# METABOLIC INTERACTIONS AMONG AMINO ACIDS, PHOSPHOLIPIDS AND FATTY ACIDS

by

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

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

September 2013

**Abstract**

Cystic fibrosis (CF) is the most common life-shortening disorder among Caucasians. Excessive faecal bile acid loss, increased oxidant stress, reduced plasma choline, increased oxidant stress, reduced glutathione and alterations in essential fatty acids are well recognized in patients with CF. It is also well-known that diabetes perturbs the methionine-homocysteine cycle. However, experimental data linking loss of amino acids in CF or decreased glucose availability in experimental diabetes to altered phospholipids and fatty acid metabolism are lacking. In the liver, bile acids are conjugated with glycine or taurine prior to secretion, and glycine *de novo* synthesis begins with glucose. Thus, the objectives of this thesis are: 1) to determine if inducing faecal bile acid loss will alter the methionine-homocysteine, and choline-betaine cycle metabolites, phospholipids and phospholipids n-6 and n-3 fatty acids, and 2) to show that experimental diabetes, which decreases glucose availability, alters methionine-homocysteine and choline-betaine cycle metabolites, phospholipids and phospholipid fatty acids in rats.

Studies to address the first objective demonstrated that inducing faecal bile acid malabsorption leads to fat malabsorption with increased faecal total lipids and phospholipid excretion. This increased excretion was accompanied by increased plasma betaine concentration, decreased plasma triacylglycerol concentration, increased plasma and liver S-adenosylhomocysteine (SAH) concentration, and changes in the fatty acid composition of hepatic phospholipids. Studies to address the second objective showed that experimental diabetes led to increased plasma betaine concentration,

decreased homocysteine concentration, increased liver phosphatidylethanolamine, decreased phosphatidylcholine, changes in the fatty acid composition of hepatic phospholipids, and abundance of the enzyme choline dehydrogenase. Thus, experimental diabetes, which reduces intracellular glucose availability, alters methionine-homocysteine and choline-betaine cycle metabolites, phospholipids and fatty acids. In conclusion, metabolism of phospholipids, their fatty acids, and the amino acids involved in the methionine-homocysteine cycle are inter-related.

**Preface**

This thesis contains the work of a research study for a master degree and was prepared in accordance to the University of British Columbia and The Faculty of Graduate and Postdoctoral Studies requirements. I was responsible for conducting the experiments and preparing the thesis which was accomplished with the assistance and guidance of my supervisor Dr. Sheila M. Innis. HPLC analysis of amino acids was done by Dr.Bernd Keller and Dr. Cyrielle Garcia. I would like to thank Roger Dyer, and Janette King for their wide range of help throughout. Ethics approval to conduct this research was obtained by the University of British Columbia Animal Care Committee; certificate number A10-0141 for studies in Chapter 2 and A09-0911 for studies in Chapter 3.

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**List of abbreviations**

BHMT: betaine-homocysteine methyltransferase

CDP-choline: cytidine diphosphosphate choline

CF: Cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CHDH: choline dehydrogenase

CTP: cytidine triphosphate

CY: cholestyramine

DHA: docosahexaenoic acid, 22:6n-3

EDTA: ethylenediaminetetraacetic acid

HFBA: heptafluorobutyric acid

GCC: Glycine cleavage complex

GC: gas chromatography

GNMT: Glycine N-methyltransferase

GSH: glutathione

LDL: Low-density lipoprotein

LPC: lysophosphatidylcholine

Hcy: homocysteine

HDL: high-density lipoprotein

HPLC: high performance liquid chromatography

kg: kilogram

kcal: kilocalorie

5,10-CH2-THF: 5,10-methylenetetrahydrofolate

MTHFR: methylenetetrahydrofolate reductase

mg: milligram

min: minute

mRNA: messenger ribonucleic acid

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PEMT: Phosphatidylethanolamine N-methyltransferase

PERT: Pancreatic enzyme replacement therapy

PI: phosphatidylinositol

PS: phosphatidylserine

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SD: standard deviation

SHMT: serine hydroxymethyltransferase

SPSS: Statistical Package for Social Sciences

STZ: streptozotocin

SPH: sphingomyelin

THF: tetrahydrofolate

TG: triacylglycerol

QTOF-MS: Quadrupole Time-of-Flight Tandem Mass Spectrometer

Rpm: revolutions per minute

# Chapter 1 Literature review, hypothesis, and objective

## 1.1 Introduction

This thesis research addresses the interaction between phospholipids, essential fatty acids, the methionine-homocysteine cycle, and glycine metabolism. Previous studies in our laboratory have shown that children with cystic fibrosis (CF) have reduced plasma choline and altered n-6 and n-3 fatty acids, with decreased docosahexaenoic acid (DHA, 22:6n-3) [1]. The reasons for these alterations are not known, but may include a combination of dietary factors and effects secondary to the disease. However, it is known that children with CF malabsorb bile acids, and that bile acids are conjugated with the amino acids glycine and taurine in the liver [2]. Other recent studies have shown that dietary n-3 fatty acid deficiency leads to decreased protein abundance and gene expression of hepatic cytosolic serine hydroxymethyltransferase (SHMT), the reversible enzyme that catalyzes conversion of serine to glycine [3]. It has been speculated that a link exists among n-6 and n-3 fatty acids, methyl metabolism, and pathways related to oxidative stress and inflammation. However, biochemical evidence for this speculation is still missing [4, 5]. Phosphatidylethanolamine (PE) is methylated to form phosphatidylcholine (PC) using methyl groups from methionine via *S*-adenosylmethionine (SAM) [6]. Studies by others have shown that synthesis of PC from PE leads to higher plasma phospholipid DHA than when PC is formed from diglyceride with CDP- choline via the CDP-pathway [7]. These studies suggest that the

metabolism of phospholipids and their fatty acids are inter-related with the methionine-homocysteine cycle. Serine and glycine are also inter-related with the methionine-homocysteine cycle, due to the major role of glycine as a source of methyl units to regenerate methionine from homocysteine [8]. This research seeks to use two approaches to determine a metabolic inter-connection among amino acids (related to the methionine-homocysteine cycle), phospholipids and their fatty acids.

## 1.2 Literature review

### 1.2.1 Methionine metabolism

Methionine is an essential amino acid that contains a methyl group ($CH_3$) and sulphur. In addition to protein synthesis, methionine plays important roles that include methyl transfer (Figure 1). The transfer of methyl groups from methionine first involves synthesis of SAM [9]. SAM donates $CH_3$ to methyl acceptors, for which over 100 different methyl donation reactions have been detected [85]. Among these, the methylation of glycine by glycine N-methyltransferase (GNMT), which leads to synthesis of N-methylglycine [10], and methylation of PE by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT), which leads to synthesis PC, are major uses of SAM [11]. PEMT activity has been estimated to account for about 30% of PC synthesis in the liver [12]. GNMT is also a major enzyme using SAM, and as noted above, this process leads to synthesis of N-methylglycine from glycine and SAM [13]. Transfer of $CH_3$ from SAM to

a methyl acceptor also generates SAH, which is then further metabolized to homocysteine (Hcy) by the enzyme SAH hydrolase. Hcy can then be remethylated to regenerate methionine, or it can irreversibly enter the trans-sulfuration pathway leading to cysteine, which requires the initial action of cystathionine β-synthase (CBS) [14]. Cysteine together with the amino acids glycine and glutamate are used to synthesize the tripeptide glutathione (GSH). GSH plays a central role in lots of processes, including protection against oxidative stress, regulation of protein and DNA synthesis, inflammatory responses and generation of nitric oxide [15]. Cysteine is also the precursor of coenzyme A and taurine (Figure 1).

Choline is an essential dietary nutrient and also functions as an important nutrient in methyl metabolism, in addition to its role as a component of PC and sphingomyelin (SPH). Choline can serve as a source of methyl groups for remethylation of homocysteine to methionine, and for the one-carbon folate pool. Choline is converted to betaine by the enzyme choline dehydrogenase (CHDH), then serves as a folate-independent source of methyl groups for remethylation of homocysteine to methionine, by the enzyme betaine-homocysteine methyltransferase (BHMT) [16] (Figure 1). BHMT is found mainly in the liver and kidney, and represents 0.5–1.6% of the total soluble protein in the mammalian liver [17].

### 1.2.2 Glycine metabolism

Glycine plays an important role in many metabolic processes, including conjugation of bile acids, synthesis of purines, porphyrins and creatine, in addition to GSH [18]. Glycine is considered as a "non-essential amino acid" because it can be synthesized in the body from serine. Glycine synthesis begins with glucose, which is further metabolized to 3-phosphoglycerate, and then oxidized by phosphoglycerate dehydrogenase to yield 3-phosphohydroxypyruvate [19]. Next, 3-phosphohydroxypyruvate is transaminated with glutamate to generate 3-phosphoserine and α-ketoglutarate, with 3-phosphoserine further metabolized to serine [19]. Glycine can then be synthesized from serine by the enzyme serine hydroxymethyltransferase (SHMT), and this process is a reversible enzymatic pathway [20]. SHMT is present in the cytosol and mitochondria. The SHMT reaction involves the transfer of the hydroxymethyl group from serine to the cofactor tetrahydrofolate (THF), producing 5,10-methylenetetrahydrofolate ($5,10\text{-}CH_2\text{-}THF$) and glycine. After that, 5,10-methylene-THF can be irreversibly reduced by methylenetetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrofolate ($5\text{-}CH_3\text{-}THF$): the primary methyl donor for remethylation of homocysteine to methionine [21].

Glycine synthesized from serine or provided in the diet can also be oxidized by glycine decarboxylase (also known as the glycine cleavage complex, GCC) to yield 5,10-methylene-THF as well as $NH_4^+$ and $CO_2$. The glycine cleavage complex (GCC), a four-protein complex composed of P protein, H protein, L protein, and T protein, is

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present only in mitochondria [18].

### 1.2.3 Phospholipid metabolism

The metabolism of the phospholipids is interrelated with the methionine homocysteine cycle through PEMT, which synthesizes PC from PE using methyl groups from SAM. Phosphatidylcholine is the most abundant phospholipid in animals and is critical for lipid absorption and transport, cell membrane structures, cell signaling, and synthesis of lipoproteins [22].

In addition to *de novo* synthesis via the PEMT pathway, PC can also be synthesized by the cytidine diphosphocholine (CDP) pathway that requires choline (Figure 1). Choline is considered to be an essential dietary nutrient. Current evidence indicates that the ability for endogenous choline synthesis via the PEMT pathway is insufficient to meet the body's needs for choline [23]. Once taken into cells, choline can be phosphorylated by choline kinase to phosphocholine, which reacts with cytidine triphosphate (CTP) to generate cytidine diphosphocholine, which then is used in phosphatidylcholine synthesis [24] (Figure 1).

As discussed, choline also serves to provide methyl groups via its role as a precursor of betaine. Transfer of a methyl group from betaine to homocysteine regenerates methionine, with dimethylglycine as the other product. Dimethylglycine can further transfer two methyl groups to the mitochondrial folate pool, thereby generating glycine

[18] (Figure 1).

### 1.2.4 Integration of phospholipid, fatty acid and amino acid metabolism

Phospholipids, specifically PC and PE, and glycine interact with the methionine-homocysteine cycle through their interrelationships in methyl groups alteration and methyl transfers (Figure 1). Briefly, glycine can serve as a substrate for methyl acceptance, leading to synthesis of SAH, and hence generate homocysteine, a precursor of cysteine. However, dimethylglycine can also be formed when betaine is used to remethylate homocysteine to methionine. Glycine is also a major source of methyl groups for the carbon folate pool [25]. Endogenous synthesis of glycine from serine requires glucose, which plays a key role in providing carbons for synthesis of non-essential amino acids. A decreased availability of glucose in cells can occur in diabetes due to insulin deficiency, or insulin resistance [26]. The effects of perturbation of glycine and glucose availability on the betaine-homocysteine cycle, choline, phospholipids, and phospholipid fatty acids are not well understood.

### 1.2.5 Evidence for a potential link between glycine, phospholipids and the methionine-homocysteine cycle

Glycine can accept the methyl group from SAM to form N-methylglycine in a reaction catalyzed by glycine N-methyltransferase (GNMT) [10]. PE can accept methyl groups

from SAM to form PC. Plasma homocysteine concentrations were significantly lower in PEMT -/-mice than in wild type mice, while plasma N-methylglycine concentrations were elevated [27]. PEMT-/- mice also showed a significant decrease in DHA species in plasma and liver PC than the wild-type mice [28], which is consistent with evidence that PC with higher amounts of long chain polyunsaturated fatty acids may be generated through the PEMT pathway (when compared to the PC synthesized via CDP-choline pathway) [7]. In GNMT -/- mice, there was a marked increase in plasma SAM and methionine [29]. These studies point to the role of GNMT and PEMT in normal methionine-homocysteine metabolism, showing that the lack of either of these enzymes results in decreased methionine conversion to homocysteine. Because homocysteine is the precursor of cysteine, this decrease may suggest that cysteine would also be decreased. Like PC synthesis, the methylation of glycine may contribute substantially to the transmethylation flux [30]. Addition of 3% glycine to a 10% casein diet, however, had no effect on hepatic SAM or PC/PE, but led to decreased 20:4n6/18:2n6 ratio and increased delta 6-desaturase activity (when compared to the 10% casein diet group) [31].

