METHYL METABOLISM IN OBESITY-RELATED CARDIAC REMODELING

by

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ABSTRACT

**Background:** Cardiovascular disease (CVD) is the second leading cause of death in Canada. Obesity is a well-established risk factor for CVD and its prevalence has increased dramatically across the globe during recent decades. In the setting of obesity, excess lipid accumulation in the heart leads to changes in cardiac function and metabolism of CVD. The molecular mechanisms contributing to these obesity-related CVDs are not well understood, and may involve DNA methylation. Methylation of DNA is a post-replication modification that provides ‘marks’ in the genome, such that genes are set to be transcriptionally activated or silenced. DNA methyltransferases are responsible for the methylation of DNA and use S-adenosylmethionine (AdoMet) as the methyl donor. The metabolism of methyl groups and the production of AdoMet involve three interrelated pathways: folate cycle, methionine cycle, and transsulfuration pathway. As such, disturbances in methyl metabolism could change DNA methylation patterns in the heart and be involved in the pathogenesis of CVD. The objective of this thesis was to test the general hypothesis that disturbances in methyl metabolism contributes to obesity-related cardiovascular pathology.

**Methodology/Results:** C57BL/6J mice with (+/-) and without (+/+ ) a heterozygous targeted disruption of the gene for cystathionine-beta-synthase (CBS), an enzyme required for the transsulfuration pathway, were used to disrupt methyl metabolism. At weaning, mice were fed either a control diet or a high-fat diet (HFD, 60% energy from fat) to induce excess adiposity (obesity). Studies in the thesis revealed three major findings. First, disturbances in methyl metabolism enhanced cardiac lipotoxicity associated with diet-induced obesity. Second,
disturbances in methyl metabolism altered cardiac energy metabolism and function associated with obesity-related cardiac remodeling. Third, disturbances in methyl metabolism contributed to a tissue-specific relationship between ‘methylation capacity’ (AdoMet/AdoHcy ratio), DNA methylation, and gene expression in Cbs +/- mice.

**Conclusion:** My findings suggest a unique role for CBS in cardiac fatty acid metabolism, possibly contributing to the pathology of obesity-related cardiac remodeling. These findings also provide first time evidence of disturbances in methyl metabolism and the functional consequences as it pertains to DNA methylation, regulation of gene expression, and cardiac remodeling in mice with diet-induced obesity.
PREFACE

All procedures were approved by the University of British Columbia (UBC) Animal Care Committee, and are in accordance with the guidelines of the Canadian Council on Animal Care using the following protocols: A09-0346 and A14-0249. All molecular biology experiments were performed in Dr. Angela M. Devlin’s Laboratory in the Child and Family Research Institute (CFRI) if not otherwise stated. The overall thesis hypothesis was established by Dr. Devlin.


A version of Chapter 3 has been published. Gosh S., Sulistyoningrum D.C., Glier M.B., Verchere B.C., and Devlin A.M. 2011. Cystathionine-Beta-Synthase Deficiency Augments Cardiac Lipotoxicity Associated with Diet Induced Obesity in Mice. Journal of Biological Chemistry. 286:42483-42493. Molecular experiments were performed by Ghosh S., Sulistyoningrum D.C, and Glier M.B. Quantification of metabolites and lipids were conducted in the Metabolomics Core of the Nutrition and Metabolism Research Program by Benny Chan (directed by Dr. Innis).

Cardiac Energy Metabolism and Remodeling in Mice. Molecular experiments were performed by M.B. Glier with assistance from Gerrard S.L. Quantification of lipids and fatty acids were performed at the University of Alberta by da Silva R.P, directed by Jacobs R.L.

Echocardiography was performed by Bohunek L. in the Genetically Engineered Models Facility, and isolated working heart experiments were executed by Wambolt R.B. (directed by Dr. Allard) at the James Hogg Research Centre. Quantification of tissue and serum thiols was conducted by Benny Chan in Dr. Lammers’ Laboratory.

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<td>ELISA</td>
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<td>FABPpm</td>
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<td>HH</td>
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<td>IP</td>
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<td>LINE</td>
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<td>NPPA</td>
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<td>NRF1</td>
<td>nuclear respiratory factor-1</td>
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</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>SDS-PAGE</td>
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<td>WHO</td>
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<tr>
<td>WHR</td>
<td>waist-to-hip ratio</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
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ACKNOWLEDGEMENTS

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CHAPTER 1:

LITERATURE REVIEW
OBESITY AND CARDIOMETABOLIC RISK

Obesity is characterized by an excess amount of body fat that presents a risk to health and in adults is defined as a body mass index (BMI) $\geq 30$ kg/m$^2$ (287);(4; 252). As of 2014, the prevalence of overweight (BMI $\geq 25$ kg/m$^2$) and obese adults in Canada has risen to 54% and 20%, respectively (Statistics Canada). What’s more alarming is the number of overweight and obese children in Canada. During 2009 to 2011, the World Health Organization (WHO) estimated that one third (31.5%) of children aged 5 to 17 years were classified as overweight or obese (228). Obesity is a problem that has expanded worldwide and has reached pandemic proportions. Obesity is also no longer a problem isolated to high-income countries, but now seen in middle- and low-income countries (251; 288). The fundamental etiology of obesity involves an imbalance between energy intake and energy expenditure; however, there are multiple factors that influence excess body weight (genetics, diet, level of physical activity/exercise, and stress) adding a layer of complexity to this public health issue (159). Obesity can have a serious impact on health and is a major risk factor for several non-communicable pathologies, such as dyslipidemia, type 2 diabetes mellitus (T2D), hypertension, cardiovascular diseases (CVD) and some types of cancers (65; 66). Therefore, prevention and treatment of obesity is necessary to lessen the burden and improve upon this public health concern.
The Public Health Agency of Canada (PHAC) has estimated that nine out of ten (90%) Canadians over the age of 20 years have at least one risk factor for CVD, with overweight and obesity being major risk factors (56). As the prevalence of obesity continues to escalate a parallel rise in the number of Canadians at risk for developing CVD is expected to occur. Like obesity, CVD has a major economic impact in Canada, costing a total of 22.2 billion dollars, as estimated by the PHAC in 2002 (56). In 2011, the second leading cause of death in Canada was CVD, representing 47,627 Canadians or 9.7% of all deaths in Canada (Statistics Canada). Therefore, understanding the link between obesity and the onset of cardiovascular pathology is crucial to the development of prevention and treatment strategies of future cardiovascular events. It’s clear that obesity poses an increased risk for the development of CVD; however, the direct relationship between obesity and CVD is unknown.

REGIONAL FAT DISTRIBUTION

Obesity is a phenotypically heterogeneous condition; for example, amongst obese individuals, body fat distribution appears to vary greatly. In 1947, French physician Dr. Jean Vague reported a difference in body fat distribution between his obese patients with T2D and clinical signs of CVD, and his obese patients without complications. Vague categorized these two body types as: android obesity to refer to fat accumulation within the abdominal region, giving an apple-like appearance, and is associated with T2D and CVD; and gynoid obesity to refer to fat accumulation within the hip and thigh region, giving a pear-like appearance and is less likely associated with comorbidities (263). These observations aroused the interest of the medical and scientific community, focusing attention on body fat distribution and health outcomes. This idea
also stimulated the use of more specific anthropometrics to better assess obesity than the most widely used anthropometric indicator, BMI. Although simple and easy to obtain, BMI doesn’t account for body fat composition or distribution, especially with respect to location (i.e. visceral adiposity) and cardiometabolic risk. Cardiometabolic risk factors include: obesity, hyperglycemia, insulin resistance, hypertension, and dyslipoproteinemia (64; 65). In an attempt to account for differences in body fat composition and distribution, the waist-to-hip ratio (WHR) was developed, and considered a better tool for predicting cardiometabolic risk compared to BMI alone (142). However, it is important to keep in mind that WHR cannot distinguish between body fat compartments i.e. between subcutaneous and visceral adipose tissue. Other measures include the use of imaging technologies such as, dual X-ray absorptiometry (DEXA), computed tomography (CT) and magnetic resonance imaging (MRI). Total body composition and fat content can be measured by DEXA scans, whereas abdominal visceral and subcutaneous adipose tissue can be assessed and measured by CT and MRI cross-sectional images (116; 255; 258). The development of more specific anthropometrics and imaging technologies has allowed for better assessment of obesity-related cardiometabolic risk. As a result, multiple case-control and small longitudinal studies have clearly confirm Vagues’ observation that visceral adiposity is more predictive of cardiometabolic risk (67; 154; 160; 162; 163; 186; 199).

VISCERAL ADIPOSITY AND CARDIOMETABOLIC RISK

Visceral adipose tissue (VAT) is associated with the greatest risk for the development of CVD, although the mechanisms underlying this association are not fully understood (253). An important question debated in the scientific community is whether VAT is a causal factor or
simply a marker of increased cardiometabolic risk. There are currently three proposed mechanisms by which VAT could be linked to CVD risk. The first theory relates to the anatomical location of VAT. Within the abdominal cavity, VAT surrounds vital organs and has direct access to the hepatic portal circulation. One theory is that as VAT accumulates it becomes hyperlipolytic and insulin resistant, exposing the liver, through portal circulation, to high concentrations of fatty acids, thus contributing to insulin resistance and excess triglyceride accumulation in the liver. Chronic exposure of high concentrations of fatty acids can promote hepatic gluconeogenesis, increase hepatic lipogenesis, and decrease insulin clearance (because of increased liver triglyceride content) (26; 28; 144; 184; 191; 195). Many population studies in men and women have reported that VAT, and not subcutaneous adipose tissue (SAT) or total adiposity, is more commonly associated with insulin resistance (87; 110; 155; 168; 219).

The second theory pertains to the fact that adipose tissue is no longer considered a passive reservoir for lipid storage and mobilization. Over recent years, adipose tissue has been recognized as a remarkable endocrine and paracrine organ releasing various proteins involved in inflammation and immunity (88; 257; 283; 293). Among others, these proteins include adipocytokines such as leptin and adiponectin, as well as pro-inflammatory cytokines such as interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1), and tumor-necrosis factor alpha (TNFα) (88; 257). As VAT accumulates in excess, the production and secretion of adipocytokines and pro-inflammatory cytokines become altered resulting in systemic inflammation and insulin resistance (64; 65).
Alternatively, the third theory alludes to the idea that VAT is merely a marker of the inability of SAT to act as a protective storage unit. During times of energy excess and reduced energy expenditure, adipose tissue adapts by increasing in adipocyte number (hyperplasia) and size (hypertrophy), to expand storage capacity and accommodate excess free fatty acids (220; 244). It has been hypothesized that excess fat is initially stored in SAT and if or when SAT loses its ability to expand, fat begins to accumulate in VAT and becomes dysfunctional and insulin resistant (11; 115; 254). A consequence of dysfunctional adipose tissue is that fat begins to accumulate in non-adipose tissues, such as the liver, muscle, pancreas and heart (33; 68). It is reasonable to consider that all three theories are involved and contribute to the link between VAT and cardiometabolic risk.

**OBESITY-RELATED CARDIAC LIPID ACCUMULATION**

Excess cardiac lipid accumulation is associated with changes in cardiac function and metabolism, which may contribute to the increased risk of heart failure in obese subjects (148; 286). The association between cardiac lipid accumulation and cardiac dysfunction in humans with obesity was first observed more than 150 years ago (271) and has gained renewed interest with the development of $^1$H-magnetic resonance spectroscopy (89). In 2006, a small human study ($n = 9$) was the first to report a 3-fold elevation in myocardial triglyceride content and 2-fold elevation in epicardial fat, quantified by MRS, in obese (mean BMI = 30 kg/m$^2$) versus healthy (mean BMI = 22 kg/m$^2$) men. Circulating serum FFA concentrations and epicardial fat content were twice as high in obese men compared to lean (146). Furthermore, the $^1$H-Magnetic Resonance Spectroscopy Study ($n = 134$) compared myocardial triglyceride content and left
ventricular (LV) function between four groups of individuals: lean (BMI < 25 kg/m²), normoglycemic (2 hour glucose < 7.8 mmol/L); obese (BMI ≥ 25 kg/m²), normoglycemic; impaired glucose tolerance (2 hour glucose 7.8-11.1 mmol/L), obese (BMI ≥ 25 kg/m²); T2D (history of diabetes or 2 hour glucose ≥ 11.1 mmol/L), obese (BMI ≥ 25 kg/m²). This study was able to confirm that myocardial triglyceride content was present in the obese group and higher in the impaired glucose tolerance and T2D groups when compared to the lean group. In addition, myocardial triglyceride content, in all 3 groups was associated with no indication of LV systolic dysfunction (% ejection fraction), suggesting that myocardial triglyceride content in humans, accumulates before the onset of T2D mellitus and LV systolic dysfunction (193). In another study using 1H-Magnetic Resonance Spectroscopy, higher myocardial triglyceride content in obese (BMI ≥ 25 kg/m²) T2D (2 hour glucose ≥ 11.1 mmol/L) men (n = 38) was also associated with no changes in LV systolic function, but was associated with impaired LV diastolic function when compared to control subjects (n = 28; same age, 45-65 years, and BMI, 25-32 kg/m²) (227). Together, these studies support the concept that myocardial triglyceride content appears to precede LV systolic dysfunction and T2D. It is still unclear if cardiac lipid accumulation causes cardiac dysfunction or is merely a marker for alteration in cardiac energy metabolism. The next section will review cardiac energy metabolism in health and in the setting of obesity.

1.2 CARDIAC ENERGY METABOLISM

The heart is a unique organ because it demands a high and constant rate of cellular energy in the form of adenosine triphosphate (ATP) to sustain recurrent contractile function. Under normal physiological conditions, the mammalian heart obtains ATP through mitochondrial oxidation of
multiple substrates including fatty acids, glucose, lactate, amino acids and ketones (203). The majority of energy produced in the cardiomyocyte is derived from the oxidation of fatty acids and glucose, although fatty acids are the preferred substrate because they generate more ATP (~70% of the total) compared to other substrates (175; 176; 205; 247; 248).

The heart relies on a continuous exogenous supply of fatty acids because it has a limited ability to synthesize and store them. Fatty acids are supplied to the heart as components of triglyceride-rich lipoproteins or as free fatty acids bound to albumin (175) (267). Fatty acids are released from triglyceride-rich lipoprotein particles (VLDL and chylomicrons) via hydrolysis by lipoprotein lipase (LPL) located on the luminal surface of coronary endothelial cells (14; 206). Fatty acids first bind to plasma membrane fatty acid binding protein (FABPpm) and enter the cardiomyocyte via passive diffusion or uptake by the following protein-mediated carriers: fatty acid translocase (FAT/CD36) or fatty acid transport protein (FATP1/6) (206; 230). The most extensively studied transporter is FAT/CD36, which is estimated to participate in 50-60% of fatty acid uptake and oxidation by the heart (161; 180). Translocation of FAT/CD36 in the sarcolemma membrane is stimulated by insulin or contraction by the heart (240). Once in the cytoplasm, the initial step of fatty acid metabolism begins by activation of fatty acids to fatty acyl-Co-enzyme A (CoA) by long-chain acyl CoA synthetase (ACSL). Long-chain fatty acyl CoAs are converted to long-chain acylcarnitine by carnitine palmitoyltranserase (CPT1) and transported into the mitochondria for β-oxidation, or redirected and used for intracellular triglyceride synthesis (for endogenous storage), when CPT1 activity is low (302).
The second most commonly used substrate used by the heart for energy is glucose, generating ~30% of the total ATP. Glucose is supplied to the heart via the circulation and uptake is dependent on two factors: the transmembrane glucose gradient; and sarcolemma glucose protein transporters (GLUT1 and GLUT4) (112; 298). The transporter GLUT1 has greater sarcolemma localization and is accountable for basal glucose uptake into cardiomyocytes; whereas GLUT4 is the dominant transporter and translocation is stimulated by insulin and heart contractions (247; 298) (179). Upon entry into the cardiomyocyte, glucose is rapidly phosphorylated to glucose 6-phosphate (G6P) by hexokinase, and then enters glycolysis for further breakdown to pyruvate or is redirected to glycogen synthesis (GS) for endogenous storage (124; 247). Glycolysis is a ten-step enzymatic pathway responsible for producing ~10% of the total ATP used by the heart (204). After glycolysis, pyruvate can also be converted to lactate by lactate dehydrogenase and then transported across the sarcolemma by monocarboxylate transporter 1 for exit outside the cell (100). However, the majority of pyruvate is transported into the mitochondria for further ATP generation by the carrier protein pyruvate translocase. Pyruvate is decarboxylated to acetyl-CoA by the multi-enzyme complex pyruvate dehydrogenase (PDH) (217; 285). Acetyl-CoA then enters the tricarboxylic acid (TCA) cycle to produce nicotinamide adenine dinucleotide (NAD to NADH) and flavin adenine dinucleotide (FAD to FADH2), along with an ATP molecule. These molecules act as electron carriers and are used to generate more ATP in the oxidative phosphorylation (electron transport chain) pathway (247). In addition, a small amount of pyruvate within the mitochondria can be converted to oxaloacetate or malate and used to replenish the TCA cycle, a process called “anaplerosis” (105).
The regulation of fatty acid uptake and metabolism in the heart is regulated, in part, by key metabolic proteins such as peroxisome proliferator-activated receptors (PPARs) (77; 90). They are a group of ligand-activated transcription factors and belong to the nuclear hormone receptor super-family (150; 295). Once activated, PPARs heterodimerize with retinoid X receptor (RXR) and bind to PPAR responsive elements (PPRE) within promoter regions of genes that encode proteins involved in fatty acid metabolism (69; 222). The family of PPARs consist of the following members: PPARα, PPARδ (also known as PPARβ) and PPARγ, each expressed by the heart (69; 107).

