CHOLINE NUTRITION, CHOLINE STATUS, AND DEVELOPMENTAL OUTCOME IN EARLY CHILDHOOD

by

BRIAN TID-FUNG WU

B.Sc., The University of British Columbia

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2014

ABSTRACT

Choline is an essential dietary nutrient that plays a key role as one of the few sources of methyl donors in the diet. Additional roles include the neurotransmitter acetylcholine and lipids such as phosphatidylcholine and sphingomyelin. Studies in adults have shown that choline deficiency results in fatty liver. In developing animals, choline deficiency also leads to impaired neural development. Despite the importance of choline as a nutrient, there is limited data on dietary intake in relation to needs, or dietary choline and its connection to human development. This project aims to determine the choline intake and plasma status of choline among children 5 years and 9 months of age, and to explore the relationship plasma free choline and its metabolites and cognitive development. This study includes a cross-sectional study with a total of 200 children at 5 years and 9 months of age. Dietary intake was collected by FFQ, 3 x 24 hr recalls and 1 x 24 hr recall. Dietary choline intake was estimated using USDA database. Venous blood was collected and plasma free choline, betaine, dimethylglycine, homocysteine, methionine and cysteine were analyzed using LC-MS/MS, and plasma tB12 and folate were analyzed using microparticle enzyme immunoassay and ion capture assay, respectively. Cognitive development was evaluated using KABC-II, PPVT-4, and Beery-VIM. The median intakes of choline and betaine were 278 mg/d and 90.2 mg/d, respectively, when estimated using the FFQ. The mean ± SD (IQR) for plasma free choline, betaine and dimethylglycine were 8.57 ± 2.08 (7.20 – 9.60) µmol/L, 45.4 ± 12.9 (37.0 – 53.1) µmol/L, and 3.26 ± 0.95 (2.60 – 3.80) µmol/L, respectively. About 34% of the children consumed below the current DRI of 250 mg/d of choline. Significant positive association was found between dietary intake of choline and plasma free choline (r =

0.198, $P = 0.014$), but dietary intake of betaine was not related to plasma betaine ($P = 0.915$). Plasma free choline was inversely associated with plasma homocysteine ($P = 0.013$). No associations were found between plasma free choline, betaine or dimethylglycine and any of the cognitive test results ($P > 0.05$). An inverse relationship between plasma free choline and homocysteine might indicate the presence of choline deficiency in the children in this study.

PREFACE

This thesis contains the work of a research study for a master degree and was prepared in accordance to the University of British Columbia and The Faculty of Graduate and Postdoctoral Studies requirements. I was responsible for database integration, blood sample processing, data and statistical analyses, and thesis preparation, which was accomplished with the assistance and guidance from my supervisor Dr. Sheila M. Innis. All biochemical analyses were done by Mr. Roger A. Dyer and Ms. D. Janette King. Ms. Kelly J. Richardson was responsible for subject enrolment, blood sample collection, and psychometric assessment of the subjects. Ms. Betina F. Rasmussen and Ms. Kelly A. Mulder were responsible for collecting dietary information and data entry. This study was conducted according to guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia (B.C.) and the B.C. Children's and Women's Hospital. Written informed consent was obtained from a parent or legal guardian for each child prior to and for themselves enrollment. A modified version of this thesis will be submitted as manuscript(s) for publication in peer-reviewed academic journal(s).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Adequate Intake
ANOVA	Analysis of variance
BADH	Betaine-aldehyde dehydrogenase
BBB	Blood-brain barrier
Beery-VMI	Beery-Buktenica Developmental Test of Visual-Motor Integration, 6th Edition
BHMT	Betaine-homocysteine *S*-methyltransferase
BMI	Body mass index
CCT	Cytidine triphosphate:phosphocholine cytidylyltransferase
CDP-choline	Cytidine 5-diphosphocholine
CHDH	Choline dehydrogenase
CHT1	High-affinity choline transporter
CK	Choline kinase
Cl^-	Chloride
CTL	Choline transporter-like
c.v.	Coefficient of variability
d	Day(s)
DNA	Deoxyribonucleic acid
DRI	Dietary Reference Intakes
EAR	Estimated average requirement
EDTA	Ethylenediaminetetraacetic acid
FFQ	Food frequency questionnaire

GPCho	Glycerophosphocholine
H_2O	Water
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
hr	Hour(s)
IQ	Intelligence quotient
IQR	Interquartile range
KABC-II	Kaufman Assessment Battery for Children, 2nd Edition
LCMS/MS	Liquid chromatography-electrospray tandem mass spectrometry
Lyso-PC	Lysophosphatidylcholine
MAT	Methionine adenosyltransferase
5-MTHF	5-methylene tetrahydrofolate
mo	Month(s)
Na^+	Sodium
NaOH	Sodium hydroxide
NHANES	National Health and Nutritional Estimation Survey
OCTs	Organic cation transporters
PAF	Platelet activating factor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase
PLA2	Phospholipase A2
PLB	Phospholipase B

PLD1	Phospholipase D1
PPVT-4	Picture Peabody Vocabulary Test-4th Edition
RDA	Recommended Dietary Allowance
RNA	Ribonucleic acid
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosyl methionine
SD	Standard deviation
SM	Sphingomyelin
SNP	Single nucleotide polymorphism
tB12	Total vitamin B12
TONI-3	Test of Nonverbal Intelligence-3
USDA	United States Department of Agriculture
VLDL	Very low-density lipoprotein
WHO	World Health Organization
WMHV	White matter hyperintensity volume
yr	Year(s)

ACKNOWLEDGEMENTS

First of all, I would like to express my greatest gratitude to my supervisor, Dr. Sheila Innis, for providing me the opportunity to pursue a MSc degree and for her supervision throughout my academic endeavour. She has shown me how to become a great scientist with the utmost integrity. Not only does her mentorship have a significant positive impact on my scientific aptitude development, it also has a fabulous influence on my personal life. I was given a vast array of incredible opportunities and exposure that I would not have attained elsewhere.

I would like to express thanks to my supervisory committee members, Dr. David Kitts and Dr. Tim Green, for their guidance throughout my study.

I would like to give a special thank you to Roger Dyer, Janette King, Kelly Richardson, Kelly Mulder, Glynis Sieber and Betina Rasmussen for their tremendous contributions to the study.

I would like to thank Alejandra Wiedeman and Sara Moukarzel for their constructive input and fruitful discussions during the preparation of my thesis.

I also thank Lynda Soberanes, Dr. Jie (Jessica) Yang, Cindy Wong, Cindy Cao, Cyrielle Garcia, Vinodha Chetty, Rong Yu, Michael George, and Julie Matheson for their assistance and friendship. Being around such extraordinary and friendly individuals has made the lab such a wonderful place to be.

Finally, I would like to thank my parents and brother for their boundless love and support. No words can express how grateful I am for having such a fantastic family.

I would like to dedicate this thesis with all my love and thanks to all the people who never stop believing in me and have been with me every step of the way in this special and challenging journey

My mother (Ma-mi), Brenda

My father (Papa), Larry

My brother, Richard

My late grandmother (Popo)

And lastly to my long-time best friend, Carol.

CHAPTER 1: BACKGROUND

1.1 General introduction and aspects of choline

1.1.1 Forms of choline

Choline is a positively-charged quaternary ammonium salt possessing three methyl groups at its *N*-terminal. Choline can be obtained by either endogenous synthesis or diet. Since endogenous synthesis is not able to generate sufficient choline for bodily needs in the absence of dietary intake, it was categorized as an essential dietary nutrient in 1998 (Institute of Medicine, 1998). Choline is present in the diet in water-soluble and lipid-bound forms. Water-soluble forms of choline include free choline, glycerophosphocholine (GPCho), and phosphocholine; lipid-bound forms of choline include phosphatidylcholine (PC) and sphingomyelin (SM) (**Figure 1.1**).

A well-known oxidized form of choline is *N,N,N*-trimethylglycine, which is commonly known as betaine (Zeisel & Blusztajn, 1994). Betaine is formed from choline by two successive, irreversible oxidation reactions via the formation of an aldehyde intermediate (Chern et al., 2000; Chi-Shui & Ru-Dan, 1986). In mammals, this irreversible process involves two enzymes: choline dehydrogenase (CHDH) and betaine-aldehyde dehydrogenase (BADH). This conversion takes place in the mitochondrial matrix following choline transport into these organelles (O'Donoghue et al., 2009).

1.1.2 Digestion and absorption of choline

Dietary free choline is absorbed in the jejunum of the small intestine (Budowski et al., 1977; Flower et al., 1972; Kuczler et al., 1977; Sanford and Smyth, 1971). Since free choline is a positively charged quaternary amine, it requires a transporter to cross cell membranes, although passive diffusion occurs at choline concentrations higher than 4 mmol/L (Kuzcler et al., 1977). Intestinal choline transporters are not active transporters as they are not energy-dependent, and they are also not sodium-dependent (Kuzcler et al., 1977; Sanford and Smyth, 1971).

PC is the major form of choline in the diet. PC must be hydrolyzed to lysophosphatidylcholine (lyso-PC) by the enzyme pancreatic phospholipase A2 (PLA2) before it is absorbed by enterocytes (De Haas et al., 1989; Parthasarathy et al., 1974). Once inside the enterocyte, lyso-PC may be re-acylated, thus regenerating PC. Alternatively, de-acylation of lyso-PC catalyzed by phospholipase B (PLB) results in the generation of GPCho. Chylomicron particles, of which PC is a component, are assembled in the endoplasmic reticulum of the enterocyte and are subsequently released into the lymphatic circulation where their remnants are cleared by the liver. Within cells, the metabolism of PC can lead to the generation of multiple signaling molecules that have important roles in cellular function which will be discussed later.

SM, GPCho and phosphocholine exist in smaller amount in the diet. SM is intactly taken up into intestinal cells and is believed to be entirely broken down in the enterocyte (Nilsson, 1968). GPCho is disintegrated by 3-glycerylphosphocholine glycerylphosphohydrolase, followed by the release of glycerophosphate and free

choline, which is subsequently transported into intestinal cells and released into the portal circulation (Parthasarathy et al., 1974). Phosphocholine is degraded by alkaline phosphatase which in turn produces free choline and phosphate (Macfarlane et al., 1932).

Betaine is a zwitterionic quaternary amine. Dietary betaine is absorbed in the duodenum of the small intestine (Kettunun and Tilhonen, 2001; Kettunen and Peuranen 2001). Cellular uptake of betaine occurs via one of the following mechanisms: 1) passive transport through the γ-aminobutyric acid transporter protein, 2) an active Na^+ or Cl^- coupled transport, 3) Na^+- independent passive transport mechanism via amino acid transport system A (Kim et al., 2003; Peters-Regehr et al., 1999; Weik et al., 1998; Wettstein et al., 1998). Betaine formed in the cells by endogenous synthesis can freely diffuse down its concentration gradient across the mitochondrial membranes from the mitochondrial matrix into the cytosol (Porter et al., 1993).

1.1.3. Transport of choline

There are three possible choline transport mechanisms in tissues: 1) a facilitated diffusion system (poly-specific organic cation transporters, OCTs), 2) a high affinity, Na^+-dependent active transport system (high-affinity choline transporter, CHT1), and 3) a lower affinity active transport system (choline transporter-like; CTL) (Michel et al., 2006). CHT1 is localized to cells that primarily use choline for acetylcholine generation specifically for cholinergic neurons. Thus, the forebrain, brain stem, striatum and spinal cord are the areas within the central nervous system with highest CHT1 expression

(Apparsundaram et al., 2000; Okuda et al., 2000; Okuda and Haga, 2000). OCTs are a family of non-specific organic cation transporters primarily expressed in brain, kidney, liver, and intestine (Michel et al., 2006).

1.1.4 General functions of choline

Choline and its metabolites are responsible for several critical physiological functions. The functions of choline include: 1) the choline-containing lipids, PC and the sphingolipids of which SM is the best known (these lipids are essential to maintaining normal cell membrane signaling and membrane structure), plasma lipoproteins, bile lipid and lung surfactant (Zeisel & Blusztajn, 1994); 2) as a precursor for the synthesis of acetylcholine which is an important neurotransmitter in the central nervous system and the peripheral nervous system (Zeisel & Blusztajn, 1994); 3) as a precursor to platelet activating factor (PAF), a choline-containing phospholipid derived from PC, imparts hormone-like activity and has been demonstrated to regulate platelet aggregation, mediating inflammatory responses, and bronchoconstriction (Demopoulos et al., 1979); and 4) as a source of methyl groups and precursor to betaine (Ueland, 2011), which is involved in the remethylation of homocysteine to methionine in the methyl transfer pathway in the liver and kidney. Betaine also functions as a crucial organic osmolyte in multiple organs and cells. Choline's function as a methyl donor is further expanded in the following section.

1.2 Metabolic importance of choline

1.2.1 Historical perspective of research on choline's roles in methionine metabolism

Upon his discovery of homocysteine via demethylation of methionine in 1932 (Butz and du Vigneaud, 1932), Nobelist Vincent du Vigneaud had taken an interest in studying the roles of methionine and relevant compounds in biological methylations and their metabolic context. In 1939, he first observed that when methionine was absent from the diet, a supply of homocysteine was necessary to support growth given that a source of methyl group such as choline was available to enable methionine generation (du Vigneaud et al., 1939; du Vigneaud 1941). After 10 years of investigation, du Vigneaud's team finally determined that a complete absence of choline as a labile source of methyl groups in the diet would perturb methionine, and substantially increase the tendency of methionine to be used as a methyl group instead of for its role in protein synthesis (Ferger & du Vigneaud, 1950; Mackenzie & du Vigneaud, 1951). This important discovery has paved the way to understand the interdependent relationships between choline and methionine, and their role in one-carbon metabolism.

1.2.2 Choline as a methyl donor: intersecting with folate and methionine metabolism

As an important source of methyl groups and a precursor to betaine, choline functions with folate, vitamin B12, and methionine in methyl transfer pathways which revolve around the methylation of homocysteine to methionine (**Figure 1.2**). The

methylation of homocysteine can be accomplished by one of two pathways. First, choline is oxidized to betaine by mitochondrial CHDH in the liver or kidney. Betaine can provide methyl groups for methylation of homocysteine to form methionine in a reaction catalyzed by betaine-homocysteine *S*-methyltransferase (BHMT), an enzyme expressed most predominantly in the liver and kidney (Park & Garrow, 1999, Sunden et al., 1997). This enzymatic pathway produces dimethylglycine as the demethylated product of methyl donation by betaine. Dimethylglycine may then be further metabolized by dimethylglycine dehydrogenase to form methylglycine (sarcosine). Sarcosine is then converted to glycine via sarcosine dehydrogenase. Each step of betaine conversion to glycine donates a methyl group that can be used for synthesis of 5-methylene tetrahydrofolate (5-MTHF). 5-MTHF can in turn donate a methyl group to homocysteine for synthesis of methionine in a reaction catalyzed by the B12-dependent enzyme methionine synthase. Important to note, glycine is also consumed in the diet and can be synthesized endogenously. Methionine possesses one methyl group. Methionine is the precursor to homocysteine, which is an intermediate and is not present in the diet. Methionine can be regenerated after donating a methyl group to form homocysteine via the remethylation of homocysteine by either 5-MTHF or betaine. Methionine can be converted by methionine adenosyltransferase (MAT) to *S*-adenosyl methionine (SAM) (Mudd & Cantoni, 1958), a crucial methyl donor for numerous cellular methylations including deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and histone methylation, conversion of norepinephrine to epinephrine, synthesis of purines and thymidylate (components of DNA and RNA), creatine (energy storage as creatine phosphate), carnitine (fatty acid transport into the mitochondria) and polyamines (cell growth),

inactivation of catecholamines (Luka & Mudd, 2009), and PC synthesis which is explained below.

1.2.3 Endogenous synthesis of choline

In mammals, endogenous synthesis of choline using methyl groups from methionine has been estimated to contribute about one third of PC biosynthesis in the liver (DeLong et al., 1999; Reo et al., 2002; Sundler et al., 1975). In most animal tissues, PC together accounts for about 40% of phospholipids in most cell membranes. SM and PC account for 95% of the total cellular choline pool, while the remaining 5% of the pool encompasses phosphocholine, free choline, GPCho, acetylcholine, and cytidine 5-diphosphocholine (CDP-choline) (Li & Vance, 2008). PC *de novo* biosynthesis is regulated by the SAM-dependent enzyme called phosphatidylethanolamine *N*-methyltransferase (PEMT), which methylates phosphatidylethanolamine (PE) thrice to form PC (Bremer & Greenberg, 1961), thereby consuming three molecules of SAM which in turn generates three molecules of *S*-adenosylhomocysteine (SAH) (**Figure 1.2**). SAH is then converted by SAH hydrolase to homocysteine (Cantoni, 1975). PC can also be hydrolyzed by phospholipase D1 (PLD1) to form free choline and phosphatidic acid (Bocckino et al., 1987).

In 2003, Zhu et al. conducted an in vivo study to elucidate the effect of the absence of *Pemt* gene in mice. Their results showed that *Pemt -/-* mice had abnormal liver function and were unable to maintain normal liver concentration of phosphocholine despite being fed with supplemental sources of choline (12 mg choline/d). The *Pemt -/-*

on the control diet (3 mg choline/d) had a significantly lower hepatic total choline, PC and phosphocholine compared to the wild-type mice fed on the same diet (Zhu et al., 2003). This suggests that regardless of diet, endogenous synthesis of PC via PEMT, and hence an intact methyl transfer pathway is important for normal liver function and plasma lipids.

1.3 Dietary sources of choline

Choline and choline metabolites can be found in many foods regularly consumed by humans. However, choline is only abundant in a limited number of foods (**Table 1.1**). Foods highest in choline content per 100 g include beef and chicken liver, eggs, fish, and wheat germ. Foods with high betaine content per 100 g include beetroot, wheat bran, quinoa, spinach, and wheat germ (USDA, 2008). It is important to take betaine content into consideration when considering diet, since the presence of betaine may spare choline from the use for betaine synthesis in a diet low in choline in chicks (Dilger et al., 2007). Experimental studies on betaine's role in sparing choline in a low choline diet have not yet been conducted in other species. To date, no dietary recommended intake for betaine has been established.