Type I Diabetes is characterized by decreased glycine *de novo* synthesis in the hyperglycemic state [32]. GSH deficiency and decreased plasma glycine level have also been found in type 2 diabetic patients [33]. Adequate provision of glycine is important for optimal GSH synthesis [34]. Sekhar *et al.* [33] found that after receiving glycine and cysteine supplementation, type-2-diabetic patients had significantly increased

concentrations of GSH, with decreased plasma biomarker of oxidative stress and lipid peroxides. The likelihood that decreased glucose availability for intracellular glycine synthesis contributes to the diabetic pathology is an attractive hypothesis.

Glycine has been reported to decrease the plasma concentration of glucose, triacylglycerols (TG) and total cholesterol (TC) in streptozotocin (STZ) -induced diabetic rats [35]. Those changes could be due to 7α-hydroxylase activation that participates in the synthesis of biliary acids [35,36]. Some evidence suggests that glycine administration can increase insulin secretion in humans has been reported [37], although little has been done in this field .


### 1.2.6 Methyl-groups and phospholipids in cystic fibrosis

In children with CF, as in adults, malabsorption of bile acids occurs despite the use of pancreatic enzyme replacement therapy [38]. In the liver, bile acids are conjugated with glycine or taurine prior to secretion. It is also known that children with CF have significantly reduced plasma choline concentrations despite normal or even high choline intakes, and that plasma n-3 fatty acids, particularly plasma DHA levels are reduced [1]. The reasons for the low plasma choline and DHA in CF are not known. However, the increased loss of glycine and/or taurine due to chronic bile acid malabsoption could be a common linkage. Notably, CF also involves a chronic inflammatory state with lower GSH [39]. Experimental data to link loss of amino acids, or decreased intracellular capacity for

glycine synthesis, to alteration in methionine-homocysteine cycle, phospholipid, fatty acids metabolism are still lacking.

### 1.2.7 Bile acids and cholestyramine

Bile acids are used to facilitate the formation of micelles, which break down large fat globules into small fat droplets in the intestine. Bile acids are synthesized in hepatocytes from cholesterol and conjugated with glycine or taurine prior to being released to the intestine [40]. During the interdigestive period, bile acids are transported to the gallbladder and then to the duodenum. In healthy individuals, more than 90% of bile acids secreted into the intestine are reabsorbed by active transport in the ileum, and subsequently returned to the liver [41]. Cholestyramine, which is a bile acid sequestrant, binds bile acids in the intestine and prevents their reabsorption [42]. This molecule results in increased demand for cholesterol conversion to bile acids to maintain the bile acid pool. At the same time, the need of glycine and taurine for bile acid conjugation should also increase.

Bile acid glycine-conjugates generally predominate in healthy adults [43]. Robb *et al.* documented significantly raised glycine/taurine bile acid conjugation ratio in duodenal juice from CF patients, with increased glycine bile acid secretion [44]. Rats conjugate bile acid with glycine as the major amino acids, when availability of intracellular taurine is limited [45]. Ide *et al.* demonstrated that dietary protein sources low in sulphur amino acids lead to a large increase in the bile acid glycine/taurine ratio (in biliary bile acids)

and a decrease in hepatic taurine concentrations [46]. Thus, dietary protein composition, specifically the availability of sulphur amino acids, impacts bile acid conjugation [47]. A casein-based diet without supplemental methionine or choline will be used and combined with cholestyramine to increase malabsorption of bile acids and their amino acid conjugates. The same diet with cholestyramine will be fed with the addition of methionine and choline. This design will therefore test if additional methionine (as a source of sulphur) or choline (as a source of methyls) can overcome any perturbation of the methionine-homocysteine cycle, or choline-betaine cycle, which results from the bile acid sequestrant.

## 1.3 Research rationale and objectives

### 1.3.1 Rationale and objectives for Chapter 2

**Rationale:**

Cystic fibrosis is caused by a mutation of the CF transmembrane conductance regulator (CFTR) and is the most common life-shortening inherited disorder among Caucasians. Chronic bile acid malabsorption, excessive faecal bile acid loss, increased proinflammatory mediators and oxidant stress, and reduced GSH, with alterations in n-6 and n-3 fatty acid compositions, are well recognized in patients with CF [1]. It is also known that children with CF have significantly reduced plasma choline concentrations, despite normal or even high choline intakes, and that plasma n-3 fatty acids, particularly

DHA levels are reduced [1]. The reasons for the low plasma choline and DHA in CF are not known. Since glycine serves as a precursor of GSH and is also a methyl group acceptor and donor in methionine-homocysteine cycle [14], one possible explanation could be the increased loss of glycine (due to chronic bile acid malabsorption in CF). Chronic loss of taurine, which is synthesized from cysteine, could also result in a net drain of methionine, possibly influencing the capacity for PC synthesis via PEMT. Since DHA composition in liver PE is higher than liver PC, PC generated through PE and PEMT pathway would contain higher DHA level than the PC synthesized via CDP-choline pathway. This fact might explain the decreased plasma phospholipids DHA also described in CF. An increased use of choline via betaine for remethylation of homocysteine (due to limited glycine) could also contribute to the low plasma choline in CF. Thus, the purpose of the first part of this research is to use an experimental model to examine whether bile acid malabsorption increases fat and phospholipid malabsorption, and leads to alterations in hepatic and plasma methionine-homocysteine, choline-betaine pathway metabolites, and PC, PE and phospholipid fatty acids.

**Objective:**

To determine if inducing faecal bile acid loss will 1) increase fat and phospholipids malabsorption and 2) alter methionine-homocysteine, choline-betaine cycle metabolites, phospholipids, and phospholipids n-6 and n-3 fatty acids in rat liver and plasma.

**Null hypothesis:**

Inducing bile acid malabsorption will not alter faecal fat and phospholipid excretion, and will not alter methionine-homocysteine, choline-betaine cycle metabolites, phospholipids, or phospholipids n-6 and n-3 fatty acids in rat liver and plasma.

## 1.3.2 Rationale and objectives for Chapter 3

**Rationale:**

Emerging evidence shows that diabetes and insulin perturb the methionine -homocysteine cycle and elevate cellular oxidative stress. GSH deficiency and decreased glycine level have also been found in type 2 diabetes [33]. Type I Diabetes is characterized by decreased glycine *de novo* synthesis in the hyperglycemic state [32]. Glycine *de novo* synthesis begins with glucose, which is metabolized to serine and then to glycine. It is unclear whether decreased glycine availability alters the choline-betaine cycle metabolites, phospholipids and phospholipid n-6 and n-3 fatty acids or not. Thus, the purpose of the second part of this research is to use an experimental diabetes model to determine whether potential metabolism in glycine, without fat and phospholipid malabsorption, alters methionine-homocysteine cycle, the choline-betaine cycle, phospholipids and their fatty acids in the rat.

**Objectives:**

To determine if alteration in methionine-homocysteine and choline-betaine cycle metabolites, phospholipids, and phospholipids n-6 and n-3 fatty acids occurs (in the absence of fat and phospholipids malabsorption) in an experimental model of diabetes that will decrease cellular glucose.

**Null hypothesis:**

Experimential diabetes will have no effect on the methionine-homocysteine and choline-betaine cycle metabolites, phospholipids, and phospholipids n-6 and n-3 fatty acids.

**1.4 Specific aims of the thesis**

This research uses two different approaches: 1) bile acid malabsorption (Chapter 2) and 2) decreased glucose availability secondary to experimental diabetes (Chapter 3) to determine a functional dependence of the methionine-homocysteine with the choline-betaine cycle metabolites, phospholipids, and phospholipids n-6 and n-3 fatty acids (using the rat as the experimental model).
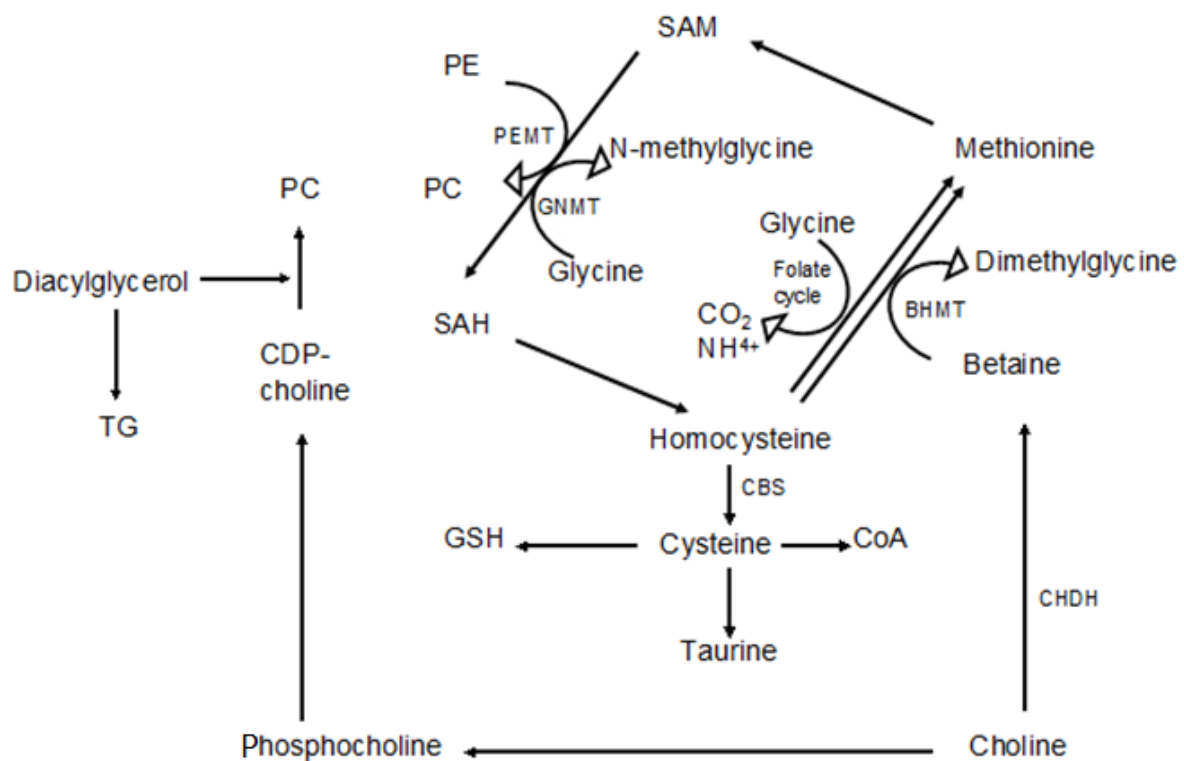
Figure 1. Schematic of methionine metabolism.

BHMT, betaine-homocysteine methyltransferase; CoA, Coenzyme A; GNMT, glycine N-methyltransferase; GSH, Glutathione; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PC, phosphatidylcholine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; CBS, cystathionine β-synthase; CHDH, choline dehydrogenase; CDP-choline, cytidine diphosphate-choline; TG, triglyeride.
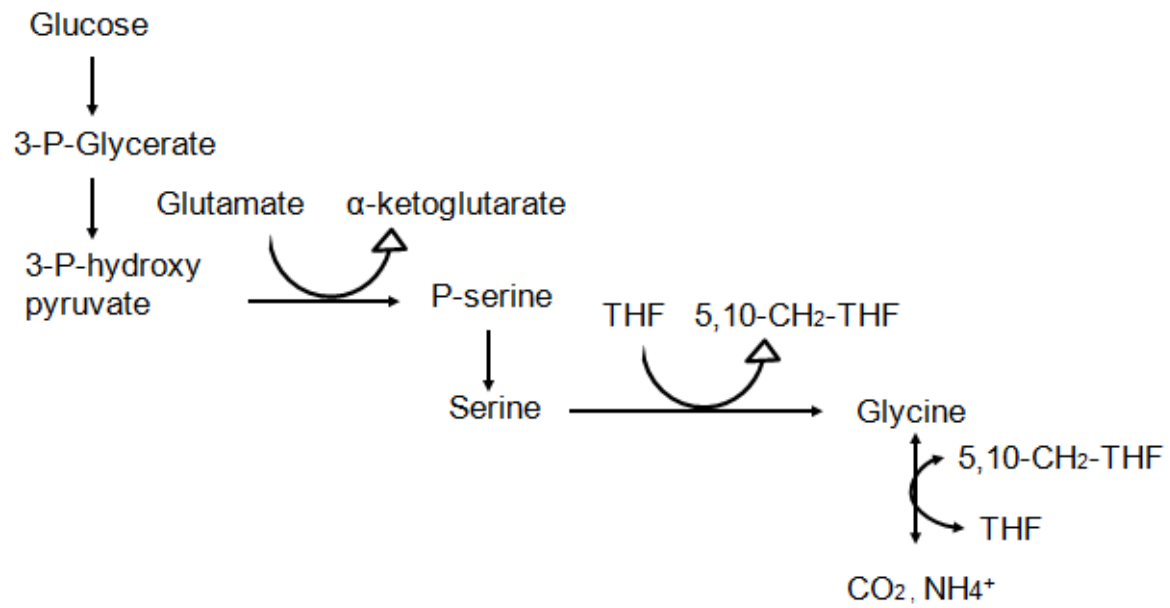
Figure 2. Schematic of glycine metabolism.