The master regulator of cellular energy (glucose and fatty acid metabolism) is adenosine monophosphate (AMP) activated protein kinase (AMPK) (80; 117). In the heart and other tissues, it acts as a fuel gauge by reacting to alterations in intracellular energy (AMP/ATP ratios) during physiological or pathological stress (117). Acetyl CoA carboxylase (ACC) is involved in the conversion of acetyl-CoA to malonyl-CoA. Activation of AMPK leads to phosphorylation and inhibition of ACC, resulting in a reduction of malonyl-CoA, which inhibits CPT1, thereby stimulating greater transport of fatty acids into the mitochondria for β-oxidation (15; 119; 177; 229).
1.3 CARDIAC REMODELING IN OBESITY

In the setting of obesity, cardiac energy metabolism is disturbed and there is an imbalance between fatty acid uptake and oxidation. When fatty acid uptake exceeds oxidation, harmful consequences eventually arise from excess lipids being shunted into non-oxidative pathways, resulting in the accumulation of toxic lipid species. These consequences promote oxidative stress, mitochondrial dysfunction and apoptosis. Together, these processes are referred to as cardiac lipotoxicity and may contribute to cardiac dysfunction and failure. This next section will review the possible mechanisms by which excess cardiac lipid accumulation leads to changes in cardiac function and metabolism. These changes are referred to as ‘cardiac remodeling’ and could play a role in in the pathogenesis of cardiomyopathies and failure.

LEASONS LEARNED FROM RODENT MODELS

Rodent models have been extensively studied in an attempt to delineate mechanisms underlying the association between obesity and cardiac remodeling.

HIGH-FAT DIET MODEL OF OBESITY

Alterations in cardiac energy metabolism have been identified early in the time course of HFD feeding mouse studies. For example, a study in male C57BL/6J mice fed a HFD (45% energy from fat) from 10 weeks of age, for 2 weeks, had reduced rates of glycolysis and glucose oxidation, in addition to greater rates of fatty acid (palmitate) oxidation in isolated working
hearts compared to mice fed the control diet (289). Although changes in cardiac energy metabolism appear quickly in mice following HFD feeding, changes to cardiac function are more robust and appear only with long-term HFD feeding. For example, a longitudinal study in male C57BL/6J mice showed no evidence of cardiac dysfunction (reduced % fractional shortening) until after 20 weeks of HFD (55% energy from fat) feeding compared to control-fed mice (212). In adult male Wistar rats, 8 weeks of HFD (50% energy from fat) feeding resulted in cardiac dysfunction (reduced % fractional shortening and ejection fraction) compared to control-fed rats (210). In contrast, another longitudinal study in male C57BL/6J mice reported no change in cardiac function at 8, 12 and 16 months of HFD (60% energy from fat) feeding compared to control-fed mice (39). Despite no change in cardiac function, mice fed the HFD at 8 and 16 months had greater measurements of heart weight/tibia length ratio, intraventricular septum and left ventricular wall thickness, and overexpression of cardiac collagen type I and collagen type III protein expression compared to control fed mice (39). Similarly, a longitudinal study in male Wistar rats also showed no change in cardiac function following HFD (49% energy from fat) for 15 and 45 weeks compared to control-fed rats (201). Therefore, it is clear that cardiac energy metabolism is altered in the setting of HFD-induced obesity and that the duration HFD feeding plays an important role in the development of obesity-related changes to cardiac function. However, it’s difficult to determine whether HFD has direct adverse effects contributing to metabolic derangements and changes in cardiac function, or if it’s just an effect of excess adiposity caused by HFD-obesity.
GENETIC MODELS OF OBESITY

This next section will review what has been reported pertaining to genetic models of obesity and the pathogenesis of obesity-related cardiomyopathy, without the use of HFD to induce obesity. A wide body of evidence has come from studies using rodents carrying homozygous recessive mutations that are associated with impaired leptin production and signaling. In 1950, the spontaneously obese mouse, *ob/ob* mouse, was discovered; it was later found to have a mutation in the leptin gene (*Lep*) in 1994 (135). Shortly after, a second spontaneously occurring obese mouse model was discovered, the *db/db* mouse, which is deficient in leptin receptor because of a variant in the leptin receptor gene (*Lepr*) (131). The *ob/ob* mice have hyperglycemia, hyperinsulinemia, and glucose intolerance at 8 weeks of age and developed overt diabetes between 10-15 weeks of age; whereas the onset of diabetes develops as early as 4-6 weeks of age in *db/db* mice (37; 192). Both *ob/ob* and *db/db* mice develop excess cardiac lipid accumulation associated with alterations in cardiac function and metabolism (1; 22; 52; 192; 232). In 2005, Abel et al. conducted a longitudinal study to determine whether the observed alterations in cardiac metabolism, gene expression, and function preceded or followed the onset of hyperglycemia in the *ob/ob* and *db/db* mice at 4, 8 and 15 weeks of age. The findings of this study indicate that excess cardiac lipid accumulation and alterations in cardiac energy metabolism preceded the onset of hyperglycemia and cardiac dysfunction in *ob/ob* and *db/db* mice (37). The studies suggest that alteration in cardiac energy metabolism may precede cardiac dysfunction. Another model of obesity is the Zucker fatty (*fa/fa*) rat, which also has a spontaneous mutation in the leptin receptor gene (304). Obese *fa/fa* rats also develop excess cardiac lipid accumulation, and this is associated with LV hypertrophy, cardiac dysfunction
(reduced % fractional shortening), greater ceramide content, and apoptosis in the heart (299). Together these studies suggest that excess lipid accumulation in the heart in rodents with obesity, due to defective leptin production and or signaling, plays a direct role in altering cardiac energy metabolism and cardiac dysfunction in cardiomyopathy.

TRANSGENIC AND KNOCKOUT MODELS

Studies in transgenic and knockout mouse models have provided more direct evidence pertaining to alterations of key proteins involved in cardiac energy metabolism (discussed above), and how this relates to the pathogenesis of cardiac remodeling, in the absence of obesity. The first mouse model evaluated the consequence of cardiac-specific overexpression of mouse ACSL (catalyzes the activation fatty acids to fatty acyl-Co-A). These mice had greater cardiac lipid accumulation associated with LV hypertrophy, cardiac dysfunction (reduced % fractional shortening), cardiomyocyte apoptosis (increased ceramide content and cytochrome c release) and developed overt heart failure as early as 4 weeks of age (50). Mice lacking the gene for ACSL1 (Acsl1-/-) had reduced cardiac long-chain acyl-CoA content, with no change in cardiac TG content associated with a 95% reduction in palmitate oxidation, a compensatory increase in glucose oxidation rates, and LV hypertrophy, with no change in cardiac function (82).

Transgenic mice with cardiac-specific overexpression of a human LPL (catalyzes the hydrolysis of triglyceride-rich lipoprotein particles, releasing their free fatty acids for entry into tissue cells) have also been studied. These mice were reported to have greater cardiac lipid accumulation associated with LV hypertrophy, cardiac dysfunction (reduced % fractional shortening) and
increased mortality (294). Mice lacking the gene for LPL ($Lpl^{-/-}$), had a loss of LPL-mediated fatty acid uptake by the heart that was associated with reduced rates of palmitate oxidation, yet higher glycolysis and glucose oxidation in isolated working hearts, with later (at 6 months) onset of cardiac dysfunction (reduced % fractional shortening) and fibrosis in older mice (13). The consequence cardiac-specific overexpression of FATP1 (involved in the transport of fatty acids into the cardiomyocyte) has also been studied. Mice with cardiac-specific overexpression of mouse FATP1 had greater fatty acid uptake in the heart accompanied by greater palmitate oxidation and reduced glucose oxidation in isolated working hearts. In addition, greater fatty acid uptake and utilization was associated with LV hypertrophy and measurements of impaired diastolic dysfunction (49). Thus far, these studies have provided a baseline knowledge pertaining to critical pathways responsible for cardiac energy metabolism; furthermore, the involvement of such pathways in the pathogenesis of cardiac dysfunction during obesity-related cardiac remodeling.

A few PPAR gene deletion and cardiac-specific transgenic mouse models have provided valuable insights into the roles of PPAR$\alpha$ and PPAR$\gamma$ in regulating cardiac energy metabolism (185). Mice lacking the gene for PPAR$\alpha$ ($Ppara^{-/-}$), had greater cardiac lipid accumulation associated with lower fatty acid oxidation rates and conversely, higher glucose oxidation rates in isolated working hearts (40; 211). Additionally, no alterations to cardiac function were observed in $Ppara^{-/-}$ mice, despite the detected changes in cardiac energy metabolism (40; 182; 211). Generalized (whole-body) PPAR$\gamma$ gene deletion causes embryonic lethality (19); therefore, cardiac-specific PPAR$\gamma$ gene deletion ($Ppar\gamma^{-/-}$) mice were developed and characterized (78).
Cardiac-specific *Ppary* -/- mice displayed evidence of mild cardiac hypertrophy, with preserved systolic cardiac function (76).

Cardiac-specific overexpression of mouse PPARα and PPARγ1 transgenic mice have also been developed and characterized. Both cardiac-specific PPARα and PPARγ1 transgenic mouse models displayed excess cardiac lipid accumulation associated with cardiac dysfunction (reduced % fractional shortening) and LV hypertrophy (91; 92; 242). Isolated working heart experiments showed higher fatty acid oxidation rates and lower glucose oxidation rates in mice with cardiac-specific overexpression of PPARα, whereas no change in fatty acid oxidation rates were reported in mice with cardiac-specific overexpression of PPARγ1 (92; 243). In both models, these data suggest that the excess lipid accumulation in the heart must be out of proportion to induction of fatty acid oxidation. Another study reported mice with cardiac-specific overexpression of mouse PPARγ1 to have altered mitochondrial structure, higher ceramide content, and apoptosis in the heart (242). These studies have demonstrated that PPARα and γ appear to play an important role in modulating energy metabolism in the heart, and have highlighted the similar and distinct actions they have in regards to cardiac energy metabolism and function.

In summary, the use of loss- and gain-of-function genetically modified mouse models has provided fundamental knowledge into the direct and indirect effects of key proteins that regulate or are involved in cardiac energy metabolism or function. Furthermore, these studies using genetically modified mouse models also provide insights linking these proteins involved in cardiac energy metabolism and cardiac remodeling to the development and progression of cardiomyopathies.
1.4 METHYL METABOLISM

Dietary factors required for methylation reactions are often referred to as ‘methyl nutrients’ and are required for the generation of S-adenosylmethionine (AdoMet), the key methyl donor for DNA, RNA, protein and lipids (93). Methyl nutrients include: vitamins, such as folate, vitamin B12, vitamin B6 and choline; and amino acids, such as methionine, cysteine, glycine and serine. AdoMet generation involves three interrelated biochemical pathways: the folate cycle, the methionine cycle, and the transsulfuration pathway. In addition, the synthesis of the major antioxidant, glutathione, is metabolically linked through the transsulfuration pathway. Below is a brief description of each pathway and their metabolic relationship is illustrated in Figure 1.

FOLATE CYCLE

Folate, a water-soluble B-vitamin, is an essential nutrient that must be obtained through dietary sources or supplements (17; 233; 249). Folate is the generic term for a variety of folate compounds found naturally in dietary sources and synthetically in supplements and fortified food sources. The naturally occurring dietary folates exist as reduced polyglutamates and require hydrolysis of their glutamate residues before absorption in the proximal small intestine. The major forms of folate found in the blood circulation are tetrahydrofolate (THF), 5-methylTHF (5-MTHF) and 10-formylTHF.

The active form of folate is tetrahydrofolate (THF) and functions as a donor of one-carbon units in the synthesis of purines, pyrimidines and amino acids. Folic acid enters the folate cycle by
first being reduced to dihydrofolate (DHF), and is then converted to THF by DHF reductase (DHFR). THF is converted to 5,10-MTHF by serine hydroxymethyltransferase (SHMT) and this reaction requires serine as a one-carbon donor and vitamin B6 as a cofactor. Next 5,10-MTHF is converted to 5-MTHF by MTHF reductase (MTHFR), a reaction that requires riboflavin (vitamin B2) as a cofactor. Remethylation of homocysteine to the amino acid methionine requires methyl donation from 5-MTHF, and is catalyzed by methionine synthase (MS) and its cofactor vitamin B12. Conversion of THF into 5,10-MTHF through 10-formylTHF and 5,10-MTHF is catalyzed by the trifunctional enzyme MTHF dehydrogenase. The 10-formylTHF can donate one-carbon groups for purines biosynthesis. A deficiency of vitamin B12, in the presence of adequate folate, results in an accumulation of 5-MTHF due to decreased MS activity. This is referred to as the ‘methyl folate trap’ hypothesis because the conversion of 5,10-MTHF to 5-MTHF is unidirectional, trapping 5-MTHF.

METHIONINE CYCLE

Methionine, an essential sulfur-containing amino acid, is converted to AdoMet by methionine adenosyltransferase (MAT). S-adenosylhomocysteine (AdoHcy) is formed after methyl donation followed by reversible liberation of adenosine and formation of homocysteine, and catalyzed by AdoHcy hydrolase (SAHH). Homocysteine is a non-protein forming amino acid with two possible fates: remethylation to methionine, or conversion to cysteine and glutathione by the transsulfuration pathway. Remethylation of homocysteine to methionine can be accomplished (as above) by the ubiquitous methionine synthase, which requires vitamin B12 as a cofactor and utilizes 5-MTHF as the methyl donor. Additionally, betaine-homocysteine methyltransferase
(BHMT) can also remethylate homocysteine to methionine and utilizes betaine as the methyl donor in the liver (93; 190). Betaine can be obtained from the diet or is endogenously synthesized from choline. Choline comes from phosphatidylcholine (PC), which can be obtained from the diet or is endogenously synthesized by the cytidine diphosphocholine (CDP-choline) pathway (268). An additional pathway of PC synthesis is present in other tissues, such as liver, and involves the methylation of phosphatidylethanolamine (PE) to PC by phosphatidylethanolamine methyltransferase (PEMT).

**TRANSSULFURATION PATHWAY**

Homocysteine is a non-essential amino acid irreversibly converted to cysteine, a semi-essential amino acid, by a two-step process involving the formation of cystathione by cystathionine-β-synthase (CBS), followed by conversion to cysteine by cystathionine-γ-lyase (CGL) (34; 231). Both enzymes require vitamin B6 as a cofactor. The transsulfuration pathway is only found in liver, pancreas, kidney, and intestine because of the tissue-specific activity of CBS and CGL. Cysteine is used for protein synthesis, or further metabolized to other compounds including taurine and glutathione.

**GLUTATHIONE SYNTHESIS**

Glutathione is a sulfur-containing tripeptide antioxidant that plays a key role in redox signaling (183). The synthesis of glutathione involves two sequential steps. The first reaction involves the rate limiting enzyme glutamate cysteine ligase (GCL), which catalyzes cysteine to γ-
glutamylcysteine. Next, glycine is added to $\gamma$-glutamylcysteine to form glutathione, catalyzed by glutathione synthase (GS) (238). Glutathione is recycled through the redox cycle in its reduced (GSH) and oxidized (GSSG) forms. The reduced GSH serves as an electron donor to glutathione peroxidase (GPx) in the reduction of hydroperoxides, whereas the oxidized GSSG can be converted back to GSH by glutathione reductase (8).
**Figure adapted from:** Glier M.B., Green T.J., and Devlin A.M. 2014. *Molecular Nutrition and Food Research.* 58:172-182.

**FIGURE 1. Methyl Metabolism Pathways**

AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-beta-synthase; CGL, cystathionine-gamma-lyase; CDP-choline, cytidyldiphosphoryl choline; choline-P, phosphoryl choline; DHF, dihydrofolate; DMG, dimethylglycine; GCL, glutamate cysteine ligase; GS, glutathione synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; MAT, methionine adenosyl transferase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidyl ethanolamine methyl transferase; SAHH, S-adenosyl-homocysteine hydrolase; THF, tetrahydrofolate.
1.5 DNA METHYLATION

DNA methylation involves the addition of methyl groups to the 5’ position of cytosine within CpG dinucleotides. It is estimated that 70-80% of CpGs within the genome are methylated (166). DNA methylation also occurs at cytosine residues not adjacent to guanine residues (termed ‘non-CG methylation’); however, further studies are required to determine their role in regulating gene expression (173). Methylation of DNA is accomplished by DNA methyl transferases (DNMTs) that use AdoMet as the methyl donor. *De novo* methylation occurs during embryogenesis and is accomplished by DNMT3A and 3B, whereas maintenance methylation occurs during somatic cell division and is accomplished by DNMT1 (27).

Immediately following fertilization, during the initial stages of embryo development, there is a genome-wide demethylation of the parental genomes and reprogramming of the methylation patterns (221; 226). The mechanisms involved in this ‘epigenetic reprogramming’ are complex and there is asymmetry in the mechanisms and degree to which this happens between maternal and paternal genomes. This phenomenon suggests the presence of heritable epigenetic memory, patterns which are transmitted during somatic cell division throughout the lifespan. Furthermore, this epigenetic reprogramming that occurs during early embryonic development may be especially vulnerable to environmental factors that can affect methylation. There are several examples in the literature whereby changes in nutrition status, both during development or in adulthood, can affect DNA methylation patterns and have profound effects on gene expression and phenotype (74; 108; 278; 279). Furthermore, epigenetic regulation of gene expression and
DNA methylation profiles are tissue and cell-specific so the vulnerability will be cell and tissue-specific and dependent on the developmental stage and sex of the organism.

Clusters of CpGs, often referred to as ‘CpG islands’, are commonly located within gene promoter regions and are usually unmethylated (63). These CpG-rich regions play a role in governing gene expression, especially in tissue-specific and developmental-specific patterns (63). CpG islands are also found in intragenic and intergenic regions, the functions of which remain to be fully elucidated. Methylation of DNA at sites within a promoter region, or at other regulatory sites, is often associated with silencing of gene expression (125). Silencing of gene expression can also occur by alteration of DNA methylation outside a promoter region and not within a CpG island, but in sequences up to 2 kb upstream, often referred to as ‘CpG island shores’ (138). In addition, DNA methylation plays an important role in X chromosome inactivation (291) and in allele-silencing of imprinted genes (134).

