Examining the essentiality of maternal choline intake during early infant development

by

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Abstract

Choline is an essential micronutrient with increased requirements during pregnancy and lactation due to a high demand by the developing fetus and infant. Animal studies have demonstrated that choline is essential for brain development, and as a component of all cell membranes it is hypothesized that choline is also required during the postnatal period for the rapidly developing immune system. Choline is not routinely consumed in supplements; therefore, food is the primary exogenous source. Despite the reliance on dietary sources, there is little research on the amount and forms of choline in the maternal diet, and the role of maternal choline intake on infant health outcomes. Therefore, the objective of this thesis was to describe pre- and early postnatal maternal choline intake and examine the consequences of consuming low dietary choline, and the differential effects of form, on offspring immune development.

A comprehensive choline database was created to estimate dietary choline, and included the choline composition of meat and pulses commercially available in Alberta. Interview and online 24-h dietary intake recalls were collected at each trimester of pregnancy and 3 months postpartum from women in the Alberta Pregnancy Outcomes and Nutrition (APrON) study using two different methods. It was found that only 23% and 10% of women met recommendations for choline during pregnancy and 3-months postpartum, respectively. Phosphatidylcholine (PC) and free choline were the forms of choline consumed in the highest proportions. Only energy-adjusted estimates of total choline had strong correlation and good agreement between the 24-h recall methods, and were comparable between methods when categorizing low choline intake.

In a parallel set of animal experiments, we sought to understand the consequences of not consuming sufficient amounts of choline during lactation and the effect of meeting choline

recommendations through different forms on offspring immune development. Lactating rats were fed diets containing varying forms and amounts of choline and the offspring immune development investigated by identifying changes in the types of immune cells and response to *ex vivo* immune challenges. A maternal diet devoid of choline during the suckling period resulted in lower body weight, lymphopenia and lower percentage of major T cell phenotypes (CD3+, CD4+, CD8+) (all $P<0.05$), compared to maternal diet sufficient in choline (1g choline as free choline/kg diet). Choline-devoid offspring had lower production of cytokines IL-1β and IFN-γ following *ex vivo* mitogen stimulation. The long-term consequences of a lower supply of choline during this critical period of development resulted in a lower expansion of the CD4+ population and a lower ability to produce IL-6 in offspring at 10 weeks of age, even after choline was fed in the weaning diet ($P<0.05$).

There was no difference in offspring growth when dams were fed choline as PC compared to free choline. However, feeding PC resulted in lower proportion of cells involved in antigen presentation and higher cytokine production following immune challenge (IL-2, IL-6, IFN- γ and TNF-α) (all $P<0.05$). In a cell culture model using isolated splenocytes, lysoPC was shown to increase T cell activation and T cell proliferation (IL-2 production). PC pups appeared more efficient at mounting a response to antigens and developmentally more mature compared to pups from free choline-fed dams and increasing the supply of PC, or lysoPC, may be responsible for these effects.

Overall, we concluded that maternal dietary choline intake is low during pregnancy and early postpartum, and choline is essential in the maternal diet to support offspring growth and immune system development. Furthermore, the forms of choline in the maternal diet differentially affect immune system development, with choline in the form of PC improving development of the offspring's T and B cells.

Preface

This thesis is original work by Erin D. Lewis. The research project involving humans, of which this thesis is a part, received research ethics approval from the University of Alberta Hearth Research Ethics Biomedical Panel and the University of Calgary Health Research Ethics Board, "Alberta Pregnancy Outcomes and Nutrition (APrON)" Pro00002954, March 4, 2009. The research project involving animals, of which this thesis is a part, received research ethics approval from the Committee of Animal Policy and Welfare of the Faculty of Agriculture, Life and Environmental Sciences at the University of Alberta, "The importance of choline in human health during pregnancy and infant development-the potential of egg yolks for dietary enrichment", Protocol #095, May 21, 2009. The contributions made by the candidate, Erin D. Lewis, and the co-authors to the completion of this work are described here.

Chapter 3 of this thesis has been published as E.D. Lewis, S.J. Kosik, Y-Y Zhao, R. L. Jacobs, J. M. Curtis, and C. J. Field, "Total choline and choline-containing moieties of commercially availabile pulses", *Plant Foods for Human Nutrition*, 69(2): 115-121. I was responsible for analyzing and performing statistical analyses of the data, and preparing the manuscript. JMC, RLJ and CJF designed the research study, obtained the specific funding for this specific analysis on this study; SJK, YYZ and JMC conducted the research. CJF and JMC also prepared the manuscript and CJF had primary responsibility for final content.

Chapter 4 of this thesis has been published as E.D. Lewis, Y-Y Zhao, C. Richard, H.L. Bruce, R. L. Jacobs, C. J. Field, and J. M. Curtis, "Measurement of the abundance of choline and the distribution of choline-containing moieties in meat", *International Journal of Food Sciences and Nutrition*, 66(7): 743-748. I was responsible for analyzing and performing statistical analyses of the data, and preparing the manuscript. CJF, JMC, and RLJ designed the research study and obtained the specific funding for this study; YYZ and JMC conducted the

research. CJF, YYZ and JMC also prepared the manuscript and JMC had primary responsibility for final content.

Chapter 5 of this thesis has been published as E.D. Lewis, F. B. Subhan, R. C. Bell, L. J. McCargar, J. M. Curtis, R. L. Jacobs, C. J. Field, "Estimation of choline intake from 24-h dietary intake recalls and the contribution of egg and milk consumption to intake among pregnant and lactating women in Alberta", *British Journal of Nutrition*, 112(1): 112-121. I was responsible for conducting the research, analyzing and performing statistical analyses of the data, and preparing the manuscript. JMC, CJF and RCB obtained the specific funding for this specific analysis on this study of APrON, CJF, RCB, LJM, JMC, and RLJ designed the research study, FB assisted with statistical analyses. CJF also prepared the manuscript and had primary responsibility for final content.

Chapter 6 of this thesis used data collected in the APrON study. I was responsible for analyzing and performing statistical analyses of the data, and writing the chapter.

Chapters 7 and 8 of this thesis have been published as E.D. Lewis, S. Goruk, C. Richard, N. S. Dellschaft, J. M. Curtis, R. L. Jacobs and C. J. Field. "Feeding a diet devoid of choline to lactating rodents restricts growth and lymphocyte development in offspring", *Britrish Journal of Nutrition*, 116(6): 1001-1012, and "Feeding phosphatidylcholine compared to free choline in the maternal diet differentially affects immune development in the suckled offspring", *Journal of Nutrition*, 146(4): 823-830, respectively. I was responsible for conducting the research, analyzing and performing statistical analyses of the data, and preparing the manuscripts. CJF, JMC, and RLJ designed the research study, SG and NSG conducted the research and CR assisted with statistical analyses. CJF also prepared the manuscript and had primary responsibility for final content.

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List of Abbreviations

24-h – 24-hour

AI – Adequate Intake

APC – antigen presenting cell

APrON – Alberta Pregnancy Outcomes and Nutrition

ChD – Choline Devoid

ChS – Choline Sufficient

ConA – concanavalin A

CTLA-4 – cytotoxic T lymphocyte antigen 4

CTP – phosphocholine cytidylyltransferase

DAG – diacylglycerol

DHA – docosahexaenoic acid

dpm – disintergrations per minute

ELISA – enzyme-linked immunosorbent assay

FFQ – food frequency questionnaire

GALT – gut associated lymphoid tissue

GPC – glycerophosphocholine

HILIC LC-MS/MS - hydrophilic interaction liquid chromatography-tandem mass spectrometry

HPA – hypothalamic-pituitary-adrenal

IFN-γ – interferon-γ

Ig – immunoglobulin

IL – interleukin

IOM – Institute of Medicine

LC-MS/MS - liquid chromatography-tandem mass spectrometry

LPS – lipopolysaccharide

lysoPC – lysophosphatidylcholine

MHC – major histocompatibility complex

MLN – mesenteric lymph nodes

NDB – Nutrient Database

NHANES III – National Health and Nutritional Examination Survey III

NK – Natural Killer

OVA – ovoalbumin

PC – phosphatidylcholine

Pcho – phosphocholine

PE – phosphatidylethanolamine

PEMT – phosphatidylcholine-*N*-methyltransferase

Th – T helper

TMA – trimeythylamine

TMAO – trimethylamine-*N*-oxide

TNF-α – tumour necrosis factor α

Treg – T regulatory cell

USDA – United States Department of Agriculture

VLDL – very low density lipoproteins

WST-1 – water-soluble tetrazolium salt

CHAPTER 1: Introduction and literature review[1]

1.1 Overview of choline

In 1998, choline was formally recognized by the Institute of Medicine (IOM) as an essential nutrient. It is required for a variety of critical processes including the synthesis of acetylcholine, formation of cell membrane components sphingomyelin and phosphatidylcholine (PC), and is a major source of methyl groups. Choline can be acquired from both dietary and *de novo* sources. *De novo* synthesis occurs primarily in the liver by the methylation of phosphatidylethanolamine (PE) to PC; however, this process is not sufficient in meeting human requirements therefore a source in the diet is also needed (Zeisel and da Costa 2009).

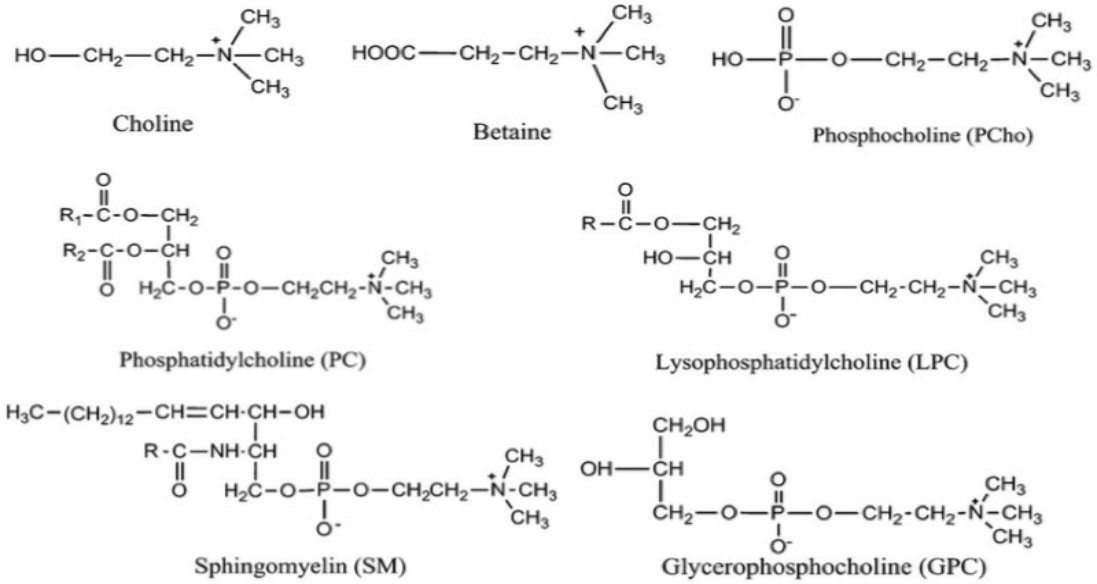
1.1.1 Forms and dietary sources

Choline can be found in a wide variety of membrane-containing foods (Zeisel 2006). Animal liver (beef and chicken), eggs, wheat germ, soybeans and pork all contained high concentrations of choline (Zeisel, Mar et al. 2003). Although dairy products are not particularly high in choline, due to their frequent consumption, dairy is also considered a major dietary source of choline (Cho, Zeisel et al. 2006, Mygind, Evans et al. 2013, Lewis, Subhan et al. 2014). The United States Department of Agriculture released a second version of the Database of the Choline Content of Common Foods in 2008 (Patterson, Williams et al. 2008)). This database contains the choline composition of approximately 630 foods commonly consumed in the United States and has been a valuable tool in estimating dietary choline in

[1]A version of this chapter has been published: Lewis ED, Jacobs RL, Field CJ. (2015) Should the forms of dietary choline also be considered when estimating dietary intake and the implications for health? *Lipid Technology*, 27(10): 227-230.

populations worldwide. Choline can be found in a food in a variety of forms including free (unesterified) choline, phosphocholine, sphingomyelin, glycerophosphocholine, PC and lysophosphatidylcholine (lysoPC), shown in **Figure 1-1**.

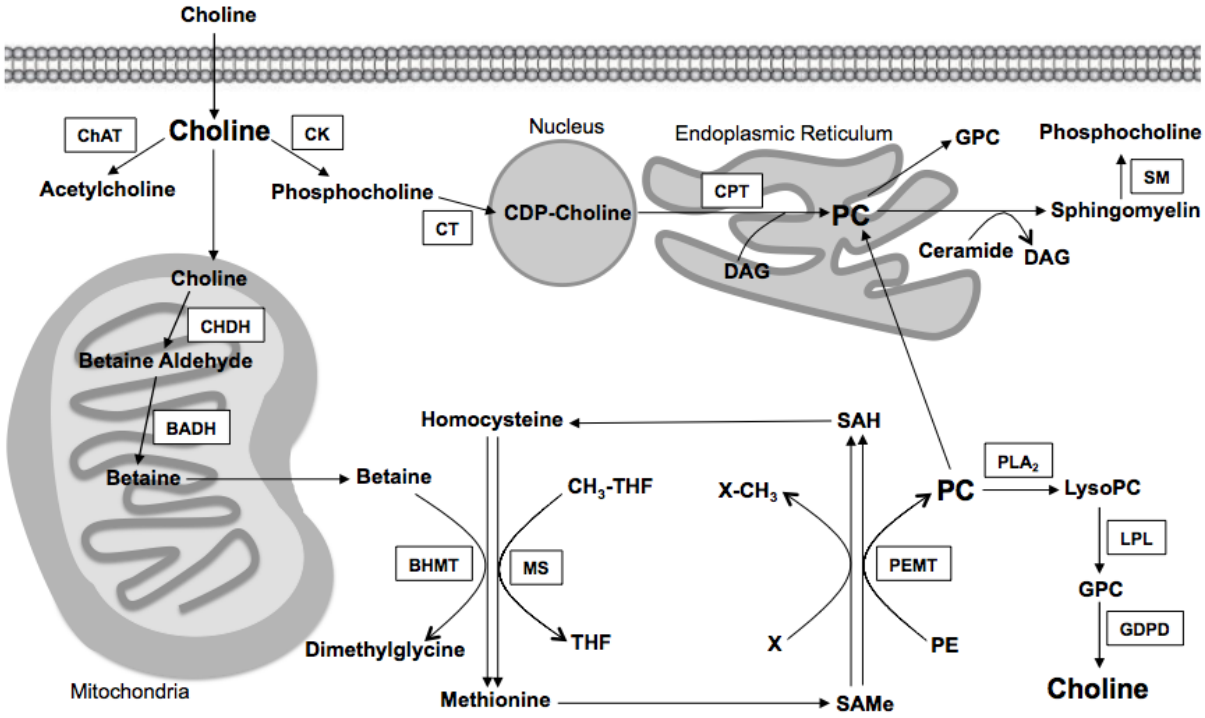
Figure 1-1: Structures of the forms of choline (modified from Zhao, Xiong et al. 2011)



1.1.2 *De novo* synthesis sources of choline

In addition to dietary source, choline can be obtained through synthesis of PC from the methylation of PE, shown in **Figure 1-2**. This reaction is catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT) (Ueland 2011). This methylation reaction uses 3 S-adenosylmethionine (SAMe) molecules and produces 3 molecules of S-adenosylhomocysteine (SAH).

Figure 1-2: The cellular metabolism of choline and PC[1] (adapted from Corbin and Zeisel 2012)



[1]Abbreviations BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; ChAT, choline acyltransferase; CHDH, choline dehydrogenase; CH_3-THF, methyl tetrahydrofolate; CK, choline kinase; CPT, choline phosphotransferase; CT, CTP:phosphocholine cytidylyltransferase; DAG, diacylglycerol; GPC, glycerophosphocholine; glycerophosphocholine phosphodiesterase, GDPD; lysophospholipase, LPL; MS, methionine synthase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylcholine-N-methyltransferase; phospholipase A_2, PLA_2; SAH, S-adenosylhomocysteine; SAMe, S-adenosylmethionine; SM, sphingomyelinase; THF, tetrahydrofolate

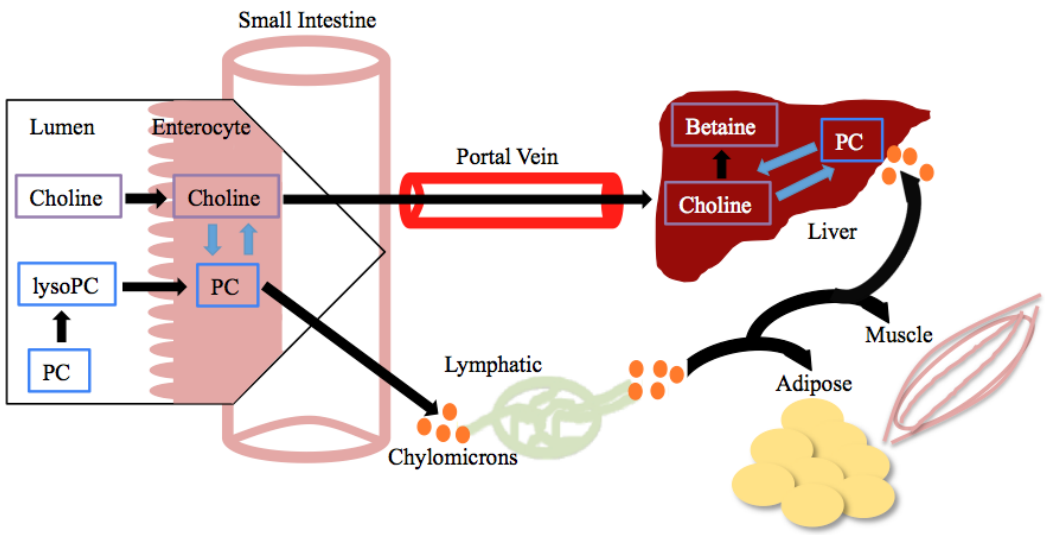
It has been estimated that approximately 30% of PC synthesized in the liver occurs via the PEMT pathway and is most active in a choline-deficient state (Shields, Agellon et al. 2001). Despite being fed choline sufficient or high choline diets, $PEMT^{-/-}$ mice have lower concentrations of choline in liver, demonstrating that the production of choline from the PEMT pathway is a significant source of choline in addition to dietary sources (Zhu, Song et al. 2003). *De novo* sources of PC (PEMT-derived) are typically enriched with long chain fatty acids including n-3 fatty acid docosahexaenoic acid (DHA) and are a major source of DHA to

extra hepatic tissues (Caudill 2010). Conversely, dietary sources of PC (CDP-choline-derived) are more commonly enriched in n-6 fatty acid linoleic acid and medium chain saturated fatty acids (16:0, 18:0) (DeLong, Shen et al. 1999).

1.1.3 Absorption and metabolism of dietary choline

Research in rodents using radiolabelled carbon has found that the dietary forms of choline are absorbed and metabolized differently in the body (Cheng 1996). Free choline, phosphocholine and GPC are water soluble, which differ in their absorption and metabolism from the lipid soluble forms of choline, PC, lysoPC and sphingomyelin. As shown in **Figure 1-3**, Free choline is absorbed in the jejunum and ileum and transported into the enterocyte by sodium-independent carrier mediated transport, or passive diffusion when free choline concentrations are high (Tsubaki and Komai 1987). Once in the enterocyte, free choline can be irreversibly oxidized to betaine or directly absorbed into the portal circulation to the liver. Unabsorbed choline goes to the large intestine where it is metabolized by intestinal bacteria to trimethylamine (TMA) where it is either excreted in the urine, or is circulated to the liver where it is converted to trimethylamine-*N*-oxide (TMAO). Choline enters hepatocytes by sodium-independent carrier mediated transport and can either be oxidized to betaine or enter the CDP-choline pathway for PC synthesis (shown in Figure 1-2).

Figure 1-3: Intestinal absorption and metabolism of dietary free choline and PC in humans



To enter the CDP-choline pathway, choline is phosphorylated to phosphocholine, which is the committed step in the conversion of free choline to PC. Hepatic concentrations of phosphocholine are strongly correlated to dietary intake, and is the form most sensitive to dietary deficiency (Pomfret, daCosta et al. 1990) Conversion of choline to PC occurs in all nucleated cells. Synthesis of PC from choline via the CDP-choline pathway is dependent on substrate availability; choline, the enzyme phosphocholine cytidylyltransferase (CTP) and diacylglycerol (DAG) are all required (Table 1-1) (Li and Vance 2008). In some cell types including the liver, kidney and intestine, free choline can be irreversibly oxidized to form betaine for use in methyl donation. The oxidation of choline is catalyzed by enzymes choline dehydrogenase and betaine aldehyde dehydrogenase. An additional fate of free choline in some tissues is acetylation to acetylcholine.

Unlike free choline, lipid soluble PC must first be hydrolyzed by phospholipase A_2 to form lysoPC in order to be absorbed by the enterocyte (Parthasarathy, Subbaiah et al. 1974). LysoPC can then be reacetylated to PC or it can be further broken down to form GPC, then free choline (Parthasarathy, Subbaiah et al. 1974). Although the aqueous forms of choline are

transported directly to the liver, the lipid-soluble forms are secreted into chylomicrons in the enterocyte and enter the bloodstream through the thoracic duct (Scow, Stein et al. 1967, Le Kim and Betzing 1976). Dietary lipid-soluble forms of choline are thereby delivered directly to peripheral organs (adipose and muscle) on route to the liver. In the liver, both dietary and endogenously synthesize source of PC can then be utilized in the synthesis of lipoproteins.

1.1.4 Major functions of choline

The main functions of choline have been reviewed by Li and Vance 2008, Zeisel and da Costa 2009, Caudill 2010, and summarized by form of choline in **Table 1-1**.

Table 1-1: The main functions of the different forms of choline (Lowe, Thien et al. 2008, Zeisel and da Costa 2009, Caudill 2010)

Choline Form	Main Functions
Free choline	• Precursor for betaine which is involved in methyl group donation • Precursor for acetylcholine, a neurotransmitter involved in sleep, learning, memory and muscle control • Forms PC via CDP-choline pathway
Phosphocholine	• Hepatic concentrations most correlated with dietary intake
GPC	• Osmolyte in the kidney used to adapt to osmotic stress
PC	• Essential membrane phospholipid • Required for bile formation • Required for very low density lipoprotein (VLDL) synthesis and secretion • Precursor for plasmalogen and platelet-activating factor
LysoPC	• Involved in cell signalling • Delivers DHA to brain • Component of oxidized low density lipoproteins in atherosclerotic lesions and plasma
Sphingomyelin	• Structural component of cell membranes • Secondary messenger in signalling cascades that regulate cell growth, differentiation and death • Required for myelination of nerve fibres in the peripheral and central nervous system

1.1.4.1 Methyl group donation

The irreversible oxidation of choline via enzyme choline dehydrogenase creates the methyl donor betaine (Figure 1-2). Betaine serves a critical role in the remethylation of homocysteine to methionine by the enzyme betaine homocysteine methyltransferase (BHMT) (Ueland, Holm et al. 2005). The metabolism of methionine, choline and folate are interrelated at the point of conversion of homocysteine to methionine. The interaction of these nutrients in metabolic pathways explains why dietary requirements for folate and choline are related and why both these nutrients are required during periods of growth and development (Zeisel and da Costa 2009). Another critical role of betaine as methyl donor, particularly in fetal and infant development, is methylation of DNA. DNA can be methylated at cytosine bases followed by guanosine, also known as CpG islands (Zeisel 2011) and in mammals, approximately 60-80% of the CpG islands in DNA are methylated (Zeisel 2007). The methylation of DNA influences the transcription of genes and genomic stability (Zeisel and da Costa 2009). CpG island methylation is normally associated with gene silencing or reduced gene expression. Once the methylation of the CpG island is complete, genes are copied and therefore the methylation and the effects that DNA methylation has persists throughout life. By altering the availability of the methyl groups from diet, changes in gene methylation can occur, altering gene expression and the predicted phenotype. Low dietary intake of choline has been demonstrated to decrease concentrations of S-adenosylmethionine in liver (Zeisel, Zola et al. 1989). In choline-deficient cell cultures and in fetal rodent brains from mothers fed a diet deficient in choline, the methylation of the CDKN3 gene promoter is decreased. This causes upregulation of the CDKN3 gene that inhibits cell proliferation. A change in gene expression such as alteration of the CDKN3 gene results in altered neurogenesis for life (Zeisel 2006).

1.1.4.2 Phospholipid and lipoprotein synthesis

PC, lysoPC and sphingomyelin are all essential components of cell membranes, with PC comprising over 50% of total membrane phospholipids in almost every mammalian cell (Zeisel 1993). Choline is also essential for the synthesis of lipoproteins as PC is the major phospholipid on the surface monolayer of VLDL. It has been demonstrated that the synthesis of PC is required for VLDL synthesis and secretion from the liver (Yao and Vance 1988, Yao and Vance 1989), in order to deliver cholesterol and lipid to other organs. Both CDP-choline and PEMT pathways make PC for VLDL synthesis. Several reviews have focused on the role of choline in lipoprotein synthesis (Vance 1990, Li and Vance 2008) and a comprehensive discussion on this topic is beyond the scope of this thesis. PC also comprises the majority of bile phospholipids and is required for bile formation (Boyer 2013).

1.1.4.3 Signalling roles

Choline metabolites provide key signalling molecules involved in cell-cell signalling and signal transduction. Products of PC hydrolysis including phosphatidic acid, lysoPC and free fatty acids act as secondary messengers (Zeisel 1993). Phosphatidic acid has the ability to act as a mitogen (Wakelam, Cook et al. 1991) and lysoPC is known to be involved in chemotaxis, cell lysis, activation of T cells and relaxation of smooth muscle (Nishizuka 1992). Sphingomyelin is synthesized from a phosphocholine group derived from PC and a ceramide. Sphingomyelin, and it's metabolites including DAG and ceramide, can act as a secondary messengers in signalling cascades that regulate cell growth, differentiation and death, with these functions reviewed extensively (Zeisel and Blusztajn 1994, Ohanian and Ohanian 2001).

1.1.4.4 Neurotransmitter synthesis

The small proportion of choline is acetylated through the enzyme choline acetyltransferase to form acetylcholine. This metabolic pathway occurs in neuronal tissue and some non-neuronal tissue including the placenta. Acetylcholine is a critical neurotransmitter, particularly in the parasympathetic nervous system, that is involved in sleep, learning, memory and muscle control. The critical functions of acetylcholine are reviewed by Picciotto, Higley et al. 2012, and will not be discussed in detail in this thesis. Sphingomyelin is also required in functioning of the nervous system, as it is required for myelination of nerve fibres in the peripheral and central nervous system.

1.1.5 The consequences of choline deficiency

In humans and rodents, a well-described consequence of choline deficiency is the development of hepatosteatosis (Buchman, Dubin et al. 1995). The lack of PC available for phospholipid synthesis results in impaired VLDL secretion from the liver (Yao and Vance 1988). Furthermore, elevated serum aminotransferase indicating liver damage is associated with choline deficiency (Albright, Liu et al. 1996). Muscle damage also occurs because muscle membranes are more fragile to deficiency (da Costa, Badea et al. 2004). The majority (80%) of men and postmenopausal women on a choline-deficient diet developed signs of subclinical organ dysfunction (fatty liver or muscle damage) (da Costa, Badea et al. 2004). However, due to the promotion of endogenous choline synthesis by estrogen, less than half of premenopausal women developed such signs. Upon consumption of a high choline diet, liver and muscle damage was reversed (da Costa, Badea et al. 2004). Insufficient choline intake has also been demonstrated to affect other organs including the immune system that will be discussed in section 1.3.

1.2 Choline intake and the need during pregnancy and lactation

Choline requirements increase in pregnancy and lactation to ensure that needs of the rapidly developing fetus and infant are met and maternal stores are maintained (Caudill 2010). In addition to higher requirements, there is increased endogenous synthesis of choline due to increased estrogen production (Fischer, daCosta et al. 2007). During pregnancy, plasma concentrations of estradiol increase from 1nM to 60nM at term and this increase in estrogen indicates that the capacity for endogenous choline synthesis is highest when the development of the fetus needs to be supported (Zeisel 2006).

1.2.1 Prenatal delivery to the fetus

Expression of PEMT is very low or absent in the fetal liver and placental tissue, therefore the availability of choline for fetal development is solely from maternal supply (Caudill 2010).

During pregnancy, choline concentrations in the plasma are significantly higher compared to nonpregnant women, 16.5 μM of free choline and 3520 μM of phospholipid-bound choline at 36-40 weeks of pregnancy compared to 10.7 μM of free choline and 2780 μM of phospholipid-bound choline (Ilcol 2002). The fetus is exposed to very high concentrations of choline delivered across the placenta by a choline transport system that pumps the choline against a concentration gradient (Zeisel 2006). Both mediated and non-mediated choline transport systems have been described in the placenta (Zeisel, Mar et al. 1995). Although there is increased *de novo* synthesis in the mother during pregnancy, the demand exceeds the choline stores, increasing the importance of adequate dietary choline intake. In newborn and fetus, plasma choline concentrations are six to seven fold higher compared to concentrations in adults suggesting a gradient towards delivery to the infant.

The placenta is one of the only other tissues, besides nervous tissue, to store choline in large amounts, in the form of acetylcholine. The large storage of acetylcholine is believed to serve as a storage reserve of choline to ensure adequate choline delivery to the fetus (Zeisel 2006).

1.2.2 Postnatal delivery to the infant

In the postnatal period, choline is delivered to the newborn via breast milk or formula. Milk contains a high concentration of choline, which further increases the demand for choline during lactation and further extends the depletion of choline in tissue stores (Zeisel 2006). *De novo* synthesis in the mammary gland (Yang, Blusztajn et al. 1988) and active transport from maternal circulation provides choline in milk (Chao, Pomfret et al. 1988). In a study conducted on lactating rats fed varying levels of choline the diet (control diet, choline-deficient diet or choline-supplemented diet) the choline content in the milk reflected the concentration of choline in the diet (Holmes-McNary, Cheng et al. 1996). It has been demonstrated in rodents that lactating females, compared to non-lactating females had greater depletion of liver choline metabolites when fed a choline-deficient diet, suggesting greater sensitivity to inadequate choline intake (Zeisel, Mar et al. 1995). These studies suggest that endogenous sources of choline are not sufficient in meeting the demands for the infant, and a source of choline in the maternal diet is also important (Zeisel, Mar et al. 1995). Human breast milk contains a high concentration of choline, containing between 104 to 156 mg/L (1 to 1.5 mmol/L) (Caudill 2010). A study conducted by Ilcol *et al*., (2005) found that maternal choline status influences the amount of free choline in breast milk, and is significantly associated with serum choline concentrations in the infant (Ilcol, Ozbek et al. 2005). GPC and phosphocholine are the major choline metabolites in rodent and human milk, with approximately seven times

more choline coming from GPC than any other form of choline (Rohlfs, Garner et al. 1993). Choline content in infant formula is based on estimates of choline concentrations in human milk based (Zeisel, Char et al. 1986); however, there are differences in the composition of choline between milk and infant formulas (both soy and cow-derived). Human milk contains a significantly higher concentration of phosphocholine compared to cow or soy-derived formulas (Holmes-McNary, Cheng et al. 1996). Although cow-milk-derived formula contains similar amounts of lipid-soluble forms of choline, soy-based formulas are higher in PC while containing lower amounts of sphingomyelin compared to human milk (Holmes-McNary, Cheng et al. 1996). Additionally, human milk contains 30-80% less free choline compared to both cow and soy-derived formulas (Holmes-McNary, Cheng et al. 1996). It is still unknown if the differences in choline forms in human milk and formula effect infant health.

1.2.3 Estimating dietary intake of choline in women of childbearing age and during pregnancy and lactation

There have been emerging studies estimating dietary choline intake in adult populations, given the recent availability of data on the choline content of common foods (Patterson, Williams et al. 2008). Several studies have estimated dietary choline intake in women of childbearing age; however, there are limited number of studies examining intake in pregnant and lactating women. A full summary of the studies examining dietary choline intake in women of childbearing age is presented in **Table 1-2**.

In women of childbearing age (18 to 40 years) in New Zealand, dietary choline was estimated using 3-day weighed food records (Mygind, Evans et al. 2013). Mean daily total choline intake was 316 ± 65 mg/d, with an energy-adjusted intake of 180 mg/1000 kcal. Of the 125 women in the study, only 16% met daily recommendations for choline (425 mg/d) (Mygind, Evans et al. 2013). The majority of choline was consumed as PC (123 ± 32 mg/d)

and free choline (65 ± 17 mg/d), with eggs, red meat and milk identified as major sources of dietary choline, similar to what has been previously reported (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Mygind, Evans et al. 2013). The results of this study may not be generalizable to other populations, given the small sample size of a relatively homogenous population, with 88% of the study population with post-secondary education and 75% identifying as New-Zealand European. Choline intake may have also been underestimated in this population as the choline content data was derived from the USDA choline database. Substitutions were made for the choline content of local foods, such as silverbeet and Marmite, but this may underrepresent the actual choline content in these foods. A quantitative food frequency questionnaire (FFQ) was used to estimate dietary choline in women (ages 45-75) enrolled in the Multiethnic Cohort Study in the United States (Yonemori, Lim et al. 2013). Similar to intake in the New Zealand population, mean total dietary choline for the women in the cohort (n=102,363) was 304 ± 153 mg/d for all ethnicities, with energy adjusted intake of 333 mg/d. PC was the main form of choline consumed, contributing approximately 50% to total choline (Yonemori, Lim et al. 2013). Choline intake was found to vary by ethnicity with Japanese American women consuming the lowest amount of choline (268 ± 110 mg/d) and Native Hawaiian consuming the greatest amount of dietary choline (358 ± 198 mg/d) (Yonemori, Lim et al. 2013). Although this study utilized a large sample size, the FFQ used in the Multi Ethnic Study was not validated for the estimation of choline. Therefore, estimation of individual intake, and the percent of the population meeting daily recommendations should be interpreted with caution. Furthermore, although this study took place in the United States, where the USDA choline database was constructed, it is likely the database was missing the choline content of ethnic foods consumed by the different ethnicities in this study. In a recent study by Coathup et al. (2015), estimation of dietary choline intake

was compared between recall methods in women of childbearing age (n=64) from the United Kingdom. Median total choline intake estimated by FFQ was 285 mg/d (IQR 221-355 mg/d) and median total choline intake estimated by an average of 3 24-h recalls was 255 mg/d (IQR 201-331 mg/d). Compared to 24-h recalls, the FFQ overestimated total choline by a mean of 15 mg/d (Coathup, Wheeler et al. 2015). However, this study only assesse total choline, with no estimation of the forms of choline, therefore it is unknown if one form of choline is responsible for the over-estimation in the FFQ method. Additionally, the FFQ used in this study was only validated for the estimation of dietary folate, not choline, which may have introduced error in the estimation given from the FFQ method. Although the authors state that caution should be given for the interpretation between dietary methods, it is unknown if an over-estimation of individual total choline intake by 15 mg/d is meaningful when analyzed on a population level.

To date, there have been four studies, two of which were published within the last year, examining dietary choline intake in pregnant women. In a case-control study, Shaw *et al.,* (2004) examined the association between neural tube defects (NTDs) and dietary choline intake in pregnancy. Mean total choline intake was 337 ± 176 mg/d in women with NTD-affected pregnancies (case group, n=424) and 409 ± 179 mg/d in women without such pregnancies (control group, n=440). Relative to the lowest quartile of intake (<290 mg/d), the odds ratio for NTD-affected pregnancy women in the highest quartile of intake (>498 mg/d) was 0.49, suggesting that increasing dietary choline intake decreases the risk for NTD. The population in this study is primarily (43%) Caucasian, however, as discussed in the study by Yonemori et al (2013), choline intake can vary with ethnicity, therefore the interpretation of these results may not be applicable to other ethnicities within the United States. Additionally, although the authors mention the 100-item FFQ used to assess intake in this population is

validated for use in epidemiologic studies, it has not been validated for the estimation of choline, which may have resulted in an underestimation of dietary choline. A study conducted by Fischer et al. (2005) found that in men and women, the use of a self-administered dietary questionnaire (3-day food record) significantly underestimated *ad libitum* intake, compared to estimating daily choline intake in a diet with a known amount of choline (Fischer, Scearce et al. 2005). Although this study had a small sample size (n=32), it demonstrates that dietary choline intake obtained from self-reported questionnaires should be interpreted with caution. Gossell-Williams et al. (2005) estimated total choline intake in a small sample (n=16) of Jamaican women in their first trimester of pregnancy using FFQs (Gossell-Williams, Fletcher et al. 2005). Mean total choline intake was 279 ± 29 mg/d, with the majority of this population (88%) not meeting daily recommendations. Dietary intake in this population was slightly lower compared to the previously mentioned studies (Shaw, Carmichael et al. 2004, Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013). As mentioned with the previous study, this study uses an FFQ that has not been validated for the estimation for dietary choline, which may have resulted in an under-reporting of intake. Similarly, this population consumed a variety of local foods in which the choline content was not available, which may have also resulted in an under-reporting of dietary intake. In a more recent study, total choline intake in a population of pregnant women (n=990) in Latvia was estimated using two 24-h dietary intake recalls (Vennemann, Ioannidou et al. 2015). Median total choline intake for this population was 330 mg/d (with a range of 200-592 mg/d). Total choline was estimated using the USDA choline database, yet there was no comment on the forms of choline consumed. Furthermore, although estimation of dietary intake is strengthened with the use of two 24-h recalls, many foods commonly consumed in Europe were absent from the USDA database, which may have resulted in an under-reporting of intake. To date, there has been one study

that has estimated dietary choline of pregnant women in a Canadian population (Masih, Plumptre et al. 2015). Dietary and supplemental choline was assessed by a semi-quantitative Block FFQ in women (n=386) during early (0-16 weeks) and late (23-37 weeks) pregnancy. Mean total choline intake was 306 ± 127 mg/d in early pregnancy and 302 ± 122 mg/d in late pregnancy, with 13% of women meeting daily choline recommendations (Masih, Plumptre et al. 2015). Mean free choline was 77 ± 28 mg/d and mean choline from PC was 49 ± 22 mg/d across early and late pregnancy, with no reported consumption of choline from supplemental sources (Masih, Plumptre et al. 2015). Although this study uses a FFQ that has been validated for the estimation of dietary folate, vitamin B_6, vitamin B_{12}, it was not validated for the estimation of choline. Furthermore, this population is primarily well educated (82% with post-secondary degree or greater) and Caucasian (45%), limiting the generalizability to other Canadian populations.

Consistently, dietary choline intake in women of childbearing age, or pregnant women, indicate that these populations are not meeting daily recommendations. There is variability in estimates of choline intake between studies due to several factors including methods used and ethnicity. Given the variation in dietary recall method used, interpretation of results with different recall methods is cautioned. It appears that choline intake may be over-estimated by FFQ compared to a 24-h recall (Coathup, Wheeler et al. 2015), but self-reported dietary intake results in an under-reporting of choline intake compared to measured intake (Fischer, Scearce et al. 2005). Five of the six of the studies used self-reported dietary intake recall methods and may have resulted in under-reporting of choline. A limitation common to all studies estimating dietary choline intake is the possibility of under-reporting due to limited food composition data. Although the USDA database contains a wide variety of choline containing foods, many dietary sources of choline may be absent from this database, depending on the location or

ethnicity of the population of study. Choline intake has been demonstrated to vary by ethnicity (Yonemori, Lim et al. 2013); however, three of the studies examined choline intake in a primarily Caucasian population. It has been demonstrated in Canada that food insecurity and low socioeconomic status is associated with nutrient inadequacies (Kirkpatrick and Tarasuk 2008, Dachner, Ricciuto et al. 2010, Tarasuk, Fitzpatrick et al. 2010). Choline intake may vary with socioeconomic status but none of the discussed studies commented on the socioeconomic status of the population. Further research needs to be conducted with multiethnic populations and populations with varying socioeconomic. Furthermore, only three studies reported intake of the forms of dietary choline, in addition to total choline (Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013). Given emerging evidence that the dietary forms of choline may differentially affect human health, it is important to report the forms, in addition to total intake. To date, there have been no studies examining dietary choline intake of women in the lactating/early postpartum period. The studies discussed would suggest that it is likely women in this period are not meeting daily recommendations, similar to pregnant and non-pregnant women, yet there have been no studies that have conclusively examined this. Choline recommendations even further in the lactating period, increasing an additional 100 mg/d from pregnancy, therefore this population may be at an even greater risk of inadequate choline intake on both mother and infant.

1.3 Overview of the immune system

The immune system plays a crucial role in the body, serving as a defense against infectious agents, identification and elimination of tumour cells and response to injury and trauma (Calder 2007). Morbidity and mortality due to infection, in part, depends on the ability of the immune system to appropriately and effectively respond to environmental challenges

including viruses, bacteria, parasites and fungi (Crockett 1995). Appropriate immune functions and responses are also necessary to distinguish "self" from "nonself", crucial in the development of tolerance to antigenic dietary and commensal bacterial challenges. The innate and acquired (also known as adaptive) immune systems are coordinated in their responses and include many cells types, chemical agents and mediators. All immune cells originate in the bone marrow and are then organized into various lymphoid organs including the thymus, lymph nodes, spleen and gut-associated lymphoid tissue (GALT).

1.3.1 The innate immune system

The innate immune system, consisting of physical barriers, soluble factors and a number of immune cells, serves as the first line of defense against foreign antigens. The main function of this system is to prevent infectious agents from entering the body and if they do enter, then the innate immune system attempts to quickly eliminate them. Phagocytic cells of the innate system include monocytes/macrophages, natural killer (NK) cells, neutrophils and dendritic cells and the granulocytes, eosinophils, basophils and mast cells (Parkin and Cohen 2001). Bacterial antigens can directly activate neutrophils, which phagocytize and neutralize invading pathogens (Alam 1998). Monocytes and macrophages produce cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 that are critical in activating other cells of the innate and adaptive immune systems. The production of these cytokines serves as the communication between the two arms of the immune system (Calder 2007). NK cells are large granular lymphocytes capable of destroying pathogens by releasing cytotoxic proteins (Calder 2007); and mast cells and eosinophils serve as a defense against parasitic infections.

1.3.1.1 Innate immune system development

The innate immune system is present before pathogen exposure and cells of the innate immune system possess no memory of prior exposures (Parkin and Cohen 2001). Thus, the innate immune system functions quite well in the infant and is the primary defense against pathogens. Although functioning, immune cells of the innate immune system still lack the functionality of adult cells that are acquired with age (Perez-Cano, Castellote et al. 2007). In humans and rodents, neutrophils lack adequate phagocytic activity and the ability to mount an adequate chemotactic response (Raju 2012). Reduced function of neutrophils is believed to be an important factor in the susceptibility to infection in infants (Wilson, Westall et al. 1986). Monocytes in the young animal or infant also have a reduced capacity to produce cytokines (Raju 2012). IL-12 expression by monocytes is correlated with age, and increased production influences the development of the Th population as the infant or young animal matures (Hartel, Adam et al. 2005). Dendritic cells are found in a higher proportion of newborns than adults, but are functionally less competent at producing cytokines (Banchereau, Briere et al. 2000). The proportion of NK cells is similar to the proportion in adults (Perez-Cano, Castellote et al. 2007), suggesting that they may have regulatory functions and a role in peripheral tolerance. However, infant NK cells have reduced activity to destroy other cells compared to older children and adults (Chirico 2005). The diminished functional capacity of NK cells gradually improves with age, and by age four adult functions are normally reached (Wilson, Westall et al. 1986).

1.3.2 The acquired immune system

The acquired immune system recognizes foreign pathogens and antigens that have been presented by antigen presenting cells (APCs) expressing major histocompatibility

complex (MHC) Class I and II molecules. The acquired immune system becomes active over several days prior to activation by the infectious antigen and persists for a period of time even after the antigen has been removed. This concept is known as immunological memory and provides a stronger and more effective immune response if the body happens to be re-exposed to the same pathogen.

1.3.2.1 B cells

B cells, or B lymphocytes, represent approximately 5-15% of total circulating lymphocytes (Crockett 1995). B cells have the ability to produce antibodies (immunoglobulins, Ig) that are antigen specific including IgG, IgA, IgM, IgD and IgE. These antibodies work in several manners including the neutralization of microorganisms and activation of complement proteins in plasma (Calder 2007). Immunity involving antibodies and extracellular antigens is known as humoral immunity. B cells can be activated with or without the interaction of T cells. Polymeric antigens, including lipopolysaccharide (LPS), are able to elicit B cell responses without T cell co-stimulation but this activation does not result in development of B cell memory (Chaplin 2003). B cells are capable of serving as APCs for T cells, and T cells reciprocally activate B cells through co-stimulation and secretion of cytokines (Chaplin 2003). B cells that are specific to that antigen proliferate, becoming plasma cells or memory B cells. The plasma B cells secrete antibodies and memory B cells are capable of responding to the recognized antigen upon future invasion (Crockett 1995).

1.3.2.2 B cell development

B cells differentiate in the liver of the fetus and then mature postnatally in the bone marrow. In rats and newborn humans, the B cell population is the earliest to appear in spleen after birth (Perez-Cano, Castellote et al. 2007). The expression of Ig of B cells occurs in

various stages of development, with only IgG capable of placental transfer, providing defense against bacterial and viral infections (Crockett 1995). Serum concentrations of IgG and IgM are detected in 5-day old rats, gradually increasing during the suckling period. The ability to produce adult levels of IgG is not reached until age five. Upon antigenic challenge, such as a neonatal infection, concentrations of IgM rapidly increase (Albers, Antoine et al. 2005) and adult levels of IgM are reached at approximately one year of age (Crockett 1995). IgA is the last Ig to appear in infant and rat (Perez-Cano, Castellote et al. 2007) serum. Breast milk is a major source of IgA and is believed to confer benefits to immune function in breast fed infants (Butler and Kehrli 2005).

1.3.2.3 T cells

T cells, or T lymphocytes, develop in the thymus and are present in the highest proportion of circulating lymphocytes and many lymph node tissues including spleen and mesenteric lymph nodes. They play a role in regulation of innate (inflammatory) and humoral responses and are a key component in the development of immune tolerance (Calder, Krauss-Etschmann et al. 2006). Immunity conferred by T cells is referred to as cell-mediated immunity.

On the T cell surface, antigen-specific receptors recognize antigens that have been presented to them by APCs. T cells can be divided into two distinct populations, CD4+ cells (T helper cells; Th and T regulatory cells; Tregs) and CD8+ (cytotoxic/suppressor T cells). Th cells recognize MHC Class II molecules that are expressed on APCs. Conventional Th cells control adaptive immunity by activating other effector cells and promoting cell-mediated immunity and can be further subdivided into T helper 1 (Th1) cells, T helper 2 cells (Th2) and T helper 17 cells (Th17). These Th cell subsets are important in promoting a specific response

in order to combat different infectious agents (Calder 2007). Differentiation of Th1 and Th2 cells depends on a variety of factors including the cytokine environment, antigen presented and co-stimulatory signals present (Kaiko, Horvat et al. 2008). Th1 cells normally develop in the presence of IL-12 and secrete IL-2 and interferon (IFN)-γ (Kaiko, Horvat et al. 2008). Th2 cells generally develop in the presence of IL-4, IL-6 and IL-10 and absence of IL-12. Th2 cells secrete cytokines including IL-4, IL-5, IL-10 and IL-13, which promote a humoral response to extracellular pathogens and a down regulation of the Th1 response. A wide range of cytokines and environmental factors including co-stimulation and presence of viral and bacterial pathogens, have a role in the differentiation of Th17 cells. Th17 cells produce IL-17, IL-22, TNF-α and GM-CSF and are involved in inflammation, pathogenesis of autoimmune diseases and defense against viral and bacterial pathogens (Mills 2008). These various cytokines secreted by Th cells have an important role in the recruitment of other immune cells such as neutrophils and monocytes to site of pathogen challenge and the activation of monocytes in order to eliminate the pathogen. Another specific subset of Th cells, known as Tregs, are identified by expression of CD4+ and CD25+ and intracellular marker Foxp3 (Josefowicz, Lu et al. 2012). The Treg population can be divided into two major types based on their site of induction; natural Treg cells originate from the thymus, and induced Treg cells develop in secondary lymphoid tissues and sites of inflammation (Bilate and Lafaille 2012). Tregs are particularly important in preventing inappropriate inflammatory response to dietary antigens and been extensively reviewed by (Bilate and Lafaille 2012).

CD8+ T cells are responsible for suppression and cytotoxic cell activity and recognize MHC II molecules expressed on all cell types. Some antigens may evade humoral immunity and require the cell-mediated immunity to combat the pathogen (Calder 2007). In order to combat invading pathogens, the T cell population must first mature and expand, a process that

begins in the thymus and then moves to the periphery (Field, Caccavelli et al. 2001). When T cells encounter pathogens it is through the APCs that recognized the pathogen, activation of the T cell occurs. The T cell receptor interacts with an antigenic peptide presented on the MHC (Class I or II) on the APC. Co-stimulatory molecules are also required for the activation of T cells, CD28 on the APC interact with CD80 or CD86 on the APC. The T cell then generates a unique receptor for that specific antigen, allowing the cell to respond to that antigen (Field, Caccavelli et al. 2001).