1.4 Choline and the brain

1.4.1 Choline metabolism in the brain

Choline plays an important role in the brain. Evidence from studies in rat has suggested that brain also has the capacity for endogenous synthesis of PC by methylation of PE via PEMT (Kewitz & Pleul, 1976). Exogenous sources of brain choline can be acquired from the blood using a carrier-mediated transport mechanism for uptake of choline from the plasma to the brain across the blood-brain barrier (BBB) via a BBB choline transporter (Diamond, 1971). After entry into the brain, free choline is metabolized quickly into acetylcholine or phosphocholine. Free choline can be phosphorylated into phosphocholine, followed by slow incorporation into brain phospholipid via the CDP-choline pathway (Blusztajn et al., 1979).

Free choline can also be converted into acetylcholine, an important neurotransmitter. Acetylcholine synthesis is catalyzed by the enzyme choline acetyltransferase (Blusztajn et al., 1987). Choline acetyltransferase is found in high concentrations in cholinergic neurons, ensuring that acetylcholine is available for release by these cells. Choline-containing phospholipids, including PC and SM, are incorporated into all cell membranes of the brain. It was also suggested that PC and SM can become a source for acetylcholine synthesis, when the demand is high (Blusztajn et al., 1986; Blusztajn & Liscovitch et al., 1987). However, it seems unlikely that important membrane structure would be sacrificed to maintain acetylcholine pools as the

consequences for the brain would be severe, unless the PC and SM are readily replaced.

1.4.2 Choline and cognition

1.4.2.1 Neurological implications in animals

Dietary choline intake has been suggested to have a significant impact on neurological development in rats and mice. Studies in this field have demonstrated that prenatal choline supplementation of the pregnant animal when compared to a choline-deficient diet improved the offspring's performance in behavioral and cognitive tests, especially visuospatial and auditory memory, in both rats and mice (Meck et al., 1999; Meck et al., 2003; Mellott et al., 2004; Schenk et al., 1995; Tees & Mohammadi, 1999; Thomas et al., 2004).

In mice, maternal choline deficiency has been linked to disrupted methylation of DNA and histones responsible for regulating cellular proliferation and apoptosis in the hippocampus (Niculescu et al., 2006), leading to a number of permanent aberrant molecular and structural changes to the developing brain. These include: 1) down-regulated expression of proteins that drive neuronal migration and a decreased rate at which neuronal precursor cells in the subventricular region migrated to the denate gyrus region of the hippocampus, after undergoing mitosis (Albright et al., 1999; Craciunescu 2003); 2) accelerated rates of apoptosis in fetal neuronal progenitor cells (Albright et al., 1999; Holmes-McNary 1997; Yen et al., 2001); 3) reduced expression of proteins

controlling angiogenesis which led to substantial decrease in blood vessels formation in fetal hippocampus (Mehedint et al., 2010). In sum, these types of studies have provided evidence that maternal choline deficiency can have an adverse impact on angiogenesis and neurogenesis in the fetal brain, which in turn has the potential for long-lasting impacts on the offsprings' neurological functions, including memory, cognitive, and learning abilities.

1.4.2.2 Neurological implication in human development

To date, relatively little information is available on choline and early cognitive development in human. An observational human study by Signore et al. (2008) found no association between maternal choline status during early, mid or late gestation and childhood intelligence measured at 5 years of age in a study of 404 American mother-child pairs in Alabama (Signore et al., 2008). However, a recent study by our group found a significant positive association between maternal plasma betaine and free choline in the first half of pregnancy and cognitive development among healthy term gestation infants assessed at 18 months of age in 154 mother-infant pairs (Wu et al., 2012). In 2013, the first published study evaluating the relationship between choline metabolites and cognitive performance among 210 children at 5 yr of age living in the Republic of Seychelles found a significant association between plasma betaine, but not free choline, and the children's language abilities (Strain et al., 2013).

Another recent epidemiological study from the U.S. found no association between mothers' dietary choline and betaine intakes in first or second trimester of

gestation and their children's performance (*n* = 1210) in verbal or overall visual motor ability at 3 yr of age (Villamor et al., 2012). However, another study by the same research group found that the maternal intake of choline in early to mid-gestation was significantly correlated to visual memory of their children at 7 yr of age (Boeke et al., 2013). The latter study also assessed other possible confounders in the maternal diets including betaine, folate, and vitamin B12, but found no significant relationships with the children's psychometric endpoints.

Very little has been published from studies on maternal choline supplementation in pregnancy and early development in infants and children. To date, one interventional study has described the impact of prenatal supplementation with PC (750 mg/d) from mid gestation (16 wk) to 70-day postpartum on early cognitive development at 10 mo and 12 mo of age in North Carolina, U.S. (Cheatham et al., 2012). This study found no difference in infant cognitive performance between the supplement group (*n* = 50) and the placebo group (*n* = 49). However, it is not known if the mothers in the study had an insufficient choline status prior to supplementation, if the sample size was adequate to find effects if present, or if the tests used were sensitive enough to detect functional effects of choline insufficiency.

The difference in socio-economical background, ethnic backgrounds, environmental factors, the age of children when the cognitive assessment was conducted, and dietary intakes of subjects in these studies might contribute to the inconsistent findings in the few studies on choline status and child neurological development to date. Further work is needed to confirm the relationship between

maternal choline status during pregnancy and its impact on early development of children, as well as the possible contributing role of the child's own dietary intake.

1.4.2.3 Neurological implications of choline in adults

Currently, there are a limited number of reports describing the relationship between choline status and cognition in adults. In 195 Dutch elderly people, Eussen et al. (2007) found that plasma betaine, but not choline, was positively associated with better cognitive functions, particularly executive function and sensomotor speed (Eussen et al., 2007). Another study examining the link between plasma choline, betaine and dimethylglycine, and cognitive performance in among elderly in Norway found similar results with evidence that low plasma free choline and betaine were associated with poor cognitive performance (Nurk et al., 2012). A recent report by Poly et al. (2011) using data from the Framingham Offspring Cohort in the U.S. explored the relationship between dietary choline intake and cognitive performance in 1391 non-demented adults (age range 36 – 83 yr of age). The results showed that a higher choline intake was associated with better cognitive performance, especially in verbal memory and visual memory (Poly et al., 2011). Studies using magnetic resonance imaging (MRI) have also demonstrated an inverse relationship between low choline intake and high white matter hyperintensity volume (WMHV), a feature linked to pathological signs, including oligodendritic apoptosis and brain atrophy (DeCarli et al., 1995; DeCarli et al., 2005; Jeerakathil et al., 2004). Previous studies have also found a positive association between WMHV and decline in cognitive performance among adults and the elderly

(DeCarli et al., 1995; Gouw et al., 2006; van der Flier et al., 2005). However, the exact mechanism of how choline affects WMHV is unknown, and no casual relationships between diet and neurological dysfunction can be drawn from these types of studies.

1.5 Consequences of choline deficiency

1.5.1 Historical perspective of research on choline functional roles in the liver

The physiological roles of choline have been a subject of interest since the 1930s. One of the pioneers in this field of research was Charles Best. Following the famous work showing the importance of insulin in pancreatetomized dogs, he also observed the development of fatty liver in these animals. The manifestation of this hepatic pathology had stimulated his interest to find a way to rectify this problem. Using depancreatized dogs as the model, Best's colleagues Hershey and Soskin observed that the fatty liver condition could be reversed by feeding either raw pancreas or PC (Hershey & Soskin, 1931). Following this discovery, Best further showed that choline was the active component of PC that prevented fatty liver in dogs and rats (Best & Huntsman, 1932a; Best & Huntsman, 1932b). Enthusiasm for investigating other physiological roles of choline remained high for the following decades and other researchers have found consistent results. The body of research on the role of choline in preventing fatty liver would eventually lead to the recognition of choline as an essential dietary nutrient for humans in 1998.

1.5.2 Hepatic steatosis

As mentioned above, decades of studies have established the best known sign of choline deficiency in mammals, which relate to the liver. The first study investigating the impact of dietary choline inadequacy in humans was done in men in the U.S. (Zeisel et al., 1991). Compared to the baseline and the control group (fed with 500 mg/d choline or 7 mg/kg/day [0.7 mmol/kg/d]), this study observed a significant decline in plasma choline and elevated alanine aminotransferase (an enzymatic marker for liver damage and fatty liver) after the subjects consumed an oral choline-deficient total parental nutrition solution (13 mg/d choline) with an adequate supply of vitamin B12, folate and methionine for 3 weeks. The signs of liver damage and elevated liver enzymes were resolved once 500 mg/day total choline (equivalent to 700 mg/day choline chloride) was provided to choline-deprived healthy adults. A subsequent study found consistent results, using a similar study design and methodology (Fischer et al, 2007).

Choline is needed to synthesize PC and SM, which are important components of very low-density lipoprotein (VLDL). VLDL particles are responsible for triglyceride transport from the liver to extra-hepatic tissues. When the choline supply is depleted, the assembly of VLDL is compromised (Li & Vance, 2008). Thus, the impaired export of triglyceride leads to fat accumulation in the liver and this ultimately leads to liver damage.

1.5.3 The importance of dietary choline and an alternate pathway of PC synthesis

As previously noted, published studies have estimated that about a third of hepatic PC is synthesized via PEMT in the liver (DeLong et al., 1999; Reo et al., 2002; Sundler et al., 1975). However, the other 70% is synthesized via the CDP-choline pathway, which requires a source of choline. In the first step of the CDP-choline (Kennedy Pathway) pathway, choline is phosphorylated by choline kinase (CK), generating phosphocholine, which is then converted to CDP-choline in a reaction catalyzed by the enzyme cytidine triphosphate:phosphocholine cytidylyltransferase (CCT); this is the rate limiting step in the CDP-choline pathway (Kennedy and Weiss, 1956). The combination of CDP-choline with diacylglycerol results in the formation of PC (**Figure 1.3**); this reaction is catalyzed by the enzyme CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Pelech & Vance, 1984).

1.5.4 Elevated homocysteine in humans

The second established clinical indicator of dietary choline deficiency is an elevated plasma homocysteine concentration (Fischer et al., 2007). The latter study indicated that dietary choline deficiency (50 mg/d choline) over a three-week period in adults led to a significant drop in plasma free choline (mean ± standard deviation (SD) at baseline: 10 ± 1.7 µmol/L, mean at depletion: 6.7 ± 1.1 µmol/L), PC (mean ± SD at baseline: 1925 ± 333 µmol/L, mean at depletion: 1700 ± 293 µmol/L), betaine (mean at baseline: 60 ± 23 µmol/L, mean at depletion: 27 ± 11 µmol/L) and dimethylglycine

(mean at baseline: 6.0 ± 2.3 µmol/L, mean at depletion: 3.1 ± 1.1 µmol/L), and a significant rise in plasma homocysteine (mean at baseline: 6.9 ± 1.7 µmol/L, mean at depletion: 8.0 ± 2.3 µmol/L) despite adequate supplies of methionine, cysteine, vitamin B12, B6, and folate over the three-week period. All the clinical signs of choline deficiency that developed on the choline-deficient diet were resolved once choline was resupplied in the diet. It should be noted that although the experiment saw an increase in plasma homocysteine, the extent of elevation did not reach the cut-off of elevated homocysteine of >13 µmol/L for adults. Regardless, the increase in homocysteine may be seen as a biochemical indicator of limited choline for the CDP-choline pathway leading to a reciprocal increase in PEMT, and hence generation of homocysteine. In addition, the decrease in betaine is markedly more pronounced than the decrease in free choline. It is possible that plasma betaine could serve as a better marker for choline deficiency, assuming that dietary betaine does not contribute to the plasma betaine pool.

1.6 Dietary Reference Intake of choline

The 1998 Dietary Reference Intakes (DRI) reported a lack of sufficient evidence to establish a DRI for choline (IOM 1998). DRIs are developed as a Recommended Dietary Allowance (RDA) based on scientific evidence of the estimated average requirement (EAR) of a group of healthy individuals. The EAR+2 standard deviations is used to generate the RDA, which is the intake sufficient to meet and 'exceed the needs' of 97.5% of healthy individuals in each gender group and life-stage. When insufficient data are available to set a RDA, then an adequate intake (AI) is used. An AI for choline

was established and this value corresponds to a recommended intake level that prevents liver dysfunction based on experimentally determined approximations in a group of apparently healthy adults (IOM, 1998). Unlike the RDA, it should be noted that the AI for choline does not imply physiological requirement and is possible to meet, fail to meet, or exceed the needs for everyone within a specific demographic group.

An intake level of 500 mg/day of choline was the intake dose that reversed elevated plasma liver enzymes among healthy men in one study (Zeisel et al., 1991). However, in the same study, the control subjects who consumed the diet with 500 mg/d choline for 3 weeks experienced a small non-significant decrease in plasma free choline (baseline at d0: 10.8 ± 1.1 µmol/L, control diet at d15: 9.6 ± 1.3 µmol/L, control diet at d20: 9.7 ± 1.2 µmol/L). The DRI expert panel then speculated that dietary intake of choline normally might be higher. Therefore, the current DRI set the AI at about 7 mg/kg/d using the reference man weighing 76 kg (550 mg/d) for men ages 19 years or older (IOM 1998). The AI adjusted for females ages 19 years or older were set at a lower value of 425 mg/d, which is derived from the adult men's AI on the basis that females have a greater ability for endogenous synthesis of choline, due to the up-regulating effect of oestrogen on PEMT. The methodology used to determine AI of dietary choline for children is explained in the following section.

1.6.1 Methodology of establishing DRI of choline among children and adolescents

As for adults, there is a lack of scientific data on which to establish an EAR or RDA for choline for children and adolescents. Therefore, the AI for children was

extrapolated from the AI for adults using the following formula, adjusting for reference body weight at different stage of growth, and an adding factor to adjust for growth:

$$AI_{child} = AI_{adult}(Weight_{child}/Weight_{adult})^{0.75} (1 + \text{growth factor})$$

The growth factor for children is listed as the following: 4- 8 years = 0.15; 9 – 13 years = 0.15; 14 – 18 years males = 0.15; 14 – 18 years females = 0.00 (FAO/WHO/UNA, 1985). Children of both sexes below the age of 14 have the same AI for choline (AI for children: age 4 – 8 years, 250 mg/d; age 9 – 13, 375 mg/day) (IOM 1998).

1.7 Dietary choline intake in children

Although data on dietary choline intakes among adults have been reported, information on dietary choline and betaine intakes among children are limited. Currently, the only available data on dietary choline intake in children are those derived from the analysis of the 2003 – 2004 National Health and Nutritional Estimation Survey (NHANES) in the US. This data indicates that “the mean choline usual intakes exceed the AI for young children” (Jensen et al., 2007). However, specific data on the range of intake were not reported in this abstract.

TABLE 1.1 Choline content (mg/100g of food) for the rich dietary source of choline[1]

Foods	Choline mg/100 g food
Egg	230
Salmon	102
Chicken Liver	330
Pork, beef	~90
Spinach	70
Whole wheat bread	27
Milk	15*

[1]Data are from the USDA Database for the Choline Content of Common Foods, Release Two (2008), and are the selected summary of choline from all sources in the food.

*38 mg choline/250 mL (1 cup) milk.

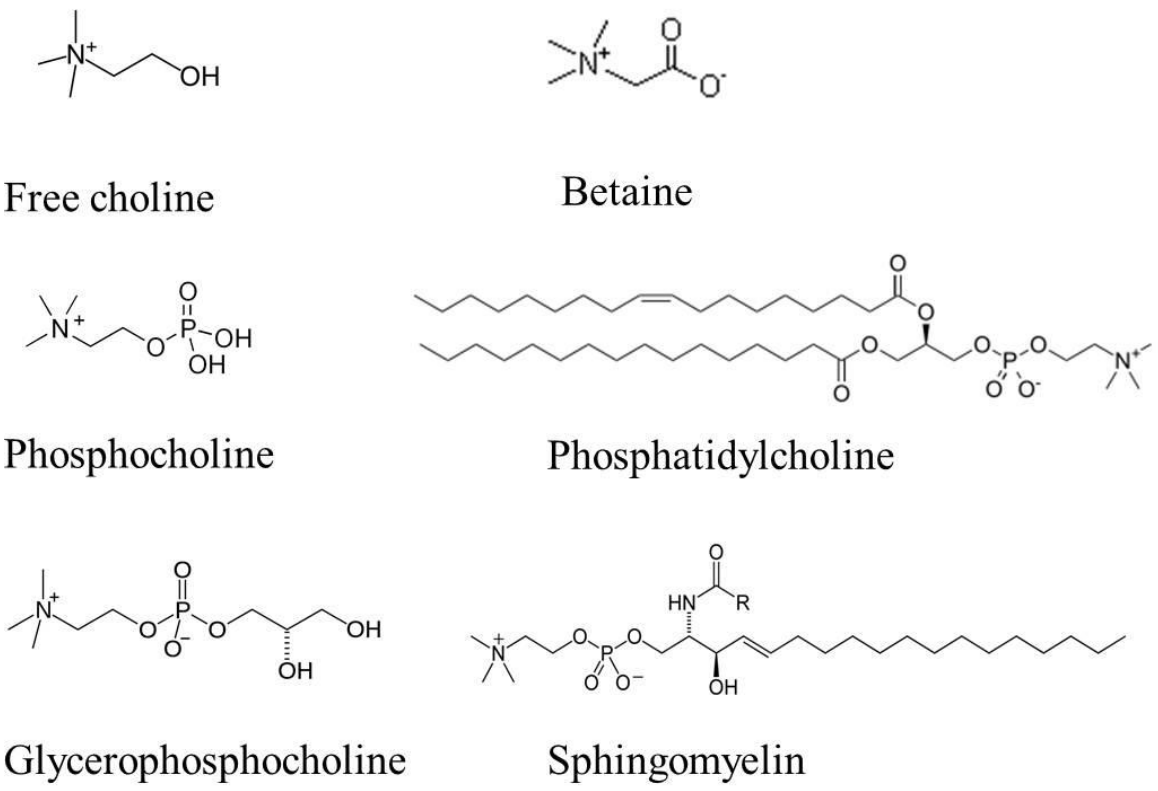

FIGURE 1.1 Structures of different forms of choline (Adapted from http://chem.ch.huji.ac.il/nmr/whatisnmr/hetcoup.htm).