3-P Glycerate, 3-phosphoglycerate; 3-P-hydroxypyruvate, 3-phospho -hydroxypyruvate;

5,10-CH2-THF, 5,10-methylenetetrahydrofolate; THF, tetrahydrofolate

**CHAPTER 2: Inducing faecal bile acid loss leads to fat-malabsorption and increased plasma betaine and SAH concentrations, and altered the fatty acid composition of liver phospholipids**

**2.1 Introduction**

Cystic fibrosis is the result of mutations in the CF transmembrane conductance regulator (CFTR), a protein that spans the plasma membrane of epithelial cells and some intracellular membranes [49]. CFTR mutations lead to abnormal transport of chloride (and consequently water and other ions) across the plasma membrane, resulting in thick mucus secretions in the pancreas, lungs, intestine, and reproductive tract. The most common clinical manifestations in CF are chronic pulmonary disease and pancreatic insufficiency [50]. Pancreatic insufficiency results in poor digestion, poor absorption of fat and fat-soluble vitamins, and loss of bile and bile acids [51]. Pancreatic enzyme replacement therapy (PERT) is a standard of care that improves, but does not completely reverse, nutrient maldigestion and malabsorption [52]. In children with CF, as in adults, malabsorption of bile acids occurs despite the use of PERT [38]. The most widely accepted view suggests that undigested dietary nutrients bind bile acids and then prevent normal absorption. In the liver, bile acids are conjugated with glycine or taurine prior to secretion. Glycine also serves as a precursor of GSH and a substrate for methyl acceptance and donation. Taurine is a sulfur amino acid synthesized from cysteine, which is formed from homocysteine.

Evidence is now accumulating to suggest that oxidative stress and abnormalities in phospholipids and fatty acid metabolism could be important factors in contributing to disease manifestations in individuals with CF [49]. It is also now known that children with CF have significantly reduced plasma choline concentration (despite normal or even high choline intakes), and that plasma n-3 fatty acids, particularly DHA levels, are reduced [1]. In addition, reduced plasma PC/PE, SAM/SAH ratios and decreased plasma methionine occur in individuals with CF [52,53,54]. Notably, cystic fibrosis also involves a chronic inflammatory state with lower GSH [39].

The reasons for the low plasma choline and phospholipids DHA in CF are not well understood. However, a common linkage could be the increased loss of glycine and taurine due to chronic bile acid malabsorption. Taurine loss could result in a net drain of methionine and might affect the PC synthesis via PEMT. Since PC generated through PE and PEMT pathway contains higher DHA level than the PC synthesized via CDP-choline pathway, this might explain the decreased CF plasma phospholipids DHA. An increased use of choline via betaine for remethylation could also lead to low plasma choline in CF. Experimental data linking bile acid malabsorption to altered phospholipid and fatty acid metabolism is lacking.

## 2.2 Experimental procedures

### 2.2.1 Animals and diets

Twenty four male Sprague Dawley rats (Charles River Laboratories), 8 weeks old with a body weight of 200-250g, were housed under constant humidity and temperature conditions with a 12-hours light-dark cycle. The rats were divided into three groups, and stratified by body weight to ensure that the body weights were similar in the groups. The three dietary groups were: 1) a semi-synthetic diet without additional methionine and choline, 2) the same diet with cholestyramine at 5% by weight of the diet, and 3) the same diet with 5% by weight cholestyramine, plus supplemental methionine and choline (Table 1). The small amount of sucrose in the cholestyramine powder was not balanced by addition of extra sucrose in diet. Body weight was measured every 2 days.

### 2.2.2 Tissue collection

After one week adaptation period and 4 weeks on the diet, rats were anesthetized (ketamine hydrochloride, 37.5 mg/kg, MTC Pharmaceuticals, Cambridge, Canada; and xylazine hydrochloride, 3.75 mg/kg, Bayvet Division, Chenango, Etobicoke, Canada). Blood samples were obtained from the aorta with a butterfly needle attached to a syringe. This needle, tube and syringe were pre-rinsed with 15% ethylene diamine tetra acetic acid (EDTA) several times before blood is drawn, to prevent clotting. Plasma and erythrocytes were separated by centrifugation at 2000g for 15 min at 4°C, and the

plasma was stored at -80°C until analyses. Then the animals were killed by a rodent-sized guillotine and organs were harvested. The liver were removed, weighed, flash-frozen in liquid nitrogen, and stored at -80°C for subsequent biochemical analyses. Due to the instability of SAM and SAH, small liver samples were homogenized immediately on collection in 10 volumes of 10% perchloric acid, then snap frozen in liquid nitrogen.

All procedures and protocols involving the animals were approved by the University of British Columbia Animal Care Committee and conformed to the guidelines of the Canadian Council of Animal Care.

### 2.2.3 Protein assay

Tissue homogenates were prepared as 1:200 dilutions with double distilled water. The 1:5 dilution of protein assay reagent (BioRad, Canada) was prepared with double distilled water and filtered using a Whatman filter paper (Grade GF/C 1.0 um). 1 mg/mL bovine serum albumin (BSA) standards and sample aliquots of 10 µL were added to each well of a 96-well plate (Immulon-1B, VWR 62402-947) then 200µL of 1:5 protein assay reagent was added to each well using the multichannel pipettor (30-300 µL, Rainin, Canada). The plate was lightly shaken and the reaction was left at room temperature for at least 10 min, and then the absorbance read at 595 nm. All analyses were done in duplicates or triplicates.

**2.2.4 Plasma triacylglycerols and cholesterol**

The triacylglycerol and cholesterol concentrations were determined using commercial reagents from BioPacific Diagnostics in 96 well plates with level 1 (QC 641) and level 2 (QC 642) BioRad Liquichek lipid control standards. Briefly, 5 µL of each sample and 5 µL of water were added to the wells of the plate. 250 µL of triacylglycerols or cholesterol colour reagent (Genzyme Triacylglycerol-SL, BioPacific Diagnotic Inc, Canada), was then added. After 10 min, the plate was read on the microplate reader (Victor, Perkin Elmer, Canada) using the assay program with 505/515 nm filter.

**2.2.5 Extraction of liver and plasma lipids**

Liver samples were homogenized in 18 volumes of saline (0.9% sodium chloride and 0.114% EDTA), then total lipids were extracted. 1 mL liver sample, depending on the assay to be done, 1.25 mL saline, 3 mL methanol and 6 mL chloroform were added and the samples vortexed briefly. Samples were centrifuged at 2000 rpm for 5 min at 4°C, and the lower layer recovered. Next, 6 mL chloroform was used to re-extract the aqueous layer. The samples were centrifuged again for 5 min at 4°C, the lower layer recovered, combined with the first extract, and dried under nitrogen [55]. Then the lipid classes were separated using high performance liquid chromatography as described below.

## 2.2.6 High performance liquid chromatography (HPLC)

Liver and plasma lipid classes, including cholesterol ester (CE), TG, PC, PE, phosphatidylinositol (PI), sphingomyelin (SPH) and lysophosphatidylcholine (LPC), were separated using a high performance liquid chromatography (Waters 2690 Alliance HPLC, Milford MA), equipped with an auto-sampler, evaporative light scattering detector (Alltech, model 2000; Mandel Scientific, Guelph, Canada). This study used a quaternary solvent system of hexane, methanol, acetone-triethylamine 1000/17 (v/v), isopropanol-acetic acid 800:40 (v/v) in a linear gradient with a flow rate of 2 mL/min [54,56]. The column used for separating lipid classes was a Waters YMC-Pack Diol120NP, 25 cm $\times$ 4.6 mm, 5 µm particle size and 12 nm pore size. The separated lipids were recovered with the use of a fraction collector (Gilson FC204, Mandel Scientific).

## 2.2.7 Gas liquid chromatography analysis of fatty acids

For the preparation of methyl esters, 1mL boron trifluoride (BF3) was added to the sample fraction, and incubated at 105°C for 10 min. Then 3 mL saline and 6 mL hexane were added to the test tube and the tube was vortexed for 15 seconds. The samples were centrifuged at 2500 rpm for 10 min at room temperature, then the top (hexane) layer was taken off and transferred to a clean test tube. The lower layer was re-extracted by adding another 3 mL of hexane to the original test tube, vortexing and centrifuging for 10 min at 2500 rpm. The top layer was recovered and combined with the first extraction

and dried under nitrogen. The separated methyl esters were recovered for analysis for fatty acids by Varian 6850 gas liquid chromatography (Agilent Technologies, US) equipped with a 100 m $\times$ 0.25 mm nonbonded fused silica SP2560 column (Supelco, Bellefonte, PA). The oven temperature began at 80°C for 2 min and then increased to 150°C by 10°C/min. After 12.5 min, the temperature increased to 180°C by 2.5°C/min. After 2 min, the temperature was increased to 245°C, which was maintained for 25 min. The injector and detector were kept at 250°C [57].

### 2.2.8 HPLC analysis of amino acids

Initial sample preparation of plasma samples involved de-proteinization via filtration through a 3 kDa molecular weight cut-off centrifugal filter (14,000 g, 40 min). The de-proteinized sample (filtrate) and protein fraction (concentrate) were then stored at -80°C until analyses. Tissue samples were first homogenized using a polytron homogenizer (100 mg liver tissue in 0.5mL distilled deionized water with stable isotope labeled internal standards, and 4% sulfosalicylic acid). Homogenization was followed by centrifugation (13,500 rpm, 15 min). A maximum of 0.5 mL supernatant was then filtered through a 3 kDa molecular weight cut off centrifugal filter (14,000 rpm, 15 min). The de-proteinized samples were then diluted five times in 0.001N HCl before derivatization and analyses. The dilute sample was derivatized by mixing with derivatization reagent (0.5 mL orthophthaldialdehyde + 2 μL mercaptopropionic acid). The derivatized sample was then loaded into an HPLC (Waters 2695 Separation Module). Before the analysis of

each sample, the column was pre-flushed for 15 min with 100% buffer C (50% methanol, 50% acetonitrile) at a flow rate of 0.45mL/min, followed by 10 min of 100% buffer B (50% 20 mM ammonium acetate, pH 7.5, 25% methanol, 25% acetonitrile), and lastly 10 min of 98% buffer A (10mM ammonium acetate, pH 7.5) and 2% buffer B. The sample injection volume was 10 µL and the column was a Zorbax Eclipse AAA reversed-phase separation column (3.5 µm particle size, 2.1 mm x 150 mm). Elution of primary amines from the column occurred through the use of a combination of buffers A and B to create an ammonium acetate buffered gradient by gradually increasing the percent B over time up to 100% B, then returning to the initial conditions (98% A, 2% B). The effluent from the column was passed through a Waters 474 scanning fluorescence detector (excitation wavelength: 330 nm, measured emission bandwidth: 465 nm +/- 10 nm) and then directly fed into the ESI source of a Waters/Micromass QTOF micro mass spectrometer via a transfer line. Primary amines were identified as they eluted from the column by comparing the masses from the mass spectrometer to a primary amine database. Their concentration was calculated by comparing the peak areas from the single ion chromatograms to those of the stable isotopes labeled glycine $^{13}C_1$, methionine $^{13}C_1$ and serine $^{13}C_1$ used as internal standards (Cambridge Isotope, UA, US) [48].

**2.2.9 Liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS)**

The concentrations of SAM and SAH were determined by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS) [58]. 10 μL internal standard (459 μM SAH $d_3$, 15 μM SAM $^{13}C_5$, CDN Isotopes, Inc., Pointe Claire, QC, Canada), were added to 50 μL of plasma or tissue homogenate once thawed. Sequentially, 20 μL 20% heptafluorobtaric acid (HFBA) $H_2O$ (v/v) and 30 μL 4% heptafluorobtaric acid (HFBA) $H_2O$ (v/v) were added to the sample. The samples were centrifuged at 13,000g for 10 min at 4°C, and then the supernatant was transferred to a clean autosampler vial. 50 μL sample volume was injected for analysis. 50 μL of sample was injected onto a trap column (2.1 X 50mm 3.5um, Agilent Aqua) using an external pump (Waters 515) with water 0.4%HFBA as mobile phase, then eluted using a gradient of water and methanol (each containing 0.4%HFBA) pumped from a Waters Acquity UPLC controlled by Waters MassLynx software. Compounds were separated using a precolumn (2.1 X 12.5mm 3.5μm, Agilent Aqua) and analytical column (2.1 X 50mm 5μm, Agilent Aqua), then postcolumn. Addition of propionic acid (Sigma #537047) in isopropanol (Fisher A451) (3:1 v:v) was used to enhance sensitivity and the eluant entered a Waters Quattro micro mass spectrometer with an electrospray source.