Silencing of gene expression by DNA methylation can occur by several means. For example, methylation can impede binding of regulatory factors that are required for transcriptional activation. Along this line, it is important to note that methylation silencing doesn’t always require the methylation of an entire CpG-rich region; methylation of one CpG site contained within an important regulatory factor binding site has been associated with silencing of gene expression (280). Methylated DNA can also serve as a target for the methyl-binding proteins (MeCP), MeCP1 and MeCP2, which can interfere with transcriptional activation (18). Furthermore, methylated DNA can influence chromatin structure such that it is in a repressive state and not transcriptionally active.
1.6 METHYL NUTRIENT IMBALANCE AND DNA METHYLATION

Most studies investigating methyl nutrient imbalance and DNA methylation have focused on folate status and hyperhomocysteinemia (HHcy). For example, Jacob et al. conducted a metabolic imbalance study in which postmenopausal women ($n = 8$) were placed on low folate diets (56 µg/d) for 91 days. From baseline to 41 days, plasma folate decreased ($19.5 \pm 4.2$ to $9.3 \pm 1.8$ nmol/L), homocysteine increased ($9.8 \pm 0.4$ to $12.5 \pm 1$ µmol/L), and lymphocyte global DNA methylation decreased (140). Similarly, lower global DNA methylation in leukocytes was reported in elderly women ($n = 33$) fed a low folate diet for 7 weeks (118 µg/d) and was accompanied by low serum folate concentrations ($12.1 \pm 7.2$ nmol/L) and elevated serum total homocysteine concentrations ($11.0 \pm 3.0$ µmol/L) (223).

Further evidence that methyl nutrient imbalance can affect DNA methylation comes from studies in subjects homozygous for the common (10-20% in Caucasian populations) $MTHFR 677C>T$ variant (rs1801133). This variant encodes for a thermolabile form of the enzyme and results in an impaired ability to synthesize 5-MTHF, and is often accompanied by elevated plasma total homocysteine (72; 98) and global DNA hypomethylation (43; 96; 241). Subjects homozygous for the $MTHFR 677C>T$ variant (TT genotype) with plasma folate concentrations < 12 nmol/L and elevated plasma total homocysteine concentrations (19.64-22.98 µmol/L) were reported to have lower global DNA methylation in leukocytes compared to subjects with the CC genotype (96). This suggests that the effect of the variant on global methylation may be dependent on the folate status of the individual. Subjects with renal failure on hemodialysis and HHcy ($n = 32$) had global DNA hypomethylation compared to control subjects without HHcy ($n = 11$), and a subset
(n = 3) with the most severe HHcy (≥ 62.5 μmol/L) had biallelic expression of the imprinted \( H19 \) gene because of changes in DNA methylation in leukocytes (136). Interestingly, lowering plasma total homocysteine concentrations with 5-methylTHF supplementation returned \( H19 \) to monoallelic expression in this study (136).

### 1.7 METHYL NUTRIENT IMBALANCE AND CARDIOVASCULAR DISEASE

**HYPERHOMOCYSTEINEMIA**

Much attention was previously focused on homocysteine and its possible role in CVD pathology. A meta-analysis of observational studies suggests that HHcy is a risk factor for CVD (128). It has been speculated that changes in DNA methylation may contribute to the vascular complications associated with HHcy. However, recent B-vitamin intervention trials designed to lower circulating total homocysteine concentrations failed to prevent secondary CVD events (29; 259). The negative findings of these trials may be a consequence of the fact that the circulating total homocysteine concentrations at baseline in these subjects was not elevated enough to affect CVD pathology, and therefore lowering the concentrations further would have no subsequent effect on CVD events. Despite these findings, several studies in animal models and humans have reported an association of HHcy with vascular endothelial dysfunction (defined in these studies as impaired vascular endothelium-dependent vasodilatation), an early indicator of vascular disease which often precedes primary cardiovascular events (194). For example, subjects (\( n = 24 \)) with acute HHcy (23.1 ± 5.4 μmol/L) following an oral methionine load were reported to
have vascular endothelial dysfunction (25). Similarly, studies in mouse models of HHcy produced by target disruption of genes encoding enzymes required for methyl nutrient metabolism, such as CBS, MTHFR, or MS and diet-induced HHcy have also reported endothelial dysfunction (58-60; 70; 169; 281). More recently, folate has also been implicated in endothelial dysfunction because of its effect on nitric oxide metabolism. A study in subjects \((n = 218)\) with coronary artery disease reported that in vascular tissue, the \(MTHFR\ 677C > T\) variant \((n = 24)\) was more strongly associated with 5-MTHF concentrations than in plasma and there was no association with vascular total homocysteine concentrations (7). This study also reported the \(MTHFR\ 677C > T\) variant to be associated with endothelial dysfunction and vascular superoxide production (oxidative stress) in vessel segments (7).

Recently, positive findings of folic acid supplementation were reported from The China Stroke Primary Prevention Trial, a randomized, double-blinded clinical trial among Chinese adults with hypertension and no history of stroke or myocardial infarction. In eligible participants, the combined use of the antihypertensive medication enalapril (10 mg) and folic acid (0.8 mg; \(n = 10,348\)) was associated with a reduced incidence of stroke compared with enalapril alone (10 mg; \(n = 10,354\)). In addition, this study is the first to stratify individuals by \(MTHFR\ C677T\) genotypes (CC, CT and TT) along with baseline folate concentrations, demonstrating the beneficial effect was more pronounced in participants with lower folate concentrations and the TT genotype (132).
A relationship between total cysteine and CVD risk was first described by two studies that identified an association between circulating total cysteine concentrations and BMI (81; 264). The Hordaland Homocysteine Study \((n = 5179)\) reported an independent positive association between plasma total cysteine concentrations and BMI and fat mass at baseline and at the 6 year follow-up (84). Increased weight gain has also been reported in animals models supplemented with high dietary cysteine or cysteine-rich protein diets (83; 85; 207). Taken together, these studies suggest that circulating total cysteine concentrations may be a marker of adiposity, which is an important risk factor for CVD.

The direct relationship between cysteine and CVD is not well understood. Case control studies have reported higher concentrations of plasma total cysteine concentrations in subjects with cerebral infarction (9), peripheral arterial disease (187), and myocardial infarction (269) compared to healthy controls. A cross-sectional study of subjects with hyperlipidemia reported higher circulating cysteine concentrations among those with CVD (139). Furthermore, the European Concerted Action Project, a case-control study in subjects with coronary heart disease, cerebrovascular disease or peripheral vascular disease \((n = 750)\) and control subjects \((n = 800)\), reported a U-shaped association between plasma total cysteine concentrations and CVDs (81). The greatest risk was observed in subjects with plasma total cysteine concentrations < 225 \(\mu\)mol/L and > 300 \(\mu\)mol/L. Similarly, a smaller case-control study assessed the relationship between plasma total cysteine concentrations and atherosclerosis (264). Cases with severe coronary atherosclerosis were defined as subjects with > 90% occlusion in one vessel and > 40%
occlusion in a second vessel (cases, \( n = 131 \)). Cases without coronary atherosclerosis were defined as subjects with < 50% occlusion in one vessel (coronary controls, \( n = 88 \)) and healthy subjects (population-based controls, \( n = 101 \)). This study reported higher plasma total cysteine concentrations in subjects with severe coronary atherosclerosis compared to control groups. However, this relationship disappeared after adjustment for other CVD risk factors including BMI. At present, the significance of the relationship between plasma total cysteine and CVD pathology is unclear, and may exist secondary to the association between adiposity and plasma total cysteine concentrations.

1.8 DNA METHYLATION AND CARDIOVASCULAR DISEASE

A few population studies have reported global changes in DNA methylation associated with CVD and cardiometabolic risk factors. For example, the Singapore Chinese Health Study (\( n = 286 \)) reported that male subjects with a history of CVD (myocardial infarction, stroke), or those with CVD risk factors (hypertension, diabetes), had higher global DNA methylation status in leukocytes (152). Findings from the Samoan Family Study of Overweight and Diabetes reported that lower LINE-1 methylation status in leukocytes was associated with higher LDL and lower HDL concentrations in fasting plasma (42).

Many studies have reported an association between altered DNA methylation status and atherosclerosis. Atherosclerosis is a major cause of many CVDs and is characterized by proliferation of vascular smooth muscle cells, lipid accumulation, connective tissue development, inflammatory cell infiltration, and calcification. (275). One study reported a
reduction in genomic DNA methylation content (5-methylcytosine) in the following: advanced atherosclerotic lesions from human subjects \((n = 55)\), atherosclerotic lesions from mice deficient in the gene for apolipoprotein E \((ApoE^{-/-})\), and aortic neointima from balloon denuded New Zealand White rabbits \((126)\). Since \(ApoE^{-/-}\) mice are genetically prone to the development of atherosclerosis, Lund et al. assessed whether changes in DNA methylation status occurs in the early stages of atherosclerosis, before the appearance of an atherosclerotic lesion \((181)\). This study observed detectable alterations in DNA methylation status, with both reduced and greater methylation, in aorta and peripheral blood mononuclear cells from \(ApoE^{-/-}\) mice with and without detectable atherosclerotic lesions \((181)\). These studies suggest a role for changes in DNA methylation status in the onset and progression of atherosclerosis.

Only a few studies have addressed gene-specific changes in atherosclerosis. Tissue collected from subjects undergoing coronary artery bypass grafting reported greater methylation of \(ESR1\) (encodes estrogen receptor alpha) in aorta, internal mammary artery, and saphenous vein; with the greater \(ESR1\) methylation observed in coronary artery atherosclerotic plaques \((218)\). Findings from the Verona Heart Project reported higher plasma activated coagulation factor VIIA concentrations and reduced promoter DNA methylation of \(F7\) (encodes coagulation factor VII) in peripheral blood mononuclear cells from men and women with coronary artery disease \((CAD)\) \((n = 165)\) compared to CAD-free subjects \((n = 88)\) \((97)\). A study in rabbits with atherosclerosis showed reduced methylation of \(Sod3\) (encodes extra-cellular superoxide dismutase) and global reduced methylation in atherosclerotic tissue from aorta \((164)\).
Currently, no studies have determined whether gene-specific changes in DNA methylation play a role in the development of obesity-related cardiac remodeling, dysfunction or lipotoxicity. Therefore, it is possible that changes in DNA methylation patterns are involved in the pathogenesis of obesity-related CVD, and may or may not involve methyl nutrient imbalance.
CHAPTER 2:

THESIS HYPOTHESIS AND SPECIFIC AIMS
HYPOTHESIS

The general hypothesis of my dissertation states: disturbances in methyl metabolism contribute to obesity-related cardiomyopathy.

SPECIFIC AIMS

This hypothesis was tested by the following specific aims:

AIM 1: To determine if disturbances in methyl metabolism enhances cardiac lipotoxicity associated with diet-induced (high-fat diet) obesity (excess adiposity).

AIM 2: To determine if disturbances in methyl metabolism alter cardiac energy metabolism and function associated with obesity-related cardiac remodeling.

AIM 3: To determine if disturbances in methyl metabolism contribute to changes in tissue-specific gene expression and DNA methylation associated with obesity-related cardiac remodeling.
CHAPTER 3:
DISTURBANCES IN METHYL METABOLISM ENHANCE
CARDIAC LIPOTOXICITY ASSOCIATED WITH DIET-
INDUCED OBESITY

### 3.1 INTRODUCTION

**AIM 1:** To determine if disturbances in methyl metabolism enhance cardiac lipotoxicity associated with diet-induced obesity.

**RATIONALE**

Over the last two decades, studies have begun to unravel signaling events involved in cardiac lipotoxicity (147; 262). However, the role of the endogenously synthesized antioxidant glutathione and factors controlling its homeostasis in cardiac lipotoxicity remains unclear. Glutathione acts to protect against oxidative stress, especially in tissues like the heart which require a great amount of energy to function (238). Given the minimal level of catalase in the heart, hydrogen peroxide, a major reactive oxygen species is mainly neutralized by glutathione peroxidase and the reduced form of glutathione, GSH (8). The rate limiting amino acid required for glutathione synthesis is cysteine (270). Although the importance of oxidative stress has been shown in multiple tissues affected by lipotoxicity (12; 48; 101), the role of cysteine provision and its capacity to maintain glutathione concentrations in the heart during lipotoxicity is not known.
Cysteine can be obtained from exogenous sources (diet, supplements) or endogenously from intracellular protein catabolism and homocysteine transsulfuration (35). Supplemental N-acetylcysteine increases plasma cysteine and tissue glutathione concentrations, and is protective in conditions associated with oxidative stress such as diabetes (62), ischemia-reperfusion (45) and obesity-related fatty liver disease (172). It has been estimated that at least 50% of the cysteine required for endogenous glutathione synthesis in the liver is supplied by the transsulfuration of homocysteine (197). The first and rate-limiting step in this pathway involves conversion of homocysteine to cystathionine, catalyzed by CBS, and followed by conversion to cysteine, accomplished by cystathionase. Current views suggest that the glutathione pool in the liver is responsible for maintaining glutathione concentrations in other tissues, such as the heart (165). For example, in studies using chronic bile duct ligated rats, cholestatic liver disease led to diminished glutathione concentrations in the liver and subsequently in the heart, brain, and kidney (20; 174). Therefore, disturbances in liver glutathione synthesis could have profound consequences on glutathione concentrations in other tissues and impair ability to combat oxidative stress.

Methyl metabolism and the transsulfuration pathway are well characterized in the liver and kidney; however, little is known about their involvement in other tissues such as the heart. Given the role of CBS in endogenous cysteine synthesis and the tie of cysteine to methyl metabolism, mice heterozygous for a targeted disruption of the gene that encodes CBS (Cbs +/- mice) were selected as a good ‘loss of function’ model to investigate the role of altered methyl metabolism in obesity-related cardiac lipotoxicity. To determine if disturbances in methyl metabolism enhance cardiac lipotoxicity associated with diet-induced obesity,
C57BL/6J mice (*Cbs* +/+ and *Cbs* +/-) were fed either a control-diet or a HFD (60% energy from fat) from weaning for 13 weeks to induce excess adiposity (obesity). I postulated that *Cbs* +/- mice with obesity would have enhanced cardiac lipotoxicity associated with lower cardiac glutathione concentrations, a consequence of disturbances in liver glutathione synthesis.

### 3.2 METHODS

**ANIMALS AND DIETS**

C57BL/6J mice, with (+/-) and without (+/+ ) heterozygous targeted disruption of the *Cbs* gene (277) were studied. Mouse colonies were housed and maintained in the Child and Family Research Institute animal facility. Male C57BL/6J mice were bred to female *Cbs* +/- mice at 8-10 weeks of age to produce *Cbs* +/- and *Cbs* +/+ littermate controls. Male C57BL/6J mice were purchased from the Jackson Laboratory and *Cbs* +/- mice were provided as a gift from Dr. Lentz (University of Iowa). Ear notches were collected from weanlings for genotyping of the disrupted *Cbs* allele and the wild-type allele by PCR as described previously (74). *Cbs* -/- mice were excluded from all studies in this thesis due to their high mortality rate during the early postnatal stages (277). At weaning, mice were randomly assigned and fed either a control diet (Pico-Vac Lab Rodent Diet, LabDiet, PMI Nutrition International), or a HFD (made in-house) *ad libitum* for 13 weeks to induce excess adiposity. Please see Table 1 for specific details pertaining to the experimental diets.
TABLE 1. Composition of Experimental Diets used in Aim 1, Chapter 3

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control Diet (Pico-Vac Lab Rodent Chow)</th>
<th>High-Fat Diet (Hand-made)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23.6% energy Whey, fish meal, etc.</td>
<td>20% energy Vit-free casein, L-cystine</td>
</tr>
<tr>
<td>Fat</td>
<td>11.9% energy Soybean oil</td>
<td>60% energy Butter, lard, shortening, soybean oil</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>64.5% energy Corn, oats, beet pulp, alfalfa, wheat, molasses</td>
<td>20% energy Cornstarch</td>
</tr>
<tr>
<td>Total Energy</td>
<td>4020 kcal/kg</td>
<td>5250 kcal/kg</td>
</tr>
</tbody>
</table>

The control diet is the Pico-Vac Lab Rodent Diet, a standard rodent control diet purchased by LabDiet, PMI Nutrition International. The HFD was handmade, and the macronutrient source and percent energy are: 20% energy from protein (vitamin-free casein, CA.160040, Harlan-Teklad), plus 3 g/kg L-cystine (30-2644, PCCA) to meet the requirements for sulfur amino acids in rodents; 60% energy from fat (mix of 30/30/30/10 of butter (Lucerne)/ lard (Tenderflake)/ vegetable shortening (Crisco)/ soybean oil (Crisco), respectively; and 20% energy from carbohydrate, cornstarch (CA.160170 Harlan-Taklad). The micronutrient source and amount in the HFD are: 10 g/kg of AIN-93 vitamin mix (TD.96254.PW, Harlan Teklad), 35.0 g/kg of AIN-93 mineral mix (TD.94046.PDW, Harlan Teklad), and 2.5 g/kg of choline bitartrate (C1629, Sigma) to meet the National Research Council (NRC) requirements for mice (224). The macronutrient composition of the HFD was based on the obesogenic diets used by the Jackson
Laboratory. All diets contained adequate concentrations of methyl nutrients (folic acid, cobalamin, pyridoxine, riboflavin and choline), as recommended for mice by the NRC (243).

The following 4 groups of mice were studied: $Cbs^{+/+}$, control diet; $Cbs^{+/-}$, control; $Cbs^{+/+}$, HFD; $Cbs^{+/-}$, HFD. An equal mix of male and female mice were used and studied per diet/genotype group except where otherwise stated. The sample size per diet/genotype group was 6-20 mice.

At the end of the feeding period, terminal cardiac punctures were performed following isofluorane gas anesthesia to collect blood (in EDTA treated tubes) and death was confirmed by cervical dislocation. Blood was immediately centrifuged at $3000 \times g$ for 20 minutes at 4 °C, and plasma was collected. Heart, liver and fat pads were immediately removed and weighed. **Figure 2** depicts the anatomical location of the subcutaneous and visceral fat pads collected. A small section of left ventricle was excised and placed in 4% paraformaldehyde for later histological analysis. Serum and tissue were flash-frozen in liquid nitrogen and stored at $-80^\circ$C until later analysis. All procedures were approved by the CFRI and the UBC Animal Care Committee, and are in accordance with guidelines of the Canadian Council on Animal Care.
FIGURE 2. Anatomical Location of Subcutaneous and Visceral Fat

A. Image shows a mouse in ventral recumbency to display the subcutaneous (inguinal) fat pads collected. Arrows point to the inguinal fat, situated at each side of the lumbar vertebrae column wrapping around and over the inguinal ligament. B. Image show a male mouse in dorsal recumbency to display the visceral (retroperitoneal, top arrows; gonadal, bottom arrows) fat pads collected. The retroperitoneal fat is connected situated in the peritoneum, connected to and behind each kidney. The term gonadal is used to identify visceral fat connected to and surrounding the male and female reproductive organs.