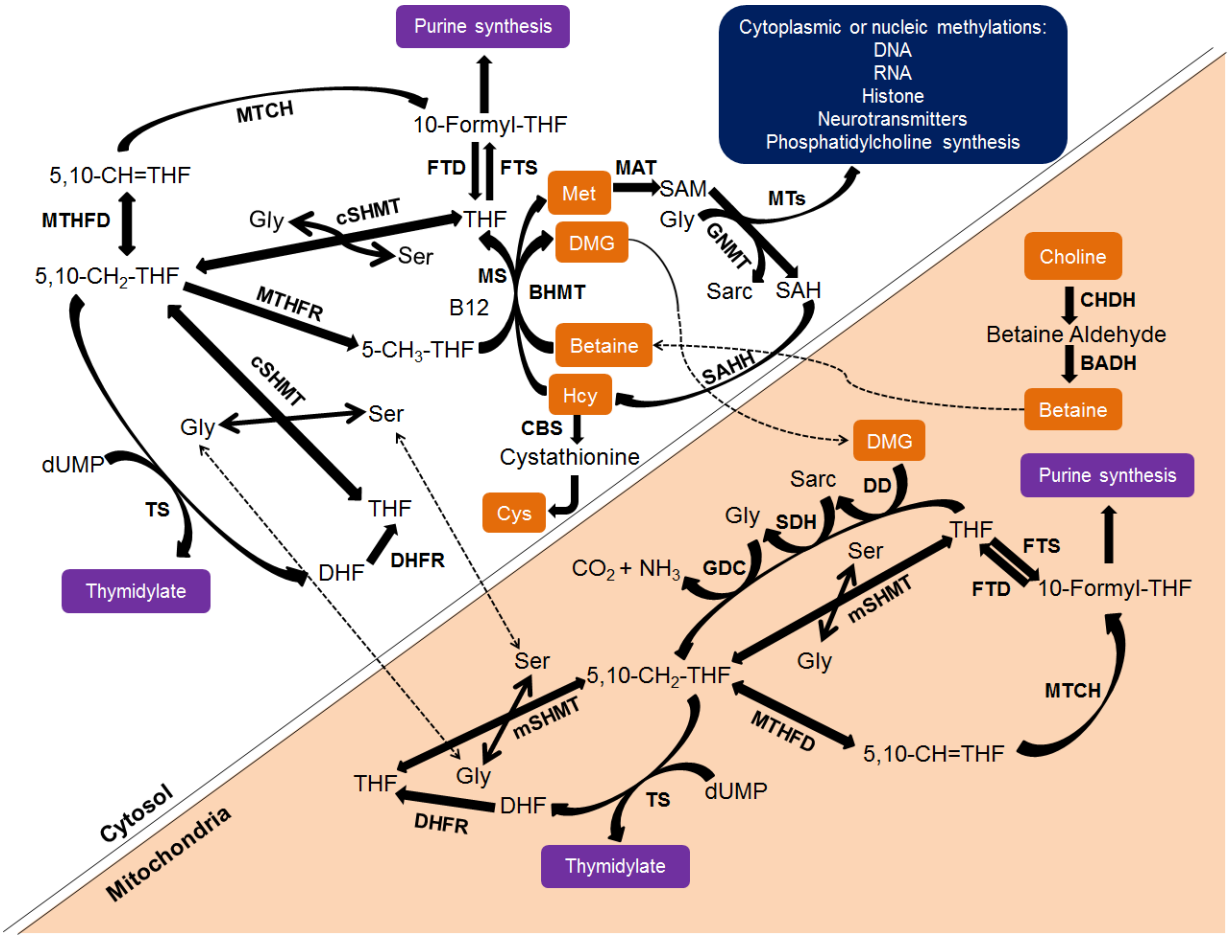

FIGURE 1.2 Schematic showing the role of choline in one-carbon metabolism intersecting with the methionine-homocysteine cycle (Adapted from Wu et al., PLoS One. 2012). 10-formyl-THF: 10-formyl-tetrahydrofolate ;5,10-CH=THF: 5,10-methenyltetrahydrofolate; 5,10-CH_2-THF: 5,10-methylenetetrahydrofolate; 5-CH_3-THF: 5-methyltetrahydrofolate; BADH: betaine aldehyde dehydrogenase; BHMT: betaine-homocysteine *S*-methyltransferase; CBS: cystathionine beta synthase; CHDH: choline dehydrogenase; Cys: cysteine; cSHMT: cytoplasmic serine hydroxymethyltransferase; DD: dimethylglycine dehydrogenase; DHF: dihydrofolate; DHFR: dihydrofolatereductase; DMG: dimethylglycine; dUMP: 2'-deoxyuridine 5'-monophosphate; FTD: 10-formyl-tetrahydrofolate dehydrogenase; FTS: 10-formyl-

tetrahydrofolate synthase; GDC: glycine decarboxylase; Gly: glycine; GNMT: glycine *N*-methyltransferase; Hcy: homocysteine; MAT, methionine adenosyltransferase; Met: methionine; MS: methionine synthase; mSHMT: mitochondrial serine hydroxymethyltransferase; MTCH: 5,10-methylenetetrahydrofolate cyclohydrolase; MTHFD: 5,10-methylenetetrahydrofolatedehydrogenase; MTHFR: 5,10-methylenetetrahydrofolate reductase; MTs, *S*-adenosyl methionine-dependent methyltransferases; SAH, *S*-adenosylhomocysteine; SAHH: *S*-adenosylhomocysteine hydrolase; SAM: *S*-adenosyl methionine; Sarc: sarcosine; SDH: sarcosine dehydrogenase; Ser: serine; THF: tetrahydrofolate; TS: thymidylate synthase. Not all enzymes and intermediates are shown in this pathway.

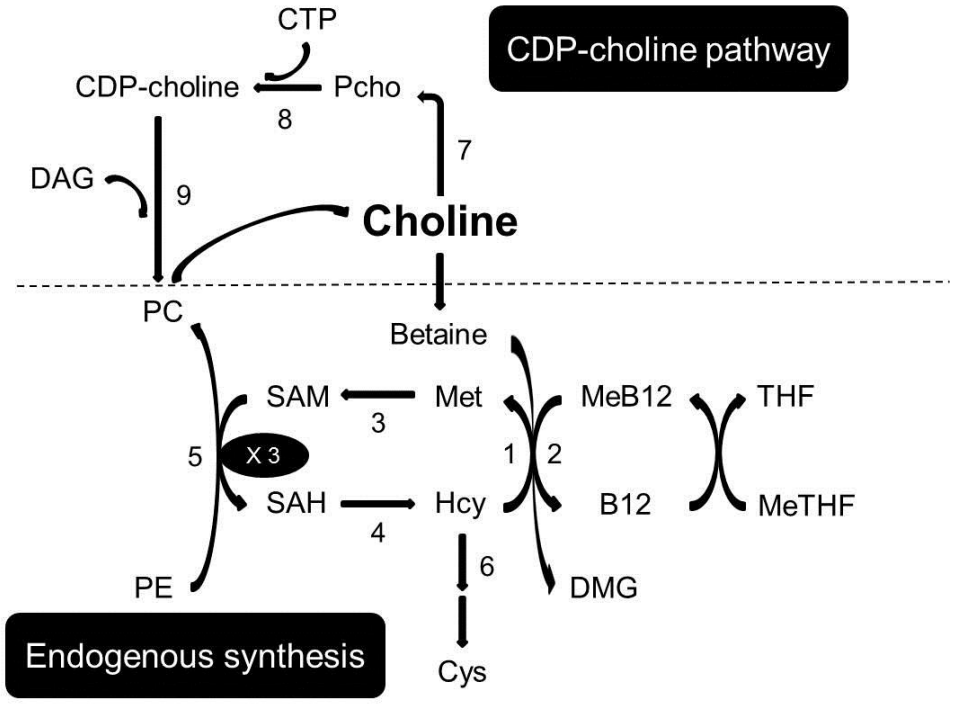

FIGURE 1.3 Simplified schematic to show dual roles of choline as the precursor of betaine and as phosphatidylcholine, but need for methyl groups for endogenous choline synthesis (Adapted and modified from Wu et al., Am J Clin Nutr. 2013). CDP-choline, cytidine 5-diphosphocholine; CTP, cytidine triphosphate; Cys, cysteine; DAG, diacylglycerol; DMG, dimethylglycine; Hcy, homocysteine; MeB12, methyl-B12; Met, methionine; MeTHF, methyl-tetrahydrofolate; PC, phosphatidylcholine; Pcho, phosphocholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate. Enzymes shown are: 1. betaine-homocysteine methyltransferase; 2. methionine synthase; 3. methionine adenosyltransferase; 4. *S*-adenosylhomocysteine hydrolase; 5. phosphatidylethanolamine *N*-methyltransferase; 6. cystathionine β-synthase; 7. Choline kinase; 8. Cytidine triphosphate:phosphocholine cytidylyltransferase; 9. CDP-choline:1,2-diacylglycerol cholinephosphotransferase.

CHAPTER 2: RATIONALE, OBJECTIVES, HYPOTHESES, SPECIFIC AIMS AND GENERAL ASSESSMENT

2.1 Rationale, objectives, hypotheses and specific aims

Rationale of the study

When the research was commenced, there were no published data on choline intakes or biochemical measures of choline status in young children in Canada. In addition, published data relating estimation of dietary choline intake to biochemical marker of choline status and insufficiency in children are few.

Objectives

The objectives of this MSc. Project are a) to determine choline status and its relationship to the estimated intake of choline from the diet in children 5 – 6 years of age; b) to use plasma homocysteine as a biomarker to determine whether children are at risk of choline insufficiency; c) to explore the relationship between plasma free choline and its metabolites and cognitive development in children 5 – 6 years of age.

Hypotheses

1. The dietary intake of choline is an important contributor to differences in plasma free choline in young children.
2. Plasma free choline will be positively associated with the plasma betaine:dimethylglycine ratio, and inversely associated with plasma homocysteine.
3. Plasma free choline and its metabolites betaine and dimethylglycine will be positively associated with tests of cognitive development in children.

Specific aims

1. To use dietary recalls for children 5 – 6 years of age to estimate the intakes and the food sources of choline and betaine.
2. To use venous blood from children 5 – 6 years of age to measure plasma free choline, betaine and dimethylglycine, in addition to homocysteine, methionine, cysteine, folate and vitamin B12.
3. To determine the association between the estimated dietary intake of choline and plasma free choline in children 5 – 6 years of age.
4. To determine the association between plasma free choline and plasma homocysteine in children 5 – 6 years of age, using homocysteine as a potential biomarker of choline insufficiency.

5. To determine the association between plasma free choline and its metabolites and cognitive development.

2.2 General assessment

2.2.1 Participants and setting

This study was a part of a larger cross-sectional study addressing the relationship between diet and development in healthy children of about 5.75 years of age, with no known health problems. All of the children were living in Vancouver, Canada. A total of 200 children were enrolled with a parent or legal guardian from the community. Because this study involved assessment of neurodevelopment, children born preterm (<37 weeks gestation), with congenital or acquired disease, or who had severe food allergies or immune disorders likely to impact growth and development were ineligible for participation. On enrollment, each child was assigned a unique, computer-generated, random code each held in a sealed opaque envelope and this code was used on all data collection forms and blood samples. This study was conducted according to guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia (B.C.) and the B.C. Children's and Women's Hospital. Written informed consent was obtained from a parent or legal guardian for each child prior to and for themselves enrollment.

2.2.2 Methods

Subject characteristics

Socio-demographic data, including maternal age, highest level of education attained, ethnic background, household income, and family size were collected by questionnaire. Data on how long the child was breastfed was also collected in a separate health questionnaire. Because of the limitations of using income and formal education as a proxy for the mother's intelligence (IQ), we assessed each mother's IQ using the Test of Nonverbal Intelligence-3 (TONI-3). The TONI-3 assesses aptitude, abstract reasoning and problem solving, but does not require any written instructions (Brown et al., 2006).

Dietary assessment and data analyses

Dietary assessment

Dietary intake was assessed using a FFQ that gathered information on the foods eaten, frequency of intake and portion sizes over the previous month, and using 24 hr recall records. On the day of blood collection, the FFQ and one 24 h recall record of all food and beverages consumed the previous day was completed by in-person interview with the parent or legal guardian, using food models and measuring utensils. Two further 24 hr recall records were collected by telephone at random over the following 14

d, all using a standardized 5-pass technique identical to that used in the in-person 24 hr record. The dietary information was entered into nutrient analysis software database with a Canadian food nutrient data base (ESHA Food Processor SQL, Version 10.10.0.0, Salem, OR: ESHA Research, 2012) and the USDA Database on the Choline Content of Common Foods, Version 2 (USDA, 2008).

Data analyses

The child weight and height were measured in light clothing without shoes. Z-scores for weight-for-age, height-for-age and body mass index (BMI)-for-age were calculated using the World Health Organization (WHO) Anthroplus anthropometric calculator software (version 1.0.4).

All statistical analyses were done using SPSS software (version 20.0; SPSS Inc, Chicago, NC). Prior to statistical analyses, all data were checked for normal distributions using the Kolmogorov-Smirnov test before carrying out further statistical analyses, as appropriate. Descriptive statistics including mean, median, SD, interquartile range (IQR), 2.5^{th} – 97.5^{th} percentile, 5^{th} – 95^{th} percentile, as appropriate, were used to summarize subject characteristics, dietary data and plasma metabolite data.

i. Descriptive summary of subject characteristics

Descriptive statistics including mean ± SD are used to report children's age, z-scores for weight-for-age, height-for-age, and BMI for age, and maternal TONI-3 scores. The proportion of boys and girls are reported as percentage. Maternal education background is given as ≤ high school, college, or university, while household income is categorized as <CAD $30,000, $30,000 – $50,000, or >$50,000. Maternal ethnic backgrounds were grouped into Caucasian, Chinese and "others." The proportion of each category was also indicated as percentage.

ii. Descriptive summary of macronutrient intake

Descriptive statistics were used to describe the intakes estimated using the FFQ, as the average of 3 x 24 hr recalls, and 1 x 24 hr recall with data analyzed for caloric intake (kcal), protein intake as g/d and % energy from protein, fat intake as g/d and % energy from fat, and carbohydrate intake as g/d and % energy from carbohydrate. One-way analysis of variance (ANOVA) with post-hoc Tukey's test was used to detect and analyze statistical difference among intakes estimated using different assessment methods.

iii. Dietary intakes of choline, betaine and methionine

The intakes of total choline, the sum of lipid-bound choline (PC and SM), and the sum of water-soluble choline (free choline, GPCholine and phosphocholine) were estimated from the data gathered with the FFQ, 3 x 24 hr recalls and 1 x 24 hr recall using the food processor software with the USDA database for choline. Descriptive statistics are given for the intakes of choline, betaine and methionine when estimated using each dietary assessment tool. To adjust for the possible overestimation of caloric intake by FFQ, data for the intakes of choline and betaine were adjusted for caloric intake (per 1,000 kcal). Mann-Whitney *U* tests were used to detect statistical differences among estimated intakes derived from the 3 assessment tools.

iv. Dietary source of choline

Descriptive statistics of the contribution of water-soluble (the sum of % free choline, % GPCholine and % phosphocholine) and lipid-bound choline (the sum of % PC and % SM) to total choline intake were calculated. One-way ANOVA or Mann-Whitney *U* test was used to determine significant differences in proportion of different forms of choline when estimated using different dietary approaches. Major food categories of dairy products, eggs, meat and seafood, vegetable and nuts, grain products, and others were used to address the contribution of different food groups to dietary choline and betaine intakes, with the results expressed as median intakes from each food group.

v. Plasma metabolites data analyses

Descriptive statistics were used to describe the plasma free choline, betaine, dimethylglycine, betaine:dimethylglyine, homocysteine, methionine, total B12 and folate for all children, as well as boys and girls separately. Student's *t*-test was used to detect differences between boys and girls in the plasma metabolite data. Skewed data were log-transformed for analyses and Pearson correlation analysis was used to determine the association among different plasma metabolites.

vi. Relationship between dietary and plasma choline and betaine

Pearson correlation coefficients were calculated to describe any potential relationship between dietary choline or betaine intake and the plasma free choline or betaine concentration, respectively.

vii. Relationship between plasma choline, betaine, dimethylglycine and cognitive test scores

To determine the relationship between plasma choline metabolites and the cognitive test scores, two sequential steps were used. First, unadjusted Pearson correlation analyses were used to determine any significant relationships between plasma free choline, betaine, or dimethylglycine and each of the cognitive performance tests. Next, a multivariate regression model adjusted for possible confounders (child sex, child's BMI for age z-score, child's ethnic background, parent's IQ, breastfeeding

duration of less than or over 3 mo, number of adults at home, and number of siblings) was used to determine the association between plasma free choline, betaine, or dimethylglycine and each cognitive test score among children. Importantly, since plasma choline and its metabolites decline with age (Ilcol et al., 2005), we studied children within the tight age window of 5 – 6 years of age, thus fulfilling the need to control for the effect of age in the analyses of plasma choline and betaine. A significant level of $P < 0.05$ was used, unless otherwise stated.

Blood sample collection

Venous blood was collected by a certified technician into tubes with ethylenediaminetetraacetic acid (EDTA) as the anti-coagulant. Immediately upon collection, the blood samples were centrifuged at 2000 × *g*, 4 °C for 10 min, then the plasma was recovered, separated into aliquots, and frozen at -70 °C until analyzed.