1.3.2.4 T cell development

In the early postnatal period, the T cell population is low in spleen, characterized by a low proportion of CD4+ and CD8+ cells, and those present display an immature phenotype (Perez-Cano, Castellote et al. 2007). There is rapid expansion of the Th cell population in the early postnatal period, with a slower, more gradual expansion of cytotoxic T cell population. This results in a high CD4/CD8 ratio at birth and then a gradual decline of this ratio with age in both rats and humans (Chirico 2005). The responses of the helper and cytotoxic T cells are low at birth and increase with age (Prescott 2003, Perez-Cano, Franch et al. 2012). Under standard activation conditions, in both humans and rodents, Th1 and cytotoxic T cell functions are poor, with a predominant Th2 function (Adkins, Ghanei et al. 1993). Reduced proliferative capacity is due to limited IL-2 secretion (Chirico 2005) that gradually increases with age in the young animal and infant. Both rodents and infants mount a Th2 dominant response, characterized by the secretion of IL-4, IL-5, IL-10 and IL-1. The production of Th1 cytokines IFN-γ and IL-4 by T cells been demonstrated to gradually increase during the suckling period in rodents, or first year of life in infants (Albers, Antoine et al. 2005, Hartel, Adam et al. 2005). The production of TNF-α is also related to age, and increases in both TNF-α and IFN-γ

represents a maturation of the immune repertoire (Hartel, Adam et al. 2005). Compared to adults, neonatal T cells require more co-stimulation in order to mount an efficient Th1 response, both *in vivo* and *ex vivo* (Adkins, Ghanei et al. 1993). The development of Tregs occurs after birth upon exposure to the external environment and rapid expansion of lymphoid tissues (Faria and Weiner 2005, Pabst 2013). The 'immaturity' of the neonatal immune system is thought to render infants more vulnerable to exogenous challenges, increasing susceptibility to infection and disease (Calder, Kremmyda et al. 2010). Furthermore, an inappropriate Th2 response is thought to increase the risk for development of atopic diseases such as allergic diseases and asthma (Prescott 2003).

1.3.3 The gut associated immune system

Gut associated lymphoid tissue (GALT) is the largest collection of lymphoid tissues within the body (Forchielli and Walker 2005), comprised of immune cells in the intraepithelial and lamina propria regions of the gut, Peyer's patches and mesenteric lymph nodes (MLN). T cells are the predominant immune cell population found in the GALT (Forchielli and Walker 2005). The GALT is the site for intestinal immune responses and critical for protection against pathogens that may gain entry through mucosal surfaces (Acheson and Luccioli 2004). Similar to other lymphoid tissues, the role of the GALT is to mount appropriate responses when recognizing pathogenic microorganisms from innocuous antigens. The responses of the GALT can be influenced by the microflora present and play an important role in the activation of T cells within the intestine (Forchielli and Walker 2005).

1.3.3.1 Gut associated immune system development

At birth, similar to the systemic immune system, the gut-associated immune system is relative naïve at birth due to low antigen exposure and stimulation by gut bacteria required for

maturation (Blumer, Pfefferle et al. 2007). In addition to the maturation of the structures and cells of the GALT, the maturation of immune responses of the GALT and development of oral tolerance is dependent on colonization of microbes (Lorenz and Newberry 2004). Oral tolerance is a state of local and systemic immune unresponsiveness induced by orally administered antigens such as food proteins and involves the recognition of 'self' and other components such as commensal bacteria. Although evidence suggests that oral tolerance may be induced at any time in the lifecycle (Garside and Mowat 2001), this process typically occurs during early infancy in humans and the suckling period in rodents (Pabst 2013). The immune system is also modulated in the postnatal period by breast milk and oral contact with the environment (Blumer, Pfefferle et al. 2007). Secretory antibodies, primarily sIgA plays a key role in defense against enteric pathogens, and commensal bacteria promote secretion of sIgA from IgA producing cells (Blumer, Pfefferle et al. 2007). The appropriate development and maturation of the GALT for the infant plays a key role in the development of food allergies (oral tolerance) and autoimmune diseases.

1.3.4 *Ex vivo* and *in vivo* assessments of immune function and development

There are several ways to assess the development and function of the immune system. Lymphocyte phenotypes in the spleen can be used as an indicator of maturation in the suckling period (Perez-Cano, Franch et al. 2012). As the B cell population is predominant during the first few weeks of life, the ratio of B and T cells can be used to indicate the phase of maturation. Furthermore, as the proportion of CD4+ and CD8+ T cells is low at birth, the presence of this phenotype can also be used to indicate maturation state. It has been demonstrated in newborn humans that the ratio of CD4/CD8 cells in blood is approximately

four, and as the newborn ages this ratio gradually decrease to adult levels, which is approximately two (Chirico 2005).

The functionality of the immune system, particularly for the suckling animal, can be assessed by response to specific T and B cell mitogens *ex vivo*. Immune responsiveness to antigens can result in a variety of biological activities within the immune system including immune cell stimulation to produce cytokines, expression of cytokine receptors, up-regulation or expression of activation-specific cell surface markers and development of effector cell functions (Collins 2000). Common antigens used to stimulate T cells include concanavalin A (ConA), anti-CD3/CD28 antibodies and phytohemaglutinen (PHA), which bind to the T cell receptor-CD3 complex (Kay 1991). Pokeweed mitogen (PWM) is capable of stimulating both T and B cells and LPS is a bacterial antigen that targets the toll-like receptor (TLR)-4 on macrophages and activates B cells (Palsson-McDermott and O'Neill 2004). Following stimulation, the production of cytokines in the supernatant can be measured using enzyme-linked immunosorbent assays (ELISA). Cytokine production, *ex vivo*, after stimulation serves as excellent measure of cellular responsiveness to immune challenge (Collins 2000) and an assessment of cellular functional capacity (Neubert, Delgado et al. 2000). The pattern of cytokines produced by cells in response to stimulation can indicate the functionality and maturity of the immune cells. IFN-γ and IL-2 are commonly used as markers for the Th1 response (Calder 2007), and IL-4, IL-5 and IL-10 as markers of Th2 response (Collins 2000). In addition to overall production of these cytokines, the ratio of Th1 and Th2 cytokine production can indicate maturation state of the lymphocytes. A higher ratio indicates skew towards a Th1 response, which is indicative of immune system maturation in the newborn (Chirico 2005). IL-2 has also been used as a marker of T cell proliferation (Boyman and Sprent 2012). Furthermore, incorporation of radiolabelled thymidine into DNA of stimulated

or unstimulated lymphocytes can be used as a measure of lymphocyte proliferation (Calder 2007). The amount of thymidine incorporated into the DNA indicates the amount of cell division in the culture. A stimulation index can be calculated as: amount of thymidine uptake (disintegrations per min, dpm) in the presence of each mitogen divided by the amount of thymidine uptake (dpm) in the absence of each mitogen and serves as a measure of cellular proliferation. Cellular metabolic activity can also be assessed using Cell Proliferation Reagent, water-soluble tetrazolium salt (WST-1). Circulating concentrations of cytokines in serum can be measured *in vivo* by using an ELISA or an electrochemiluminescent multiplex cytokine kit, which is more sensitive than an ELISA. Elevated circulating cytokine concentrations would indicate systemic inflammation, however, in the serum, the source of the cytokines measured is unknown, which limits the applicability (Calder 2007).

1.4 Choline and the immune system

1.4.1 Cell culture studies

Several studies have examined the effect of choline on the function of immune cells including macrophages and lymphocytes *in vitro*. A full summary of the cell culture studies examining the role of choline in immune cell function is presented in **Table 1-3.**

Tian *et al.,* (2008) demonstrated that an exogenous source of choline is required for cytokine production in macrophages deficient in CCT-α, the rate-limiting enzyme in *de novo* PC synthesis. When stimulated with LPS, CCT-α deficient macrophages produced less TNF-α and IL-6 compared to wild-type macrophages (Tian, Pate et al. 2008). Incubation for 18 hours with 100 μM palmitic acid (16:0)-lysoPC increased the production of TNF-α and IL-6 in the CCT-α deficient macrophages. Yet, Tian *et al*., (2008) used 16:0-lysoPC and without attempting to balance for the 16:0. Concentrations of 20-200 μM of palmitate have been

shown to induce the secretion of TNF-α and IL-1β in cultured macrophages (Haversen, Danielsson et al. 2009, Nakakuki, Kawano et al. 2013), therefore the independent effect of the fatty acid cannot be excluded. Asaoka *et al.,* 1992 hypothesized that lysoPC may be involved in the activation of T lymphocytes. Incubation of human T lymphocytes for 30 hours with increasing concentrations (0-100 μM) of 16:0-lysoPC increased the expression of the IL-2 receptor in a dose-dependent manner, and [H^3]thymidine incorporation following CD3 stimulation (Asaoka, Oka et al. 1992). A maximum effect on IL-2 receptor expression was observed at 100 μM 16:0-lysoPC, with concentrations above 100 μM resulting in cell lysis. However, similar to the previously mentioned study (Tian, Pate et al. 2008), this study did not use a control of 16:0 fatty acid alone, which may have affected T cell activation in this study. Further, similar concentrations of 16:0 used in this study (50 μM) have been shown to induce cytokine production (TNF-α, IL-6, IL-1β, IL-2 and IL-10) of cultured CD4 T lymphocytes from humans (Stentz and Kitabchi 2006). Therefore, it is unknown if the affect on cytokine production is attribute to the choline moiety, or the fatty acid. Additionally, the effect of 16:0-lysoPC on T cell stimulation was only observed in the presence of DAG and calcium ionophore (ionomycin) confirming that lysoPC alone is not sufficient to activate T cells. Huang *et al.,* (1999) also examined the effect of varying concentrations of 16:0-lysoPC and egg yolk derived lysoPC on cytokine production and antibody production of human peripheral blood mononuclear leukocytes (Huang, Schafer-Elinder et al. 1999). Concentrations of 5 and 25 μM of 16:0-lysoPC induced secretion of IFN-γ and TNF-α, and increased antibody production by leukocytes from healthy adults incubated for 72 hours. Similar to Asaoka *et al.,* (1992), this study demonstrated that lysoPC had a stimulatory effect on T cell function, but at a lower concentration than previously reported (25 μM compared to 100 μM). This study used a control of culture medium alone, without controlling for the potential effect of the lysoPC's

fatty acid composition and only commented that the fatty acid composition of the egg yolk-derived lysoPC contained both unsaturated and saturated fatty acids.

Collectively, these studies demonstrated that lysoPC may activate T cells, but further studies should provide a control balanced for fatty acid composition in order to investigate the effect of the choline molecule on lymphocyte proliferation. The lysoPC (or PC) used in these studies would have provided additional fatty acids that are metabolized by immune cells (reviewed by (Calder 2008). In particular, palmitic acid (16:0) (Asaoka, Oka et al. 1992, Huang, Schafer-Elinder et al. 1999, Tian, Pate et al. 2008) has been demonstrated to independently modulate immune responses, including cellular proliferation (Calder 2008). An additional variable that should be explored is the time dependent activity of the lysoPC or PC. Of the mentioned studies, incubation time ranged from 16 hours (Asaoka, Oka et al. 1992) to 72 hours (Huang, Schafer-Elinder et al. 1999). As some studies reported cell toxicity, it may be useful to examine if shorter incubation times resulted in less cell lysis. Therefore the efficacy of low doses of PC and lysoPC, containing a neutral fatty acid (oleic), for varying incubation lengths, on immune cell function should be investigated further.

1.4.2 Rodent studies

There have been several rodent studies that have demonstrated that a maternal diet containing lower amounts of choline, in addition to other lipotropes (folate, methionine and vitamin B_{12}) negatively affect immune function. A full summary of the animal studies examining the role of choline in immune cell function is presented in **Table 1-4.**

Newberne at el., (1970), examined the effect of varying amounts of lipotropes in the maternal and weaning diet on response of *Salmonella typhimurium* infection in the offspring. Dams were fed one of four diets varying in choline, methionine and vitamin B_{12} content for 3

months prior to pregnancy until the end of suckling: “deficient” (0.7g choline from choline chloride, 1g methionine, 0g B_{12}/kg diet), “marginally deficient” (0.7g choline from choline chloride, 1g methionine, 45µg B_{12}/kg diet), “moderate” (2.2g choline from choline chloride, 3g methionine, 0g B_{12}/kg diet) or control (2.2g choline from choline chloride, 3g methionine, 45µg B_{12}/kg diet). At the end of suckling, offspring were randomized to receive either their mother’s diet, or control diet until 100 days post-weaning. The pups from the “deficient” and “marginally deficient” groups (both 0.7g choline) had lower birth and weaning weights and higher mortality rates from *Salmonella typhimurium* infection compared to pups from the control group. The pups from the dams fed “deficient” and “marginally deficient” diets that were switched to a control diet at weaning had increased their body weight to that of controls at the time of infection, but still had a greater mortality rate compared to pups fed control diet throughout the study. The “deficient” pups also had the lowest white blood cell count, or leukopenia, a common symptom of malnourishment (Carrillo, Jimenez et al. 2014), regardless of the weaning diet. This indicates that despite a choline sufficient diet (control diet) at weaning, choline deficiency during pregnancy and suckling has lasting effects on the leukocyte population into the weaning period. This study demonstrated that low intake of lipotropes during pregnancy and lactation predisposes offspring to bacterial infection, regardless of intake in the post-weaning period. This study did not address the effect of choline alone, or the differential effects of choline forms, as the “deficient”, “marginally deficient” and “moderate” diets contained a range of free choline, methionine and vitamin B_{12}, the authors cannot conclude low intake of which nutrient is responsible for the observed effects. Furthermore, this study examines the essentiality of choline, and other lipotropes, in the prenatal, suckling and weaning periods together, yet these developmental periods were not examined separately so it was not possible to distinguish if one developmental period is more

sensitive to choline deficiency. These are critical periods of development for the immune system with acquisition of function and competency occurring with age (Perez-Cano, Castellote et al. 2007, Perez-Cano, Franch et al. 2012), therefore research should focus on determining the effect of choline on these distinct developmental periods alone.

Williams *et al.,* (1979) examined the effect of a low maternal methionine-choline deficient diet (2.5g methionine and 1g choline/kg diet) during gestation, lactation or both periods on offspring immunity. At weaning, all pups were fed the control diet (4.5g methionine and 3g choline/kg diet) and at 5 months of age were infected with *Salmonella typhimurium* bacteria. This study does not report the form of choline that was used in their diets. Mortality was higher in pups from the low methionine-choline diet groups, regardless of when the low diet was fed (gestation, lactation or both), compared to pups from the control diet groups. Splenocytes from pups from dams fed the low methionine-choline diet during the lactation period had the lowest stimulation index ([^{3}H]thymidine uptake) to ConA and PHA stimulation at 5 months of age (Williams, Gebhardt et al. 1979). This study suggests that the development of the offspring's immune system may be most sensitive to low availability of choline and methionine during the lactation period, compared to pregnancy. However, the experimental diet used was low in both methionine and choline compared to the control diet, thus this study did not address low choline alone and choline has biological functions that are not the same as methionine. Although this diet may be considered lower (68%) in choline compared to control, 1g choline/kg diet is considered the minimum requirement to meet the needs of rodents, therefore this study did not establish if choline is essential to the functioning of the immune system. Similar to the previously discussed study (Williams, Gebhardt et al. 1979), Nauss et al. (1982) also examined effect of a low methionine-choline diet (1g methionine and 0.7g choline from choline chloride/kg diet), in addition to the effect of age in

weaned male rats. Although there was no difference in body weights as reported by the previous study, rats who were fed the low methionine-choline diet for 3 weeks to 3 months had lower spleen lymphocyte proliferation (assessed by [^{3}H]thymidine uptake) following ConA and PHA stimulation compared to rats fed the control diet (3g methionine and 2.2 choline from choline chloride/kg diet) (Nauss, Connor et al. 1982). There was also decreased spleen follicle cellularity in the rats from the marginal methionine-choline deficient diet compared to control (Nauss, Connor et al. 1982), which is an histopathology indicator of decreased number of T and B cells, following immune challenge (Elmore 2006). There was no difference in immune parameters measured between rats fed the marginal methionine-choline deficient diet and control diet at 12 months, suggesting that younger animals are more sensitive to low intake of methionine and choline. Nauss *et al.,* (1982) demonstrated that low intake of both methionine and choline in young animals (3 weeks to 3 months) lowers proliferative response to lymphocytes and alters spleen histology. However, the experimental diet used in this study was low in both choline and methionine, therefore the effects of choline on immune function cannot be isolated from that of methionine. Furthermore, this study used choline chloride as the source of dietary choline, thus the effects of providing a different dietary source of choline such as PC cannot be determined. All of the studies reviewed examined the effect of a low choline (relative to control) diet, however, no studies to date have examined the effect of a choline-devoid diet on the functioning of the immune system. The use of a choline-devoid diet would examine if endogenous synthesis were sufficient in meeting the needs of the immune system, without providing dietary choline, while providing significant stress on the system in a nutrient deficient state.

Although no studies in offspring have investigated the effects of a diet devoid only in choline on immune system development, James *et al.,* (1989) examined the effects of a

methionine and choline-devoid diet (both 0g/kg diet) on lymphocyte DNA in young (90-110g) male rats. Splenocytes from rats fed a choline and methionine-devoid diet for 3 weeks when stimulated with PHA had higher [H^3]thymidine uptake and lower [6-^{3}H]deoxyuridine uptake compared to splenocytes from rats fed a control diet (6g methionine, 2.2g choline from choline chloride, 2mg folate/kg diet). This study suggests that a diet devoid of choline and methionine alters DNA synthesis and may contribute to the immune dysfunction observed with low choline and methionine intake (James and Yin 1989). Although this study does use a devoid diet (0g/kg) to examine the effect on the immune system, it assumes that the only function of choline is as an alternative methyl donor. Both choline and methionine have an essential role in the transmethylation pathway, essential for DNA methylation (see Figure 1-2) (Niculescu, Craciunescu et al. 2006). This study also uses only male rats, which have been demonstrated to be more susceptible to the acute effects (such as fatty liver) of a devoid-choline diet compared to females (Saito, Palomba et al. 1991). Therefore, it would be beneficial to conduct an experiment using both male and females to examine any sex differences in the examined outcomes. Additionally, although this study demonstrates that alterations in DNA synthesis occur after feeding the devoid choline-methionine diet for 3 weeks, therefore longer study duration is recommended to examine if these effects continue and if providing choline back into the diet can reverse them.

To date, there have only been two studies that have examined the effect of choline alone, with sufficient intake of other lipotropes such as methionine, vitamin B_{12} and folate. Courreges *et al.,* 2003 examined the effect of a choline-devoid diet on the immune function in adult female rodents. Rats fed a choline-devoid diet (0g choline/kg diet) for 2 months had lower delayed-type hypersensitivity (DTH) response and ex vivo proliferation of splenocytes after stimulation with ConA compared to rats fed a control diet (2.6g choline from choline

chloride/kg diet) (Courreges, Benencia et al. 2003). This study suggests that in female rats, a diet devoid of choline modulates T cell function and response to *ex vivo* stimulation. However, similar to the limitation discussed in the previous study (James and Yin 1989), this study used only female rats. As it has been established that dietary choline deficiency differentially affects the sexes (Saito, Palomba et al. 1991), future studies should use both male and female rodents. Furthermore, similar to all the reviewed studies, the control diet used in this study provided choline from choline chloride, as free choline. The differential effects of choline form (specifically PC) on T cell function should be explored. Dellschaft *et al.,* (2015) also examined the effect of a choline-devoid diet on immune function in female rats but in this study the female rats were lactating dams (Dellschaft, Ruth et al. 2015). Dams were fed a choline-devoid diet (0g/kg diet) or varying amounts of choline from choline bitartrate (1, 2.5 and 6g/kg diet) for the duration of the lactating period (21 days). Choline-devoid dams had lower final body and pup weights compared to dams fed 1g and 2.5g choline. Splenocytes from choline-devoid dams had lower production of cytokines following immune challenges (ConA, LPS and CD3/CD28) and a lower proportion of mature T cells and activated suppressor cells. Dams fed 6.2g choline had a higher cytokine production following CD3/CD28 production compared to 1g and 2.5g choline dams, with no difference between 1 and 2.5g choline dams. This study suggests that choline is essential in the maternal diet for immune function, with excess dietary choline having little effect on immune function. With the exception of pup weight, the effect of the lower choline intake (maternal dietary choline deficiency and lower breast milk content) on offspring immune development was not reported. Further, this study examined the effect of varying doses of choline from choline bitartrate (free choline). Therefore, future studies should examine the effect of different choline forms, in varying doses, on both maternal immune function, and the offspring immune development.

Similar to the previous study, Rivera *et al.,* 1998 examined the effect of high intake of dietary choline (4.5g of choline from choline chloride) on response to immune challenge; this study used an *in vivo* model of immune response. Compared to a control diet containing 1.5g of choline from choline chloride, feeding a high choline diet to female adult rats (weighing 250-275g) for 3 days prior to LPS injection (5 mg/kg) improved survival by 56% (Rivera, Wheeler et al. 1998). Lung pathology (cellularity and alveolar filling) was improved and TNF-α production by alveolar macrophages and serum AST levels were lower in the high choline diet group, suggesting improvements in lung inflammation and liver damage associated with LPS infection (Rivera, Wheeler et al. 1998). In this study, the authors reported that following LPS injection, respiratory failure was the most common cause of mortality, providing a rationale for examining lung immune cells. This study reports improvements in lung and liver health, however, LPS was injected into the periphery (via tail vein) therefore other immune tissues, including peripheral immune tissues and gut-associated lymphoid tissues could have been affected by the bacterial infection but were not examined. Furthermore, similar to previously mentioned animal studies, the form of choline used in this study is choline chloride. The authors hypothesize that choline may prevent lung damage by altering LPS binding to macrophages, possibly by increasing membrane PC content. It is possible that feeding a different form of choline, such as PC, may have differential effects on the immune outcomes measured in this study thus warranting further study.

Collectively, these animal studies provide evidence that dietary choline has a role in the modulation of immune functions. However, only two studies (Courreges, Benencia et al. 2003, Dellschaft, Ruth et al. 2015) examined the effect of choline alone, while providing an otherwise nutritionally complete diet. Both studies suggest that for adult female rats, both non-lactating and lactating, a source of dietary choline is essential for T cell function and response

to immune challenge. Similar to findings from Dellschaft *et al.,* (2015) Williams *et al.,* (1979) suggests that the lactation period is sensitive to a low choline diet and methionine diet (Williams, Gebhardt et al. 1979). Additionally, younger animals appear more sensitive to a low choline and methionine diet, suggesting that a supply of choline is needed during immune system maturation (Nauss, Connor et al. 1982). However, to date, there have been no studies examining the effect of dietary choline on offspring immune function. Choline and methionine both play a role in methylation reactions, therefore it is unknown if the effects on immune function observed (Williams, Gebhardt et al. 1979, Nauss, Connor et al. 1982) were attributed to choline, similar to what was observed in studies by Courreges *et al., (*2003) and Dellschaft *et al.,* (2015), or simply to more methylation capacity, or the other dietary lipotropes, or a combination. Additionally, all of the studies with a low or "deficient" choline diet use a dose of choline of 0.7-1g choline/kg diet (Newberne, Wilson et al. 1970, Williams, Gebhardt et al. 1979, Nauss, Connor et al. 1982). The current recommendation for choline is 1g/kg diet (equivalent to 2.5 g of choline bitartrate/kg diet) during periods of growth, pregnancy and lactation in rodents (Reeves, Nielsen et al. 1993). Although these diets are 68% lower in choline compared to the control diets used, there have been no studies to date that have examined the effect of a devoid choline diet on the functioning of the immune system. The use of a choline devoid diet would examine if endogenous synthesis is sufficient in meeting the needs of the immune system, without providing dietary choline, while providing significant stress on the system in a nutrient deficient state. All of the discussed animal studies used choline in the form of free choline, either from choline chloride or choline bitartrate. Rivera *et al.* (1998) suggests that providing excess choline could enhance PC synthesis and maintain proper membrane fluidity. However, there have been no investigations into providing PC compared to free choline and it is unknown if feeding different forms of choline would have

similar effects on immune function as previously discussed. Thus, the effect of a choline-devoid diet and the potential differential effects of the forms of choline in the maternal diet on offspring immune development warrant further investigation.

1.4.2 Human studies

In humans, the effects of low choline intake have been described for organs including the brain (Shaw, Carmichael et al. 2004), liver (Zeisel, Da Costa et al. 1991, IOM 1998) and muscle (da Costa, Badea et al. 2004) with few studies examining the effects on the immune system. A full summary of the human studies examining the role of choline in immune function is presented in **Table 1-5.**

Similar to what has been demonstrated in rodents (James and Yin 1989), when adult men and women were fed a diet low in choline (50 mg/70 kg body weight) for 42 days, there was higher amounts of caspase-3, a marker of cell apoptosis, and DNA damage in peripheral lymphocytes (da Costa, Niculescu et al. 2006). Cell apoptosis also occurs in other cell types including hepatocytes (Albright, Liu et al. 1996) and fetal neurons (Yen, Mar et al. 2001) when a choline-devoid diet was fed, thus providing indirect evidence that choline is essential for lymphocyte survival. In addition, apoptotic markers in lymphocytes were associated with other indices of choline deficiency, such as increased serum levels of creatine phosphokinase, a measure of muscle damage and fatty liver, assessed by MRI (da Costa, Niculescu et al. 2006). However, although this study demonstrated that lymphocyte apoptosis was increased, there were no functional measures of lymphocytes that would indicate if the lymphocytes were functionally impaired by the low choline diet. Detopoulou et al., 2008, examined the association between inflammatory markers and dietary choline intake in a cross-sectional study. In healthy adult men and women, with no history of cardiovascular disease, choline

intake below 250mg/day was associated with higher plasma concentrations of CRP, IL-6 and TNF-α compared to adults consuming choline above 310 mg/day, suggesting that low choline intake may be associated with higher levels of systemic inflammation. However, higher choline intake was also associated with higher consumption of fruits and vegetables and legumes per week, which also have been demonstrated to be independently negatively associated with concentrations of inflammatory markers (including IL-6 and TNF-α)(Holt, Steffen et al. 2009). Although this was a large cohort with a large power to assess differences in intake, diet was assessed by semi-quantitative FFQ. There are limitations associated with the FFQ dietary recall method, in particular the concern that estimates from FFQ has been demonstrated to over-estimate choline intake compared to a estimates from 24-hour dietary recalls (Coathup, Wheeler et al. 2015). Additionally, this study did not account for the choline content in foods commonly consumed in Greece, and relied on choline estimates from the USDA choline database. Based on the use of an FFQ and limited choline data available, estimated intake may not be a good estimation of intake in this population and is a concern for interpretation of the study given the small range in the teriles of choline intake.

In humans, one study has examined the effect of choline supplementation during pregnancy and lactation on the epigenetic state and expression of components of the hypothalamic-pituitary-adrenal (HPA) axis (Jiang, Yan et al. 2012). The HPA axis is involved in communication with and regulation of the immune system and in the stress response (Tsigos and Chrousos 2002). Pregnant women were supplemented with 930 mg or 480 mg of choline per day as choline chloride for 12 weeks beginning in the third trimester. Women consuming the high choline diet had decreased expression of corticotropin releasing hormone (CRH), a main regulator of the HPA axis, in the placenta. Cord plasma cortisol concentrations and cord leukocyte CRH methylation was lower in babies from women consuming the high

choline diet (Jiang, Yan et al. 2012). Cortisol is the main glucocorticoid produced from the HPA axis, known to suppress immune responses and induce stress (Silverman, Pearce et al. 2005). This study suggests that a higher maternal choline intake may lower fetal and neonatal cortisol concentrations and activation of the HPA axis, which may reduce the stress-induced vulnerability to infection and illness (Marchetti, Morale et al. 2001). Although this study suggests that cord leukocyte CRH methylation may be lower when a maternal diet high in choline is consumed, this study focuses on fetal development, not the critical period in early postnatal life. Furthermore, this study mentions that higher maternal choline intake may reduce the stress-induced vulnerability to infection, yet no outcomes were examined to measure this. It is also unknown if the same outcomes would have been achieved had the women been supplemented with choline in other forms, such as PC.

Overall, the limited number of human studies suggests that low choline intake, below what is currently recommended, may negatively affect the functioning of the immune system, increasing lymphocyte apoptosis (da Costa, Niculescu et al. 2006) and systemic inflammation (Detopoulou, Panagiotakos et al. 2008). Dietary choline intake exceeding recommendations (930mg/d) may confer benefits to both mother and infant in reducing activation of the HPA axis and stress-induced illness. However, as the limitations of each of these studies discussed, none of these studies examine the role of dietary choline on development in the early postnatal period, another critical period for infant development. Further, there have been no studies in humans to date that have examined the role of dietary choline in the early postnatal period on maternal immune function, or infant immune development.

Collectively, several studies conducted in cell, animal and human models suggest that dietary choline intake may impact on immune function. Similar to what occurs in other organ systems (Zeisel, Da Costa et al. 1991, IOM 1998), low choline appears to be detrimental to the

functioning of the immune system, and ensuring sufficient intake of choline may provide a benefit. However, there have been no studies to date examining the independent effect of choline on the development and function of the immune system in young, suckled offspring. The lactation period represents a critical period of immune system development where there is rapid development, which may be sensitive to choline intake. Similarly, there have been no studies to date examining the potential differential effects of the forms of dietary choline. The majority of scientific research has focused on total choline amount, primarily providing choline as free choline in rodent diets. However, given the structural and metabolic differences between the forms of choline, the effect and comparison of different choline forms should be explored.

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Tables

Table 1-2: Summary of studies examining choline intake in women of childbearing age

Study population	Country	Demographic and anthropometric information	Dietary and supplement intake collected	Assessment tools used	Results	Reference
Healthy men (n=16) and women (n=16)	United States	Ages 18-67 81% and 50% of men and women, respectively, were Caucasian	*Ad libitum* intake of measured diets Dietary folate, vitamin B_{12}, methionine, cysteine, choline and betaine estimated	3-day food record before study, daily food intake recalls during study Analyzed by Food Processor SQL, choline and betaine manually entered	Mean total choline intake measured from 3-day food records was 398 ± 152 mg/d; 180 ± 6 mg/1000 kcal (men) and 258 ± 91 mg/d; 170 ± 6 mg/1000 kcal (women) Intake was significantly different between men and women Mean total choline intake measured from measured food record was 631 ± 157 mg/d; 220 ± 5 mg/1000 kcal (men) and 443 ± 88 mg/d; 200 ± 6 mg/1000 kcal (women)	(Fischer, Scearce et al. 2005)
Case-control cohort of pregnant women (n=864)	United States	43% and 53% of case and controls, respectively, were Caucasian 23% and 33% of case and controls, respectively, with >high school education	Dietary folate, methionine, energy, total choline and betaine estimated Supplement intake of folic acid	100-item food FFQ Validated for use in epidemiologic studies, but not validated for choline	Mean total choline intake was 377 ± 176 mg/d in NTD cases and 409 ± 179 mg/d in controls Compared to lowest quartile of intake (<290 mg/d), decreasing risk for three increasing choline intake quartiles Highest quartile of intake (>498 mg/d) had odds ratio of 0.49 relative to lowest quartile of intake	(Shaw, Carmichael et al. 2004)

Women in first trimester of pregnancy (n=16)	Jamaica	Ages 18-38 No comments on other variables	Dietary total choline Fasted plasma choline concentration	FFQ, not validated for choline Choline intake estimated from USDA database	Mean total choline intake was 279 ± 29 mg/d, with 88% of the population below recommendations Means plasma choline concentration was 8.4 ± 0.4 μmol/L	(Gossell-Williams, Fletcher et al. 2005)
Healthy women (n=125)	New Zealand	Ages 18-40 88% with post-secondary education 75% were New Zealand Europeans 69% were normal weight	Dietary energy, choline (total choline and forms) and betaine	3-day weighed food records Choline intake estimated from USDA database	Mean total choline intake was 316 ± 65 mg/d and 180 mg/1000 kcal 16% of participants met AI (425 mg/d) Mean free choline was 65 ± 17 mg/d and mean PC was 123 ± 32 mg/d Top food sources of choline were eggs, red meat and milk	(Mygind, Evans et al. 2013)
Men (n=85,784) and women (n=102,363) in the Multiethnic Cohort	United States	Ages 45-75 25% of men and women were Caucasian	Dietary energy, choline (total choline and forms) and betaine	Quantitative FFQ Not validated for choline	Mean total choline intake was 372 ± 187 mg/d; 337 mg/d energy adjusted (men) and 304 ± 153 mg/d; 333 mg/d energy adjusted (women) Mean free choline was 93 ± 56 mg/d (men) and 75 ± 39 mg/d (women) Mean PC was 184 ± 104 mg/d (men) and 149 ± 85 mg/d (women) 14% of men and 16% of women met AI Mean choline intake was significantly different races/ethnicities	(Yonemori, Lim et al. 2013)

Women <16 weeks gestation (n=386)	Canada	82% with post-secondary education or greater 45% were Caucasian	Dietary folate, vitamin B_{12}, vitamin B_6, methionine, energy, choline (total choline and forms) and betaine estimated Supplement intake of folic acid, vitamin B_{12}, vitamin B_6	Semi-quantitative Block FFQ Validated for folate, vitamin B_6, vitamin B_{12} but not validated for choline	Mean total choline intake was 306 ± 127 mg/d in early pregnancy and 302 ± 122 mg/d in late pregnancy Mean free choline was 77 ± 28 mg/d and mean PC was 49 ± 22 mg/d 13% of women met AI None of the prenatal multivitamins contained choline	(Masih, Plumptre et al. 2015)
Healthy women (n=64)	United Kingdom	Mean age 26 ± 4 57% with post-secondary education or greater 84% were Caucasian	Dietary intake of folate, vitamin C, carotenoids, total choline and betaine Supplement intake of folic acid and vitamin C	FFQ validated for folate Average of three 24-h recalls	Median total choline intake measured from FFQ was 285 mg/d (IQR 221-355 mg/d) Median total choline intake measured from 24-h recalls was 255 mg/d (IQR 201-331 mg/d) FFQ overestimated choline by a mean of 15 mg/d with correlation coefficient of 0.34	(Coathup, Wheeler et al. 2015)
Pregnant women (n=990)	Latvia	Age range 18-45	Total dietary choline	Two 24-h recalls Choline intake from USDA database	Median total choline intake was 330 mg/d (200-592 mg/d) Top food sources of choline in all females in study were meat and meat products, milk and dairy products and egg and egg products	(Vennemann, Ioannidou et al. 2015)

Table 1-3: Summary of cell culture studies examining the role of choline on parameters of immune function

Cell type	Choline concentrations used	Incubation length	Immune parameters measured	Results	Reference
CCT-α deficient macrophages isolated from mice	100 μM 16:0-lysoPC	18 hours	Cytokine production following LPS stimulation Immunohistochemistry staining of intracellular TNF-α and IL-6	Secretion of TNF-α and IL-6 was ↓ in CCT-α macrophages LysoPC restored cytokine section by macrophages	(Tian, Pate et al. 2008)
Human peripheral resting T lymphocytes	0, 10, 30, 50, 100 μM [C^{14}] 16:0-lysoPC	16-30 hours (+6 hours with [C^{14}]thymidine)	IL-2Rα expression Lymphocyte proliferation ([C^{14}]thymidine incorporation)	Increasing concentrations of lysoPC ↑ number of T cells expressing IL-2 receptor, with maximum effect at 100 μM lysoPC 50 μM lysoPC ↑ thymidine incorporation	(Asaoka, Oka et al. 1992)
Peripheral blood mononuclear cells (PBMC) isolated from human buffy coats	Doses of 0, 5, and 25 μM of lysoPC from egg yolk, 16:0-lysoPC or platelet-activating factor	72 hours	Cytokine secretion Number of IFN-γ producing cells Number of Ig producing cells	LysoPC induced IFN-γ secretion and the number of IFN-γ producing cells from PBMC LysoPC ↑ number of Ig producing cells (IgG, IgA and IgM), most effective at 5 μM	(Huang, Schafer-Elinder et al. 1999)

Table 1-4: Summary of animal studies examining the role of choline on parameters of immune function

Animal and age	Choline dose used	Length of feeding	Immune parameters measured	Results	Reference
Weaned, female rats and male offspring	1) Deficient (0.7g choline from choline Cl, 1g methionine, 0g B_{12}/kg diet) 2) Marginal (0.7g choline from choline Cl, 1g methionine, 45µg B_{12}/kg diet) 3) Moderate (2.2g choline from choline Cl, 3g methionine, 0g B_{12}/kg diet) 4) Control (2.2g choline from choline Cl, 3g methionine, 45µg B_{12}/kg diet)	Dams fed for 3 months prior to pregnancy Offspring fed either mothers diet or control diet from weaning to 100 days post-weaning	Response to *Salmonella typhimurium* infection (survival, WBC count)	↓ birth and weaning weights and ↑ mortality rates following infection in deficient and marginal groups Switch to control diet at weaning ↑ body weight, but no effect on mortality rate Regardless of post-weaning diet, pups from deficient maternal diet had lowest WBC count	(Newberne, Ahlstrom et al. 1970)
Weaned male Sprague-Dawley rats	1) Control (3g methionine, 18mg folate and 2.2g choline from choline Cl/kg diet) 2) Folate deficient (3g methionine, 0 mg folate and 2.2g choline from choline Cl /kg diet) 3) Methionine-choline deficient (1g methionine, 18 mg folate and 0.7g choline from choline Cl/kg diet)	3 weeks, 3 months or 12 months	Lymphocyte proliferation ([^{3}H]thymidine uptake) following PHA, ConA and PWM stimulation RNA and protein synthesis following PHA, ConA and PWM stimulation ([^{3}H]uridine and [^{3}H]leucine uptake Spleen and liver histology following *Salmonella typhimurium* infection	No difference in anthropometric measurements between groups at all ages ↓ cellularity in follicles of spleen in methionine-choline deficient rats ↓ spleen lymphocyte proliferation following ConA and PHA stimulation in folate and methionine-choline deficient rats at 3 weeks and 3 months, but not at 12 months ↓ MLN lymphocyte proliferation with folate or methionine-choline deficient diet only at 3 weeks	(Nauss, Connor et al. 1982)

Female WL rats and their offspring	1) Control (3g choline and 4.5g methionine/kg diet) 2) Marginal methionine-choline (MC) deficient (1g choline and 2.5g methionine/kg diet); fed during gestation, lactation or both 3) Control diet in post-weaning period	Gestation to 5 months of age	Pup survival following *Salmonella typhimurium* infection [^{3}H]uridine uptake [^{3}H]thymidine uptake following PHA, ConA and PWM stimulation	Mortality ↑ for all deficient groups (deficient during gestation, lactation or both) at 7-10 after infection ↓ response to ConA by splenic lymphocytes in group deficient during both gestation and lactation) Rats fed CM deficient diet during lactation had the most significant ↓ in ConA and PHA response (↓[^{3}H]thymidine uptake)	(Williams, Gebhardt et al. 1979)
Young male Fischer 344 rats, (90-110g)	1) Control diet (6 g methionine, 2.2 g choline from choline Cl and 2mg folate/kg diet) 2) MCFD devoid diet (0g methionine, choline or folate) 3) Folate devoid diet, FD (6 g methionine and 2.2 g choline from choline Cl/kg diet, 0g folate) 4) Methionine and choline devoid diet, MC (2 mg/kg folic acid)	Fed *ab libitum* for 3 weeks	Splenocytes isolated and stimulated with PHA and measurements assessed DNA strand breakage [H^3]thymidine and [6^{3}H]deoxyuridine incorporation Intracellular NAD content	DNA strand breakage was highest in MCFD group [H^3]thymidine incorporation was ↑ and [6-^{3}H]deoxyuridine incorporation was ↓ in devoid diet groups NAD levels were ↓ in devoid diet groups, with negative correlation between NAD levels and DNA strand breakage	(James and Yin 1989)
2 month old female Wistar rats	1) Choline devoid (CD) (0 g choline) 2) Control (2.6g choline from choline Cl/kg)	Fed *ab libitum* for 2 months	Antibody production and DTH response following immunization with SRBC or OVA Cell proliferation in	↓ SRBC antibody and IgG production in CD (shift in Th1 response) ↓ DTH response to OVA ↓ cell proliferation in response to	(Courreges, Benencia et al. 2003)

			spleen after ConA or LPS stimulation (MTT assay)	ConA stimulation in CD group, no difference with LPS stimulation	
Female Sprague-Dawley rats (250-275g)	1) Control (1.5g choline from choline Cl/kg diet) 2) Choline supplemented (4.5g choline from choline Cl/kg diet) 3) Glycine and choline supplemented (5g glycine and 4.5g choline from choline Cl/kg diet)	Rats were fed diets for 3 days	Survival after LPS injection Cytokine secretion in serum or in supernatant of cultured alveolar macrophages AST levels Lung pathology	↑ survival to 100% after LPS injection with choline supplementation Lung pathology was improved by choline (↓cellularity, alveolar filling and inflammation) Choline ↓ TNF-α secretion by alveolar macrophages and AST levels in serum	(Rivera, Wheeler et al. 1998)
Female Sprague-Dawley rats	1) Choline devoid (D) (0g choline/kg diet) 2) C1 (1g choline from choline bitartrate/kg diet) 3) C2.5 (2.5g choline from choline bitartrate/kg diet) 4) C6 (6.2g choline from choline bitartrate/kg diet)	Rats were fed diets for 21 days of lactation	Spleen immune cell phenotypes Cytokine production following ConA, LPS or CD3/CD28 stimulation	D and C6 dams had lower body and pup weight D spleens had a lower proportion of CD28+ T cells and higher proportion of CD8 cells expressing CD25, CD152 and CD71 D dams produced ↓ cytokines following stimulation C6 dams produced ↑ cytokines following stimulation	(Dellschaft, Ruth et al. 2015)

Table 1-5: Summary of human studies examining the role of choline on parameters of immune function

Study design	Study population	Choline dose used	Immune parameters measured	Results	Reference
Randomized control trial	Men (n=31) and women (n=35) ranging in age from 18-70	1) First received 500 mg/70 kg bodyweight) (control diet) for 10 days 2) Control diet or CD (50 mg/70kg) diet for 42 days 3) Control diet for 40 days	Lymphocyte DNA damage and apoptosis	65% of subjects developed organ dysfunction (assessed by serum creatine phosphokinase) and fatty liver (assessed by MRI) on CD diet ↑ capsase-3 (marker of cell apoptosis) in lymphocytes of subjects with organ dysfunction after CD diet At the end of CD period, all subjects had lymphocyte DNA damage	(da Costa, Niculescu et al. 2006)
ATTICA study, cross-sectional survey	Men (n=1528) and women (n=1514)	None	Plasma levels of inflammatory markers Diet assessed by semi-quantitative FFQ	Mean choline intake was 291 mg/d (men) and 285 mg/d (women) Higher choline intake associated with ↑ fruit, veggies, legumes and red meat consumption per week Participants who consumed >310 mg/day choline had 22% lower IL-6, 6% lower TNF-α	(Detopoulou, Panagiotakos et al. 2008)
12 week randomized control trial	Pregnant women in third trimester (26-29 wks; n=24)	1) 480 mg choline/day (100 mg choline Cl/day) 2) 930 mg choline/day (550 mg choline Cl/day) Meals were provided	Blood samples taken at baseline and 12 weeks Maternal and umbilical cord plasma cortisol concentrations DNA methylation	↑ placental promoter methylation of cortisol-regulating genes, CRH and glucocorticoid receptor in high choline group ↓ placental CRH transcript abundance in high choline group	(Jiang, Yan et al. 2012)

CHAPTER 2: Research plan

2.1 Rationale

Choline is an essential nutrient necessary for the normal function of rapidly growing cells (Li and Vance 2008). Choline requirements increase during pregnancy and lactation to meet the demands by the fetus and infant for rapidly dividing and developing tissues, and to account for the depletion of maternal stores (Ilcol, Ozbek et al. 2005, Caudill 2010). Choline is not commonly included in prenatal multivitamins, therefore must be obtained primarily through the diet. Currently, the USDA Database for the Choline Content of Common Foods, Release 2 is the only available food composition database to estimate choline intake from dietary records. Although this database contains the choline composition of over 600 foods, estimating dietary choline is challenging when the choline content of commonly consumed regional foods are absent. Of the few studies examining dietary choline intake, studies in women of childbearing age (Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013), results indicate that that the majority of non-pregnant women are not meeting daily choline recommendations. Despite increased recommendations and reports of low intake in non-pregnant women, little is known about the dietary patterns and choline consumption of pregnant and lactating women.

The early postnatal period is also critical for the development of the immune system in the infant (Perez-Cano, Franch et al. 2012). There is a large amount of cellular expansion and development that occurs within the immune system, therefore it is hypothesized that choline plays a critical role in the development and function of the immune system. Adequate choline intake appears to play a role in the functioning of the adult immune system (Rivera, Wheeler

et al. 1998, Mehta, Singh et al. 2010) and insufficient choline intake appears to have detrimental effects of the functioning of an effective immune system in older animals (Newberne, Wilson et al. 1970, Courreges, Benencia et al. 2003) and adult humans (Detopoulou, Panagiotakos et al. 2008). However, the essentiality dietary choline on immune system development and function during the early postnatal period has not been examined. Furthermore, there has been no research examining the roles of different dietary forms of choline on the immune system. Due to the metabolic differences that exist between the forms of choline, it is likely that they will have differential affects on immune system development and function.

2.2 Objectives and hypotheses

The overall objective of this research was to describe pre- and early postnatal maternal choline intake and examine the consequences of consuming low dietary choline, and the differential effects of form, on offspring immune development. To address this overall objective, two specific and several sub-objectives and their hypotheses were established as follows:

1. The first objective of this research was to use the comprehensive choline database to estimate prenatal and postnatal intake in a cohort of Albertan women. This objective was investigated using the following sub-objectives and hypotheses:
    a) The first sub-objective of objective 1 was to quantify the choline content of commercially available pulses and meats from Alberta in order to expand the current choline composition database.
    b) The second sub-objective was to use the expanded choline composition database to estimate prenatal and postnatal intake in a cohort of Albertan women and examine the

contribution of two food sources of choline to dietary intake. We hypothesized that during pregnancy and early postpartum, women will consume below current choline recommendations and consumption of major dietary sources of choline (eggs and milk) will assist in meeting daily recommendations.

c) The third sub-objective was to compare two methods of 24-h dietary recall used in the APrON study to estimate choline intake. We hypothesized that estimates of choline intake would be similar between interview and online 24-h recall methods.

d) The fourth sub-objective was to examine the correlation between maternal prenatal choline intake and infant birth weight. We hypothesized that there would be a positive correlation between maternal energy-adjusted total choline intake in pregnancy and infant birth weight.

2. The second objective of this research was to use an animal model to examine the effect of maternal choline, both amount and form, on the development of the infant's immune system. This objective was investigated using the following sub-objectives and hypotheses:

   a) The first sub-objective of objective 2 was to examine the effect of a maternal diet devoid in choline on offspring immune system development. We hypothesized that a maternal diet devoid of choline would be insufficient in supporting the development of the offspring's immune system, with lasting effects on the offspring's immune system, even with a choline-sufficient diet post-weaning.

   b) The second objective was to examine the differential effects of the two major forms of choline in the maternal diet on offspring immune development. We hypothesized that the form of choline, phosphatidylcholine compared to free choline, in the maternal diet will differentially affect immune development in the offspring.

c) The third objective was to elucidate a potential mechanism of phosphatidylcholine on immune function modulation. We hypothesized that providing phosphatidylcholine in the maternal diet would increase the availability of phosphatidylcholine for membrane synthesis and promote immune cell expansion and proliferation, resulting in greater immune system maturation.

2.3 Chapter format

The objectives and hypotheses stated above were tested in a series of studies. These studies were organized into thesis chapters that have been submitted and/or accepted for publication as individual manuscripts.

Chapter 3 reports the choline composition (total and forms) of 32 varieties of pulses commercially available in North America in order to expand the current food composition database available for dietary choline. The effect of cooking on pulse choline concentration and the potential contribution of pulses to dietary choline intake was examined. Objective 1 and sub-objective 1(a) were addressed in this chapter.

Chapter 4 reports the choline composition of twenty meat samples available in Alberta in order to expand the current food composition database available for dietary choline. The choline content of analyzed meat and meat present in the current food composition database (USDA) was compared, in addition to the correlation between total choline and fat content in meat samples. Objective 1 and sub-objective 1(a) were addressed in this chapter.

Chapter 5 examines dietary choline intake of the first cohort of women in the APrON study using the reported data from Chapters 3 and 4 to expand the current choline composition database (USDA database). Major food sources of choline were described, and the contribution of some frequently consumed food sources of choline to overall daily intake were

identified. This is the first study to describe choline intake in women during pregnancy and early postpartum. Objective 1 and sub-objective 1(b) were addressed in this chapter.

Chapter 6 compares two methods of dietary assessment used in the APrON study in the estimation of choline intake. The two methods were used to estimate dietary choline in cohorts two and three of women in the APrON study and the association between maternal choline intake in pregnancy and infant birth weight was examined. This is the first study to examine the agreement between two methods of 24-h recall in the estimation of dietary choline in women of childbearing age. Objective 1 and sub-objectives 1(c) and 1(d) were addressed in this chapter.

Chapter 7 examines the effect of a choline-devoid maternal diet on immune system development and function in the suckled offspring and in offspring later in life in a rodent model. This is the first study examining the programming effects of a maternal devoid choline diet on offspring immune function. Objective 2 and sub-objective 2(a) were addressed in this chapter.