For analysis of plasma homocysteine, methionine and cysteine, 50 μL of plasma was transferred to an Eppendorf tube containing 10 μL of each 500 μM homocystine $d_8$, 1000 μM methionine $d_4$ and 1000 μM cysteine $d_2$ as internal standards, and 10 μL

dithiothreitol (500 mmol/L in 0.1 mol/L NaOH) was added. Then the mixture was vortexed, and the samples were kept at room temperature for 15 min. Afterwards 100 µL acetonitrile containing 0.2% (v:v) HFBA were added to the sample. Then, the samples centrifuged at 13,000g for 10 min at 4°C and 20 µL supernatant and 100 µL 0.2 % HFBA/$H_2O$ (v/v) were transferred into a 150 µL HPLC vial insert. A sample volume of 5 µL was used for analyses, with a total analytical time of 6.2 min. Separation of compounds was achieved using a Zorbax SB Aqua column (2.1 × 100mm 3.5um), with a precolumn (2.1 × 12.1mm 5um, Agilent Technologies). 0.2 % HFBA/$H_2O$ (v/v) were used as mobile phase at a flow of 0.5 mL/min [59].

Choline, betaine, dimethylglycine were also determined by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS). 10 µL internal standard (20 µM Choline $d_9$, 10 µM Betaine $d_9$, 10 µM DMG $d_6$, CDN Isotopes, Inc., Pointe Claire, QC, Canada), were added to 50 µL of plasma once thawed. Then, 100 µL of protein precipitate solution (acetonitrile containing 0.1% formic acid) was added to the sample. Then the mixture was vortexed and centrifuged at 13,000g for 10 min at 4°C, and 20 µL supernatant and 100 µL mobile phase (19% 15 mmol/l ammonium formate, 0.1% formic acid in $H_2O$, and 81% acetonitrile) were transferred into a 150 µL HPLC vial insert. The LC was equipped with a Rx-Sil column (2.1 × 150 mm 5 µm) and a precolumn (2.1 × 12.1mm 5um, Agilent Technologies). The chromatographic separation was carried out isocratically at a flow of 0.5 mL/min with the mobile phase. The autosampler and column were maintained at 10°C and 25°C, respectively. The sample volume used for analysis

was 4 µL, with a total analytical time of 7.0 min [60].

## 2.2.10 Western blot

Liver tissue homogenates were diluted and boiled with sample buffer [0.5 M Tris-Hydrochloride, pH 6.8, glycerol, 10% SDS and 0.5%(w/v) bromphenol blue] with β-mercaptoethanol. Samples corresponding to 50 µg protein were used in SDS-PAGE, transferred to nitrocellulose membranes, and dried overnight at room temperature. Membranes were incubated with rabbit choline dehydrogenase polyclonal antibody (CHDH, Proteintech Group, Chicago,IL) for 2 hours at room temperature. After three washes in TBS-T, the membranes were incubated for 1 hour at room temperature with secondary goat anti-rabbit, and visualized using CDP-Star substrate (Perkin-Elmer, Woodbridge, Ontario) with the Chemigenius detection system using GeneTools software (Syngene, Frederick, MD) for densitometric analysis.

## 2.2.10 Statistical analyses

The means, standard deviation and standard error of the mean for all measurements were calculated using descriptive statistics. Independent sample t-test was used to determine the difference between the control group and cholestyramine group, as well as the difference between the cholestyramine (CY) group and cholestyramine plus supplemental methionine and choline (CY + Meth/Chol) group. The level of significance for all tests was $p = 0.05$. All statistical analysis was performed using the Statistical

Package for the Social Sciences (SPSS, Version 20, Chicago, IL).

**2.3 Results**

**2.3.1 Effect of cholestyramine diet on rat body weight, liver and faeces.**

The cholestyramine diet resulted in a decrease in body weight (Figure 3), liver weight and the liver weight/body weight ratio (Table 2), $p<0.05$. In the liver, decreased triacylglycerol and cholesterol ester concentrations were also found in the cholestyramine group (CY), $p<0.05$. The liver phospholipid content was significantly higher in rats fed with cholestyramine, when compared to control diet (when described as mg/g wet weight), with the difference between the groups increasing when the data was expressed as mg/g protein, $p<0.05$. Adding methionine and choline to the cholestyramine diet (CY + Meth/Chol) had no statistically significant effect on growth, liver weight, or liver lipid content (Table 2).

Next, the rat faeces were analyzed in this study. Increased faeces weight and faecal total lipid and phospholipid contents were found in rats fed both the cholestyramine diet and the cholestyramine diet with additional methionine and choline (Table 3), $p<0.05$. The increase in faecal lipid excretion due to cholestyramine demonstrated that the intervention successfully mimicked fat malabsorption, thus giving a model to explore the linkage of fat malabsorption to altered methionine and choline. The HPLC chromatograms showed cholesterol ester, diglyceride, monoglyceride and free fatty acids in the faecal fat. This study therefore attempted analyzing the composition of the

free fatty acids in the faecal lipid. However, some of the peaks could not be identified and therefore resulting in unreliable data. This may be in part due to further metabolism by intestinal microflora.

**2.3.2 Effect of cholestyramine diet and methionine and choline supplementation on liver phospholipids**

Since the liver plays a major role in phospholipid metabolism and is a major source of plasma phospholipids, we also analyzed liver phospholipid composition. The liver lysophosphatidylcholine was significantly lower in the cholestyramine group, $p < 0.05$, but no significant differences in liver PE, PS, PI, PC, or SPH were found among different diet groups when expressed as a percentage of the total phospholipids (Table 4).

However, analyses of the phospholipid fatty acids showed that feeding the CY compared to control diet resulted in lower PC 18:1n7 ($3.91 \pm 0.67$ compared to $4.67 \pm 0.60$), 20:3n6 ($1.59 \pm 0.29$ compared to $1.97 \pm 0.24$), higher 20:5n3 ($3.12 \pm 0.80$ compared to $2.14 \pm 0.38$), and 22:5n3 ($0.99 \pm 0.20$ compared to $0.62 \pm 0.18$) in the liver PC fatty acids (Table 5), $p < 0.05$. The addition of supplemental methionine and choline to the cholestyramine diet did not cause any changes in the liver PC fatty acids.

A similar alteration of the fatty acid composition was found in the liver PE of rats fed cholestyramine. The liver PE 18:1n7, 20:2n6, 20:4n6 were significantly lower and 20:5n3, and 22:5n3 were higher in the CY group compared to the control group (Table 6), $p < 0.05$.

Again, the addition of supplemental methionine and choline to the diet had no significant effect on the liver PE fatty acids of rats fed CY.

### 2.3.3 Effect of cholestyramine diet and supplemental methionine and choline on plasma lipids

Plasma triacylglycerol and total cholesterol were significantly lower in rats fed the diet with cholestyramine than in those fed with the control diet (Table 7), $p < 0.05$. Again, supplemental methionine and choline did not change plasma triacylglycerol and total cholesterol levels. Our results showed that feeding the CY diet increased plasma PE and decreased the plasma PC/PE ratio, when compared to the control diet, due to an apparent increase in plasma PE with no change in PC (Table 8). However, adding methionine and choline to the CY diet led to a plasma PE level lower than in the CY group, and a plasma PC/PE ratio higher than the CY group ($p < 0.05$). The plasma PC, SPH and LPC were also not significantly affected by either cholestyramine or the inclusion of supplemental methionine and choline in the CY diet, $p > 0.05$. Analysis of the plasma PC fatty acids showed lower 18:0 and higher 16:1n9 and 22:5n3 in the CY, when compared to control group, with a lowering of 18:2n-6 in the group given the CY diet with supplemental methionine and choline (Table 9), $p < 0.05$.

## 2.3.4 Effect of cholestyramine diet and supplemental methionine and choline on methyl metabolites

To further understand if increasing faecal fat excretion alters methyl metabolism, we also considered the effects of the cholestyramine diet on the plasma methyl metabolites. Plasma betaine was increased from $54.4 \pm 5.64$ to $70.0 \pm 8.55$ in rats fed with the cholestyramine diet (Table 10), $P<0.05$. As important components of methyltransferase reactions, SAM and SAH were also measured in plasma, together with methionine, homocysteine and cysteine. The results showed that SAM/SAH ratio was reduced in rat plasma ($15.9 \pm 2.65$ compared to $19.2 \pm 3.32$) in response to cholestyramine feeding, with no change in methionine, homocysteine or cysteine, and no significant effect of supplemental methionine and choline. The decrease in the SAM/SAH was explained by an increase in SAH with no effect on SAM (Table 10), $P<0.05$. Analysis of SAM and SAH in the liver of CY fed rats showed an increase in SAH similar to that found in plasma, with no evidence of any difference in the liver serine, glycine or methionine and no effect of supplemental methionine and choline (Table 11), $p<0.05$. Since choline dehydrogenase catalyzes the oxidation of choline to betaine, its gene expression was performed by Western blot. However, there was no statistically significant difference between the control group and the CY group.

## 2.4 Discussion

This work sought to determine whether bile acid malabsorption will increase fat malabsorption and if this is associated with the decreased plasma choline, as well as the alteration of phospholipids and fatty acids in the plasma and the liver. The results showed that the bile acid binding resin, cholestyramine, was efficacious in causing fat malabsorption as shown by the significant elevations in faecal total lipids and phospholipids (Table 3). Similarly, it is known that children with CF malabsorb fat and phospholipids [53, 61]. These significant elevations in faecal fat and phospholipid excretion suggest that bile acid malabsorption is accompained by loss of phospholipids, as well as dietary energy. This result likely explains the lower body weight of rats fed the CY than control diet. Dietary fat malabsorption secondary to impaired exocrine pancreatic function in patients with CF results in reduced digestion and absorption of phospholipids [53, 61]. In addition, CF patients commonly display faecal bile salt wasting, which is related to fat malabsorption [62]. In this study, the bile acids sequestrate cholestyramine bound bile acids in the intestine and prevented their reabsorption. The increased faecal total lipids and phospholipids in the animals fed cholestyramine suggested that the cholestyramine diet group successfully mimicked fat malabsorption in CF patients.

The finding of no significant difference in liver glycine concentrations between the groups in our study was unexpected. In the liver, bile acids are conjugated with glycine and taurine prior to secretion [63]. About 90% of excreted bile acids are reabsorbed by

active transport in the ileum and are moved back to the liver and the gall bladder through enterohepatic circulation [41]. As a result, the increased faecal bile acid loss in the rats given cholestyramine was expected to have resulted in an increased drain on liver glycine. However, we found no significant difference in the glycine concentrations in the rat liver. There might be several reasons for this, including compensatory up-regulation of glycine synthesis to maintain the liver concentrations. Unfortunately, despite several attempts, we were not able to quantify faecal glycine or taurine loss, hence it is possible that although fat malabsorption was increased, the bile acid conjugating amino acids were reabsorbed in the small intestine.

Although decreased plasma choline was not achieved by the cholestyramine-feeding model, we found that fat malabsorption, including total fat and phospholipid, was associated with change in phospholipids, an increase in liver and plasma SAM, and an increase in plasma betaine. In our study, the plasma and liver triacylglycerol concentrations were significantly decreased in rats fed cholestyramine. The increased faecal phospholipid excretion is expected to result in a decrease in the absorption and return of PC, the major bile acid phospholipid, to the liver. The primary route for reabsorption of dietary and biliary PC is as LPC, which is transported to the liver. LPC can also be re-esterified in the intestinal enterocyte and be secreted in chylomicron or HDL. There are two major biosynthetic pathways for PC in the liver: the CDP-choline pathway (which has been showed to contribute 70% of hepatic PC), and the PEMT pathway (which contributes the remaining 30% of PC) [12]. The demand for PC to

support secretion of bile acid could be met by enhanced *de novo* generation of PC from diacylglycerol and CDP-choline, or the PEMT pathway. Alternatively, increased hepatic uptake of LDL would provide plasma PC. Cholestyramine is a bile acid binding resin that is used clinically as a way to lower plasma cholesterol. This is achieved because cholesterol is utilized in the synthesis of bile acids and faecal bile acid excretion is increased. The increased need for bile acid synthesis from cholesterol in the liver results in increased expression of liver LDL receptors and increased uptake of LDL, which would provide PC in addition to cholesterol. This mechanism is consistent with the lower plasma TG and cholesterol, some of which are carried in LDL, in rats fed cholestyramine, when compared to the control diet. Alternatively, the lower plasma TG could be due to the overall increased fat malabsorption, or the use of diglyceride for PC synthesis in the liver resulting in decreased secretion.