Biochemical analyses

Plasma free choline, betaine, and dimethylglycine were measured by liquid chromatography-electrospray tandem mass spectrometry (LCMS/MS) (Friesen et al., 2007). 10 µmol/L internal standard (20 µmol/L Choline d_9, 10 µmol/L Betaine d_9, 10 µmol/L DMG d_6, CDN Isotopes, Inc., Pointe Claire, QC, Canada), were added to 50 µL of plasma once thawed. Then, 100 µL of acetonitrile containing 0.1% formic acid was added to the sample to precipitate the proteins, the mixture vortexed, then was

centrifuged at 13,000 x *g* for 10 min at 4°C. Next, 20 µmol/L supernatant was added to 100 µL high performance liquid chromatography (HPLC) mobile phase (19% 15 mmol/L ammonium formate, 0.1% formic acid in H_2O, and 81% acetonitrile) in a 150 µL HPLC vial insert. Separation was accomplished with a Rx-Sil column (2.1 × 150 mm 5 µm) and a pre-column (2.1 × 12.1 mm 5 µm) both from Agilent Technologies, Santa Clara, CA, isocratically, with at a flow of 0.5 mL/min. The autosampler and column were maintained at 10°C and 25°C, respectively. The sample volume used for analysis was 4 µL, with a total analytical time of 7.0 min.

Plasma homocysteine, methionine and cysteine analyses were also analyzed by LCMS/MS (Innis & Hasman, 2006). First, 50 µL of plasma was transferred to an Eppendorf tube containing 10 µL of each of 500 µmol/L homocysteine d_8, 1000 µmol/L methionine d_4 and 1000 µmol/L cysteine d_2 as internal standards, and 10 µL dithiothreitol (500 mmol/L in 0.1 mol/L NaOH) was added. The mixture was then vortexed and the samples were kept at room temperature for 15 min. Afterwards 100 µL acetonitrile containing 0.2% (v:v) heptafluorobutyric acid (HFBA) were added to each sample, and the samples centrifuged at 13,000g for 10 min at 4°C. Next, 20 µL supernatant and 100 µL 0.2 % HFBA/H_2O (v/v) were transferred into a 150 µL HPLC vial insert. Separation of compounds was achieved using a Zorbax SB Aqua column (2.1 × 100 mm 3.5 µm), with a pre-column (2.1 × 12.1 mm 5 µm) both from Agilent Technologies, Santa Clara, CA. The mobile phase was 0.2 % HFBA/H_2O (v/v) at a flow of 0.5 mL/min with a sample volume of 5 µL was used for analyses, with a total analytical time of 6.2 min.

Plasma folate was measured by ion capture assay, and total vitamin B12 (tB12) was quantified by microparticle enzyme immunoassay using an AxSym Analyzer (Abbott Laboratories, Abbott Park, IL), as per the manufacturer's instructions. The intra- and inter-assay c.v. were for choline, 2.5% and 3.8%, respectively; betaine, 2.2% and 3.5%, respectively; dimethylglycine, 2.4% and 3.8%, respectively; homocysteine, 2.3% and 1.7%, respectively; methionine, 1% and 2%, respectively; cysteine, 1.2% and 1.4%, respectively; folate, 2.9% and 3.5%;, respectively; and tB12, 1.7% and 3.2%, respectively. All biochemical analyses were done by experienced laboratory technologists.

Child neurodevelopmental assessments

Child development was assessed using a) the Kaufman Assessment Battery for Children, 2nd Edition (KABC-II), b) the Picture Peabody Vocabulary Test-4th Edition (PPVT-4), and c) the Beery-Buktenica Developmental Test of Visual-Motor Integration, 6th Edition (Beery-VMI). The KABC-II is designed as a test of IQ for children 2.5 to 12 y of age (Kaufman & Kaufman, 2004). The test involves a sequential processing scale and simultaneous processing scale, with scores from parts of these two scales used to give a mental composite scale (Mental Process Index, MPI). The sequential processing scale measures a child's ability to solve problems requiring ordered arrangement of stimuli, while the simultaneous processing scale requires the child to solve organizational/spatial problems that require arrangement of multiple stimuli at one time. Child development was also assessed using the PPVT-4 which provides age-based

scores for receptive and expressive language (Dunn & Dunn, 2007). Children were further evaluated using the Beery-VMI, which offers age-based scores for their visual and motor abilities. The tests present drawings of geometric forms arranged in order of increasing difficulty that the individual is asked to copy (Beery et al., 2010). All cognitive tests were administered by a clinical research staff specialized in child psychology.

CHAPTER 3: RESULTS

A. Subjects

The current study enrolled 200 subjects. Three subjects who were born preterm infants (defined as <37 wk gestation) and the second twin from a set of twins enrolled was subsequently excluded from the data analysis, giving n = 196 children for whom data could be analyzed.

The subject characteristics for the 196 children are summarized in **Table 3.1**. The variability in age among the children was low due to the enrollment inclusion criteria requiring recruitment between age 5 yr 9 mo and 5 yr and 11 mo of age. The number of girls was slightly higher than boys (53% and 47%). Most of the mothers of the children in this study had completed at least a university degree, and most households had an annual income of $50,000 CAD, or above. The majority of the mothers in this study were of Caucasian background (70%), followed by Chinese (13%), and other ethnic backgrounds (17%) including First Nations, Black African, East Indians/South Asian and others.

B. Dietary intakes

Three FFQ were excluded due to incomplete or unreliable data, 16 of the 3 x 24 hr recalls, and five of the 1 x 24 hr recalls were excluded from analysis for similar reasons. **Table 3.2** shows the estimated dietary intakes of energy and macronutrients

for the children. The mean ± SD % of dietary energy from protein, fat and carbohydrate were 16.1 ± 2.4%, 32.4 ± 4.4%, and 54.3 ± 5.5%, respectively, when estimated using the FFQ; 16.0 ± 2.7%, 32.8 ± 5.4% and 53.5 ± 6.3%, respectively, when estimated as the average of three 24 hr recalls; 15.7 ± 3.6%, 33.2 ± 8.6% and 53.5 ± 9.3%, respectively, when estimated using a single 24 hr recall. There were no significant differences in the percent dietary energy from protein, fat or carbohydrate estimated using the three different assessment approaches (FFQ, 3 x 24 hr recalls, and 1 x 24 hr recall, $P > 0.05$). The intakes of energy, protein, fat and carbohydrate were 1840 ± 494 kcal, 74.1 ± 23.6 g/d, 66.8 ± 21.9 g/d, 248 ± 65.8 g/d, respectively, when estimated using the FFQ; 1510 ± 336 kcal, 60.5 ± 17.2 g/d, 55.6 ± 16.5 g/d, 202 ± 47.8 g/d, respectively, when estimated using the average of three 24 hr recalls; and 1550 ± 445 kcal, 60.4 ± 20.8 g/d, 58.2 ± 24.5 g/d, 53.5 ± 9.3 g/d when estimated using a single 24 hr recall. The amount of protein, fat and carbohydrate and total energy consumed was higher when estimated using the FFQ than when estimated as the average of 3 x 24 hr recalls ($P < 0.05$).

Table 3.3 shows the intakes of choline, betaine and methionine for the children. The median intakes of choline, betaine, and methionine were 278 mg/d, 90.2 mg/d and 1.36 g/d, respectively, when estimated using the FFQ; 235 mg/d, 75.5 mg/d, and 1.18 g/d, respectively, when estimated using a single 24 hr recall; and 228 mg, 80.9 mg/d and 1.13 g/d, respectively, when estimated as the average of three 24 hr recalls. Choline, betaine and methionine intakes were significantly higher when estimated by FFQ than using either a single 24 hr recall or as the average of three 24 hr recalls ($P < 0.05$). After adjusting for energy intake, the median intake of choline, betaine were 162

mg/1,000 kcal and 51.6 mg/1,000 kcal, 0.80 g/1,000 kcal, respectively, when estimated using the FFQ; 145 mg/1,000 kcal, 48.9 mg/1,000 kcal, 0.76 g/1,000 kcal, respectively, when estimated using a single 24 hr recall; and 155 mg/1,000 kcal, 53.0 mg/1,000 kcal, and 0.79 g/1,000 kcal, respectively, when estimated using the average of three 24 hr recalls ($P > 0.05$). Therefore, after adjusting for energy intake, there were no significant differences in the intakes of choline, betaine or methionine estimated using the three different approaches ($P > 0.05$).

Table 3.4 shows the percent contribution of water-soluble and lipid-bound choline to the choline intake of the children in this study. As shown by the data, water-soluble and lipid-bound choline each contributed 51.4 ± 9.5% and 48.6 ± 9.5, respectively, to the total choline intake. The free choline, GPCholine and phosphocholine each contributed 46.2 ± 7.5%, 43.5 ± 7.5%, and 10.3 ± 1.6%, respectively, to the total water-soluble choline intake. PC and SM each contributed 89.3 ± 2.4% and 10.7 ± 2.4% to the intake of lipid-bound choline.

Figure 3.1 shows the frequency distributions of total choline intake as mg/d and mg/1,000 kcal/d for the children. Using the current Adequate Intake (AI) of choline intake of 250 mg/d for children 4 – 8 yr of age, 34% of children in this study consumed less than the recommended intake of choline. Dividing the AI for choline by the average recommended energy intake for children 4 – 8 yr of age (~1500 kcal), a similar 38% of the children in the study consumed less than the extrapolated recommend choline intake of 150 mg/1,000 kcal.

B1. Food sources of dietary choline and betaine

Table 3.5 shows the % contribution of different food to the total dietary choline and betaine intakes of the children. The results for the contribution of different foods to choline intake were skewed, thus median intakes were used to interpret the data. For this analysis, foods were grouped into dairy products, eggs, meat and seafood, vegetable and nuts, grain products, and others, and then the intake of choline from each food group was determined. Dairy products provided the largest contribution to total choline intakes (median: 29.2 %), followed by animal products (21.2%), vegetables (18.2 %), grain products (14.7%), eggs (12.1 %), and others (median: 0.23 %). For water-soluble choline intake, dairy products contributed half (49.9 %), with vegetables (23.0 %) and grain products (17.9 %) also being major sources. Meat and seafood, and eggs contributed to majority of the lipid-bound choline (36.3 % and 24.7 %, respectively), with grain products and vegetables intakes contributing a median of 10.5 % and 14.0 %, respectively. Grain products contributed 84.8% of the betaine consumption, followed by meat and seafood (5.12 %), dairy (3.57%), vegetables and nuts (2.94 %).

C. Choline, homocysteine, cysteine, B12 and folate status among children

Of 196 subjects, 163 plasma samples were analyzed for plasma free choline, betaine and dimethylglycine, 120 samples were analyzed for homocysteine and methionine, and 132 samples were analyzed for total vitamin B12 and folate. Data on plasma choline and its metabolites, homocysteine, methionine, cysteine, B12 and folate

are shown in **Table 3.6**. Plasma free choline, betaine and dimethylglycine, and cysteine were normally distributed. On the other hand, homocysteine (P = 0.003) and methionine (P = 0.045) were skewed to the right, and folate was skewed to the left (P = 0.04) of the mean. The mean ± SD and median (IQR) for plasma free choline, betaine, dimethylglycine, cysteine and total B12 were 8.57 ± 2.08 and 8.20 (7.20 – 9.60) µmol/L, 45.4 ± 12.9 and 43.2 (37.0 – 53.1) µmol/L, 3.26 ± 0.95 and 3.10 (2.60 – 3.80) µmol/L, and 594 ± 158, 222 ± 29.2 (201 – 236) µmol/L, and 608 (491 – 725) pmol/L, respectively. The median (IQR) for plasma homocysteine, methionine and folate were 4.65 (4.10 – 5.20) µmol/L, 28.1 (23.4 – 33.8) µmol/L, and 33.1 (31.2 – 34.6) nmol/L, respectively. No child had a B12 <148 pmol/L or folate <6.8 nmol/L, used as the cut off for deficiency of B12 and folate, respectively (Miller et al., 2006; Macfarlane et al., 2011). Only 1 child in our study had a plasma B12 concentration lower than 220 pmol/L, which is the cut-off for B12 sufficiency (Macfarlane et al., 2011). Similarly, no child had a plasma homocysteine >13 µmol/L, a reference value for elevated homocysteine, although this is an adult cutoff vale (Macfarlane et al., 2011).

Comparison of plasma metabolites between boys and girls revealed no significant differences or trends with gender (P > 0.10). Interestingly, the girls had a significantly higher ratio of betaine:dimethylglycine than the boys (P < 0.001).

D. Associations among dietary intakes and plasma choline and betaine

Figure 3.2 shows the scatter plots and correlation analysis of the association between the estimated dietary intake of choline and plasma free choline. Total choline

intake showed a positive association with plasma free choline (r = 0.198, *P* = 0.014), which remained significant when choline intake was expressed as mg/1,000 kcal (r = 0.181, *P* = 0.025). **Figure 3.3** shows the scatter plots of the relationship between the estimated dietary intake of betaine and plasma betaine concentration. Neither betaine intake nor betaine intake/1,000 kcal was significantly associated with plasma betaine (r = 0.008, *P* = 0.915 and r = 0.005, *P* = 0.950, respectively).

Figure 3.4 shows the scatter plots of the relationships between estimated intakes of different forms of choline (lipid-bound form and water-soluble form) and plasma free choline. Further analysis shows that intake of lipid-bound choline (the sum of intakes of PC and SM) showed a positive, significant association with plasma free choline (r = 0.220, *P* < 0.005). The intake of water-soluble forms of choline (the sum the intakes of free choline, GPCho and phosphocholine), on the other hand, was not associated with plasma free choline (r = 0.121, *P* = 0.131). Dietary intake of lipid-bound choline was positively associated with dietary intake of water-soluble choline (r = 0.43, *P* < 0.001) (**Figure 3.5**). All dietary data used for analyses in this section were derived from FFQ. However, dietary choline intake (total choline, lipid-bound choline, and water-soluble choline) estimated using 3 x 24 hr recall or 1 x 24 recall showed either much weakened association or a lack of association with plasma free choline. It is possible that FFQ could estimate choline intake better than the 24 hr recalls since it covers foods high in choline content that are not consumed on a regular basis.

E. Relations among choline, choline metabolites, homocysteine, methionine, total vitamin B12 and folate in children

Statistical associations between choline metabolites, homocysteine, B12 and folate are shown in **Table 3.7**. Pearson correlation analyses revealed significant positive associations between choline and betaine (r = 0.47, *P* < 0.001), choline and dimethylglycine (r = 0.29, *P* < 0.001), choline and methionine (r = 0.34, *P* < 0.001), betaine and dimethylglycine (r = 0.29, *P* < 0.001), dimethylglycine and methionine (r = 0.33, *P* < 0.001), cysteine and homocysteine (r = 0.57, *P* < 0.001), dimethylglycine and B12 (r = 0.19, *P* = 0.029), and B12 and folate (r = 0.29, *P* = 0.001). Significant inverse associations were found between choline and homocysteine (*P* = 0.013) (**Figure 3.6**), betaine and homocysteine (r = -0.37, *P* < 0.001) (**Figure 3.7**), cysteine and free choline (r = -0.25, *P* = 0.006), cysteine and betaine (r = -0.22, *P* = 0.017), cysteine and dimethylglycine (r = -0.33, *P* < 0.001), homocysteine and B12 (r = -0.25, *P* = 0.005), and dimethylglycine and homocysteine (r = -0.19, *P* = 0.036). The ratio of betaine:dimethylglycine was significantly, inversely associated with homocysteine (r = -0.18, *P* = 0.049) (**Figure 3.8**) and methionine (r = -0.24, *P* = 0.009). However it should be noted that the significant association between plasma free choline and homocysteine disappeared when the analysis was done on data exclusive of 6 data points all of which were outside of the 5^{th} – 95^{th} percentile of plasma free choline (3 data points below the 5^{th} percentile and 3 data points were above the 95^{th} percentile) (r = 0.16, *P* = 0.088). Mean (minimum – maximum) for plasma betaine, dimethylglycine, homocysteine, methionine, cysteine, B12, and folate for the mentioned 3 subjects below the 5^{th}

percentile plasma free choline were 31.4 (21.5 – 46.7) µmol/L, 2.73 µmol/L (2.20 – 3.40), 9.57 (7.9 – 12.1) µmol/L, 34.3 (28.5 – 39.2) µmol/L, 296 (218 – 344) µmol/L, 588 (522 – 344) pmol/L, and 37.2 (35.7 – 40.0) nmol/L, respectively; mean (minimum – maximum) for plasma betaine, dimethylglycine, homocysteine, methionine, cysteine, B12, and folate for the mentioned 3 subjects above 95th percentile free choline were 78.5 (47.5 – 119) µmol/L, 4.33 (3.50 – 4.80) µmol/L, 4.03 (3.20 – 5.10) µmol/L, 56.4 (51.8 – 65.1) µmol/L, 200 (178 – 224) µmol/L, 651 (556 – 772) pmol/L, and 33.1 (31.9 – 34.9) nmol/L (**Table 3.8**).

F. Relationships between plasma choline metabolites of children and their psychometric test results

Cognitive abilities in the children were evaluated using the PPVT-4, Beery VMI and KABC-II. The summary of the scores of each test and subtests is described in **Table 3.9**. Briefly, the (mean ± SD) were PPVT-4, 117 ± 18.2; Beery VMI: 16.8 ± 2.13; KABC-II, Learning Ability, 21.9 ± 4.93; Sequential Process, 21.6 ± 4.44; Simultaneous Process, 34.6 ± 5.36; Mental Process Index, 78.1 ± 11.1. There were no statistical significant differences between boys and girls for any of the test scores ($P > 0.05$).

The first analysis used unadjusted univariate analyses with the Pearson correlation coefficient to determine the relationships between the cognitive measures and each of the plasma choline metabolites (namely plasma free choline, betaine and dimethylglycine) (**Table 3.10**). Significant positive associations were found between plasma betaine and PPVT-4 ($r = 0.18$, $P = 0.02$), and betaine and the KABC-II

Simultaneous Process (r = 0.17, *P* = 0.03). A trend was also found between plasma betaine and the KABC-II Mental Process Index (r = 0.16, *P* = 0.05). No significant associations were detected between choline or dimethylglycine and any cognitive test score (*P* > 0.05).

Next, a multivariate regression model was used to determine possible associations between cognitive performance and plasma choline metabolites (plasma free choline, betaine and dimethylglycine), adjusting for possible confounding variables of the cognitive performance including child sex, BMI for age z-score, ethnic background, mother's IQ, breastfeeding duration (less than or over 3 mo), number of adults at home, and number of siblings (**Table 3.11**). Contrary to the unadjusted analyses, all the statistical significant associations between betaine and the cognitive domains disappeared. Nonetheless, borderline significant associations remained between plasma dimethylglycine and the KABC-II Mental Process Index (B = 2.04, SE = 1.09, *P* = 0.06).