GLUCOSE TOLERANCE TEST

One week prior to the end of the feeding period, an intraperitoneal (IP) glucose tolerance test (IP-GTT) was performed (6). Following a 5 hour fast, mice were given an IP injection of 50%
dextrose at a dosage of 2 g/kg body weight. Blood (20 µl) was collected from the saphenous vein at 0 minutes (baseline) and at 15, 30, 60, 90 and 120 minutes post glucose injection. Blood glucose concentrations were quantified using a blood glucose meter (Breeze2, Bayer). Fasting insulin concentrations were quantified in plasma samples collected at baseline (time = 0 minutes) measurement during the IP-GTT, using the mouse ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics).

**BIOCHEMICAL ASSESSMENTS**

Total lipids were extracted from liver and heart by the method of Folch and Stanley (94). The organic phase was evaporated under nitrogen, the lipids were solubilized in chloroform-methanol-acetone-hexane (2.0:3.0:0.5:0.5, v/v/v/v). Individual classes of lipids were separated by high performance liquid chromatography (HLPC) (Waters 2690 Alliance, Waters Ltd). The separated lipid classes were detected and quantified by evaporative light scattering detection (Model 2000, Alltech, Mandel Scientific) as described previously (137). Calibration curves to determine the linear range of the analysis were established by using standard curves for each lipid class, and samples were quantified by using the external standard method. Three analyses were conducted for each sample.

The following plasma metabolites: total homocysteine, total cysteine, and methionine were quantified by HPLC-mass spectrometer (MS)/MS as described previously (95). The MS/MS was a Quattro Micro tandem MS configured with an electrospray source and operated in a positive ion mode, coupled to an Acquity HPLC equipped with a thermostatted autosampler (Waters
Corporation). Briefly, an internal standard for each metabolite, and dithiothreitol (500 mmol/L in 0.1 mmol/L of NaOH) was added to plasma samples (50 μL). The mixture was vortexed, and the samples were kept at room temperature for 15 minutes, to allow reduction of the disulfide bonds. Proteins were precipitated with an acetonitrile mixture containing 0.2% (v/v) heptafluorobutyric acid. The samples were centrifuged and an aliquot of supernatant (20 μL) was transferred to an autosampler vial containing (100 μL) the HPLC mobile phase. Chromatographic separation of each metabolite was completed by using a Zorbax SB Aqua column maintained at 25°C (Agilent Technologies, Canada) with a mobile phase of H₂O and 0.2% heptafluorobutyric acid. Three analyses were conducted for each sample. Quantification of lipids and metabolites were conducted in the Metabolomics core of the Nutrition and Metabolism Research Program (directed by Dr. Innis) at the CFRI.

**OXIDATIVE STRESS ASSAYS**

Total glutathione [reduced (GSH) + oxidized (GSSG)] and GSH concentrations and glutathione reductase (GR) activity were quantified using the HT Glutathione Assay kit (Trevigen). Oxidative stress-induced lipid peroxidation was estimated by direct quantification of lipid hydroperoxides in heart tissue using a commercial kit (Cayman Chemicals) as described previously (102). Protein concentrations were determined using a commercial protein assay kit (BioRad) based on the method of Bradford (31).
RELATIVE mRNA EXPRESSION

Total RNA was extracted from the left ventricle using the Fibrous RNeasy Mini Kit (Qiagen) with DNase I-treatment to remove contaminating genomic DNA. Integrity of the RNA was assessed by confirming the presence of 18s and 28s rRNA on agarose gels. RNA (500 ng) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) and real-time PCR performed using the TaqMan Universal Master Mix II, gene-specific TaqMan gene expression probes, and the 7500 Real-Time PCR System (Applied Biosystems). Relative expression of mRNAs were quantified by real-time PCR using the comparative delta delta Ct method (ΔΔCt) (49). The endogenous reference gene was 18s rRNA. Each sample was run in duplicate and each target gene was assessed twice.

IMMUNOBLOT ANALYSIS

 Samples of left ventricle were sonicated in ice-cold phosphatase inhibitor RIPA Lysis Buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Roche). Homogenates were centrifuged at 4°C, the pellets were discarded and the concentration of the supernatant was determined using a commercial protein assay kit (BioRad) based on the method of Bradford (31). Samples were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (10%) and electro-transferred to nitrocellulose membranes (Millipore). Membranes were blocked with 5% nonfat dry milk overnight at 4°C.

The following primary antibodies were used to detect the respective proteins: goat anti-human
glutamate cysteine ligase, catalytic subunit (GCLc) (Santa Cruz Biotechnology (sc)-2267), goat anti-human glutathione peroxidase 1 (GPX-1) (sc-22146), rabbit anti-human acyl CoA oxidase 1 (ACOX1) (sc-98499), rabbit anti-human PPARγ coactivator-1α (PGC1α) (sc-13067), rabbit anti-human beta-actin (sc-130657), rabbit anti-nitrotyrosine (sc-55256), mouse anti-human X-linked inhibitor of apoptotic factors (XIAP) (BD Biosciences), rabbit anti-human cleaved caspase-3 (Cell Signaling Technologies). The secondary antibodies used were mouse anti-goat (sc-2355), goat anti-mouse (sc-2047), and donkey anti-rabbit (sc-2315) antibodies conjugated to alkaline phosphatase. For loading controls, rabbit anti-human beta actin (sc-130656) was used. Two different blots were used for the study and corresponding actin bands are depicted as beta actin-1 (blot #1) and beta actin-2 (blot #2) in figures. GPX-1, caspase-3, and PGC-1α were probed on blot #1 using Restore Plus stripping buffer (Thermo Scientific) for 10 minutes each time. Proteins: GCLc, ACOX1, nitrotyrosine, and XIAP were probed on blot #2.

Fluorescence was detected using the CDP-Star chemiluminescence detection reagent (Perkin Elmer) and densities were quantified using ChemiGenius gel imaging system (Perkin-Elmer). The obtained images were analyzed using Image J (National Institutes of Health) and expressed as a ratio to beta actin levels. Immunoblots were quantified in collaboration with Dr. Ghosh.

MITOCHONDRIAL DENSITY

Mitochondrial density was estimated by quantifying the mitochondrial encoded cytochrome b gene (mt-Cytb) copy number relative to the nuclear encoded beta-actin gene (Actb) copy number using Real-time PCR (234). Genomic DNA was extracted from heart using the DNeasy Kit.
(Qiagen). Copy numbers were quantified using TaqMan Universal Master Mix II, gene-specific TaqMan gene probes, and the 7500 Real-Time PCR System (Applied Biosystems). Each sample was run in duplicate and each target gene was assessed twice.

STATISTICAL ANALYSIS

Results are reported as mean ± standard error (SEM). Two-way analysis of variance (ANOVA) was used to detect statistical differences between groups, with diet and genotype as the independent variables. If an interaction between diet and genotype was found, the effect of genotype was analyzed separately in mice fed the control diet and the HFD by a one-way ANOVA. Analyses were accomplished by SPSS statistics, version 16.0 (SPSS Inc.).

3.3 RESULTS

BODY WEIGHT AND FAT DISTRIBUTION

As expected, Cbs +/+ and Cbs +/- mice fed the HFD had greater ($P < 0.01$) body weight gain and final body weight compared to mice fed the control diet (Figure 3A and B). To assess body fat distribution, visceral and subcutaneous fat pads were removed and weights were obtained for each. Mice fed the HFD had greater ($P < 0.01$) visceral (gonadal and retroperitoneal) and subcutaneous (inguinal) fat pad mass compared to mice fed the control diet (Figure 3C-E). These findings demonstrate that Cbs +/- mice are susceptible to diet-induced obesity to a similar
extent as \( Cbs +/+ \) mice.

**GLUCOSE HOMEOSTASIS**

The effect of feeding a HFD for 12 weeks on glucose homeostasis was assessed by conducting an IP-GTT. An interaction \((P < 0.01)\) was found between diet and genotype on fasting blood glucose concentrations (**Figure 4A**). In mice fed the HFD, only \( Cbs +/- \) mice had higher \((P < 0.01)\) fasting plasma glucose concentrations compared to \( Cbs +/+ \) mice. As expected for C57BL/6J mice (5), both \( Cbs +/+ \) and \( Cbs +/- \) mice fed the HFD had similar elevations \((P < 0.001)\) in fasting plasma insulin concentrations at baseline compared to mice fed the control diet (**Figure 4B**). In addition, \( Cbs +/+ \) and \( Cbs +/- \) mice fed the HFD had mild glucose intolerance compared to mice fed the control diet (**Figure 4C**). The area under the curve (AUC) for glucose measurements during the 2 hr. IP-GTT was greater \((P < 0.001)\) in \( Cbs +/- \) mice than \( Cbs +/+ \) mice and this was further amplified in \( Cbs +/- \) mice fed the HFD (**Figure 4D**). These results suggest that diet-induced obesity in \( Cbs +/- \) mice is associated with mild changes in glucose homeostasis.

**PLASMA AND HEPATIC LIPID ACCUMULATION**

To begin to address whether \( Cbs +/- \) mice with diet-induced obesity are more susceptible to ectopic lipid accumulation, I quantified major lipid classes in plasma and liver (**Figure 5 and Table 2**). No effect of \( Cbs +/- \) genotype or diet on plasma triglyceride concentrations was observed (**Figure 5A**). As expected, mice fed the HFD had higher \((P < 0.01)\) triglyceride
concentrations in liver compared to mice fed the control diet (Figure 5B). Liver cholesteryl ester concentrations were lower \((P < 0.01)\) in mice fed the HFD compared to mice fed the control diet (Figure 5C). I observed no effect of genotype or diet on liver total cholesterol concentrations (Figure 5D). *Cbs* +/- mice had higher \((P < 0.05)\) phosphatidylethanolamine and phosphatidylinositol concentrations in liver compared to *Cbs* +/- mice (Table 2). Whereas mice fed the HFD had lower \((P < 0.01)\) phosphatidylinositol, phosphatidylserine, and cardiolipin concentrations in liver compared to mice fed the control diet (Table 2).

**CARDIAC LIPID ACCUMULATION**

To determine if *Cbs* +/- mice are more susceptible to ectopic cardiac lipid accumulation, I quantified the major lipid classes in the heart. Interestingly, *Cbs* +/- mice had higher \((P < 0.05)\) triglyceride concentrations in heart compared to *Cbs* +/- mice (Figure 6A). As expected, triglyceride concentrations in heart were higher \((P < 0.05)\) in mice fed the HFD compared to mice fed the control diet (Figure 6A). Independent of diet, *Cbs* +/- mice had higher \((P < 0.05)\) concentrations of cholesteryl ester, free cholesterol, phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine and sphingosine in heart compared to *Cbs* +/- mice (Figure 6 and Table 3). These results confirm that *Cbs* +/- are more susceptible to excess lipid accumulation in the heart at 16 weeks of age, an effect exacerbated by HFD feeding and therefore are a good model to test the role of methyl metabolism in obesity-related cardiac lipotoxicity.
CARDIAC APOPTOSIS AND OXIDATIVE STRESS

I further determined whether the greater triglyceride deposition in heart from Cbs +/- mice with diet-induced obesity was accompanied by indicators of lipotoxicity by assessing markers of apoptosis and oxidative stress (Figure 7). To assess apoptosis, I quantified protein expression of cleaved caspase-3 and X-linked inhibitor of apoptosis (XIAP). Cleaved caspase-3 expression in the heart was higher (P < 0.001) in Cbs +/- mice compared to Cbs +/+ mice and higher (P < 0.005) in mice fed the HFD compared to mice fed the control diet (Figure 7A). An interaction (P < 0.001) was found between diet and genotype on XIAP expression in the heart (Figure 7B). Expression of XIAP in the heart was lower (P < 0.01) in Cbs +/- mice compared to Cbs +/+ mice when fed the control and HFD. Nitrotyrosine expression and lipid hydroperoxides concentration were quantified in the heart as indicators of oxidative stress. Nitrotyrosine expression in the heart was higher (P < 0.001) in Cbs +/- mice compared to Cbs +/+ mice and higher (P < 0.005) in both groups of mice fed the HFD (Figure 7C). An interaction (P < 0.01) was found between diet and genotype on concentrations of lipid hydroperoxides in the heart (Figure 7D). Cbs +/- mice fed the control and HFD had higher concentrations of lipid hydroperoxides (P < 0.01) in the heart compared to Cbs +/+ mice.

Effects of the lipid deposition in Cbs +/- mice with diet-induced obesity on mitochondrial damage were assessed in the heart. Mitochondrial density was estimated by quantifying the mitochondrial-encoded cytochrome b gene (mt-Cytb) copy number relative to the nuclear-encoded beta-actin gene (Actb) (10). Cbs +/- mice fed the control diet and HFD had fewer (P < 0.01) copies of mt-Cytb/Actb compared to Cbs +/+ mice (Figure 8A). Next, protein expression
of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), a prime regulator of mitochondrial biogenesis was quantified in the heart (10). Also independent of diet, PGC-1α expression in heart was higher ($P < 0.001$) in $Cbs +/-$ mice compared to $Cbs +/-$ mice (Figure. 8B). These results indicate an up-regulation of PGC-1α expression as a compensatory mechanism to induce mitochondrial biogenesis (250). I assessed if the higher expression of PGC-1α in $Cbs +/-$ mice was accompanied by altered expression of acyl-CoA oxidase 1 (ACOX1) in the heart, which is required for peroxisomal fatty acid β-oxidation (38), but found no effect of $Cbs$ genotype or HFD (Figure. 8C). Together these findings suggest that $Cbs +/-$ mice demonstrate mitochondria-specific damage and are more susceptible to obesity-related cardiac lipotoxicity and oxidative stress.

**CARDIAC GENE TARGETS OF OXIDATIVE STRESS**

I quantified relative mRNA expression of genes involved in oxidative stress and fatty acid metabolism in the heart (Figure 9). The following gene targets were selected and analyzed: $Pgcla$ (peroxisome proliferator-activated receptor gamma coactivator-1α), widely known to stimulate mitochondrial biogenesis (290); $Acox1$ (acyl-CoA oxidase 1); $Cpt1b$ (carnitine palmitoyltransferase 1b), mitochondrial fatty acid transporter (265); $Nrf1$ (nuclear respiratory factor-1), regulates genes in mitochondrial replication and biogenesis (292); $Rela$ (p65 NF Kappa B subunit), regulates genes involved in oxidative stress and inflammation (225); $Ppara$ (peroxisome proliferator-activated receptor alpha), regulates genes involved in fatty acid oxidation (185). $Cbs +/-$ mice had higher ($P < 0.01$) mRNA expression of $Pgc1a$, $Acox1$, $Cpt1b$ and $Nrf1$ expression in the heart compared to $Cbs +/-$ mice (Figure. 9A-D). An interaction ($P <$
0.01) was found between diet and genotype on mRNA expression of *Ppara* and *Rela* in the heart (Figure. 9E-F). In mice fed the control diet, mRNA expression of both *Ppara* and *Rela* were higher in *Cbs +/−* mice compared to *Cbs +/+* mice (Figure 9E-F). These findings further demonstrate that *Cbs +/−* mice are more susceptible to obesity-related cardiac oxidative stress.

**CARDIAC GLUTATHIONE HOMEOSTASIS**

As a first step towards assessing the mechanisms underlying the augmented cardiac lipotoxicity and oxidative stress in *Cbs +/−* mice with diet-induced obesity, I assessed changes in cardiac glutathione homeostasis. *Cbs +/−* mice had lower total glutathione (GSH + GSSG) (*P* < 0.01) and GSH (*P* < 0.01) concentrations in heart compared to *Cbs +/+* mice, an effect observed in both control-fed and HFD-fed mice (Figure 10A and B). No effect of diet or genotype on GSSG was observed (Figure 10C). To investigate this further, I assessed expression of proteins involved in glutathione synthesis and metabolism. Synthesis of glutathione is catalyzed by the rate-limiting enzyme glutamate cysteine ligase, a heterodimer that consists of a catalytic subunit (GCLc), with GCLc activity upregulated by oxidative stress (130). An interaction (*P* < 0.01) was found between diet and genotype on GCLc expression in heart (Figure 10D). Expression of GCLc in the heart was lower (*P* < 0.05) in *Cbs +/−* mice fed the control diet, but higher (*P* < 0.005) in *Cbs +/−* mice fed the HFD compared to *Cbs +/+* mice. An additional pathway important in maintaining tissue glutathione homeostasis is recycling of GSSG to GSH, catalyzed by GR (113). *Cbs +/+* and *Cbs +/−* mice fed the HFD had lower (*P* < 0.005) GR concentrations compared to those fed control (Figure 10E). I also assessed expression of glutathione peroxidase 1 (GPX-1), an antioxidant enzyme that utilizes GSH (238). An interaction (*P* < 0.01) was found
between diet and genotype on GPX-1 expression in heart (Figure 10F). In mice fed the HFD, GPX-1 expression in heart was lower ($P < 0.01$) in $Cbs$ +/- mice compared to control fed mice (Figure 10F). Taken together, these findings suggest that a reduced pool of glutathione and reduced ability to utilize glutathione as an antioxidant in the heart may contribute to the enhanced cardiac lipotoxicity in $Cbs$ +/- mice with diet-induced obesity.

LIVER GLUTATHIONE METABOLISM

Given that the glutathione pool in the liver is thought to be responsible for maintaining glutathione concentrations in other tissues such as the heart (20; 165; 174), the effect of diet-induced obesity in $Cbs$ +/- mice on liver glutathione concentrations was determined. Interestingly, no effect of diet or genotype on total glutathione, GSH, and GSSG were found in liver (Figures 11A-C). These findings, in addition to the observed higher hepatic triglyceride concentrations in mice fed the HFD, suggest that the liver is likely able to utilize glutathione as an antioxidant.