The results of this study also show that feeding cholestyramine altered choline-betaine metabolism, specifically with an increase in plasma betaine concentrations. Increased betaine could be explained by a decreased turnover to supply methyl groups, or the increased endogenous synthesis via choline dehydrogenase (CHDH). However, we found no significant difference in CHDH gene expression or in plasma choline, which could mean that the increased betaine was not due to increased synthesis. Decreased homocysteine remethylation due to increased homocysteine metabolism via CBS to cysteine, and hence taurine, is a possible explanation (Figure 1). An increased demand for taurine would result in increased use of homocysteine for

taurine synthesis, decreasing homocysteine remethylation. Our study also found that feeding cholestyramine increased plasma and liver SAH, which resulted in a decreased SAM/SAH ratio to 15-16 compared to about 20 in the control group. It has been demonstrated that SAH is a product inhibitor of most SAM-dependent methyltransferase enzymes by binding to the active site of cellular methyltransferases with higher affinity than SAM [64], with the suggestion that the SAM/SAH ratio reflects the methylation potential [65]. The increased liver SAH in the CY group could reflect increased SAH synthesis or decreased conversion of SAH to homocysteine. SAH and homocysteine should change in parallel because SAH hydrolase is reversible. The action of SAH hydrolase in the forward direction depends on the rate of removal of homocysteine. It seems most likely that the modest increase in SAH was due to increased synthesis. Further studies are needed to understand why betaine was elevated in the CY fed animals.

Another objective of this study was to examine whether bile acid malabsorption altered the phospholipid composition and the phospholipids fatty acids in the liver and plasma. The fatty acid composition of the liver PE and PC were altered by feeding the cholestyramine diet, and this involved lower 20:3n6, 20:4n6 and higher 20:5n3 and 22:5n3 in the liver PE and PC. However, no significant differences in DHA of the liver PE, liver PC and plasma PC were found among different diet groups. PC generated through PE and PEMT pathway would contain higher DHA level than the PC synthesized via CDP-choline pathway, since DHA composition in liver PE was higher than liver PC. The

results in this study suggested that bile acid malabsorption was not the reason of decreased plasma DHA, as found in CF children.

In summary, the results showed that the bile acid binding resin cholestyramine in the diet increased faecal total lipids and phospholipid excretion. However, bile acid malabsorption did not contribute to the significantly reduced plasma choline and DHA levels, which were shown in children with CF. This study also provided evidence that the metabolism of betaine, SAH, phospholipids and fatty acids were all altered following feeding with the bile acid binding resin, cholestyramine. Further studies are needed to consider the implications of the altered methionine- homocysteine cycle related to bile acid malabsorption.

Table 1. Nutrient content of diets with cholestyramine and supplemental methionine and choline (Meth/Chol)

| | Control | Cholestyramine | |
| --- | --- | --- | --- |
| | | --- | Meth/Chol |
| | | g/1000g | |
| Protein | 190 | 190 | 190 |
| Sucrose | 205 | 155 | 155 |
| Corn starch | 431 | 431 | 431 |
| Total carbohydrate | 636 | 586 | 586 |
| Canola oil | 60 | 60 | 60 |
| Mineral mix | 50 | 50 | 50 |
| Vitamin mix | 10 | 10 | 10 |
| Choline chloride | 0 | 0 | 1 |
| Methionine | 0 | 0 | 3 |
| Cellulose | 62.5 | 62.5 | 62.5 |
| Cholestyramine | 0 | 50 | 50 |
| $SeO_2$ | 0.084 | 0.084 | 0.084 |
| Manganese chloride | 0.299 | 0.299 | 0.299 |

Meth/Chol group means the control diet with 5% by weight cholestyramine plus supplemental methionine and choline.

Table 2. Effect of cholestyramine and supplemental methionine and choline on rat body and liver weight, and liver lipids[1]

| | Diet group | | |
| --- | --- | --- | --- |
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| Body wgt, g | 563±42.4 | 495±17.0 * | 500±47.5 |
| Liver wgt, g | 22.8±3.40 | 18.0±1.76 * | 18.1±2.96 |
| Liver wgt / body wgt, mg/g | 40.3±3.60 | 36.2±2.69 * | 36.1±4.40 |
| Liver TG, mg/g wet wgt | 10.9±2.90 | 5.61±2.88 * | 6.51±2.18 |
| Liver CE, mg/g wet wgt | 2.62±0.26 | 1.75±0.50 * | 1.57±0.49 |
| Liver phospholipid[2], mg/g wet wgt | 22.2±1.65 | 24.0±0.95 * | 23.4±2.35 |
| Liver protein, mg/g wet wgt | 192±22.5 | 182±28.2 | 173±19.6 |
| Liver TG, mg/g protein | 57.2±13.5 | 28.1±13.7 * | 38.2±12.8 |
| Liver CE, mg/g protein | 13.9±1.91 | 8.83±2.32 * | 9.42±3.63 |
| Liver phospholipid[2], mg/g protein | 117±15.8 | 134±16.4 * | 136±14.4 |

[1] Values are means $\pm$ SD, n=8. Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline.

* Significant difference between the control group and cholestyramine group, p<0.05.

[2] $\sum$ phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH), and lysophosphatidylcholine (LPC). TG, triacylglycerols; CE, cholesterol ester.

Table 3. Effect of cholestyramine and supplemental methionine and choline on rat faecal

lipid[1]

|  | Diet group | | |
|  | Control | Cholestyramine | |
|  |  | --- | Meth/Chol |
| Faeces wgt, g | 5.31±1.40 | 7.53±0.68 * | 7.52±0.87 |
| Faeces total lipids, mg/g | 43.0±7.35 | 125±43.1* | 146±39.6 |
| Faecesphospholipids, mg/g | 1.83±0.79 | 3.23±0.67* | 2.98±0.35 |

[1] Values are means $\pm$ SD, n=8.

* Significant difference between the control group and cholestyramine group, p<0.05.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental

methionine and choline.

Table 4. Effect of cholestyramine and supplemental methionine and choline on rat liver phospholipid composition[1]

| | Diet group | | |
| --- | --- | --- | --- |
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| | % total liver phospholipids | | |
| PC | 57.1±1.91 | 57.5±1.89 | 59.5±4.33 |
| PE | 15.7±0.74 | 16.9±1.88 | 15.9±1.63 |
| SPH | 10.6±1.52 | 9.43±1.85 | 8.96±2.30 |
| PI | 10.3±0.35 | 10.6±0.44 | 9.77±1.44 |
| PS | 3.78±0.24 | 3.57±0.26 | 3.54±0.44 |
| LPC | 2.58±0.23 | 2.03±0.31* | 2.19±0.22 |

[1] Values are means $\pm$ SD, n=8.

* Significant difference between the control group and cholestyramine group, p<0.05.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline.

PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table 5. Effect of cholestyramine and supplemental methionine and choline on rat liver phosphatidylcholine fatty acid composition[1]

| | Control | Cholestyramine | |
| --- | --- | --- | --- |
| | | --- | Meth/Chol |
| Saturated | | | |
| 16:0 | 19.2±1.39 | 18.9±1.21 | 18.5±1.52 |
| 18:0 | 22.2±2.02 | 23.0±2.26 | 23.0±2.69 |
| Monounstaturated | | | |
| 16:1n9 | 0.19±0.08 | 0.23±0.10 | 0.21±0.09 |
| 16:1n7 | 1.66±0.44 | 1.60±0.45 | 1.51±0.53 |
| 18:1n9 | 7.14±1.53 | 6.86±1.00 | 7.55±1.50 |
| 18:1n7 | 4.67±0.60 | 3.91±0.67* | 4.09±0.59 |
| n6 polyunsaturated | | | |
| 18:2n6 | 9.99±0.95 | 10.2±1.06 | 9.69±1.29 |
| 18:3n6 | 0.13±0.06 | 0.16±0.07 | 0.13±0.06 |
| 20:2n6 | 0.27±0.03 | 0.29±0.05 | 0.25±0.05 |
| 20:3n6 | 1.97±0.24 | 1.59±0.29* | 1.61±0.40 |
| 20:4n6 | 20.7±2.02 | 20.1±3.18 | 21.2±3.17 |
| 22:4n6 | 0.13±0.03 | 0.11±0.06 | 0.16±0.11 |
| 22:5n6 | 0.21±0.10 | 0.18±0.07 | 0.17±0.10 |
| n3 polyunsaturated | | | |
| 18:3n3 | 0.10±0.04 | 0.12±0.03 | 0.07±0.05 |
| 20:5n3 | 2.14±0.38 | 3.12±0.80* | 2.57±0.86 |
| 22:5n3 | 0.62±0.18 | 0.99±0.20* | 1.07±0.35 |
| 22:6n3 | 8.19±0.69 | 8.10±0.79 | 7.63±1.46 |

[1] Values are means $\pm$ SD, n=8, g/100g fatty acid

* Significant difference between the control group and cholestyramine group, $p < 0.05$.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline.

Table 6. Effect of cholestyramine and supplemental methionine and choline on rat liver phosphatidylethanolamine fatty acid composition[1]

| | Control | Cholestyramine | |
| --- | --- | --- | --- |
| | | --- | Meth/Chol |
| Saturated | | | |
| 16:0 | 16.5±1.01 | 17.0±0.74 | 15.9±1.60 |
| 18:0 | 23.4±1.19 | 23.1±1.23 | 23.7±1.34 |
| Monounstaturated | | | |
| 16:1n9 | 0.13±0.04 | 0.21±0.17 | 0.26±0.32 |
| 16:1n7 | 0.84±0.24 | 1.05±0.46 | 0.65±0.30 |
| 18:1n9 | 3.86±0.29 | 4.42±0.93 | 4.77±0.95 |
| 18:1n7 | 3.14±0.42 | 2.65±0.45 * | 2.72±0.41 |
| n6 polyunsaturated | | | |
| 18:2n6 | 3.82±0.45 | 4.40±0.81 | 4.43±0.80 |
| 20:2n6 | 0.06±0.03 | 0.13±0.07 * | 0.11±0.05 |
| 20:3n6 | 0.65±0.41 | 0.53±0.10 | 0.58±0.13 |
| 20:4n6 | 23.9±1.07 | 21.6±1.55 * | 22.9±1.67 |
| 22:4n6 | 0.53±0.38 | 0.44±0.07 | 0.47±0.14 |
| 22:5n6 | 0.28±0.13 | 0.26±0.09 | 0.24±0.12 |
| n3 polyunsaturated | | | |
| 18:3n3 | 0.11±0.05 | 0.18±0.10 | 0.12±0.08 |
| 20:5n3 | 1.97±0.36 | 2.94±0.71 * | 2.89±0.64 |
| 22:5n3 | 1.55±0.40 | 2.40±0.49 * | 2.88±0.62 |
| 22:6n3 | 18.9±0.88 | 18.3±2.19 | 16.9±2.55 |

[1] Values are means $\pm$ SD, n=8, g/100g fatty acid

* Significant difference between the control group and cholestyramine group, $p<0.05$.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline.

Table 7. Effect of cholestyramine and supplemental methionine and choline on rat plasma lipid concentrations[1]

| | Diet group | | |
|---|---|---|---|
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| Triacylglycerols, mmol/l | 0.91±0.18 | 0.36±0.06 * | 0.31±0.05 |
| Total cholesterol, mmol/l | 2.11±0.32 | 1.61±0.22 * | 1.46±0.25 |
| Cholesterol ester, % of TC | 75.1±1.55 | 77.3±1.83 | 75.9±2.88 |
| Free cholesterol, % of TC | 24.9±1.55 | 22.7±1.83 | 24.1±2.88 |

[1] Values are means $\pm$ SD, n=8.

* Significant difference between the control group and cholestyramine group, p<0.05.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline. TC, Total Cholesterol.

Table 8. Effect of cholestyramine and supplemental methionine and choline on rat plasma phospholipid composition[1]

| | Diet group | | |
| --- | --- | --- | --- |
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| | % total phospholipids | | |
| PC | 73.2±5.41 | 71.1±4.42 | 75.7±5.46 |
| SPH | 22.5±5.24 | 24.6±7.13 | 23.4±5.35 |
| LPC | 4.53±0.87 | 3.82±0.72 | 3.67±0.85 |
| PE | 0.68±0.15 | 1.23±0.32* | 0.70±0.12 |
| PC/PE | 121±24.7 | 70.0±26.0* | 125±35.2 [#] |

[1] Values are means $\pm$ SD, n=8. * Significant effect of cholestyramine, p<0.05.