TABLE 3.1 Subject characteristics

Child characteristics	Mean ± SD, %
Age (mo), *n*= 196	68.7 ± 0.7
Child sex (Boys/Girls) %, *n* = 196	47.4/52.6
Weight for age, z-score, mean ± SD, *n* = 196	0.19 ± 0.96
Height for age, z-score, mean ± SD, *n* = 196	0.19 ± 0.97
BMI for age, z-score[1], mean ± SD, *n* = 196	0.08 ± 0.99
Family characteristics	
Mother's education, *n* = 191	
≤ High school/College/University, (*n*)	9/50/132
(%)	4.7/26.2/69.1
Maternal TONI[2], *n* = 167	35.2 ± 6.85
Household income, *n* =195	
< $30,000/$30,000 – $50,000/>$50,000, (*n*)	9/19/167
(%)	4.6/9.7/85.6
Maternal ethnic backgrounds	
Caucasian/Chinese/Others[3] (%)	70/13/17

[1]Z-scores were calculated using the World Health Organization (WHO) Anthroplus anthropometric calculator. BMI: Body mass index.

[2]TONI: Test of non-verbal intelligence

[3]Others: Other ethnic backgrounds include East Indians/South Asians, First Nations, Black Africans and others.

TABLE 3.2 Dietary intakes of energy and macronutrients among children 5 – 6 yr of age[1]

	FFQ (n = 193)	3 x 24 hr recalls (n = 180)	1 x 24 hr recall (n = 191)
kcal	1840 ± 494[2]	1510 ± 336	1550 ± 445
Protein, g/d	74.1 ± 23.6[2]	60.5 ± 17.2	60.4 ± 20.8
% energy	16.1 ± 2.39	16.0 ± 2.73	15.7 ± 3.57
Fat, g/d	66.8 ± 21.9[2]	55.6 ± 16.5	58.2 ± 24.5
% energy	32.4 ± 4.35	32.8 ± 5.44	33.2 ± 8.62
CHO[3], g/d	248 ± 65.8[2]	202 ± 47.8	205 ± 65.1
% energy	54.3 ± 5.48	53.5 ± 6.31	53.5 ± 9.31

[1] Data are mean ± SD and were normally distributed, by Kolmogorov-Smirnov test, $P < 0.05$

[2]Significantly different from 3 x 24 hr recalls and 24 hr recall before visit, by One-way ANOVA, post-hoc Tukey's HSD test, $P < 0.05$.

[3]CHO: Carbohydrate.

TABLE 3.3 Dietary intakes of choline, betaine and methionine among children 5 – 6 yr of age

	FFQ	1 x 24 hr recall	3 x 24 hr recalls
Choline (mg/d)[1]			
Mean ± SD	302 ± 99.6	246 ± 108	246 ± 90.8
Median (IQR)	278 (225 – 366)[2]	235 (164 – 301)	228 (185 – 284)
2.5^{th} – 97.5^{th}	162 – 556	88.6 – 491	124 – 485
Choline (mg/1,000 kcal)			
Mean ± SD	165 ± 35.5	162 ± 63.1	163 ± 44.4
Median (IQR)	162 (140 – 184)	145 (117 – 189)	155 (130 – 187)
2.5^{th} – 97.5^{th}	111 – 247	78.7 – 331	98.8 – 279
Betaine (mg/d)[1]			
Mean ± SD	100 ± 40.8	88.9 ± 60.2	88.0 ± 39.8
Median (IQR)	90.2 (71.1 – 118)[2]	75.5 (50.6 – 109)	80.9 (60.5 – 106)
2.5^{th} – 97.5^{th}	46.6 - 206	19.0 - 238	33.5 – 208
Betaine (mg/1,000 kcal)[1]			
Mean ± SD	54.8 ± 18.0	58.2 ± 35.0	58.5 ± 23.6
Median (IQR)	51.6 (42.3 – 61.0)	48.9 (34.9 – 72.3)	53.0 (42.6 – 71.5)
2.5^{th} – 97.5^{th}	31.9 – 111	16.1 – 146	26.8 – 122
Met (g/d)[1,3]			
Mean ± SD	1.48 ± 0.53	1.18 ± 0.50	1.20 ± 0.42
Median (IQR)	1.36 (1.08 – 1.79)[2]	1.11 (0.80 – 1.53)	1.13 (1.07 – 1.78)
2.5^{th} – 97.5^{th}	0.77 – 2.65	0.38 – 2.31	0.57 – 2.30
Met (g/1,000 kcal)			
Mean ± SD	0.80 ± 0.17	0.77 ± 0.27	0.80 ± 0.20
Median (IQR)	0.80 (0.67 – 0.91)	0.76 (0.35 – 0.58)	0.79 (0.50 – 0.64)
2.5^{th} – 97.5^{th}	0.50 – 1.22	0.27 – 1.36	0.42 – 1.28

[1] Data are skewed, by Kolmogorov-Smirnov test, $P < 0.05$.

[2] Significantly different from 3 x 24 hr recalls and 1 x 24 recall, (Mann-Whitney *U* test, $P < 0.05$)

[3] Met: Methionine

TABLE 3.4 Contributions of % water-soluble and lipid-bound choline to total choline intake[1]

	% Total choline	% Water-soluble	% Lipid-bound
Water-soluble choline	51.4 ± 9.5	-	-
Free choline	-	46.2 ± 7.5	-
Glycerophosphocholine	-	43.5 ± 7.4	-
Phosphocholine	-	10.3 ± 1.6	-
Lipid-bound choline	48.6 ± 9.5	-	-
Phosphatidylcholine	-	-	89.3 ± 2.4
Sphingomyelin	-	-	10.7 ± 2.4

[1]Results are mean ± SD, *n* = 193 for dietary intakes determined by food frequency questionnaire (FFQ). The distribution of choline intake from different sources was normally distributed by Kolmogorov-Smirnov test, *P*>0.05. There were no differences in the sources of intake when measured using FFQ compared to 3 x 24 hr recalls or 1 x 24 hr recall before blood draw, by 1-way ANOVA, post-hoc Tukey's HSD test, *P* > 0.05.

TABLE 3.5 Contribution of different food to dietary choline and betaine intakes[1]

% Intake from	Choline			Betaine
	Total choline	Water-soluble	Lipid-bound	
Dairy	29.2	49.9	8.78	3.57
	(8.75 – 52.3)	(16.4 – 67.8)	(1.89 – 24.7)	(0.45 – 9.21)
Eggs	12.1	0.09	24.7	0.04
	(0 – 30.7)	(0 – 0.56)	(0 – 51.5)	(0 – 0.32)
Meat and seafood	21.2	6.85	36.3	5.12
	(3.72 – 40.8)	(0.76 – 18.3)	(5.96 – 37.2)	(1.02 – 15.0)
Vegetables and nuts	18.2	23.0	14.0	2.94
	(9.53 – 39.3)	(11.6 – 50.9)	(5.77 – 37.2)	(0.27 – 24.2)
Grain	14.7	17.9	10.5	84.8
	(6.36 – 26.9)	(8.76 – 33.3)	(3.84 – 28.2)	(61.8 – 94.5)
Others	0.23	0.34	0	0
	(0 – 2.11)	(0 – 3.39)	(0 – 1.45)	(0 – 0.22)

[1]Results are median (5^{th} – 95^{th} percentile), *n* = 193 for dietary intakes. The intake of choline from different food sources was calculated as % total choline and % of each choline component for each child. The intake distribution was skewed, by Kolmogorov-Smirnov test, *P* > 0.05.

TABLE 3.6 Plasma free choline, betaine and dimethylglycine concentrations among children 5 – 6 yr of age

Plasma	Mean ± SD	Median (IQR)	2.5^{th} – 97.5^{th}
Free choline (μmol/L)			
All (*n* = 163)	8.57 ± 2.08	8.20 (7.20 – 9.60)	5.51 – 13.8
Boys (*n* = 77)	8.69 ± 1.87	8.40 (7.30 – 9.70)	5.60 – 13.0
Girls (*n* = 86)	8.46 ± 2.26	8.05 (7.08 – 9.23)	5.30 – 16.6
Betaine (μmol/L)			
All (*n* = 163)	45.4 ± 12.9	43.2 (37.0 – 53.1)	26.1 – 69.3
Boys (*n* = 77)[2]	42.9 ± 10.9	42.0 (34.1 – 49.7)	25.1 – 67.4
Girls (*n* = 86)	47.7 ± 14.1	44.6 (38.3 – 56.2)	28.3 – 87.4
Dimethylglycine (μmol/L)			
All (*n* = 163)	3.26 ± 0.95	3.10 (2.60 – 3.80)	1.80 – 5.68
Boys (*n* = 77)	3.38 ± 0.99	3.30 (2.70 – 3.80)	1.78 – 5.95
Girls (*n* = 86)	3.15 ± 0.89	3.00 (2.50 – 3.80)	1.55 – 5.10
Bet:DMG			
All (*n* = 163)	14.7 ± 4.66	13.8 (11.5 – 17.1)	6.77 – 25.5
Boys (*n* = 77)[2]	13.3 ± 3.47	12.8 (11.0 – 16.3)	6.01 – 20.9
Girls (*n* = 86)	16.0 ± 5.21	14.7 (12.8 – 20.2)	7.71 – 29.4
Homocysteine (μmol/L)			
All (*n* = 120)[1]	4.81 ± 1.23	4.65 (4.10 – 5.20)	3.30 – 8.10
Boys (*n* = 58)	4.95 ± 1.24	4.70 (4.10 – 5.30)	3.25 – 8.42
Girls (*n* = 62)	4.69 ± 1.22	4.50 (3.98 – 5.03)	3.26 – 9.34
Methionine (μmol/L)			
All (*n* = 120)[1]	30.4 ± 9.97	28.1 (23.4 – 33.8)	17.0 – 64.8
Boys (*n* = 58)	29.3 ± 7.15	28.1 (23.9 – 33.1)	17.0 – 46.2
Girls (*n* = 62)	31.5 ± 12.0	28.1 (23.0 – 36.8)	15.8 – 66.4
Cysteine (μmol/L)			
All (*n* = 120)	222 ± 29.2	220 (201 – 236)	170 – 280
Boys (*n* = 58)	227 ± 34.7	227 (206 – 248)	162 – 335
Girls (*n* = 62)	218 ± 22.3	219 (197 – 231)	182 – 276
Total B12 (pmol/L)			
All (*n* = 132)	594 ± 158	608 (491 – 725)	252 – 862
Boys (*n* = 64)	616 ± 162	609 (521 – 759)	230 – 869
Girls (*n* = 68)	573 ± 153	596 (466 – 395)	261 – 856
Folate (nmol/L)			
All (*n* = 132)[1]	32.7 ± 3.39	33.1 (31.2 – 34.6)	24.7 – 39.2
Boys (*n* = 64)	32.4 ± 3.68	32.9 (30.3 – 34.1)	20.6 – 40.4
Girls (*n* = 68)	33.0 ± 3.09	33.6 (31.7 – 34.9)	25.2 – 39.1

[1] Data are skewed, by Kolmogorov-Smirnov test, $P < 0.05$. Bet:DMG: Betaine:Dimethylglycine.

[2] Significant difference between boys and girls, by student's *t*-test, $P < 0.01$.

TABLE 3.7 Relations among choline metabolites, homocysteine, total vitamin B12 and folate among children 5 – 6 y of age[1]

	Bet	DMG	Bet:DMG	Hcy	Met	Cys	tB12	Folate
FC	0.47[2]	0.29[2]	0.11	-0.28[2]	0.34[2]	-0.25[2]	0.06	-0.02
Bet	-	0.29[2]	0.56[2]	-0.37[2]	0.11	-0.22[3]	0.05	0.05
DMG	0.29[2]	-	-0.57[2]	-0.19[3]	0.33[2]	-0.33[2]	0.19[3]	0.05
Hcy	-0.37[2]	-0.19[3]	-0.18[3]	-	0.14	0.57[2]	-0.25[2]	-0.09
Met	0.11	0.33[2]	-0.24[3]	0.14	-	-0.13	0.16	-0.05
Cys	-0.22[3]	-0.33[2]	0.10	0.57[2]	-0.13	-	-0.05	0.16
tB12	0.05	0.19[3]	-0.13	-0.25[2]	0.16	-0.05	-	0.29[2]
Folate	0.05	0.05	-0.03	-0.09	-0.05	0.16	0.29[2]	-

[1]Data are Pearson r correlation coefficient. Bet: Betaine; Bet:DMG: Betaine:Dimethylglycine; Cys: Cysteine; DMG: Dimethylglycine; FC: Free choline; Hcy: Homocysteine; Met: methionine; tB12: Total vitamin B12. Log-transformed data of skewed variables were used for analysis

[2]Significant association between indicated variables, $P < 0.01$.

[3]Significant association between indicated variables, $P < 0.05$.

Table 3.8 Summary of plasma betaine, dimethylglycine, methionine, cysteine, folate and B12 of 3 subjects with plasma choline below 5th percentile and 3 subjects with plasma choline above 95th percentile[1]

Plasma metabolites	Outlier children		All children
	Low choline, high hcy[2] (*n* = 3)	High choline, low hcy (*n* = 3)	Group means
Betaine (µmol/L)	31.4 (21.5 – 46.7)	78.5 (47.5 – 119)	45.4
DMG (µmol/L)	2.73 (2.20 – 3.40)	4.33 (3.50 – 4.80)	3.26
Met (µmol/L)	34.3 (28.5 – 39.2)	56.4 (51.8 – 65.1)	30.4
Cysteine (µmol/L)	296 (218 – 344)	200 (178 – 225)	222
B12 (pmol/L)	588 (522 – 635)	651 (556 – 772)	594
Folate (nmol/L)	37.2 (35.7 – 40.0)	33.1 (31.9 – 34.9)	32.7

[1]Data are Mean (Minimum – Maximum). DMG: Dimethylglycine; Hcy: Homocysteine; Methionine: Methionine.

TABLE 3.9 Summary of cognitive test results in children

Cognitive test score	Mean ± SD (*n*)		
	All	Boys	Girls
PPVT4	117 ± 18.2 (160)	114 ± 20.3 (75)	119 ± 16.0 (85)
Beery	16.8 ± 2.13 (163)	16.8 ± 2.17 (77)	16.8 ± 2.11 (86)
KABC-II, Mental Process Index	78.1 ± 11.1 (158)	77.2 ± 11.7 (74)	79.0 ± 10.5 (84)
KABC-II, Learning ability	21.9 ± 4.93 (160)	21.2 ± 4.99 (75)	22.5 ± 4.84 (85)
KABC-II, Sequential process	21.6 ± 4.44 (161)	21.5 ± 4.56 (75)	22.5 ± 4.84 (86)
KABC-II, Simultaneous process	34.4 ± 5.74 (161)	34.8 ± 5.01 (76)	34.6 ± 5.36 (85)

[1]MPI: Mental Process Index, the global IQ that comprises learning ability, sequential process and simultaneous process.

TABLE 3.10 Associations between plasma choline and its metabolites and neurodevelopmental outcome assessed using KABC-II and PPVT-4 among children 5- 6 yr of age (*n* = 163) by univariate regression analysis using Pearson correlation coefficient

	Free choline		Betaine		DMG	
	r	P	r	P	r	P
PPVT4	0.03	0.71	**0.18**[2]	**0.02**	-0.07	0.38
Beery	-0.08	0.34	-0.12	0.15	0.13	0.10
KABC-II						
MPI[1]	-0.03	0.71	0.16	0.05	0.11	0.18
Learning	0.02	0.98	0.13	0.10	0.04	0.65
Sequential	-0.14	0.09	0.03	0.68	0.11	0.15
Simultaneous	0.05	0.71	**0.17**[2]	**0.03**	0.13	0.12

[1]MPI: Mental Process Index, the global IQ that comprises learning ability, sequential process and simultaneous process.

[2]Significant association, $P < 0.05$.

TABLE 3.11 Associations between plasma choline and its metabolites and neurodevelopmental outcome assessed using KABC-II and PPVT-4 among children 5 – 6 yr of age (*n* = 163) by multivariate regression analysis[1]

	Free choline			Betaine			DMG		
	B[3]	SE	P	B[3]	SE	P	B[3]	SE	P
PPVT4	0.07	0.65	0.91	0.12	0.11	0.26	-0.34	1.49	0.82
Beery	-0.11	0.09	0.24	-0.03	0.02	0.09	0.30	0.21	0.15
KABC-II									
MPI[4]	-0.13	0.48	0.79	0.10	0.08	0.22	2.04	1.09	0.06
Learning	0.04	0.21	0.86	0.04	0.04	0.25	0.82	0.48	0.09
Sequential	-0.22	0.18	0.22	0	0.03	0.94	0.28	0.42	0.50
Simultaneous	0.04	0.23	0.86	0.06	0.04	0.13	0.86	0.53	0.11

[1]Adjusted for child sex, child's BMI for age z-score, child's ethnic background, parent's IQ, breastfeeding duration (less than or over 3 mo), number of adults at home, and number of child's siblings.

[2]Unstandardized coefficient.

[3] MPI: Mental Process Index, the global IQ that comprises learning ability, sequential process and simultaneous process.