CARDIAC CBS EXPRESSION AND PLASMA METABOLITES

It has been estimated that at least 50% of the cysteine required for endogenous glutathione synthesis in the liver is supplied by the transsulfuration of homocysteine (197). However, little transsulfuration enzyme CBS expression is thought to be present in heart (35) and therefore, endogenous glutathione synthesis in heart is dependent on cysteine availability. As such, I questioned whether the reduced glutathione concentrations and cardiac lipotoxicity in heart from
Cbs +/- mice with diet-induced obesity were a result of decreased cysteine availability for endogenous glutathione synthesis by heart. As a first step, to determine if this is the case, mRNA expression of Cbs was quantified in the heart relative to other tissues (brain, liver) and between diet and genotype groups (Figure 12). The mRNA expression of Cbs was detectable in heart, but at very low levels relative to other tissues (Figure 12A). No differences were observed for mRNA expression of Cbs between diet or genotype groups (Figure 12B). As the mRNA expression of CBS in the heart is minimal, the functional significance remains to be determined.

Methionine, total homocysteine, total cysteine and total glutathione concentrations in plasma were quantified (Figure 13). Interestingly, mice fed the HFD had lower methionine concentrations in plasma compared to mice fed the control diet (Figure 13A). Cbs +/- mice fed the control and HFD had higher ($P < 0.05$) total homocysteine concentrations in the plasma compared to Cbs +/- mice (Figure 13B). An interaction ($P < 0.01$) was found between diet and genotype on cysteine concentrations in plasma. Total cysteine concentrations in the plasma were higher ($P < 0.01$) in Cbs +/- mice fed the control diet but lower ($P < 0.01$) in Cbs +/- mice fed the HFD, when compared to Cbs +/- mice (Figure 13C). For the reason that plasma homocysteine and total cysteine concentrations were higher in Cbs +/- mice fed the control diet, could suggest that the transsulfuration pathway is up-regulated in the liver of these mice. The finding that Cbs +/- mice fed the HFD had lower plasma total cysteine, could suggest there is a reduced availability of circulating cysteine for glutathione synthesis by the heart in Cbs +/- mice with diet-induced obesity.
FIGURE 3. Diet-induced Obesity at 13 Weeks

A. Body weight gain from weaning for 13 weeks. B. Terminal body weight at 13 weeks.

Visceral adiposity assessed by C. gonadal and D. retroperitoneal fat pad weights. E.

Subcutaneous adiposity assessed by inguinal fat pad weights. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 9-20 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.05.
FIGURE 4. Glucose Homeostasis at 12 Weeks Post-Weaning

Fasting (5 hour) baseline measurements of **A.** blood glucose and **B.** plasma insulin concentrations. **C.** Blood glucose concentrations at baseline and following the intraperitoneal (IP) injection of 50% dextrose at 2.0 g/kg body weight. **D.** Area under the curve (AUC) for glucose concentrations during the glucose tolerance test (GTT). Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-6 male mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, *P* < 0.01; *Effect of diet, P* < 0.01; **Effect of genotype, P* < 0.01.
FIGURE 5. Plasma and Hepatic Lipid Accumulation

A. Plasma triglyceride concentrations. Hepatic lipid accumulation assessed by B. triglyceride, C. cholesteryl ester, D. free cholesterol and E. total phospholipids concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/− mice, (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.01; **Effect of genotype, P < 0.01.
TABLE 2. Hepatic Phospholipid Classes

<table>
<thead>
<tr>
<th>Lipid Classes</th>
<th>Control</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cbs +/+</td>
<td>Cbs +/-</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>57.48 ± 1.11</td>
<td>59.20 ± 1.10</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.98 ± 1.40</td>
<td>31.60 ± 1.02**</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>12.54 ± 0.28</td>
<td>13.18 ± 0.28**</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>5.60 ± 0.09</td>
<td>5.74 ± 0.20</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>12.18 ± 1.60</td>
<td>11.18 ± 0.60</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>7.36 ± 0.14</td>
<td>7.68 ± 0.30</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>6.89 ± 0.12</td>
<td>7.26 ± 0.19</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 5-6 mice per diet/genotype group). *Effect of diet, P < 0.01; **Effect of genotype, P < 0.01.
FIGURE 6. Cardiac Lipid Accumulation

Cardiac lipid accumulation assessed by A. triglyceride, B. cholesteryl ester, C. free cholesterol, and D. total phospholipids concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.05; **Effect of genotype, P < 0.05.
### TABLE 3. Cardiac Phospholipid Classes

<table>
<thead>
<tr>
<th>Lipid Classes</th>
<th>Control</th>
<th></th>
<th>HFD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cbs +/+</td>
<td>Cbs +/-</td>
<td>Cbs +/+</td>
<td>Cbs +/-</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>9.88 ± 0.29</td>
<td>12.04 ± 1.63</td>
<td>9.87 ± 0.14</td>
<td>11.28 ± 1.42</td>
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<tr>
<td>Phosphatidylethanolamine</td>
<td>6.04 ± 0.38</td>
<td>7.10 ± 0.85</td>
<td>7.63 ± 0.12</td>
<td>7.73 ± 1.16</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1.14 ± 0.04</td>
<td>1.46 ± 0.22**</td>
<td>1.13 ± 0.02</td>
<td>1.48 ± 0.16**</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>1.04 ± 0.07</td>
<td>1.56 ± 0.31**</td>
<td>0.87 ± 0.06</td>
<td>1.45 ± 0.29**</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>2.42 ± 0.13</td>
<td>4.28 ± 1.34**</td>
<td>1.93 ± 0.11</td>
<td>3.73 ± 0.95**</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>0.98 ± 0.05</td>
<td>1.36 ± 0.27**</td>
<td>0.80 ± 0.04</td>
<td>1.35 ± 0.23**</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>3.54 ± 0.16</td>
<td>4.54 ± 0.68</td>
<td>3.63 ± 0.11</td>
<td>3.95 ± 0.63</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 4-6 mice per diet/genotype group). **Effect of genotype, P < 0.05.
FIGURE 7. Cardiac Apoptosis and Oxidative Stress

Markers of cardiac apoptosis were assessed by A. cleaved caspase-3 expression and B. XIAP protein expression. Indicators of cardiac oxidative stress were assessed by C. nitrotyrosine protein expression and D. lipid hydroperoxide concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, P < 0.01; *Effect of diet, P < 0.01; **Effect of genotype, P < 0.01.
FIGURE 8. Cardiac Mitochondrial Dysfunction

Cardiac mitochondrial dysfunction was assessed by A. mitochondrial-encoded cytochrome b gene (mt-Cytb) copy number, and B. PGC-1α and C. ACOX-1 protein expression. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. **Effect of genotype, P < 0.01.
FIGURE 9. Cardiac Gene Targets Involved in Oxidative Stress

Cardiac mRNA expression of genes involved in oxidative stress: A. 

Pgc1a, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; B. Acox1, acyl-CoA oxidase 1; C. Cpt1b, carnitine palmitoyltransferase 1b; D. Nrf1, nuclear respiratory factor 1; E. Rela, p65 NF Kappa B subunit; and F. Ppara, peroxisome proliferator-activated receptor alpha. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, P < 0.01. **Effect of genotype, P < 0.01.
FIGURE 10. Cardiac Glutathione Metabolism

A. Total glutathione, B. reduced glutathione (GSH) and C. oxidized glutathione (GSSG) concentrations in the heart. D. Glutamate cysteine ligase catalytic subunit (GCLc) expression, E. glutathione reductase concentrations and F. Glutathione peroxidase 1 (GPX-1) expression in the heart. Open bars, Cbs +/+ mice; closed bars, Cbs +/− mice, (n = 5 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, P < 0.01; *Effect of diet, P < 0.01; **Effect of genotype, P < 0.01.
FIGURE 11. Hepatic Glutathione Metabolism

Hepatic glutathione metabolism assessed by A. total glutathione, B. reduced glutathione (GSH), and C. oxidized glutathione (GSSG) concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 7-11 mice per diet/genotype group). Data presented as mean ± SEM.

FIGURE 12. Expression of Cbs in Various Organs

A. Expression of mRNA in heart, aorta, brain and liver from Cbs +/- mice. B. Expression of mRNA in heart. Open bars, Cbs +/- mice; closed bars, Cbs +/- mice, (n = 5 mice per diet/genotype group). Data presented as mean ± SEM.
FIGURE 13. Plasma Methionine, Homocysteine, and Cysteine

Plasma metabolites assessed by A. total methionine, B. homocysteine, and C. cysteine concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-9 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, $P < 0.01$; *Effect of diet, $P < 0.01$; **Effect of genotype, $P < 0.01$.

3.5 DISCUSSION

Disturbances in glutathione homeostasis have been reported in Cbs +/- mice (272) and over expression of the glutathione utilizing enzyme, GPX-1, in these mice attenuates the endothelial dysfunction found in this model (282). However, the direct role of CBS in maintaining cardiac glutathione status has not been reported. The goal of this study was to test the hypothesis that Cbs +/- mice fed a HFD to induce obesity will have decreased glutathione concentrations and lipotoxicity in the heart because of disturbances in liver glutathione synthesis. There are three main findings in this study. First, Cbs +/- mice are susceptible to diet-induced obesity to a similar degree as Cbs +/+ mice but have greater glucose intolerance. Cbs +/- mice had lower total glutathione and GSH concentrations in heart and this occurred to the greatest extent in Cbs
+/- mice fed the HFD. The disturbances in glutathione homeostasis in heart were accompanied by triglyceride accumulation, enhanced oxidative stress, and markers of pro-apoptotic signaling. Lastly, total glutathione concentrations in liver were unaffected by Cbs +/- genotype or the HFD. However, Cbs +/- mice with diet-induced obesity had lower plasma total cysteine concentrations suggesting lower provision of cysteine for heart glutathione synthesis. Taken together, these findings suggest an important role for CBS in the maintenance of cardiac glutathione and in the prevention of cardiac lipotoxicity associated with diet-induced obesity in young adult mice.

Cysteine and glutathione are linked to lipid metabolism through the methionine cycle. Previous studies have demonstrated disturbances in lipid metabolism in liver from Cbs +/- mice with hyperhomocysteinemia, (74) (200, 284) but little is known regarding lipid metabolism in the heart of Cbs +/- mice or the effect of feeding a HFD. Interestingly, impaired glutathione homeostasis in heart from Cbs +/- mice was accompanied by triglyceride accumulation in heart, and this occurred to the greatest extent in Cbs +/- mice fed the HFD. The underlying mechanism to account for this finding is unknown but could be linked to reduced mitochondrial density. A decrease in glutathione status, that was observed in the heart from Cbs +/- mice fed the HFD, can lead to an accumulation of hydrogen peroxide, which decomposes to form a highly reactive hydroxyl radical, causing membrane lipid peroxidation (246) and damages the mitochondria (103). Higher concentrations of lipid hydroperoxides in heart from Cbs +/- mice were observed, suggesting oxidative stress. This was also accompanied by increased nitrotyrosine concentrations in the heart. These findings suggest that there may be increased peroxynitrite, a reactive nitrogen species, which nitrates tyrosine residues of proteins to form nitrotyrosine and is in accordance
with previous reports that showed increased concentrations of nitrotyrosine in aorta from C57BL/6J mice fed a HFD (196).

Given that the pool of glutathione in liver is thought to regulate glutathione concentrations in extra-hepatic tissues (174), I hypothesized that Cbs +/- mice fed the HFD would be more susceptible to cardiac lipotoxicity because of diminished liver glutathione. I found no effect of the Cbs +/- genotype or HFD-feeding on liver total glutathione concentrations, however I did find an effect in heart. Prior studies have either failed to detect CBS expression (200) or found marginal CBS activity (46) in heart. I postulated that if CBS is expressed in vascular endothelial cells (274) and smooth muscle cells (46), which are present in the heart (from coronary arteries), it would be present in heart homogenates. I did find Cbs mRNA expression in heart, but at very low levels relative to other tissues. This could suggest that the heart does not have a functional transsulfuration pathway.

Whether disturbances in glutathione homeostasis could account for the lower concentrations of reduced GSH and total glutathione in heart was also assessed. In this regard, the higher GCLc expression in heart from Cbs +/- mice fed the HFD was expected as this enzyme is known to be upregulated under conditions associated with reduced GSH depletion (178). Glutathione reductase activity, the primary enzyme responsible for recycling reduced GSH from its oxidized form (GSSG), was the lowest in the Cbs +/- mice fed the HFD, which could be an important factor underlying the lower reduced GSH concentrations in heart from these mice. In addition, I also observed decreased GPX-1 expression in heart from Cbs +/- mice fed the HFD, which may simply be the result of a decreased requirement for the enzyme because of decreased GSH
concentrations, the enzyme substrate, and may result in ineffective removal of reactive oxygen species. Such mechanisms may explain lower reduced GSH concentrations \textit{per se}, that does not explain lower total glutathione concentrations in the heart from \textit{Cbs} +/- mice fed either diet, which is reflective of tissue glutathione biosynthesis. This suggests that other factors, such as diminished cysteine availability, may also contribute to the vastly reduced glutathione status in the heart.

Furthermore, given that the heart most likely lacks a functional transsulfuration pathway, I predict that it is more reliant on circulating cysteine provision to maintain glutathione concentrations, to combat oxidative stress. Interestingly I found lower plasma total cysteine concentrations in \textit{Cbs} +/- mice with diet-induced obesity compared to \textit{Cbs} +/+ mice. These findings are in contrast to reports in humans that have shown a positive relationship between plasma total cysteine concentrations and BMI and fat mass (84). The mechanism underlying the decreased plasma cysteine concentrations in \textit{Cbs} +/- mice fed the HFD is unknown, but may involve the liver and oxidative stress. A prior study showed that the transsulfuration pathway in hepatocytes is up-regulated under conditions of oxidative stress (273). Given that \textit{Cbs} +/- mice have diminished capacity to endogenously synthesize cysteine because they only have one functioning \textit{Cbs} allele, I postulate that in these mice, the endogenously synthesized cysteine may be consumed by the liver to maintain glutathione concentrations to combat oxidative stress associated with HFD-feeding (41). If this were the case, it could account for the lack of differences in liver total glutathione concentrations between \textit{Cbs} +/+ and \textit{Cbs} +/- mice fed the HFD and lower levels of plasma cysteine concentrations in \textit{Cbs} +/- mice fed the HFD. As a result, less cysteine is available in the circulation and for glutathione synthesis in other tissues,
such as heart. Given that $Cbs^{+/-}$ mice with HFD-induce obesity have decreased glutathione concentrations and lipotoxicity in the heart, suggest these hearts are dependent on cysteine provision for glutathione synthesis, and therefore more susceptible to oxidative stress.
CHAPTER 4:
DISTURBANCES IN METHYL METABOLISM ALTER CARDIAC ENERGY METABOLISM AND FUNCTION ASSOCIATED WITH OBESITY-RELATED CARDIAC REMODELING
4.1 INTRODUCTION

AIM 2: To determine if disturbances in methyl metabolism alter cardiac energy metabolism and function associated with obesity-related cardiac remodeling.

RATIONALE

Obesity-related excess cardiac lipid accumulation is associated with changes in cardiac function and metabolism, which may contribute to the increased risk of heart failure in obese subjects (148; 286). In the setting of obesity, excess lipid accumulation leads to an imbalance between fatty acid uptake and oxidation, which eventually results in cardiac remodeling and dysfunction (32; 262). Little is known about methyl metabolism in the heart and whether disturbances in methyl metabolism can alter cardiac lipid metabolism, like what has previously shown for liver by Devlin et al (73; 74). In Aim 1 (Chapter 3), Cbs +/- mice with diet-induced obesity had greater glucose intolerance and changes in methyl metabolism, which was accompanied by diminished cardiac glutathione concentrations and greater cardiac lipid accumulation and lipotoxicity (104). The molecular mechanisms underlying obesity-related cardiac lipotoxicity are still unclear.

Therefore, I postulate that CBS may have an indirect role in cardiac lipid metabolism and contribute to the pathology of obesity-related cardiometabolic dysfunction. This study is an extension of the preliminary findings found in Aim 1. As a next step towards assessing my overall thesis hypothesis, my second AIM is to determine if disturbances in methyl metabolism
are associated with alterations in cardiac energy metabolism and function in obesity-related cardiac remodeling. Similar to Aim 1, young adult C57BL/6J Cbs +/+ and Cbs +/- mice were fed either a control-diet or a HFD (60% energy from fat) from weaning for 20 weeks to induce obesity-related cardiac dysfunction. This feeding time is longer than in Aim 1/Chapter 3 (mice were fed for 13 weeks) and was selected based on published work by others that report 20 weeks of HFD feeding is sufficient to produce cardiac dysfunction, as indicated by a reduction in ventricular fractional shortening (212).