Chapter 8 examines the effect of two major forms of choline in the maternal diet, phosphatidylcholine and free choline, on offspring growth and immune system development in a rodent model. Additionally, a potential mechanism of immune system modulation by phosphatidylcholine was examined using an *in vitro* model. This is the first study to demonstrate that a maternal diet provided choline as phosphatidylcholine, compared to free choline, differentially affects immune development and function in offspring. Objective 2 and sub-objectives 2(b) and 2(c) were addressed in this chapter.

Chapter 9 summarizes results from each objective and hypothesis with an overall discussion and directions for future research.

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CHAPTER 3: Total choline and choline-containing moieties of commercially available pulses[1]

3.1 Introduction

Choline is needed in the body for a variety of critical processes including the synthesis of the neurotransmitter acetylcholine, lipid transport by lipoproteins and methyl group donation (via the choline metabolite, betaine) (Zeisel 2006). The IOM Standing Committee on the Scientific Evaluation of Dietary Reference Intakes has set current dietary recommendation for choline as AI values, 550 mg/d for men and 425 mg/d for women (IOM 1998). The recommendation increases during pregnancy and lactation to 450 mg/d and 550 mg/d, respectively (IOM 1998). There is emerging epidemiological data estimating dietary choline intake in populations worldwide including North America (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Chiuve, Giovannucci et al. 2007, Guerrerio, Colvin et al. 2012, Yonemori, Lim et al. 2013), Jamaica (Gossell-Williams, Fletcher et al. 2005), the Netherlands (Dalmeijer, Olthof et al. 2008), New Zealand (Mygind, Evans et al. 2013) and Norway (Konstantinova, Tell et al. 2008). In the North American population, choline intake has been examined in a number of large cohorts including the Framingham Offspring Study (Cho, Zeisel et al. 2006), the Atherosclerosis Risk in Communities (ARIC) Study (Bidulescu, Chambless et al. 2007) and the Multiethnic Cohort study (Yonemori, Lim et al. 2013). All of these cohorts that have attempted to estimate choline intake report that intake is below the AI. Free (unesterified) choline, phosphocholine, glycerophosphocholine, phosphatidylcholine,

[1] A version of this chapter has been published: Lewis ED, Kosik SJ, Zhao Y-Y, Jacobs RL, Curtis JM, Field CJ. (2014) Total choline and choline-containing moieties of commercially availabile pulses. *Plant Foods for Human Nutrition*, 69(2): 115-121.
[2] Content in this chapter was presented at the Alberta Institute of Agrology, Banff, AB, April 2015

lysophosphatidylcholine and sphingomyelin are the different choline-containing moieties forms that contribute to total dietary choline. In a rodent model, the forms of choline have been shown to differ in absorption and metabolism (Cheng 1996), suggesting that an understanding of the different forms of choline rather than only total choline in a diet is needed.

Prior to 2004, a particular challenge in estimating choline intake was the absence of food composition information. In 2004, the USDA released the Database for the Choline Content of Common Foods (Release 1) which was later updated in 2008 (Release 2) (Patterson, Williams et al. 2008). The second release of the USDA database included betaine and total choline and forms (free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine and sphingomyelin) for 634 foods, grouped into 22 food categories. Although this database serves as a source of choline composition for a wide variety of foods, other commonly consumed foods that provide dietary choline are not contained in the database. Specifically, in the "Legume and Legume Products" food category there is currently only choline composition data available for seven bean samples; other pulse varieties consumed in North America are absent from this database. It has also been reported that the major choline-containing moieties content of vegetables can vary with cooking due to enzymatic activity (Zeisel, Mar et al. 2003) and there is limited data on the choline content after cooking. Using the available data in the USDA database, pulses contain a mean of 49 mg total choline per 100 g, with major choline-containing moieties being free choline and phosphatidylcholine. A serving (3/4 cup, 175 mg, according to Canada's Food Guide) from this food category would provide approximately 20% of the recommended daily intake.

The objectives of the study described in this report were to 1) quantify the choline-containing moieties and the total choline content of a variety of pulses that are commercially

available in North America and 2) use the expanded compositional database to determine the potential contribution of pulses to dietary choline intake.

3.2 Materials and Methods

3.2.1 Materials

L-α-Phosphatidylcholine (from egg yolk, ≥99%), sphingomyelin (from egg yolk, >95%), choline chloride (>98%), choline-trimethyl-d_9 (Cho-d_9) chloride, phosphocholine chloride calcium salt tetrahydrate (Sigma grade), betaine hydrochloride (>99%) were purchased from Sigma (St. Louis, MO); 1,2-distearoyl-sn-glycero-3-phosphocholine-N,N,N-trimethyld_9 (PC-d_9), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl (16:0 monomethyl-PE, MMPE), and L-α-lysophosphatidylcholine (egg, chicken) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Glycerophosphocholine was supplied by Bachem Americas Inc. (Torrance, CA). Phosphocholine-N, N, N-trimethyl-d_9 (Pcho-d_9) chloride calcium salt was purchased from C/D/N Isotopes Inc. (Quebec, Canada). HPLC-grade ammonium formate (≥99%) and formic acid were supplied by Sigma (St. Louis, MO). Acetonitrile and water were of LC/MS grade from Fisher Scientific Company (Ottawa, ON, Canada). All other solvents were of HPLC grade.

3.2.2 HILIC LC-MS/MS method

The content (mg/100 g food) of choline-related compounds including free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and betaine in samples were determined using the hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC LC-MS/MS) previously described (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). Briefly, an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (AB SCIEX, Concord, ON, Canada)

under turbospray positive mode was used to analyze standard and sample solutions. The separation was carried out on an Ascentis Express 150 mm×2.1 mm HILIC column, 2.7 μm particle size (Sigma, St. Louis, MO). The column temperature was controlled at 25°C. The mobile phase A was acetonitrile and B was 10 mM ammonium formate in water at pH 3.0, adjusted using formic acid. The gradient was as follows: 0–0.1 min, 8% B; 0.1–10 min, from 8% to 30% B; 10–17 min, 95% B; and then back to 8% B at 17.1 min for column re-equilibrium prior to the next injection. The flow rate of mobile phase was 400 μl/min for the period from 20 min to 27 min and 200 μl/min for all other periods. The injection volume was 2 μl and the cycle time was 30 min/injection. Nitrogen was used as curtain gas, nebulizing gas and drying gas. Several scan modes, including precursor ion scan, neutral loss scan and multiple reaction monitoring were used in order to quantify the target compounds, as described elsewhere (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). A valve was programmed by the data system to divert the LC effluent to waste before and after the selected retention time window from 2.5 min to 19 min. All other instrumental parameters were the same as previously reported (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012).

3.2.3 Sample preparation and extractions of phospholipids from pulses

To provide a more comprehensive table of the choline values of 32 samples of pulses were selected. To determine if the choline content of a pulse varied with cooking, the pulses were analyzed after different preparation methods including raw, canned and boiled. The analysis of 3 pulse varieties (kidney, pinto and soybean) was included for comparative purposes as these pulses are already included in the 2008 USDA Database for the Choline Content of Common Foods. The choline content of an additional 17 pulse food items that were not included in the USDA database were analyzed. Dry pulses, canned pulses and cooked

pulses were purchased from local supermarkets. Dry pulses were ground and about 12 g of each was freeze dried. Canned pulses were drained, ground and freeze-dried. For measurements on cooked pulses, either dry pulses or canned pulses were cooked following the directions on the package. Cooked pulses were drained, ground and freeze-dried. The extraction procedure was described previously (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). Briefly, 100 mg of freeze-dried sample was homogenized in 2 mL of extraction solvent (chloroform:methanol:water, 1:2:0.8, v/v/v) for 5 min using a Polytron PT 1300D homogenizer at 10,000 rpm (Kinematica AG, Switzerland), then centrifuged at 1106 RCF (relative centrifuge force) for 5 min. The supernatant was collected and the extraction procedure repeated twice. The combined supernatant was diluted with methanol to a final volume of 10 mL in a volumetric flask. Around 1mL of this solution was filtered using syringe filter into a 2-mL HPLC vial and stored at -20°C prior to analysis.

After analysis was completed, the pulses were assigned a nutrient database number that corresponds with the USDA Nutrient Database for Standard Reference. For foods with no corresponding food description in the USDA Nutrient Database (no nutrient database number), they were not assigned a specific number and identified only by descriptive name. This occurred for the lentil varieties that were analyzed (green, red and canned lentils) as the only nutrient composition available was for raw lentils and cooked (boiled) lentils, lentil variety is not specified in the USDA database.

3.2.4 The application of updated choline content values in a meal rich in pulses

To examine the application of the updated choline database, we examined the effect of substituting the choline values available in the USDA database and the values obtained in our analyses in a group of pulse-based recipes (a gift of Dr. Rhonda Bell, University of Alberta).

These recipes are currently being used in an intervention trial examining the effects of regular pulse consumption on blood lipid profiles. One serving of each recipe provides 120g of pulses, either beans or peas. The recipes includes in the analysis were for vegetable soup and tortellini soup. The choline values available from our analysis for black beans and yellow peas were used in estimating the choline content of the bean-based vegetable soup and the pea-based vegetable soup, respectively. The choline content of canned navy beans was used to estimate the choline content of these foods based on the USDA database values because navy beans were felt to be the most comparable bean given available data. Choline values for pinto beans were available from both our analysis of choline and the USDA database and this was used as the pulse ingredient in the tortellini soup.

3.2.5 Statistical Analysis

All data are presented as means ± SD, unless otherwise indicated. Choline content from each choline-containing moiety was expressed in milligram per 100 grams of sample. A two-tailed paired *t*-test was used to compare changes in choline-containing moieties from cooking. A P-value of <0.05 was considered statistically significant for all analyses.

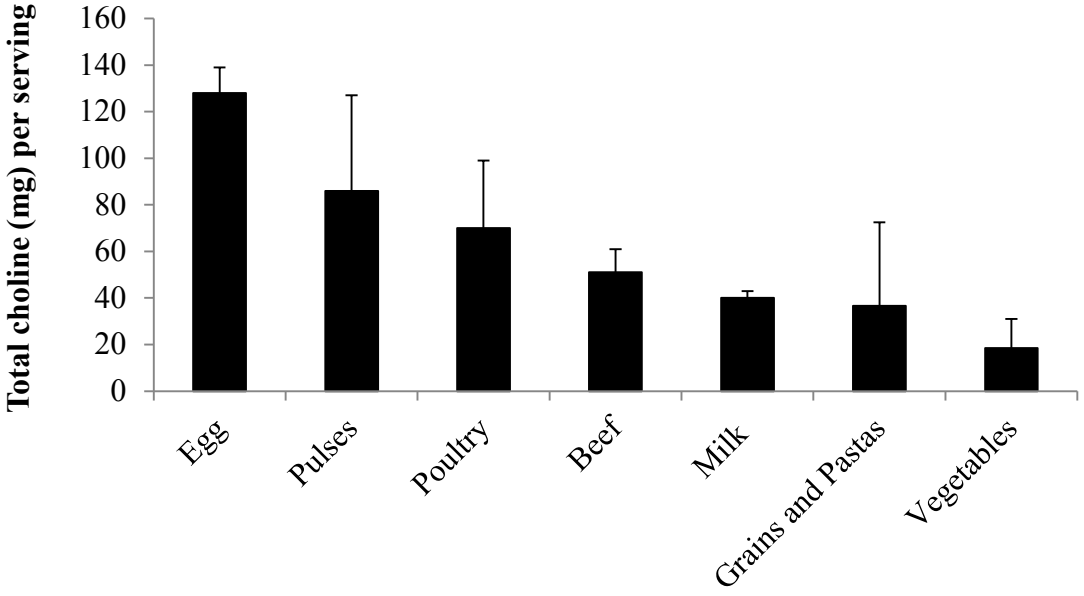
3.3 Results and Discussion

3.3.1 Choline content of commercially available pulses in Alberta

A total of 32 samples of commonly consumed pulses were analyzed for choline and choline moiety content. Included in the table is the USDA nutrient database number (if available), food description, moisture content (5%), choline content arising from each choline-containing moiety (mg) and total choline (mg) per 100 g of sample (**Table 3-1**). Many health organizations recommend pulse consumption as part of a healthy diet (Leterme 2002) and

regular pulse consumption has been associated with reduced risk of cardiovascular disease and risk factors of metabolic syndrome (Anderson, Smith et al. 1999, Anderson and Major 2002). In rodent studies, pulses have been shown to exhibit hypolipidemic effects (Zulet and Martinez 1995) and a meta-analysis of clinical trials examining the effect of pulses on serum lipoproteins concluded that consumption of approximately 100 g of uncooked pulses/day resulted in lowering of serum cholesterol (Anderson and Major 2002). In addition to being associated with positive effects on risk factors of cardiovascular disease and metabolic syndrome, pulses may also be a major dietary source of choline when consumed as a meat alternative. A 100 g daily serving of pulses, shown to positively effect cardiovascular disease risk, provides 15% of the current daily choline recommendation. A serving of pulses provides more dietary choline compared to many other reported major dietary sources of choline reported (dairy and meat) (Bidulescu, Chambless et al. 2007, Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013) (**Figure 3-1**).

Figure 3-1: Comparison of total choline in one serving of selected food items and food categories[1]



[1]Food categories corresponding to the USDA Choline Content of Common Foods and one serving size corresponding to Canada's Food Guide, with the exception of eggs which is half a serving [1]Pulses including kidney, navy, black and soybeans, one serving equal to 175 g (3/4 cup); Poultry including chicken and turkey, one serving equal to 75 g (½ cup); Beef excluding beef liver, one serving equal to 75 g (½ cup); Grains and Pastas including rice, bulgur and buckwheat, egg noodles and spaghetti, one serving equal to 125 g (½ cup); Milk including skim, 1%, 2%, whole and chocolate varieties, one serving equal to 250 g (1 cup); Vegetable including many varieties, with one serving equal to 125 g (½ cup).

3.3.2 Comparison of the choline content between the raw and cooked form of pulses

To examine changes with cooking, the proportion of choline arising from each of the choline-containing moieties in raw and cooked preparation methods as a percentage of total choline is presented in **Table 3-2**. Absolute values of each choline-containing moiety are included in **Appendix 1**. In order to directly compare raw and cooked pulses, the choline contents of raw pulses were adjusted for moisture content and determined on a dry weight basis. Cooking significantly reduced ($P<0.05$) the relative percentage water- soluble free choline to total choline in each of the pulse varieties (-9.7 ± 2.4%), with the exception of

soybeans and green lentils. Cooking of soybeans or green lentils resulted in small increases in the relative abundance of choline that is present as free choline by about 2% in each case. Correspondingly, the contribution of phosphatidylcholine to total choline significantly increased ($P<0.05$) with cooking pulses, with the exception of soybeans and green lentils. Cooking of soybeans and whole green lentils decreased the percentage of choline arising from phosphatidylcholine by approximately -12% and -4%, respectively (see Table 3-2). For all pulse varieties, the contribution of lysoPC to total choline increased by 1.3 ± 0.2% ($P<0.05$) with cooking but it still remains a minor component of the overall choline content. The present study demonstrated that the cooking of pulses and discarding the cooking water resulted in a loss of water-soluble compounds such as free choline. This is consistent with the work on vegetables by Zeisel, Mar et al. 2003. It remains unclear as to why anomalous results were obtained for soybeans and green lentils, for which cooking results in a small increase in the relative contribution of free choline to total choline content along with a corresponding decrease in the relative contribution of phosphatidylcholine to total choline content.

3.3.3 Application of choline content of pulses to a meal rich in pulses

To determine the value of the expanded database on pulses, the choline content of a meal containing pulses as the main protein source was used. We examined the difference in choline content that resulted in substitutions of the nutrient values for pulses between the USDA database and our analyzed pulses for two recipes (vegetable soup and tortellini soup). **Table 3-3** lists the name of the pulse-containing meal used in this study, the pulse in the meal and the estimated choline content for one serving of the recipe. The estimate includes the choline content of the pulses and other food ingredients (calculated using USDA database).

For two recipes, the USDA database overestimated total choline content by 23% and 6%. For a recipe using the same pulse (NDB No 16043), the USDA database underestimated total choline content for one serving by 30%. These results suggest that when pulses are a large part of a meal or diet, the use of accurate food composition data should be used. Pulse consumption varies between regions (Phillips 1993) and in Western countries consumption is considered low with consumption less than 3.5 kg/ capita per year (Mudryj, Yu et al. 2012). However, in other populations with high pulse consumption (Africa, South America and India) where intakes range from 5 kg/ capita to 40 kg/ capita per year (Mudryj, Yu et al. 2012; Ofuya and Akhidue 2005, Leterme 2002) pulses could make a major contribution to choline intake and the expanded database would be valuable in estimating intake. This was illustrated by the analysis of the vegetarian (pulse and pea) meals from the clinical trial, taking place at the University of Alberta. Using meals from a study that relies heavily on pulses (120g pulses per serving) we observe a difference in the estimation of total choline based on available choline content from the USDA database and our values. To our knowledge there have been no published studies examining choline intake in vegetarian or vegan populations.

3.4 Conclusions

Our research adds valuable choline composition data for 32 pulses. A common limitation of studies examining dietary choline intake is the lack of choline composition values available for certain foods specific to the population. This occurred in a population of women from New Zealand (Mygind, Evans et al. 2013), a multiethnic population in the United States (Yonemori, Lim et al. 2013) and a population of pregnant women from Jamaica (Gossell-Williams, Fletcher et al. 2005). The USDA Database for the Choline Content of Common Foods includes 634 foods, and the expansion of the current choline composition database will

allow researchers to more accurately estimate usual intake and work towards establishing EAR and RDA values for choline.

In conclusion, the generation of accurate and comprehensive food composition data is essential for estimating usual dietary intake in populations that consume pulses as meat alternatives. The expanded pulse database in this manuscript will be useful in future research and in positioning pulses as a source of dietary choline.

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Table 3-1: Choline content of commercially available pulses

NDB No[1]	Description	Moisture	Free Cho	GPC	Pcho	Ptd Cho	Lyso PC	SM	Total Cho[2]
		%	mg choline moiety/ 100 g of food						
16316[3]	Beans, black, canned	68.8	14.8	1.8	0.5	14.9	0.9	0.0	32.9
16015[3]	Beans, black, canned, cooked	66.7	13.7	1.7	0.4	14.3	0.9	0.0	31.0
[5]	Beans, black, cooked	63.8	14.8	3.1	0.5	24.4	0.9	0.0	43.7
[5]	Beans, black, raw	11.1	59.1	8.5	1.5	58.6	1.6	0.0	129.3
16358[3]	Beans, chickpeas (garbanzo), canned	69.1	5.8	1.3	0.6	23.9	3.2	0.0	34.8
16057[3]	Beans, chickpeas (garbanzo), canned, cooked	65.8	6.4	1.5	0.3	25.1	2.4	0.0	35.7
[5]	Beans, chickpeas, (garbanzo), whole, cooked	61.4	7.2	2.9	2.9	35.8	1.9	0.0	50.7
[5]	Beans, chickpeas, (garbanzo), whole, raw	7.6	23.5	5.6	1.9	88.4	3.3	0.0	122.7
16034[4]	Beans, kidney, red, canned	72.8	12.1	1.2	0.4	13.4	2.4	0.0	29.5
16071[3]	Beans, lima, raw	6.6	45.9	1.5	7.5	65.7	2.0	0.0	122.6
16146[3]	Beans, pinto, canned	70.7	8.4	0.8	2.3	11.8	1.5	0.0	24.8
[5]	Beans, pinto, canned, cooked	74.2	4.1	0.2	0.7	11.0	1.6		17.6
16043[4]	Beans, pinto, cooked	60.1	18.1	3.6	0.6	23.3	1.4	0.0	47.0
16042[4]	Beans, pinto, raw	7.2	59.9	8.0	1.3	56.3	3.0	0.0	128.5
[5]	Beans, red kidney, canned, cooked	70.8	13.8	1.4	0.3	13.4	1.6	0.0	30.5
[5]	Beans, red kidney, cooked	65.2	11.4	2.7	1.1	20.8	1.2	0.0	37.2
[5]	Beans, red kidney, raw	7.5	59.7	10.3	2.2	52.5	1.7	0.0	126.4
16045[3]	Beans, small white, raw	12.3	61.5	7.2	2.6	57.7	2.7	0.0	131.7
[5]	Beans, white kidney, canned	-	18.3	2.3	0.0	14.7	3.0	0.0	38.3

NDB No[a]	Description	Moisture	Free Cho	GPC	Pcho	Ptd Cho	Lyso PC	SM	Total Cho[b]
		%	mg choline moiety/ 100 g of food						
[5]	Beans, white kidney, canned	-	18.3	2.3	0.0	14.7	3.0	0.0	38.3
[5]	Lentils, canned	-	2.8	1.6	2.2	19.3	2.2	0.0	28.1
[5]	Lentils, green, whole, cooked	71.0	6.0	1.5	1.1	24.7	1.6	0.0	34.9
[5]	Lentils, green, whole, raw	7.4	18.4	4.4	4.2	90.9	3.2	0.0	121.1
[5]	Lentils, red, split, cooked	69.5	2.8	0.8	0.6	24.7	1.3	0.0	30.2
[5]	Lentils, red, split, raw	7.6	20.0	4.3	4.9	87.5	2.7	0.0	119.4
11813[3]	Peas, green, canned,	81.6	5.1	1.9	1.5	24.3	6.5	0.0	39.3
11811[3]	Peas, green, canned, cooked	79.7	4.9	1.9	1.6	27.6	6.6	0.0	42.6
[5]	Peas, green, split, cooked	63.8	7.8	3.6	1.4	26.2	1.5	0.0	40.5
[5]	Peas, green, whole, raw	7.9	18.1	8.4	3.0	68.0	6.3	0.0	103.8
16086[3]	Peas, yellow, split, cooked	63.7	6.6	4.0	1.1	28.2	1.4	0.0	41.3
16085[3]	Peas, yellow, split, raw	7.8	30.1	8.2	2.8	86.1	3.0	0.0	130.2
16109[3]	Soybeans, cooked	50.8	38.5	4.5	2.3	31.8	2.8	0.0	79.9
16108[3]	Soybeans, raw	6.7	71.5	10.6	3.1	66.8	3.9	0.0	155.9

[1]NDB number is a numerical code used in the USDA Nutrient Database for Standard Reference

[2]Total choline refers to the sum of free choline, GPC, phosphocholine, PC, lysoPC and SM and does not include betaine

[3]Indicates a pulse that is not included in the 2008 USDA Database for Choline Content of Common Foods where the accompanying choline values have been added to the assigned nutrient database number

[4]Indicates a pulse that is included in the 2008 USDA Database for Choline Content of Common Foods where the accompanying choline values have been modified based on analysis

[5]There was no specific NDB number for this pulse and only has description of pulse analyzed.

Table 3-2: Contribution percentage from choline-containing moieties to total choline between 100 g of raw and cooked pulses included in our analysis

Pulse	Prep[1]	Moisture[2]	Free Cho	PC	LysoPC	GPC	Pcho
		%	% contribution of choline to total choline in 100g of pulses				
Black bean	Raw	11.1	38.6	51.2	1.4	7.5	1.3
	Cooked	63.8	27.7	61.0	2.3	7.8	1.3
Chickpeas	Raw	7.6	17.1	73.8	2.8	4.7	1.6
	Cooked	61.4	11.4	75.3	4.0	6.1	3.2
Dark red kidney beans	Raw	7.5	40.2	47.3	1.5	9.2	1.8
	Cooked	65.2	24.8	60.6	3.4	7.9	3.2
Pinto beans	Raw	7.2	43.3	46.6	2.5	6.6	1.1
	Cooked	60	34.1	53.2	3.2	8.2	1.4
Soya beans	Raw	6.7	46.0	42.6	2.7	7.1	1.6
	Cooked	50.8	48.3	39.7	3.5	5.6	2.9
Green lentils, whole	Raw	7.4	11.8	78.0	2.8	3.8	3.6
	Cooked	71	13.5	74.0	4.8	4.5	3.3
Red lentils, split	Raw	7.6	13.1	76.5	2.3	3.8	4.3
	Cooked	69.5	7.1	83.7	4.4	2.7	2.1
Green peas, split	Raw	6.3	21.0	66.8	2.8	8.0	1.5
	Cooked	63.8	14.7	68.4	3.9	9.4	3.7
Yellow peas, split	Raw	7.8	18.3	70.2	2.5	6.7	2.3
	Cooked	63.7	12.5	71.2	3.6	10.1	2.7

[1]Prep refers to the preparation method used for each pulse variety. Cooked pulses were boiled prior to analysis of choline content.
[2]Choline content has been adjusted based on moisture content of 100g of sample.

Table 3-3: Comparison of the choline content between the USDA Choline Database and the expanded database (Alberta) of one serving of meat substitute recipe using pulses as the main protein source[1]

Meal[1]	Pulse Used	Database	Bet	Free Cho	GPC	Pcho	PC	Lyso PC	SM	Total Cho
			mg	mg choline moiety per serving						
Vegetable Soup	Black Beans[2]	USDA	0.6	37.5	3.0	3.0	32.2	0.0	0.0	76.3
		Alberta	0.5	28.7	2.8	3.5	23.0	1.1	0.0	59.0
Vegetable Soup	Yellow Peas[3]	USDA	0.6	37.5	3.0	3.0	32.2	0.0	0.0	75.7
		Alberta	0.5	20.2	5.5	4.3	39.7	1.7	0.0	71.4
Tortellini Soup	Pinto Beans[4]	USDA	9.3	18.2	3.9	1.7	30.2	0.0	0.6	54.5
		Alberta	9.1	28.5	6.6	2.3	31.4	1.7	0.6	71.0

[1]Each recipe used for the meal contains 120 g of beans or peas per serving
[2]For USDA database pulse used was beans, navy, mature seeds, cooked, boiled, without salt (NDB No. 16038) and Alberta database used beans, black, canned, cooked (16015).
[3]For USDA database pulse used was beans, navy, mature seeds, cooked, boiled, without salt (NDB No. 16038) and Alberta database used peas, yellow, split, cooked (16086).
[4]For USDA database and Alberta database pulse used was beans, pinto, mature seeds, cooked, boiled, without salt (16043).
[*] Indicates significant difference in total choline from values estimated using expanded choline database (includes additional analysis of pulses).

CHAPTER 4: Measurement of the abundance of choline and the distribution of choline-containing moieties in meat[1,2]

4.1 Introduction

Choline is an essential nutrient required for many metabolic processes including methyl group donation, lipoprotein metabolism, neurotransmitter synthesis and cell membrane signalling (Zeisel 2006). The current daily choline recommendations, or AI, values are 550 mg/d for men and 425 mg/d for women, with recommendations increasing during pregnancy and lactation to 450 mg/d and 550 mg/d, respectively (IOM 1998). These recommendations for total choline intake include dietary choline in all major forms including free (unesterified) choline, phosphocholine, GPC, PC, lysophosphatidylcholine and sphingomyelin (Zeisel, Mar et al. 2003). Recently, epidemiological data examining usual dietary choline intake in populations worldwide, including North America, has been presented (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Guerrerio, Colvin et al. 2012, Yonemori, Lim et al. 2013, Lewis, Subhan et al. 2014) All of these studies identify eggs, dairy, and meat as the major dietary sources of choline. Typically, estimation of dietary choline intake and identification of food sources of choline requires the use of food composition databases. The USDA released the Database for the Choline Content of Common Foods in 2004 (Release 1) then updated it in 2008 (Release 2) which contains the choline content of 634 foods and is the only choline database publically available to estimate choline intake (Patterson, Williams et al. 2008). Although this database contains a wide variety of choline containing foods, there are many

[1] A version of this chapter has been published: Lewis ED, Zhao YY, Richard C, Bruce HL, Jacobs RL, Field CJ, Curtis JM. (2015) Measurement of the abundance of choline and the distribution of choline-containing moieties in meat. *International Journal of Food Sciences and Nutrition*, 66(7): 743-748.

[2] Content in this chapter was presented at the Canadan Nutrition Society, St. John's NL, June 2014

dietary sources of choline absent from this database. In particular from the meat and meat product category, there are many meats varieties, including lamb, duck, pork and bison, that are absent from the current USDA choline database. The availability of comprehensive data on the choline composition of the widest possible range of foods is an important requirement of the expanding research effort in choline and nutrition. Recently, our group published data on the choline composition of over 30 pulse products with the objective of expanding this current food composition database (Lewis, Kosik et al. 2014). The objective of the work presented here is to quantify both the total choline content and the choline-containing moieties from a range of meat types and cuts that are available in Alberta in order to expand the current food composition database available for dietary choline. In addition, some meat samples were selected that are present in the USDA choline database to enable a comparison, given the possibility of regional differences in agricultural practices, animal breeds and conditions.

4.2 Materials and methods

4.2.1 Materials

L-α-Phosphatidylcholine (from egg yolk, ≥99%), sphingomyelin (from egg yolk, >95%), choline chloride (>98%), choline-trimethyl-d_9 (Cho-d_9) chloride, and phosphocholine chloride calcium salt tetrahydrate (Sigma grade) were purchased from Sigma (St. Louis, MO); 1,2-distearoyl-sn-glycero-3-phosphocholine-N,N,N-trimethyl-d_9 (PC-d_9), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl (16:0 monomethyl-PE, MMPE), and L-α-lysophosphatidylcholine (lysoPC) (egg, chicken) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Glycerophosphocholine (GPC) was supplied by Bachem Americas Inc. (Torrance, CA). Phosphocholine-N, N, N-trimethyl-d_9 (Pcho-d_9) chloride calcium salt was purchased from C/D/N Isotopes Inc. (Quebec, Canada). HPLC-grade ammonium formate

(≥99%) and formic acid were supplied by Sigma (St. Louis, MO). Acetonitrile and water were of LC/MS grade from Fisher Scientific Company (Ottawa, ON, Canada). All other solvents were of HPLC grade.

4.2.2 HILIC LC-MS/MS method

The content (mg/100 g food) of choline-related compounds including free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin in samples were determined using HILIC LC-MS/MS as previously described (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). Briefly, an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (AB SCIEX, Concord, ON, Canada) under turbospray positive mode was used to analyze standard and sample solutions. The separation was carried out on an Ascentis Express 150 mm×2.1 mm HILIC column, 2.7 μm particle size (Sigma, St. Louis, MO, USA). The column temperature was controlled at 25°C. The mobile phase A was acetonitrile and B was 10 mM ammonium formate in water at pH 3.0, adjusted using formic acid. The gradient was as follows: 0–0.1 min, 8% B; 0.1–10 min, from 8% to 30% B; 10–17 min, 95% B; and then back to 8% B at 17.1 min for column re-equilibrium prior to the next injection. The flow rate of mobile phase was 400 μl/min for the period from 20 min to 27 min and 200 μl/min for all other periods. The injection volume was 2 μl and the cycle time was 30 min/injection. Nitrogen was used as curtain gas, nebulizing gas and drying gas. Several scan modes, including precursor ion scan, neutral loss scan and multiple reaction monitoring were used in order to quantify the target compounds, as described elsewhere (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). A valve was programmed by the data system to divert the LC effluent to waste before and after the selected

retention time window from 2.5 min to 19 min. All other instrumental parameters were the same as previously reported (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012).

4.2.3 Sample collection, moisture content determination and extraction of phospholipids and total lipid

Twenty samples of meat were obtained from a butcher in Edmonton (Real Deal Meats, Edmonton, AB, Canada) specializing in providing meat from locally raised animals. The analysis of 4 meat samples (lean and regular ground beef, and pork shoulder and tenderloin) was included for comparative purposes to the 2008 USDA Database for the Choline Content of Common Foods. All of the meat samples were analyzed in duplicate. The meat samples were trimmed to remove excess fat and bones leaving just muscle tissue remaining and cut into small chunks. A weight of the muscle tissue to be freeze-dried was taken and placed in a tray for freeze-drying for a minimum of 60 hours. Then the freeze-dried samples were weighed and calculated for moisture content. The moisture content of the meat samples was subsequently used to convert the fat content into wet based values. The freeze-dried samples were then ground and stored at -20°C prior to phospholipid extraction and total fat content determination. The extraction procedure was described previously (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012). Briefly, 100 mg of freeze-dried sample was homogenized in 2 mL of extraction solvent (chloroform:methanol:water, 1:2:0.8, v/v/v) for 5 min using a Polytron PT 1300D homogenizer at 10,000 rpm (Kinematica AG, Switzerland), then centrifuged at 1106 RCF (relative centrifuge force) for 5 min. The supernatant was collected and the extraction procedure repeated twice. The combined supernatant was diluted with methanol to a final volume of 10 mL in a volumetric flask. Around 1mL of this solution was filtered using syringe filter into a 2-mL HPLC vial and stored at -20°C prior to analysis. The total lipid content in samples were determined gravimetrically after total solvent evaporation under vacuum in a

rotary evaporator (40°C). After analysis was completed, the meat cuts were assigned a nutrient database number that corresponds with the USDA Nutrient Database for Standard Reference. For meat samples with no corresponding food description in the USDA Nutrient Database (no nutrient database number), they were not assigned a specific number and identified only by descriptive name. This occurred for the Heritage Angus beef varieties that were analyzed as they were not found in the Nutrient Database.

4.2.4 Statistical analysis

All data are presented as means ± SD, unless otherwise indicated. The choline content from each choline-containing moiety was expressed in milligram per 100 grams of sample (wet or dry weight). Spearman correlation analyses were used to examine the association between total choline content and fat content in meat samples using SAS (v9.4, Cary, NC, USA). A percentage difference calculation was used to determine the agreement in total choline values between our measured samples and previously available data from the USDA database. Percentage difference in total choline calculated by USDA value subtracted from our data (of same NDB number), divided by mean of two values, presented as a percentage using Microsoft Excel 2011 (version 14.4.7; Microsoft Corporation, Mississauga, ON, Canada). A *P*-value of <0.05 considered statistically significant for all analyses.

4.3 Results

4.3.1 Measurement of choline content of commercially available meat samples

A total of twenty samples of various cuts of meat from Alberta, Canada were analyzed for choline and choline moiety content (**Table 4-1**). Sixteen samples analyzed were absent from the current USDA database and four samples were included for comparison. Included in Table 4-1 is the USDA nutrient database (NDB) number (if available), a description of the

food, moisture content (%), the choline content arising from each choline-containing moiety (mg), the total choline (mg) per 100 g of sample and the total choline (mg) based on a 75 g serving. The choline content was calculated based on the wet weight of each sample, as this has most relevance to dietary intake and to the previously published data (Patterson, Williams et al. 2008). Considering all of the meats analyzed in this study, for each 75 g serving of meat (as defined in Canada's Food Guide), the average total choline content was found to be 50 ± 12 mg. Consistent with previous reports, the majority of choline in the meat cuts analyzed was in the form phosphatidylcholine, contributing 82.6% ± 5.5% to total choline.

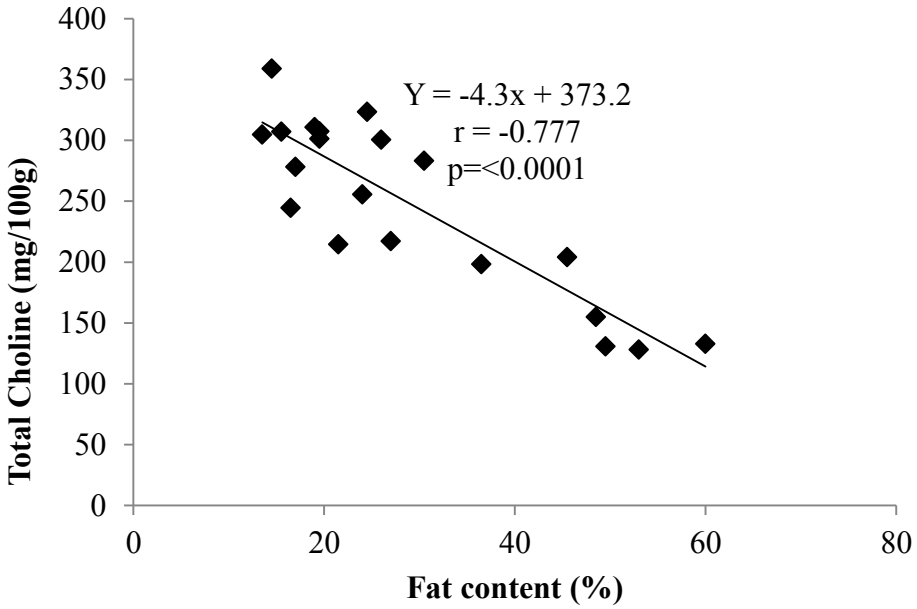
4.3.2 Agreement between samples and previously published data

Our data generally agrees well with previously published data from the USDA Database for the Choline Content of Common Foods (Patterson, Williams et al. 2008). USDA values and our data show similar phosphatidylcholine and total choline content in samples of ground beef (70% and 80% lean), with USDA values representing a percentage difference in total choline of -2.8% and 0.5%, respectively (**Table 4-2**). Greater differences were found between the USDA values for the pork samples measured, in which the total choline in pork tenderloin and pork shoulder were found to be lower by 10.9 and 17.4 mg/100g meat, respectively. For chicken thigh, although there was a difference in the preparation method (raw vs. cooked), similar values for the total choline content were measured; 58.4 mg/100g of raw meat, compared to the USDA value of 65.0 mg/100g of cooked meat. The raw chicken drumstick total choline content was found to be 82.9 mg/100g compared to the USDA value of 84.0 mg/100g for cooked meat.

4.3.3 Association between choline content and fat content between cuts of meat

In order to examine the potential correlation between the measured total choline content and the total fat content of the meat samples, the lipid content of each cut of meat was measured. A comparison of the fat content and mg of total choline content of meat samples were expressed both on a dry and wet weight basis (**Table 4-3**). It was found that when the total choline and fat content was expressed on a dry weight basis for each meat sample, there is a clear correlation between the total choline content and fat content. From **Figure 4-1** it can be seen that there is a strong negative correlation ($r=-0.777$, $p<0.001$) between the total choline content and the fat content, such that low fat cuts of meat had a higher choline content compared to high fat cuts of meat. The leaner cuts of meat analysed include chicken breast (14.5% fat), trimmed lamb chops (24.5% fat) and duck (19.5% fat) which had relatively higher total choline contents, while higher fat cuts of meat including ground bison (53.0% fat), ground beef (49.5% fat) and pork ribs (60.0% fat) were found to contain lower amounts of total choline, on a dry weight basis.

Figure 4-1: Spearman correlation[1] of total choline content and fat content in commercially available meat samples[2] (dry weight)



[1]Spearman correlation analysis was used to examine the association between choline content and fat content in meat samples.
[2]Samples were freeze-dried and then total choline content of twenty meat samples analyzed by LC-MS/MS and total fat content in meat samples were determined gravimetrically. Total Cho (total choline) refers to the sum of free choline, GPC, phosphocholine, PC, lysoPC and SM and does not include betaine.

4.4 Discussion

Many epidemiological studies have identified meats as a major dietary source of choline (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Yonemori, Lim et al. 2013, Lewis, Subhan et al. 2014), as estimated using the USDA Database for the Choline Content of Common Foods, Release 2 (Patterson, Williams et al. 2008). Since meat contributes a large proportion to dietary choline, it is important to have complete food compositional data to account for all types of meat, thus accurately reflecting their actual dietary contributions. This research adds the choline composition of twenty meat products, sixteen of which are absent from the current USDA Database for the Choline Content of Common Foods, Release 2 (Patterson, Williams et al. 2008). Our analysis includes the choline content of game meats

including elk, bison and duck, all of which are absent from the current USDA database. The meat samples examined contained a mean of 50 ± 12 mg of total choline per Canada's Food Guide serving (75 g). Consistent with their fat contents, boneless, skinless chicken breasts contained the most total choline per serving (70.8 mg/75 g) and regular ground beef contained the least amount of total choline per serving (34.6 mg/75 g). Also consistent with what is found in other animal products (Zeisel, Mar et al. 2003), we found that the form phosphatidylcholine comprised the majority of total choline in all of the meat samples, providing a mean of 82.6% ± 5.5% of the total choline. We estimated the amount of choline from lysophosphatidylcholine, a form of choline that is currently not included in the USDA database. However, in the meat samples lysophosphatidylcholine contributes only a small proportion to total choline, on average 1.5% ± 1.1% of total choline. Research has suggested the various species that choline are metabolized differently (Cheng 1996) and may have different biological functions, therefore it is important to have estimates of the intake of both total choline content as well as the different moieties of choline.

An important consideration in the evaluation of our current data is the agreement between our measured values for four cuts of meat that are included in the USDA database (Table 4-2) based on the NDB number. For fresh ground beef 70% lean (NDB 13498) and fresh ground beef 80% lean (NDB 23572) there is excellent agreement between the total choline and phosphatidylcholine values measured in our work compared to the USDA method; with the USDA being 2.8% and -0.5% different in total choline content, respectively.

In the case of fresh pork tenderloin (NDB 10060) there is good agreement between phosphatidylcholine values, but due to the higher GPC content in the USDA data, the total choline content in our data was lower by 14.4%. It was noted that the GPC content in the USDA pork tenderloin is also higher compared to other cuts of pork contained in the USDA

database (Patterson et al., 2008). A somewhat higher deviation was found between our measured choline content for fresh pork shoulder (NDB 10084) and the USDA database value. The percentage difference of total choline content was 24.8% and in this case, significant differences were evident across all of the choline containing moieties measured. The fat content of pork shoulder was high (45.5% fat) in this study (Table 4-3) and given the good agreement between analytical methods observed and the negative correlation established between fat content and choline content it seems that a reasonable explanation for this discrepancy lies in a difference between the fat content of the samples used for the two measurements. Unfortunately, the USDA database does not list the fat content of the sample so this cannot be confirmed but for such a fatty cut of meat, differences could arise due to different trimming procedures. Considering the above, the difference between the value determined in this work (61.6 mg choline/100g pork) and the USDA database value (79.0 mg choline/100g pork) is within the variation to be expected for such a comparison.

In this work, the choline content of three cuts of raw chicken were measured, and the results are listed in Table 4-1. The USDA choline database lists the choline composition of cooked cuts of chicken. Although there are no exact matches between the cuts of poultry used (raw skinless breast meat, raw drumstick meat and raw thigh meat) and the USDA database, similar cuts of chicken prepared as cooked are listed. For comparison, these are given in Table 4-3 with their associated NDB numbers, and it can be seen that for chicken drumstick meat and thigh meat there is good agreement between the measured raw values and the cooked values from the USDA database. Surprisingly, the database value for skinless breast meat is lower than the values given for thigh or drumstick meat which have considerably higher fat levels. This value is also considerably lower than that measured in this work (94.3 mg choline/100g food) compared to cooked skinless breast meat in the USDA database (62 mg

choline/100g food). It has been previously reported in vegetables (Zeisel, Mar et al. 2003) and pulses (Lewis, Subhan et al. 2014) that water-soluble compounds, mainly free choline, are lost with cooking. However, it appears in the meat samples, this loss of water-soluble compounds does not occur and free choline content is higher in the USDA values for cooked chicken samples compared to raw values. The effect of cooking on choline composition still needs to be elucidated and in future, our analysis will include measurement of cooked varieties of meat samples in order to conduct a direct comparison of poultry samples.

Our research found that meat samples with a lower fat content contained more choline on a per gram dry weight basis. For example, the lamb chops (100g sample) had a fat content of 48.5% and contained 54.5 mg of total choline, but when the lamb chops were trimmed of fat, their fat content reduced to 24.5% and total choline content was 86.0 mg per 100g wet weight sample. Thus, trimming the fat from these lamb chops resulted in a proportionate increase in the total amount of choline per fixed weight of meat. Note that the correlation between total choline content and fat content per dry weight basis was examined because there was a large variation in the moisture content of the meat samples. It appears that the choline content is correlated with lean muscle, containing a large proportion of phosphatidylcholine, which is primarily found in cell membranes. Using the dry weight of each sample ensures that the solid components (fat) could be accurately correlated, without the confounding variation in moisture content. This is of great nutritional importance as both Health Canada and the National Institute of Health recommend that when choosing meat and alternatives, lean meats and lean and extra lean cuts of meat should be selected in order to limit the intake of total fat, saturated fat, and trans fat. It should be mentioned that although lean meats appear to be higher in dietary choline compared to higher fat cuts of meats, there is emerging literature suggesting limited consumption of choline rich foods (Wang, Klipfell et al. 2011). Recently, plasma

choline metabolites, including choline and trimethylamine-oxide (TMAO) have been associated with increased risk of cardiovascular disease. It has been postulated that dietary choline is converted to TMAO, which promotes the development of various cardiovascular diseases (Wang, Klipfell et al. 2011, Tang, Wang et al. 2013, Tang, Wang et al. 2015). However, plasma choline concentrations appear to be an unreliable marker of dietary choline (Abratte, Wang et al. 2009) thus the relationship between dietary choline and TMAO needs further investigation.

4.5 Conclusions

As meat products are a major source of dietary choline, the generation of comprehensive food composition data is essential in estimating intake. This research adds valuable choline composition data to a food group that is a major source of dietary choline. Our data agrees strongly with the currently available data in the USDA Database for the Choline Content of Common Foods. We also found a strong negative correlation between fat and choline content, suggesting that lower fat cuts of meat are a better source of dietary choline. The data presented here provide additional values to those already listed in the current USDA choline database and will allow for more comprehensive and accurate estimations of usual dietary intake than available at present. Epidemiological studies of usual intake will be used to work towards establishing Estimated Average Requirements and Recommended Dietary Allowance values for choline.

4.6 References

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Tables

Table 4-1: Choline content of commercially available meat and cuts of meat in Alberta

NDB No[1]	Description	Moisture	Free Cho	GPC	Pcho	PC	Lyso PC	SM	Total Cho[2]	Total Cho
		%	mg choline moiety/ 100 g of food						mg/ 100g	Per serv[3]
Beef										
13498[5]	Beef, ground, 70% lean meat/30% fat, raw	64.7	0.3	1.8	3.4	37.6	0.4	2.6	46.1	34.6
23572[5]	Beef, ground, 80% lean meat/20% fat, raw	71.6	0.3	1.9	3.2	47.5	0.6	2.8	56.3	42.2
[6]	Beef, heritage angus rib eye	70.7	0.3	2.6	2.7	54.9	0.4	2.9	63.7	47.8
[6]	Beef, heritage angus striploin	71.8	0.2	1.6	2.3	53	0.4	3	60.5	45.4
[6]	Beef, heritage angus top sirloin	73.4	0.3	2.6	2.1	59.3	0.4	3.4	68.0	51.0
Chicken and Duck										
5062[4]	Chicken, broilers or fryers, breast, skinless, boneless, meat only, raw	73.7	0.8	1.4	7.2	77.4	2.3	5.2	94.3	70.8
5071[4]	Chicken, broilers or fryers, dark meat, drumstick, meat only, raw	72.5	2.8	1.6	5.9	64.4	1.5	6.7	82.9	62.2
5096[4]	Chicken, broilers or fryers, dark meat, thigh, meat only, raw	76.1	2.3	0.8	5.4	47.8	1.3	0.8	58.4	43.8
5139[4]	Duck, meat and skin, wing, raw	72.7	1.3	0.4	2.9	72	1.6	5.7	83.9	62.9
Lamb and Game										
17330[4]	Game meat, bison, ground, raw	63.2	0.3	2.2	2.1	38.3	0.9	3.4	47.1	35.3
17268[4]	Game meat, bison, ribeye	72.7	0.4	3.8	2	70.9	1.4	3.5	82.0	61.5

17267[4]	Game meat, bison, top sirloin	73.2	0.1	2.5	1.5	74.2	0.5	3.4	82.3	61.7
17342[4]	Game meat, elk, tenderloin	76.1	0.1	2.3	2.1	56.3	2.4	3.3	66.5	49.9
[6]	Lamb, chops	64.8	1.4	4.9	2.1	41.3	0.5	4.3	54.5	40.9
[6]	Lamb, chops, fat trimmed	73.4	1.8	11.9	1.9	64.5	0.6	5.3	86.0	64.5
17015[4]	Lamb, leg, shank, raw	75.6	0.9	6	1.6	62.6	0.5	4.2	75.8	56.9
17043[4]	Lamb, shoulder, arm, raw	87.5	1.1	2.3	1	28.7	0.3	2	35.4	26.6
Pork										
10060[5]	Pork, fresh, loin, tenderloin	77	0.3	13	2.1	50.5	1.5	2.8	70.1	52.6
10084[5]	Pork, fresh, shoulder	69.8	1	8.5	1.8	47	0.5	2.8	61.6	46.2
[6]	Pork, fresh, side ribs	61.9	2.1	7.6	2.1	35.6	0.3	2.9	50.6	38.0

[1]NDB number is a numerical code used in the USDA Nutrient Database for Standard Reference.
[2]Total choline refers to the sum of free choline, GPC, phosphocholine, PtdCho, lysoPC and SM and does not include betaine.
[3]Total choline per serving (Per serv) (75 g) according to Canada's Food Guide and USDA My Plate.
[4]Indicates a meat sample that is not included in the 2008 USDA Database for Choline Content of Common Foods where the accompanying choline values have been added to the assigned nutrient database number.
[5]Indicates a meat sample that is included in the 2008 USDA Database for Choline Content of Common Foods where the accompanying choline values have been modified based on analysis.
[6]There was no specific NDB number for and only has description of meat sample analyzed.

