Peanuts (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Almonds (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Walnuts? (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Other nuts/ Mixed Nuts (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Trail Mix (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Chocolate Bars	O	O	O	O	O	O	O	O	O	O	O
Chocolate Candies (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Jelly Beans (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Hard Candies (1/4 Cup)	O	O	O	O	O	O	O	O	O	O	O
Chewy Candies/ Gummies (1/4 Cup))	O	O	O	O	O	O	O	O	O	O	O
Freezies/ Popsicles	O	O	O	O	O	O	O	O	O	O	O
Jello (1/2 Cup)	O	O	O	O	O	O	O	O	O	O	O

Appendix B: Comparison of the concentration of water-soluble forms of choline and betaine in milk between trials

Comparison of the concentration of water-soluble forms of choline and betaine in milk per treatment group in the first Canadian trial

Milk metabolites	**Dose 1** (*n* = 79)	**Dose 2** (*n* = 68)	***P*-value**
	Concentrations (µmol/L)[1]		
Free choline	153 ± 84	139 ± 57	0.533
Phosphocholine	514 ± 192	524 ± 155	0.706
Glycerophosphocholine	394 ± 121	388 ± 131	0.653
Water-soluble choline[2]	1061 ± 204	1053 ± 211	0.772
Betaine	5.2 ± 2.3	5.3 ± 2.2	0.687

[1]Milk concentrations were quantified by liquid chromatography-tandem mass spectrometry. Data are presented as mean ± SD.
[2]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

Comparison of the concentration of water-soluble forms of choline and betaine in milk per treatment group in the second Canadian trial

Milk metabolites	Dose 1 (n = 22)	Dose 2 (n = 26)	Dose 3 (n = 19)	*P*-value
		Concentrations (µmol/L)[1]		
Free choline	143 ± 93	152 ± 104	164 ± 90	0.086
Phosphocholine	554 ± 240	546 ± 216	571 ± 238	0.626
Glycerophosphocholine	449 ± 159	425 ± 160	445 ± 211	0.673
Water-soluble choline[2]	1146 ± 349	1122 ± 324	1181 ± 391	0.634
Betaine	3.8 ± 2.0	4.1 ± 2.1	4.4 ± 2.8	0.102

[1]Milk concentrations were quantified by liquid chromatography-tandem mass spectrometry. Data are presented as mean ± SD.
[2]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

Comparison of the concentration of water-soluble forms of choline and betaine in milk per treatment group in the Cambodian trial

Milk metabolites	Dose 1 (n = 22)	Dose 2 (n = 26)	Dose 3 (n = 19)	*P*-value
		Concentrations (µmol/L)[1]		
Free choline	135 ± 68	150 ± 109	146 ± 75	0.110
Phosphocholine	541 ± 194	560 ± 186	579 ± 244	0.751
Glycerophosphocholine	393 ± 126	387 ± 153	389 ± 134	0.990
Water-soluble choline[2]	1069 ± 253	1098 ± 287	1125 ± 274	0.536
Betaine	4.8 ± 4.0	4.4 ± 3.6	6.6 ± 5.6	0.223

[1]Milk concentrations were quantified by liquid chromatography-tandem mass spectrometry. Data are presented as mean ± SD.
[2]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

Comparison of the concentration of water-soluble forms of choline and betaine in milk between the Canadian trials

Milk metabolites	Canadian trial 1 (n = 147)	Canadian trial 2 (n = 154)	*P*-value
	Concentrations (µmol/L)[1]		
Free choline	146 ± 73	162 ± 103	0.604
Phosphocholine	519 ± 175	551 ± 232	0.169
Glycerophosphocholine	391 ± 125	426 ± 177	0.078
Water-soluble choline[2]	1057 ± 242	1139 ± 367	0.140
Betaine	5.3 ± 2.2	4.4 ± 2.4	0.058

[1]Milk concentrations were quantified by liquid chromatography-tandem mass spectrometry. Data are presented as mean ± SD.
[2]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

Association between the concentration of water-soluble forms of choline and betaine in milk and weeks postpartum in the Cambodian trial

	Cambodian trial (*n* = 67)	
Milk metabolites	*r*	*P-value*
Free choline	0.074	0.550
Phosphocholine	-0.173	0.161
Glycerophosphocholine	0.202	0.101
Water-soluble choline[1]	0.011	0.930
Betaine	-0.107	0.390

[1]Water-soluble choline corresponds to the sum of free choline, phosphocholine, and glycerophosphocholine.

Appendix C: Dietary choline and betaine intakes at 16 and 36 weeks of gestation in one of the Canadian trials

	Canadian participants (*n* = 143)		Difference	Correlation	
Dietary intakes	16 weeks gestation	36 weeks gestation	*P-value*	*r*	*P-value*
	Intakes (mg/d)[1]				
Total choline[2]	412 ± 103	408 ± 111	0.927	0.590	< 0.001
Free choline	93 ± 27	87 ± 24	0.057	0.587	< 0.001
Phosphocholine	25 ± 10	24 ± 11	0.321	0.438	< 0.001
Glycerophosphocholine	89 ± 35	92 ± 35	0.087	0.493	< 0.001
Phosphatidylcholine	185 ± 64	185 ± 66	0.899	0.513	< 0.001
Sphingomyelin	20 ± 7	20 ± 6	0.095	0.593	< 0.001
Betaine	137 ± 67	142 ± 69	0.147	0.442	< 0.001

[1]Dietary intakes were estimated using Food Frequency Questionnaire and choline content in common foods USDA database. Data are presented as mean ± SD.

[2]Total choline corresponds to the sum of individual forms of choline.

Appendix D: Plasma concentrations of free choline, betaine, and dimethylglycine in the mothers of the children enrolled in the study

	Women (*n* = 281)[1]	
Plasma metabolites	*Mean ± SD*	*Median (IQR)*
	Concentration (µmol/L)	
Free choline	7.9 ± 2.0	7.6 (6.5 – 8.9)
Betaine	38.6 ± 12.3	37.3 (29.8 – 46.7)
Dimethylglycine	2.4 ± 0.9	2.2 (1.8 – 2.7)

[1] Plasma concentrations were quantified by liquid chromatography-tandem mass spectrometry.