4.2 METHODS

ANIMALS AND DIETS

A second set of Cbs +/- and Cbs +/- mice were used this Aim that were from the same colony of mice used in Aim 1 (Chapter 3). The experimental design is similar to that of Aim 1 (Chapter 3), but with two changes. First, Cbs +/- and Cbs +/- mice were fed either the HFD or the control diet from weaning, for 20 weeks instead of 13 weeks to induce excess adiposity. Secondly, only male mice were studied (n = 5-8). Given tissue limitations, a second set of mice was used in the isolated working heart experiments, glucose tolerance, and quantification of serum, liver and heart thiol concentrations. In this subset, a hand-made control diet was used instead of the purchased control diet (Pico-Vac Lab Rodent Diet, LabDiet) to control for the levels of cysteine in the diet. Please see Table 4 below, for specific details pertaining to the macronutrient contents.
Table 4. Composition of Experimental Diets used in Aim 2, Chapter 4

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<th>Nutrient</th>
<th>Source</th>
<th>Control Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>Vitamin-free test casein (CA.160040, Harlan-Teklad)</td>
<td>20% energy Vit-free casein</td>
<td>20% energy Vit-free casein</td>
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<tr>
<td><strong>Fat</strong></td>
<td>Mixed (details below)</td>
<td>16% energy Soybean oil</td>
<td>60% energy Butter, lard, shortening, soybean oil</td>
</tr>
<tr>
<td><strong>Carbohydrate</strong></td>
<td>Cornstarch (CA.160170, Harlan-Teklad)</td>
<td>64% energy Cornstarch</td>
<td>20% energy Cornstarch</td>
</tr>
<tr>
<td>Cellulose</td>
<td>CA.160390, Harlan-Teklad</td>
<td>50.0 g/kg</td>
<td>77.5 g/kg</td>
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<tr>
<td>AIN Vitamin Mix</td>
<td>AIN-93 (TD.96254.PWD, Harlan-Teklad)</td>
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<td>10.0 g/kg</td>
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<tr>
<td>AIN Mineral Mix</td>
<td>AIN-93G (TD.94046.PWD, Harlan-Teklad)</td>
<td>35.0 g/kg</td>
<td>35.0 g/kg</td>
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<tr>
<td>Choline</td>
<td>Choline Bitartrate (C1629, Sigma)</td>
<td>2.5 g/kg</td>
<td>2.5 g/kg</td>
</tr>
<tr>
<td>Cysteine</td>
<td>L-cystine (30-2644, PCCA)</td>
<td>3.0 g/kg</td>
<td>3.0 g/kg</td>
</tr>
<tr>
<td><strong>Total Energy</strong></td>
<td></td>
<td>3948 kcal/kg</td>
<td>5250 kcal/kg</td>
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The control diet is identical to the HFD in all aspects except for the energy provided by macronutrients. The control diet in Chapter 4 was almost identical to the control diet used in Chapter 3. The control diet was handmade and the details are below. The macronutrient source and percent energy in the control diet are: 20% energy from protein, vitamin-free casein (Harlan-Teklad), supplemented with 3 g/kg of L-cystine (PCCA) to meet the requirements for sulfur amino acids in rodents; 16% energy from fat, soybean oil (Crisco); 64% energy from carbohydrates, cornstarch (Harlan-Teklad). The micronutrient sources and amount in the control diet are: 10 g/kg of AIN-93 vitamin mix (Harlan-Teklad); 35.0 g/kg of AIN-93 mineral mix (Harlan-Teklad); and 2.5 g/kg of choline bitartrate (Sigma) to meet the NRC (224). The HFD used in Chapter 4 was identical to the HFD in Chapter 3. All diets contained adequate concentrations of methyl nutrients (folic acid, cobalamin, pyridoxine, riboflavin and choline), as recommended for mice by the NRC (243). The methyl nutrients for diets used in Chapters 3 and 4 were identical.

At the end of the feeding challenge, the procedures for terminal blood collection and tissue harvest were performed as in Aim 1 (Chapter 3). Following cardiac puncture, blood sat at room temperature for 15 minutes, centrifuged at 3000 × g for 10 min and serum was collected flash-frozen in liquid nitrogen and stored at −80°C until later analysis.

WHOLE BODY COMPOSITION ANALYSIS

At 1 week prior to the end of the feeding period total body composition was quantified in conscious animals by quantitative magnetic resonance (QMR) technology (EchoMRI), as
previously described (109). The QMR technology distinguishes differential proton states
between fat and lean tissues, and free water. Mice were individually placed into the QMR system
for about 1 minute while the machine quantified fat and lean mass.

**GLUCOSE TOLERANCE TEST**

Two weeks prior to the end of the feeding period an IP-GTT was performed as previously
reported in Aim1 (Chapter 3). In this Aim the dose of dextrose was lowered to 1.5 g/kg body
weight to account for predicted higher glucose readings as a result of greater body weights in
mice fed a HFD for 18 weeks as opposed to 13 weeks.

**BIOCHEMICAL ASSESSMENTS**

Commercial ELISAs (Alpco) were used to quantify serum total and high molecular weight
(HMW) adiponectin concentrations and insulin concentrations. Fasting serum non esterified fatty
acid (NEFA) concentrations were quantified using a commercial colorimetric assay (Wako).

Total lipids in hearts were quantified as trimethyl-silyl ester (TME) derivatives by high
temperature gas lipid chromatography (GC) using the method of Myher and Kuksis (198). To
dephosphorylate phospholipids, heart homogenates (5 mg) were digested with a phospholipase-C
solution (17.5mM Tris, pH 7.3; 10mM CaCl₂; 2 units of phospholipase-C (*Clostridium welchii*)
and 2ml of diethyl ether). Samples were shaken vigorously at 30°C for 2 hours. The mixture was
diluted with 2:1 chloroform:methanol and tridecanoin was used as an internal standard in each
sample extracted. Extracts were separated into lipid fractions by thin-layer chromatography, and either dried with nitrogen gas or stored in a phosphate buffered saline for later use. Dried extracts were derived with of BSTFA (N,O-(bis-trimethylsilyl)trifluoroacetamide) and thoroughly mixed. Samples were then dried with nitrogen gas and re-dissolved in hexane. Profiles of lipids were quantified by GC (Agilent GC model 6890) equipped with a hydrogen flame ionization detector and column.

Total fatty acids were measured as fatty acid methyl esters (FAMEs) (55). Briefly, homogenates of frozen heart tissue were made in phosphate buffered saline. Lipids were extracted from a volume of homogenate equivalent to 5 mg of wet tissue mass using a modification of a Folch-type extraction employing 2:1 chloroform:methanol (94). Pentadecanoic acid was added to each extract as an internal standard. Samples were hydrolyzed by adding potassium hydroxide, in methanol, and refluxed for 1 hour at 110°C, followed by cooling. Hexane and boron trifluoride, in methanol, was added to samples and further refluxed at 110°C for 1 hour. After cooling, H2O was added and samples were thoroughly mixed. After phase separation the top layer was removed, dried and re-dissolved in hexane. Derivatized fatty acids were separated by (Agilent GC model 6890) equipped with a flame ionization detector. Quantification of both tissue lipids and fatty acids were conducted in Dr. Jacobs’s laboratory by Dr. da Silvia at the University of Alberta.

Total homocysteine, total cysteine and total glutathione concentrations in tissue and serum were quantified by HPLC with fluorescence detection using N-(2-mercaptopropionyl) glycine as internal standard (214). Tissue concentrations were quantified with the use of aqueous standards
and in serum with the use of serum spiked standard curves, as previously described in Aim 1, Chapter 3. AdoMet and AdoHcy concentrations in heart were quantified by HPLC using ultraviolet detection, as described previously (30). Samples were deproteinized in ice-cold 0.4 mol/L perchloric acid, homogenized, and centrifuged. The supernatant fraction was immediately frozen and stored at -80°C for later analysis of AdoMet and AdoHcy concentrations. Quantification of tissue and serum thiols was conducted in Dr. Lammer’s laboratory by Benny Chan at UBC.

**RELATIVE QUANTIFICATION OF MRNA EXPRESSION**

Similar materials and methods were used as previously described in Aim 1 (Chapter 3)

**IMMUNOBLOT ANALYSIS**

Similar materials and methods were used as previously described in Aim 1 (Chapter 3) unless otherwise stated here. The following commercial rabbit anti-mouse antibodies were used: Adenosine monophosphate activated protein kinase alpha (AMPKα) (Cell Signaling Technology (sc) -2603), phosphorylated-AMPKα (cs-2535), collagen type I alpha 1 (COL1A1) (sc-25974), and collagen type 3 alpha 1 COL3A1 (sc-28888). The alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as a secondary antibody (sc-2030). The loading control used was rabbit anti-human beta actin (sc-130656). Each protein target plus the loading control were probed on individual polyvinylidene fluoride (PVDF) membranes (Millipore).
TRANSTHORACIC ECHOCARDIOGRAPHY

At the end of the feeding period, transthoracic 2D echocardiography was performed on mice lightly anesthetized with 2% isoflurane, using a VEVO 770 High Resolution Imaging System equipped with an ultrasound 30-MHz transducer (RMV 716; Visual Sonics). M-mode, or motion mode, was used to visualize the heart’s structures (cross-sectional images) during systole and diastole. Cardiac structure was assessed by the following measurements at end diastole and end systole: left ventricular internal diameter (LVID, mm), left ventricular posterior wall thickness (LVPW, mm), and internal ventricular septum wall thickness (IVS, mm). Cardiac function was estimated by the following calculations generated by the manufacturer’s software: fractional shortening % = [(diastolic LVID − systolic LVID)/diastolic LVID] x 100%; left ventricular volume (LVV, µl) by using the Teichholz formula at end diastole and end systole = 7.0/(2.4 + LVID) x LVID^3; ejection fraction % = [(diastolic LVV − systolic LVV)/diastolic LVV] x 100%. All values represent an average of 3 measurements for each animal. These experiments were conducted in the Genetically Engineered Models Facility at the Centre for Heart and Lung Research/James Hogg Research Centre at St. Paul’s Hospital (Vancouver).

ISOLATED WORKING HEART FUNCTION

Heart function and substrate utilization were measured in isolated working mouse hearts, as previously described (2; 23; 36). At the end of the feeding period, mice were anesthetized by 4% isoflurane gas anesthesia and the hearts were isolated and perfused as working heart preparations using a modified Krebs-Henseleit solution, supplemented with substrates at physiologically
relevant concentrations. The Krebs Henseleit solution consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 0.6 mM palmitate bound to 3% BSA, 11 mM glucose, 1.5 mM lactate, 0.15 mM pyruvate, and 20 mU/ml insulin was oxygenated with 95%O2 and 5% CO2 and was maintained at 37°C throughout.

Hearts were perfused in working heart mode with a preload of 11.5 mm Hg and an afterload of 60 mm Hg for 30 minutes. Heart rate and peak systolic pressure were measured by means of a pressure transducer (Viggo-Spectramed) inserted into the afterload line. Cardiac output and aortic flow were measured using external flow probes (Transonic Systems Inc.) attached to the preload and aortic outflow lines, respectively. From these measurements the following were calculated: coronary flow = cardiac output – aortic flow; rate pressure product = heart rate x peak systolic pressure; and hydraulic work = cardiac output x peak systolic pressure.

**ISOLATED WORKING HEART SUBSTRATE UTILIZATION**

Radiolabelled substrates were added to the Krebs-Henseleit solution in order to determine the rates of palmitate oxidation and glycolysis, as previously described (2). Palmitate oxidation was determined by quantitative collection of 14CO2 released from either [1-14C]-palmitate as a gas and dissolved in the Krebs-Henseleit solution as [14C]-bicarbonate. Rates of glycolysis were determined by quantitatively measuring the rate of 3H2O production released into the perfusate from [5-3H]-glucose. After completion of perfusion, hearts were freeze clamped, weighed, and stored at -80°C for further analysis.
HISTOLOGICAL ASSESSMENT

Sections of left ventricle were fixed in 4% paraformaldehyde solution overnight and then paraffin-embedded, followed by staining with hematoxylin and eosin (H&E) or Masson’s trichrome for detection of collagen. A total of five photomicrographs were captured using an Olympus BX61 microscope at 400X and 40X magnification and morphometric analysis of blue stained (fibrotic sections) was accomplished by Image Pro Plus software.

STATISTICAL ANALYSIS

Statistical analysis is identical to that described in Aim 1 (Chapter 3).

4.3 RESULTS

DIET-INDUCED OBESITY AT 20 WEEKS

As expected, Cbs +/+ and Cbs +/- mice fed the HFD had similar body weight gain \( (P < 0.001) \) over the 20 week-feeding period compared to mice fed the control diet (Figure 14A). Cbs +/- mice had less \( (P < 0.05) \) fat mass, compared to Cbs +/+ mice, irrespective of diet. Furthermore, mice fed the HFD had greater \( (P < 0.001) \) fat mass compared to mice fed the control diet (Figure 14B). No effect of genotype or diet on lean mass was observed (Figure 14C). Interestingly, I found an interaction \( (P < 0.05) \) between diet and genotype on epididymal and retroperitoneal fat pad size (Figure 14D-E). In mice fed the control diet, Cbs +/- mice had smaller \( (P < 0.05) \)
retroperitoneal and epididymal fat pads compared to $Cbs$ $+/+$ mice (Figure 14D-E). Mice fed the HFD had larger ($P < 0.001$) inguinal fat pads compared to mice fed the control diet (Figure 14F). Reduced visceral adiposity in control-fed $Cbs$ $+/-$ mice is consistent with the lean phenotype reported in human subjects with congenital CBS deficiency (106) and in another mouse model of CBS deficiency ($TgI278T Cbs$ $-/-$ mice) (114).

**GLUCOSE HOMEOSTASIS**

I assessed the effects of feeding the HFD for 18 weeks on glucose homeostasis. As predicted, mice fed the HFD had greater ($P < 0.001$) glucose intolerance and AUC for glucose measurements during the 2 hour IP-GTT compared to mice fed the control diet (Figure 15A). However, an interaction ($P < 0.01$) was found between diet and genotype on blood glucose concentrations at baseline and at 15 min post IP glucose injection (Figure 15B). Interestingly, control-fed $Cbs$ $+/-$ mice had lower ($P < 0.05$) blood glucose concentrations at baseline and 15 min compared to $Cbs$ $+/$ mice; this genotype effect was not observed in mice fed the HFD (Figure 15B). Fasting serum insulin concentrations were higher in $Cbs$ $+/$ and $Cbs$ $+/-$ mice fed the HFD compared to mice fed the control diet (Figure 15C).

**PLASMA AND HEART ADIPONECTIN**

Adiponectin is well known for its insulin-sensitizing properties and is unique among other adipocytokines in that it circulates inversely proportional to adiposity (54; 129; 215). Irrespective of diet, serum total adiponectin concentrations were lower ($P < 0.05$) in $Cbs$ $+/-$ mice compared
to Cbs +/− mice (Figure 16A). Adiponectin circulates as multiple isoforms, with the high molecular weight (HMW) isoform being the most abundant and demonstrating the greatest insulin-sensitizing properties (118). An interaction between diet and genotype (P < 0.05) on serum HMW adiponectin concentrations was observed; however, no significant differences were detected between genotype groups when analyzed separately by diet group (Figure 16B). No effect of diet or genotype on low molecular weight (LMW) adiponectin was observed (Figure 16C). Moreover, recent attention has been focused on the role of adiponectin in the heart, specifically on its cardioprotective properties and its possible role in CVD (208). Next, I quantified mRNA expression of adiponectin (Adipoq) and its receptor (Adipor1) in the left ventricle. I observed no effect of diet or genotype on mRNA expression of Adipoq and Adipor1 in the left ventricle (Figure 16D-E).

**IN VIVO CARDIAC FUNCTION**

As a first step to determine whether Cbs +/- mice with diet-induced obesity have cardiac dysfunction, cardiac function and structure were measured indirectly by 2D transthoracic echocardiography (Figure 17 and Table 5). Cbs +/- mice had a greater (P < 0.01) heart weight (HW) (Figure 17B) and no differences in nose-to-anus (NA) length, resulting in a higher (P < 0.01) HW/NA ratio compared to Cbs +/+ mice (Figure 17C). These results could suggest that Cbs +/- mice may have cardiac hypertrophy, a phenotype not exacerbated by HFD feeding. This was accompanied by greater (P < 0.05) interventricular septum (IVS) wall thickness during systole and diastole (Figure 17D-E). Interestingly, mice fed the HFD had enhanced contractile function as indicated by greater (P < 0.05) percent ejection fraction and greater (P < 0.05)
fractional shortening compared to mice fed the control diet. No effect of diet or genotype was observed for the remainder of the echocardiography parameters (Table 5). I further analyzed expression of genes that are indicators of heart failure (297). I observed no effect of genotype or diet on Nppa (encodes atrial natriuretic peptide), Nppb (encodes brain natriuretic peptide), Acta1 (encodes alpha skeletal actin 1) and Myh7 (encodes myosin heavy chain beta isoform 7) mRNA in the left ventricle (Table 6).

EX VIVO CARDIAC FUNCTION

I further assessed cardiac function using the isolated working heart model (23). In this subset of mice, I also observed that Cbs +/- mice had greater ($P < 0.01$) heart weight and HW/NA length ratio compared to Cbs +/- mice (Figure 18A-B). Of interest, an interaction ($P < 0.01$) between diet and genotype on heart rate and cardiac output was observed (Figure 18C-D). Cbs +/- mice fed the control diet had a lower ($P < 0.05$) heart rate and lower ($P < 0.005$) cardiac output compared to Cbs +/- mice; this effect was not observed in mice fed the HFD (Figure 18C-D). Independent of diet, Cbs +/- mice had lower ($P < 0.005$) aortic flow compared to Cbs +/- mice (Figure 18E). An interaction ($P < 0.005$) was also found between genotype and diet on coronary flow with Cbs +/- mice fed the HFD having greater ($P < 0.01$) coronary flow compared to Cbs +/- mice (Figure 18F). Given that cardiac output was unaffected in mice fed the HFD; this finding indicates more blood flow redirected to the coronary arteries. Mice fed the HFD had less ($P < 0.01$) rate pressure product compared to mice fed the control diet (Figure 18G). No effect was observed for diet or genotype on peak systolic pressure or hydraulic work (Figure 18H-I). These results suggest that the heart is working hard but ultimately compensating.
CARDIAC MORPHOLOGY

Hematoxylin and eosin staining of left ventricle sections revealed no differences between diet and genotype groups (Figure 19).

CARDIAC COLLAGEN DEPOSITION

Alterations in the cardiac collagen network, often observed during cardiac remodeling, is associated with several pathophysiological conditions in the heart (158). Morphometric examination of Masson’s trichrome-stained sections of left ventricle revealed no differences in total collagen deposition between genotype/diet groups (Figure 20A). One limitation of histological examination with Masson’s trichrome stain is that it stains total collagen. Approximately 85% of total collagen in the heart is type I, which forms thick fibres to provide rigidity and strength. The next most abundant is type III (11% of total), which forms thin fibres that are laid down during early tissue remodeling to maintain elasticity (53). As such, I quantified collagen type I alpha 1 (COL1A1) and collagen type III alpha 1 (COL3A1) expression in left ventricle by immunoblot analysis (Figure 20B-C). I found no significant effects of diet or genotype on COL1A1 expression. However, I did find that mice fed the HFD had less ($P < 0.005$) COL3A1 protein expression compared to mice fed the control diet, an effect independent of genotype. Interestingly, an interaction ($P < 0.01$) between genotype and diet was observed for the ratio of COL1A1/COL3A1 expression (Figure 20D). Cbs +/- mice fed the HFD had a higher ($P < 0.01$) ratio of COL1A1/COL3A1 expression compared to Cbs +/- mice fed the HFD; an
effect not observed in mice fed the control diet. These findings suggest there may be greater cardiac tissue remodeling in Cbs +/- mice fed the HFD.