Table 4-2: Comparison between the choline content of meat samples in Alberta and the USDA database values for the same NDB number or similar NBD number

Data Source	NDB No[1]	Description	Free Cho	GPC	Pcho	PC	Lyso PC	SM	Total Cho[2]	% Diff Total Cho[3]
			mg choline moiety/ 100 g of food						mg/ 100g	
Alberta	13498	Beef, ground, 70% lean meat/30% fat, raw	0.3	1.8	3.4	37.6	0.4	2.6	46.1	
USDA[4]	13498	Beef, ground, 70% lean meat/30% fat, raw	2.6	2.4	0.5	36	-	5.1	47.4	
	Difference in mg choline		-2.3	-0.6	2.9	1.6	0.4	-2.5	-1.3	-2.8%
Alberta	23572	Beef, ground, 80% lean meat/20% fat, raw	0.3	1.9	3.2	47.5	0.6	2.8	56.3	
USDA[4]	23572	Beef, ground, 80% lean meat/20% fat, raw	2.6	2.8	0.5	45	-	5.6	56	
	Difference in mg choline		-2.3	-0.9	2.7	2.5	0.6	-2.8	0.3	0.5%
Alberta	10060	Pork, fresh, loin, tenderloin	0.3	13	2.1	50.5	1.5	2.8	70.1	
USDA[4]	10060	Pork, fresh, loin, tenderloin, separable lean only, raw	1.6	24	0.6	49	-	6.1	81	
	Difference in mg choline		-1.3	-11	1.5	1.5	1.5	-3.3	-10.9	-14.4%
Alberta	10084	Pork, fresh, shoulder	1	8.5	1.8	47	0.5	2.8	61.6	
USDA[4]	10084	Pork, fresh, shoulder, blade, separable lean only, raw	5.4	11	1	55	-	6	79	
	Difference in mg choline		-4.4	-2.5	0.8	-8	0.5	-3.2	-17.4	-24.8%
Alberta	5096	Chicken, broilers or fryers, thigh, meat only, raw	2.3	0.8	5.4	47.8	1.3	0.8	58.4	
USDA[4]	98088	Chicken, broilers or fryers, thigh, meat only, cooked, rotisserie	5.8	0.8	2.8	46	-	9.7	65	
	Difference in mg choline		-3.5	0	2.6	1.8	1.3	-8.9	-6.6	-10.7%
Alberta	5071	Chicken, broilers or fryers, drumstick, meat only, raw	2.8	1.6	5.9	64.4	1.5	6.7	82.9	
USDA[4]	98086	Chicken, broilers or fryers, drumstick,	6	1.1	3	62	-	12	84	

		meat only, cooked								
	Difference in mg choline		-3.2	0.5	2.9	2.4	1.5	-5.3	-1.1	-1.3%
Alberta	5062	Chicken, broilers or fryers, breast, meat only, skinless, raw	0.8	1.4	7.2	77.4	2.3	5.2	94.3	
USDA[4]	98085	Chicken, broilers or fryers, breast, meat only, cooked, rotisserie	3.2	1.6	2.1	46	-	8.9	62	
	Difference in mg choline		-2.4	-0.2	5.1	31.4	2.3	-3.7	32.3	41.3%

[1]NDB number is a numerical code used in the USDA Nutrient Database for Standard Reference
[2]Total choline refers to the sum of free choline, GPC, phosphocholine, PtdCho, lysoPC and SM and does not include betaine.
[3]% difference in total choline calculated as difference between Alberta value subtract the USDA value, divided by the mean of two values, shown as a percentage.
[4]Data from the 2008 USDA Database for Choline Content of Common Foods (Patterson, Williams et al. 2008).

Table 4-3: Comparison of the fat content and mg of total choline content of meat samples per dry and wet weights (100 g)

		Dry Weight (100 g)		Wet Weight (100 g)	
Description	Moisture content (%)	Fat content (%)	Total choline (mg)	Fat content (%)	Total choline (mg)
Beef, ground, 70% lean meat/30% fat, raw	64.7	49.5	130.6	17.5	46.1
Beef, ground, 80% lean meat/20% fat, raw	71.6	36.5	198.2	10.4	56.3
Beef, heritage angus rib eye	70.7	27.0	217.3	7.9	63.7
Beef, heritage angus strip loin	71.8	21.5	214.5	6.1	60.5
Beef, heritage angus top sirloin	73.4	24.0	255.6	6.4	68.0
Chicken, broilers or fryers, breast, meat only, skinless	73.7	14.5	358.7	3.8	94.3
Chicken, broilers or fryers, drumstick, meat only, cooked	72.5	19.5	301.4	5.4	82.9
Chicken, broilers or fryers, thigh, meat only, cooked	76.1	16.5	244.4	3.9	58.4
Duck, meat and skin, raw	72.7	19.5	307.2	5.3	83.9
Game meat, bison, ground, raw	63.2	53.0	128.1	19.5	47.1
Game meat, bison, rib eye	72.7	26.0	300.5	7.1	82.0
Game meat, bison, top sirloin	73.2	15.5	307.0	4.2	82.3
Game meat, elk, tenderloin	76.1	17.0	278.0	4.1	66.4
Lamb, chops	64.8	48.5	154.8	17.1	54.5
Lamb, chops, fat trimmed	73.4	24.5	323.3	6.5	86.0
Lamb, leg, shank, raw	75.6	19.0	310.6	4.6	75.8
Lamb, shoulder, arm, raw	87.5	30.5	283.1	3.8	35.4
Pork, fresh, loin, tenderloin	77.0	13.5	304.7	3.1	70.1
Pork, fresh, shoulder	69.8	45.5	203.9	13.7	61.6
Pork, fresh, side ribs	61.9	60.0	132.7	22.9	50.6

CHAPTER 5: Estimated choline intake from 24-h records and the contribution of egg and milk consumption to intake among pregnant and lactating women in Alberta[1,2]

5.1 Introduction

Choline was recognized by the IOM as an essential nutrient in 1998 (IOM 1998). The need for choline increases during periods of rapid growth and development including pregnancy and lactation (Zeisel 2006) as it plays a vital role in fetal development, particularly in the brain (Holmes-McNary, Loy et al. 1997, Zeisel 2000 2004, Craciunescu, Albright et al. 2003, Shaw, Carmichael et al. 2004, Zeisel and Niculescu 2006, Zeisel 2011). Choline is transferred by placental transporters (Molloy, Mills et al. 2005) with umbilical cord concentrations of choline approximately 3 times higher than maternal plasma concentrations (Molloy, Mills et al. 2005) and newborn infants have higher serum free choline concentrations compared to their mothers (Ozarda Ilcol, Uncu et al. 2002). In a rodent model, pregnancy diminished choline stores in the liver compared to non-pregnant controls (Zeisel, Mar et al. 1995). When fed a choline-deficient diet, lactating rodents had greater depletion of liver choline metabolites compared to non-lactating rodents, suggesting greater sensitivity to choline deficiency during lactation (Zeisel, Mar et al. 1995). Dietary choline intake has been indicated to influence milk composition in a rodent mode (Holmes-McNary, Cheng et al. 1996). In humans, large amounts of choline are present in breast milk (Holmes-McNary,

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[1] A version of this chapter has been published: Lewis ED, Subhan FB, Bell RC, McCargar LJ, Curtis JM, Jacobs RL, Field CJ. (2014) Estimation of choline intake from 24-h dietary intake recalls and the contribution of egg and milk consumption to intake among pregnant and lactating women in Alberta. *British Journal of Nutrition,* 112(1): 112-121.

[2] Content in this chapter was presented at the Women and Children's Health Research Institute Research Day, Edmonton, AB, November 2013 and the Alberta Institute of Human Nutrition, Edmonton, AB, February 2012.

Cheng et al. 1996) and lost from maternal stores through breast-feeding (Ilcol, Ozbek et al. 2005).

Due to the lack of human dietary studies, there is currently no estimated human requirement for choline, and the IOM Standing Committee on the Scientific Evaluation of Dietary Reference Intakes (IOM 1998) has determined an AI of choline during pregnancy and lactation as 450 mg/d and 550 mg/d, respectively. The AI has been established for total choline, which may comprise six forms of choline including free (unesterified) choline, phosphocholine, GPC, PC, lysoPC and sphingomyelin (Zeisel, Mar et al. 2003). Research in rodents suggests that different forms of choline may be absorbed and metabolized differently (Cheng 1996).

Several studies have estimated dietary choline intake in women (Cho, Zeisel et al. 2006, Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013) however, there are a limited number of studies that have examined dietary choline intake in pregnant and lactating women, where choline recommendations are increased. A case-control cohort study of pregnant women in the United States investigated the association between maternal dietary choline intake and infant NTDs and found that consuming >290 mg/day of choline was associated with decreased risk of NTDs (Shaw, Carmichael et al. 2004). Higher dietary choline intake was associated with increased use of choline as a methyl donor, which is essential for several fetal development processes that involve DNA methylation (Jiang, Yan et al. 2012).

Currently the USDA Database for the Choline Content of Common Foods, Release 2, is the only available food composition database, containing 634 foods, to estimate choline intake from dietary records (Patterson, Williams et al. 2008). The aims of this study were to examine dietary choline intake in a cohort of pregnant and lactating women in Alberta, Canada, to identify major food sources of choline, and to characterize the contribution of some

frequently consumed food sources of choline to overall daily choline intake. The contribution of consuming eggs and milk to choline intake were further studied in this population. Eggs are one of the richest dietary sources of choline containing approximately 130 mg of choline per medium (50 g) egg, mainly in the form of PC. Although compared to eggs, milk is not a rich source of choline (16 ± 2 mg of total choline per 100g of milk, varying depending on milk fat percentage), milk is consumed in significant quantities by the majority of pregnant (Mannion, Gray-Donald et al. 2006) and lactating women (Mannion, Gray-Donald et al. 2007) in North America therefore could represent a major food source of choline.

5.2 Participants and methods

5.2.1 Participants and study design

The APrON study is a Canadian cohort of women and infants that enrolled women during their first or second trimester of pregnancy (Kaplan, Giesbrecht et al. 2014). Included participants were pregnant women >16 years of age, <27 weeks gestation and living in or near Edmonton or Calgary. Participants were excluded if they planned to move outside the region during the time of the study or if they were unable to answer questions in English. Dietary information for the first 600 women enrolled in the APrON cohort was available at the time of this study. The sample size of approximately 600 participants is a convenience sample that includes the available data at the time of writing this manuscript. Women were recruited from Edmonton and Calgary (Alberta, Canada) and surrounding areas between June 2009 and June 2010 through media advertisements and physicians' offices. Women were recruited early in pregnancy and interviewed during each trimester and at 3-months postpartum. Women who were recruited at ≤13 weeks gestation were assessed once during each trimester. Women recruited at 14-27 weeks gestation were assessed in the second and third trimesters only.

Further analysis was conducted at 3-months postpartum (lactation period). Detailed description of the overall study design, recruitment and collection methods have been previously published (Kaplan, Giesbrecht et al. 2014). This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the University of Alberta Health Research Ethics Biomedical Panel and the University of Calgary Health Research Ethics Board. Written informed consent was obtained from all subjects.

5.2.2 Estimation of choline intake during pregnancy and lactation

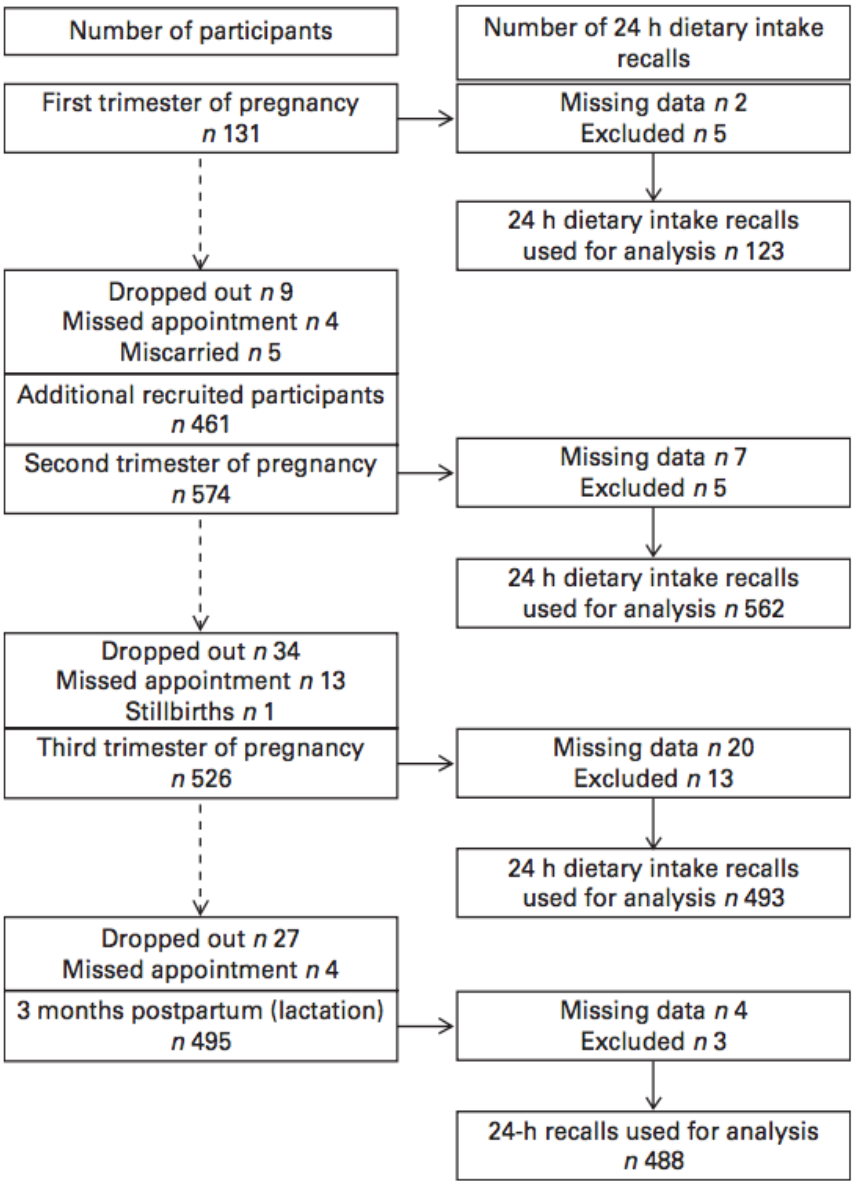
Dietary choline intake during each trimester of pregnancy and at 3-months postpartum was estimated by 24-h food recall interviews. At each study visit, women were asked to describe in detail the quantity and type of food and dietary supplements consumed in the previous 24-h period. These dietary recalls were conducted during face-to-face interviews by trained nutrition educated research assistants using a "Multiple Pass Method" (Conway, Ingwersen et al. 2003). Food models were used to help women estimate portion sizes and probes including details regarding cooking method and food brand names were used. The 24-h dietary intake recalls were entered into Food Processor Standard Query Language (SQL) (ESHA Research, Salem, OR, USA) to estimate macronutrient intake. All output from Food Processor was checked for completeness by research assistants who reviewed the data for entry errors, which were corrected by referring back to original paper copies of 24-h recalls. The data was further checked for completeness and cleaned based on outliers in energy intake, 24-h recalls that reported >14654 kJ/d (3500 kcal/d) and <3349 kJ/d (800 kcal/d) were checked for entry errors to ensure correct dietary data had been entered. If this data was

correct, outliers were removed from analysis, as discussed in the exclusion criteria for our analysis.

A comprehensive choline database (Alberta Database) was developed for use with the 24-h food recall data to estimate the choline content of foods consumed by APrON participants. The Standard Operating Procedure on the creation and use of the Alberta Database is detailed in **Appendix 2**. In brief, this database contained information on total choline content in food, as well as the five most common dietary forms of choline (free choline, glycerophosphocholine, phosphocholine, PC, sphingomyelin) and betaine. The Alberta Database began with the available choline values for food items from the USDA Database for the Choline Content of Common Foods Release 2 (634 foods) by matching the nutrient database number used in the USDA choline database and USDA National Nutrient Database for Standard Reference (Zeisel, Mar et al. 2003, Patterson, Williams et al. 2008). Foods not in the USDA choline database were substituted with nutritionally comparable foods, substitutions being made based on similar energy and appearance. Overall, 2576 substitutions were made and added to the Alberta database. Recipes were constructed for foods with multiple food items where choline composition data was available for individual ingredients. In total, 105 new recipes were added to the database. In addition, data for 26 new foods were added to the database through analysis by LC-MS/MS, as described by Xiong et al. (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012) including several varieties of pulses (chickpeas, black beans and lima beans), shellfish and seafood (shrimp). Samples were analyzed by LC-MS/MS using an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QSTAR mass spectrometer (AB SCIEX, Concord, ON, Canada). Foods that were predicted to contain negligible amounts of choline (e.g. water, hard candies, and beverage syrups) were not added to the database. The completed Alberta database included

choline content values for 2707 foods that were consumed by APrON participants (Appendix 2). Foods that had no appropriate substitution, or for which a recipe could not be formulated, were not included in the analysis (e.g. almond milk, rice milk, hemp hearts, bee pollen). The accuracy of the database in automatically estimating choline content was validated by a comparison of total choline intake between the automated database and estimation through manual calculations. Choline intake from 24-h dietary intake recalls for 18 participants selected at random were estimated and the difference was found to be non-significant using a paired t-test. Dietary choline intake was estimated for each trimester of pregnancy and 3-months postpartum (lactation). The number of participants included in the analysis was determined based on the availability of dietary information. Not every participant who completed a visit for each time point completed a 24-h recall, in some cases entry of 24-h recalls into Food Processor had not been conducted therefore was listed as missing from the analysis of choline intake. We then examined 24-h recalls for representativeness of intake; done by excluding from the analysis any recalls where estimated energy, fibre or choline intake was ± 3 SD from the mean. This represented a small number of recalls and the number is identified as 'excluded' in **Figure 5-1**.

Figure 5-1: Study participants enrolled and included in the analysis at each time point



5.2.3 Choline supplement intake in the APrON cohort

Maternal supplement intake was assessed using a questionnaire that was administered by interview during each trimester of pregnancy and at the 3-month postpartum visit, as previously described (Leung, Kaplan et al. 2013).

5.2.4 Estimation of major food sources of total dietary choline

To determine important food sources of choline, foods were classified based on the 22 food categories listed in the USDA Database (Patterson, Williams et al. 2008). The "Dairy and Eggs" category was further subdivided into "Dairy" and "Egg" to specifically identify the contribution that foods in each of these groups made to choline intake. The "Dairy" category included all dairy products such as butter, cheese, milk and yogurt. The "Meat" category combined the following: "Lamb, Veal and Game", "Sausages and Luncheon Meats", "Pork and Pork Products" and "Beef and Beef Products". A total of 20 food categories were used to analyze the contribution of food categories to total choline intake. For each time point, the total amount of choline consumed from foods in each category was reported as percent of total dietary choline (Subar, Krebs-Smith et al. 1998, Cho, Zeisel et al. 2006, Mygind, Evans et al. 2013). The ranking of the food categories that contributed to total choline was consistent across all trimesters of pregnancy, therefore only the second trimester of pregnancy and lactation time points are reported.

5.2.5 Estimation of the importance of egg and milk consumption to choline intake during pregnancy and lactation

Egg and milk consumption was assessed for each trimester of pregnancy but the second trimester was chosen to represent intake during pregnancy. There was no significant difference in milk and egg intake from each trimester of pregnancy, and similar results were found in the first and third trimesters. The second trimester was used to assess egg and milk intake due to the largest sample size, thereby having greater statistical power.

The contribution of daily egg consumption to choline intake was assessed using reported egg consumption from 24-h recalls collected during the second trimester of pregnancy and lactation. Intake days were grouped into two categories: egg consumers (women who reported consuming at least 1 egg, as a separate food, in a day), and non-egg

consumers (women who did not consume at least one egg in a day). Total choline intake, choline from choline-containing moieties and the proportion of the group meeting the choline AI at each time point was compared between the two categories.

The contribution of fluid milk to daily choline intake during the second trimester of pregnancy and during lactation was assessed using 24-h dietary intake recalls. Milk consumers were participants who reported consuming any amount of fluid milk (as a beverage, in cold cereal or in coffee/tea) in a 24-h recall period. Milk consumption included whole milk, 2% milk, 1% milk and skim/nonfat milk. This analysis only included the contribution of fluid milk intake and did not include any other form of dairy. The contribution of servings of milk to choline intake was assessed from the total daily reported intake of fluid milk from the 24-h dietary intake recalls that were collected in the second trimester of pregnancy and lactation. The frequency of milk consumption was categorized as: <250 mL/day (includes women who did not report consuming any milk in the 24-h recall period), 250 mL to <500 mL/day, and ≥500 mL/day.

5.2.6 Statistical analyses

All data are presented as means ± SD, unless otherwise noted. All data were tested and found to be normally distributed. Differences in choline intake based on egg consumption and milk consumption during pregnancy and lactation were assessed using a two-tailed *t*-test or one-way ANOVA with Bonferroni corrections where appropriate. Differences in choline intake between milk consumption groups were assessed using one-way ANOVA with a Bonferroni correction or a Kruskal-Wallis non-parametric test and Wilcoxon rank-sum test as appropriate. Multinomial logistic regression was performed to identify the likelihood of meeting the AI based on egg or milk consumption. Data were analyzed using STATA Version

11 (StataCorp LP, College Station, TX, USA) with a P-value of <0.05 considered statistically significant for all analyses.

5.3 Results

5.3.1 Population characteristics, enrollment and analysis at each time point

The population characteristics of the cohort are described in **Table 5-1**. The number of participants enrolled and included in the analysis at each time point is presented in Figure 5-1**.** The majority of subjects were recruited in their second trimester of pregnancy. For the 3-month postpartum visit, dietary information was available for 488 of the 491 women who attended their clinic visit. At 3-months postpartum, 90.5% of the cohort (n=410) reported breast feeding their infant (54% exclusively breastfed), as previously reported (Jessri, Farmer et al. 2013). For participants who were missing 24-h dietary data or excluded from analysis, age was 31.4 ± 4.3 years, BMI 22.2 ± 3.4, 62% Caucasian and 45% had high family income (≥$ 100 000). The characteristics of participants who did not have dietary information available (missing) or excluded from analysis of choline intake did not differ from the cohort reported.

5.3.2 Total choline, choline requirements and supplement intake

Prior to estimating dietary choline intake, the accuracy of the automated choline database to estimate choline intake was validated. Mean total choline intake using the automated database (378 ± 194 mg/d) was compared to mean total choline intake using manual calculation (358 ± 199 mg/d), P=0.11.

Mean intakes of total choline and choline containing moieties across pregnancy and lactation are presented in **Table 5-2**. The estimated daily choline intake was 340 ± 148 mg/d,

349 ± 154 mg/d, and 353 ± 144 mg/d in the first, second and third trimester, respectively, with 23% of participants meeting the AI for choline intake (450 mg/d) across pregnancy. During lactation, mean total choline intake was 346 ± 151 mg/d and 11% of women met the AI of 550 mg/d. The major form of choline in the diet of the APrON women was PC, which contributed 48 ± 2% of total estimated choline in pregnancy and lactation (Table 5-2).

A total of 36 women reported consuming a supplement that contained choline, at least one time during the study period. Choline bitartrate was the most common form of supplemental choline (n=35), providing a mean of 70 ± 44 mg choline bitartrate (29 ± 18 mg of choline) and was included in their prenatal multivitamin or B-complex vitamin. One woman reported consuming a PC supplement providing 420 mg of PC (58 mg of choline) during her second and third trimesters of pregnancy. Supplement intake was included in the estimation of total choline intake (Table 5-2). However, due to the limited number of participants consuming supplemental choline and the small amounts of choline provided, supplement intake did not significantly change the proportion of participants meeting daily recommendations when compared to considering only dietary sources. Women in the lowest quintile of dietary choline ingested approximately half of the AI for choline in pregnancy or lactation. Only women in the highest quintile met or slightly exceeded the AI during pregnancy and lactation.

5.3.3 Food sources of choline

Major food categories contributing to total choline intake during pregnancy and lactation are shown in **Table 5-3**. The three food categories that made the largest contributions to choline intake in the cohort were dairy, eggs and meat and they accounted for approximately 50% of total dietary choline. Seven other food categories (poultry, vegetables,

baked products, fruits, legumes, finfish and shellfish and mixed dishes (pregnancy) or fast foods (lactation)) contributed 35% to total choline intake. The remaining 10 food groups (not listed) included beverages, snacks, nuts, seeds, fats and oils, which together contributed 15% to choline intake during pregnancy and lactation.

5.3.4 Contribution of egg and fluid milk consumption to daily choline intake

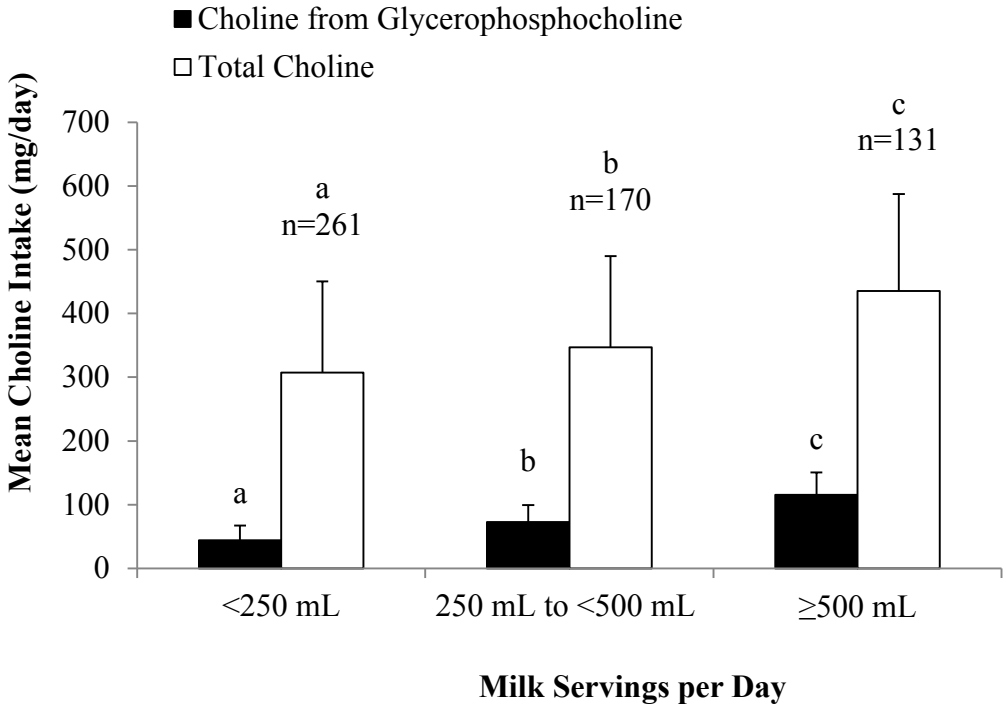
During pregnancy and lactation, egg consumption in a 24-h recall period was found to significantly affect total choline intake for the day. During the second trimester and lactation, consuming at least one egg, as a single food item, was associated with significantly ($P<0.001$) higher daily intake of choline (**Table 5-4**). This increase in daily choline intake was due to a significantly higher content of PC and sphingomyelin in the diet (Table 5-4). During pregnancy, women who reported consuming one or more eggs during the 24-h period of recall were more likely to meet daily choline recommendations compared to women who did not consume an egg (OR=8.1, 95% CI=5.2-12.6) (Table 5-4). During lactation, women who reported consuming one or more eggs during a 24-h period of recall were more likely to meet daily choline recommendations compared to women who did not consume eggs (OR=10.8, 95% CI=5.7-20.7).

During pregnancy and lactation, milk consumption in a 24-h period was associated with significantly ($P<0.05$) higher daily choline intake (**Table 5-5**). This higher daily choline intake in a 24-h recall period reporting milk consumption was due to a significantly higher intake of free choline, glycerophosphocholine and phosphocholine in the diet (Table 5-5).

Further, **Figure 5-2** examines the contribution of servings of fluid milk to total daily choline intake and glycerophosphocholine intake in 24-h dietary recalls from the second trimester of pregnancy (n=562). Compared to women who did not consume milk and who

consumed <250 mL of milk, women who consumed ≥500 mL of milk, equal to 2 servings of milk per day (as defined by Health Canada and the USDA), were 2.8 times more likely to meet daily choline recommendations (95% CI=1.7-4.8). This higher total choline intake was due to a significantly higher intake of glycerophosphocholine (P<0.05). During lactation women who consumed ≥500 mL of milk, equal to 2 servings of milk in the 24-h recall were 3.1 times more likely to meet daily choline recommendations (95% CI=1.5-6.2).

Figure 5-2: Contribution of servings of fluid milk to total daily choline intake and glycerophosphocholine intake in 24-h dietary recalls from the second trimester of pregnancy



Within either the total choline or glycerophosphocholine datasets, bars that do not share a letter are significantly different (P<0.05).

5.4 Discussion

The increased demand for choline during pregnancy and lactation has been demonstrated (Zeisel 2006, Zeisel 2006). We estimate that mean choline intake is below the

current daily recommendations and less than one quarter of participants met the choline recommendations in pregnancy and only 10% of women met the increased recommendations during the lactation period. Although no difference in total choline intake between pregnancy and lactation, the proportion of women meeting daily recommendations in lactation is less than half that of pregnancy. This is due to the change in recommendation, which is 100 mg/d higher in lactation compared to pregnancy. The percentage meeting daily recommendations is consistent with other studies in non-pregnant women (Yonemori, Lim et al. 2013), and pregnant women (Shaw, Carmichael et al. 2004). In a previous study in adults with non-alcoholic fatty liver disease (Guerrerio, Colvin et al. 2012), the authors defined less than one-half of the AI as deficient intake. Using this criterion, approximately 20% and 40% of the cohort, during pregnancy and lactation, respectively, were consuming a diet possibly deficient in choline. Reporting the proportion of the population meeting choline recommendations must be interpreted with caution due to the fact that the current recommendations for choline may not be appropriate. The AI values were established based on a metabolic study conducted in men, which was then extrapolated to non-pregnant and then pregnant and lactating women. Although considering the increased requirement for choline during pregnancy and lactation, there are concerns as to the accuracy of the AI (Yonemori, Lim et al. 2013). Nevertheless, individuals with dietary choline intake severely below recommendations (e.g. less than one-half of the recommended daily intake) may be at the highest risk of choline insufficiency. The implications on the women's and their infant's health requires further study to determine if the current recommendation is appropriate for healthy pregnant and lactating women. An intervention study examining the effects of dietary choline during pregnancy reported that choline metabolism is altered during pregnancy, as the circulation of choline-derived methyl donors (betaine) were much lower compared to nonpregnant women. A higher maternal

choline intake (930 mg/d) increased the use of choline as a methyl donor compared to low choline intake (480 mg/d) (Jiang, Yan et al. 2012). This could have major health implications for the fetus and infant as a supply of methyl groups is essential for development as they are necessary to support cellular proliferation and DNA methylation.

Our findings on total choline intake during pregnancy and lactation are consistent with a study of a cohort of primarily Caucasian (42.7%) pregnant women in California (n=864) between 1989 and 1991 (Shaw, Carmichael et al. 2004). The main aim of that study was to examine the association between NTDs and dietary choline, and choline intake was assessed using an interviewer-administered FFQ (Shaw, Carmichael et al. 2004). However, our choline intake estimates are higher than much of what has been reported in the female population enrolled in the Atherosclerosis Risk in Communities (ARIC) study (Bidulescu, Chambless et al. 2007) and a cohort of women of child-bearing age in New Zealand (Mygind, Evans et al. 2013). The ARIC Study and the research from New Zealand did not comment on the socioeconomic status of their populations. In Canada, food insecurity and low socioeconomic status have been associated with other nutrient inadequacies (Kirkpatrick and Tarasuk 2008, Dachner, Ricciuto et al. 2010, Tarasuk, Fitzpatrick et al. 2010). However, over half of participants in APrON had high family income (≥$100,000/year), which limits the applicability of our results to populations with lower incomes. As our population appears to be of high socioeconomic status, we may hypothesize that our population may have higher dietary choline intake compared to a population of lower socioeconomic status. An additional limitation of our study is the lack of ethnic diversity (87% Caucasian) within our large study population. Intake of both choline (Yonemori, Lim et al. 2013) and milk and milk products (Fulgoni, Nicholls et al. 2007) have been shown to vary based on ethnicity. Hence, the

interpretation of our results may not be directly applicable to multiethnic populations, including data previously reported (Yonemori, Lim et al. 2013).

The methodologies used to collect dietary intake data can also influence the estimation of choline intake. A study conducted by Fischer et al in 2005 (Fischer, Scearce et al. 2005) suggests that under-reporting during the use of self-administered dietary questionnaires may underestimate dietary choline intake. The 24-h dietary intake recalls in our study were conducted in face-to-face interviews by trained study personnel using food models, which may have helped reduce the chance of missing foods or underestimating portion size. The problem of portion size is supported by the energy-adjusted choline intakes across pregnancy (0.041 mg/KJ) and lactation (0.043 mg/kJ) in this study that are consistent with previous reports in a New Zealand population of women of reproductive age (Mygind, Evans et al. 2013) and a population of healthy individuals in the United States (Fischer, Scearce et al. 2005) where the absolute choline intake was lower.

In our population of study, 50% of the choline in the diet was provided by dairy, eggs and meat. Previous studies have also shown these food categories to be among the top contributors to dietary choline (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Mygind, Evans et al. 2013). Consumption of eggs or milk significantly increased the daily choline intake. Eggs are one of the richest sources of choline (Patterson, Williams et al. 2008), with an average whole medium egg containing approximately 130 mg choline, according to the USDA database (Patterson, Williams et al. 2008) and unpublished data. The difference in total choline intake between egg and non-egg consumers was due to a significantly higher intake of PC and sphingomyelin. Results from the most recent National Health and Nutritional Examination Survey III (NHANES III) found that nearly 20% of the American population were egg consumers (subjects that reported consuming an egg or egg product in the 24-h recall

period) (Song and Kerver 2000). Egg consumption of Albertan women during pregnancy and lactation was consistent with this estimation with 23% and 24%, respectively, reporting egg consumption in their 24-h recall. The NHANES III study reported that egg consumers had greater daily nutrient intake (except vitamin B_6 and fibre, nor did they measure choline) compared to non-egg consumers (Song and Kerver 2000). In addition to being a rich source of choline, eggs are a good source of vitamins B_{12}, D and A, folate, phosphorus and an excellent source of riboflavin, vitamin K and selenium (Applegate 2000) and an inexpensive source of high quality protein (Herron and Fernandez 2004). In this population, where choline intake is suboptimal, egg consumption is associated with higher mean total choline intake, and where egg consumption is not highly prevalent, the promotion of egg consumption may assist in increasing daily choline intake during a period when choline demand is high. The promotion of egg consumption may also contribute other nutritional benefits during a period of rapid growth and development.

Milk has also been reported to be a major contributor to total choline intake in other populations (Cho, Zeisel et al. 2006, Bidulescu, Chambless et al. 2007, Mygind, Evans et al. 2013). Milk is not considered a rich source of choline, containing an average of 16 ± 2 mg of choline per 100g of milk. However, milk was consumed by the majority of participants with 69% reporting fluid milk consumption in their 24-h recalls. Women who consumed ≥500 mL of milk per day within a day had a higher mean daily choline intake compared to women who consumed <250 mL of milk (including no milk) the day before their study visit. According to Eating Well with Canada's Food Guide and the USDA MyPlate, 250 mL of milk (preferably skim or low fat) is one serving of milk and alternatives, indicating that two servings of milk (500 mL) per day can impact choline intake. The difference in total choline intake was due to higher dietary content of free choline, glycerophosphocholine and phosphocholine. Similar to

egg consumption, milk consumption has been shown to positively affect nutrient intake and is an important source of vitamin D, calcium, potassium and magnesium in the American diet (Subar, Krebs-Smith et al. 1998, Nicklas, O'Neil et al. 2009, Fulgoni, Keast et al. 2011, Rice, Quann et al. 2013).

Although this study utilizes a large Canadian cohort to examine dietary choline intake and the contribution of egg and milk consumption to intake there are various limitations that should be discussed. Despite a reasonable sample size, this population is fairly homogenous in ethnicity, education and socioeconomic status, all of which influence nutritional status therefore these results may not be directly related to populations with differing characteristics. Also affecting the interpretation of the results may be the use of an AI as a recommendation. Due to limited data, the establishment of the AI for choline during pregnancy and lactation was set using a metabolic study in men. Dietary intake in the current study was estimated using one 24-h recall and was not validated with a biomarker. Unfortunately, there is no good biomarker of choline status as plasma choline is homeostatically regulated (Abratte, Wang et al. 2009). There are also limitations associated with the collection method used, as the use of only one 24-h recall may not fully capture dietary patterns during each trimester as they only represent dietary intake in a 24-h period. This could present a problem for foods that are less frequently consumed, including eggs, which may not be consumed every day but a person may consume them on a regular basis. Not considering eggs present in other dishes and not including lysoPC from eggs (absent in the USDA database) may have resulted in an underestimation of the contribution of eggs to total choline intake in this population. Similarly, our study only examined the importance of fluid milk consumption and not other dairy products (yogurt, cheese) or dairy alternatives limiting our ability to determine the contribution of all dairy to choline intake.

5.5 Conclusion

In conclusion, this is the first study examining dietary choline intake in a Canadian population. Our findings indicate that choline intake is below recommendations in this population, during a period where choline demand is high. The consumption of egg and fluid milk increases daily choline intake and the promotion of both egg and milk consumption may assist in meeting daily dietary choline recommendations. In addition, progress should be made to expand the current database of food choline content values to more accurately estimate dietary intake of this essential nutrient.

5.6 References

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Tables

Table 5-1: Characteristics of women enrolled in the first cohort of the APrON study

Characteristic[1]	n	%[2]
Age		
17-30	274	45.8%
31-45	324	54.2%
Pre Pregnancy BMI[3]		
Underweight	16	2.8%
Normal weight	374	65.0%
Overweight	109	19.0%
Obese	76	13.2%
Unknown	23	-
Weight gain during pregnancy[4]		
Inadequate	159	33.2%
Adequate	192	40.2%
Excessive	127	26.6%
Unknown	120	-
Parity		
0	305	56.0%
1	184	33.8%
≥2	56	10.3%
Unknown	53	-
Marital Status		
Married/Common law	546	96.5%
Other	20	3.5%
Unknown	32	-
Education		
≤ High school	56	10.0%
Trade	117	20.9%
Undergraduate/Post Graduate	391	69.8%
Unknown	34	-
Family Income		
Low (≤ $69 000)	108	19.4%
Medium ($ 70 000-99 000)	141	25.3%
High (≥$ 100 000)	308	55.3%
Unknown	41	-
Ethnicity		
Caucasian	490	86.9%
Other	74	13.1%
Unknown	34	-
Breastfeeding		
>12 weeks	410	90.5%
≤12 weeks	43	9.5%
Unknown	42	-

[1]Baseline characteristics collected at the time of study enrollment.
[2]% of each characteristic based on available data, does not include the percentage of unknown values, in which data was not collected or obtained from participants.
[3]Pre-pregnancy BMI was assessed based on self-reported pre-pregnancy weight (kg) and height (m). BMI (kg/m^2) was categorized using the World Health Organization (WHO) classifications for underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9) and obese (≥30) (WHO 2013).
[4]Weight gain during pregnancy was assessed using calculated pre-pregnancy BMI and total weight gain during pregnancy (3rd trimester visit). Weight gain classified inadequate, adequate and excessive based on the recommended range of total weight gain by BMI category from the Canadian Gestational Weight Gain Recommendations (Health Canada 2010).

Table 5-2: Estimated daily intake of total choline, choline containing moieties and betaine using 24-h dietary intake recall across pregnancy and lactation (3-months postpartum) in the APrON cohort[1]

	First Trimester	Second Trimester	Third Trimester	Lactation
n	123	562	493	488
Consuming choline in supplement (n) (%)	5 (4%)	13 (2%)	13 (3%)	16 (3%)
Betaine (mg/d)	231 ± 116	213 ± 137	215 ± 141	252 ± 208
Choline Containing Moieties	mg of choline per day			
Free Choline	79 ± 46	82 ± 37	81 ± 36	77 ± 35
Glycerophosphocholine	57 ± 29	69 ± 40	71 ± 40	63 ± 36
Phosphocholine	16 ± 7	17 ± 10	17 ± 9	15 ± 8
Phosphatidylcholine	166 ± 110	161 ± 112	163 ± 106	170 ± 114
Sphingomyelin	20 ± 12	20 ± 12	20 ± 11	20 ± 12
Total Choline	340 ± 148	349 ± 154	353 ± 144	346 ± 151
Adequate Intake (mg/d)	450	450	450	550
Meeting AI (n)	28 (23%)	130 (23%)	113 (23%)	52 (11%)
Energy Intake (kcal/d)	2066 ± 759	2219 ± 876	2212 ± 741	2010 ± 733

[1]Values are number of participants, mean ± SD

Table 5-3: Most commonly reported food categories contributing to total dietary choline during pregnancy and lactation in the APrON cohort

	Pregnancy		Lactation	
Rank	Food Category[1]	Contribution (%)[2]	Food Category[1]	Contribution (%)[2]
1	Dairy	20.9	Dairy	16.9
2	Eggs	12.4	Meat	15.0
3	Meat	11.1	Eggs	13.8
4	Poultry	8.9	Vegetables	8.3
5	Vegetables	8.5	Poultry	8.2
6	Baked products	7.7	Baked products	7.2
7	Fruits	6.6	Fruits	4.8
8	Legumes	4.1	Legumes	3.5
9	Finfish and Shellfish	2.7	Finfish and Shellfish	3.2
10	Mixed Dishes	2.6	Fast Foods	3.1
Sum[3]		85.4		84.0

[1]Food categories are based on the USDA Database for the Choline Content of Common Foods Release 2 categories: Dairy (butter, cheese, milk, yogurt), Eggs (egg and egg products), Meat (lamb, veal and game, sausages and luncheon meats, pork and pork products and beef and beef products), Poultry (chicken and turkey), Vegetables (vegetables and vegetable products), Baked Products (bagels, bread, crackers and muffins), Fruits (fruits and fruit juices including bananas, orange juice, oranges, berries and apples), legumes (peanuts, pulses, soy products), finfish and shellfish (crab, salmon, tuna), Mixed Dishes (stew, chili and lasagna), and Fast Foods. The remaining food categories (not shown) include: Sugars and Sweets, Soups, Sauces and Gravies, Beverages, Cereal Grains, Pastas and Snacks, Breakfast Cereals, Snacks, Nut and Seed Products, Fats and Oils, and Spices and Herbs.

[3]Percent contribution calculated by summing the total amount of choline consumed for each food category then dividing by total choline from all food categories for all participants. These values were then multiplied by 100 to generate the percent contribution to total dietary choline by each food category at each time point. Only second trimester and lactation time points are reported.

[4]Sum represents the total percent contribution to total choline intake by the top 10 food categories consumed during pregnancy and lactation.

Table 5-4: Association of egg consumption to mean total choline intake and choline containing moieties in a 24-h recall period during pregnancy and lactation in the APrON cohort[1]

	Pregnancy		Lactation	
	Egg Consumed[2]	No Egg Consumed[3]	Egg Consumed[2]	No Egg Consumed[3]
n	132	430	118	371
Choline containing moieties	mg choline per day		mg choline per day	
Free Choline	81 ± 27	82 ± 40	76 ± 34	77 ± 33
Glycerophosphocholine	68 ± 39	69 ± 40	56 ± 30	65 ± 38*
Phosphocholine	17 ± 7	17 ± 11	15 ± 8	15 ± 8
Phosphatidylcholine	296 ± 124	119 ± 67*	309 ± 125	127 ± 69*
Sphingomyelin	28 ± 12	17 ± 10*	27 ± 13	17 ± 10*
Total Choline	491 ± 154	305 ± 126*	482 ± 161	302 ± 118*
Meeting AI (%)[4]	56	13**	31	4**

[1]Values are number of participants, mean ± SD

[2]Defined as participants who reported consuming at least one egg (as a single food item) in a 24-h recall period. Egg consumption included various preparation methods (fried, poached, scrambled and boiled).

[3]Defined as participants who did not report consuming an egg in a 24-h recall period.

[4]% meeting AI for the 24-h recall day for each time point, 450 mg/d during pregnancy and 550 mg/d during lactation.

* indicates significant difference from egg consumed group, $P<0.05$. Independent sample *t*-tests were used to test differences in choline intake between groups.

** indicates significant difference from egg consumed group, $P<0.05$. A chi-square test was used to test for differences in the proportion of participants meeting daily recommendations between each group.

Table 5-5: Intake of total choline and choline containing moieties among women who consumed or did not consume liquid milk during pregnancy and lactation in the APrON cohort[1]

	Pregnancy		Lactation	
	Milk Consumed[2]	No Milk Consumed[3]	Milk Consumed[2]	No Milk Consumed[3]
n	391	171	316	172
Choline containing moieties	mg choline per day		mg choline per day	
Free Choline	84 ± 33	78 ± 44*	81 ± 34	71 ± 35*
Glycerophosphocholine	82 ± 38	39 ± 22*	76 ± 35	38 ± 22*
Phosphocholine	20 ± 11	12 ± 6*	17 ± 8	11 ± 7*
Phosphatidylcholine	158 ± 108	170 ± 120	167 ± 109	176 ± 123
Sphingomyelin	21 ± 12	18 ± 12	20 ± 11	19 ± 13
Total Choline	363 ± 152	316 ± 154*	361 ± 147	315 ± 147*
Meeting AI (%)[4]	23	18	12	8

[1] Values are number of participants, mean ± SD

[2]Defined as participants who reported consuming any amount of fluid milk (as a beverage, in cold cereal or in coffee/tea) in a 24-h recall period. Milk consumption included whole milk, 2% milk, 1% milk and skim/nonfat milk.

[3]Defined as participants who did not report consuming milk in a 24-h recall period.

[4]% meeting AI for the 24-h recall day for each time point, 450 mg/d during pregnancy and 550 mg/d during lactation.

*indicates significant difference from milk consumed group, $P<0.05$. Independent sample *t*-tests were used to test differences in choline intake between groups.

CHAPTER 6: Comparison of choline intake from two 24-h dietary intake recall methods in the APrON study and the relationship of maternal choline intake to infant birth weight

6.1 Introduction

Choline is essential for a variety of critical processes in the body including neurotransmitter synthesis, lipoprotein formation and methyl group donation. The need for exogenous choline increases in pregnancy and the postnatal period to meet the high demands by the infant to support developing tissues, and account for the depletion of maternal stores (Ozarda Ilcol, Uncu et al. 2002). To meet this increased need, maternal dietary requirements dietary choline (set as AI values) increase from 425 mg/d for non-pregnant, non-lactating women to 450 mg/d and 550 mg/d for pregnant and lactating women, respectively.

Previous studies examining dietary choline intake in women of childbearing age have consistently shown that intake is below recommendations. In a cohort of healthy women of childbearing age in New Zealand, dietary choline was estimated by 3-day weighed food records with mean intake of 316 ± 65 mg/d, and only 16% of participants meeting the daily recommendation (425 mg/d) (Mygind, Evans et al. 2013). A recent study in a Canadian cohort of pregnant women estimated choline intake by FFQ and reported mean total choline intake of 306 ± 127 mg/d in early pregnancy and 302 ± 122 mg/d in late pregnancy (Masih, Plumptre et al. 2015). Of the 386 women enrolled in this cohort, only 13% of women met daily choline recommendation for pregnancy (Masih, Plumptre et al. 2015). Similar results were found in our initial study in first cohort of pregnant and lactating women (n=600) in the APrON study, where choline intake was assessed by interview 24-h recalls (Lewis, Subhan et al. 2014). Mean total intake during pregnancy and 3 months postpartum was 347 ± 149 mg/d and 346 ± 151 mg/d, respectively, with only 23% of women meeting recommendations during pregnancy, and 10% meeting recommendations during 3 months postpartum (Lewis, Subhan et al. 2014). We

also estimated the forms of choline in the diet, with approximately 50% of total choline as PC, followed by 23% as free choline.

Within the APrON study, dietary data was collected using two methods of 24-h recall, consisting of interview or online 24-h recalls. The first 1000 participants completed an interview administered face-to-face 24-h recalls, and approximately 1000 of the remaining participants completed an online version of the 24-h recall. 24-h dietary intake recalls are a commonly used dietary assessment tool as it provides detailed intake data that is relatively inexpensive with low respondent burden (Shim, Oh et al. 2014). Additionally, as dietary recalls are conducted after the food has been consumed, they are less likely to interfere with dietary behaviour, in contrast to dietary records (Carroll, Midthune et al. 2012). Interview 24-h recalls administered by trained personnel aids in participant completion, and overcomes the issue of participant literacy (Carroll, Midthune et al. 2012). However, with the use of a trained interviewer, interview 24-h recalls are associated with interview bias, and this method of recall may be more expensive and time consuming compared to other delivery methods (Shim, Oh et al. 2014). Conversely, online, or web-based, 24-h recalls are self-administered, without the presence of trained personnel. The online method offers the advantage of further limiting respondent burden and reducing the cost of research personnel and resources (Touvier, Kesse-Guyot et al. 2011, Shim, Oh et al. 2014). The online Food Behaviour Questionnaire was used in the APrON study, and has been validated for estimations of macronutrient and micronutrient intake compared to the interview 24-h recall method for use in a population of school-aged children (Hanning, Royall et al. 2009). Hanning *et al.,* (2009), found that the online recall generally underreported nutrients, including energy (10.5% lower) and fat (15.6% lower) intakes, compared to the interview recall (Hanning, Royall et al. 2009). The use of the online 24-h recall has not been validated for estimations of dietary choline intake compared to the

interview 24-h recall method. As both recall methods were used in the APrON study, examining the agreement between dietary recall methods is critical. In order to combine all participants, researchers must be aware of the potential differences in dietary estimates resulting from the use of the two methods.