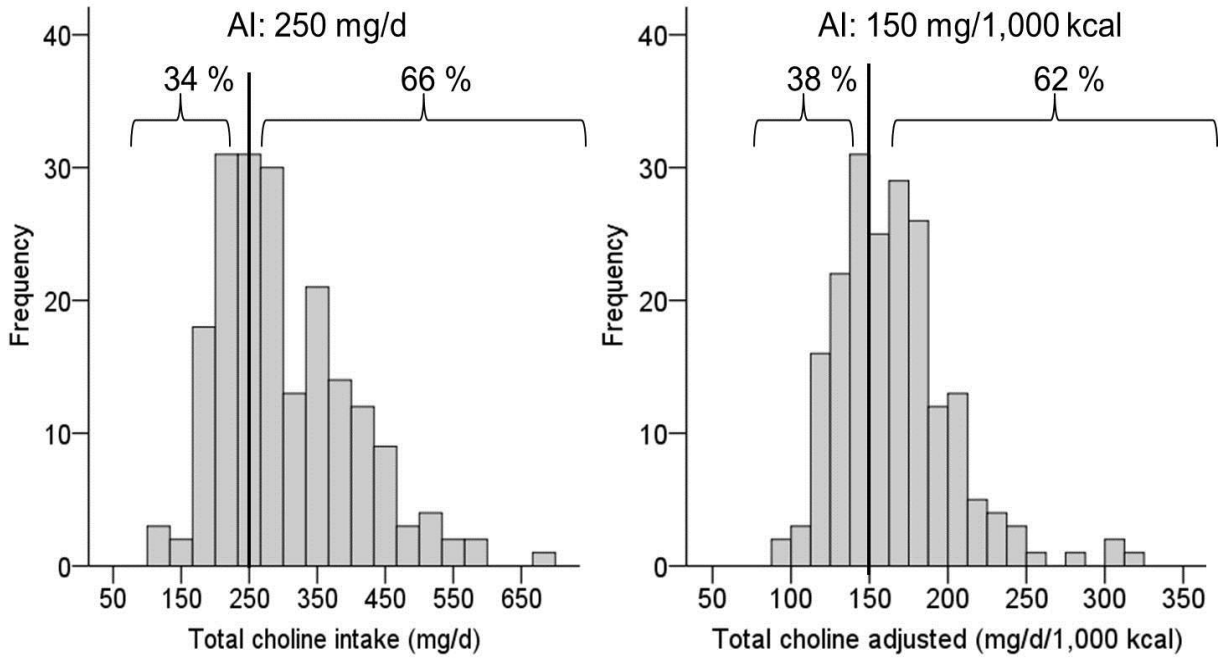

FIGURE 3.1 Histograms to show the distribution of total choline intake (mg/d) and choline intake adjusted for energy (mg/1,000 kcal). *n* = 193 for all data**.**

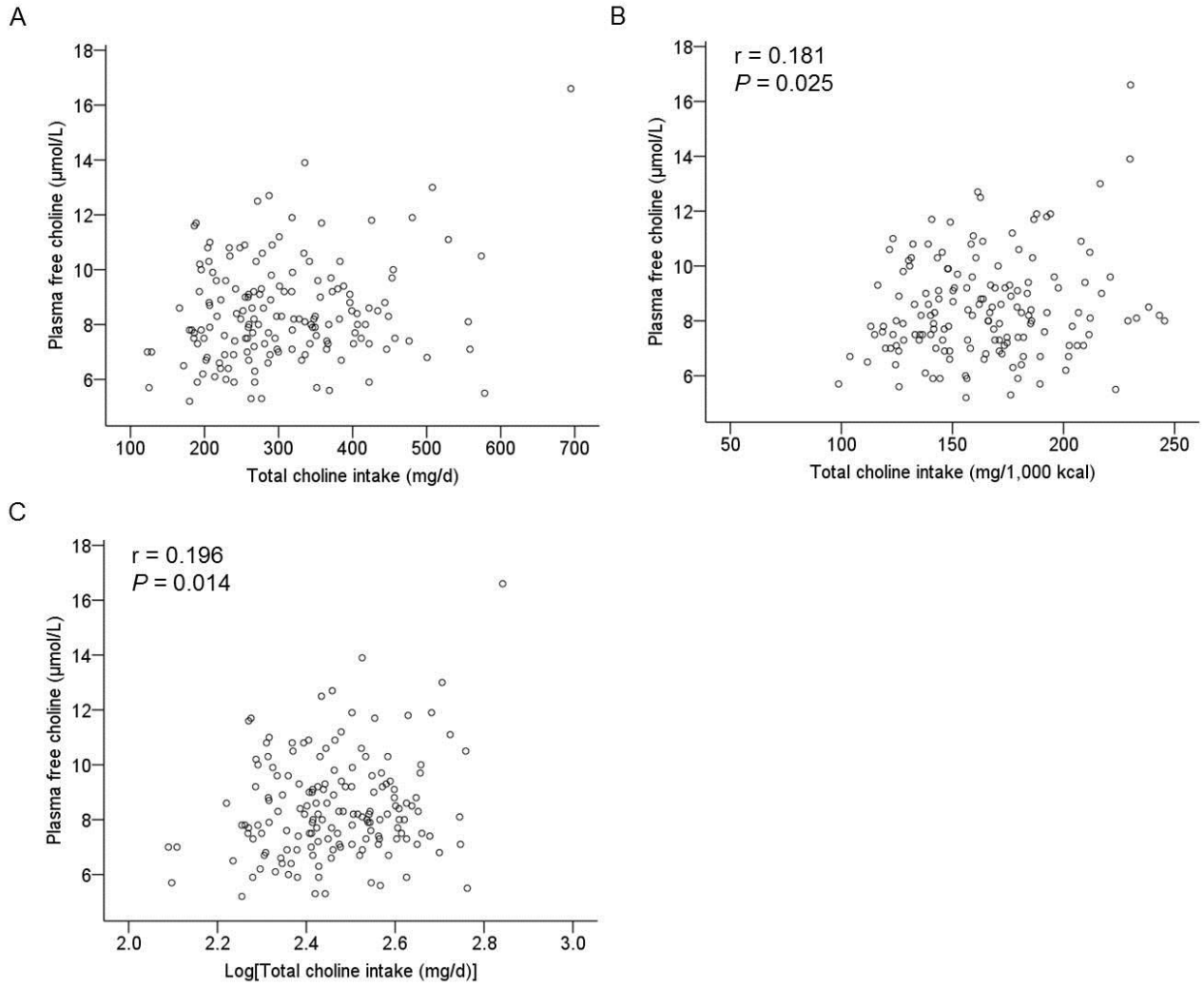

FIGURE 3.2 Scatter plots to show the relationships between dietary choline intake and plasma free choline among children 5 – 6 y of age. Choline intake in mg/d and in mg/1,000 kcal; Panels A and B show the plots of untransformed data between plasma choline and choline intake and between plasma choline and choline intake adjusted for energy, respectively. Panel C shows the correlation using log-transformed data (*n* = 163 for all data).

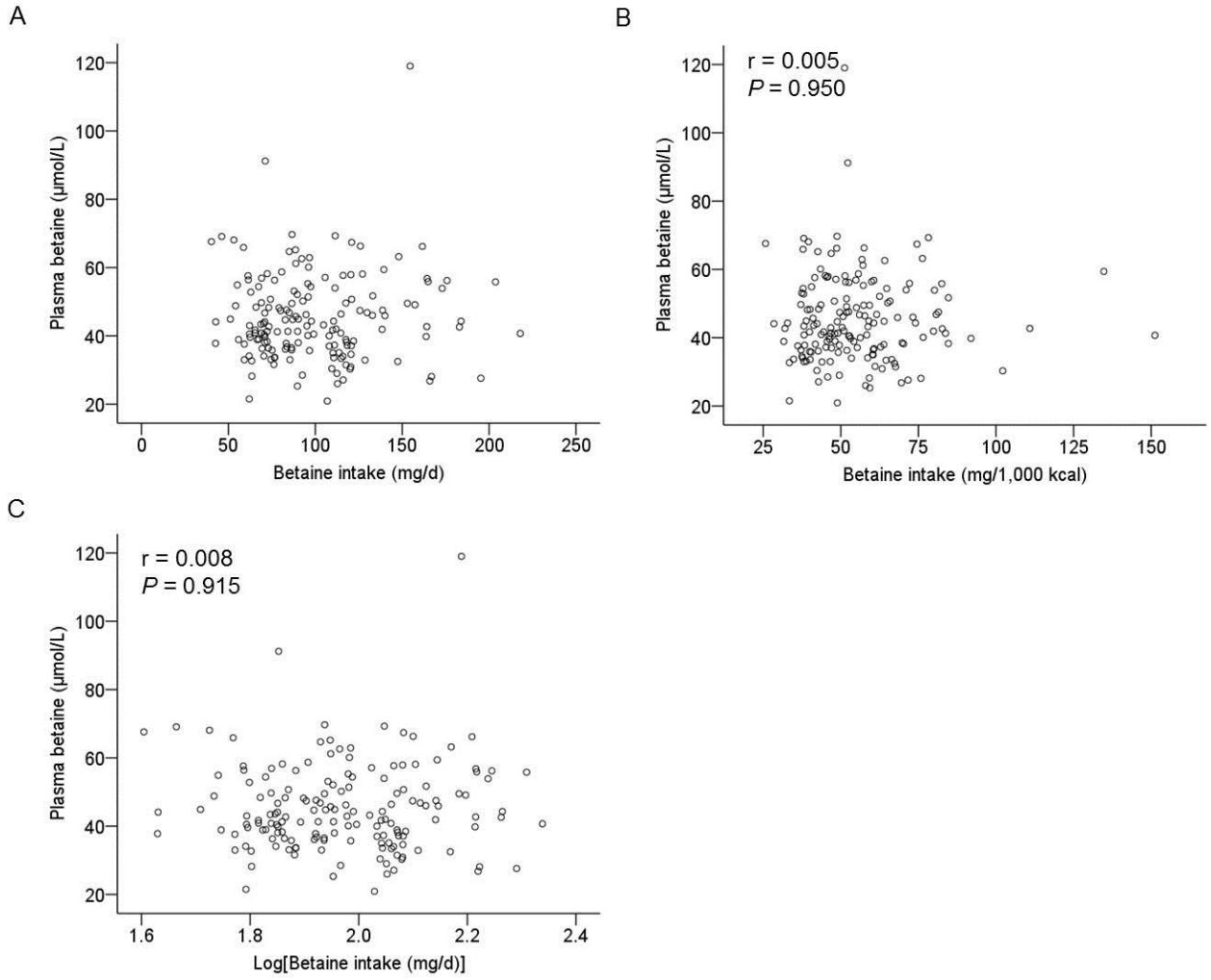

FIGURE 3.3 Scatter plots to show the relationships between dietary betaine intake and plasma betaine among children 5 – 6 y of age. Betaine intake in mg/d and in mg/1,000 kcal; Panels A and B show the plot of untransformed data between plasma betaine and betaine intake and between plasma betaine and betaine intake adjusted for energy, respectively. Panel C shows the correlation using log-transformed data (*n* = 163 for all data).

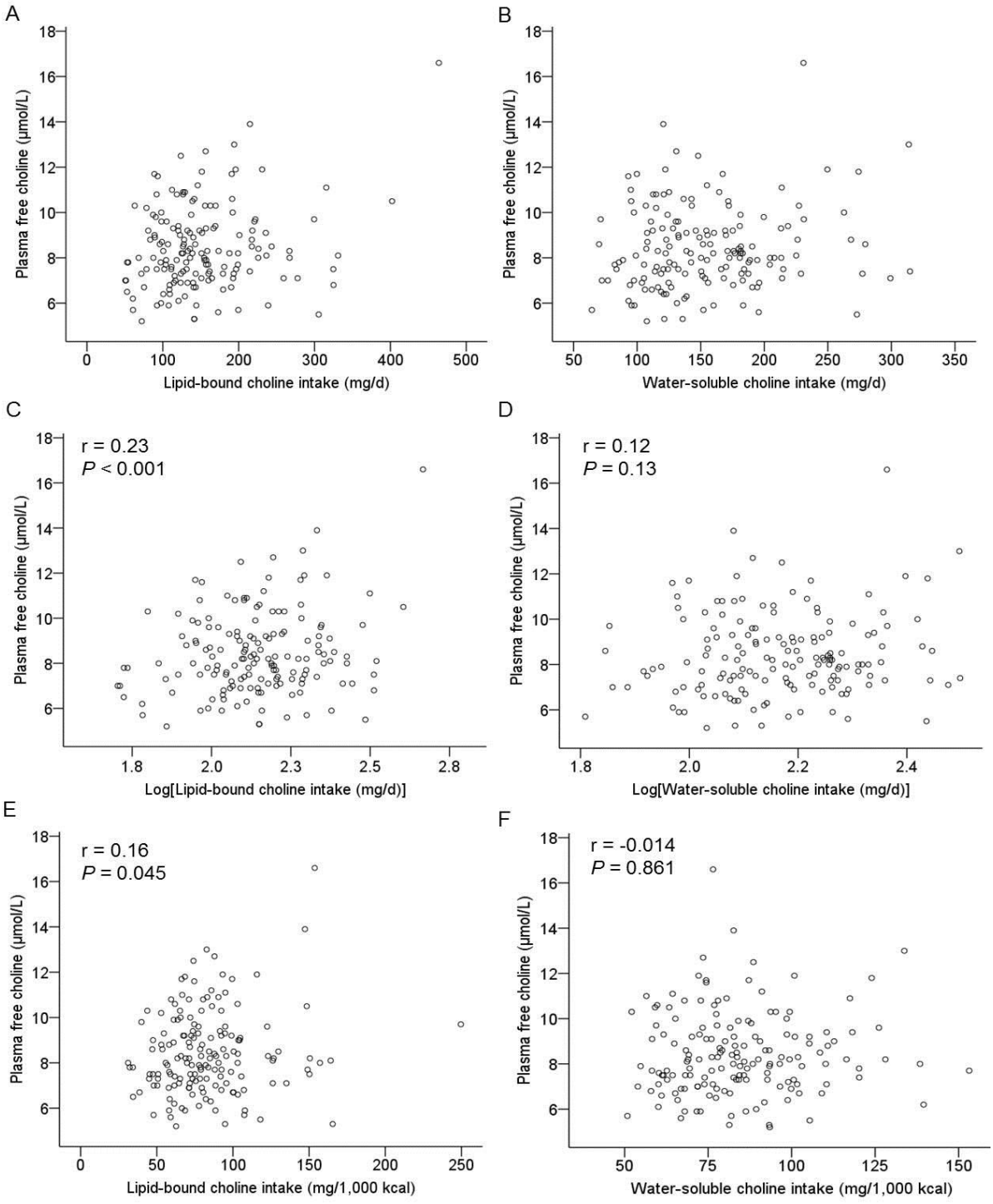

FIGURE 3.4 Scatter plots to show the relationships between estimated intakes of water-soluble choline and lipid-bound choline among children 5 – 6 y of age.

Panels A and B show the plots of untransformed data between plasma free choline and

lipid-bound choline intake, and between plasma free choline and water-soluble choline intake, respectively. Panels C and D show the correlations using log-transformed data. Panels E and F show the correlations using intake data adjusted for caloric intake (*n* = 163 for all data).

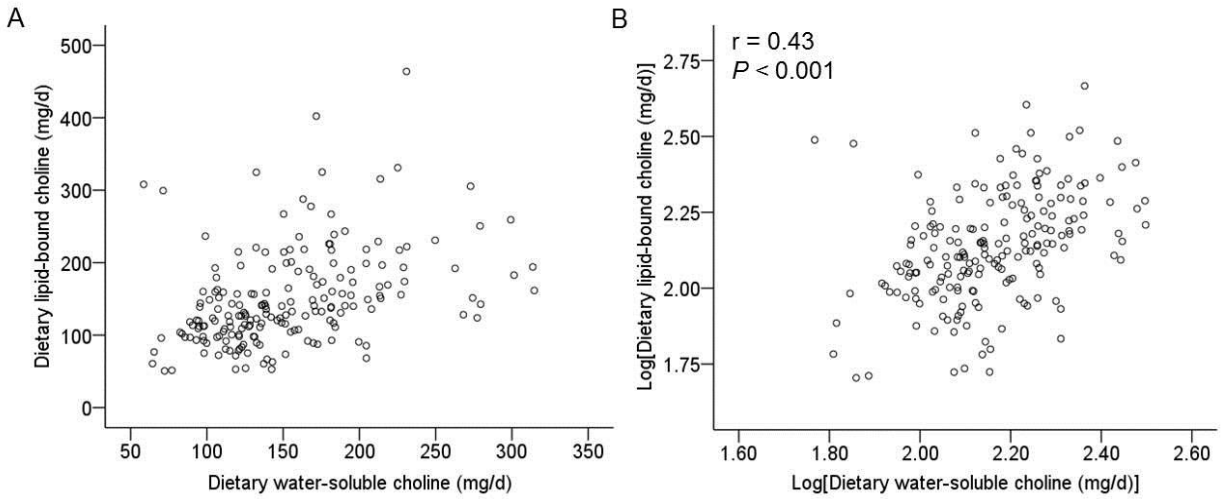

FIGURE 3.5 Scatter plots to show the relationships between estimated intakes of water-soluble choline and lipid-bound choline among children 5 – 6 y of age.

Panel A shows the plot of untransformed data between estimated lipid-bound choline intake and estimated water-soluble choline intake. Panel B shows the correlation using log-transformed data (*n* = 193 for all data).

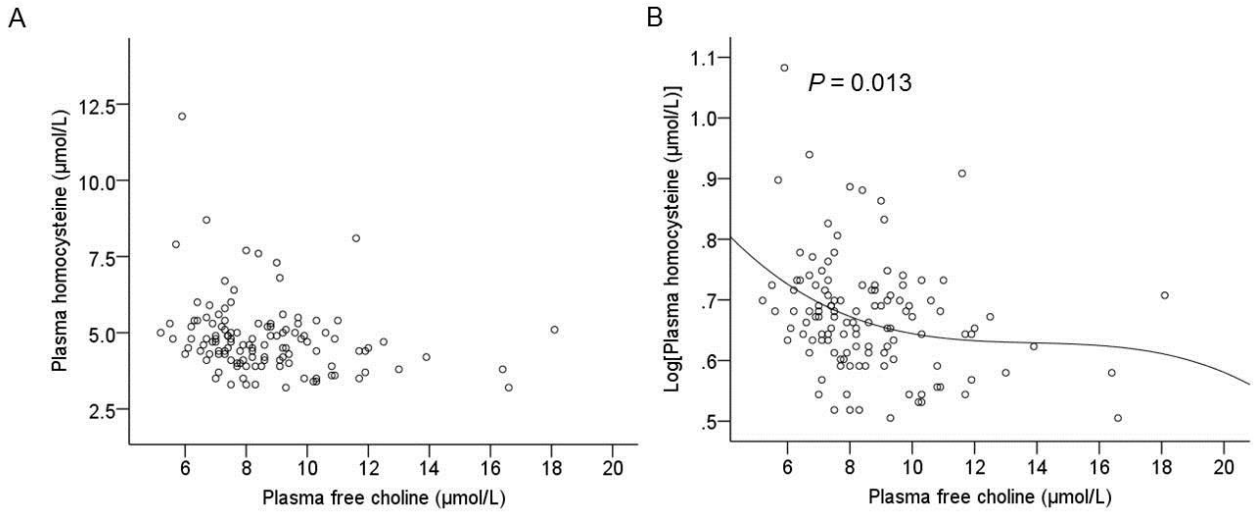

FIGURE 3.6 Scatter plots to show the relationship between plasma homocysteine and plasma free choline among children 5 – 6 y of age. Panel A shows the plot of untransformed data between plasma homocysteine and plasma free choline. Panel B shows the significance of cubic relationship using curvilinear estimation (*n* = 120 for all data).