**CARDIAC LIPIDS**

Similar to what was reported in Aim 1 (Chapter 3), Cbs +/- and Cbs +/- mice fed the HFD had greater \( (P < 0.05) \) triglyceride concentrations in the heart compared to mice fed the control diet (Table 7). However, no effect of diet or genotype on monoacylglycerol, cholesteryl ester, free cholesterol and phospholipids was observed in the heart (Table 7).

**SERUM AND CARDIAC FATTY ACIDS**

Obesity is commonly associated with greater circulating concentrations of non-esterified free fatty acids (NEFA), which may contribute to ectopic lipid accumulation in the heart (303). I observed an interaction \( (P < 0.001) \) between genotype and diet on serum NEFA concentrations (Table 8). In mice fed the control diet, Cbs +/- mice had higher \( (P < 0.005) \) serum NEFA concentrations than Cbs +/- mice. The opposite was observed in mice fed the HFD, where Cbs +/- mice had lower \( (P < 0.01) \) serum NEFA concentrations compared to Cbs +/- mice (Table 8).

I further investigated whether there were differences in individual fatty acid species (Table 8 and Figure 21), which has been previously described in pressure overload cardiac hypertrophy (167; 266). Mice fed the HFD had higher \( (P < 0.05) \) stearic acid (18:0) and oleic acid (18:1n-9)
concentrations in the heart compared to mice fed the control diet (Table 8). Interestingly, I found greater ($P < 0.05$) linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-6), docosapentanoic acid (DPA, 22:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and total fatty acid concentrations in heart from $Cbs$ +/- mice compared to $Cbs$ +/- mice (Figure 21A-D). Mice fed the HFD also had greater ($P < 0.001$) DPA and AA concentrations, but lower ($P < 0.001$) DHA concentrations in the heart compared to mice fed the control diet (Figure 21C-E).

Delta-6-desaturase (D6D) is the rate-limiting enzyme responsible for the synthesis of AA and DHA from the precursors, LA and alpha-linolenic acid (ALA, 18:3n-3), respectively (Figure 22A). D6D hyperactivity has been implicated in the pathology of multiple cardiomyopathies in humans (189; 276), and mouse models (167). The ratios of AA/LA and DHA/DPA were calculated to estimate D6D activity (Figure 22B-C). Interestingly, I found that mice fed the HFD had higher ($P < 0.05$) ratios of AA/LA, but lower ($P < 0.001$) ratios of DHA/DPA in heart compared to mice fed the control diet. These findings suggest altered D6D activity in heart from mice fed the HFD. These findings could suggest changes in D6D activity in the heart from mice fed the HFD, although further studies, directly measuring D6D activity, are required to confirm these findings.

**CARDIAC ENERGY METABOLISM**

To characterize the underlying mechanisms accounting for the lipid accumulation and differences in fatty acid concentrations in the heart, I directly measured palmitate oxidation and glycolysis rates in isolated working hearts. Interestingly, mice fed the HFD had greater rates of
both palmitate oxidation ($P < 0.005$) and glycolysis ($P < 0.001$) compared to control-fed mice (Figure 23A-B). $Cbs +/-$ mice had lower ($P < 0.05$) palmitate oxidation rates compared to $Cbs +/+ $ mice, with the lowest rates of palmitate oxidation observed in $Cbs +/-$ mice fed the control diet (Figure 23A). I next determined whether the lower rates of palmitate oxidation in $Cbs +/-$ mice was accompanied by changes in expression of genes involved in fatty acid metabolism in the left ventricle (Figure 23C). $Cbs +/-$ mice, compared to $Cbs +/+ $ mice, had higher ($P < 0.01$) mRNA expression of: $Cd36$, which encodes fatty acid translocase and plays a major role in fatty acid uptake into the heart; $Cpt1$, which encodes carnitine palmitoyltransferase 1 and functions to transport fatty acids into the mitochondria for oxidation; and $Dgat1$, which encodes diacylglycerol acyltransferase 1 and is involved in triglyceride synthesis (Figure 23C).

Left ventricle expression of genes involved in cardiac glucose metabolism were assessed to determine if differences accompanied the greater glycolysis rates in mice fed the HFD (Figure 23D). I observed no effect of diet or genotype on mRNA expression of: $Glut4$ which encodes glucose translocase 4 and regulates glucose uptake into the cardiomyocyte; and $Gys1$, which encodes glycogen synthase 1 and functions to synthesize glycogen. In mice fed the HFD, mRNA expression of $Pdk4$, which encodes pyruvate dehydrogenase kinase 4 and functions to negatively control the activity of the pyruvate dehydrogenase complex, was higher ($P < 0.01$) compared to mice fed the control diet.

Moreover, I assessed the expression of other genes involved in cardiac energy metabolism in the left ventricle (Figure 24A-C). I observed no effect of diet or genotype on mRNA expression of $Acca$, which encodes acetyl CoA carboxylase $\alpha$ and the first and rate limiting step in endogenous fatty acid synthesis (151) (Figure 24A). $Cbs +/-$ mice compared to $Cbs +/+ $ mice had higher ($P$
< 0.01) mRNA expression of Acsl1, which encodes long-chain acyl coenzyme A synthetase 1 and functions to activate fatty acids with coenzyme A (Figure 24A). I next assessed mRNA expression of mitochondrial uncoupling proteins, UCP2 and UCP3, because of their role in regulating energy expenditure in the heart (202). Cbs +/- mice had higher (P < 0.05) mRNA expression of Ucp2 in heart compared to Cbs +/+ mice (Figure 24B). Mice fed the HFD had higher (P < 0.001) mRNA expression of Ucp3 in heart compared to mice fed the control diet (Figure 24B). Expression of PPARα and PPARγ was assessed because of the central role in regulating cardiac lipid metabolism (185). Cbs +/- mice had higher (P < 0.01) Ppara mRNA expression compared to Cbs +/+ mice (Figure 24C). Lastly, no effect of diet or genotype on Pparg was observed. Taken together, these findings indicate that Cbs +/- may have altered cardiac fatty acid metabolism, with a reduced capacity for palmitate oxidation compared to Cbs +/- mice (Figure 24C).

Altered expression of AMP-activated protein kinase alpha (AMPKα), a regulator of cellular energy homeostasis, has been implicated in the development of cardiac hypertrophy (80; 80; 120). I observed higher (P < 0.01) AMPKα protein expression in left ventricle from Cbs +/- mice compared to Cbs +/+ mice, independent of diet (Figure 25A). Mice fed the HFD had lower (P < 0.001) phosphorylated (p) AMPKα protein expression compared to mice fed the control diet (Figure 25B). These data resulted in a lower (P < 0.01) ratio of pAMPKα/AMPKα expression in left ventricle from Cbs +/- mice compared to Cbs +/+ mice, with the greatest effect observed in Cbs +/- mice fed the HFD (Figure 25C). These findings suggest that AMPKα is less active in the left ventricle from Cbs +/- mice, and to a greater extent in those fed the HFD, further confirming altered cellular energy homeostasis.
SERUM AND TISSUE-SPECIFIC THIOL CONCENTRATIONS

In Aim 1, Chapter 3, Cbs+/- mice with diet-induced obesity had lower circulating cysteine concentrations. As such, I postulated that Cbs +/- mice may have a diminished availability of cysteine for glutathione synthesis in heart tissue contributing to cardiac lipotoxicity. To confirm this, I next measured serum, liver and heart thiol concentrations (Table 9).

As expected, Cbs +/- mice had higher ($P < 0.001$) serum total homocysteine concentrations compared to Cbs +/+ mice, independent of diet. An interaction ($P < 0.05$) was observed between genotype and diet on serum total cysteine and GSH concentrations. In mice fed the HFD, Cbs +/- mice had higher ($P < 0.05$) serum total cysteine and GSH concentrations compared to Cbs +/+ mice, an effect not observed in mice fed the control diet. These findings are in contrast to what was observed in Aim 1 and likely a factor of time/duration of HFD feeding. The HFD feeding challenge for mice in Aim 1 was 13 weeks compared to 20 weeks for the current mice. As a result, serum and tissue thiols measured in the current study will not be compared or associated with findings in Aim 1.

Next, we quantified thiol concentrations in the liver and heart tissue (Table 9). In liver, an interaction ($P < 0.005$) was observed between genotype and diet on total homocysteine and cysteine concentrations. In mice fed the control diet, Cbs +/- mice had higher ($P < 0.01$) total homocysteine and cysteine concentrations in liver compared to Cbs +/+ mice; however, no effect was observed in mice fed the HFD. No effects of diet or genotype on GSH concentrations in the liver were observed. In heart, Cbs +/- mice had higher ($P < 0.05$) total cysteine concentrations.
compared to mice Cbs +/+ mice. Total cysteine concentrations in the heart were slightly lower ($P < 0.05$) in mice fed the HFD compared to mice fed the control diet. Although these changes were significantly different, they were minimal and the biological relevance is questionable. Lastly, mice fed the HFD had lower ($P < 0.05$) GSH concentrations in the heart compared to mice fed the control diet. Together, these results suggest that cysteine provision is unaltered and available in the heart. Though glutathione concentrations in heart were low from mice fed the HFD, suggesting the heart is using glutathione and unable to maintain glutathione concentrations to combat oxidative stress associated with HFD-feeding.
FIGURE 14. Diet-induced Obesity at 20 Weeks

A. Body weight gain from weaning for 20 weeks. Body composition assessed by quantifying B. fat mass and C. lean mass. Visceral adiposity assessed by D. gonadal and E. retroperitoneal fat pad weights. F. Subcutaneous adiposity assessed by inguinal fat pad weights. Open bars, $Cbs^{+/+}$ mice; closed bars, $Cbs^{+/-}$ mice, ($n = 8$ mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, $P < 0.05$; *Effect of diet, $P < 0.001$; **Effect of genotype, $P < 0.05$. 
FIGURE 15. Glucose Homeostasis at 18 Weeks

A. Blood glucose concentrations at baseline and following the intraperitoneal (IP) injection of 50% dextrose at 1.5 g/kg body weight, and the area under the curve (AUC) for glucose concentrations during the glucose tolerance test (GTT). Fasting (5 hour) measurements of B. blood glucose concentrations at baseline (0 minutes) and 15 minute, and C. serum insulin concentrations at baseline during the GTT. Open bars, *Cbs +/+* mice; closed bars, *Cbs +/−* mice. *(n = 6-8 mice per diet/genotype group).* Data presented as mean ± SEM. Diet x genotype = interaction, *P < 0.05; *Effect of diet, *P < 0.001; **Effect of genotype, *P < 0.05.*
FIGURE 16. Adiponectin in Serum and Left Ventricle

Fasting (5 hour) circulating adiponectin assessed by A. total adiponectin, B. high molecular weight (HMW) adiponectin, and C. low molecular weight (LMW) adiponectin concentrations in serum. Cardiac mRNA expression of D. adiponectin, Adipoq; and the E. adiponectin receptor, AdipoR1 in left ventricle. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 6-8 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, P < 0.05; **Effect of genotype, P < 0.05.
FIGURE 17. Cardiac Size and Cardiac Function In Vivo

A. Representative transthoracic echocardiography M-mode images. B. Heart weight (HW) and C. HW/nose-to-anus (HW/NA) length ratio. Interventricular septal wall thickness during D. systole and E. diastole. F. Percent ejection fraction. G. Percent fractional shortening. Open bars, $Cbs^{+/+}$ mice; closed bars, $Cbs^{+/-}$ mice. ($n = 6$ mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, $P < 0.01$; **Effect of genotype, $P < 0.05$. 
TABLE 5. Cardiac Function *In Vivo*

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>HFD</th>
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</thead>
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<tr>
<td></td>
<td><em>Cbs +/+</em></td>
<td><em>Cbs +/-</em></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>410 ± 20</td>
<td>411 ± 12</td>
</tr>
<tr>
<td>Respiration rate, bpm</td>
<td>149 ± 9.0</td>
<td>124 ± 6.5</td>
</tr>
<tr>
<td>Temperature, C</td>
<td>35.1 ± 0.2</td>
<td>34.5 ± 0.3</td>
</tr>
<tr>
<td>Diastole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>0.9 ± 0.6</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>LVID, mm</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Systole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.9</td>
</tr>
<tr>
<td>LVID, mm</td>
<td>2.9 ± 0.2</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>

Value are mean ± SEM (*n* = 5-6 mice per diet/genotype group). LVPWT, left ventricular posterior wall thickness; LVID, left ventricular internal diameter; IVS, interventricular septum thickness.

TABLE 6. Cardiac mRNA Expression of Gene Markers of Heart Failure

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Cbs +/+</em></td>
<td><em>Cbs +/-</em></td>
</tr>
<tr>
<td><em>Nppa</em></td>
<td>3.15 ± 1.95</td>
<td>4.16 ± 4.01</td>
</tr>
<tr>
<td><em>Nppb</em></td>
<td>7.19 ± 0.90</td>
<td>9.37 ± 1.99</td>
</tr>
<tr>
<td><em>Acta1</em></td>
<td>0.87 ± 0.11</td>
<td>1.26 ± 0.40</td>
</tr>
<tr>
<td><em>Myh7</em></td>
<td>0.84 ± 0.14</td>
<td>0.53 ± 0.19</td>
</tr>
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</table>

Value are mean ± SEM (*n* = 6 mice per diet/genotype group). *Nppa*, atrial natriuretic factor; *Nppb*, brain natriuretic factor; *Acta1*, alpha skeletal actin; *Myh7*, myosin heavy chain beta isoform.
FIGURE 18. Cardiac Size and Cardiac Function Ex Vivo

A. Heart weight (HW) and B. HW/nose-to-anus (HW/NA) length ratio. Cardiac function assessed by C. heart rate, D. cardiac output, E. aortic flow, F. coronary flow, G. rate pressure product, H. peak systolic pressure, and I. hydraulic work quantified during isolated working heart perfusions. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 6-8 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype interaction, $P < 0.05$; *Effect of diet, $P < 0.05$; **Effect of genotype, $P < 0.05$. 
FIGURE 19. Cardiac Tissue Morphology

A. Representative histological images of left ventricle transverse cross sections, visualized by hematoxylin and eosin staining. Arrows point to the cardiac muscle nucleus. Scale bars = 50µm. Images taken at 400x. ($n = 4-6$ mice per diet/genotype group).
FIGURE 20. Cardiac Fibril Collagen Deposition

A. Representative histological images of the left ventricle transverse cross sections, visualized by Masson’s trichrome staining to visualize total cardiac fibril collagen deposition. Scale bars = 100µm. Images taken at 40x. B. Collagen type I (COL1A1) and C. collagen type III (COL3A1) protein expression and D. COL1A1/COL3A1 ratios in left ventricle. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 4-6 mice per diet/genotype group). Data presented as mean ± SEM. Diet x genotype = interaction, $P < 0.05$; *Effect of diet, $P < 0.05$; **Effect of genotype, $P < 0.05$. 
### TABLE 7. Cardiac Lipids

<table>
<thead>
<tr>
<th>Heart (µg/mg tissue)</th>
<th>Control</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cbs +/+</td>
<td>Cbs +/-</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.59 ± 0.81</td>
<td>1.63 ± 0.36</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>0.02 ± 0.004</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>0.09 ± 0.03</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>Free Cholesterol</td>
<td>0.56 ± 0.03</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>3.47 ± 0.48</td>
<td>4.20 ± 0.79</td>
</tr>
</tbody>
</table>

Value are mean ± SEM (n = 5-6 mice per diet/genotype group). *Effect of diet, P < 0.05.