Furthermore, there are well-known determinants for infant birth weight, including maternal weight gain, parity, smoking status, infant sex and gestational age (Oken, Kleinman et al. 2003, Frederick, Williams et al. 2008). Choline has been demonstrated to be required for the growth and development of the fetal brain (Albright, Liu et al. 1996, Zeisel 2006), and more recently postnatal development of the immune system (Dellschaft, Ruth et al. 2015). However, few studies have examined the potential consequences of low maternal choline intake on infant growth. In pregnancy, maternal plasma free choline concentrations were reported to increase from 6.6 μmol/L at 9 weeks gestation to 10.8 μmol/L at 36 weeks gestation (Velzing-Aarts, Holm et al. 2005), with umbilical cord blood concentrations approximately 3 times greater than maternal blood (36.6 μmol/L vs. 12.3 μmol/L) (Molloy, Mills et al. 2005). This gradient towards choline in the placenta likely reflects the importance of choline delivery to the fetus. Two studies have found no association between maternal dietary choline or plasma choline concentrations and overall infant growth. In a 12-week randomized control trial, there was no difference in infant birth weight from women who were supplemented with 550 mg/d of choline, compared to women who were supplemented 100 mg/d of choline during the third trimester of pregnancy (Jiang, Bar et al. 2013). Hogeveen *et al.,* (2013) found no association between infant birth weight and maternal plasma free choline concentrations at 30-34 weeks gestation (Hogeveen, den Heijer et al. 2013).

Therefore, the primary objective of this study was to compare the use of two methods of 24-h dietary assessment, interview and online methods, for the estimation of dietary choline

in women of childbearing age. Upon comparison of these two methods, recommendations could be made in order to use these dietary assessment methods together in a cohort of pregnant and lactating women in Alberta. A secondary objective of this study was to examine the association between maternal prenatal choline intake and infant birth weight.

6.2 Participants and methods

6.2.1 Validation of estimating dietary choline using interview and online recall methods

The comparison of two 24-h recall methods was conducted in a group of women of childbearing age, between the ages of 18-40 (n=58). A randomized, crossover design was utilized to compare the food and beverage intake and nutrient estimates for a 24-h period between face-to-face interview recall and APrON web-based dietary recall. Trained research assistants conducted the interview 24-h recalls, detailed in Section 5.2.2 and entered recalls into Food Processor SQL (ESHA 10.6 Research, Salem, OR, USA). For the web 24h recall participants completed the online recall on their own, after instructions from research assistants (see below in section 6.2.4).

6.2.2 Participants and study design

The APrON study is Canadian cohort of women and infants that enrolled women during their first or second trimester of pregnancy. The inclusion criterion has been previously discussed in Chapter 5 (Section 5.2.1), and by Kaplan *et al.,* (2014) (Kaplan, Giesbrecht et al. 2014). Recruitment began June 2009, with women recruited in early pregnancy and interviewed during each trimester and 3-months postpartum, representing the 3 months postpartum period. Detailed description of the overall study design, recruitment and collection

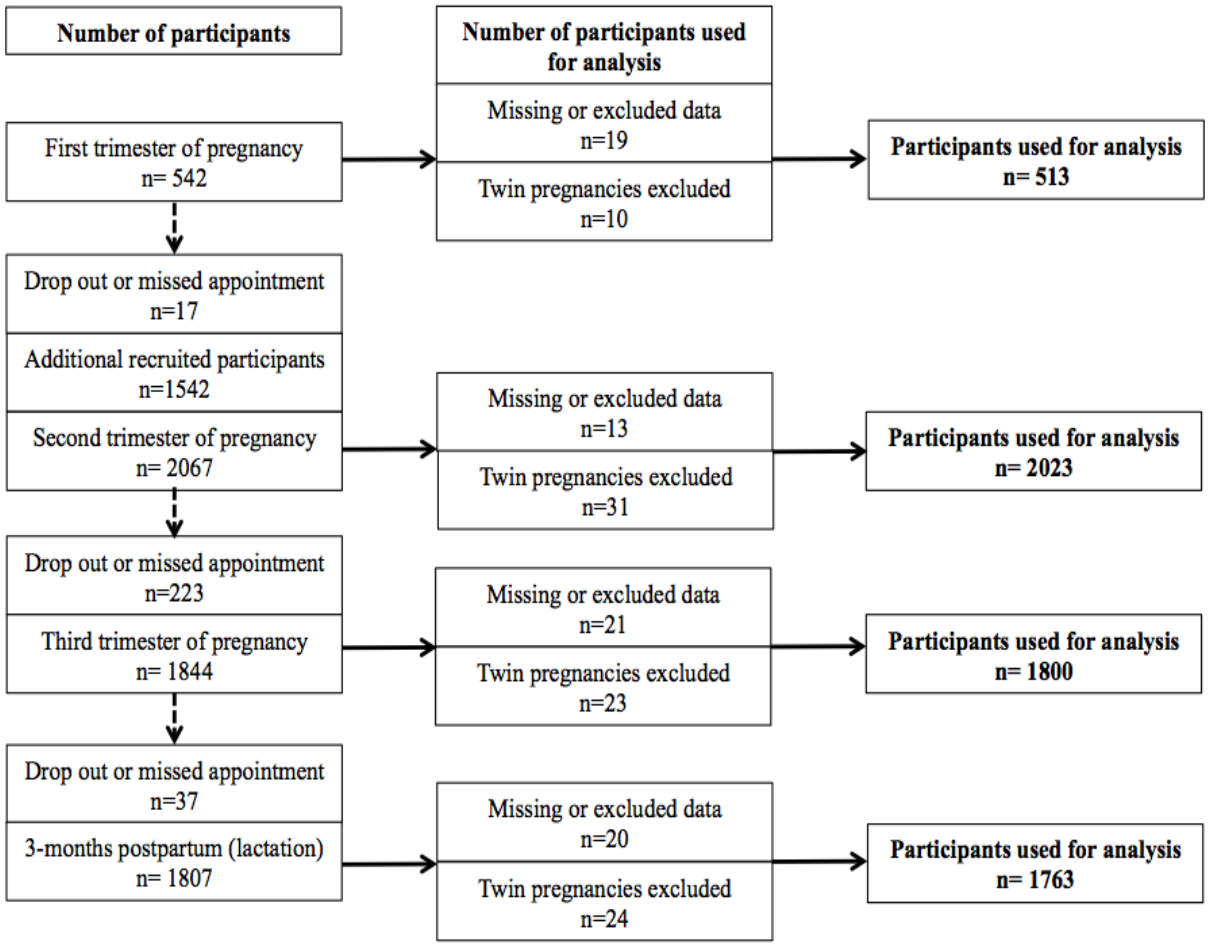
methods have been previously published by Kaplan *et al.,* 2014 (Kaplan, Giesbrecht et al. 2014).

6.2.3 Estimation of choline intake during pregnancy and 3 months postpartum from interview dietary recalls

For women recruited between June 2009 and July 2010, 24-h food recalls were conducted using interview dietary recalls. This method of dietary data collection, also referred to as face-to-face interviews, has been described in Chapter 5 (Section 5.2.2). For this method of dietary recall, at each study visit women were asked to describe in detail the quantity and type of food and dietary supplements consumed in the previous 24-h period. These face-to-face interviews were conducted by trained nutrition educated research assistants using a multiple pass method (Conway, Ingwersen et al. 2003). Interview recalls were entered into Food Processor SQL (ESHA 10.6 Research, Salem, OR, USA) and checked for completeness as described in section 5.2.2. Dietary choline intake was estimated for each trimester of pregnancy and 3-months postpartum using output from Food Processor. The Alberta choline Database was then used with this data to estimate the choline content of food consumed by participants, which has been previously described in section 5.2.2 and in Appendix 2. Described in section 5.2.2, not every participant who completed a visit for each time point completed a 24-h recall, in some cases entry of 24-h recalls into Food Processor had not been conducted therefore was listed as 'missing or excluded data'. Data was further checked for completeness and cleaned based on outliers in energy intake, 24-h recalls that reported >3500 kcal/d and <800 kcal/d were checked for entry errors to ensure correct dietary data had been entered. If this data was correct, outliers were removed from analysis, and included in 'missing or excluded data' in **Figure 6-1**. The number of participants included in the analysis

was determined based on the availability of dietary information. Not every participant who completed a visit for each time point completed a 24-h recall, in some cases entry of 24-h recalls into Food Processor had not been conducted therefore was listed as 'missing or excluded data'. Any recalls where estimated energy or choline intake that was ± 3 SD from the mean was also excluded, and listed as 'missing or excluded data' in Figure 6-1. This data represented a small number of recalls, combined for both interview and online methods, and the number for study visit listed as 'missing or excluded data' is given in Figure 6-1.

Figure 6-1: Study participants enrolled in APrON cohort with available dietary data and included in the analysis at each time point



6.2.4 Estimation of choline intake during pregnancy and 3 months postpartum from online dietary recalls

Beginning August 2010, 24-h food recalls were completed using the validated online Food Behaviour Questionnaire that was developed at the University of Waterloo (Hanning, Woodruff et al. 2007, Hanning, Royall et al. 2009). The web-based online dietary recall contained over 800 food items and utilized a multiple-pass based method. Built in cues and response options such as food pictures and pictorial depictions of portion sizes were used in the online questionnaire to help women estimate portion sizes. Additional visual cues, including a virtual meal plate and meal summary, were used to provide a summary of online recall and the opportunity to revise items before final submission. Upon completion of the online recall, participants were provided a summary of their reported intake, according to food groups in Canada's Food Guide to Healthy Eating. Dietary choline intake was estimated for each trimester of pregnancy and 3-months postpartum using output from the Food Behaviour Questionnaire. The Alberta choline database was then used with this data to estimate the choline content of food consumed by participants, which has been previously described in section 5.2.2. Described above in section 6.2.3 and section 5.2.2, missing and excluded data included dietary data in which energy or choline intake was ± 3 SD from the mean, or if a participant did not complete an online 24-h recall. This data represented a small number of recalls, combined for both interview and online methods, and the number for study visit listed as 'missing or excluded data' is given in Figure 6-1.

6.2.5 Statistical analyses

All data are presented as means ± SD, unless otherwise noted. All data were tested and found to be normally distributed using Kolmogorov-Smirnov test. For the validation study,

differences in intake and energy-adjusted intake for betaine and choline, based on recall method, was assessed by independent samples *t*-test. Pearson's correlation analysis was used to test the strength and association between interview and online dietary recall methods. Bland-Altman analysis was used to test the agreement and bias at the group level. Participants were grouped into tertiles of total choline, PC and free choline intake and energy-adjusted intake (Tertile 1-Tertile 3). The number of participants who stayed in the same quartile when arranged percent of retention based on method was calculated using the formula: (online method/interview method)*100%. Partial correlation analysis was used to assess the correlation between infant birth weight and maternal prenatal energy-adjusted total choline (both recall methods) and unadjusted PC intake (interview method), adjusting for covariates gestational weight gain and gestational age. Participants consuming below recommendations (<425 mg/d) were stratified into tertiles based energy-adjusted total choline intake (213 mg/1000 kcal) and ANCOVA was used to assess differences in infant birth weight between first and third tertiles of total choline intake, adjusting for covariates gestational weight gain and gestational age. Data were analyzed using SPSS Version 21.0 (IBM Corporation, Armonk, NY, USA) with a *P-value* of <0.05 considered statistically significant for all analyses.

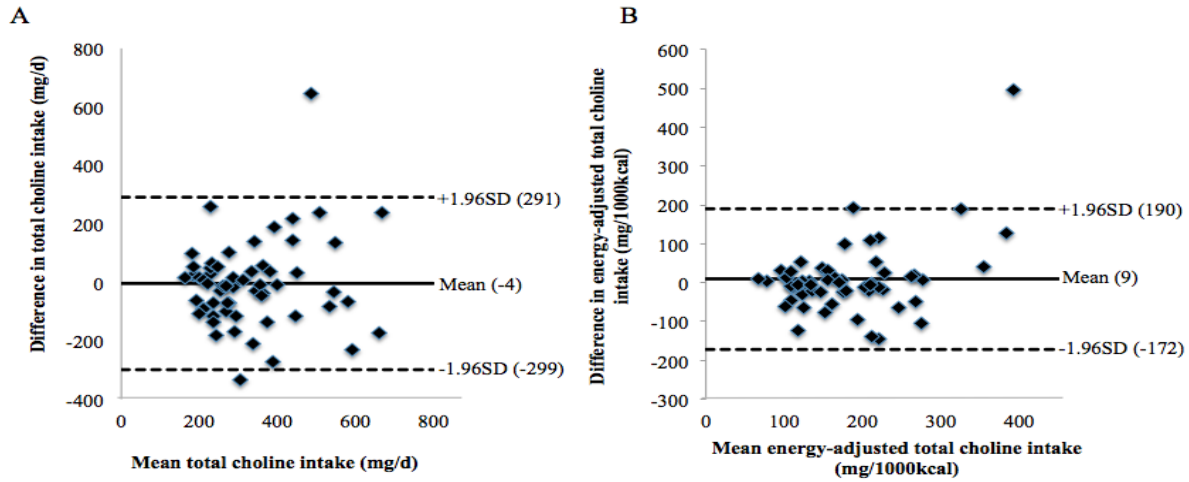
6.3 Results

6.3.1 Comparison of estimated total choline between interview and online recall methods

Unadjusted and energy-adjusted estimates of mean total choline and choline-containing moieties for interview and online recall methods are presented in **Table 6-1**. Interview and online estimates of unadjusted total choline were 333 ± 139 mg/d (95% CI=296-369) and 332 ± 156 mg/d (95% CI=290-372), respectively. The Bland-Altman plots were similar between unadjusted and energy-adjusted estimates of total choline, with the online

method, compared to the face-to-face, underestimating unadjusted total choline by -4 ± 151 mg/d (p=0.883) (**Figure 6-2A**), and overestimating energy-adjusted total choline by 9 ± 93 mg/d (p=0.524) (Figure 6-2B). For both unadjusted and energy-adjusted total choline intake, 97% and 95% of values were within the limit of agreement, respectively (Figure 6-2).

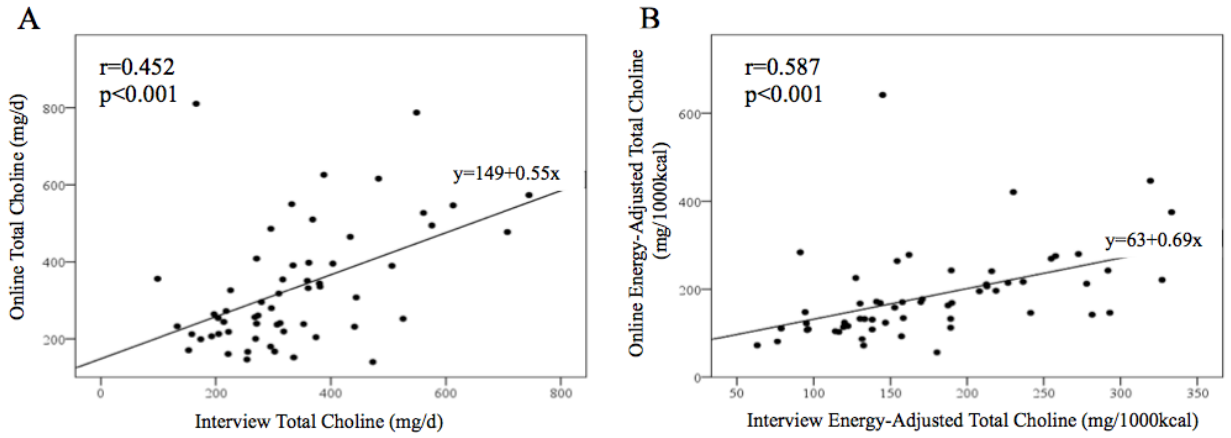
Figure 6-2: Bland-Altman plots assessing agreement and difference between interview and online 24-h recall methods for (A) total choline, and (B) energy-adjusted total choline[1]



[1]A mean of 0 indicates the two methods are in perfect agreement. Mean intake on the x-axis is a mean of the two methods for each participant.

There was a slightly stronger correlation between the methods when energy-adjusted total choline intake was used (r=0.587, p<0.001), compared to unadjusted total choline intake (r=0.452, p<0.001) (**Figure 6-3**).

Figure 6-3: Scatterplot and regression analysis[1] between interview and online recall methods for (A) total choline, and (B) energy-adjusted total choline intake

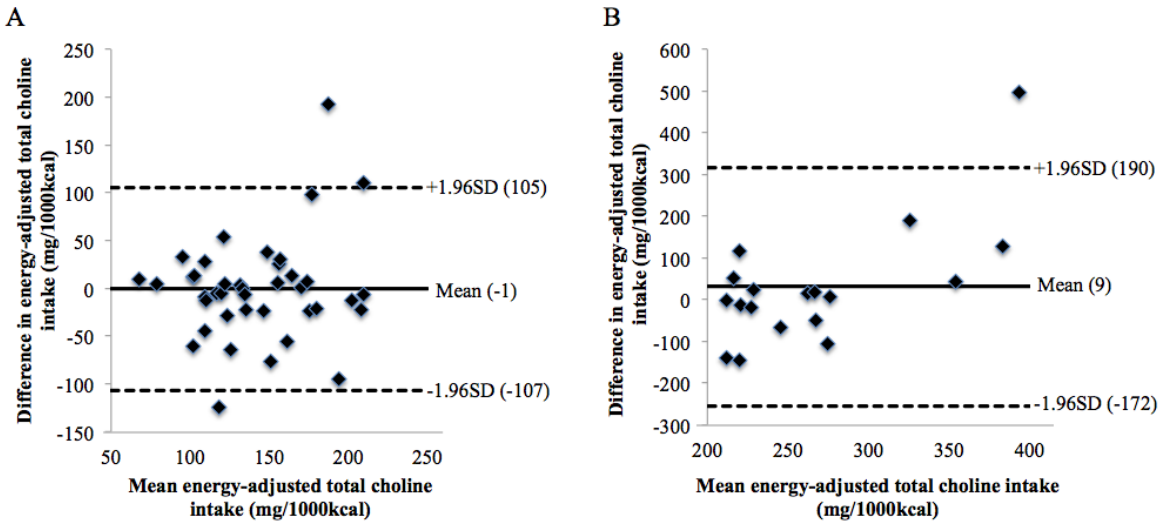


[1]Pearson correlation coefficient (r). *P-value* calculated from Pearson correlation between interview and online methods

Energy-adjusted total choline intake was also more consistent at grouping participants consuming below daily recommendations between dietary recall methods (**Table 6-2**). Approximately 84% of participants were classified as not meeting recommendations (<425 mg) for energy-adjusted total choline for both online and interview methods, compared only 44% who were classified as not meeting recommendations using unadjusted total choline (Table 6-2). Yet, the methods did not consistently classify participants consuming above daily recommendations, with only 50% consistently classified as consuming >425 mg/day for energy-adjusted total choline. This was further illustrated when Bland-Altman plots were constructed for energy-adjusted total choline intake below or above recommendations (213 mg/1000 kcal, equivalent to daily recommendations of 425 mg/d) (**Figure 6-4**). There was a low mean difference between methods (-1 mg/1000kcal) and a narrow limit of agreement (-107 to 105 mg/1000kcal when intakes were below recommendations (Figure 6-4A). Conversely, when intakes were above daily recommendations, there was much greater

variability between methods, with a higher mean difference (9 mg/1000kcal) and a wide limit of agreement (-172 to 190 mg/1000kcal) (Figure 6-4B).

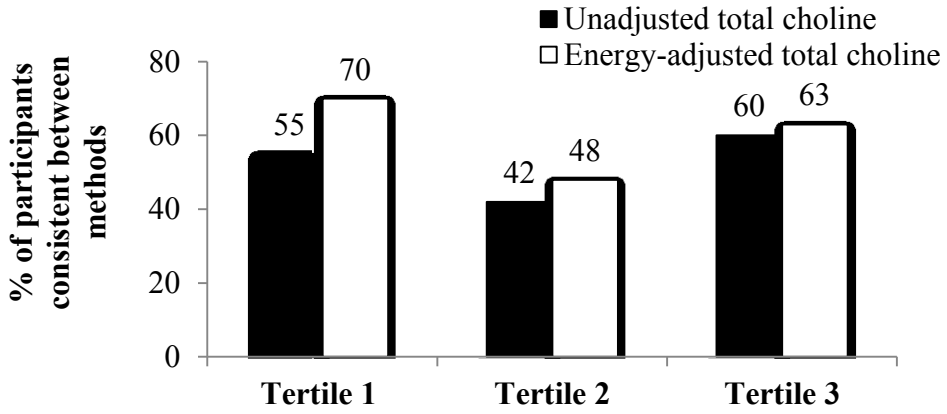
Figure 6-4: Bland-Altman plots assessing agreement and difference between interview and online 24-h recall methods for (A) energy-adjusted total choline below recommendations[1], and (B) energy-adjusted total choline above recommendations (425 mg/d)[2]



[1]The daily recommendation (425 mg/d) for energy-adjusted total choline was calculated based on a 2000 kcal diet, in which recommendations were 212.5/1000 kcal.
[2]A mean of 0 indicates the two methods are in perfect agreement. Mean intake on the x-axis is a mean of the two methods for each participant.

There was a large amount of variability between the methods when total choline was stratified by tertiles of intake **(Figure 6-5)**. For energy-adjusted total choline, 70% of participants grouped in tertile 1 using the interview method were also grouped in tertile 1 using the online method. This is compared to only 55% of participants that were grouped in tertile 1 for both recall methods when unadjusted total choline intake was used (Figure 6-5). This was consistent with the variability between methods for classification of participants in tertiles 2 and 3 (Figure 6-5).

Figure 6-5: Percentage of participants consistently grouped into same tertiles[1] of total choline intake and energy-adjusted total choline intake between interview and online dietary recall methods

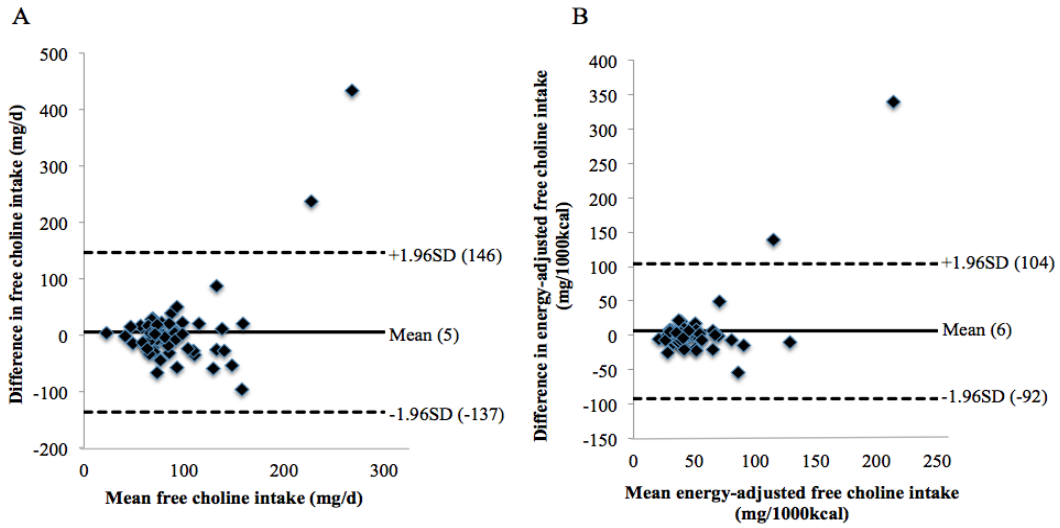


[1]Participants were grouped into tertiles based on total choline intake (Tertile 1-Tertile 3). The number of participants who stayed in the same quartile when arranged percent of retention based on method was calculated using the formula: (online method/interview method)*100%.

6.3.2 Comparison of major dietary forms of choline between interview and online recall methods

We have previously shown (Lewis, Subhan et al. 2014) that the majority of choline, approximately 75% of total choline, is consumed as PC and free choline. The Bland-Altman plots showed that for unadjusted estimates of free choline, the online method overestimated free choline by 5 ± 72 mg/d (p=0.883) (**Figure 6-6A**). When adjusted for energy, with the online method overestimated free choline by 6 ± 50 mg/d (p=0.524) (Figure 6-6B). For both unadjusted and energy-adjusted free choline intake, 98% of values were within the limit of agreement (Figure 6-6).

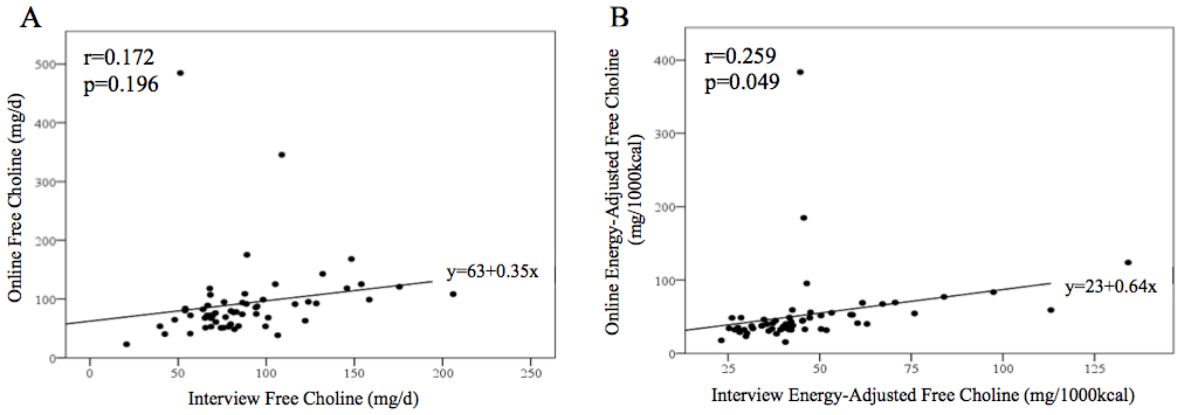
Figure 6-6: Bland-Altman plots assessing agreement and difference between interview and online 24-h recall methods for (A) free choline, and (B) energy-adjusted free choline[1]



[1]A mean of 0 indicates the two methods are in perfect agreement. Mean intake on the x-axis is a mean of the two methods for each participant.

Although the Bland-Altman plots demonstrated agreement between methods, there was no correlation between methods for free choline using unadjusted (r=0.172, *p*=0.196) and only a weak correlations for energy-adjusted (r=0.259, *p*=0.049) values between recall methods (**Figure 6-7**).

Figure 6-7: Scatterplot and regression analysis[1] between interview and online recall methods for (A) free choline, and (B) energy-adjusted free choline intake

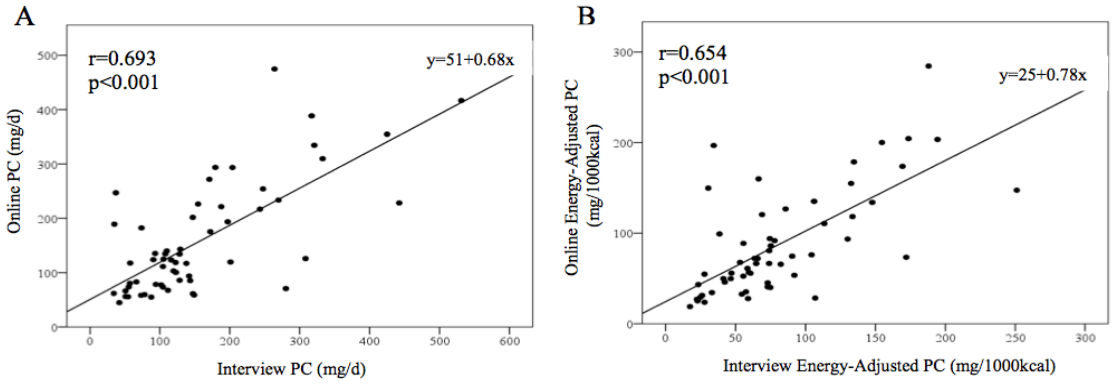


[1]Pearson correlation coefficient (r). *P-value* calculated from Pearson correlation between interview and online methods

Similar to total choline, the methods did not consistently classify participants based on tertiles of intake. Only 50% of participants grouped in tertile 1 using the interview method were also grouped in tertile 1 using the online method, and when unadjusted free choline was stratified by tertiles of intake, only 45% of participants were consistently classified in tertile 1 (**Appendix 3**).

Adjusting PC intake for energy did not improve the correlation between dietary recall methods. There was a reasonable correlation between recall methods for both unadjusted (r=0.654, p<0.001) and energy-adjusted PC intake (r=0.693, p<0.001) (**Figure 6-8**).

Figure 6-8: Scatterplot and regression analysis[1] between interview and online recall methods for (A) PC, and (B) energy-adjusted PC intake

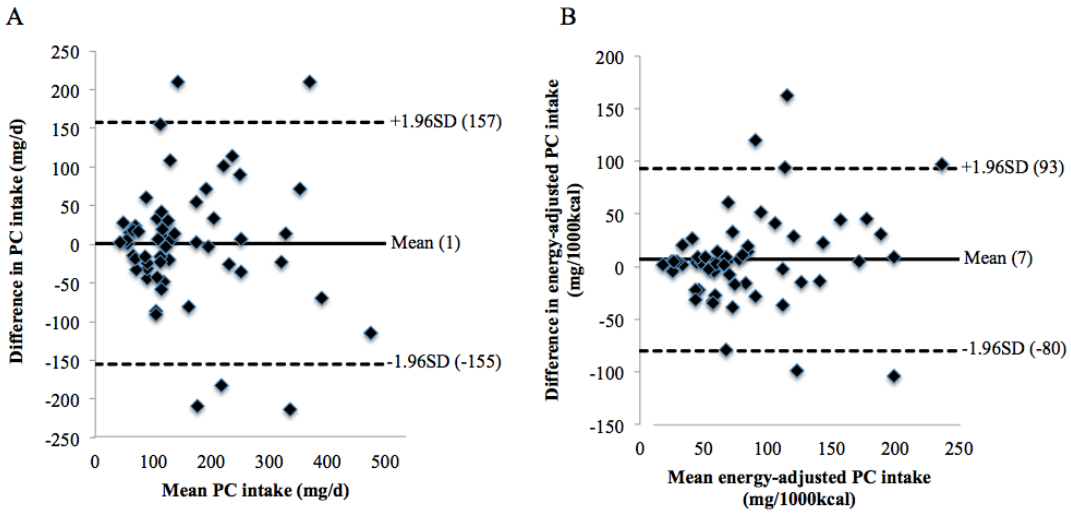


[1]Pearson correlation coefficient (r). *P-value* calculated from Pearson correlation between interview and online methods

The Bland-Altman analysis showed that for unadjusted estimates of PC, the online method overestimated PC by 1 ± 80 mg/d (p=0.883) (**Figure 6-9A**). When adjusted for energy, the online method overestimating PC by 7 ± 44 mg/d (p=0.524) (Figure 6-9B). Yet, the Bland-Altman analysis indicated both unadjusted and energy-adjusted values for PC intake

had a larger number of outliers and variability, with only 91% of values within the limit of agreement (Figure 6-9).

Figure 6-9: Bland-Altman plots assessing agreement and difference between interview and online 24-h recall methods for (A) PC, and (B) energy-adjusted PC[1]



[1]A mean of 0 indicates the two methods are in perfect agreement. Mean intake on the x-axis is a mean of the two methods for each participant.

Unlike total and free choline, there were a higher proportion of participants consistently classified in respective tertiles of intake when unadjusted estimates of PC were used (**Appendix 4**). Yet, there was still considerable variability in the proportion of participants classified by tertiles. Only 65%, 63% and 79% of participants were consistently classified in tertiles 1, 2 and 3, respectively, between methods (Appendix 3).

6.3.3 Comparison of minor dietary forms of choline between interview and online recall methods

The minor forms of choline, contributing approximately 25% of total choline, are GPC, phosphocholine and sphingomyelin. For unadjusted estimates, the online method

underestimated GPC and phosphocholine by -8 ± 30 mg/d (p=0.156) and by -2 ± 7 mg/d (p=0.120), respectively (Table 6-1). Similarly, when adjusted for energy, the online method yielded a lower estimation of GPC (-4 ± 18 mg/d; p=0.202) and phosphocholine (-1 ± 3 mg/d; p=0.397) compared to the interview method (Table 6-1). For both unadjusted and energy-estimates of sphingomyelin, the online method overestimated mean intake by 0.4 ± 11 mg/d (p=0.614) and 1 ± 7 mg/d (p=0.863).

6.3.4 Maternal and infant characteristics, enrolment and analysis of APrON study

The population characteristics of the APrON study based on recall methods are described in **Table 6-3**. For the full cohort, regardless of dietary recall method, the majority of the subjects were recruited in their second trimester of pregnancy (Figure 6-1). The number of participants for each time point (first, second and third trimester and 3-months postpartum) was determined based on availability of dietary information. Between the two cohorts that completed interview and online recall methods, there was slightly greater ethnic diversity among the online recall cohort. Of the cohort that completed interview recalls, 75.5% identified themselves as Caucasian, compared to 85.2% who completed the on-line 24h recall. Mean weight gain in pregnancy was significantly higher (P<0.05) in the interview cohort compared to the online cohort (15.5 ± 6.3 kg vs. 14.9 ± 5.4 kg) (Table 6-3). The majority (94%) of infants had normal birth weights (2500-4499 g) according to Health Canada's Canadian Perinatal Surveillance System. The mean gestational age, birth weight and head circumference of infants from women in the interview cohort were significantly higher compared to the online cohort (P<0.05) (Table 6-3).

6.3.5 Maternal dietary choline intake during pregnancy and at 3-months postpartum and correlation between prenatal choline intake and infant birth weight

Mean intake of total choline and choline-containing moieties in pregnancy and at 3-months postpartum, stratified by dietary recall method, is presented in **Appendix 5**. The correlation between infant birth weight and maternal prenatal energy-adjusted total choline intake was assessed in all participants (both interview and online cohorts). After adjusting for gestational weight gain and gestational age, there was no association between maternal choline intake and infant birth weight (r=-0.01, p=0.754). There was no significant difference in infant birth weight between tertiles of energy-adjusted total choline intake in women consuming below recommendations (**Appendix 6**). Furthermore, there was no correlation between maternal PC intake and infant birth weight after adjusting gestational weight gain and gestational age in women who completed interview 24-h recalls during pregnancy (r=-0.02, p=0.536).

6.4 Discussion

This study demonstrated that the online recall is valid dietary assessment tool when compared to the interview recall method for assessing total choline intake in a population of women of childbearing age in Alberta. However, caution should be used when assessing high total choline intake (above recommendations) and when comparing the major forms of choline, including free choline and PC between methods.

For total choline, the Bland-Altman analysis is commonly used to assess the agreement and presence of bias between dietary assessment methods at a group level (Lombard, Steyn et al. 2015). For total choline (both unadjusted and energy-adjusted), the Bland-Altman analysis indicated there was good agreement between recall methods, with low mean difference and

few outliers (95% of values within limit of agreement). Similar to findings by Hanning *et al.,* and others (Vereecken, Covents et al. 2008), we found the online questionnaire underestimated total choline (2% lower) compared to interview recall. In a population of school-aged children, the online questionnaire appeared to under estimate nutrients, including energy (10.5% lower) and fat (15.6% lower) intakes (Hanning, Royall et al. 2009). The absence of interview probing in the online method may have contributed to the underreporting of choline by online recall method, which has been reported to result in an underreporting of approximately 25% compared to 24-h recalls when interview probing is used (Campbell and Dodds 1967). Vance *et al.,* (2009) demonstrated that females underreported energy intake compared to males using the web-based dietary questionnaire (Vance, Woodruff et al. 2009), which may partly explain a small underreporting of choline in our study. However, the under or overestimation of total and all forms of choline by the online recall method was of low relevance to assessment of choline intake. For example, the underestimation of total choline by online method is equivalent to 4 mg/day, which has little relevance to the daily recommendations of 450 mg/d.

Although the agreement was similar for unadjusted and energy-adjusted values, we observed a stronger correlation between dietary recall methods when total choline was adjusted for energy intake (r=0.59), compared to unadjusted (r=0.45). Previous studies commonly use correlation analyses to assess the strength and association at an individual level (Lombard, Steyn et al. 2015). In school-aged children, Hanning et al., observed a similar positive correlation between the web-based questionnaire and interview recall method for estimates of energy, macronutrient and micronutrient intake, with energy (r=0.56) and other B-vitamins (excluding choline) with a correlation of r=0.35 (vitamin B_6) to 0.92 (vitamin B_{12}) (Hanning, Royall et al. 2009). In a cohort of women from France (n=84) there was a strong

correlation between a similar web-based recall used in our study and interview recall for energy intake (0.85) and B-vitamins ranging from r=0.67 (vitamin B_{12}) to 0.91 (vitamin B_1) (Touvier, Kesse-Guyot et al. 2011). Although neither study examined choline, both studies concluded that given the correlation between methods, the online method was a valid tool in the assessment of dietary intake. Previous studies examining the correlation of choline intake between other dietary assessment methods in women of childbearing age have reported similar correlations found in our study. The correlation for energy-adjusted total choline in our study was similar to the correlation reported by Coathup *et al.,* (2015) between interview 24-h recall and FFQ methods (r=0.60), in addition to a narrow limit of agreement (-150 to 190 mg) and few outliers (Coathup, Wheeler et al. 2015). Pauwels, *et al.,* (2014) reported a correlation of r=0.63 for total choline between 7 day food records and FFQ methods (r=0.63) (Pauwels, Dopere et al. 2014). Previous studies in Canadian adults that have validated the use of FFQ compared to 24-h recalls have reported associations for energy and nutrients (excluding choline) ranging from r=0.32 to 0.70 (Jain, Rohan et al. 2003, Shatenstein, Nadon et al. 2005).

Additionally, energy-adjusted total choline intake study appeared to be the most effective way to examine the number of participants not meeting daily recommendations between both methods. In our study, the majority of participants (84%) were consistently classified as not meeting recommendations (<AI of 425 mg/d) between methods when energy-adjusted total choline estimates were used. Yet, participants were inconsistently classified when consuming above recommendations, and there was poor agreement and wide limit of agreement between methods with high choline intake. Furthermore, less than 70% of participants were consistently classified in their respective tertiles based on total choline between methods. This suggests that energy-adjusted intake is the better way to combine interview and online recall data to describe low total choline intake, and identifying

participants who may be at risk for consuming below recommendations, but caution is advised when combining individuals consuming diets high in choline, exceeding 425 mg/d.

Regarding the forms of choline, the Bland-Altman analysis displayed good agreement between methods with only two outliers and a relatively narrow agreement limit (98% of values within limit of agreement) for free choline, with the online method overestimate intake 5 mg/d. Yet, there was poor correlation between methods ($r=0.26$, $p=0.049$) for energy-adjusted free choline compared to total choline, and previous reports (Hanning, Royall et al. 2009, Coathup, Wheeler et al. 2015). The poor correlation is likely attributed to the narrow range of values for free choline, which has been shown to contribute to a weak correlation (Lombard, Steyn et al. 2015). However, taken together this suggests that while on a population basis, there may be agreement between recall, at an individual level, free choline may not be comparable between dietary assessment methods.

Adjusting for energy intake did not improve the correlation between recall methods suggesting that intake of PC may be independent of energy intake, unlike total choline and free choline. Although PC intake was strongly correlated between methods ($r=0.69$) and the mean difference for unadjusted or energy-adjusted PC intake was <7 mg, the Bland-Altman analysis indicated there was considerable variation in the estimation of dietary PC. There was a wide limit of agreement, with 9% of values outside the limit of agreement, suggesting that there is great inter-variability between methods for PC. For both free choline and PC, a low proportion of participants were consistently grouped in their respective tertiles of intake, suggesting that the two methods are not comparable on an individual level.

For the minor forms of choline, it appears that phosphocholine and GPC are comparable between dietary recall methods. Although there was a 30% difference between

estimates of sphingomyelin between recall methods, sphingomyelin only represents a 5% contribution to total choline intake.

There are several limitations and challenges when comparing dietary assessment methods. Issues of portion size estimation, nutrient content of mixed foods and food substitutions have been shown to contribute to discrepancies in reporting nutrient intake in self-reported dietary recalls (Kye, Kwon et al. 2014, Shim, Oh et al. 2014). Examining dietary choline intake in this population of pregnant Albertan women, it appears that discrepancies between methods results primarily from estimation of portion sizes, particularly with beverages, and reporting of mixed foods and substitutions of foods. When reporting mixed foods, interview recalls appear to report the individual components of a mixed dish, compared to the online recall in which mixed foods would just be reported as a whole. For example, a noodle dish with chicken may be reported on the online recall, but the interview recall lists all the individual ingredients separately, which results in discrepancies in estimates of choline between methods. When combining dietary recall methods, particular care should be given when estimating the choline intake of mixed foods. An additional factor to consider when using different dietary assessment methods is the number of recalls or assessments needed to accurately estimate intake. A study by Ma *et al.,* (2009) suggested that completion of three 24-h dietary recalls was optimal for the estimation of energy intake, with one 24-h recall resulting in an underestimation of energy, and additional 24-h recalls not improving estimation (Ma, Olendzki et al. 2009). Pertaining to choline, when only one 24-h recall is used, it may not fully capture dietary patterns and result in underreporting of less frequently consumed sources of choline, such as eggs. Using three 24-h dietary recalls instead of just one, may provide the best estimate of dietary choline intake (Ma, Olendzki et al. 2009), and be the most useful in comparing methods of 24-h recalls.

Using the results from this study, recommendations and relevance for practice can be discussed. A study by Fischer *et al.,* (2005) found that choline intake was correlated to energy intake, using either self-reported or weighed food dietary recall methods, suggesting adjusting for energy may normalize data between methods (Fischer, Scearce et al. 2005). There was a stronger correlation between methods for energy-adjusted values of total choline, and a stronger agreement between methods when assessing participants consuming diets low in choline (<425 mg/d). This suggests that at the population level and those consuming below recommendations, the methods of dietary recall are comparable and can be used together. Yet, assessing individuals consuming diets high in choline (>425 mg/d) and allocation of participants based on tertiles of intake was not consistent between methods for unadjusted or adjusted total choline. Therefore, caution is advised when analyzing choline intake between methods at an individual level. Additionally, researchers should focus on total choline (comprised of five forms) rather than individual forms if using both dietary methods together. There was a weak correlation between methods for free choline, and low agreement between methods for PC, therefore if examining these two major forms of dietary choline, only the interview recall method should be used.

Furthermore, we found no association between energy-adjusted maternal choline intake in pregnancy and infant birth weight. This is consistent with two other studies that also found no association between maternal dietary choline or plasma choline concentrations and overall infant growth (Jiang, Bar et al. 2013, Hogeveen, den Heijer et al. 2013). Although we found no association with maternal energy-adjusted total choline intake and infant birth weight, this research question requires further investigation. We demonstrated in a rodent model that a maternal choline-devoid diet during lactation reduced offspring growth at the end of suckling by approximately 30% (Lewis, Goruk et al. 2016) Future research should examine if low

dietary choline during pregnancy and early postpartum is associated with low infant birth weight, compared to high dietary intake during these periods, while controlling for other factors (maternal, dietary and infant) known to influence growth. A detailed review by Caudill *et al.,* (2014), discusses the improvements to offspring health with maternal choline supplementation.

6.5 Conclusion

Overall, the correlation between online and interview 24-h recall methods was similar to previous studies. If only assessing the individual forms of choline including free choline and PC, only the interview recall method should be used. However, energy-adjusted estimates of total choline had strong correlation and good agreement between methods, and were comparable between methods when examining low choline intake.

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Tables

Table 6-1: Intake of choline containing moieties and total choline estimated by online and interview 24-h recalls (n=58)

	Interview (Mean ± SD)	95% CI	Online (Mean ± SD)	95% CI	Mean difference[1]	P-value[2]	Correlation (r)[3]	P-value[4]
Energy (kcal)	1984 ± 603	1826 - 2143	1892 ± 471	1768 - 2016	-92 ± 502	0.361	0.629	<0.001*
Choline-containing moieties								
Free Choline (mg/d)	89 ± 35	79 - 97	93 ± 70	74 - 111	5 ± 72	0.661	0.172	0.196
Energy-adjusted free choline (mg/1000 kcal)	47 ± 21	41 - 52	53 ± 51	39 - 66	6 ± 50	0.413	0.259	0.049*
Glycerophosphocholine (mg/d)	57 ± 31	49 - 65	49 ± 28	41 - 56	-8 ± 30	0.156	0.491	<0.001*
Energy-adjusted glycerophosphocholine (mg/1000 kcal)	30 ± 19	25 - 35	26 ± 15	22 - 30	-4 ± 18	0.202	0.484	<0.001*
Phosphocholine (mg/d)	16 ± 8	14 - 18	14 ± 6	12 - 16	-2 ± 7	0.120	0.608	<0.001*
Energy-adjusted phosphocholine (mg/1000 kcal)	9 ± 5	7 - 10	8 ± 4	7 - 9	-1 ± 3	0.397	0.723	<0.001*
Phosphatidylcholine (mg/d)	154 ± 107	125 - 182	157 ± 103	131 - 185	1 ± 82	0.845	0.693	<0.001*
Energy-adjusted phosphatidylcholine (mg/1000 kcal)	80 ± 51	66 - 93	88 ± 58	73 - 103	7 ± 46	0.433	0.654	<0.001*
Sphingomyelin (mg/d)	18 ± 12	15 - 21	18 ± 13	15 - 22	0.4 ± 11	0.863	0.614	<0.001*
Energy-adjusted sphingomyelin (mg/1000 kcal)	10 ± 8	8 - 12	11 ± 11	8 - 14	1 ± 7	0.542	0.779	<0.001*
Total Choline (mg/d)	333 ± 139	296 - 369	332 ± 156	290 - 372	-4 ± 151	0.883	0.452	<0.001*
Energy-adjusted total choline (mg/1000 kcal)	175 ± 68	157 - 193	185 ± 102	158 - 212	9 ± 93	0.524	0.587	<0.001*

[1]Mean difference between methods=Online – Interview

[2]*P-value* calculated using paired *t*-test to test mean difference between interview and online methods

[3]Pearson correlation coefficient (r)

[4]*P-value* calculated from Pearson correlation between interview and online methods

*indicates significant Pearson correlation coefficient ($P<0.05$).

Table 6-2: Percentage of participants consistently grouped into meeting or not meeting daily recommendations (AI) for women between interview and online dietary recall methods for unadjusted and energy-adjusted total choline intake[1]

	% of participants classified between recall methods	
	Unadjusted Total choline	Energy-Adjusted Total Choline[2]
Not Meeting AI (<425 mg/d)	44	84
Meeting AI (≥425 mg/d)	60	50

[1]Participants for each method were arranged based on total choline intake and divided into groups based on meeting or not meeting recommendations. The number of participants who stayed in the same group when arranged percent of retention based on method was calculated using the formula: (online method/interview method)*100%.
[2]The daily recommendation (425 mg/d) for energy-adjusted total choline was calculated based on a 2000 kcal diet, in which recommendations were 212.5/1000 kcal.

Table 6-3: Characteristics of women and infants in the APrON study stratified into cohorts based on dietary recall method (n=1763)

Characteristic[1]	Dietary Recall Method	
	Interview	Online
Age (years), n (%[2])		
16-30	379 (36.8%)	448 (41.0%)
31-45	651 (63.2%)	644 (59.0%)
Unknown	2	1
Pregnancy Weight Gain (kg)	15.4 ± 6.3	14.9 ± 5.4*
Pre-pregnancy BMI Categories[3], n (%[2])		
Underweight	30 (3.5%)	39 (3.8%)
Normal Weight	554 (63.9%)	639 (62.7%)
Overweight	183 (21.1%)	228 (22.3%)
Obese	100 (11.5%)	113 (11.1%)
Unknown	165	74
Pregnancy Weight Gain Categories[4], n (%[2])		
Inadequate	123 (15.7%)	157 (17.5%)
Adequate	260 (33.1%)	309 (34.4%)
Excessive	402 (51.2%)	430 (48.0%)
Unknown	247	197
Education, n (%)		
≤ High school	102 (10.3%)	148 (14.4%)
Trade	209 (21.0%)	185 (18.0%)
Undergraduate/postgraduate	682 (68.7%)	694 (67.6%)
Unknown	39	66
Family income, n (%[2])		
Low (≤ $69 000)	191 (23.1%)	263
Medium ($70 000- 99 000)	222 (21.5%)	230 (22.3%)
High (≥ $100 000)	572 (55.4%)	538 (52.1%)
Unknown	47	62
Ethnicity, n (%[2])		
Caucasian	844 (85.2%)	784 (75.5%)
Latin American	35 (3.5%)	35 (3.4%)
Korean	13 (1.3%)	94 (9.1%)
Other	99 (10.0%)	125 (12.0%)
Unknown	41	51
Infant Gestational Age (weeks), mean ± SD	39.2 ± 1.7	39.0 ± 2.1*
Infant Birth Length (cm), mean ± SD	55.2 ± 3.6	50.8 ± 3.3
Infant Head Circumference (cm), mean ± SD	34.5 ± 1.9	34.3 ± 2.0*
Infant Birth Weight (g), mean ± SD	3372 ± 517	3321 ± 554*
Birth Weight Categories[5], n (%[2])		
Low birth weight (≤ 2499 g)	43 (4.6)	45 (4.9)
Normal birth weight (2500-4499 g)	864 (93.9)	861 (93.8)
High birth weight (≥ 4500 g)	14 (1.5)	12 (1.3)
Unknown	111	175

[1]Baseline characteristics collected at the time of study enrolment.