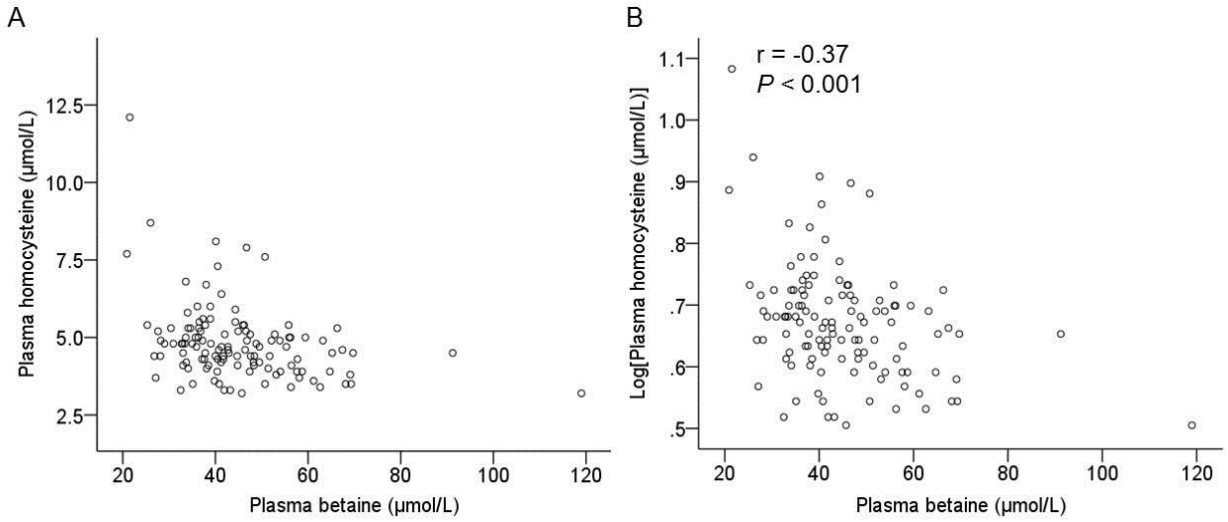

Figure 3.7 Scatter plots to show the relationship between plasma homocysteine and plasma betaine among children 5 – 6 y of age. Panel A shows the plot of untransformed data between plasma homocysteine and plasma betaine. Panel B shows the correlation using log-transformed data (*n* = 120 for all data).

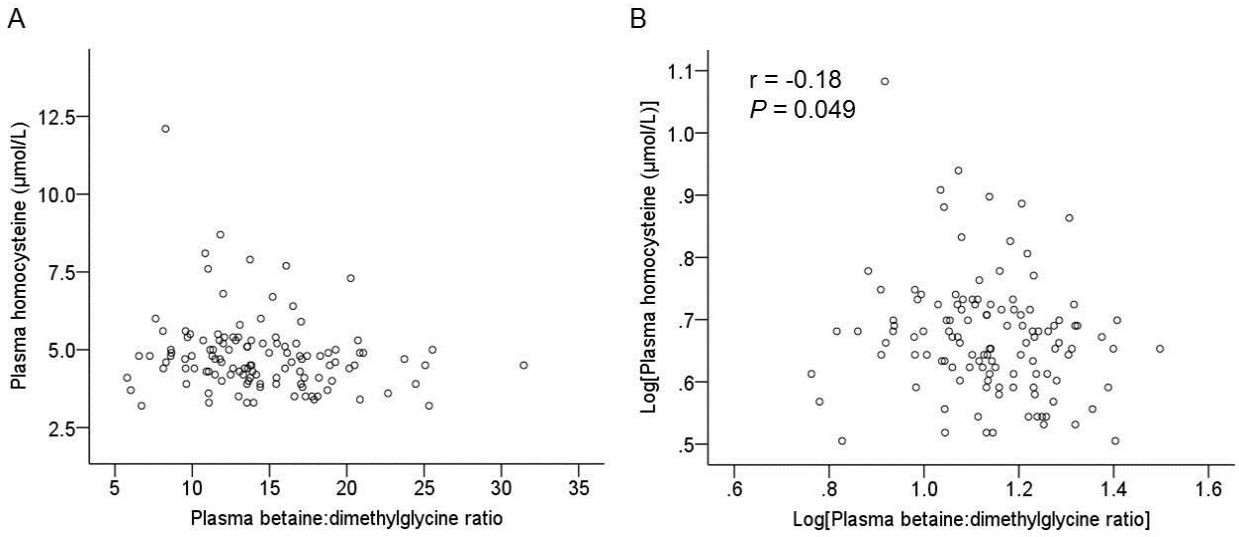

Figure 3.8 Scatter plots to show the relationship between plasma homocysteine and plasma betaine:dimethylglycine ratio. Panel A shows the plot of untransformed data between plasma homocysteine and plasma betaine:dimethylglycine ratio. Panel B shows the correlation using log-transformed data (*n* = 120 for all data).

CHAPTER 4: DISCUSSION, LIMITATIONS, AND FUTURE DIRECTION

4.1. Discussion

The objectives of this thesis were 1) to determine choline status and its relationship to the estimated intake of choline from the diet in children 5 – 6 years of age; 2) to use plasma homocysteine as a biomarker to determine whether children are at risk of choline insufficiency; 3) to explore the relationship between plasma free choline and its metabolites and cognitive development in children 5 – 6 years of age.

4.1.1. Choline status and its relationship to intake of choline from the diet

4.1.1.1 Dietary intakes of choline among children 5- 6 years of age

Our current study reveals that about one-third of the children in our study population consumed below the current recommended intake of 250 mg/d of choline, although the group mean and median choline intake were above the current recommendation. The results of this study are consistent with a previous abstract which examined choline intake using data from NHANES survey 2003 – 2004 among 7581 people including adults and children, with the mean choline intake of young children in the U.S. reported above the current AI (Jensen et al., 2007). The abstract did not report specific data, such as the mean and the range of choline intake in children.

In terms of the dietary sources of total choline, the highest contribution to the total choline intake of children in the present study was from dairy products (29%),

followed by meat and seafood (21%), and then by vegetables and fruits (18%). As noted above, data on the dietary choline intake among children, including food sources, are very limited. However, when compared to data on the contribution of different foods to the total intakes of choline in adults, as results indicate that the major dietary source of choline may differ. In adults, data gathered in the Framingham Offspring study in the U.S. analyzed for choline found that about 32% of the total choline intake came from meat and seafood, while dairy products contributed only about 9% of the total choline intake (Cho et al., 2006). The latter study also reported that half of the total choline intake came from lipid-bound choline and the other half came from water-soluble choline, a finding that is consistent with the present results for children. Dietary betaine intake among the children in the present study was primarily derived from grain products (85%), which is similar to that reported for American adults (Cho et al., 2006). The latter study by Cho et al. in the U.S. adults also reported a lack of association between dietary intakes of lipid-bound choline and water-soluble choline, whereas these two forms of choline were significantly associated in the children's diet in the present study.

4.1.1.2 Plasma free choline and related metabolites in children 5 – 6 yr of age

Plasma free choline of the children in this study (Mean ± SD: 8.57 ± 2.08 µmol/L) is similar to those reported in the study done on children of the same age group (5 yr of age) in the Republic of Seychelles (Mean ± SD: 9.17 ± 2.09 µmol/L) (Strain et al., 2013), but significantly lower than children from a study in Turkey (Mean ± SD: 12.9 ± 2.75

µmol/L) (Ilcol et al., 2005). The lower plasma choline in the children in our study and that of Strain et al (2013) could be due to factors such as difference in dietary pattern and ethnic background, or analytical methods. It should be noted that the sample size in the study by Ilcol study was only *n* = 21, which is considerably smaller than our study and the study conducted in the Republic of Seychelles (Strain et al., 2013) in which the number of children was 210.

Although no children met the criteria for B12 or folate deficiency, or elevated plasma homocysteine in the present study, it is important to note that the cut-off values used are for adults. Currently, there are no established deficiency cut-offs for children. It is known that plasma choline and B12 tend to decline with increasing age (Ilcol et al., 2005; Macfarlane et al., 2011). On the other hand, plasma homocysteine tends to increase over the lifespan, with the plasma homocysteine in children being significantly lower than in adults (Macfarlane et al., 2011). Therefore, it is possible that homocysteine cut-offs could be lower, and the B12 deficiency cut off might be higher in children than in adults.

4.1.1.3 The extent to which dietary choline consumption contributes to variability in plasma free choline in children

The analysis in this study indicated a significant positive association between total dietary choline intake and plasma free choline in the children. No other studies of similar nature have been published in this area in young children. Noteworthy, we found that intake of lipid-bound choline shared a much stronger relationship with plasma free

choline (r = 0.22, *P* < 0.001) than dietary water-soluble choline did (r = 0.12, *P* = 0.13). To cite a specific case from our study, one of the children had a very high dietary choline intake (~800 mg/d) and this child also had a high consumption of eggs (3 eggs/day) which are high in PC (105 mg PC/standard egg). This child also had the highest plasma free choline (18 µmol/L) of any child in the study. This is consistent with an earlier study on Norwegian elderly men and women that showed that egg intake was the strongest predictor of plasma free choline (Konstantinova et al., 2008).

More evidence for an effect of dietary lipid-bound choline on plasma free choline is apparent in recent studies involving choline supplementation. Supplementation with 5.4 g/d PC (equivalent to 750 mg/d total choline) in pregnant women for 6 weeks, North Carolina resulted in a significant increase in plasma free choline in the supplemented women compared to the placebo group (Fischer et al., 2010). Interestingly, when supplemented with a water-soluble form of choline (550 mg/d) with a mixed diet providing both water-soluble (142 mg/d) and lipid-bound (236 mg/d), women in the supplemental group did not show higher plasma free choline than women in the control group (Yan et al., 2012). These studies suggest that the specific form of dietary choline may have different effects on plasma free choline in adults and possibly children.

We found a modest positive association between plasma free choline and total choline intake (r^2 = 0.04). A higher choline intake than observed in the present study, particularly the lipid-bound choline, could potentially result in a stronger association between dietary choline intake and plasma free choline. The presence of a small positive association between plasma free choline and dietary intake of choline may be in part due to inaccuracy and incomplete data on food sources of choline in the current

dietary database on choline from USDA. Since plasma free choline represents a small fraction of the total choline pool in plasma, it is also possible that plasma free choline might not be the ideal biomarker reflecting dietary choline consumption.

Considering factors that might lead to dietary choline intake accounting for small variability in plasma free choline, it is plausible that the strength of the relationship is partially hindered by using single time-point blood sample for analysis. Specifically, the intra-individual variability in plasma free choline might affect the strength of the relationship between dietary intake and choline. From unpublished data for 40 men and women from our lab, we found that the mean intra-individual day-to-day c.v was 14%, with a range of 1% to 31%. There was a significant difference between plasma free choline measured in blood collected at 2 different time points, especially fast versus fed on the same day using student's *t*-test (t_1 = 7.1 ± 2.1 μmol/L, t_2 = 8.3 ± 2.4 μmol/L, P < 0.001). In addition, functional single nucleotide polymorphisms (SNPs) of genes encoding for enzymes pertinent to choline metabolism might contribute to variability in plasma free choline. Previous report showed that *CHDH* variants rs9001 and rs12676, and *BHMT* variant rs3733890 increased susceptibility of fatty liver as a proxy of choline deficiency in humans (da Costa et al., 2006). However, the report did find statistical difference in any forms of choline or betaine in plasma between subjects with SNPs and those without.

4.1.2 *Evidence of choline "deficiency" as an inverse relation between plasma free choline and homocysteine*

Using homocysteine as a potential biomarker for finding choline deficiency, we found a significant inverse relationship between plasma homocysteine and plasma free choline ($P = 0.009$). However, this relationship was driven by 6 subjects with a plasma free choline below ($n = 3$) or above ($n = 3$) the 5th and 95th percentile values, respectively. No evidence for relationship was found when the analyses only were done using data for values falling between 5th – 95th percentiles for plasma free choline. Thus, it is possible that 3/120 of the children had insufficient choline. The dietary total choline intakes of these children were 125.04 mg/d, 267.79 mg/d and 201.95 mg/d, respectively.

Compared to plasma free choline, plasma betaine showed an even more pronounced inverse relationship with homocysteine ($r = -0.37$, $P < 0.001$). In addition, a moderate inverse relationship was also found between betaine:dimethylglycine ratio and homocysteine ($r = -0.18$, $P = 0.049$). In adults a folate-fortified population, plasma homocysteine is not associated with plasma betaine or betaine:dimethylglycine ratio (Wu et al., 2013). Based on the evidence presented in the current study, it is likely that BHMT-dependent remethylation of homocysteine is more active in children than in adults.

The only other study which has reported the relationship between plasma free choline and homocysteine in children found a significant positive relationship between plasma free choline and homocysteine ($n = 210$, $r = 0.19$, $P = 0.006$) in the children in The Republic of Seychelles (Strain et al., 2012), which is the exact opposite of what we

reported. However, the scatter plot of the association was not given in the paper. The Republic of Seychelles does not have mandatory folate fortification in their food supply, thus it is possible the folate nutrition is an important mediator of choline needs and status. In a study done with adults in the Netherlands, another country without mandatory folate fortification their food supply, has also reported a similar moderate positive relationship between plasma free choline and homocysteine (n = 896, r = 0.14, P < 0.001) (Eussen et al., 2007). However, as noted, plasma free choline and homocysteine change with age. Therefore, caution is needed when using data generated in adults to interpret those for children. Nonetheless, the implementation of folate fortification of the food supply in Canada may have enhanced our capacity to remethylate homocysteine to methionine, thus decreasing needs for betaine. On the other hand, populations where folate fortification is not mandated may have an increased dependence on methyl groups from choline via betaine for homocysteine remethylation. Also, the choline-betaine pool alone might not be sufficient for maintaining a low homocysteine in the presence of insufficient dietary folate.

4.1.3 The relationship between plasma free choline and the cognitive development of children

Our results provided some evidence of a significant positive association between plasma betaine, but not free choline, and cognition in an unadjusted univariate analysis. There are several possibilities for this observation. Since plasma free choline has a normal range around 7 – 20 µmol/L and betaine has a range between 10 to 100 µmol/L,

the wider physiological range of betaine might allow a greater degree of variability to find an association. However, in a multivariate regression analysis, we found no association between plasma free choline, betaine, or dimethylglycine and child developmental outcome. Given the absence or limited number of children (3/120) with possible choline insufficiency, it is expected that a positive association would not be present between choline and cognition in our population. However, our sample size in examining the relationship between plasma free choline and cognition (n = 163) is also relatively small. An increase in study population might find a possible existing association. The results of this study contradict the Seychelles study which found a positive relationship between plasma betaine and cognitive ability among children 5 years of age, with a sample size of 210. The possible factors leading to such discrepancy include difference in ethnic background, socio-economic status, sample size, culture, different dietary pattern, maternal intelligence, lack of folate fortification in Seychelles, deficiency of other associated nutrients in the Seychelles. Nonetheless, it is still unclear whether betaine or choline could facilitate post-natal neural development.

4.2 Limitations

1. We have poor representation of low-income families in this study. This can be due to the cost and time needed to travel to the Children's Hospital on top of their busy schedule.
2. Situated along the coastline, the dietary patterns among British Columbians could be different from Canadians from other provinces or in the interior B.C. Therefore, the results of this study regarding dietary sources intake of choline might not be extrapolated to the entire Canadian population.
3. We were not able to collect blood samples from all the children (n = 37). The need for blood samples could be daunting for both the parent and child.
4. Possible errors and limitations stemming from the current database on the choline content of foods might affect the accuracy in our estimation of the dietary choline intake among the children in this study. On top of the possible limitations of the data on the choline content of the foods and beverages, the limited number of food items analyzed and reported in the current database required us to make substitutions, extrapolations and assumptions about some "ethnic" foods, and to use recipes to estimate choline content of some mixed foods and meals. It should also be noted that the current database for the choline content of foods is based on the American food supply. It is uncertain how different it would be compared to the choline content of the foods from the Canadian food supply.

5. The inherent inaccuracies in dietary methodologies and recall bias might also lead to errors in estimating dietary choline intake in the children.

6. This study utilized a single-time point, non-fasting blood sample. The unpublished data from our group has shown a degree of day-to-day variability in fasting blood samples taken on different days (c.v. = 13%) with a range of 1% to 31% among 40 healthy adults. Such variability might have an impact in our research finding.

7. The sample size for (*n* = 163) may be too small to find a relationship between plasma choline metabolites and cognitive test scores. If such a relationship were to exist, a larger study population might permit us to find an association between these two variables.

4.3 Future direction

1. Further studies will be needed to enrich the data regarding the distribution of dietary intake of choline in young children in other demographics.

2. There is a need to develop a better and more complete database for the choline content of foods. In particular, having a database on choline content in foods in Canadian food supply will immensely benefit and enable any future research related to dietary choline and related needs in Canada.

3. Address whether the relationship between dietary choline and plasma free choline can be improved using blood analysis of multiple time points instead of a single-time point blood sample.

4. Future studies should be directed to investigate whether consumption of different forms of choline, including the lipid-bound forms and the water-soluble forms would have any difference in different impact on plasma free choline. A supplementation study addressing potential effects of different forms of choline supplements (lipid-bound form versus water-soluble form) on plasma free choline may be useful.