* Significant difference between the control group and cholestyramine group, p<0.05.

[#]Significant difference between the cholestyramine group and Meth/Chol group, p<0.05.

PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table 9. Effect of cholestyramine and supplemental methionine and choline on the fatty acid composition of rat plasma phosphatidylcholine composition[1]

| | Control | Cholestyramine | |
| --- | --- | --- | --- |
| | | --- | Meth/Chol |
| Saturated | | | |
| 16:0 | 18.7±1.85 | 19.7±1.11 | 19.0±2.80 |
| 18:0 | 24.4±2.12 | 21.8±1.87 * | 24.8±3.23 |
| Monounstaturated | | | |
| 16:1n9 | 0.17±0.05 | 0.25±0.03 * | 0.20±0.05 |
| 16:1n7 | 0.92±0.29 | 0.74±0.39 | 0.70±0.25 |
| 18:1n9 | 8.61±0.63 | 8.19±0.96 | 8.82±1.03 |
| 18:1n7 | 3.89±0.44 | 3.90±0.65 | 3.29±0.51 |
| n6 polyunsaturated | | | |
| 18:2n6 | 14.0±1.22 | 15.1±0.30 | 13.3±1.58[#] |
| 18:3n6 | 0.09±0.08 | 0.11±0.06 | 0.07±0.03 |
| 20:2n6 | 0.32±0.12 | 0.27±0.15 | 0.31±0.07 |
| 20:3n6 | 1.84±0.64 | 1.39±0.26 | 1.38±0.29 |
| 20:4n6 | 17.4±1.86 | 17.1±1.87 | 17.7±2.50 |
| 22:4n6 | 0.24±0.10 | 0.26±0.04 | 0.37±0.17 |
| 22:5n6 | 0.22±0.06 | 0.18±0.04 | 0.17±0.08 |
| n3 polyunsaturated | | | |
| 18:3n3 | 0.17±0.03 | 0.17±0.14 | 0.19±0.08 |
| 20:5n3 | 1.52±0.30 | 1.93±0.64 | 1.89±0.60 |
| 22:5n3 | 0.59±0.13 | 1.18±0.15 * | 1.13±0.18 |
| 22:6n3 | 6.25±0.92 | 7.00±1.14 | 6.17±1.01 |

[1] Values are means $\pm$ SD, n=8, g/100g fatty acid

* Significant difference between the control group and cholestyramine group, p<0.05.

[#] Significant difference between the cholestyramine group and Meth/Chol group, p<0.05.

Table 10. Effect of cholestyramine and supplemental methionine and choline on plasma

methyl metabolite concentrations[1]

| | Diet group | | |
|---|---|---|---|
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| Methionine, µmol/L | 76.5±8.36 | 78.6±9.80 | 90.7±14.4 [#] |
| Homocysteine, µmol/L | 6.23±0.43 | 7.02±1.41 | 7.28±1.54 |
| Cysteine, µmol/L | 208±20.6 | 201±20.3 | 202±22.1 |
| SAM, nmol/L | 377±54.7 | 370±65.7 | 350±53.4 |
| SAH, nmol/L | 19.9±3.19 | 23.3±3.09 * | 22.6±3.31 |
| SAM/SAH | 19.2±3.32 | 15.9±2.65 * | 15.6±1.75 |
| Choline, µmol/L | 8.21±1.22 | 7.80±0.76 | 8.09±1.27 |
| Betaine, µmol/L | 54.4±5.64 | 70.0±8.55 * | 63.5±7.15 |
| Dimethylglycine, µmol/L | 3.83±0.95 | 4.06±0.99 | 3.71±0.80 |

[1] Values are means $\pm$ SD, n=8.

* Significant difference between the control group and cholestyramine group, $p < 0.05$.

[#] Significant difference between the cholestyramine group and Meth/Chol group, $p < 0.05$.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental

methionine and choline; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine.

Table 11. Effect of cholestyramine and supplemental methionine and choline on rat liver methionine, serine, glycine, SAM and SAH concentrations [1]

| | Diet group | | |
| --- | --- | --- | --- |
| | Control | Cholestyramine | |
| | | --- | Meth/Chol |
| Methionine, ug/mg protein | 0.25±0.07 | 0.22±0.06 | 0.18±0.08 |
| Serine, ug/mg protein | 0.86±0.22 | 0.94±0.24 | 0.67±0.20 |
| Glycine, ug/mg protein | 0.91±0.26 | 0.90±0.14 | 0.82±0.21 |
| SAM, nmol/g | 118±13.8 | 113±18.8 | 129±23.3 |
| SAH, nmol/g | 14.6±1.68 | 20.7±3.96 * | 20.9±3.66 |
| SAM/SAH | 8.08±0.94 | 5.57±1.05 * | 6.25±0.88 |

[1] Values are means ± SD, n=8.

* Significant difference between the control group and cholestyramine group, $p < 0.05$.

Meth/Chol: the control diet with 5% by weight cholestyramine plus supplemental methionine and choline. SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine.
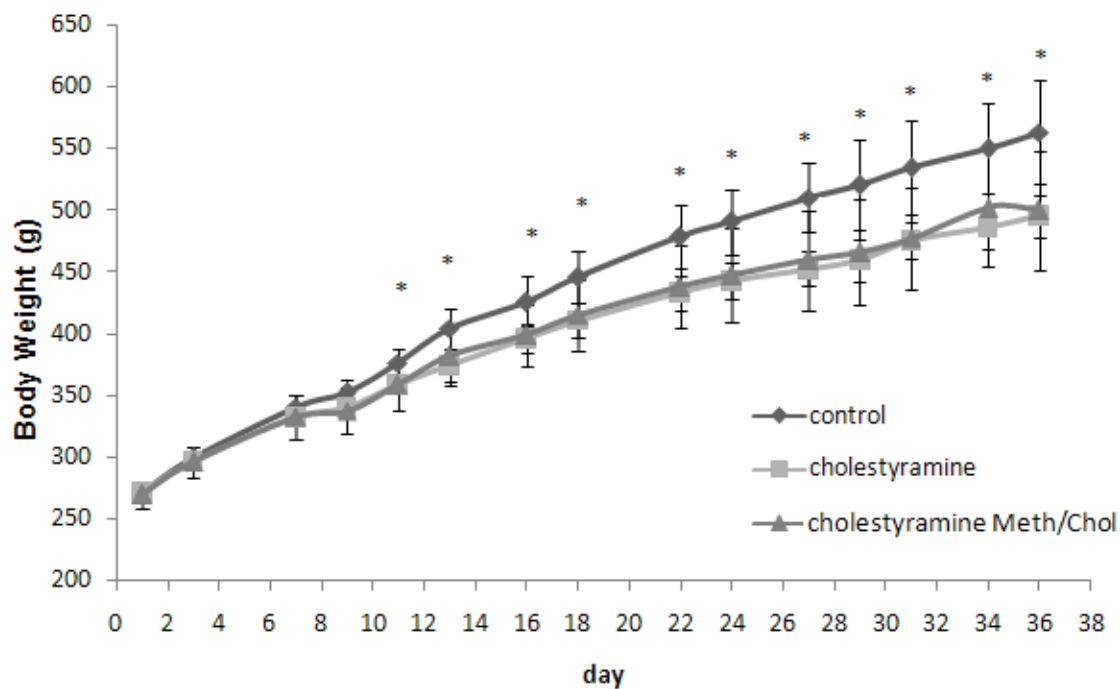
Figure 3. Effect of cholestyramine on body weight gain.

Cholestyramine without or with supplemental methionine and choline (Meth/Chol) diets were started on day 7. Values are means ± SD, n=8.

* Significant difference between the control group and cholestyramine group, p<0.05.

**CHAPTER 3: Experimential diabetes alters the methionine-homocysteine cycle, as well as phospholipids and fatty acids in the liver**

**3.1 Introduction**

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia and observed with clinical symptoms, such as alterations in lipid and amino acid metabolism and changes in hormone levels [66]. The hyperglycemia is caused by a deficiency in insulin in type 1 diabetes and results from insulin resistance in type 2 diabetes. Emerging evidence shows that diabetes and insulin perturb the methionine-homocysteine methylation cycle and elevate cellular oxidative stress [67]. GSH deficiency and decreased glycine have also been found in type 2 diabetes [33].

A type 1 diabetic state has been identified as a pathological factor in the modulation of methyl group in homocysteine metabolism [11]. The STZ induced diabetic rat model is commonly used to study type 1 diabetes [68]. STZ is a toxin that can selectively destruct the pancreatic beta cells and result in insulin deficiency and hyperglycemia [69].

It has been reported in the literature that the hepatic activities of GNMT, folate-independent homocysteine remethylation enzyme BHMT, and SAM-dependent PEMT are elevated in the STZ-induced diabetic rat model, whereas the folate-dependent remethylation enzyme MS is diminished [70]. The abundance of GNMT mRNA and BHMT mRNA were also reported to increase in diabetic rats [71]. At the same time, reduced plasma homocysteine levels, lower hepatic SAM/SAH ratio, decreased hepatic

methionine, and increased hepatic level of cysteine were found in the STZ-induced diabetic rats [71]. Those data revealed increased homocysteine catabolism via CBS in STZ-induced diabetic rat model.

Type I Diabetes is characterized by decreased *de novo* glycine synthesis in the hyperglycemic state [32]. Glycine *de novo* synthesis begins with glucose that is further metabolized to serine and then glycine (Figure 3). An attractive hypothesis is that decreased glucose availability for intracellular glycine synthesis contributes to the diabetic pathology. Some evidence has been reported that glycine administration can increase the response to insulin in humans [37], although little has yet been done in this field. The purpose of this study is to determine that decreased glucose availability alters methionine-homocysteine and choline-betaine cycle metabolites, phospholipids and phospholipid fatty acids.

## 3.2 Experimental procedures

Male Sprague Dawley rats (n=16) (Charles River Laboratories), 8 weeks old with an approximate weight of 200-250 g, were housed under constant humidity and temperature conditions with a 12-hours light-dark cycle. The rats were divided into two groups, and were fed the same diet (Table 12). The groups were stratified by body weight to ensure similar body weights. 55 mg/kg body weight STZ was administered via tail vein to one of the two groups after 4 weeks on the diet. The rats were monitored for

signs of hyperglycemia, including systemic distress, excess drinking, and urination. Blood glucose levels were determined by a CONTOUR® NEXT EZ meter and CONTOUR® NEXT test strip in the control group and diabetic group 30-hours after STZ-injection to confirm successful induction of diabetes. Blood and tissues were collected 4 days after STZ administration. Tissue collection, and analytical methods were similar, as described in Chapter 2. The total lipids were extracted from the liver and plasma, and the composition of phospholipids (including PE, PC, SPH and LPC) were determined using HPLC. The separated lipid was recovered for analysis of fatty acids by GC. The concentrations of SAM, SAH, homocysteine, methionine, choline, betaine, dimethylglycine were determined by LC – MS/MS. The expression of choline dehydrogenase was determined by Western blot.

### 3.2.1 Statistical analyses

The means and standard deviations of the mean for all measures were calculated, and differences between groups were determined using independent sample t-tests. The level of significance for all tests was set at $p = 0.05$. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 20, Chicago, IL).

### 3.3 Results

### 3.3.1 Effect of STZ injection on rat body weights and liver lipids

All the rats appeared healthy, behaved normally, and gained weight before the

injection of STZ. The animals given the STZ were clearly diabetic with elevated blood glucose levels at 30-hours post injection (Figure 5), but did not have severe clinical symptoms at that time.

Decreased body weight, liver weight, liver weight/body weight ratio, and liver protein concentration were present in the diabetic group (Figure 4, Table 13), p<0.05. In the liver, the triacylglycerol concentration was lower in the control group (156±26.0 mg/g protein) than in the diabetic group (109±11.8 mg/g protein) (Table 13), p<0.05. However, no statistically significant difference was found in liver cholesterol ester or total phospholipid per gram of liver wet weight. Analysis of the liver phospholipid composition showed increased liver PE and decreased PC, which resulted in a decreased PC:PE ratio in the diabetic rats (Table 14), p<0.05. No significant differences were found in the liver PI, SPH and LPC between the groups (Table 14).

The liver PC and PE fatty acid compositions are shown in Table 15. These analyses showed that PC 20:3n6 and 20:5n3 were decreased and 18:2n6 was increased in PC (p<0.05). The liver PE 16:0, 16:1n7, 18:1n7, 20:3n6, 20:5n3 and 22:4n6 were decreased but 18:2n6 and 20:4n6 were increased in the diabetic group, when compared to the control group (Table 15), p<0.05.