[2]% of each characteristic based on available data, does not include the percentage of unknown values, in which data was not collected or obtained from participants.
[3]Pre-pregnancy BMI Categories assessed based on self-reported pre-pregnancy weight (kg) and height (m). BMI (kg/m^2) was categorized using the World Health Organization (WHO) classifications for underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9) and obese (≥30) (WHO 2013).
[4]Pregnancy Weight Gain Categories assessed using calculated pre-pregnancy BMI and total weight gain during pregnancy (3rd trimester visit). Weight gain classified inadequate, adequate and excessive based on the recommended range of total weight gain by BMI category from the Canadian Gestational Weight Gain Recommendations (Health Canada 2010).
[5]Birth Weight Categories assessed based on Health Canada's Canadian Perinatal Surveillance System.
*indicates value within a row that is significantly different from Interview cohort *(P*<0.05*)*.

CHAPTER 7: Feeding a diet devoid of choline to lactating dams restricts growth and lymphocyte development in offspring[1, 2]

7.1 Introduction

Choline is an essential nutrient required for a variety of critical processes including synthesis of membrane phospholipids and acetylcholine, lipoprotein formation and methyl group metabolism (Zeisel and da Costa 2009). It can be obtained from both dietary and *de novo* sources, with subclinical symptoms of fatty liver and/or muscle dysfunction resulting from insufficient intake of choline across species (Hove and Copeland , Zeisel). Choline is widely distributed in food sources, with major sources in the diet reported to include eggs, meat (beef and chicken) and dairy products (Lewis, Subhan et al. 2014). In the early postnatal period, the need for exogenous choline increases to meet the demands by the infant for rapidly dividing and developing tissues, and to account for the depletion of maternal stores (Gossell-Williams, Fletcher et al. 2005). Both the umbilical cord (Molloy, Mills et al. 2005) and newborn plasma (Ilcol, Ozbek et al. 2005) choline concentrations are significantly higher than concentrations in mother's plasma, suggesting delivery against a concentration gradient to the fetus and infant. Pregnancy and lactation are reported to increase *de novo* synthesis in rodents (Gwee and Sim 1979, Burdge, Hunt et al. 1994), suggesting a reliance on dietary sources during this period may be reduced. However, it has been demonstrated that lactating female

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[2] Content in this chapter was presented at Experimental Biology, San Diego, CA, April 2016, the Canadian Nutrition Society, St. John's, NL, June 2014, and Experimental Biology, Boston, MA, April 2013

rodents had greater depletion of liver choline metabolites when fed a choline-deficient diet as compared to non-lactating females. This suggests that although there is greater *de novo* choline synthesis, this period may result in greater sensitivity to changes in dietary choline intake (Zeisel, Mar et al. 1995). Despite increased requirements, our recent cohort study found that the majority (90%) of lactating women were consuming far below the current daily AI recommendations (Lewis, Subhan et al. 2014) and it is not clear what effects this may have on their children's long-term health. The essentiality of choline in the early postnatal period is well established for many organs including brain and liver (reviewed by (Caudill 2010). Given the large amount of cellular expansion occurring within the immune system, it is likely that choline plays a critical role in the development of the immune system; however, the effects of maternal choline deprivation during this critical period has not yet been examined.

The early postnatal period is a critical stage of immune system development, with rapid immune cell expansion and proliferation in the rodent (Perez-Cano, Franch et al. 2012) and human (Field 2005). It is recognized that nutrients modify immune function (Calder 2007) and this may be of particular importance during the critical development that occurs during the suckling period (Field 2005). A study in adult rodents found that feeding a diet without choline (compared to a diet containing 3.5g choline/kg) for 8 weeks resulted in lower DTH response and proliferation of splenocytes after stimulation with ConA (Courreges, Benencia et al. 2003). Similarly, we demonstrated that feeding a diet without exogenous choline during the lactation period significantly impaired the function of the maternal immune system and reduced growth of the suckling offspring (Dellschaft, Ruth et al. 2015). However, the effect of a maternal diet devoid of choline on the pup's immune system was not measured.

The concept of nutritional programming refers to the effect of environmental factors including diet during a critical period of development on immediate and long-term functions

and responses. This concept has not been well established for the development of the immune system, and the potential programming effects of maternal choline intake during the early postnatal period has not been studied. An earlier study found that maternal dietary lipotrope deficiency (choline, methionine and vitamin B_{12}) during suckling resulted in depressed T cell function in the offspring and that it predisposed offspring to bacterial infection (*Salmonella typhimurium*) in the post weaning period (Newberne, Wilson et al. 1970). Another study examining the effects of age on a marginally methionine-choline deficient diet (67% lower in methionine and choline compared to control diet) in weaned rats found that animals at 3-weeks post weaning were most sensitive to methionine-choline deficiency, compared to older animals (12 months post weaning) (Nauss, Connor et al. 1982). These studies suggest that lipotrope deficiency during suckling may result in increased susceptibility to infection. However, there are no reported studies examining the effects of choline insufficiency (independent of other methyl donors in the diet) during suckling on immune function later in life.

Our previous study demonstrated that dams fed a diet devoid of choline during lactation negatively affected maternal immune function and impaired pup growth (Dellschaft, Ruth et al. 2015). Therefore, the first objective of the present study was to determine the effect of maternal choline deficiency on immune system development in suckled offspring. The second study objective was to determine if the intake of choline during lactation would have a programming effect on the development and function of the offspring's immune system later in life. We hypothesized that a choline-deficient maternal diet would be insufficient in supporting the development of the offspring's immune system, and would have lasting effects on the offspring's immune system even if provided a choline sufficient diet post weaning.

7.2 Experimental methods

7.2.1 Animals and diets

Experiment 1. To examine the effect of maternal choline deficiency on offspring immune system development and function, female Sprague-Dawley rats (n=19) were obtained at 14 days gestation from Charles River Laboratories (Montreal, QC, Canada). All dams were fed a standard rat chow diet (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA; containing 1 g choline/kg, Harlan Teklad, Indianapolis, IN, USA) for the duration of pregnancy until parturition. Within 24-h of birth, dams were randomized to one of two isocaloric, isonitrogenous and nutritionally adequate diets (**Table 7-1**). Diets were fed *ad libitum* for the duration of the lactation period until 3 weeks postnatal. Animals had free access to food throughout the study period and feed cups were refilled every 2-3 days.

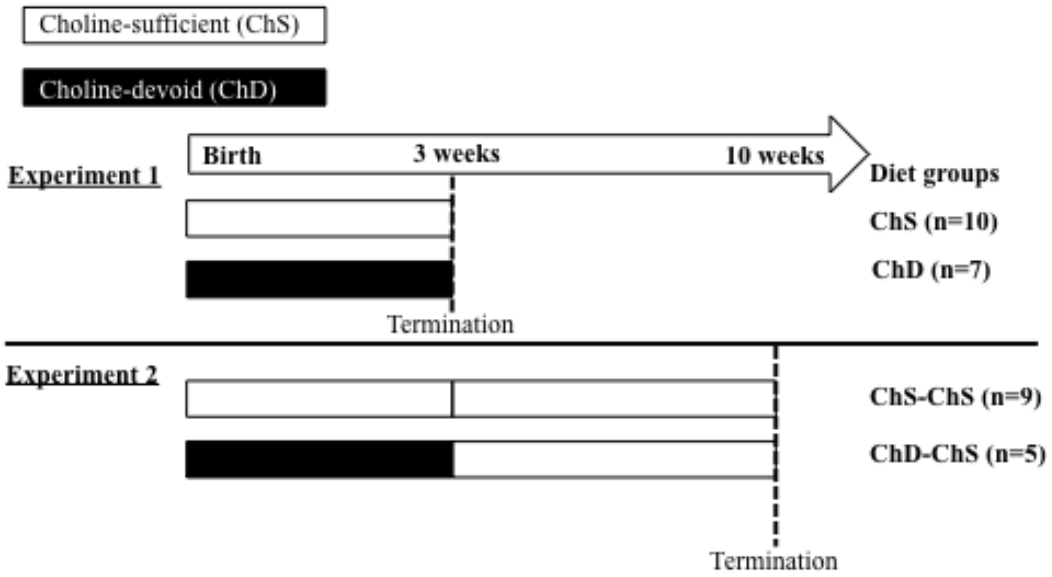
The two diets differed only in the amount of choline provided (Table 7-1): choline-deficient (0 g choline/kg diet; ChD, n=9) and choline-sufficient (1.0 g choline /kg diet; ChS, n=10). The choline-sufficient diet contains the recommended amount of choline by The Nutrient Requirements of Laboratory Animals (Reeves 1997). This amount of choline (1g/kg diet) is included in AIN-93 standard rodent diet mix (Reeves 1997). In humans, the daily recommendation (which is reported as an adequate intake) of choline for lactating women is 550 mg/d. Mean intake in this population of humans is approximately 350 mg/d. We have previously reported that approximately the majority of lactating women (90%) are estimated to be consuming below what is thought to be the adequate intake (Lewis, Subhan et al. 2014). The diet used in the current study contains what is recommended to meet the needs of pregnant and lactating and growing rodents. At birth, litters were culled to ten offspring (5 males and 5 females when possible) per dam. Dietary intake of dams was recorded throughout the lactation period and body weight was recorded regularly throughout the study period. This

study concluded at the end of the suckling period (3 weeks) and two offspring from each dam were euthanized, with the study design presented in **Figure 7-1.** Two of the dams and their litters from the ChD group were euthanized before the end of the experiment due to significant weight loss and not included in data analysis making the final n=7 for the ChD group. Experiment 1 was conducted in two complete blocks in two separate years. The experimental unit in the study was the dam. For each n in the study we used a mean of the measure from two offspring (including one male and one female offspring), and for each offspring measure that we report as a unit (n) was the mean of two rodents. The sex of the offspring was not recorded; however, the covariant of variability between the two offspring that we combined for each dam was <10% for all the parameters. This suggests that there was not a significant effect of sex at this age, similar to what we have previously reported (Field 1999).

Experiment 2. To investigate if the amount of choline provided during suckling has a programming effect on immune development and function, a new set of dams were randomized (within 24-h of giving birth) to the choline-deficient (n=7, ChD-ChS) or choline-sufficient (n=9, ChS-ChS) diet (Table 7-1) for the 3 week lactation/suckling period, as described in Experiment 1. At 3 weeks of age, two female offspring per dam from both diet groups were weaned to the ChS diet for 7 weeks. All offspring were euthanized at 10 weeks of age (Figure 7-1). Rats reach sexual maturity at about 4 to 5 weeks of age, therefore female rats in this experiment were sexually mature. Two dams and their litters from the ChD-ChS group were euthanized before the end of the experiment due to significant weight loss and not included in data analysis making the final n=5 for the ChD-ChS group. The institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Committee of Animal Policy and Welfare

of the Faculty of Agriculture, Life and Environmental Sciences at the University of Alberta, Edmonton, AB, Canada.

Figure 7-1: Experimental study design[1]



[1]Dams were randomly assigned to the choline-sufficient (ChS) or choline-devoid (ChD) diet for the duration of the lactation/suckling period. For experiment 1, two offspring (one female and one male) from each dam were terminated at the end of the 3-week suckling period. For experiment 2, two female offspring from each dam were then fed a ChS diet for an additional seven weeks. At 10 weeks, offspring were terminated. The dam represents the experimental unit in this design therefore the number of observations within each group is equal to the number of dams, with an average of two offspring per dam for every measurement.

7.2.2 Tissue collection

At both time points, two offspring from each dam were weighed and euthanized by CO_2 asphyxiation in the morning hours. Blood was collected in EDTA-containing tubes via cardiac puncture. Plasma was collected following centrifugation at 3000g for 10 minutes. Liver and spleen weight and intestine length were recorded. Spleens were collected aseptically, weighed and immune cells were isolated for further processing (see below).

Offspring stomach content and livers were collected aseptically, weighed, snap frozen using liquid nitrogen then stored at -80°C until analysis.

7.2.3 Choline metabolite analyses of offspring stomach content

The stomach content of offspring was analyzed to reflect the choline content of dam's milk and determine the effect of maternal diet on milk content. Frozen stomach content was ground on liquid nitrogen and extracted using a modified Bligh and Dyer method that has been described in detail elsewhere (Zhao, Xiong et al. , Xiong, Zhao et al.). Extracts were quantified for all significant choline-containing metabolites and total choline content by HILIC LC-MS/MS (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012) using an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP mass spectrometer (AB SCIEX, Concord, ON, Canada).

7.2.4 Immune cell isolation

Isolation of immune cells from spleen has been previously described (Field, Wu et al. 1990). Briefly, single cell suspensions were obtained by disrupting tissue through a nylon mesh screen in sterile Krebs-Ringer HEPES buffer with bovine serum albumin (5 g/l; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, 10mM $KHCO_3$; Fisher Scientific, Edmonton, AB, Canada) was used to lyse erythrocytes. Cells were washed then re-suspended in complete culture medium (RPMI 1640 media; Life Technologies, Burlington, ON, Canada), supplemented with 5% (v/v) heat-inactivated fetal calf serum, 25 mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic (pH 7.4; Fischer Scientific, see above). A haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich, as above) and diluted to 1.25 x 10^6 cells/ml.

7.2.5 Immune cell phenotype analysis

Immune cell subsets present in freshly isolated splenocytes were identified by direct immunofluorescence assay, as previously described (Field, Wu et al. 1990, Field, Thomson et al. 2000). Briefly, 200,000 immune cells were incubated for 30 mins at 4°C with pre-labelled monoclonal antibodies applied in combination in order to quantify immune cell phenotypes. The use of four-colour flow cytometry allowed identification of the following combinations of surface molecules in Experiment 1 (Exp 1) and/or Experiment 2 (Exp 2): CD28/CD3/CD8/CD4 (Exp 1 and 2), CD3/CD45RA/CD27/OX12 (Exp 1 and 2), OX62/OX6/CD8 (Exp 1 and 2), CD68/CD284/CD11b/c (Exp 1 and 2), CD161/CD3 (Exp 1 and 2), IgG/IgM, IgA, CD3/FOXP3/CD25/CD4 (Exp 1 and 2), CD25/CD152/CD8/CD4 (Exp 1), CD71/CD3/CD8/CD4 (Exp 1), CD80/CD152/CD28 (Exp 1), OX12/OX6/CD27 (Exp 2), CD25CD3/CD4/CD8 (Exp 2), CD45RA (Exp 2). The CD45RA isoform, also known as leukocyte common antigen, is present only on B cells in rats (Woollett, Barclay et al. 1985). All antibodies with the exception of IgG, IgM and OX6 (BD Biosciences, Mississauga, ON, Canada) were purchased from Cedarlane Laboratories, (Burlington, ON, Canada). After incubation, cells were washed and fixed in paraformaldehyde (10 g/l; ThermoFisher, Edmonton, AB, Canada) in phosphate-buffered saline. To identify the intracellular protein FOXP3, an indicator of T regulatory cells (Treg), isolated cells were permeabilized prior to antibody addition, according to manufacturer's directions (Cedarlane Laboratories, Burlington, ON, Canada). All of the samples were acquired within 72 h of preparation by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) according to the relative fluorescence intensity determined using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada). From the samples acquired, the total lymphocyte population was gated from the

forward-scattered light (FSC) vs. side-scattered light (SSC) plot, and the data presented in the results section represents the percent of total cells from that mononuclear cell population gate.

7.2.6 Ex vivo cytokine production by mitogen-stimulated splenocytes

The measurement of the production of cytokines by mitogen-stimulated splenocytes has been previously described (Blewett, Gerdung et al. 2009). Briefly, splenocytes (1.25×10^6 cells/ml) were cultured in 3 ml RMPI-1640 medium (as above) for 48 h at 37°C and 5% CO_2 without mitogen (unstimulated) or with mitogen ConA (2.5 μg/ml; MP Biomedicals, Montreal, QC, Canada), LPS, 100 μg/ml; Sigma-Aldrich, as above), or both CD3 (1 μg/ml) and CD28 (5 μg/ml; both from e-Bioscience Inc., San Diego, CA). ConA is a polyclonal T-cell stimulant and monoclonal antibodies CD3/CD28 activate the T cell receptor, with both CD3/CD28 and ConA stimulating the T cell population. LPS activates the antigen-presenting cell population, including dendritic cells, macrophages and B cells by binding to their Toll-like receptor (CD284). After incubation, cells were centrifuged for 10 minutes at 1000 rpm and supernatants collected and stored at -80°C until analyses. Concentrations of cytokines interleukin (IL)-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ were measured by commercial ELISA kits according to manufacturer's instructions and as previously described (Blewett, Gerdung et al. 2009). The detection limits for all cytokines were 15.6-4000 pg/ml, except for IFN-γ in which the detection limit was 9.8-2500 pg/ml (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%. The amount of IL-2 in the media after LPS stimulation was below detection levels. IL-1β was only measured in the supernatant of LPS stimulated cells. To express cytokine response by specific lymphocyte population, the % of immune cell

phenotype (i.e. CD4+CD25+) was multiplied by $1.25x10^6$ (number of total cells added to culture). Then cytokine production (pg/ml) (i.e. IL-2) was divided by the number of lymphocytes added to culture (ie. $x10^6$ of CD4+CD25+ cells) to express the amount of IL-2 in the media per a specific lymphocyte subset.

7.2.7 Plasma concentrations of cytokines, chemokines and haptoglobin

To determine circulating concentrations of cytokines and chemokines in plasma, electrochemiluminescent multiplex cytokine kit (Proinflammatory Panel 1 V-PLEX, MesoScale Discovery, Rockville, MD, USA) was used. IFN-γ, IL-10, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α and KC/GRO (keratinocyte chemoattractant/human growth-regulated oncogene also know as CXCL1, chemokine ligand 11) were measured according to the manufacturer's instructions. Briefly, standards and samples (50 µl/well) were added in duplicate to a plate pre-coated with capture antibody for each cytokine. Plates were incubated for 2 hours at room temperature on a plate shaker. Plates were then washed with wash buffer (3 x PBS with 0.05% Tween 20). Detection antibody was added and incubated for an additional 2 hours. Plates were then washed with wash buffer (3x) and Read Buffer was added. Plates were read on the MSD Sector Image 6000 (MesoScale Discovery, as above) and all measurements were conducted in duplicates. The lower level of detection limits were 0.65 pg/ml (IFN-γ), 16.4 pg/ml (IL-10), 1.97 pg/ml (IL-13), 6.92 pg/ml (IL-1β), 0.69 pg/ml (IL-4), 14.1 pg/ml (IL-5), 13.8 pg/ml (IL-6), 0.72 pg/ml (TNF-α), 1.04 pg/ml (KC/GRO).

Plasma haptoglobin concentrations were determined using a colorimetric assay according to manufacturer's instructions (Tridelta Development, Wicklow, Ireland). The detection limit for haptoglobin was 0.312 mg/ml (Tridelta Development, see above). All measurements were conducted in duplicates, with CV <10%. For the 3-week offspring,

discrepancies between total number of offspring (n=17) and the number available for measurements (n=12) of haptoglobin, TNF-α and KC/GRO are due to clotting of the sample, preventing the processing of those samples.

7.2.8 Estimation of lymphocyte proliferation in the absence and presence of mitogens in spleen

In a subset of 3-week old animals (n=5 dams), proliferation was estimated by the rate of [^{3}H]thymidine uptake by spleen lymphocytes (1.25 x 10^6/ml) in the presence or absence of mitogens. Lymphocytes were cultured for 48 h in 96-well plates with or without mitogens, as previously described (Field, Van Aerde et al. 2008). The mitogens used were ConA (2.5 μg/ml; MP Biomedicals, as above), LPS (100 μg/ml; Sigma-Aldrich, as above) or both CD3 (1 μg/ml) and CD28 (5 μg/ml; both from e-Bioscience Inc., as above). Each well was pulsed with 0.037 MBq of [^{3}H]thymidine (Amersham/Pharmacia Biotech, Montreal, QC, Canada) for 18 h before collecting cells. Proliferation to LPS was not measured as in our ex vivo conditions as it does not stimulate IL-2 production by splenocytes. All assays were conducted in triplicate for each offspring and two offspring were pooled to obtain a measure of each dam. Stimulation index for each mitogen condition was calculated as: amount of [^{3}H]thymidine uptake (dpm) in the presence of each mitogen divided by the amount of [^{3}H]thymidine uptake (dpm) in the absence of each mitogen. Lymphocyte proliferation was not measured in 10-week old animals as there was no difference in IL-2 production following ConA stimulation.

7.2.9 Statistical analyses

All data are presented as means ± standard errors of means (SEM), unless otherwise noted. As the dam was the experimental unit (n), each n in the data is the mean of two offspring per dam. All measurements were conducted in duplicate or triplicate for each

offspring. All data sets were tested for normal distribution using a Kolmogorov-Smirnov test. Parametric data was then analyzed for differences using two-tailed *t* test. Non-parametric data was log-transformed prior to analysis, then a two-tailed *t* test performed. In some cases, log-transformation of the data did not lead to normal distribution therefore groups were analyzed using the Mann-Whitney *U* test. Experiment 1 was conducted over two years, therefore we included year as a blocking variable in all analyses. Year was a blocking variable as it was not a variable of interest, but the variability due to the experiments being conducted over two years must be taken into account. There was no difference between years (in the block) therefore data is illustrated as a combination of both years. Statistical analyses were performed using SPSS Statistics (version 21; IBM, Armonk, NY, USA) and SAS statistical software (version 9.3; SAS Institute Inc, Cary, NC, USA) with a *P*-value of <0.05 considered statistically significant for all analyses.

7.3 Results

7.3.1 Anthropometric characteristics and total choline stomach content

For Experiment 1, two of the dams and their litters from the ChD group were euthanized before the end of the experiment due to significant weight loss and not included in data analysis making the final n=7 for the ChD group. In Experiment 1, three-week-old offspring from ChD dams that survived had mean lower body weight, liver weight and intestine length compared to the offspring from the ChS dams (**Table 7-2**). Although spleen weight was not significantly different between diet groups, ChD offspring had indications of lymphopenia as demonstrated by a significantly lower number of splenocytes compared to ChS offspring ($P<0.05$) (Table 7-2). ChD dams began to consume significantly lower amounts of food beginning on postnatal day 9, which is likely when they started to be deficient in

choline. As previously reported, at 21 days postnatal, the ChD dams weighed 17% less and their food intake was 29% lower compared to ChS dams (Dellschaft, Ruth et al. 2015).

ChD offspring had a significantly lower total choline concentration in the stomach content compared to ChS offspring (Table 7-2). The relative proportion of choline metabolites that contributed to total choline was also different between diets and has been reported by Dellschaft *et al.,* (2015) (Dellschaft, Ruth et al. 2015).

For Experiment 2, two dams and their litters from the ChD-ChS group were euthanized before the end of the experiment due to significant weight loss and not included in data analysis making the final n=5 for the ChD-ChS group. ChD-ChS offspring had lower body weight at weaning ($P<0.05$) (**Table 7-3**). However, after being placed on a choline sufficient diet for the remaining 7 weeks, final body weight was not significantly different compared to ChS-ChS offspring. Food intake, spleen and liver weight and intestine length was not significantly different between diet groups at the end of 10 weeks (Table 7-3).

7.3.2 Splenocyte immune cell phenotypes

Consistent with the indications of lymphopenia, ChD offspring had 27% lower total number of T lymphocytes (CD3+), and 47% lower total number of B lymphocytes (CD45RA+) in the spleen compared to ChS offspring ($P<0.05$) (**Table 7-4).** When examined as a proportion of gated cells, ChD offspring had a higher proportion of CD4+ and CD8+ T cells, which resulted in a significantly higher ratio of T cells (CD3+): B cells (CD45RA+) (Table 7-4). Although having a significantly lower total amount of lymphocytes, assessment of activation markers on T cells (CD25, CD28, CD152) indicated that ChD offspring had a higher proportion of CD4+ T cells expressing CD28+ and a higher proportion of CD8+ T cells expressing C28 and CD152 cells compared to ChS offspring ($P<0.05$) (**Table 7-5**). There was

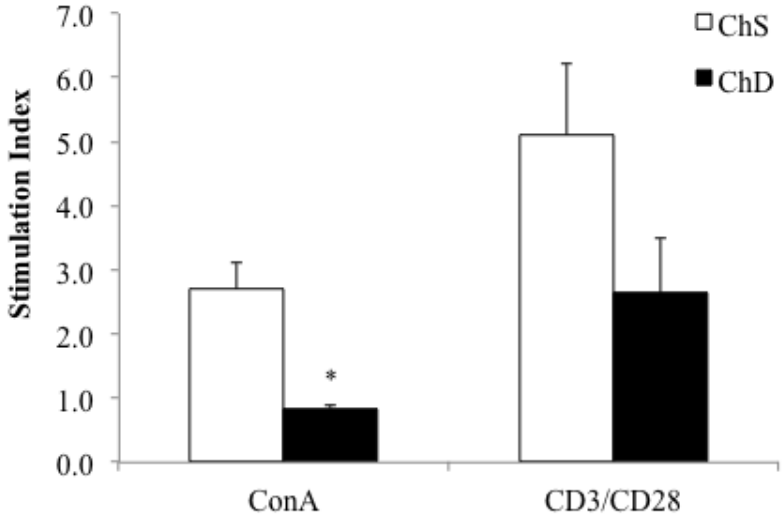
also a significantly higher proportion of CD4+ and CD8+ cells expressing the proliferation marker CD71 (transferrin receptor) compared to ChS offspring ($P<0.05$) (Table 7-5). There was no significant difference in the proportion of FoxP3+CD25+ cells within the CD4+ T cell population, representing putative Treg cells, between diet groups (Table 7-5). There was also a higher proportion of B cells (CD45RA+) with antigen presenting capacity (OX6+) in ChD offspring compared to ChS offspring ($P<0.05$) (Table 7-5).

In Experiment 2, feeding a choline-sufficient diet to offspring fed a choline-devoid during the suckling period (ChD-ChS offspring) rescued the lymphopenic status, as demonstrated by similar numbers of splenocytes (Table 7-3) and proportion of total T cells (CD3+) in the spleen between both groups of 10-week old offspring (**Table 7-6**). However, ChD-ChS offspring still had a slight shift in lymphocyte proportions as there was a lower proportion of CD4+ T cells, compared to ChS-ChS offspring ($P<0.05$) **(**Table 6**)**. ChD-ChS offspring also had a significantly lower proportion of CD8+ T cells expressing activation marker CD28 (co-stimulatory molecule) (**Table 7-7**). ChD-ChS offspring also had a lower proportion of IgA+ cells in spleen, yet there was no significant difference in the proportion of total B cells (CD45RA+) cells between groups at 10 weeks (Table 7-6).

7.3.3 Ex vivo response to stimulation by splenocytes

At 3 weeks, the absolute rate of [^{3}H]thymidine uptake in the presence of mitogen (ConA or CD3/CD28) or absence of mitogen (unstimulated) was used to calculate the stimulation index. ChD offspring had a significantly lower stimulation index following ConA stimulation compared to ChS offspring ($P<0.05$) (**Figure 7-2**).

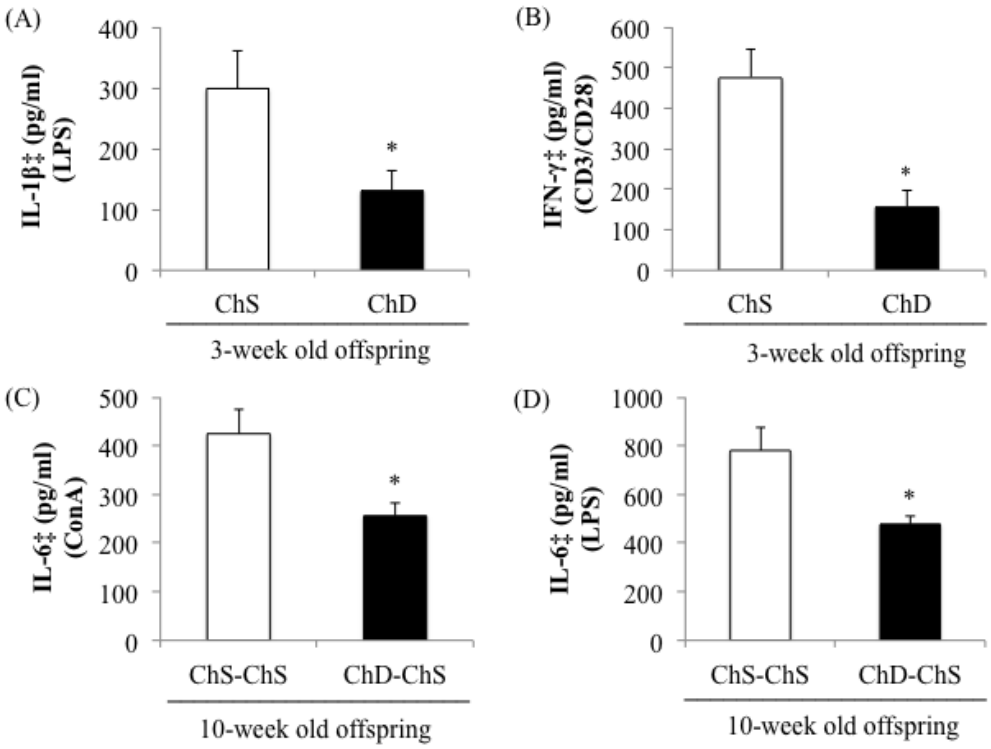
Figure 7-2: Response of spleen lymphocytes[1] from subset[2] of 3-week old offspring from dams fed choline-sufficient (as free choline) (ChS; n=3) or choline-devoid (ChD; n=2) diets during the suckling period.



[1]Response measured as stimulation index (SI): amount of [^{3}H]thymidine uptake (disintegrations per min, dpm) in the presence of each mitogen divided by the amount of [^{3}H]thymidine uptake (dpm) in the absence of each mitogen
ConA, concanavalin A
[2]n refers to the number of dams as they are the experimental unit. This includes two offspring (one male and one female) pooled to obtain a measure for each dam, with measurements from each offspring conducted in triplicate.

When equal numbers of splenocytes were stimulated *ex vivo* with LPS, ChD offspring produced significantly lower amounts of IL-1β following LPS stimulation ($P<0.05$) (**Figure 7-3A**), with no difference in other cytokines (IL-6, IL-10, IFN-γ and TNF-α) following LPS stimulation (data not shown). Following stimulation with CD3/CD28, a T cell antigen, ChD offspring produced significantly lower amounts of IFN-γ compared to ChS offspring (Figure 7-3B).

Figure 7-3: Ex vivo mitogen-stimulated splenocyte cytokine production from 3-week old offspring (ChS and ChD) (A, B) and 10-week old offspring (ChS-ChS and ChD-ChS) (C, D)[1,2]



[1]CD3/CD28 stimulation performed in subset of animals: ChS (n=5), ChD (n=4). The amount of IL-2 in the media after LPS stimulation was below detection levels. IL-1β was only measured in the supernatant of LPS stimulated cells.
[2]Cytokine concentrations in spleen supernatant (pg/ml) after 48-h culture with mitogen. Each of the measures were conducted in duplicate for each of the two offspring for a dam (CV <10%). The n in this figure represents the mean of two offspring of a dam.
* indicates value within a panel that is significantly different from choline sufficient group (ChS or ChS-ChS) *(P<0.05).*

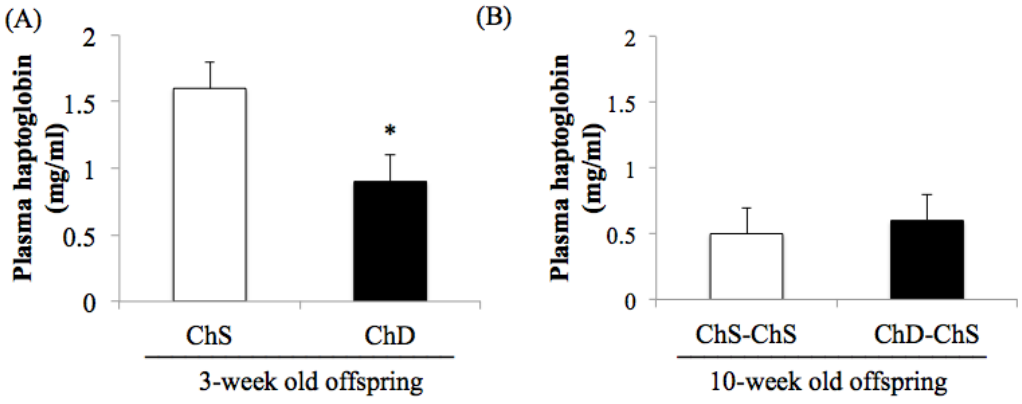
There was no significant difference in other cytokines (IL-2, IL-6, IL-10 and TNF-α) following CD3/CD28 stimulation (data not shown). With mitogen ConA, there was no significant difference in cytokine production (IL-2, IL-6, IL-10, IFN-γ and TNF-α) between the ChD and ChS diet groups (data not shown). At 10 weeks, when splenocytes were stimulated *ex vivo* with ConA or LPS, ChD-ChS offspring produced significantly lower amounts of IL-6 compared to ChS-ChS offspring (P<0.05) (Figure 3C and 3D). There was no

significant difference in cytokine production (IL-1β, IL-2, IL-10, IFN-γ and TNF-α) when stimulated with ConA or LPS between the ChD-ChS and ChS-ChS diet groups (data not shown). There was no significant correlation between IL-2 production and lymphocyte subsets (**Appendix 7**). However, when IL-2 was expressed based on the number of lymphocytes thought to be involved in producing this cytokine, there was a significantly lower amount of IL-2 when expressed in relation to the number of CD4+CD25+ cells and total T cells (CD4+ and CD8+ cells) expressing CD25+ in the ChD offspring (**Appendix 8**).

7.3.4 Plasma concentrations of cytokines, chemokines and haptoglobin

Plasma haptoglobin concentrations were significantly lower in ChD offspring compared to ChS offspring ($P<0.05$) (**Figure 7-4A**); however there was no difference in plasma haptoglobin between ChD-ChS and ChS-ChS offspring at 10 weeks (Figure 7-4B).

Figure 7-4: Plasma concentrations of haptoglobin in 3-week old offspring (ChS and ChD) (A) and 10-week old offspring (ChS-ChS and ChD-ChS) (B)[1]



TNF-α, tumour necrosis factor-α; KC/GRO, Keratinocyte chemoattractant/human growth-regulated oncogene

[1]Discrepancies between total number of offspring and the number available for measurements of haptoglobin, TNF-α and KC/GRO are due to sample errors.

* indicates value within a panel that is significantly different from ChS group ($P<0.05$).

Plasma concentrations of TNF-α and KC/GRO were not significantly different between ChS and ChD offspring at 3 weeks of age nor were concentrations different between ChS-ChS and ChD-ChS offspring at 10 weeks of age (data not shown). Circulating plasma concentrations of IFN-γ, IL-10, IL-13, IL-1β, IL-4, IL-5 and IL-6 were all below detection for both 3-week and 10-week old offspring.

7.4 Discussion

We have demonstrated that endogenous choline synthesis is not sufficient to meet the offspring's needs for growth and immune development, and a dietary source is needed in the maternal diet. We provided a choline-devoid maternal diet as lactation, when there is a reported increase *de novo* synthesis of choline in rodents, suggesting reliance on dietary choline sources during this period may be reduced (Gwee and Sim 1979, Burdge, Hunt et al. 1994). Despite the maternal diet being devoid of choline, the suckled offspring did receive some choline in maternal milk from endogenous synthesis by the dams. Consistent with findings from Dellschaft et al. (2015) (Dellschaft, Ruth et al. 2015), the absence of choline in the diet during the lactation period resulted stunted growth likely due to both the reduced supply of choline and reduced delivery of other essential nutrients due a lower intestinal surface area in the pup (da Silva, Kelly et al. 2015). In support of this, plasma haptoglobin concentrations were lower in ChD offspring, suggestive of general malnutrition (Sadrzadeh and Bozorgmehr 2004). Although conducted in two separate experiments and therefore invalid for assessment of statistical differences, our studies suggest that providing a choline-sufficient diet after weaning reversed some of the negative effects on growth with a choline-devoid diet in early life. A choline-devoid diet during suckling significantly reduced offspring growth, but providing choline in the weaning diet enabled catch up growth to occur, as the mean final

body weights of the ChD-ChS offspring were similar to those of the ChS-ChS offspring at 10 weeks of age. This is consistent with a previous study in which maternal dietary lipotrope deficiency (choline, methionine and vitamin B12) resulted in low birth and weaning weights, but a switch to a lipotrope-sufficient diet at weaning corrected body weights at 14 weeks post-weaning (Newberne, Ahlstrom et al. 1970). Similarly, a choline-sufficient diet in the weaning period appeared to reverse the symptoms of malnutrition (lymphopenia and lower mean haptoglobin concentration) that occurred with a choline-devoid diet in the suckling period. A choline-devoid during in the suckling period reduced the proportion of B cells in the spleen of the offspring. However, a choline-sufficient diet in the weaning period appeared to reverse these reductions, with similar proportion of B cells in spleen of offspring in both groups at the end of 10 weeks. ChD-ChS also had similar IFN-γ and IL-1β production compared to ChS-ChS offspring. This suggests that the effect of a lower dietary supply of choline during suckling on B cells is reversible by providing what is currently believed to be adequate choline in the weaning diet. Further research is needed to identify the mechanism for the role of choline in B cell function and development during the suckling period.

This study demonstrated that not providing choline in the maternal diet resulted in indicators of lymphopenia in 3-week old offspring, demonstrated by 34% and 46% lower number of CD4+ T lymphocytes and B lymphocytes, respectively, similar to what has been reported for other micronutrient deficiencies (zinc (Hosea, Rector et al. 2004) and folate (Williams, Shoot et al. 1975). As the suckling period is a time of rapid cellular expansion (Perez-Cano, Franch et al. 2012), this may have significant consequences on later immune function. Interestingly, despite a lower total number of lymphocytes, assessment of T cell activation markers (CD25, CD28, CD152) revealed a more activated phenotype in ChD offspring compared to the ChS offspring. ChD offspring had a higher proportion of the CD4+

cells expressing CD28. CD28 expression is critical for the interaction of T cells with antigen presenting cells (Thompson, Lindsten et al. 1989) and suggests a more activated and stimulated CD4+ population. There was a higher proportion of CD8+ and CD4+ cells from ChD offspring that expressed the transferrin receptor (CD71), which is involved in cellular iron uptake and expression, also suggesting cellular activation (Reddy, Eirikis et al. 2004). However, despite an activated phenotype, splenocytes from ChD offspring had a significantly lower *ex vivo* proliferative response (stimulation index) to ConA, a polyclonal T cell mitogen. ChD offspring had a higher proportion of activated T cells and T cells expressing the IL-2 receptor (CD25), yet there was no difference in IL-2 production after either ConA or CD3/CD28 stimulation. Higher proportions of CD8+CD25+ have been shown to supress IL-2 response (McNally, Hill et al. 2011), therefore this immunosuppression may explain why there was no difference in IL-2 production after stimulation. When the production of IL-2 produced was expressed based on the number of T lymphocytes expressing the IL-2 receptor (CD25), we observed a significantly lower IL-2 per CD4+CD25+ lymphocytes in ChD offspring. This suggests that the lymphocytes from ChD offspring may also have reduced ability to synthesize IL-2. Furthermore, ChD offspring had a higher proportion of CD8+ T cells expressing CD152 (cytotoxic T-lymphocyte antigen-4 (CTLA-4), which is a well-characterized co-receptor involved in the regulation of T cell response (Rudd 2009, Rudd, Taylor et al.) that could negatively affect the development of the immune system. CTLA-4 can also be expressed intracellularly, and recently has been demonstrated to play a role in the function of Tregs (Wing, Onishi et al. 2008), therefore future studies should examine both surface and intracellular expression of this protein.

As the proliferative response to CD3/CD28, a mitogen that directly stimulates T cells via the T cell receptor, was not significantly different, our results suggest that the altered *ex*

vivo function may be related to both impaired T cells that do not function as well (on a cell per cell basis) and the lower number of B cells (accessory cells). Similar to what we reported in dams fed the choline-devoid diet (Dellschaft, Ruth et al. 2015), ChD offspring have reduced capacity to produce IFN-γ following CD3/CD28 stimulation. Production of IFN-γ is a critical cytokine for inhibiting the differentiation and activation of T helper 2 (Th2) cells and promoting a Th1 response. Skewing towards a Th1 response is indicative of immune system maturation in both humans and rodents (Perez-Cano, Franch et al. 2012). Inappropriate Th2 response in early development has been indicated to play a role in the production of allergic inflammation, and predisposition towards allergy (Calder 2007). Insufficient IFN-γ is also associated with increased incidence of infection among newborns (Kotiranta-Ainamo, Rautonen et al. 2004). This combined with a higher Th2 response would be predicted to impair the ability of the immune system to respond appropriately to challenges. This is supported by previous research that has shown that choline insufficiency results in impaired T cell functions. More specifically, in adult rodents, dietary choline deficiency has been shown to reduce lymphocyte proliferation (Courreges, Benencia et al. 2003), which was accompanied by depression of T cell function. Similarly, 5-month old offspring from dams fed a choline-methionine deficient diet during lactation had a decreased stimulation index with ConA or PHA stimulation and greater mortality from a bacterial infection of *Salmonella typhimurium* (Williams, Gebhardt et al. 1979).

We also have evidence from this study that not providing choline in the maternal diet during suckling may alter the ability of offspring to respond to bacterial antigens. Following *ex vivo* stimulation with LPS, a bacterial antigen, ChD offspring produced a lower amount of IL-1β. Inadequate production of IL-1β would increase the susceptibility to intracellular pathogens (Hunter, Chizzonite et al. 1995) and reduce the Th1 and Th17 response by T cells

(Reddy, Eirikis et al. 2004). As LPS stimulates antigen-presenting cells including macrophages, B cells and dendritic cells, it is possible that cytokine production in response to LPS stimulation can be attributed to other immune cells, in addition to T cells. However, as the observed changes in immune cell populations (Table 4 and 5) at 3 weeks of age were primarily in the T lymphocyte population, it is likely that the lymphocyte population had the most profound effect on cytokine production.

Furthermore, this study demonstrates for the first time that insufficient maternal dietary choline during the suckling period results in alterations in the expansion and maturation of the T cell population and the ability to respond to mitogens later in life. Feeding a choline-sufficient diet during weaning rescued the lymphopenic status of the ChD offspring at 3 weeks. However, female offspring from dams fed a choline-deficient diet during suckling had a slight shift in lymphocyte proportions at 10 weeks, displaying a lower proportion of CD4+ T cells and lower IL-6 production after ConA or LPS stimulation. Expansion of the CD4+ population is a critical developmental process that occurs during the suckling period (Perez-Cano, Castellote et al. 2007) and IL-6 has been demonstrated to play a role in this process. IL-6 induces proliferation of T cells in an IL-2 independent manner (Lotz, Jirik et al. 1988), promoting differentiation of the CD4+ and CD8+ populations (Okada, Kitahara et al. 1988) and the ability of T cells to communicate with accessory cells (B cells) (Eddahri, Denanglaire et al. 2009). Therefore it appears that it is not only the number of CD4+ cells, but also their ability to produce an important cytokine, that is affected by maternal choline intake during suckling. Additionally, ChD-ChS offspring had a lower proportion of cytotoxic T cells expressing co-stimulatory molecule CD28, also used as a maturation marker. The reduced proportion of mature T cells may be in part responsible for the impaired *ex vivo* IL-6 response observed when stimulated. A source of choline has been demonstrated to be essential for

macrophage production of IL-6 (Tian, Pate et al. 2008). Although the proportion of macrophages (CD284+CD11+) was not altered between diet groups, reduced IL-6 production may be attributed to impaired macrophage function in addition to T cell function.

A limitation in the experiment examining the effects of a maternal diet devoid of choline in 3-week old offspring was that we did not examine the possible influence of sex, as one male and one female offspring was used for each dam. To our knowledge, there have been no studies examining the difference in choline requirements between sexes in young animals (i.e. during suckling) but previously we did not find an effect of sex on immune cell phenotypes in animals at this age (Field 1999). The experiment examining the long-term effects of choline-devoid maternal diet during lactation was conducted on female offspring only. It has been shown that female mice, at sexual maturity, have greater synthesis of PC through the PEMT pathway (Noga and Vance 2003), and mature female rodents are less susceptible to the consequences of low dietary choline compared to male rodents (Saito, Palomba et al. 1991, Tessitore, Sesca et al. 1995). It would be logical to follow up our findings with male offspring in both studies as we may see a larger programing effect in an animal that has a lower ability to endogenously synthesize choline.

The impaired T cell-mediated functions and maturation observed in both ChD and ChD-ChS offspring is supported by previous studies demonstrating that choline (Dellschaft, Ruth et al. 2015) or lipotrope deficiency (including choline) (Courreges, Benencia et al. 2003) has the most profound effect on the T cell component of the immune system. This impaired T cell function may increase the susceptibility to infection later in life. Newberne et al. (1970) demonstrated that when offspring from dams fed a marginally choline deficient diet during suckling were placed on a choline sufficient diet after weaning, they were still more susceptible to *Salmonella typhimurium* infection at 100 days post weaning compared to

offspring fed a choline-sufficient diet. However, Nauss et al. (1982) suggests that age plays a role in the function of the immune system in the context of a methionine-choline deficient diet. Rats fed a marginally methionine-choline deficient diet (67% lower in methionine and choline compared to control diet) during weaning were most susceptible to *Salmonella typhimurium* infection at a younger age (3 weeks post weaning) compared to older animals (3 and 12 months post weaning) (Nauss, Connor et al. 1982). Younger animals also had the lowest amount of lymphocyte proliferation compared to older animals on the same marginally deficient diet. Our results suggest that maternal choline deficiency during the suckling period may impair the development of the T cell population later in life, perhaps with greater effects in the early post-weaning period. Our study focused primarily on surrogate markers of immune function (*ex vivo* cytokine production, [^{3}H]thymidine uptake and cellular proliferation). As previous studies have found an effect of choline deficiency on T-cell mediated functions (Courreges, Benencia et al. 2003), future studies should examine not only surrogate markers of immune function, but more specific immune challenges such as susceptibility to infection and DTH response to examine T cell functions, and tests that have greater applicability to humans. Additionally, we did not perform the proliferation assay on the 10-week old offspring. In the 10-week offspring, there was lower IL-6 production in the offspring from the dams fed the choline devoid diet, with similar amounts of IL-2 produced compared to the offspring from dams fed the choline sufficient diet during suckling. IL-6 has been demonstrated as a potent regulator and stimulator of lymphocyte proliferation, independent of the proliferation actions associated with IL-2 (Lotz, Jirik et al. 1988, Holsti and Raulet 1989). Additionally, the ChD-ChS offspring had a lower proportion of CD3+CD4+ and markers indicating activation (CD28+). Therefore, we would predict that the ChD-ChS

offspring would have a lower proliferative response following stimulation that is associated with the lower IL-6 production when stimulated with ConA or LPS.

7.5 Conclusion

We demonstrated that choline is essential in the maternal diet to support offspring growth and development of the immune system. Maternal choline deficiency during a critical period of development stunted growth and resulted in lymphopenia, which may have contributed to the impaired T and B cell responses in the offspring at the end of suckling. The long-term consequences of a lower supply of choline during this critical period of development resulted in a lower expansion of the CD4+ population and a lower ability to produce IL-6, even after choline was fed in the weaning diet. Together, with findings of a possible suboptimal intake during the lactation period in humans, future research should focus on identifying potential benefits of increasing total choline in the maternal diet to support immune development in the infant.

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Tables

Table 7-1: Composition of experimental diets

Component (g/kg diet)[1]	Choline Sufficient (ChS)	Choline Devoid (ChD)
Casein	270	270
Starch	241	244
Sucrose	126	126
Vitamin mix[2]	19	19
Mineral mix[3]	50	50
Calcium phosphate dibasic	3.4	3.4
Inositol	6.3	6.3
Cellulose	80	80
L-cysteine	1.8	1.8
Flax oil	7	7
Olive oil	48	48
Sunflower oil	67	67
Canola stearin	78	78
DHAsco[4]	1.5	1.5
ARAsco[5]	1.5	1.5
Choline bitartrate	2.5	0
% energy from protein	25	25
% energy from carbohydrate	34	34
% energy from fat	41	41
Total choline (g/kg) diet	1.0	0

[1]All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were purchased from Safeway (Edmonton, AB, Canada), and canola stearin was donated by Richardson Oilseed Limited (Lethbridge, AB, Canada).
[2]AIN-93-VX Vitamin mix (Reeves 1997)
[3]Bernhart-Tomarelli salt mixture (Bernhart and Tomarelli 1966)
[4]DHAsco is a microalgae source of docosahexaenoic acid (DHA)
[5]ARAsco is a fungal source of arachidonic acid (ARA)

Table 7-2: Anthropometric data of 3-week old offspring from dams fed choline-sufficient (as free choline) (ChS) or choline-devoid (ChD) diets during the suckling period[1]

	ChS	ChD
n[2]	10	7
	Mean ± SEM	Mean ± SEM
Body weight (g)	40 ± 3	28 ± 2*
Spleen weight (g)	0.2 ± 0.02	0.2 ± 0.01
Splenocytes/g spleen (x10^6)	744 ± 46	539 ± 13*
Liver weight (g)	1.7 ± 0.1	1.1 ± 0.1*
Liver fat weight (mg/g liver)	37 ± 5	34 ± 1
Intestine length (cm)	63 ± 2	54 ± 2*
Stomach total choline content (mg/100g)	13.1 ± 1.2	6.2 ± 1.0*

[1]At birth, litters were standardized to ten offspring per dam consisting of five males and five females when possible. Measurements collected at 3 weeks were pooled from one male and one female per dam.