5. Future efforts should also focus on how SNPs of genes encoding for key enzymes pertinent to choline metabolism and increased susceptibility to choline deficiency in human, such as CHDH rs9001 and rs12676, and BHMT rs 3733890, could contribute to variability in plasma free choline concentration.

6. Future research is needed to investigate whether betaine is essential, perhaps leading to establishing a dietary recommendation for betaine.

7. Future studies should investigate the relationship between plasma free choline, or related metabolites, and developmental outcomes in children with a larger sample size.

REFERENCES

Albright CD, Tsai AY, Friedrich CB, Mar MH, Zeisel SH. Choline availability alters embryonic development of the hippocampus and septum in the rat. Brain Res. 1999;113:13–20.

Apparsundaram S, Ferguson SM, George AL Jr, Blakely RD. Molecular cloning of a human, hemicholinium-3-sensitive choline transporter. Biochem Biophys Res Commun. 2000. 276:862–867.

Beery KE, Buktenica NA, Beery NA. Beery-Buktenica Developmental Test of Visual-Motor Integration, 6th Edition (BEERY™ VMI). San Antonio, TX: Pearson Education, 2010.

Best CH, Huntsman ME. Effects of choline on liver fat of rats in various states of nutrition. J Physiol. 1932;75:405–412.

Best CH, Huntsman ME. The effects of the components of lecithine upon the deposition of fat in the liver. J Physiol. 1932;75:405-412.

Blusztajn JK, Holbrook PG, Lakher M, Liscovitch M, Maire JC, Mauron C, Richardson UI, Tacconi M, Wurtman RJ. "Autocannibalism" of membrane choline-phospholipids: physiology and pathology. Psychopharmacol Bull. 1986;22:781–786.

Blusztajn JK, Liscovitch M, Mauron C, Richardson UI, Wurtman RJ. Phosphatidylcholine as a precursor of choline for acetylcholine synthesis. J Neural Transm Suppl. 1987;24:247–259.

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;84:5474–5477.

Blusztajn JK, Zeisel SH, Wurtman RJ. Synthesis of lecithin (phosphatidylcholine) from phosphatidylethanolamine in bovine brain. Brain Res. 1979;179:319–327.

Bocckino SB, Blackmore PF, Wilson PB, Exton JH. Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. J Biol Chem. 1987;262:15309–15315.

Boeke CE, Gillman MW, Hughes MD, Rifas-Shiman SL, Villamor E, Oken E. Cholineintake during pregnancy and childcognition at age7years. Am J Epidemiol. 2013;177:1338–1347.

Bremer J, Breenberg D. Methyl transferring enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholine). Biochim Biophys Acta. 1961;46:205–216.

Brown L, Sherbenou RJ, Johnsen SK. Test of Nonverbal Intelligence, Third Edition (TONI-3). Austin TX: Pro Ed, 1997.

Budowski PI. Kafri I, Sklan D. Utilization of choline from crude soybean lecithin by chicks. 2. Absorption measurements. Poultry Sci. 1977;56:754–757.

Butz LW, du Vigneaud V. The formation of a homologue of cysteine by the decomposition of methionine with sulfuric acid. J Biol Chem. 1932;99:135–142.

Cantoni GL. Biological methylation: selected aspects. Annu Rev Biochem. 1975;44:435–451.

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;96:1465–1472.

Chern MK, Gage DA, Pietruszko R, Betaine aldehyde, betaine, and choline levels in rat livers during ethanol metabolism. Biochem Pharmacol. 2000;60:629–637.

Chi-Shui L, Ru-Dan W. Choline oxidation and choline dehydrogenase. J Prot Chem. 1986;5:193–200.

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;83:905–911.

Craciunescu CN, Albright CD, Mar MH, Song J, Zeisel SH. Choline availability during embryonic development alters progenitor cell mitosis in developing mouse hippocampus. J Nutr. 2003;133:3614–3618.

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;20:1336-44.

de Hass GH, van Oort MG, Dijkman R, Verger R. Phospholipase A2 inhibitors: monoacyl, monoacylamino- glycero-phosphocholines. Biochem Soc Trans. 1989;17:274–276.

DeCarli C, Fletcher E, Ramey V, Harvey D, Jagust WJ. Anatomical mapping of white matter hyperintensities. Stroke. 2005;36:50–55.

DeCarli C, Murphy DG, Tranh M, Grady CL, Haxbym JV, Gillette JA, Salerno JA, Gonzales-Aviles A, Horwitz B, Rapoport SI. The effect of white matter hyperintensity volume on brain structure, cognitive performance, and cerebral metabolism of glucose in 51 healthy adults. Neurology. 1995;45:2077–2084

DeLong CJ, Shen YJ, Thomas MJ, Cui Z. Molecular distinction of phosphatidylcholine synthesis between the CDPcholine pathway and phosphatidylethanolamine methylation pathway. J Biol Chem. 1999;274:29683–29688.

Demopoulos CA, Pinckard RN, Hanahan DJ. Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). J Biol Chem. 1979;254:9355–9358.

Diamond I. Choline metabolism in brain. The role of choline transport and the effects of phenobarbital. Arch Neurol. 1971;24:333–339.

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;137:2224–2228.

du Vigneaud V, Chandler JP, Moyer AW, Keppel, DM. The effect of choline on the ability of homocystine to replace methionine in the diet. J Biol Chem. 1939;131,57–76.

du Vigneaud V, Cohn M, Chandler JP, Schenck JR, Simmonds S. The utilization of the methyl group of methionine in the biological synthesis of choline and creatine. J Biol Chem. 1941;140:625-–641.

Dunn LM, Dunn DM. Peabody Picture Vocabulary Test, Fourth Edition. San Antonio, TX: Pearson Education, 2007.

Eussen SJ, Ueland PM, Clarke R, Blom HJ, Hoefnagels WH, van Staveren WA, de Groot LC. The association of betaine, homocysteine and related metabolites with cognitive function in Dutch elderly people. Br J Nutr. 2007;98:960–968.

FAO/WHO/UNA (Food and Agriculture Organization of the United Nations/World Health Organization/United Nations). 1985. Energy and Protein Requirements Reports of a joint FAO/ WHO/UNA Expert Consultation. Technical Report Series. No. 724. Geneva: World Health Organization.

Ferger MF, du Vigneaud V. Oxidation in vivo of the methyl groups of choline, betaine, dimethylthetin, and dimethyl-beta-propiothetin. J Biol Chem. 1950;185:53–57.

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;92:1113–1119.

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;85:1275–1285.

Flower RJ, Pollitt RJ, Sanford PA, Smyth DH. Metabolism and transfer of choline in hamster small intestine. J Physiol. 1972;226:473–489.

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;137:2641–2646.

Gouw AA, Van der Flier WM, van Straaten EC, Barkhof F, Ferro JM, Baezner H, Pantoni L, Inzitari D, Erkinjuntti T, Wahlund LO, Waldemar G, Schmidt R, Fazekas F, Scheltens P; LADIS Study Group. Simple versus complex assessment of white matter hyperintensities in relation to physical performance and cognition: The LADIS study. J Neurol. 2006;253:1189–1196.

Hershey JM, Soskin S. Substitution of "lecithin" for raw pancreas in the diet of the depancreatized dog. Am J Physiol. 1931;98:74–85.

Holmes-McNary MQ, Loy R, Mar M-H, Albright CD, Zeisel SH. Apoptosis is induced by choline deficiency in fetal brain and in PC12 cells. Dev Brain Res. 1997;101:9–16.

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;16:489–499.

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;136:2226–2231.

Institute of Medicine, National Academy of Sciences. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B 6, folate, vitamin B 12, pantothenic acid, biotin, and choline. Washington, DC: The National Academies Press, 1998.

Jeerakathil T, Wolf PA, Beiser A, Massaro J, Seshadri S, D'Agostino RB, DeCarli C. Stroke risk profile predicts white matter hyperintensity volume: The Framingham Heart Study. Stroke. 2004;35:1857–1861.

Jensen HH, Batres-Marquez SP, Carriquiry A, Schalinske KL. Choline in the diets of the U.S. population: NHANES, 2003-2004. FASEB J. 2007;21:219.

Kaufman AS, Kaufman NL. Kaufman Assessment Battery for Children, Second Edition (KABC-II). San Antonio, TX: Pearson Education, 2004.

Kennedy EP, Weiss SB. The function of cytidine coenzymes in the biosynthesis of phospholipids. J Biol Chem. 1956;222,193–214.

Kettunen H, Peuranen S, Tiihonen K, Saarinen M. Intestinal uptake of betaine in vitro and the distribution of methyl groups from betaine, choline, and methionine in the body of broiler chicks. Comp Biochem Physiol A Mol Integr Physiol. 2001;128:269–278.

Kettunen H, Tiihonen K, Peuranen S, Saarinen MT, Remus JC. Dietary betaine accumulates in the liver and intestinal tissue and stabilizes the intestinal epithelial structure in healthy and coccidia-infected broiler chicks. Comp Biochem Physiol A Mol Integr Physiol. 2001;130:759–769.

Kewitz H, Pleul O. Synthesis of choline from ethanolamine in rat brain. Proc Natl Acad Sci U S A. 1976;73:2181–2185

Kim SK, Choi KH, Kim YC. Effect of acute betaine administration on hepatic metabolism of S-amino acids in rats and mice. Biochem Pharmacol. 2003;65:1565–1574.

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;88:1663–1669.

Kovacheva VP, Davison JM, Mellott TJ, Rogers AE, Yang S, O'Brien MJ, Blusztajn JK. Raising gestational choline intake alters gene expression in DMBA-evoked mammary tumors and prolongs survival. FASEB J. 2009;23:1054–1063.

Kuczler, FJ, Nahrwold DL, Rose RC. Choline influx across the brush border of guinea pig jejunum. Biochim. Biophys. Acta. 1977;465:131–137.

Li Z, Vance D. Phosphatidylcholine and choline homeostasis. J Lipid Res. 2008;49:1187–1194.

Luka Z, Mudd SH, Wagner C. Glycine N-methyltransferase and regulation of S-adenosylmethionine levels. J Biol Chem. 2009;284:22507–22511

MacFarlane AJ, Greene-Finestone LS, Shi Y. Vitamin B-12 and homocysteine status in a folate-replete population: results from the Canadian Health Measures Survey. Am J Clin Nutr. 2011;94:1079–1087.

Macfarlane MG, Patterson LM, Robison R, The phosphatase activity of animal tissues. Biochem J. 1934;28:720–724.

Mackenzie CG, du Vigneaud V. Effect of choline and cysteine on the oxidation of the methyl group of methionine. J Biol Chem. 1952;195:487–491.

Meck WH, Williams CL. Choline supplementation during prenatal development reduces proactive interference in spatial memory. Brain Res Dev Brain Res. 1999;118:51–59.

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;2:385–399.

Mehedint MG, Craciunescu CN, Zeisel SH. Maternal dietary choline deficiency alters angiogenesis in fetal mouse hippocampus. Proc Natl Acad Sci U S A. 2010;107:12834–12839.

Mellott TJ, Williams CL, Meck WH, Blusztajn JK. Prenatal choline supplementation advances hippocampal development and enhances MAPK and and CREB activation. FASEB J. 2004;18:545–547.

Michel V, Yuan Z, Ramsubir S, Bakovic M. Choline transport for phospholipid synthesis. Exp Biol Med (Maywood). 2006;231:490–504.

Miller JW, Garrod MG, Rockwood AL, Kushnir MM, Allen LH, Haan MN, Green R. Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. Clin Chem. 2006;52:278–285.

Mudd SH, Cantoni GL. Activation of methionine for transmethylation. III. The methionine-activating enzyme of Bakers' yeast. J Biol Chem. 1958;231:481–492.

Niculescu M, Zeisel. Diet, methyl donors and DNA methylation: Interactions between dietary folate, methionine and choline. J Nutr. 2002;132:2333S–2335S.

Niculescu MD, Craciunescu CN, Zeisel SH. Dietary choline deficiency altersglobal and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. FASEB J. 2006;20:43–49.

Nurk E, Refsum H, Bjelland I, Drevon CA, Tell GS, Ueland PM, Vollset SE, Engedal K, Nygaard HA, David Smith A. Plasma free choline, betaine and cognitive performance: the Hordaland Health Study. Br J Nutr. 2012 [Epublication May 1, 2012].

O'Donoghue N, Sweeney T, Donagh R, Clarke KJ, Porter RK. Control of choline oxidation in rat kidney mitochondria. Biochim Biophys Acta. 2009;1787:1135–1139.

Okuda T, Haga T, Kanai Y, Endou H, Ishihara T, Katsura I. Identification and characterization of the high-affinity choline transporter. Nat Neurosci. 2000;3:120–125.

Okuda T, Haga T. Functional characterization of the human high affinity choline transporter. FEBS Lett. 2000;484:92–97.

Park EI, Garrow TA. Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. J Biol Chem. 1999. 274:7816–7824

Parthasarathy S, Subbaiah PV, Ganguly J. The mechanism of intestinal absorption of phosphatidylcholine in rats. Biochem J. 1974;140:503–508.

Pelech SL, Vance DE. Regulation of phosphatidylcholine biosynthesis. Biochim Biophys Acta. 1984;779:217–251.

Peters-Regehr T, Bode JG, Kubitz R, Häussinger D. Organic osmolyte transport in quiescent and activated rat hepatic stellate cells (Ito cells). Hepatology. 1999;29:173–180.

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;94:1584–1591.

Porter RK, Scott JM, Brand MD, Characterization of betaine efflux from rat liver mitochondria. Biochim. Biophys. Acta. 1993;1141:269–274.

Reo NV, Adinehzadeh M, Foy BD. Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine biosynthesis using (13)C NMR spectroscopy. Biochim Biophys Acta. 2002;1580:171–188.

Ridgway ND, Vance DE. Kinetic mechanism of phosphatidylethanolamine N-methyltransferase. J Biol Chem. 1988;263:16856–16863.

Sanford PA, Smyth DH. Intestinal transfer of choline in rat and hamster. J Physiol. 1971;215:769–788.

Sauter M, Moffatt B, Saechao MC, Hell R, Wirtz M. Methionine salvage and S-adenosylmethionine: essential links between sulfur, ethylene and polyamine biosynthesis. Biochem J. 2013;451:145–154.

Schenk F, Brandner C. Indirect effects of peri- and postnatal choline treatment on place-learning abilities in rat. Psychobiology.1995;23:302–313.

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;87:896–902.

Strain JJ, McSorley EM, van Wijngaarden E, Kobrosly RW, Bonham MP, Mulhern MS, McAfee AJ, Davidson PW, Shamlaye CF, Henderson J, Watson GE, Thurston SW, Wallace JM, Ueland PM, Myers GJ. Choline status and neurodevelopmental outcomes at 5 years of age in the Seychelles Child Development Nutrition Study. Br J Nutr. 2013;110:330–336.

Sunden SL, Renduchintala MS, Park EI, Miklasz SD, Garrow TA. Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. Arch Biochem Biophys. 1997;345:171–174.

Sundler R, Akesson B. Regulation of phospholipid biosynthesis in isolated rat hepatocytes. Effect of different substrates. J Biol Chem. 1975;250:3359–3367.

Tees RC, Mohammadi E. The effects of neonatal choline dietary supplementation on adult spatial and configural learning and memory in rats. Dev Psychobiol. 1999;35:226–240.

Teng YW, Mehedint MG, Garrow TA, Zeisel SH. Deletion of betaine-homocysteine S-methyltransferase in mice perturbs choline and 1-carbon metabolism, resulting in fatty liver and hepatocellular carcinomas. J Biol Chem. 2011;286:36258–36267.

Thomas JD, Garrison M, O'Neill TM. Perinatal choline supplementation attenuates behavioral alterations associated with neonatal alcohol exposure in rats. Neurotoxicol. Teratol. 2004;26:35–45.

Ueland PM. Choline and betaine in health and disease. J Inherit Metab Dis. 2011;34:3–15.

US Department of Agriculture, Database for the Choline Content of Common Foods, Release Two. January 2008. Internet: http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/Choline/Choln02.pdf (accessed 24 April 2013).

van der Flier WM, van Straaten EC, Barkhof F, Ferro JM, Pantoni L, Basile AM, Inzitari D, Erkinjuntti T, Wahlund LO, Rostrup E, Schmidt R, Fazekas F, Scheltens P; LADIS study group. Medial temporal lobe atrophy and white matter hyperintensities are associated with mild cognitive deficits in non-disabled elderly people: the LADIS study. J Neurol Neurosurg Psychiatry. 2005;76:1497–1500.

Vance DE, and Ridgway ND. The methylation of phosphatidylethanolamine. Prog Lipid Res. 1988;27:61–79.

Villamor E, Rifas-Shiman SL, Gillman MW, Oken E. Maternalintake of methyl-donornutrients and childcognition at 3 years of age. Paediatr Perinat Epidemiol. 2012;26:328–335.

Weik C, Warskulat U, Bode J, Peters-Regehr T, Häussinger D. Compatible organic osmolytes in rat liver sinusoidal endothelial cells. Hepatology. 1998;27:569–575.

Wettstein M, Weik C, Holneicher C, Häussinger D. Betaine as an osmolyte in rat liver: metabolism and cell-to-cell interactions. Hepatology. 1998;27:787–793.

Wu BTF, 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;98:1209–1217.

Wu BTF, Dyer RA, King DJ, Richard 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:e43448.

Wu G, Fang Y-Z, Yang S, Lupton J, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134:489–492

Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Devapatla S, Pressman E, Vermeylen F, Stabler SP, Allen RH, Caudill MA. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am J Clin Nutr. 2012;95:1060–1071

Yen CL, Mar MH, Meeker RB, Fernandes A, Zeisel SH. Choline deficiency induces apoptosis in primary cultures of fetal neurons. FASEB J. 2001;15:1704–1710.

Zeisel SH, Blusztajn JK. Choline and human nutrition. Ann Rev Nutr. 1994;14:269–296.

Zeisel SH, da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, Beiser A. Choline, an essential nutrient for humans. FASEB J. 1991;5:2093–2098.

Zhu X, Song J, Mar MH, Edwards LJ, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (*PEMT*) knockout mice have hepatic steatosis and abnormal hepatic choline metabolite concentrations despite ingesting a recommended dietary intake of choline. Biochem J. 2003;370:987–993.