### 3.3.2 Effect of STZ injection on rat plasma lipids and phospholipid fatty acids

The plasma triacylglycerol concentration was significantly increased in the diabetic group (Table 16), p<0.05. However, the STZ injection had no significant effect on plasma

total cholesterol or on the proportion of cholesterol (present as free or esterified form). Analysis of the plasma phospholipid composition showed increased plasma PE, resulting in giving decreased PC/PE ratio in the diabetic, when compared to the control group (Table 17). At the same time, the proportion of PC, SPH, and LPC in the plasma were not altered. Similarly, no changes were found in fatty acid composition of plasma PC following STZ injection, with the exception of decreased plasma PC 18:3n6, 20:2n6 and 20:5n3 in the diabetic group (Table 18), $p<0.05$.

### 3.3.3 Effect of STZ injection on methyl metabolites

Despite no change in plasma choline or dimethylglycine, plasma betaine increased from $62.1\pm15.3$ µmol/L (in the control group) to $150\pm27.9$ µmol/L (in the diabetic group) (Table 19), $p<0.05$. In contrast, homocysteine decreased from $9.10\pm18.4$ µmol/L to $5.34\pm0.91$ µmol/L, with a trend towards lower methionine (p= 0.06), with higher SAH in the diabetic group, when compared to control group. The increase in plasma SAH resulted in a lower plasma SAM/SAH ratio. The increase in plasma betaine suggested that the expression or activity of choline dehydrogenase was increased. Therefore, we also assessed the protein abundance of this enzyme in the liver by Western blot and found significantly elevated liver choline dehydrogenase expression (Figure 6), $p<0.05$. Analysis of the liver SAM and SAH showed elevated SAH with a decrease in the SAM/SAH ratio from $6.94\pm0.81$ to $10.8\pm1.35$ in the diabetic group compared to control

group (Table 20), p<0.05.

## 3.4 Discussion

The purpose of this study was to determine whether experimental diabetes, with hyperglycemia, would alter methionine-homocysteine and choline-betaine cycle metabolites, phospholipid and their fatty acids. The results showed that the STZ significantly elevated blood glucose levels and this was associated with elevated plasma triacylglycerols. The higher plasma triacylglycerols concentration could be explained by increased hepatic triacylglycerol biosynthesis, which has been reported in a vivo STZ-injected rat study [72]. Around 10% of total body weight loss was found in diabetic group, as well as decreased liver weight and liver protein concentration. The depressed protein synthesis in the liver of STZ-injected rats might be a reason of the low liver protein level in diabetic rats [84].

This study found lower hepatic PC/PE ratios, due to higher levels of liver PE with lower liver PC, in diabetic, when compared to control rats. This might be due to decreased methylation of PE to produce PC. The higher PE was accompanied by increased liver SAH, which resulted in a lower SAM/SAH ratio. The significant increase in hepatic SAH is important because this is a potent inhibitor of many methyltransferases [73]. SAH is removed by a reversible enzyme SAH hydrolase, which converts it to homocysteine and adenosine [73]. Agents that impair the removal of adenosine can result in increased SAH levels [64,65]. Therefore, the increased hepatic SAH levels

might be due to the impaired removal of adenosine in diabetic rats. The ratio of SAM/SAH, which has been used as an indicator of cellular methylation capacity [64,65], was decreased in diabetic rat liver due to an increased SAH. The results of the recent study show that increased SAH and decreased SAM/SAH ratio might indicate reduced methylation capacity in the diabetic rat liver, which might result in reduced liver PE methylation to PC, and hence increased PE.

In the diabetic group, the increased plasma SAH and decreased plasma SAM/SAH ratio were consistent with the change in the liver. At the same time, similar to other studies [71], lower plasma homocysteine was found in the diabetic group in this study. This lower plasma homocysteine could result from increased conversion via cystathione $\beta$-synthase to generate cysteine for synthesis of glutathione, since elevated cellular oxidative stress [67] and glutathione deficiency have been found in diabetes [33]. Consistent with the results of another study in the literature [77], plasma triacylglycerol was increased in experimental diabetes in our study. The increased plasma triacylglycerol could be explained by the impaired clearance of chylomicron triacylglycerol and accompanied by a reduction in the activity of adipose tissue lipoprotein lipase in experimental diabetes [78]

However, experimental diabetes in the recent study also resulted in a two-fold elevation in circulating betaine concentration. Betaine plays an important role in the remethylation of homocysteine [74]. In a recent study, plasma betaine concentrations were found to be inversely associated with fasting plasma homocysteine concentrations

in healthy humans [75]. The inverse association was similar to what was observed in our study. The increased betaine concentration in diabetic animals could be explained by a decreased turnover to supply methyl groups, or the increased endogenous synthesis via choline dehydrogenase (CHDH). Further, our data showed that the abundance of the enzyme CDHD, which catalyzes the first of two successive oxidation steps in the biosynthetic conversion of choline to betaine, also increased in the diabetic rats [76]. Although Shobhitha *et al.* [76] reported that the activity of hepatic CDHD increased in the diabetic rat, our study is the first report to show that the protein abundance of hepatic CDHD is also increased in diabetic rats. Further studies are needed to address the interrelation of experimental diabetes with methionine remethylation and the homocysteine-transsulfuration pathway.

Another major finding is that STZ-induced diabetic rats have altered long-chain polyunsaturated fatty acid metabolism in liver. Specifically, compared to normal rats, STZ-induced diabetic rats had a significantly increased level of 20:4n6 in liver PE but not in liver PC. This may be a reflection of reduced liver PC synthesis via the PEMT pathway. PEMT prefers to utilize PE acylated with long chain polyunsaturated fatty acids (for example 20:4n6 and 22:6n3) as substrates for synthesis of PC [7]. It has been reported in the literature that a defect in fatty acid desaturation is associated with experimental diabetes [79]. However, another study showed that decreased 20:4n6 and increased 18:2n6 in diabetic animals due to depressed activity of liver microsomal fatty acid desaturases [80]. Several other factors, such as membrane lipid turnover, fatty acid

chain elongation and oxidation might have contributed to the changes in membrane lipid composition.

In summary, this study showed that experimental diabetes resulted in increased liver and plasma SAH, increased liver PE, decreased plasma homocysteine, decreased liver PC as well as altered liver phospholipid fatty acid composition. This evidence suggests that hyperglycemia induced by experimental diabetes cannot lead to the decreased plasma choline and phospholipid DHA. Further studies are needed to determine the interrelation of experimental diabetes with *de novo* generation of PC and CHDH.

Table 12. Nutrient content of the diet fed in the study of experimental diabetes

|                    | diet     |
|--------------------|----------|
|                    | g/1000g  |
| Protein            | 200      |
| Sucrose            | 200      |
| Corn starch        | 413      |
| Canola oil         | 70       |
| Mineral mix        | 50       |
| Vitamin mix        | 10       |
| Choline chloride   | 1        |
| Methionine         | 3        |
| Cellulose          | 50       |
| $SeO_2$            | 0.084    |
| Manganese chloride | 0.299    |

Table 13. Effect of streptozotocin (STZ) on body and liver weight and liver lipids[1]

|  | Control | STZ |
| --- | --- | --- |
| Body wgt, prior to STZ, g | 456±43.0 | 440±29.2 |
| Body wgt, 4 d after STZ, g | 469±44.1 | 402±34.4 * |
| Wgt change in 4 d, g | +13.0±3.61 | - 37.1±4.76 |
| Liver wgt, g | 20.8±3.77 | 14.5±2.62 * |
| Liver / body wgt, mg/g | 45.3±4.47 | 35.9±4.06 * |
| Liver protein, mg/g wet wgt | 157±9.20 | 136±13.4 * |
| Liver TG, mg/g wet wgt | 17.2±2.15 | 21.2±4.57 |
| Liver CE, mg/g wet wgt | 1.62±0.42 | 1.87±0.58 |
| Liver phospholipid[2], mg/g wet wgt | 24.8±3.84 | 27.7±4.58 |
| Liver TG, mg/g protein | 156±26.0 | 109±11.8 * |
| Liver CE, mg/g protein | 10.3±1.63 | 13.9±4.58 |
| Liver Phospholipid[2], mg/g protein | 158±14.5 | 205±39.5 |

[1] Values are means $\pm$ SD, n=8 /group. * Significant effect of streptozotocin (STZ), p<0.05. TG, triacylglycerols; CE, cholesterol ester.

[2] $\sum$ phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH), and lysophosphatidylcholine (LPC).

Table 14. Effect of streptozotocin (STZ) induced diabetes on the composition of phospholipids in rat liver [1]

| | Control | STZ |
|---|---|---|
| | % total phospholipids | |
| PC | 61.3±1.50 | 56.3±2.68 * |
| PE | 19.2±2.83 | 24.9±3.15 * |
| PI | 10.1±1.44 | 10.3±0.53 |
| SPH | 8.38±1.43 | 7.59±0.90 |
| LPC | 1.03±0.13 | 0.93±0.16 |
| PC/PE | 3.26±0.52 | 2.30±0.42 * |

[1] Values are means $\pm$ SD, n=8 /group, % total phospholipids.

* Significant effect of streptozotocin (STZ), $p < 0.05$.

PE, Phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table 15. Effect of streptozotocin induced diabetes on rat liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acid composition[1]

| | PC | | PE | |
|---|---|---|---|---|
| | Control | STZ | Control | STZ |
| Saturated | | | | |
| 16:0 | 19.9±1.80 | 19.2±3.32 | 17.4±1.71 | 15.0±1.05 * |
| 18:0 | 25.3±3.32 | 24.8±3.61 | 28.3±2.59 | 29.8±1.68 |
| Monounstaturated | | | | |
| 16:1n9 | 0.19±0.07 | 0.15±0.05 | 0.05±0.03 | 0.04±0.01 |
| 16:1n7 | 1.19±0.60 | 0.46±0.17 | 0.66±0.19 | 0.27±0.04 * |
| 18:1n9 | 6.49±0.77 | 7.94±0.95 | 4.30±0.36 | 4.71±0.79 |
| 18:1n7 | 4.15±1.14 | 3.13±0.63 | 3.73±0.80 | 2.37±0.49 * |
| n6 polyunsaturated | | | | |
| 18:2n6 | 9.99±1.33 | 12.3±2.07 * | 5.05±0.48 | 6.86±1.09 * |
| 20:2n6 | 0.24±0.12 | 0.21±0.11 | 0.12±0.06 | 0.11±0.07 |
| 20:3n6 | 1.80±0.50 | 0.82±0.40 * | 0.73±0.17 | 0.47± 0.16 * |
| 20:4n6 | 20.9±2.53 | 23.3±1.70 | 21.2±1.49 | 23.9±1.76 * |
| 22:4n6 | 0.19±0.03 | 0.23±0.12 | 0.68±0.20 | 0.37±0.09 * |
| 22:5n6 | 0.22±0.09 | 0.07±0.02 * | 0.27±0.11 | 0.10±0.03 * |
| n3 polyunsaturated | | | | |
| 20:5n3 | 1.30±0.50 | 0.09±0.07 * | 1.38±0.59 | 0.59±0.18 * |
| 22:5n3 | 0.58±0.15 | 0.62±0.10 | 1.39±0.43 | 1.39±0.28 |
| 22:6n3 | 6.93±0.87 | 5.98±1.20 | 14.4±1.57 | 13.6±1.13 |

[1] Values are means $\pm$ SD, n=8 /group, g/100g fatty acid.

* Significant effect of streptozotocin (STZ), $p < 0.05$.

Table 16. Effect of streptozotocin induced diabetes on rat plasma lipid concentrations[1]

|                             | Control    | STZ          |
| --------------------------- | ---------- | ------------ |
| Triacylglycerols, mmol/l    | 1.02±0.36  | 2.03±0.34 *  |
| Total cholesterol, mmol/l   | 2.81±0.25  | 3.37±1.11    |
| Cholesterol ester, % of TC  | 60.1±5.08  | 63.5±5.61    |
| Free cholesterol, % of TC   | 39.9±5.08  | 36.5±5.61    |

[1] Values are means $\pm$ SD, n=8 /group.

* Significant effect of streptozotocin (STZ), $p < 0.05$. TC: total cholesterol.

Table 17. Effect of streptozotocin induced diabetes on rat plasma phospholipid composition[1]

|  | Control | STZ |
| --- | --- | --- |
|  | % total phospholipids | |
| PC | 72.3±2.88 | 75.6±3.30 |
| SPH | 22.2±2.60 | 18.4±3.86 |
| LPC | 4.80±1.33 | 4.55±1.38 |
| PE | 0.66±0.13 | 1.44±0.35 * |
| PC/PE | 114±27.0 | 55.1±12.3 * |

[1] Values are means $\pm$ SD, n=8 /group.

* Significant effect of streptozotocin (STZ), p<0.05.

PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

Table 18. Effect of streptozotocin induced diabetes on rat plasma phosphatidylcholine fatty acid composition[1]

|  | Control | STZ |
| --- | --- | --- |
| Saturated |  |  |
| 16:0 | 21.3±2.74 | 22.8±3.44 |
| 18:0 | 20.1±3.13 | 18.9±2.86 |
| Monounstaturated |  |  |
| 16:1n9 | 0.17±0.03 | 0.16±0.07 |
| 16:1n7 | 0.82±0.46 | 0.49±0.17 |
| 18:1n9 | 7.95±0.45 | 8.38±0.76 |
| 18:1n7 | 4.29±1.11 | 3.32±0.38 |
| n6 polyunsaturated |  |  |
| 18:2n6 | 19.3±1.85 | 19.7±3.80 |
| 18:3n6 | 0.08±0.02 | 0.04±0.02 * |
| 20:2n6 | 0.32±0.09 | 0.18±0.08 * |
| 20:3n6 | 0.30±0.03 | 0.31±0.17 |
| 20:4n6 | 16.0±2.68 | 18.4±3.12 |
| 22:4n6 | 0.11±0.04 | 0.09±0.03 |
| n3 polyunsaturated |  |  |
| 20:5n3 | 0.81±0.40 | 0.27±0.10 * |
| 22:5n3 | 0.60±0.17 | 0.64±0.11 |
| 22:6n3 | 7.02±0.89 | 5.93±0.95 |

[1] Values are means $\pm$ SD, n=8 /group, g/100g fatty acid.

* Significant effect of streptozotocin (STZ), $p < 0.05$.

Table 19. Effect of streptozotocin induced diabetes on plasma concentrations of methionine, choline and their metabolites[1]

|  | Control | STZ |
| --- | --- | --- |
| Methionine, µmol/L | 97.0±18.4 | 78.7±13.1 |
| Homocysteine, µmol/L | 9.10±1.46 | 5.34±0.91 * |
| Cysteine, µmol/L | 219±21.7 | 245±19.0 |
| SAM, nmol/L | 198±14.4 | 176±22.7 |
| SAH, nmol/L | 16.1±4.67 | 29.1±6.48 * |
| SAM/SAH | 13.3±4.17 | 6.24±1.30 * |
| Choline, µmol/L | 11.3±2.47 | 10.4±1.63 |
| Betaine, µmol/L | 62.1±15.3 | 150±27.9 * |
| Dimethylglycine, µmol/L | 5.62±1.16 | 6.66±2.04 |

[1] Values are means $\pm$ SD, n=8 /group.

* Significant effect of streptozotocin (STZ), $p<0.05$.

SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine.

Table 20. Effect of streptozotocin induced diabetes on rat liver concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) [1]

|  | Control | STZ |
|---|---|---|
| Liver |  |  |
| SAM, nmol/g | 128±13.1 | 124±12.8 |
| SAH, nmol/g | 12.0±1.89 | 18.2±3.71* |
| SAM/SAH | 10.8±1.35 | 6.94±0.81* |

[1] Values are means $\pm$ SD, n=8 /group.

* Significant effect of streptozotocin (STZ), $p<0.05$.

SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine.
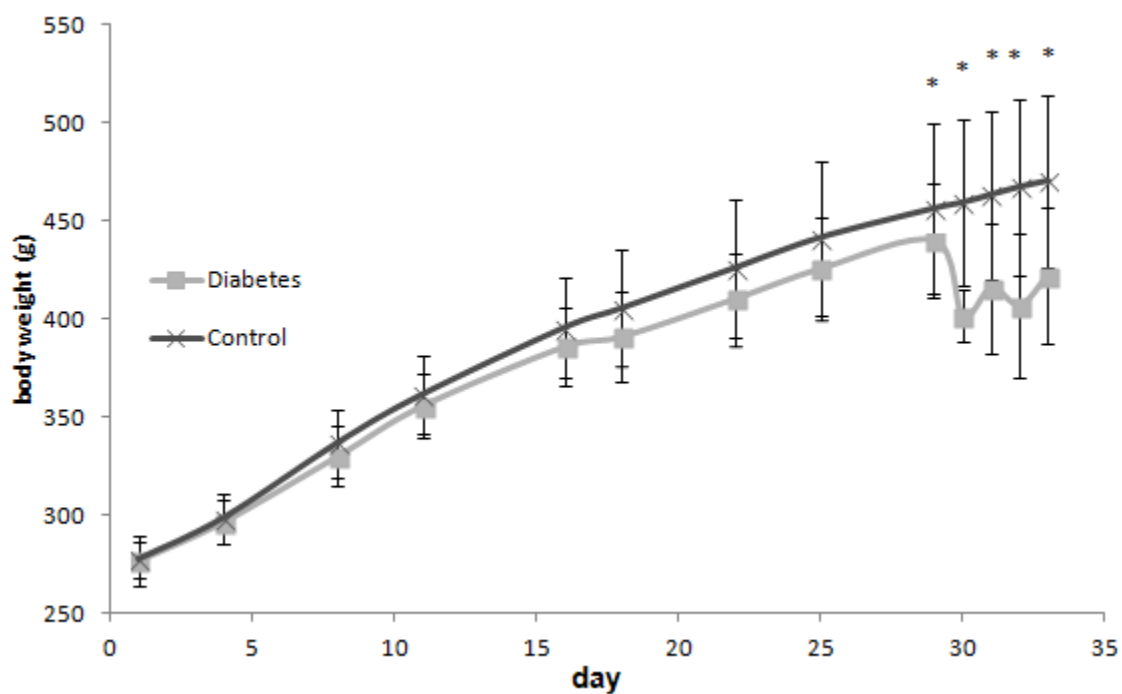
Figure 4. Effect of streptozotocin (STZ) on body weights.

Streptozotocin (STZ) was administered via tail vein to the diabetes group at day 30.

Values are means $\pm$ SD, n=8. * Significant effect of streptozotocin, p<0.05.

Figure 5. Effect of streptozotocin (STZ) on blood glucose.

The blood glucose level was measured at 30 hours after STZ injection.

Values are means $\pm$ SD, n=8. * Significant effect of streptozotocin, p<0.05.

Figure 6. Effect of streptozotocin (STZ) on liver choline dehydrogenase (CHDH) abundance by Western blot analysis.

Values are means $\pm$ SD, n=8. * Significant effect of streptozotocin, p<0.05.
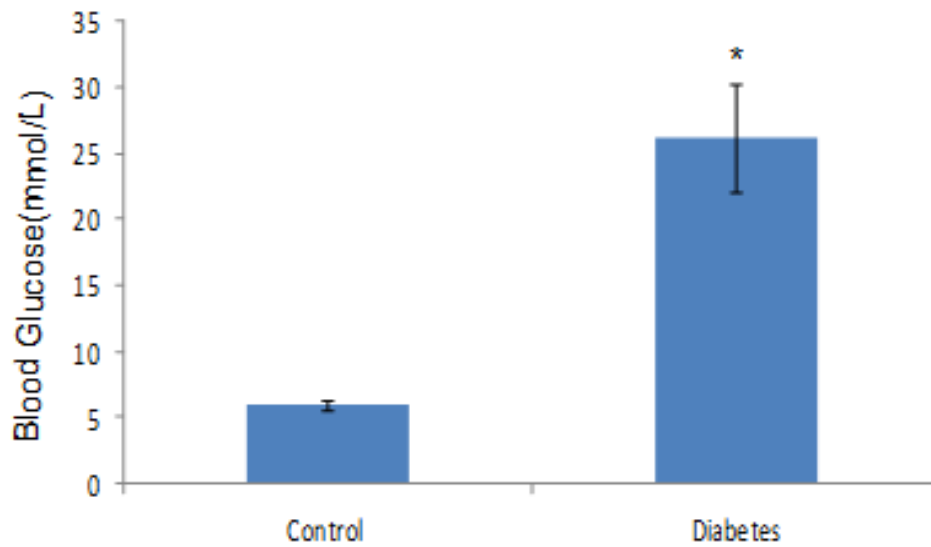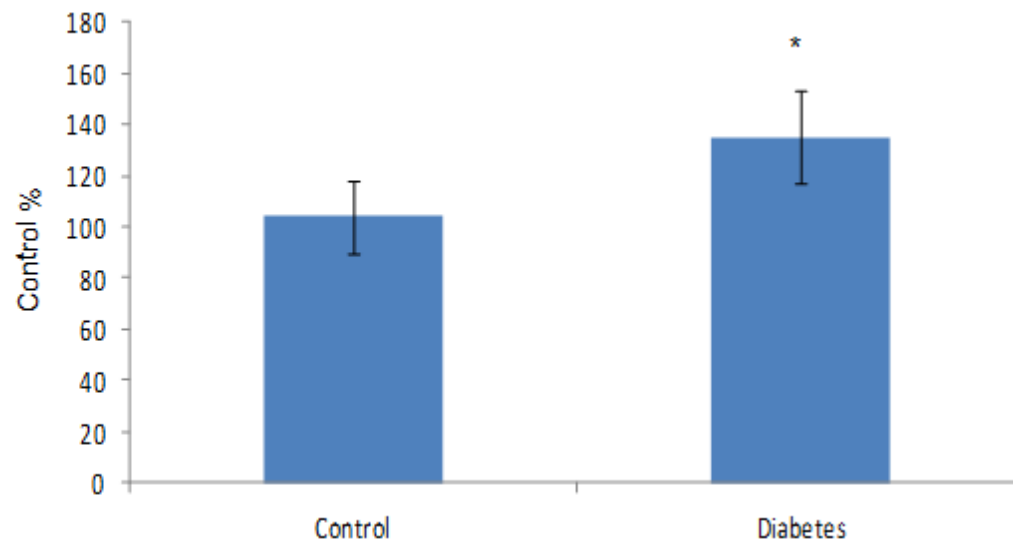
**Chapter 4 Conclusion, strengths and future**

**4.1 Conclusion**

Our research used two different approaches: 1) bile acid malabsorption (Chapter2) and 2) experimental diabetes (Chapter 3) to determine if inducing faecal bile acid loss and decreased glucose availability alter the methionine-homocysteine, and choline-betaine cycle metabolites, phospholipids and phospholipid n-6 and n-3 fatty acids. The results of Chapter 2 showed that inducing faecal bile acid loss (resulting from including the bile acid binding resin, cholestyramine in the diet) resulted in increased faecal total lipid and phospholipid excretion. This was accompanied by increased plasma betaine, decreased plasma triacylglycerol, decreased plasma and liver SAM/SAH ratio, and changes in the fatty acid composition of hepatic phospholipids. However, bile acid malabsorption did not cause the decreased plasma choline and DHA levels, which were found in CF children. The results of Chapter 3 also showed that decreased glucose availability in the absence of fat and phospholipid malabsorption in experimental diabetes altered the methionine-homocysteine, and choline-betaine cycle metabolism, as well as phospholipids and fatty acids. Increased liver and plasma SAH, increased liver PE, decreased plasma homocysteine, decreased liver PC and altered liver phospholipid fatty acid composition were found in experimental diabetic rats. In conclusion, these studies provide new knowledge to determine that amino acids, phospholipids and fatty acids are interdependent.

## 4.2 Strengths

This research is designed to use biochemical approaches to show that lipids, specifically phospholipids, fatty acids, amino acids, and glucose are interconnected though the methionine homocysteine cycle. It has provided insight into the possible significance of bile acid malabsorption and decreased glucose availability in human disease, in contributing to altered methyl group and fatty acid status. At the same time, the animal model used in this study successfully mimicked the fat malabsorption in CF patients by feeding cholestyramine. This model successfully tested the effect of whether or not faecal bile acid loss caused decreased plasma choline and DHA levels. Further studies in metabolic pathways related to fat malabsorption in CF patients could be addressed by this model. This study is also the first to show that plasma betaine is increased (following feeding with the bile acid binding resin) and the protein abundance of hepatic CHDH is increased in diabetic rats.

## 4.3 Limitation and future directions

An obvious limitation was that we were examining rats and not humans. As the rats have different nutritional requirements and metabolism than humans, our rat model cannot completely mimic human disease. We also made the animals diabetic by using the drug (streptozotocin) that damaged part of the beta cells of the pancreas. The

limitations of the method, although extensively used, for human type 2 diabetes is well known [81]. The free radical generation has been reported in STZ diabetogenecity [82].

Another main limitation of this study was that the samples were collected at only one point in time. Data in this study could not suggest that the alteration of the hepatic SAM/SAH and lipid profile induced by specific intervention could be the cause or the result of plasma changes. Samples collected at multiple time points would provide more insight to the understanding of the metabolic interactions, especially among different organs.

Biomarkers in methionine-homocysteine and choline-betaine cycle metabolites, lipids and fatty acids were measured in this study. The decreased plasma choline and DHA in CF children were not due to the faecal bile acids loss or hyperglycemia. Other explanations should be addressed in the future studies. For example, decreased intestinal fluid and impaired pancreatic $HCO_3^-$ secretion, which result in low pH in the duodenum and jejunum [83], might cause the phospholipid malabsorption with alteration in plasma choline. Future studies may include enzymatic approaches (for example enzymes and protein activity assays) to further study the metabolic interactions.

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