[2]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.

*indicates mean within a row that is significantly different from ChS group ($P<0.05$).

Table 7-3: Anthropometric data from 10-week old offspring fed a choline-sufficient (as free choline) diet for the duration of the 10 weeks (ChS-ChS) or offspring from dams fed a choline-devoid diet during the suckling period then fed a choline-sufficient diet for the remaining 7 weeks (ChD-ChS)[1]

	ChS	ChD
n[2]	9	5
	Mean ± SEM	Mean ± SEM
Body weight at weaning (4 weeks) (g)	76 ± 4	61 ± 4*
Final body weight (10 weeks) (g)	267 ± 9	266 ± 15
Food intake (g/d)	23 ± 1	23 ± 2
Spleen weight (g)	0.62 ± 0.03	0.65 ± 0.1
Splenocytes/g spleen ($x10^6$)	464 ± 40	493 ± 40
Liver weight (g)	9.0 ± 0.3	9.1 ± 0.4
Intestine length (cm)	118 ± 2	117 ± 1

[1]At birth, litters were standardized to ten offspring per dam consisting of five males and five females when possible. Measurements collected at 10 weeks were from females only.

[2]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.

*indicates mean within a row that is significantly different from ChS-ChS group *($P<0.05$).*

Table 7-4: Proportion and total number of T and B lymphocyte populations in spleen from 3-week old offspring from dams fed choline-sufficient (as free choline) (ChS) or choline-devoid (ChD) diets during the suckling period

	ChS	ChD	ChS	ChD
n[1]	10	7	10	7
	% of total lymphocytes		Total number of immune cells ($x10^6$)[2]	
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Total CD3+	22.3 ± 1.1	27.7 ± 1.1*	32.3 ± 3.0	23.7 ± 2.0*
CD3+CD4+	11 ± 0.8	13.9 ± 0.9*	16.3 ± 2.1	12.3 ± 1.8
CD3+CD8+	9.3 ± 0.5	12.7 ± 0.6*	14.1 ± 2	10.9 ± 1.1
Total CD45RA+	59.7 ± 0.8	54.9 ± 1.7*	88.9 ± 11.1	47.5 ± 4.6*
CD3+: CD45+ Ratio	0.37 ± 0.01	0.51 ± 0.02*	0.38 ± 0.03	0.51 ± 0.02*
Total IgM+	63.8 ± 1.3	63.1 ± 2.8	85.0 ± 7.8	54.4 ± 5.3*
Total IgG+	7.0 ± 0.9	10.9 ± 0.8*	11.9 ± 3.4	9.2 ± 4.6

[1]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.

[2]The total number of immune cells was calculated by multiplying the % of immune cell phenotype in the spleen by the total number of splenocytes isolated ($x10^6$).

*indicates mean within a row that is significantly different from ChS group *(P*<0.05*)*.

Table 7-5: Splenocyte T and B lymphocyte phenotypes from 3-week old offspring from dams fed choline-sufficient (as free choline) (ChS) or choline-devoid (ChD) diets during the suckling period

	ChS	ChD	
n[1]	10	7	
	Mean ± SEM	Mean ± SEM	Representative population
	% of CD4+ T cells		
CD25+	11.7 ± 2.2	14.3 ± 3.1	CD4+T cells expressing IL-2 receptor
CD28+	77.8 ± 3.9	91.2 ± 3.3*	CD4+T cells expressing co-stimulatory molecule
CTLA-4+ (CD152+)	9.5 ± 1.3	12.3 ± 1.1	CD4+T cells expressing cytotoxic T lymphocyte associated protein 4 (CTLA-4)
CD71+	20.9 ± 1.4	27.4 ± 1.3*	CD4+ T cells expressing the transferrin receptor
αεβ7+ (OX62+)	9.5 ± 1.2	10.5 ± 0.7	CD4+T cells with gut homing potential expressing the α E2 integrin
CD25+FoxP3+	10.5 ± 3.7	12.1 ± 4.7	Regulatory T cells (Treg)
	% of CD8+ T cells		
CD25+	12.5 ± 1.2	16.9 ± 2.4*	CD8+T cells expressing IL-2 receptor
CD28+	72.8 ± 1.9	75.5 ± 3.0	CD8+T cells expressing co-stimulatory molecule
CTLA-4+ (CD152+)	19.3 ± 2.7	29.2 ± 5.6	CD8+T cells expressing cytotoxic T lymphocyte associated protein 4 (CTLA-4)
CD71+	34.9 ± 2.4	50.3 ± 4.7*	CD8+ T cells expressing the transferrin receptor
αεβ7+ (OX62+)	10.6 ± 1.2	10.3 ± 0.8	CD8+T cells with gut homing potential expressing the α E2 integrin
	% of CD45RA+ B cells		
CD27+	94.1 ± 1.2	94.0 ± 1.4	B cells expressing a member of the TNF-superfamily indicating memory cells
MHCII+ (OX6+)[2]	79.6 ± 2.5	91.9 ± 2.9*	B cells with antigen presenting capacity expressing MHC Class II

[1]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.

[2]% of CD45RA+ cells expressing OX6+ calculated using Total OX6+ cells – OX6+OX62+ to represent population of CD45RA+ cells expressing OX6+

*indicates mean within a row that is significantly different from ChS group *(P*<0.05*)*.

Table 7-6: Proportion and total number of T and B lymphocyte populations in spleen from 10-week old offspring fed a choline-sufficient (as free choline) diet for the duration of the 10 weeks (ChS-ChS) or offspring from dams fed a choline-devoid diet during the suckling period then fed a choline-sufficient diet for the remaining 7 weeks (ChD-ChS)

	ChS-ChS	ChD-ChS	ChS-ChS	ChD-ChS
n[1]	9	5	9	5
	% of total lymphocytes		Total number of immune cells ($x10^6$)[2]	
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Total CD3+	41.0 ± 2.0	40.7 ± 1.5	115.8 ± 9.6	127.9 ± 13.5
CD3+CD4+	20.5 ± 1.2	16.5 ± 1.3*	58.4 ± 5.5	52.6 ± 8.0
CD3+CD8+	14.7 ± 1.0	14.5 ± 2.6	32.3 ± 3.6	47.7 ± 10.3
Total CD45RA+	41.8 ± 1.8	45.3 ± 3.5	123.6 ± 12.9	141.3 ± 15.5
CD3+: CD45+ Ratio	1.04 ± 0.09	0.84 ± 0.06	0.94 ± 0.06	0.93 ± 0.10
Total IgM+	52.0 ± 1.8	52.8 ± 4.1	129.2 ± 15.4	171.2 ± 32.8
Total IgG+	11.9 ± 1.6	6.4 ± 1.9	45.5 ± 4.8	21.4 ± 3.8

[1]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.
[2]The total number of immune cells was calculated by multiplying the % of immune cell phenotype in the spleen by the total number of splenocytes isolated ($x10^6$).
* indicates mean within a row that is significantly different from ChS group ($P<0.05$).

Table 7-7: Splenocyte T and B lymphocyte phenotypes from 10-week old offspring fed a choline-sufficient (as free choline) diet for the duration of the 10 weeks (ChS-ChS) or offspring from dams fed a choline-devoid diet during the suckling period then fed a choline-sufficient diet for the remaining 7 weeks (ChD-ChS)

	ChS	ChD	
n[1]	10	7	
	Mean ± SEM	Mean ± SEM	Representative population
	% of CD4+ T cells		
CD25+	30.7 ± 1.0	27.2 ± 2.2	CD4+T cells expressing IL-2 receptor
CD28+	90.2 ± 0.8	79.5 ± 5.4	CD4+T cells expressing co-stimulatory molecule
αεβ7+ (OX62+)	9.5 ± 1.2	10.5 ± 0.7	CD4+T cells with gut homing potential expressing the α E2 integrin
CD25+FoxP3+	9.8 ± 1.0	11.4 ± 1.0	Regulatory T cells (Treg)
	% of CD8+ T cells		
CD25+	9.3 ± 0.8	11.7 ± 3.5	CD8+T cells expressing IL-2 receptor
CD28+	85.2 ± 2.5	54.0 ± 4.0*	CD8+T cells expressing co-stimulatory molecule
αεβ7+ (OX62+)	10.6 ± 1.2	10.3 ± 0.8	CD8+T cells with gut homing potential expressing the α E2 integrin
	% of CD45RA+ B cells		
MHCII+ (OX6+)[2]	88.7 ± 3.7	89.5 ± 3.4	B cells with antigen presenting capacity expressing MHC Class II

[1]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate.

[2]% of CD45RA+ cells expressing OX6+ calculated using Total OX6+ cells – OX6+OX62+ to represent population of CD45RA+ cells expressing OX6+

*indicates mean within a row that is significantly different from ChS group ($P<0.05$).

CHAPTER 8: The form of choline in the maternal diet affects immune development in the suckled rat offspring[1, 2]

8.1 Introduction

Choline is an essential nutrient required for a variety of critical processes including synthesis of membrane phospholipids, neurotransmitters and lipoproteins and methyl group metabolism (Zeisel and da Costa 2009). PC and free (unesterified) choline comprise approximately 65% of total choline in the diet (Mygind, Evans et al. 2013, Lewis, Subhan et al. 2014). Previous research in rodents using ^{14}C-labelled choline metabolites demonstrated differential uptake by tissues between lipid soluble PC and water soluble free choline (Cheng 1996). Approximately 50% of dietary PC enters the lymphatic circulation and reaches the peripheral organs prior to the liver, with the remaining 50% absorbed similarly to free choline. PC is an essential membrane phospholipid and is required for lipoprotein formation and signal transduction (Li and Vance 2008). Conversely, water soluble free choline is absorbed and enters hepatic circulation, reaching the liver on first pass (Cheng 1996), where it can be oxidized to betaine, or metabolized to other forms of choline, including PC. Human milk differs in the content of free choline and PC compared to infant formulas (cow and soy-based) (Zeisel, Char et al. 1986) and it has been demonstrated that serum free choline concentrations in breast-fed infants are correlated with total choline, free choline and glycerophosphocholine (GPC) breast milk concentrations (Ilcol, Ozbek et al. 2005). These findings suggest that

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[2] Content in this chapter was presented at Experimental Biology, San Diego, CA, April 2016, the Canadian Nutrition Society, St. John's, NL, June 2014, Experimental Biology, Boston, MA, April 2013, and the Canadian Nutrition Society, Vancouver, BC, May 2012

consumption of infant formula or breast milk with different choline compositions can directly alter serum levels in breast-fed and formula fed infants.

Maternal choline requirements increase in the postnatal period to enable breast milk to meet the demand by the infant to support developing tissues (Ilcol 2002). Despite increased requirements, our recent cohort study (Lewis, Subhan et al. 2014), consistent with others (Shaw, Carmichael et al. 2004, Yonemori, Lim et al. 2013), found that the majority (90%) of lactating women are not meeting current recommendations (adequate intake) for choline at 3-months postpartum. In a rodent model, a choline-deficient diet during lactation resulted in greater depletion of liver choline metabolites compared to non-lactating females, suggesting dams were more sensitive to choline inadequacy during lactation (Zeisel, Mar et al. 1995). We have demonstrated that feeding a choline-deficient diet during the lactation period significantly impaired maternal immune function, and reduced offspring growth (Dellschaft, Ruth et al. 2015). In an earlier study by Gebhardt *et al.,* (1974) offspring from dams fed a diet devoid or marginally deficient in lipotropes (including choline and folate) were more susceptible to infection in early postnatal life, an effect which was corrected by postnatal supplementation (Gebhardt and Newberne 1974).

Previous studies examining the essentiality of choline on immune function and development have focused on providing choline as free choline (the common form of choline in rodent diets). There is some immune system development that occurs during fetal development (Kelly and Coutts 2000) however the early postnatal (suckling period) is a critical stage where the acquired immune system expands and matures (Perez-Cano, Castellote et al. 2007). Although it appears choline is required in the diet during lactation to ensure optimal immune development and offspring growth (Gebhardt and Newberne 1974, Dellschaft, Ruth et al. 2015), there has been no research examining the roles of different

dietary forms of choline on immune development. Due to the metabolic differences between the two most common forms of dietary choline, we hypothesize that they will differentially affect immune function. The objectives of the present study were to 1) use an animal model to compare parameters of growth and immune function of offspring at the end of suckling from dams fed diets containing either PC or free choline 2) use an *in vitro* study to elucidate the potential effect of an increased supply of lysoPC, as a form of PC, on immune cells from young animals.

8.2 Methods

8.2.1 Animal experiment

8.2.1.1 Animals and diets

Female Sprague-Dawley rats at 14 days gestation (n=10) were obtained from Charles River Laboratories (Montreal, QC, Canada). All dams were fed a standard rat chow diet (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA; containing 1 g choline/kg, Harland Teklad) for the duration of pregnancy until 24-48 hours prior to parturition. Dams were then randomized to one of two isocaloric, isonitrogenous and nutritionally adequate diets (**Table 8-1**, Bernhart and Tomarelli 1966, Reeves 1997). Diets were fed *ad libitum* for the duration of the lactation period until the end of study at 21 days postnatal. Animals had free access to food throughout each 24-h period and feed cups were refilled every 2-3 days.

The diets differed only in the form of choline provided, either as free choline in the form of choline bitartrate (Harlan Teklad) or as PC extracted from egg yolks (Table 8-1). The two diets prepared contained free choline (1 g of choline from choline bitartrate/kg diet; C, n=5) or choline from the egg PC concentrate for which 92% of the choline was in the phosphatidylcholine form (1 g of choline from PC/kg diet; PC, n=5) (**Table 8-2**). As the PC

provided some lipids, the lipid content and composition was adjusted in the free choline diet to ensure that the diets contained a similar fat content (% w/w) and fatty acid composition (Table 8-1).

To prepare the PC concentrate, egg yolks were stirred with ethanol (8:1 v/v ethanol:yolk) for 2 h then stored overnight at 4°C. Then cold ethanolic solution was separated from the precipitate by filtration. All moisture was removed from the extract by the addition of anhydrous sodium sulfate and finally the ethanol was removed using a rotary evaporator. The neutral lipids were largely removed by dissolving the yolk extracts in cold acetone, then removing the acetone from the supernatant using a rotary evaporator. Final traces of solvent were removed from PC rich extracts under vacuum. This procedure resulted in an extract that contained 570 mg/g PC, with smaller concentrations of sphingomyelin (20 mg/g) and lysoPC (19 mg/g), contributing to a total choline concentration of 84 mg/g. The choline and phospholipid composition of the egg PC concentrate was analyzed by HILIC liquid chromatography-tandem mass spectrometry (LC-MS/MS) (as previously described (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012)) and is presented in Table 8-1**.** The fatty acid composition of the egg PC concentrate and experimental diets was analyzed by gas liquid chromatography (GLC), as previously described (Field, Ryan et al. 1988, Cruz-Hernandez, Deng et al. 2004) and presented in Table 1 and Appendix 9.

At birth, litters were standardized to ten pups (5 males and 5 females when possible) per dam. Dietary intake of dams was recorded throughout the lactation period and body weight was recorded regularly throughout the study period. The experiment and protocol was reviewed and approved by the Committee of Animal Policy and Welfare of the Faculty of Agriculture, Life and Environmental Sciences at the University of Alberta, Edmonton, AB, Canada.

8.2.1.2 Tissue collection

At weaning (21 days after parturition), 2 pups from each dam were weighed and euthanized by CO_2 asphyxiation in the morning hours. Spleens were collected aseptically, weighed and immune cells were isolated for further processing (see below). Pups' stomach contents and livers were collected aseptically, weighed, snap frozen using liquid nitrogen then stored at -80°C until analysis.

8.2.1.3 Choline metabolite analyses of pup stomach content, tissue and plasma

Stomach contents of pups were analyzed to reflect the choline concentration of dam's milk and determine the effect of maternal diet on stomach content concentrations. The choline concentrations of frozen stomach contents, liver, splenocytes and plasma were quantified by HILIC LC-MS/MS as previously described (Zhao, Xiong et al. 2011, Xiong, Zhao et al. 2012, Dellschaft, Ruth et al. 2015).

8.2.1.4 Immune cell isolation and phenotype analysis

Immune cells from spleen were isolated as previously described (Field, Wu et al. 1990, Dellschaft, Ruth et al. 2015). Immune cell subsets present in freshly isolated splenocytes were identified by direct immunofluorescence assay, as previously described (Field, Wu et al. 1990, Field, Thomson et al. 2000). The use of four-colour flow cytometry allowed identification of the following combinations of surface molecules: CD28/CD3/CD8/CD4, CD28/CD152/CD8/CD4, CD25/CD152/CD8/CD4, CD25/CD127/CD8/CD4, CD27/CD8/CD4, CD27/OX12/OX6/CD45ra, CD71/CD8/CD4, OX12/OX6/CD80, CD86/CD80/CD45ra, CD68/CD284/CD11b/c, OX62/CD25/OX6, CD161/OX62/CD3, IgG/IgM, IgA, CD3/FOXP3/CD25/CD4. All antibodies with the exception of IgG, IgM and OX6 (BD Biosciences, Mississauga, ON, Canada) were purchased from Cedarlane

Laboratories, (Burlington, ON, Canada). After incubation, cells were washed and fixed in paraformaldehyde (10 g/L; ThermoFischer, Edmonton, AB, Canada) in phosphate-buffered saline. All of the samples were acquired within 72 h of preparation by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) according to the relative fluorescence intensity determined using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada).

8.2.1.5 Ex vivo cytokine production by mitogen-stimulated lymphocytes

The measurement of the production of cytokines by mitogen-stimulated splenocytes has been previously described (Blewett, Gerdung et al. 2009, Dellschaft, Ruth et al. 2015). Concentrations of cytokines interleukin (IL)-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ were measured in the supernatant from stimulated splenocytes by commercial ELISA kits according to manufacturer's instructions and as previously described (Blewett, Gerdung et al. 2009). IL-1β was only measured in the supernatant of LPS-stimulated cells. Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%.

8.2.1.6 Plasma cytokine concentrations

To determine circulating concentrations of cytokines in plasma, electrochemiluminescent multiplex cytokine kit (Proinflammatory Panel 1 V-PLEX) (Meso Scale Discovery, Rockville, MD, USA) was used. Cytokines IFN-γ, IL-10, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6 and TNF-α were measured in plasma according to the manufacturer's instructions. Briefly, on a plate pre-coated with capture antibody for each cytokine, standards and samples (50 µL/well) were added in duplicate. Plates were incubated for 2 hours at room temperature on a plate shaker. Plates were then washed with wash buffer (3 x PBS with 0.05% Tween 20). Detection antibody was added and incubated for an additional 2 hours. Plates were

then washed with wash buffer (3x) and Read Buffer was added. Plates were read on the MSD Sector Image 6000 (MesoScale Discovery, as above) and all measurements were conducted in duplicates.

8.2.2 In vitro administration of lysoPC to splenocytes

Spleens were collected, as above, from 21-day old offspring from dams (n=4) fed the free choline diet (Appendix 9) *ad libitum* for the duration of the suckling period. Immune cells were isolated, as above and previously described (Field, Wu et al. 1990). A haemocytometer was used to count cells using trypan blue dye exclusion (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and diluted to 1.25 x 10^6 cells/mL. Splenocytes (from offspring from each dam) were then suspended in complete culture medium (RPMI-1640 media; Life Technologies, Burlington, ON, Canada) (1.25 x 10^6 cells/mL), supplemented with 5% (v/v) heat-inactivated fetal calf serum, 25mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic (pH 7.4; Fisher Scientific, Edmonton, AB, Canada) and placed in 24-well plates. Another cell aliquot suspended in RMPI-1640 (2.5 x 10^6 cells/mL) complete medium, as above, was placed in 96-well plates for water-soluble tetrazolium salt (WST-1) assay (Roche Applied Science, Laval, QC, Canada). It was estimated that the RMPI-1640 medium with fetal calf serum (5% w/w) contained 15.9 μM of choline. One of the two treatments was added to the splenocytes: 10 μM 18:1n-9 (oleic acid; octadecenoic acid; Matreya LLC, State College, PA, USA) (Control, with balanced fatty acid composition) or 10 μM 18:1 lysoPC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids Inc, Alabaster, AL, USA). LysoPC was used to simulate the PC treatment based on our findings that we observed a higher concentration of lysoPC in the stomach content of the pups from PC-fed dams. LysoPC is the form of PC most readily taken up by cells in culture (Martin,

Wysolmerski et al. 1987). We hypothesized that the higher supply of lysoPC (from PC) may be responsible for the effects on immune function that we observed between diet groups. The concentration of lysoPC used was based on a previous study demonstrating T cell activation at concentrations of 10-100 μM, and cell lysis at lysoPC concentrations >100 μM (Asaoka, Oka et al. 1992). All cells were co-cultured with mitogen concanavalin A (ConA, 5.0 μg/mL; MP Biomedicals, see above). Cells were incubated for 48 h at 37°C and 5% CO_2.

After incubation, cells were centrifuged for 10 minutes at 450 x g and supernatants collected and stored at -80°C until analyses. Immune cell subsets present in post-culture splenocytes were identified by direct immunofluorescence and IL-2 measured as described above. The use of four-colour flow cytometry allowed identification of the following combinations of surface molecules: CD25/CD152/CD8/CD4, CD28/CD152/CD8/CD4, CD71/CD3/CD8/CD4). Cellular metabolic activity was assessed using Cell Proliferation Reagent, WST-1 (Roche Applied Science, see above), according to the manufacturer's instructions.

8.2.3 Statistical analyses

All data are presented as means ± standard errors of means (SEMs), unless otherwise noted. All measurements were conducted in duplicate or triplicate for each pup to get a measure of a pup, and the mean of two pups were used for each dam as the dam was the experimental unit. All data sets were tested for normal distribution using a Kolmogorov-Smirnov test. Parametric data was then analyzed for differences using two-tailed *t* test. Non-parametric data was first log-transformed prior to analysis then a two-tailed *t* test performed. In some cases, log-transformation of the data did not lead to normal distribution therefore groups were analyzed using the Mann-Whitney *U* test. Statistical analyses were performed

using SPSS Statistics (version 21; IBM, Armonk, NY, USA) and SAS statistical software (version 9.3; SAS Institute Inc, Cary, NC, USA) with a *P*-value of <0.05 considered statistically significant for all analyses.

8.3 Results

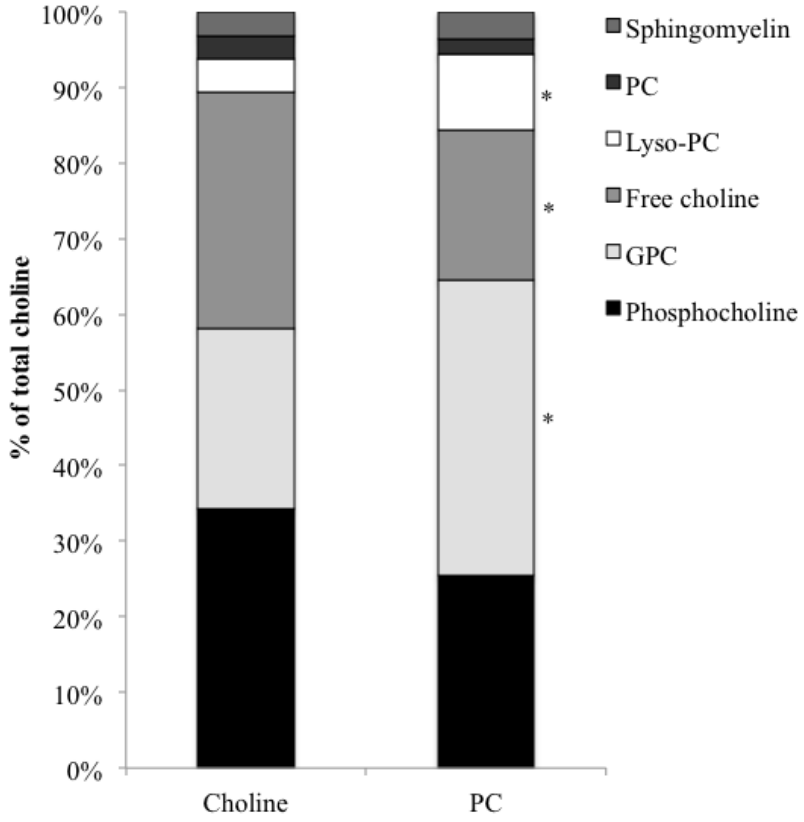
8.3.1 Anthropometric characteristics and daily food intake

There were no significant differences in body or organ weights, or intestinal length, in the pups from dams fed choline in the form of PC compared to pups from dams fed choline in the form of free choline (choline) (**Table 8-3**). There was no difference in the relative number of splenocytes (number of splenocytes/g spleen) between diet groups (Table 8-3). Mean daily food intake of the dams in each group for the duration of the suckling (21-d) period was 40 ± 1 g/day (mean of all the dams, n=10) and did not differ between diets.

8.3.2 Choline metabolites in pups' stomach content

Total choline concentration in pups' stomach content (used as a surrogate representation of breast milk composition) was not significantly different between diet groups. Total choline concentration was 14 ± 1.0 mg/100g and 14 ± 1.7 mg/100g for choline and PC pups, respectively. GPC, phosphocholine (Pcho) and free choline contribute the largest proportion to total choline (**Figure 8-1**).

Figure 8-1: Contribution of choline-containing metabolites to total choline from 3-week old rat offspring's stomach content from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]



[1]Values are means ± SEMs, n=10. *Different from choline group, (P<0.05).

The relative contribution of free choline to total choline was significantly higher in the stomach content of pups from the dams fed the free choline diet (P<0.05) and the relative contribution of lysoPC and GPC to total choline was significantly higher in the stomach content of the pups from PC-fed dams (P<0.05) (Figure 8-1). Mean betaine concentration was 0.1 ± 0.02 mg/100g (mean of all the pups, n=10) and did not differ between diet groups.

8.3.3 Choline metabolites in pups' plasma

Plasma total choline concentrations were not significantly different between diet groups. However, pups from PC-fed dams had a higher concentration of PC in plasma, and a

significantly higher concentration of total plasma choline coming from PC compared to pups from free choline-fed dams ($P<0.05$) (**Table 8-4**). Pups from free choline-fed dams had a significantly higher concentration of phosphocholine in plasma, and a significantly higher concentration of total choline coming from phosphocholine compared to pups from PC-fed dams ($P<0.05$) (Table 8-4). Plasma betaine concentration was 10 ± 0.4 μg/ml plasma (mean of all the pups, n=10) and was not significantly different between diet groups.

8.3.4 In vivo splenocyte immune cell phenotypes

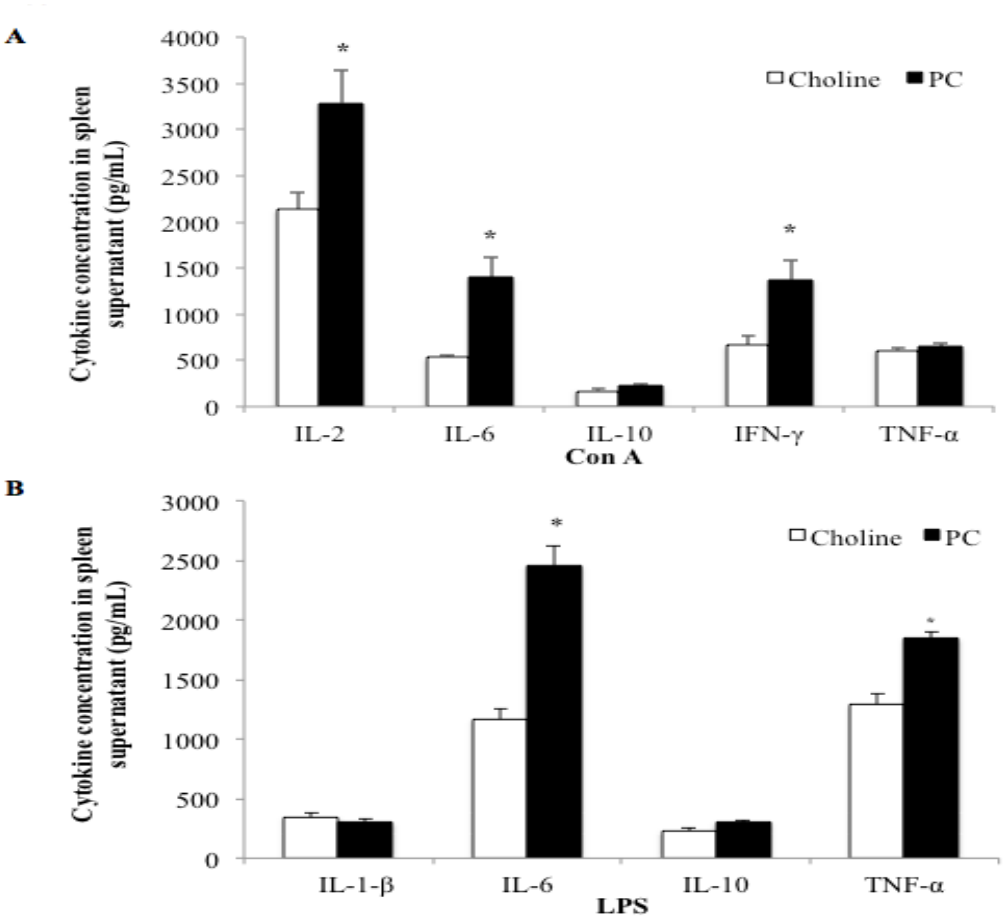
Splenocyte numbers were similar between diet groups, and there was similar distribution of the major T lymphocyte subsets between diet groups including total T cells (CD3+), helper T cells (CD3+CD4+), cytotoxic/suppressor T cells (CD3+CD8+) and T regulatory cells (CD3+CD4+CD25+FoxP3+) (**Table 8-5**). Additionally, there was no significant difference in the proportion of T cells expressing proliferation and activation markers including the IL-2 receptor (CD25+) or the transferrin receptor (CD71+) ($P<0.05$). Compared to pups from free choline-fed dams, pups from PC-fed dams had a significantly lower proportion of total B cells (Ig+ cells, OX12+) ($P<0.05$) (Table 8-5). Pups from PC-fed dams had a significantly lower proportion of activated macrophages (CD68+CD11b/c+) and total cells expressing MHC class II (OX6+) ($P<0.05$) (Table 8-5). The remaining phenotypes that did not differ significantly between diet groups are presented as means in the footnote of Table 8-5.

8.3.5 Ex vivo cytokine production by stimulated splenocytes and plasma cytokine concentrations

Following stimulation with T cell mitogen ConA, splenocytes from pups from PC-fed dams produced 54% more IL-2, 163% more IL-6 and 107% more IFN-γ compared to pups

from free choline-fed dams (P<0.05) (**Figure 8-2A**). There was no significant difference in IL-10 and TNF-α production following stimulation with ConA between diet groups. Splenocytes from pups from PC-fed dams produced 110% more IL-6 and 43% more TNF-α compared to pups from choline-fed dams (P<0.05) following stimulation with LPS (**Figure 8-2B**). There was no significant difference in IL-1β and IL-10 production following stimulation with LPS between diet groups and the concentration of IL-2 was below detectable levels in the supernatant after LPS stimulation.

Figure 8-2: Cytokine production by splenocytes following *ex vivo* stimulation with (A) concanavalin A (ConA) or (B) lipopolysaccharide (LPS) from 3-week old rat offspring from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]



[1]Values are means ± SEMs, n=10. *Different from choline group, (P<0.05).

Circulating plasma concentrations of IFN-γ, IL-10, IL-13, IL-1β, IL-4, IL-5, IL-6 were all below detection in the 3 week old pups. Mean plasma concentration of TNF-α was present at a very low circulating concentration of 2 ± 0.1 pg/mL (mean of all the pups, n=10) and did not differ between diet groups.

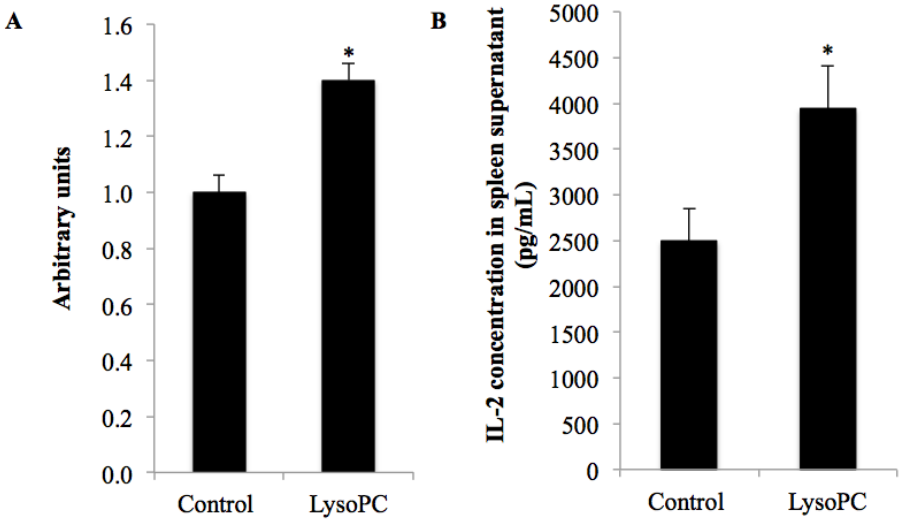
8.3.6 Choline metabolites in pups' spleen

Free choline, GPC and phosphocholine comprised the major forms of choline in pup spleen (**Table 8-6**). There was no significant difference in the concentration of total choline in splenocytes between diet groups, however pups from the PC-fed dams had a higher concentration of PC and sphingomyelin ($P<0.05$) in spleen. There was also a higher proportion of choline from PC and free choline in the splenocytes of the PC-fed pups compared to the free choline pups ($P<0.05$) (Table 8-6).

8.3.7 In vitro administration of lysoPC to splenocytes

Splenocytes had a 40% higher rate of proliferation (**Figure 8-3A**), assessed using the WST-1 assay, and produced 58% more IL-2 (**Figure 8-3B**) when cultured with lysoPC compared to control fatty acid ($P<0.05$).

Figure 8-3: (A) Cell proliferation, as measured by WST-1 assay and (B) IL-2 production from rat splenocytes following 48 h cultured with Control (18:1n-9) or LysoPC (18:1-lysophosphatidylcholine)[1]



[1]Values are means ± SEMs, n=8. *Different from control group, (P<0.05).

For the lysoPC treated splenocytes, there was a higher proportion of helper T cells (CD3+CD4+) and cytotoxic/suppressor T cells (CD3+CD8+) and higher proportion of CD4+CD25+, CD4+CD28+ and CD4+CD71+ cells (P<0.05) post culture (P<0.05) (**Table 8-7**). There was also a higher proportion of CD8+CD28+, CD8+CD71+ and CD8+CD152+, with CD152+ being the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), compared to control (P<0.05) (Table 8-7). Of the total proportion of CD8+ cells, there were a significantly higher proportion of the CD8+ population expressing CD25+ (IL-2 receptor), CD28+ (co-stimulatory molecule), CD71+ (transferrin receptor) and CD152+ (P<0.05) (Table 8-7). The remaining phenotypes that did not differ significantly between diets are presented as means ± SEM (both groups combined) in the footnote of Table 8-7.

8.4 Discussion

Feeding choline as PC or as free choline (choline salt) did not significantly affect the total choline concentration in pups' stomach content or total choline concentration in the

spleen or liver (data not shown) of the offspring. This is consistent with an absence of a significant difference in pup weight at the end of suckling. However, altering the form of choline in the maternal diet significantly changed the choline composition in the offspring's stomach content. Providing choline in the form of free choline in the maternal diet resulted in a higher proportion of total choline as free choline in the offspring's stomach content. This is consistent with our previous study in which we observed a linear relationship between feeding choline as a choline salt and the free choline content in the offspring's stomach content (Dellschaft, Ruth et al. 2015). However, this direct relationship between dietary form and stomach content form was not observed for PC as the proportion of total choline from PC in pups' stomach content was not different between diet groups. Instead, feeding choline as PC in the maternal diet resulted in higher proportion of choline coming from lysoPC and GPC. Thus it is possible that in the mammary gland, there is increased conversion of PC to lysoPC via the action of phospholipase A. This higher lysoPC content in stomach content may have contributed to the observed changes in the choline composition in the offspring's plasma and splenocytes. The higher concentration PC in plasma and splenocytes, and higher sphingomyelin concentration in splenocytes of pups from PC-fed could have also provided a larger pool of PC, that may have altered the response of the splenocytes. It is possible that the pups could have consumed a small amount of the experimental diets, however we are unable to separate the dam from the pups and the pups had access to the mothers fed cups. If this occurred then the contribution would have been minimal as the pups from the PC-fed dams did not have a higher concentration of PC in the stomach, which would be predicted if they consumed a significant amount of their mother's diet.

This is the first study to demonstrate that the form of choline in the maternal diet alters both T and B cell responses to immune challenge, suggesting that the choline form modulates

the function of the offspring's immune system. *In* vivo, pups from PC-fed dams had a lower proportion of total B cells (Ig+ cells, OX12+) and total cells expressing MHC class II (OX6+). These immune cells are involved in antigen presentation, and expression of MHC class II complexes on the B cell surface allows interaction with T cells, which activates both B and T cell responses (Patel, Arnold et al. 1999). However, when stimulated *ex vivo* with polyclonal T cell mitogen ConA, splenocytes from offspring of PC-fed dams had a higher production of cytokines IL-2, IFN-γ and IL-6 compared to splenocytes from pups from the choline-fed dams. The ability to proliferate (produce IL-2) when stimulated is one of the critical functions that increases during the early postnatal period in the rodent (Perez-Cano, Franch et al. 2012) and infant (Adkins, Ghanei et al. 1993). Neonates are also reported to have a reduced ability to produce IFN-γ (Wilson, Westall et al. 1986, Hartel, Adam et al. 2005), however pups from PC-fed dams produced 107% more IFN-γ. Insufficient production of IFN-γ has been associated with a lower protection against infection in newborn infants (Kotiranta-Ainamo, Rautonen et al. 2004). Additionally, IL-6 production was higher in splenocytes following stimulation in pups from PC-fed dams but IL-10 production was similar between diet groups. This pattern of cytokines produced by pups from the PC-fed dams is in the direction of a T helper 1 (Th1) type response. At birth, both rodent and human immune systems are characterized by a predominant T helper 2 (Th2) response (Adkins, Bu et al. 2001). The maturation the immune system shifts this response towards a Th1 dominated response (Perez-Cano, Franch et al. 2012), which is important for mounting an appropriate response to eliminate bacterial and viral pathogens (Calder 2007). Additionally, pups from PC-fed dams had a lower proportion of activated macrophages (CD68+CD11b/c+ cells), another type of antigen-presenting cell, and produced 110% more IL-6 and 43% more TNF-α when stimulated with LPS compared to pups from choline-fed dams. LPS activates macrophages and other

antigen-presenting cells leading to the activation of innate and acquired immune system responses. It has been demonstrated that PC is required for cytokine production by macrophages following LPS stimulation (Tian, Pate et al. 2008), suggesting PC may be the form of choline assisting with response to LPS, compared to free choline. Greater response to mitogen stimulation, with a lower proportion of antigen-presenting cells suggests a greater functional capacity of the antigen-presenting cells in the pups from the PC-fed dams. These cells are capable of activating T cells in order to mount an appropriate response to both T cell and B cell mitogens. Plasma circulating concentrations of cytokines were either below detection or present at very low concentrations in the 3-week old pups, therefore there is no evidence that there was systemic activation of the immune system (i.e. chronic inflammation). This allows us to conclude that the functional responses we observed in the pups from the PC-fed dams are a result of improved immune system development. The differential effects between forms of dietary choline on immune system development are likely attributed to the differences in absorption between water soluble free choline and lipid soluble PC. Upon absorption, free choline enters hepatic circulation and first seen by the liver (Cheng 1996). Conversely, approximately 50% of dietary PC is deacylated to GPC in the gut lumen and enterocyte and absorbed by the portal vein (Le Kim and Betzing 1976). The remaining PC enters the lymphatic circulation and reaches the peripheral organs, such as the spleen, prior to the liver.

LysoPC is readily taken up by tissues and can be utilized in PC synthesis (Stein and Stein 1966) which likely accounts for the higher PC in concentrations plasma and membranes of pups in the PC group. As we observed changes in the concentration of lysoPC in stomach content, and PC in plasma and spleen, we hypothesized that the higher supply of PC, or lysoPC, may be responsible for the effects on immune function that we observed between diet

groups. Consequently, we designed an *in vitro* study where we provided lysoPC to splenocytes. Although PC was used in the feeding experiment, lysoPC was used in the *in vitro* experiment as this is the form of PC most readily taken up by cells in culture (Martin, Wysolmerski et al. 1987), therefore representing PC in these conditions. We observed an increased proportion of T cells expressing the markers of activation (CD25 and CD71) as well as the co-stimulatory molecule (CD28) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152). Similar results have been previously demonstrated in adult human peripheral resting T lymphocytes, indicating that lysoPC activates T cells (assessed by IL-2α expression and thymidine incorporation into DNA) (Asaoka, Oka et al. 1992). In the current study, splenocytes provided with lysoPC resulted in a greater amount of metabolically active cells that proliferated better (produced more IL-2). PC in the diet could increase the supply of PC in splenocytes, thus activating T cells. It is also possible that lysoPC could have independent effects on T cell activation, but the *in vitro* experiment does not assess the independent actions of lysoPC or PC. Both possibilities support our hypothesis that the higher supply of PC to immune cells may have contributed to the better response to mitogens in the pups from the dams fed choline as PC. It possible that other components of the PC diet or products of PC hydrolysis, such as phosphatidic acid, contribute the changes in immune function observe, therefore future studies will explore the immune effects of other dietary components including PC, sphingomyelin, phosphocholine.

8.5 Conclusion

In summary, a lower proportion of cells involved in antigen presentation, and higher production of IL-2, IFN-γ, IL6, and TNF-α suggests that the immune cells of the pups from PC-fed dams are more efficient at mounting a response to antigens. PC pups appear

developmentally more mature compared to pups from free choline-fed dams and increasing the supply of PC, or lysoPC may be responsible for these effects. The present study demonstrates for the first time that providing choline in the form of PC, compared to free choline, to lactating dams altered the form of choline in the stomach content and promoted improved function of the offspring's T and B cells.

8.6 References

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Tables

Table 8-1: Composition of experimental diets

	Choline	PC
Casein (high protein), g/kg diet	270	270
Starch, g/kg diet	241	241
Sucrose, g/kg diet	126	126
Vitamin mix[2], g/kg diet	19	19
Salt mix[3], g/kg diet	50	50
Calcium phosphate dibasic, g/kg diet	3.4	3.4
Inositol, g/kg diet	6.3	6.3
Cellulose, g/kg diet	80	80
L-cysteine, g/kg diet	1.8	1.8
Flax oil, g/kg diet	7	7
Olive oil, g/kg diet	48	42
Sunflower oil, g/kg diet	67	64
Canola stearin, g/kg diet	78	72
DHAsco[4], g/kg diet	1.5	0.0
ARAsco[5], g/kg diet	1.5	0.0
Choline bitartrate, g/kg diet	2.5	0.0
Egg PC concentrate[6], g/kg diet	0.0	15
Energy from protein, %	25	25
Energy from carbohydrate, %	34	34
Energy from fat, %	41	41
Total choline, g/kg diet	1.0	1.2
Fatty acid, g/100g of total fatty acids[7]		
16:0	7.0	7.7
18:0	33.0	33.7
18:1t11	1.7	2.1
18:1n-9	31.3	29.8
18:1c11	0.4	0.5
18:2n-6	20.8	21.1
20:0	0.9	0.8
18:3n-3	2.2	2.1
20:3n-6	0.4	0.4
20:4n-6	0.2	0.2
20:5n-3	0.2	0.2
22:6n-3	0.2	0.1
Total SFA	40.9	42.2
Total PUFA	24.0	24.0
Total n-6	21.5	21.6
Total n-3	2.6	2.4
Total MUFA	33.2	32.2

[1] All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were purchased from Safeway (Edmonton, AB, Canada), and canola stearin was donated by Richardson Oilseed Limited (Lethbridge, AB, Canada). Choline, choline diet (1 g choline as free choline/kg diet); MUFA, monounsaturated fatty acids; PC, phosphatidylcholine diet (1.2 g choline as phosphatidylcholine); PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
[2]AIN-93-VX vitamin mix (Reeves 1997)
[3]Bernhart-Tomarelli salt mixture (Bernhart and Tomarelli 1996)
[4]DHAsco is a microalgae source of docosahexaenoic acid (DHA)
[5]ARAsco is a fungal source of arachidonic acid (ARA)
[6]Egg PC concentrate extracted from egg yolks and described in methods and Table 8-2.
[7]Analysis by gas-liquid chromatography, GLC of n=2 batches

Table 8-2: Phospholipid, choline and fatty acid composition of egg PC concentrate

Egg PC concentrate component	
Phospholipid content, mg/g	
PC	570
PE	170
Sphingomyelin	20
LysoPC	19
PI	10
LysoPE	7.9
Choline content, mg/g	
PC	77
Sphingomyelin	3.0
LysoPC	3.8
Free Choline	0.2
Total Choline, mg/g	84.1
Fatty acid composition, g/100g of total fatty acids	
Total SFA	26
Total PUFA	58
Total n-6	52
Total n-3	5.9
Total MUFA	16

Table 8-3: Anthropometric measurements of 3-week old rat offspring from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]

	Choline	PC
Body weight, g	60 ± 0.8	55 ± 1.5
Spleen weight, g	0.3 ± 0.01	0.3 ± 0.01
Splenocytes/g of spleen, $x10^6$	1130 ± 140	930 ± 48
Liver weight, g	2.5 ± 0.10	2.4 ± 0.10
Intestine length, cm	70 ± 2	72 ± 1

Choline, choline diet (1 g choline as free choline/kg diet); PC, phosphatidylcholine diet (1 g choline as phosphatidylcholine).

[1]Values are means ± SEMs, n=10.

*Different from choline group, ($P<0.05$).

Table 8-4: Choline-containing metabolites in 3-week old rat offspring's plasma from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]

	Choline	PC
Choline-containing metabolite, nmol/mL		
Free choline	16 ± 1.2	16 ± 1.1
LysoPC	490 ± 13	510 ± 17
PC	2220 ± 110	2530 ± 72*
Sphingomyelin	100 ± 9	100 ± 3
GPC	2.5 ± 0.1	2.6 ± 0.05
Phosphocholine	5.1 ± 0.5	3.7 ± 0.03*
Choline from each choline-containing metabolite, µg/mL		
Free choline	2 ± 0.1	2 ± 0.1
LysoPC	51 ± 1.3	53 ± 1.8
PC	230 ± 11	260 ± 7.5*
Sphingomyelin	14 ± 1.3	15 ± 0.4
GPC	1.0 ± 0.0	1.0 ± 0.0
Phosphocholine	3 ± 0.3	2 ± 0.02*
Total choline, µg/mL	300 ± 13	340 ± 8.4

Choline, choline diet (1 g choline as free choline/kg diet); GPC, glycerophosphocholine; LysoPC, Lysophosphatidylcholine; PC, phosphatidylcholine diet (1 g choline as phosphatidylcholine).

[1]Values are means ± SEMs, n=10. *Different from choline group, ($P<0.05$).

Table 8-5: Splenocyte immune cell phenotypes from 3-week old rat offspring from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]

	Choline	PC
Cell phenotype[2], % of total cells		
Total CD3+	25 ± 1.0	23 ± 0.50
CD3+CD4+	11 ± 0.80	10 ± 0.70
CD3+CD8+	8 ± 0.3	8 ± 1
CD4+CD25+	4 ± 0.3	5 ± 0.3
CD8+CD25+	4 ± 0.4	3 ± 0.2
CD4+CD28+	10 ± 0.7	10 ± 1
CD8+CD28+	16 ± 2.1	14 ± 0.3
CD4+CD71+	9 ± 0.3	8 ± 0.3
CD8+CD71+	6 ± 0.4	7 ± 1.0
CD4+CD152+	2 ± 0.3	3 ± 0.4
CD8+CD152+	5 ± 0.7	6 ± 1
CD3-CD161+	9 ± 0.9	11 ± 0.8
OX6+ (MHC class II+)	44 ± 2.1	38 ± 1.5*
OX12+ (Ig+ B cells)	42 ± 2.4	33 ± 2.5*
CD68+CD11b/c+ (macrophages)	3 ± 0.1	2 ± 0.1*
IgA+	13 ± 1.0	8.2 ± 0.7
IgG+	9 ± 0.7	7 ± 0.3
IgM+	46 ± 1	43 ± 0.9
CD45RA+	43 ± 2	40 ± 0.6

Choline, choline diet (1 g choline as free choline/kg diet); PC, phosphatidylcholine diet (1 g choline as phosphatidylcholine).

[1]Values are means ± SEMs. *Different from choline group, ($P<0.05$).

[2]There were no differences among groups in the following phenotypes (mean ± SEM % of total cells, n=10): CD3+CD4+CD25+FoxP3+ (2 ± 0.1), CD68+CD11- (5 ± 0.3), CD68+CD284+ (7 ± 0.3), CD3-CD161+ (10 ± 0.8), OX62+OX6+ (3 ± 0.2), CD8+CD127+ (8 ± 0.4), CD4+CD127+ (9 ± 0.5), CD4+CD27+ (6 ± 0.4), CD8+CD27+ (10 ± 0.4), OX12+CD27+ (16 ± 1.5), CD11+CD284+ (6 ± 0.2), OX62+OX6+ (3 ± 0.2).

Table 8-6: Choline-containing metabolites in 3-week old rat offspring's splenocytes from dams fed free choline (choline) or phosphatidylcholine (PC) diets[1]

	Choline	PC
Choline-containing metabolite, nmol/mg protein		
Free choline	19 ± 1.5	23 ± 1.6
LysoPC	0.6 ± 0.2	0.3 ± 0.1
PC	0.3 ± 0.1	1 ± 0.3*
Sphingomyelin	0.3 ± 0.0	0.4 ± 0.0*
GPC	16 ± 1.0	16 ± 0.89
Phosphocholine	9 ± 0.3	9 ± 0.5
Total choline, μg/mg protein	4 ± 0.4	5 ± 0.3
Choline-containing metabolite, % of total choline		
Free choline	41 ± 2	46 ± 3*
LysoPC	1 ± 0.3	0.6 ± 0.3
PC	0.8 ± 0.1	2 ± 0.5*
Sphingomyelin	0.8 ± 0.1	1 ± 0.1
GPC	35 ± 1.0	32 ± 1.0
Phosphocholine	21 ± 1.5	18 ± 1.0

Choline, choline diet (1 g choline as free choline/kg diet); phosphatidylcholine diet (1 g choline as phosphatidylcholine).

[1]Values are means ± SEMs, n=10. *Different from choline group, ($P<0.05$).

Table 8-7: Immune cell phenotypes from rat splenocytes cultured for 48 hours with 18:1n-9 (Control) or 18:1-lysophosphatidylcholine (LysoPC)[1]

	Control	LysoPC
Cell phenotype[2], % of total cells		
Total CD3+	24 ± 1.0	27 ± 0.6*
CD3+CD4+	13 ± 0.3	14 ± 0.4*
CD3+CD8+	17 ± 0.6	20 ± 0.7*
CD4+CD25+	14 ± 1.1	17 ± 0.4*
CD4+CD28+	14 ± 0.7	19 ± 0.7*
CD3+CD71+	18 ± 0.6	22 ± 0.7*
CD4+CD71+	13 ± 0.3	16 ± 0.8*
CD8+CD71+	22 ± 1.4	26 ± 0.7*
CD8+CD152+	12 ± 0.8	15 ± 0.9*
CD28+CD152+	12 ± 0.9	15 ± 0.8*
Proportionate analysis		
% of CD8+ cells that also express CD25+	78 ± 1.5	83 ± 0.3*
% of CD4+ cells that also express CD28+	84 ± 1.9	93 ± 0.7*
% of CD8+ cells that also express CD28+	74 ± 2.1	84 ± 0.8*
% of CD8+ cells that also express CD71+	76 ± 2.5	84 ± 2.0*
% of CD8+ cells that also express CD152+	40 ± 1.3	48 ± 1.5*

Control, 10 μM 18:1n-9; LysoPC, 10 μM 18:1 lysoPC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine).

[1]Values are means ± SEMs, n=8. *Different from control group, ($P<0.05$).

[2]There were no differences among groups in the following phenotypes (mean ± SEM, n=8): % of total cells: CD8+CD25+ (24 ± 1.3), CD8+CD28+ (23 ± 1.0), CD4+CD152+ (8 ± 0.5) and % of CD4+ cells that also express CD25+ (82 ± 3.6), % of CD4+ cells that also express CD71+ (79 ± 1.8), % of CD4+ cells that also express CD152+ (45 ± 1.9).

CHAPTER 9: Final discussion

9.1 General objectives

The overall objectives of this thesis were to 1) develop a comprehensive choline database to examine prenatal and postnatal maternal choline intake in a population of Albertan women and 2) examine the effect of the amount and form of early postnatal maternal choline on the development of the offspring's immune system. The choline composition of commercially available pulses and meats from Alberta were added to a comprehensive food composition database to estimate intake in a population of Albertan women during pregnancy and 3 months postpartum. Findings from this human study were used to develop a series of animal experiments to examine the effect of maternal choline on immune system development and function in the infant. The effect of a maternal diet devoid of choline, and the differential effects of the two most commonly consumed choline forms in humans, on immune system development were examined.

9.2 Summary of Results

1. **The first objective of this research was to use the comprehensive choline database to estimate prenatal and postnatal intake in a cohort of Albertan women. This objective was investigated using the following sub-objectives and hypotheses:**

    e) **The first sub-objective of objective 1 was to quantity the choline content of commercially available pulses and meats from Alberta in order to expand the current choline composition database. This objective was investigated in Chapters 3 and 4.**

In Chapter 3, the choline composition of 32 commercially available pulse varieties was quantified and in meals rich in pulses, there were differences in the choline content from the available USDA values compared to values measured in this work. Total choline content of the pulses was reduced with cooking due to a loss of water-soluble choline moieties with discarding of the cooking water. In Chapter 4, the choline composition of twenty meat samples available in Alberta was quantified, with similar total choline and phosphatidylcholine content between values measured in this work compared to values available from the USDA. There was a strong negative correlation between fat and choline content, suggesting lower fat cuts of meat are better dietary sources of choline.

f) **The second sub-objective was to estimate prenatal and postnatal intake in a cohort of Albertan women and examine the contribution of two food sources of choline to dietary intake. We hypothesized that during pregnancy and early postpartum, women will consume below current choline recommendations and consumption of major dietary sources of choline (eggs and milk) will assist in meeting daily recommendations. This objective and hypothesis was investigated in Chapter 5.**

This hypothesis was supported by results presented in Chapter 5. During pregnancy and 3 months postpartum, the majority of women were not meeting daily choline recommendations. Choline was consumed primarily from dietary sources, not supplements, with the majority of choline consumed as PC and free choline. Women who reported consuming eggs or two servings of milk (500 mL) per day had higher total choline intake and were more likely to meet daily choline recommendations.

g) **The third sub-objective was to compare two methods of 24-h dietary recall used in the APrON study to estimate choline intake. This objective was investigated in Chapter 6. We hypothesized that estimates of choline intake would be similar between interview and online 24-h recall methods.**

This hypothesis is partly supported by the results discussed in Chapter 6. There was a significant positive correlation between estimates of total choline and forms of choline, with the exception of free choline, when interview and online 24-h recall methods were compared in a subset of 60 women. We observed that energy-adjusted estimates of total choline and free choline increased the correlation between the two recall methods. Adjusting for energy intake improved the percentage of participants classified in their respective tertiles of intake (tertile 1, 2 or 3) between recall methods. This suggests that energy-adjusted estimates of total and free choline are the best way to express choline intake when using methods together.

e) **The fourth sub-objective was to examine the correlation between maternal prenatal choline intake and infant birth weight.**

The results presented in Chapter 6 did not support this hypothesis. After adjusting for gestational weight gain and gestational age, there was no correlation between maternal energy-adjusted choline intake during pregnancy and infant birth weight. Furthermore, there was no correlation between maternal phosphatidylcholine intake and infant birth weight in women who completed interview dietary recalls.

2. **The second objective of this research was to use an animal model to examine the effect of maternal choline, both amount and form, on the development of the infant's**

immune system. This objective was investigated using the following sub-objectives and hypotheses:

d) **This first sub-objective of objective 2 was to examine the effect of a maternal diet devoid in choline on offspring immune system development. We hypothesized that a maternal diet devoid of choline would be insufficient in supporting the development of the offspring's immune system, with lasting effects on the offspring's immune system, even with a choline-sufficient diet post-weaning.**

The results presented in Chapter 7 support this hypothesis. Offspring from dams fed a choline-devoid diet for the duration of the suckling period had reduced growth and lymphopenia and impaired T and B cell responses. The effects of a choline-devoid diet during a critical development period persisted into the post-weaning period, even with a choline-sufficient diet at weaning to six weeks of age. Offspring from dams fed a choline-devoid diet during suckling had impaired expansion of the helper T cell population, and reduced production of IL-6, a key regulatory cytokine.

e) **The second objective was to examine the differential effects of the two major forms of choline in the maternal diet on offspring immune development. We hypothesized that the form of choline, phosphatidylcholine compared to free choline, in the maternal diet will differentially affect immune development in the offspring**.

This hypothesis was supported by the results presented in Chapter 8. Offspring from dams fed a diet that provided choline in the form of phosphatidylcholine had a lower proportion of antigen presenting cells, and a higher proportion of cytokines with stimulation, compared

offspring from dams fed a diet containing free choline during suckling. This suggests that feeding phosphatidylcholine resulted in greater efficiency in response to antigens and immune system maturation during a key development period, compared to free choline.

f) **The third objective was to elucidate a potential mechanism of phosphatidylcholine on immune function modulation. We hypothesized that providing phosphatidylcholine in the maternal diet would increase the availability of phosphatidylcholine for immune cell expansion and proliferation, resulting in greater immune system maturation.**

This hypothesis is partly supported by the results presented in Chapter 8; however, other hypotheses still remain. In an animal model, a maternal diet containing choline in the form of phosphatidylcholine increased phosphatidylcholine in the plasma and splenocytes in offspring, suggesting a greater availability of phosphatidylcholine available for membrane synthesis. In a cell culture model using isolated splenocytes, lysoPC, the form of choline readily taken up by cultured cells, increased T cell activation and T cell proliferation IL-2 production. However, phosphatidylcholine may have other actions other than increasing membrane synthesis that may have affected immune development, which have not been explored.

9.3 General discussion and future directions

9.3.1 Expanding the choline composition database

Our research identified dietary sources of choline absent from the current choline composition database, the USDA Choline Content of Common Foods, and was the first to report the choline content of commercially available pulses and meat samples from Alberta. Each of the food items were analyzed in triplicate, similar to the stringent methods set out by

the USDA in the estimation of choline content for their own database (Patterson, Williams et al. 2008). In addition to the foods we already quantified, we identified other foods in which there were no suitable substitutes in the current choline database to estimate choline content that could be quantified to further aid in the estimation of dietary choline. This includes many dairy alternatives such as almond, soy, rice and hemp beverage, unique food items (bee pollen, chia and hemp seed), seafood items (shrimps, lobster, scallops and clams) and many ethnic or mixed ingredient foods that are not listed in the USDA choline database. Studies examining dietary choline intake in populations worldwide commonly report the limitation that the current choline database does not contain foods traditional to their ethnicity or region (Gossell-Williams, Fletcher et al. 2005, Konstantinova, Tell et al. 2008, Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013, Vennemann, Ioannidou et al. 2015). Specifically within Alberta, we have begun to follow up our published reports with the choline composition dairy products available in Alberta (Richard, Lewis et al. 2016). This study included 48 commercially available dairy and dairy alternative products, many of which were absent from the USDA choline database. This work will hopefully be continued with other populations to include a greater variety of mixed foods and regionally diverse foods samples.

In addition to supplementing the current choline composition database with unanalyzed foods absent from the database, it is important to compare the choline composition of foods within the current USDA database. Our data agreed with previous reports on the choline content of pulses and meat that were analyzed. However, Richard *et al.,* (2016) reported discrepancies between the choline composition of some dairy (milk and butter) and dairy alternatives (soy beverage) analyzed, compared to previous reports in the USDA database (Richard, Lewis et al. 2016). These differences could be attributed to differences in industrial processing methods, or regional and geographical differences. Sphingomyelin content in milk

can vary by cow breed, lactation stage and season (Graves, Beaulieu et al. 2007) and lipid content has been reported to differ based on cow's diet, which could also affect choline content (Lopez, Briard-Bion et al. 2014). The USDA database collected samples all within the United States, with over 95 food items collected locally from Chapel Hill, North Carolina. A limitation of our current work is that our samples were taken from Edmonton and surrounding area, a relatively narrow geographical range. Recognizing that differences in choline content could arise based on geography, our future work should focus on samples not only commercially available in Alberta, but also samples from a wide variety of locations.

9.3.2 Choline intake in women of childbearing age

Our study is the first to describe choline intake in women during pregnancy and early postpartum and demonstrate that consumption of eggs and milk can assist in meeting recommendations. Additionally, we examined for the first time, the agreement between two methods of 24-h dietary recall in the estimation of choline. There was good agreement between dietary assessment methods when total choline intake was adjusted for energy.. Further, the majority of women of childbearing age in Alberta are consuming total choline below recommendations, similar to populations in other regions (Mygind, Evans et al. 2013, Masih, Plumptre et al. 2015, Vennemann, Ioannidou et al. 2015), and our results indicate that there is good agreement between methods for estimates of low intake (below recommendations). Establishing recommendations for using the two dietary methods together will allow future researchers to use all participants in the APrON study and examine the potential consequences of low intake, particularly during periods with high choline needs. Although we found no association with maternal energy-adjusted total choline intake and infant birth weight, this analysis and further research questions requires further investigation.

A detailed review by Caudill *et al.,* (2014), discusses the improvements to offspring health with maternal choline supplementation, particularly for brain development. A review by McCann et al. (2006) provides a summary of the evidence of the relationship between choline and cognitive function in offspring (McCann, Hudes et al. 2006). In humans, there have been mixed findings on the association between maternal choline intake and infant cognitive functions (Cheatham, Goldman et al. 2012, Boeke, Gillman et al. 2013, Strain, McSorley et al. 2013). In the APrON study, approximately 200 children have completed cognitive tests at three years of age, assessing aspects of hippocampal function including spatial learning, memory and attention. A future research objective for this study could be to examine if there is an association between dietary choline intake and infant visuospatial memory development.

Although we demonstrated agreement between methods for energy-adjusted total choline intake, there was poor correlation and agreement between methods for estimating the forms of choline, specifically free choline and PC. With the growing body of literature examining dietary choline intake in populations worldwide, it would be beneficial for future researchers to consider the validity of their dietary assessment tools in the estimation of choline. The majority of previously published studies examining dietary choline used dietary assessment tools that were not validated for the estimation of choline (Mygind, Evans et al. 2013, Yonemori, Lim et al. 2013, Masih, Plumptre et al. 2015, Vennemann, Ioannidou et al. 2015). Researchers should consider examining dietary choline adjusted for energy intake, and should particularly focus on foods that have proven to be difficult to estimate between dietary recall methods, such as mixed foods and beverages.

9.3.3 Maternal choline deficiency and offspring growth, immune and intestinal function

Using an animal model, we demonstrated for the first time that a maternal diet devoid of choline, while sufficient in all other nutrients, during suckling, negatively affected growth and development of the immune system in the offspring. Additionally, we are the first to report that the effects of a maternal choline-devoid diet persisted into the post-weaning period, even with a choline-sufficient diet in the post-weaning period. This demonstrates that choline is essential in the suckling period to support the development and function of the immune system in the offspring.

This study also demonstrated the profound effect a maternal diet devoid of choline had on offspring growth, consistent with previous studies demonstrating a low choline or choline-devoid diet significantly impacts growth, both in adult (Courreges, Benencia et al. 2003, Dellschaft, Ruth et al. 2015) and young animals (Newberne, Wilson et al. 1970). At the end of the lactation period, the choline-devoid dams had significantly lower body weights, and their offspring were 30% smaller compared to those on the choline sufficient diet. This maternal weight loss and stunted growth in the offspring could have independent effects on the development of the immune system (van der Most, de Jong et al. 2010). In future, a pair-feeding study could be designed to determine the independent effects of choline on changes on immune function separate from those attributed to the reduced growth. Furthermore, it has been demonstrated in our cohort of women in Alberta that consumption of total choline in the early postpartum period is approximately half of what is recommended daily (Lewis, Subhan et al. 2014). Future rodents studies could use a diet containing 0.5 g choline/kg diet (half the recommendation for rodents) in order to assess the biological relevance to humans during the lactation period. This may also help offset some weight loss observed in the dams and offspring resulting from a diet that contains no choline.

In this study, we did not account for possible sex differences in response to choline deprivation. Previous reports demonstrated that estrogen increases PEMT gene expression (Resseguie, Song et al. 2007, Fischer, da Costa et al. 2010), increasing the synthesis of PC through the PEMT pathway (Noga and Vance 2003), decreasing the susceptibility of the consequences of low dietary choline in pre-menopausal women (Fischer, daCosta et al. 2007). We demonstrated that a maternal choline-devoid diet during the suckling period reduced the expansion of the CD4+ T cell population and IL-6 production with *ex vivo* stimulation in 10-week old female offspring. Given the reported sex differences with choline deficiency on other organ systems (Kashireddy and Rao 2004, Fischer, daCosta et al. 2007), future research should examine if the immune system in male offspring are more susceptible to maternal choline-devoid diet during suckling.

As mentioned, a maternal diet devoid of choline had effects on offspring growth. In the dams fed a choline-devoid diet, there was impaired intestinal function, notably reduced absorptive area (villus length) and intestinal PC content (da Silva, Kelly et al. 2015). Consistent with these findings, pups from dams fed a diet without choline, folate and vitamin B_{12} from pregnancy to weaning (21 days postnatal) had decreased crypt depth and absorptive area, and poor barrier function in their intestine (Bressenot, Pooya et al. 2013). Reduced growth of the offspring in our study may be due to reduced nutrient absorption in the intestine as a result of the choline-devoid diet. We could examine if a maternal choline-devoid diet affects intestinal absorptive capacity and function in the offspring, similar to what occurs in lactating dams (da Silva, Kelly et al. 2015). It is hypothesized that maternal choline deficiency during the suckling period would reduce the absorptive capacity of the offspring's intestine, which may decrease absorption of nutrients, possibly due to reduced production of bilary PC, contributing the reduced growth. In order to examine possible changes in intestinal

morphology we could analyze villus length, crypt depth, thickness and density of jejenum sections from the offspring. Additionally we could examine if there are changes in intestinal PC, or alterations in bilary PC necessary for the absorption of lipids.

9.3.4 Differential effects of choline forms on immune system development

We demonstrated for the first time that the forms of choline in the maternal diet differentially affect immune development and function in the offspring. We reported that providing choline as PC in the maternal diet promotes maturation of the offspring's immune system, compared to feeding free choline. In our previous study in humans, we reported that in the diet, these choline forms are consumed as a mixture, approximately 50% as PC, 25% as free choline and the remaining 25% comprised of minor dietary forms (GPC, phosphocholine and sphingomyelin). An interesting follow-up study from our initial observations in rodents to assess the biological relevance to humans would be to use a diet containing a mixture of choline forms, reflective of human dietary pattern.

We demonstrated that splenocytes provided with lysoPC had a higher amount of metabolically active cells, with greater proliferation (IL-2 production). In order to further investigate the hypothesis that increasing substrate availability is promoting expansion of the immune cell population, this experiment would need to be repeated with the addition of a group of splenocytes provided the same concentration of choline, but in the form of free choline. This would help elucidate if these two forms of choline have differential affects at the membrane level, or if other possible mechanisms are involved. Further to this proposed mechanism, we recognize that other mechanisms are likely involved regarding the differential affects of choline form. Additionally, we did not investigate the potential effects of other forms of choline on lymphocyte proliferation. Other dietary components may also have

contributed to the observed changes in immune function. Therefore, future studies should also explore the potential immunomodulatory effects of other forms of choline including GPC, sphingomyelin and phosphocholine. It is also possible that lysoPC could have independent effects on lymphocyte activation, which were not assessed in this model. Previous experiments conducted *in vitro* using human peripheral T lymphocytes have demonstrated that lysoPC, in the presence of DAG, increases IL-2 receptor expression and [^{3}H]thymidine incorporation (Asaoka, Oka et al. 1992). LysoPC has also been demonstrated to up regulate CD86 and CD40 expression on dendritic cells, and initiate IFN-γ production by T lymphocytes (Bach, Perrin-Cocon et al. 2010). Additionally, increasing the availability of PC may also increase the hydrolysis of PC, producing metabolites DAG and PKC, which have been demonstrated as mediators of T cell activation, increasing IL-2 receptor expression and IL-2 secretion from isolated human peripheral T lymphocytes (Asaoka, Oka et al. 1991).

9.4 Conclusion

Overall, the research presented in this thesis demonstrated that women during pregnancy and early postpartum were consuming below what is recommended, and in rodents, a maternal diet with no choline negatively affected offspring growth and immune system development. This thesis reported that total choline was consumed primarily as free choline and PC, and showed for the first time that a maternal diet providing choline as PC, compared to free choline, had a beneficial effect on the development of the offspring's immune system. To date, few studies have examined the effect of consuming low amounts of choline, or the differential affects of choline form, on infant health. Future research should consider not only the amount but also the forms for choline in the diet when considering the implications for health.

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APPENDIX

Appendix 1: Comparison of choline-containing moieties by pulse variety between raw and cooked pulses

Pulse	Prep[1]	Moisture[2]	Free Cho	PtdCho	LPC	GPC	Pcho
		%	mg choline moiety/100g of food				
Black bean	Raw	11.1	49.7 ± 3.8	65.8 ± 2.5	1.8 ±0.1	9.6 ± 1.5	1.7 ± 0.0
	Cooked	63.8	30.6 ± 1.1	67.4 ± 3.0	2.5 ± 0.2	8.6 ± 0.2	1.4 ± 0.0
Chickpeas	Raw	7.6	22.2 ± 4.5	95.7 ± 5.8	3.6 ± 0.5	6.1 ± 0.7	2.1 ± 0.2
	Cooked	61.4	14 ± 0.3	92.6 ± 3.6	4.9 ± 0.3	7.5 ± 0.8	3.9 ± 0.0
Dark red kidney beans	Raw	7.5	48.3 ± 7.4	56.8 ± 5.4	1.8 ± 0.0	11.1 ± 0.1	2.2 ± 0.0
	Cooked	65.2	24.5 ± 1.3	59.8 ± 5.8	3.4 ± 0.5	7.8 ± 0.3	3.2 ± 0.0
Pinto beans	Raw	7.2	56.4 ± 11.4	60.7 ± 8.4	3.2 ± 1.3	8.6 ± 1.2	1.4 ± 0.3
	Cooked	60.0	37.4 ± 2.9	58.4 ± 5.0	3.5 ± 0.4	9.0 ± 1.0	1.5 ± 0.0
Soya beans	Raw	6.7	77.2 ± 9.1	71.4 ± 1.7	4.5 ± 0.2	11.9 ± 0.6	2.7 ± 0.1
	Cooked	50.8	78.6 ± 1.3	64.6 ± 1.3	5.7 ± 0.4	9.1 ± 0.3	4.7 ± 0.2
Green lentils, whole	Raw	7.4	14.9 ± 1.0	98.2 ± 7.5	3.5 ± 0.1	4.8 ± 0.1	4.5 ± 0.0
	Cooked	71.0	15.5 ± 0.5	85.2 ± 2.9	5.5 ± 0.5	5.2 ± 0.1	3.8 ± 0.0
Red lentils, split	Raw	7.6	16.2 ± 2.4	94.7 ± 7.7	2.9 ± 0.2	4.7 ± 0.3	5.3 ± 0.0
	Cooked	69.5	6.9 ± 0.4	81.0 ± 1.7	4.3 ± 0.4	2.6 ± 0.1	2.0 ± 0.0
Green peas, split	Raw	6.3	25.9 ± 10.1	82.2 ± 11.9	3.4 ± 0.1	9.8 ±3.2	1.8 ± 0.8
	Cooked	63.8	15.5 ± 0.9	72.4 ± 6.9	4.1 ± 0.2	9.9 ± 0.9	3.9 ± 0.0
Yellow peas, split	Raw	7.8	24.4 ± 0.3	93.4 ± 0.4	3.3 ± 0.1	8.9 ±0.3	3.0 ± 0.0
	Cooked	63.7	13.6 ± 0.9	77.7 ± 3.5	3.9 ± 0.1	11.0 ± 0.6	3.0 ± 0.0

[1]Prep refers to the preparation method used for each pulse variety. Cooked pulses were boiled prior to analysis of choline content.
[2]Choline content has been adjusted based on moisture content of 100g of sample.

Appendix 2: Standard Operating Procedures for use of the University of Alberta Database for the Choline Content of Common Foods (Alberta Database)

Introduction

The University of Alberta Choline Database (Alberta Database) is an Excel spreadsheet that contains information on the choline content of common foods and estimated daily choline intake of Edmonton and Calgary participants of the Alberta Pregnancy Outcomes and Nutrition (APrON) study.

In March 2004, the United States Department of Agriculture (USDA) developed the Database for the Choline Content of Common Foods (USDA Database). In January 2008, the USDA Database was updated to include approximately 630 food items (Patterson, Williams et al. 2008). This database was developed in collaboration with the Nutrient Data Laboratory of the USDA in Beltsville, MD, and the University of North Caroline in Chapel Hill, NC. The foods samples analyzed were collected nationally from the United States, with the majority of food items collected locally from Chapel Hill (Patterson, Williams et al. 2008).

The USDA Database serves as an excellent starting point to develop a database inclusive of foods commonly consumed in Alberta. The Alberta Database was developed to: 1) include the choline content of foods collected from regions in Alberta (Edmonton and surrounding areas) and 2) estimate dietary choline intake in Albertan women participating in the APrON study. The Alberta database was first constructed by a group of NUTR 400/401 students using Microsoft Office Excel 2007. The database was expanded to include 3814 foods.

Alberta Database Worksheets

The following section will review the various Excel worksheets that are contained within the Alberta Database. The worksheets pertain to the estimation of the choline intake by women in the APrON cohort but can be applied to any population with slight alteration of the worksheets. Words that are **bolded** throughout this section refer to names of worksheets or titles of columns within the worksheets. Words that are "quoted" are words coming directly from figures within this section or from the worksheets themselves. The figures shown in the following sections are exerts from the Alberta Database and are included to serve as a guide for further understanding of the database.

a) Choline Database Worksheet

The first worksheet in the Alberta Database is **Choline Database** and includes the choline content of all foods included in the database. This database is used throughout the following worksheets for estimation of dietary choline intake. **Figure 1** is an excerpt of the Choline Database worksheet.

Figure 1. Choline Database worksheet

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Assigned NDB	Food Processor Description	Assigned NDB	USDA Description	Food Category	Dairy or Eggs	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline	a.NDB
2							(mg/100g of food)	mg choline moiety/100 g of food						
3	1121	Activia Vanilla Yogurt	1121	Yogurt, 1% to 2% MF	Dairy and Eggs	Dairy	0.8	2.1	7.8	1.6	1.5	1.1	14.1	1121
4	98009	Agave, nectar, all natural	98009	Agave, cooked	Vegetables and vegetable products		0.4	5.2	0.7	0	2.8	0.1	8.8	98009
5	14006	ALCOHOLIC, BEER, LIGHT, 4% ALCOHOL BY VOLUME	14006	Alcoholic beverage, beer, light	Beverages		6.3	5.4	2.5	0	0	0	7.9	14006
6	14096	ALCOHOLIC, DESSERT WINE, SWEET, 18.8% ALCOHOL BY VOLUME	14096	Alcoholic beverage, wine, table, red	Beverages		0.3	4.5	1.1	0	0	0	5.6	14096

Column A and C, **Assigned NBD**, refers to the assigned Nutrient Database (NDB) numbers that have been given to the particular food that was recorded. The NDB number assigned to each food is a five digit numerical code that is used in the USDA Nutrient

Database for Standard Reference (SR). Foods that were included in the USDA choline database that did not have a corresponding SR were given NDB numbers beginning with "98___". These assigned NDB numbers were included in the Alberta Database. Many foods that were added to the Alberta Database did not have a NDB number. In this case, foods were given a five or six digit assigned NDB beginning with "888__". **Assigned NDB** of "0" is assigned if data is missing from that food that is listed. If this occurs then there is a problem with the **Choline Database** worksheet that needs to be resolved. **Assigned NDB** of "1" indicates that no choline is present in the food item that was entered. This would occur for foods such as sugar or table salt in which dietary choline is not present. If a "1" occurs in the **Assigned NDB** column then values of "0" will be seen in the columns containing the choline content information (columns G to M). **Assigned NDB** of "2" indicates that the food item has not been analyzed for choline content and the choline content cannot be accurately estimated. This occurs for foods such as almond milk in which there is no nutritionally comparable food to assume the same choline content but it is consumed often, therefore included in the database. In this case "0" will be seen in the columns containing the choline content information. It is hoped that these unanalyzed foods items will be analyzed in the future and their choline content information can be added to the University of Alberta choline database.

All of the foods entered into the Alberta Database have an assigned NDB number, which is used to link the choline content of the food entered in the P column (**Item Name**) in the **Timepoint (A-E) Worksheets**. The **Assigned NDB** column is the most important aspect when estimating choline intake as it uses a value-lookup (v-lookup) Excel formula to fill in the choline values for each of the foods entered in the **Item Name** column. The v-lookup formula links the appropriate assigned NDB in the **Food Records** worksheet to the appropriate assigned NDB number in the **Food Processor** worksheet. The choline information from that

specific food is then transferred from the **Food Processor** worksheet back to the **Food Records** worksheet with the help of a conversion factor, which is calculated to fit the quantity of the food recorded.

Column B, **Food Processor Description**, is a comprehensive list of almost every food that was encountered while analyzing the 24-hour dietary recalls of the APrON participants. The food description is used by Food Processor and includes a wide variety of very similar foods, for example, chicken has many different options which include with or without skin, dark or light meat, roasted or fried and breast, drumstick, thigh or ground.

Column D, **USDA Description**, is a description of the food item that matches the description that is listed in the USDA Database and can be applied to many foods that are similar by have varying wording in **Food Processor Description** column.

Column E, **Food Category**, categorizes the food listed in the **Assigned NDB** column into food groups. Options include baked products, fruit and fruit products, vegetables and vegetable products, fast food, snacks, chicken and turkey etc.

Column F, **Dairy or Eggs**, is specific to the interests of the study that the Alberta Database was designed for. This column further divides the **Food Category** column, into either "Dairy", "Eggs" or blank. The **Dairy or Eggs** column uses another v-lookup formula to first identify if the food listed in the **Assigned NBD** column falls under the "dairy and eggs" food group, and then identifies if the food is either a dairy product or an egg product. For the study that was conducted using the database, there was particular interest into the "dairy" and "egg" foods as major sources of dietary choline.

The choline and betaine content for each food in each row is listed in columns G to M including **betaine**, free choline (**Free Cho**), glycerophosphocholine (**GPC**), phosphocholine (**Pch**), phosphatidylcholine (**Ptdcho**), sphingomyelin (**SM**) and total choline (**TC**).

b) Timepoint (A-E) Worksheets

This worksheet contains both the **Choline Database** and 24-hour dietary recalls that were entered into Food Processor Structure Query Language (SQL). Food Processor SQL is a nutrient analysis software program that has been used by the APrON study to analyze macro and micronutrient intake of participants. These worksheets are arranged by APrON visit (A, first trimester; B, second trimester; C, third trimester; E, three months postpartum). These worksheets are used to enter data in order to estimate choline intake. **Figure 2** is an exert of a Timepoint worksheet, with participant data that must be entered highlighted in red columns and the estimated choline content for each food item consumed calculated in columns in green.

Figure 2. Timepoint Worksheets: Participant Data (solid) and Choline Content Data (dotted)

O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF
Participant ID	Item Name	Quantity	Measure	Wgt (g)	Time	Day	Assigned NDB	Food Category	Dairy or Eggs	Factor	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline
											(mg/100g of food)	mg choline moiety/100 g of food					
C0001-01-1	BEEF, SIRLOIN, PATTIES	125	Gram	125.0	A	1	23563	Beef Products	0	1.25	9.88	2.75	3.88	0.38	88.13	9.63	104.75
C0001-01-1	BREAD, WHOLE WHEAT, COMMERCIAL, TOAS	2	Slice	50.0	A	1	18065	Baked products	0	0.50	46.20	7.10	1.80	0.15	2.00	0.00	11.05
C0001-01-1	BROCCOLI, RAW, SPEARS	4	Each	124.0	A	1	11090	egetables and vegetable product	0	1.24	0.12	22.44	0.00	0.50	0.12	0.12	23.19
C0001-01-1	BUTTER, REGULAR	1	Teaspoon	4.7	A	1	1001	Dairy and Eggs	Dairy	0.05	0.01	0.02	0.06	0.03	0.52	0.26	0.89

Figure 3. Timepoint Worksheets: Participant Data (solid)

O	P	Q	R	S	T	U	V	W	X	Y
Participant ID	Item Name	Quantity	Measure	Wgt (g)	Time	Day	Assigned NDB	Food Category	Dairy or Eggs	Factor
C0001-01-1	BEEF, SIRLOIN, PATTIES	125	Gram	125.0	A	1	23563	Beef Products	0	1.25
C0001-01-1	BREAD, WHOLE WHEAT, COMMERCIAL, TOAS	2	Slice	50.0	A	1	18065	Baked products	0	0.50
C0001-01-1	BROCCOLI, RAW, SPEARS	4	Each	124.0	A	1	11090	egetables and vegetable product	0	1.24
C0001-01-1	BUTTER, REGULAR	1	Teaspoon	4.7	A	1	1001	Dairy and Eggs	Dairy	0.05

Figure 3 illustrates one section of the worksheet in which data is entered.

Column O, **Participant ID**, corresponds to the identification numbers assigned to participants in the APrON study. "C" corresponds to participants that were recruited in Calgary and "E" indicates participants that were recruited in Edmonton.

Column P, **Item Name**, is the column that food data is entered. In order for the database to calculate choline content of the appropriate food, the wording must correspond to the wording according to Food Processor. For example, in the **Item Name** column "beef, sirloin, patties" is a food that was consumed by participant "C0001-01-1". This wording is the same wording that was used when the original 24-hour dietary recall was entered into Food Processor SQL for nutrient analysis.

Column Q, **Quantity**, refers to the amount of food that was consumed. For example, for participant "C0001-01-1' consumed "2 slices of bread, whole wheat, commercial, toast". The **Quantity** refers to a number value, such as 0.5 for a half, or 500 when the quantity is measured in millilitres.

Column R, **Measure**, is correlated with **Quantity,** which identifies what classification the **Quantity** column was measured in, for example "2 slices of bread, whole wheat, commercial, toast" refers to "2 slices". Options for the user to enter data in the **Measure** column can vary to include, but not limited to, cups, teaspoons, tablespoons, ounces, grams, millilitres, litres, each or slices.

Column S, **Wgt (g)** or weight (grams), lists the weight of the individual foods that have been entered. In Food Processor, this information is automatically generated based on the quantity given so this is easily imported into the worksheet. It is very important that the gram measurement of the food be correct as the weight variable is used to calculate choline content from the foods listed in the **Choline Database** worksheet. For example, for participant

"C0001-01-1", "2 slices of bread, whole wheat, commercial, toast" was consumed, which equates to "50 g".

Column T, **Time**, refers to the time point that the data was collected. For the APrON study, there were several time points representing various stages in pregnancy and post-partum, ranging from A to G. The current data that has been entered includes data from timepoints A-E.

Column U, **Day**, is the day in which the data was collected. For a 24-hour dietary recall, data was only collected on one day, therefore the data entered was "1". For studies that collect data on multiple days, for example a 3-day food journal, more than one number will be listed. This could be useful in sorting the data by days to compare intake and see the variation between days of the same participant.

Column V, **Assigned NDB**, refers to the assigned Nutrient Database (NDB) numbers that have been given to the particular food that was recorded. The NDB number assigned to each food is a five digit numerical code that is used in the USDA Nutrient Database for Standard Reference (SR) (1) and discussed above in the **Choline Database** section. The **Assigned NDB** column is the most important aspect when estimating choline intake as it uses a value-lookup (v-lookup) Excel formula to fill in the choline values for each of the foods entered in the **Item Name** column. The v-lookup formula links the appropriate assigned NDB in the **Choline Database** to the appropriate assigned NDB number in the **Timepoint** worksheets. The choline information from that specific food is then calculated using a conversion factor, which is calculated to fit the quantity of the food recorded.

Column W, **Food Category**, categorizes the food listed in the **Item Name** column into food groups. Options include baked products, fruit and fruit products, vegetables and

vegetable products, fast food, snacks, chicken and turkey etc. This also uses a v-lookup formula to assign the appropriate food group to the assigned NDB number that was given.

Column X, **Dairy or Eggs**, further divides the **Food Category** column, as either "Dairy", "Eggs" or blank. The **Dairy or Eggs** column uses another v-lookup formula to first identify if the food listed in the **Item Name** column falls under the "dairy and eggs" food group, and then identifies if the food is either a dairy product or an egg product.

Column Y, **Factor**, is the conversion factor that was used to convert the choline information from the **Choline Database**. For each food in the **Choline Database** worksheet, the choline content is listed as mg of choline per 100 g of food. The foods listed, in the **Item Name** column on the **Timepoint** worksheets, are consumed in varying amounts; the conversion factor in the **Factor** column converts the choline information from 100 g of food to the amount of food (in grams) listed in the **Item Name** column. For example, participant "C0001-01-1" consumed "beef, sirloin, patties" (**Item Name** column) which was equal to "125 g" (**Wgt (g)** column) therefore the conversion factor was 1.25 (**Factor** column) which means that the choline information will be multiplied by 1.25 to convert the information from mg/100g to mg/125g. The conversion factor is important in translating the choline content information from the choline database to the accurate amount of food that was listed by each participant.

Figure 4 illustrates the second half of the **Timepoints (A-E)** worksheets, which is the choline content data from all of the foods that have been entered in the **Item Name** column.

Figure 4. Timepoint Worksheets: Choline Content Data (dotted)

Z	AA	AB	AC	AD	AE	AF
Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline
(mg/100g of food)	mg choline moiety/100 g of food					
9.88	2.75	3.88	0.38	88.13	9.63	104.75
46.20	7.10	1.80	0.15	2.00	0.00	11.05
0.12	22.44	0.00	0.50	0.12	0.12	23.19
0.01	0.02	0.06	0.03	0.52	0.26	0.89

The food item is entered in the **Item Name** column, in addition to the amount of food in grams, and then the conversion factor in the **Factor** column converts the choline content information from **Choline Database** worksheet to the choline content information appropriate to the foods recorded in the 24-hour dietary recalls in the **Timepoints** worksheets. The choline content that has been estimated for each food recorded in the **Item Name** column is listed in columns Z to AF including **betaine**, free choline (**Free Cho**), glycerophosphocholine (**GPC**), phosphocholine (**Pch**), phosphatidylcholine (**Ptdcho**), sphingomyelin (**SM**) and total choline (**TC**).

c) Choline Summary

Within the **Timepoints** worksheets there is a section (columns AH-AP) that summarizes the choline intake for each participant. **Figure 5** is an excerpt of the summary of choline intake by participant.

Figure 5. Choline Intake by ID

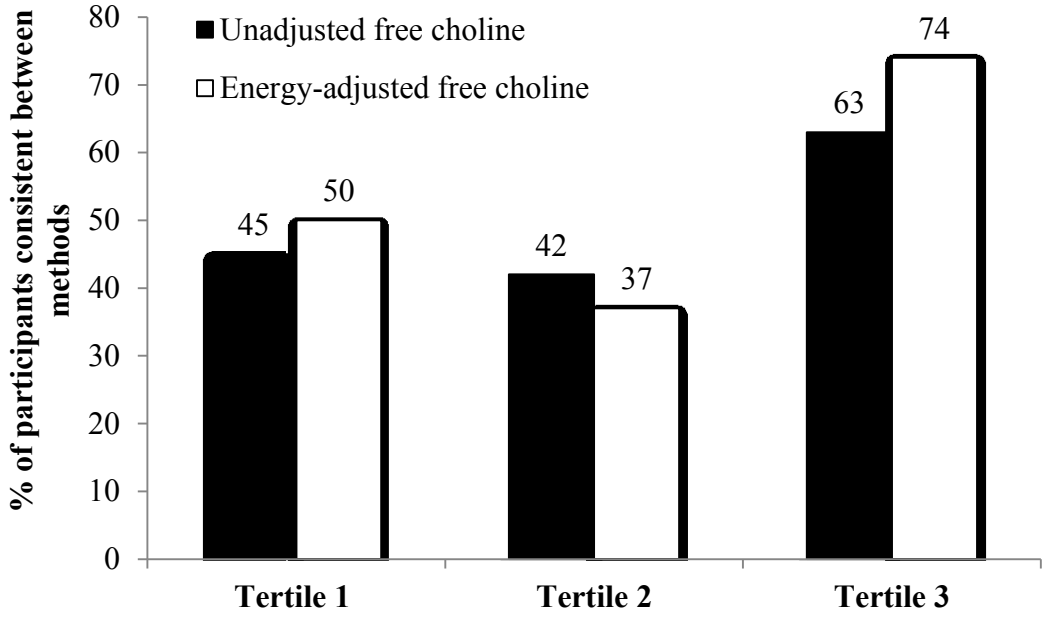
AH	AI	AJ	AK	AL	AM	AN	AO	AP
Particpant ID	Betaine	Free Cho	GPC	Pch	Ptdcho	SM	Total Choline	Meeting AI (<450 mg/day)
	(mg/100g of food)	mg choline moiety/100 g of food						n=989
C0001-01-1	209.55	112.74	91.27	21.77	258.87	28.31	512.95	TRUE
C0002-01-1	170.83	84.12	71.64	21.97	195.09	19.15	391.96	FALSE
C0003-01-1	47.70	75.51	11.03	17.28	57.00	7.01	167.83	FALSE
C0004-01-1	193.82	60.32	50.90	16.73	131.92	18.34	278.21	FALSE

Betaine, all five forms of choline and total choline has been estimated for each participant. Each nutrient total is measured in milligrams. Column AP, **Meeting AI**, lists whether each participant met the Adequate Intake (AI) values for choline in pregnancy (450 mg/d) or lactation (550 mg/d). An Excel formula is used to indicate whether the participant is below the AI (listed as **FALSE**) or above the AI (listed as **TRUE**). This information is particularly useful when describing the average intake of the population of study, and examining whether the population is meeting daily nutrient requirements.

d) Choline Database Substitutions

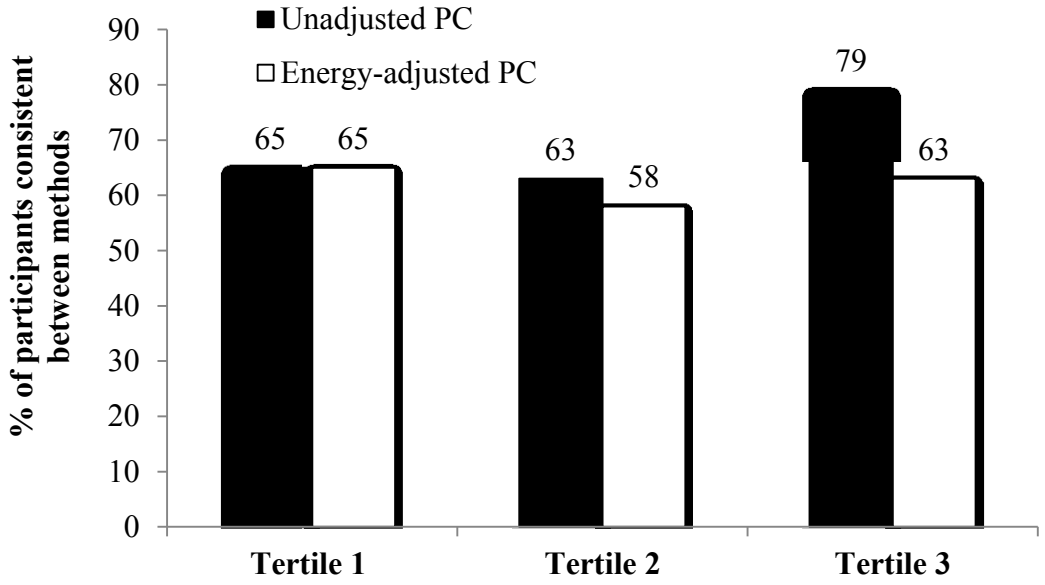
The final worksheet is the **Choline Database Substitutions,** which lists all of the additions to the **Choline Database**. This worksheet serves as a check for any new food items that have been added to the working version of the **Choline Database** and will continue to be updated. The list thus far includes pulses and meat but will be expanded to include milk products and shrimp. The format of this worksheet is the same as the **Choline Database** worksheet.

Appendix 3: Percentage of participants consistently grouped into same tertiles[1] of free choline intake and energy-adjusted free choline intake between interview and online dietary recall methods



[1]Participants were grouped into tertiles based on total choline intake (Tertile 1-Tertile 3). The number of participants who stayed in the same quartile when arranged percent of retention based on method was calculated using the formula: (online method/interview method)*100%.

Appendix 4: Percentage of participants consistently grouped into same tertiles[1] of PC intake and energy-adjusted PC intake between interview and online dietary recall methods



[1]Participants were grouped into tertiles based on total choline intake (Tertile 1-Tertile 3). The number of participants who stayed in the same quartile when arranged percent of retention based on method was calculated using the formula: (online method/interview method)*100%.

Appendix 5: Estimated daily intake of total choline, choline containing moieties and betaine based on dietary recall method during pregnancy and 3-months postpartum in the APrON cohort

	Pregnancy[1]		3-months postpartum	
Dietary recall method	Interview	Online	Interview	Online
n	1032	1093	849	914
Betaine (mg/d)	216 ± 124	235 ± 165	245 ± 209	243 ± 252
Choline-containing moieties			mg choline/d	
Free Choline	82 ± 33	82 ± 34	68 ± 39	84 ± 54
Glycerophosphocholine	67 ± 32	67 ± 33	61 ± 36	61 ± 34
Phosphocholine	17 ± 7	15 ± 6	15 ± 8	14 ± 7
Phosphatidylcholine	166 ± 89	179 ±110	173 ± 130	190 ± 140
Sphingomyelin	20 ± 9	21 ± 11	20 ± 13	22 ± 15
Total Choline	354 ± 127	364 ± 142	351 ± 166	371 ± 184
Meeting AI (%)[2]	20	23	12	15
Energy intake (kcal/d)	2214 ± 531	2162 ± 525	2085 ± 622	2080 ± 615
Energy adjusted choline intake (mg/kcal)	162 ± 52	169 ± 56	173 ± 85	182 ± 85

Abbreviations: APrON, Alberta Pregnancy Outcomes and Nutrition; AI, Adequate Intake

[1]Pregnancy represents mean of three combined 24-h recalls for first, second and third trimester of pregnancy.

[2]Meeting AI is the percent of participants meeting daily recommendations for pregnancy or lactation, which are 450 mg/d and 550 mg/d, respectively.

Appendix 6: Choline intake, infant characteristics and gestational weight gain of women consuming below recommendations in the APrON study, stratified into tertiles[1] of energy-adjusted total choline intake[2]

	Tertile 1	Tertile 2	Tertile 3
n	707	707	707
Energy adjusted total choline intake (mg/1000 kcal)	115 ± 18	158 ± 11	225 ± 48
Infant birth weight (g)	3331 ± 523	3351 ± 555	3356 ± 531
Gestational age (weeks)	38.9 ± 2.0	39.1 ± 1.9	39.1 ± 1.9
Maternal gestational weight gain (kg)	14.9 ± 5.7	15.3 ± 5.9	15.2 ± 5.9

Abbreviations: APrON, Alberta Pregnancy Outcomes and Nutrition

[1]Participants were grouped into tertiles based on energy-adjusted total choline intake (Tertile 1-Tertile 3).

[2]ANCOVA was used to assess differences in infant birth weight between first and third tertiles of total choline intake, adjusting for covariates gestational weight gain and gestational age.

Appendix 7: Correlation analysis between the number of specific cell types placed in culture and e*x vivo* IL-2 production following ConA stimulation

	Correlation between IL-2 production and lymphocyte subset (pg/x10^4 cells[1])					
	All groups		ChS		ChD	
	r_s^2	P-value	r_s^2	P-value	r_s^2	P-value
CD4+CD25+	-0.007	0.982	-0.317	0.406	0.600	0.285
CD8+CD25+	0.187	0.522	0.333	0.381	0.300	0.624
CD4+CD71+	0.433	0.122	0.600	0.088	0.600	0.285
CD8+CD71+	-0.108	0.714	-0.183	0.637	-0.100	0.873

[1]The total number of immune cells was calculated by multiplying the % of immune cell phenotype in the spleen by the total number of splenocytes isolated (x10^6).

[2]Spearman's correlation coefficient used to assess correlation between lymphocyte population and IL-2 production.

* indicates mean within a row that is significantly different from ChS group ($P<0.05$).

Appendix 8: *Ex vivo* IL-2 production by the number of specific cell types placed in culture following ConA stimulation

	ChS		ChD	
n[1]	10		7	
	Mean	SEM	Mean	SEM
	IL-2 production (pg/x10^4 cells of lymphocyte subset[2])			
CD4+CD25+	1043	154	702*	70
CD8+CD25+	1113	160	603	95
CD4+CD71+	542	44	362	38
CD8+CD71+	407	62	197	26
CD4+CD25+ and CD8+CD25+	523	74	323*	41
CD4+CD71+ and CD8+CD71+	228	26	127	15

[1]n refers to the number of dams as they are the experimental unit. This includes two offspring pooled to obtain a measure for each dam, with measurements from each offspring conducted in duplicate

[2]To express cytokine response by specific lymphocyte population, the % of lymphocyte population was multiplied by $1.25x10^6$ (number of total cells added to culture). Then IL-2 production (pg/ml) was divided by the number of lymphocytes added to culture to express the amount of IL-2 in the media per a specific lymphocyte subset

* indicates mean within a row that is significantly different from ChS group ($P<0.05$)