### TABLE 8. Serum and Cardiac Fatty Acids

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Control</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cbs +/+</td>
<td>Cbs +/-</td>
</tr>
<tr>
<td>Serum (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free Fatty Acids †</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.1**</td>
</tr>
<tr>
<td>Heart (ng/mg tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>5381 ± 358</td>
<td>6056 ± 847</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>3232 ± 111</td>
<td>3948 ± 326</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2252 ± 395</td>
<td>2436 ± 307</td>
</tr>
<tr>
<td>EPA</td>
<td>84.0 ± 16</td>
<td>64.3 ± 11</td>
</tr>
</tbody>
</table>

Value are mean ± SEM (n = 5-6 mice per diet/genotype group). † Diet x genotype interaction, P < 0.05; *Effect of diet, P < 0.05; **Effect of genotype, P < 0.05. 16:0, palmitic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:3n-3, alpha linolenic acid; 20:5n-3, eicosapentaenoic acid.
FIGURE 21. Cardiac n6 and n3 Fatty Acids

Cardiac fatty acids assessed by A. linoleic acid (LA, 18:2n-6), B. α-linolenic acid (ALA, 18:3n-3), C. arachidonic acid (AA, 20:4n-6), D. docosapentanoic acid (DPA, 22:5n-3), E. docosahexaenoic acid (DHA, 22:6n3), and F. total fatty acids concentrations. Open bars, Cbs +/+ mice; closed bars, Cbs +/− mice. (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.001; **Effect of genotype, P < 0.05.
FIGURE 22. Cardiac n-6 and n-3 Fatty Acid Ratios

A. Diagram to show the desaturation and elongation of the n-6 and n-3 fatty acid series. Cardiac ratios of B. AA/LA and C. DHA/DPA. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 5-6 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.01.
FIGURE 23. Cardiac Fatty Acid and Glucose Metabolism

A. Palmitate oxidation and B. glycolysis rates quantified during isolated working heart perfusions. Cardiac mRNA expression of gene targets involved in C. fatty acid metabolism: *Cd36, Cpt1, Dgat1* and D. glucose metabolism: *Glut4, Pdk4, Gys1* in left ventricle. Open bars, *Cbs +/+* mice; closed bars, *Cbs +/−* mice. (*n* = 6-8 mice per diet/genotype group). Data presented as mean ± SEM. *Significant effect of diet* *P* < 0.01, **Effect of genotype, *P* < 0.05.
FIGURE 24. Cardiac Gene Targets of Fatty Acid Metabolism

Cardiac mRNA expression of gene targets involved in A. Fatty acid metabolism: *Acca, Acs1*; B. mitochondrial uncoupling: *Upc2, Upc3*; and C. regulation of fatty acid metabolism: *Ppara, Pparg* in left ventricle. Open bars, Cbs ++ mice; closed bars, Cbs +/- mice. (n = 6-8 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet P < 0.01, **significant effect of genotype, P < 0.05.
FIGURE 25. Cardiac AMP-Activated Protein Kinase α Expression

Cardiac regulation of cellular energy assessed by A. AMPKα and B. phosphorylated (p) AMPKα protein expression, and C. the pAMPKα/AMPKα ratio in left ventricle. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice. (n = 6-8 mice per diet/genotype group). Data presented as mean ± SEM. *Effect of diet, P < 0.01; **Effect of genotype, P < 0.05.
TABLE 9. Serum, Hepatic and Cardiac Thiols

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Cbs +/+</th>
<th>Control Cbs +/−</th>
<th>Control Cbs +/+</th>
<th>Control Cbs +/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (µmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Homocysteine</td>
<td>5.8 ± 1.0</td>
<td>7.8 ± 1.3**</td>
<td>4.6 ± 0.4</td>
<td>10.6 ± 1.2**</td>
</tr>
<tr>
<td>Total Cysteine †</td>
<td>231 ± 16</td>
<td>206 ± 13</td>
<td>219 ± 8.8</td>
<td>256 ± 13**</td>
</tr>
<tr>
<td>Total Glutathione †</td>
<td>49.0 ± 9.3</td>
<td>40.1 ± 5.4</td>
<td>36.3 ± 3.7</td>
<td>52.4 ± 3.9**</td>
</tr>
<tr>
<td>Liver (µmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Homocysteine †</td>
<td>0.8 ± 0.1</td>
<td>2.0 ± 0.2**</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>Total Cysteine †</td>
<td>4.1 ± 0.6</td>
<td>13.4 ± 1.7**</td>
<td>5.7 ± 0.6</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Total Glutathione</td>
<td>57.7 ± 2.2</td>
<td>68.3 ± 7.2</td>
<td>73.5 ± 8.9</td>
<td>62.7 ± 8.0</td>
</tr>
<tr>
<td>Heart (µmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cysteine</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.2**</td>
<td>0.6 ± 0.1*</td>
<td>1.2 ± 0.1* **</td>
</tr>
<tr>
<td>Total Glutathione</td>
<td>20.6 ± 0.2</td>
<td>20.0 ± 3.2</td>
<td>14.0 ± 1.8*</td>
<td>14.2 ± 0.8*</td>
</tr>
</tbody>
</table>

Value are mean ± SEM (n = 5-6 mice per diet/genotype group). † Diet x genotype interaction, P < 0.05; *Effect of diet, P < 0.05; **Effect of genotype, P < 0.05.

4.5 DISCUSSION

The objective of this Aim was to determine the functional consequences and to delineate the underlying mechanisms of cardiac lipotoxicity in Cbs +/− mice with diet-induced obesity (104). I postulated that Cbs +/− mice would have cardiac dysfunction and disturbances in cardiac fatty acid metabolism because of diminished capacity of the heart to synthesize GSH from cysteine. I report three major findings in this study. First, larger hearts and reduced ex-vivo cardiac function was observed in Cbs +/− mice compared to Cbs +/+ mice. This was accompanied by disturbances in energy and fatty acid metabolism. Higher AA and DHA concentrations, lower
palmitate oxidation rates, and less AMPKα activity were found in hearts from \( Cbs +/− \) mice compared to \( Cbs +/+ \) mice. Together these findings suggest that \( Cbs +/− \) mice have cardiac hypertrophy accompanied by changes in cardiac function and alterations to fatty acid metabolism, which are unlikely attributable to effects on GSH synthesis from cysteine.

Cardiac hypertrophy has been considered a useful physiological adaptation that develops in response to chronic increased workload by the heart (16). Although, this compensatory adaptation ensures adequate heart function, over time it develops into a pathological state with less desirable outcomes, such as heart failure (3). Remarkably, larger hearts observed in \( Cbs +/− \) mice was not exacerbated by HFD-induced obesity, and this phenotype may be a result of a congenital defect arising from disruption of the CBS gene. Further studies are required to confirm cardiac hypertrophy in \( Cbs +/− \) mice and if this phenotype is a physiological adaptation or pathological.

Both cardiac hypertrophy and obesity-related lipid accumulation in the heart are associated with cardiac dysfunction and eventually failure (148). I observed altered \textit{in-vivo} contractile function of the heart by echocardiography in mice with diet-induced obesity, which may suggest a compensatory improvement in contractile performance by the heart in the setting of obesity. This phenomenon has also been reported by others, with greater ejection fraction and fractional shortening reported in human subjects that are overweight (BMI 25-29.9 kg/m\(^2\)) and obese (BMI 30-34.9 kg/m\(^2\)) compared to subjects with healthy BMIs (<25 kg/m\(^2\)) (213). Cardiac dysfunction associated with obesity is likely dependent on the amount and the location of excess adiposity and the length of time an individual is obese. The compensatory mechanisms eventually become
exhausted, resulting in diminishing indicators of cardiac function and progression to heart failure. Cardiac hypertrophy is often characterized by greater heart mass and thickening of the IVS wall. I did observe greater IVS wall thickness in mice with diet-induced obesity, but no differences were detected between $Cbs^{+/+}$ and $Cbs^{+/-}$ mice, despite finding larger hearts in $Cbs^{+/-}$ mice. These findings may be attributed to the 2D echocardiography I used for the assessments, as it has limited sensitivity compared to techniques such as 3D echocardiography or magnetic resonance imaging (57). With this knowledge, I also assessed cardiovascular function in isolated working hearts. I found reduced function of hearts ex-vivo in $Cbs^{+/-}$ mice compared to $Cbs^{+/+}$ mice, a difference that disappears with HFD-induced obesity. Interestingly, this reduction in cardiac function was associated with more blood flow redirected to the coronary arteries in $Cbs^{+/-}$ mice with HFD-induced obesity.

Cardiac fibrotic remodeling is common in most cardiomyopathies, as the heart has a limited ability to regenerate cardiomyocytes resulting in cardiac dysfunction (158). No gross differences or abnormal collagen deposition were observed upon morphometric examination of total collagen by staining left ventricle sections with Masson’s trichrome. The synthesis of collagen subtypes I and III has also been associated with cardiac fibrotic remodeling in the early stages of repair (158). I found no significant effect of diet or genotype on COL1A1 protein expression; however, I did find less COL3A1 protein in heart from mice fed the HFD, independent of genotype. Interestingly, $Cbs^{+/-}$ mice with diet-induced obesity had a higher ratio of COL1A1/COL3A1 expression indicating early stages of cardiac remodeling in mice with diet-induced obesity, especially in $Cbs^{+/-}$ mice.
Lipotoxicity occurs when there is an imbalance between fatty acid uptake and oxidation. This imbalance leads to accumulation of long chain fatty acids which, are incorporated into triglycerides, phospholipids, and other lipid subspecies (111). Changes in cardiac fatty acid concentrations and phospholipid remodeling have been reported in many mouse models of cardiac dysfunction (26, 31), as well as in human subjects with heart failure (122; 167; 245). Our findings of higher total AA and DHA concentrations in heart from Cbs +/− mice are consistent with these reports by others and provide supporting evidence of pathological alterations in lipid metabolism in these mice.

This is the first report of an association between changes in cardiac long-chain polyunsaturated fatty acids and diet-induced obesity in mice. Others have reported higher concentrations of AA in heart from mouse models of ischemia/reperfusion injury (266) and heart failure (167). It is well known that elevations in tissue AA concentrations affect eicosanoid production, ion homeostasis, protein and enzyme transport, and signal transduction, which could affect cardiac function and metabolism (266). Given that the HFD and the control diet did not contain AA, the elevated AA in mice with diet-induced obesity could be attributed, in part, to the greater amount of LA, the precursor of AA, in the HFD compared to the control diet. Delta-6-desaturase catalyzes the synthesis of AA and DHA from their respective precursors, and there have been suggestions that the serum ratio of these fatty acids to their precursors reflect systemic D6D activity and may be useful predictors of CVD (189; 276). Our finding that mice with diet-induced obesity have a higher AA/LA ratio, but a lower DHA/DPA ratio in heart compared to mice fed the control diet suggest altered D6D activity with a preference towards n-6 polyunsaturated fatty acids. This is consistent with a recent report that suggests a role for D6D in
the phospholipid remodeling observed in cardiac hypertrophy induced by transverse aortic constriction (167).

Furthermore, studies previously reported by Devlin et al. confirm direct effects of disturbances in methyl metabolism on lipid and fatty acid metabolism. *Cbs* +/- with diet-induced obesity had higher triglycerides and lower AA (20:4n-6) and DHA (22:6n-3) concentrations in liver (74). These results were accompanied by methylation-silencing of liver *Fads2* (encodes delta-6 desaturase) expression, associated with reduced methyl capacity in liver (AdoMet/AdoHcy ratios) (74). Lipid metabolism is linked to methyl metabolism through the methionine cycle and the synthesis of phosphatidylcholine (PC) (268). This pathway involves the methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine-\(N\)-methyltransferase (PEMT, EC 2.1.117) and requires 3 AdoMet molecules (268). Therefore, further studies are warranted to determine if altered cardiac fatty acid metabolism in *Cbs* +/- mice with obesity-related remodeling is accompanied by alterations in AdoMet and AdoHcy concentrations and gene-specific changes in DNA methylation in the heart.

As a step towards determining the underlying mechanisms accounting for the lipid accumulation and differences in fatty acid concentrations in the heart, I directly quantified palmitate oxidation and glycolysis rates in perfused isolated working hearts. As expected, greater rates of palmitate oxidation were observed in hearts from mice with diet-induced obesity. However, hearts from *Cbs* +/- mice had lower rates of palmitate oxidation compared to *Cbs* +/-/+ mice. Together, reduced *ex-vivo* cardiac function and lower rates of palmitate oxidation suggests that *Cbs* +/- mice are unable to sufficiently meet the myocardium energy requirements to function properly.
Glycolytic rates were also elevated in hearts of mice with diet-induced obesity. Simultaneous elevation of both glycolysis and palmitate oxidation was unexpected, and may be a reflection of metabolic inefficiency arising because of increased uncoupling protein expression.

Disturbances in adiponectin metabolism may also play a role in the cardiac dysfunction associated with obesity. Adiponectin has well-known insulin-sensitizing properties (54; 235), but recent attention has been focused on the role of adiponectin in the heart, specifically on its cardioprotective properties (209), and its ability to stimulate AMPK (235). Our findings of lower serum total adiponectin concentrations and a lower ratio of the active to inactive forms of AMPKα in the heart from Cbs +/- mice are consistent with these findings reported in Adipoq -/- mice. Greater cardiac hypertrophy following left anterior artery ligation has been reported in Adipoq -/- mice, with improvements found following adenoviral delivery of Adipoq to these mice (235). Further studies in Adipoq -/- mice reported diminished AMPKα phosphorylation and enhanced extracellular signal-regulated kinase (ERK) phosphorylation (237), suggesting adiponectin may also be an important stimulus of AMPK in the heart. Collectively, lower circulating adiponectin in Cbs +/- mice could lead to reduced AMPKα stimulation in the heart, resulting in changes in cardiac fatty acid metabolism and function.
CHAPTER 5:

DISTURBANCES IN METHYL METABOLISM CONTRIBUTE TO CHANGES IN TISSUE-SPECIFIC GENE EXPRESSION AND DNA METHYLATION STATUS

**PART 1**

**5.1 INTRODUCTION**

**AIM 3:** To determine if disturbances in methyl metabolism contribute to changes in tissue-specific gene expression and DNA methylation associated with obesity-related cardiac remodeling.

This aim will be addressed in two parts:

1) I propose to determine if disturbances in methyl metabolism contribute to a tissue-specific relationship between methylation capacity (AdoMet/AdoHcy ratio), DNA methylation, and gene expression in *Cbs* +/- mice.

2) I propose to determine if gene-specific changes in DNA methylation and expression are associated with reduced methylation capacity (AdoMet/AdoHcy ratio) in heart, and contribute to the obesity-related cardiac remodeling observed in *Cbs* +/- mice.
RATIONALE

As previously stated in Chapter 1, AdoMet is the principal methyl group donor used in methylation reactions, with DNA being one of the many methyl acceptors (93). AdoMet is produced from methionine and production of AdoHcy follows methyl donation, and the formation of homocysteine occurs through the reversible liberation of adenosine from AdoHcy (141; 143). Elevations of homocysteine, independent of the causal factor(s) contributing to HHcy, consequential changes in metabolites in the methionine cycle occur. For example, many studies in humans and animals have reported HHcy to be associated with higher intracellular AdoHcy concentrations, resulting in a lower AdoMet/AdoHcy ratio (44; 59; 70; 71; 74; 296). In vitro studies demonstrated that elevated AdoHcy concentrations and a reduced AdoMet/AdoHcy ratio inhibits DNA methyltransferase reactions in liver (127) and in cultured human fibroblasts (61). This leads to the concept that the AdoMet/AdoHcy ratio may be indicative of the methylation capacity of a cell, with a lower AdoMet/AdoHcy ratio as a marker of reduced DNA methylation capacity.

However, the relationship between higher intracellular AdoHcy concentrations and a lower AdoMet/AdoHcy ratio with DNA methylation status have yielded conflicting findings in both animal and human studies. For example, studies in subjects with HHcy reported that elevated plasma and lymphocyte AdoHcy concentrations were associated with reduced global DNA methylation in leukocytes and lymphocytes, respectively (44; 296), whereas other studies have reported no relationship (99; 123). One study has reported gene-specific associations of HHcy
with DNA methylation in human subjects (136). Similar discrepancies have been reported in mouse studies.

For example, *Cbs* -/- mice with severe HHcy were reported to have elevated concentrations of AdoHcy in liver, kidney, and brain, with reduced global DNA methylation in liver and kidney, but not in brain (51). Additionally, in liver from *Cbs* +/- mice with HHcy, higher AdoHcy concentrations and a lower AdoMet/AdoHcy ratio was associated with gene-specific changes in DNA methylation (71; 74). Conflicting reports in the literature suggest that the relationship between higher intracellular AdoHcy concentrations and a lower AdoMet/AdoHcy ratio with DNA methylation are tissue-specific. Since this work has been reported there have been significant developments in the methods used to quantify DNA methylation status and the relationship should be revisited with current methods available, such as pyrosequencing.

DNA methylation also plays a role in allele-specific silencing of genomically imprinted genes (125). Allele-specific expression of the well characterized reciprocally imprinted genes, insulin-like growth factor 2 (*Igf2*) and *H19* is regulated by DNA methylation (134). Below, Figure 27 illustrates the proposed mechanisms involved in the regulation of the *Igf2/H19* imprinting region.
Figure 26. Schematic Representation of the Igf2/H19 Imprinting Region

In both mice and humans, \(H19\) is usually expressed from the maternal allele and \(Igf2\) is expressed from the paternal allele (24; 256). A differentially-methylated domain (DMD) within the imprinting control region is believed to function as a boundary/insulator element (24; 121; 145). The proposed model suggests that the zinc-finger DNA binding protein, CCCTC-binding factor (CTCF), binds to the unmethylated maternal \(H19\) DMD allele, enabling distal enhancers to activate \(H19\) transcription, while blocking enhancer access to \(Igf2\) (24; 121). In contrast, the paternal allele \(H19\) DMD is methylated, which prevents CTCF binding and enhancer activation of \(H19\) transcription. Figure adapted from: Ideraabdullah F.Y., Vigneau S., and Bartolomei M.S. (2008). Genomic imprinting mechanisms in mammals. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 647:77-85.

As a proof of concept, I sought to determine if disturbances in methyl metabolism contributes to a tissue-specific relationship between methylation capacity (AdoMet/AdoHcy ratio), DNA methylation, and gene expression in Cbs +/- mice with HHcy. To induce HHcy and disturbances
in methyl metabolism, a combined diet and genetic approach was used. Cbs +/+ and Cbs +/- mice were fed either a control diet or a high methionine/low folate diet (HH) for 3 weeks to induce HHcy. The HH diet was selected as it has been previously used to induce HHcy and has shown to effect methylation capacity in the liver (70; 71; 74). In this study, liver and brain were studied to determine tissue-specific relationships. Two mouse strains (C57BL/6J and CAST/EiJ) were crossbred to yield hybrid Cbs +/+ and Cbs +/- offspring mice. The C57BL/6J and CAST/EiJ mouse strains were selected due to the presence of a strain-specific single nucleotide polymorphism (SNP) within the H19 coding region exists. The presence of this strain-specific SNP can be used to quantify allele-specific DNA methylation such that the C57BL/6J H19 allele can be distinguished from the CAST/EiJ allele in hybrid Cbs +/+ and Cbs +/- mice. The following groups of hybrid mice were studied: Cbs +/+ mice fed the control diet; Cbs +/+ mice fed the HH diet; and Cbs +/- mice fed the HH diet.

5.2 METHODS

ANIMALS AND DIETS

For Part 1, Cbs +/- hybrid [C57BL/6J x CAST/EiJ] offspring mice were fed either a control diet or a high methionine/low folate diet (HH) for 3 weeks to induce HHcy. Genotyping of the disrupted Cbs allele and the wild-type allele was done by PCR as previously described in Aim 1, Chapter 3. At weaning, mice were fed either a control diet (custom made, TD.05108, Harlan-Teklad) or a high methionine/low folic acid diet to induce HHcy (HH diet) (custom made, TD.00205, Harlan-Teklad) for 3 weeks. The HH diet has been previously used to induce
HHcy (70; 71; 74) and contained double the amount of methionine (8.6 g/kg) and low amounts of folic acid (0.2 mg/kg) compared to the control diet (Table 10). The HH diet also contained succinyl sulfathiazole (5.0 g/kg) to inhibit growth of intestinal bacteria, another source of folate. The following groups of hybrid mice were studied: Cbs +/+ mice fed the control diet; Cbs +/- mice fed the HH diet; and Cbs +/- mice fed the HH diet. At 6 weeks of age, mice were anesthetized with 1% Avertin (2,2,2-tribromoethanol) (0.3 ml/10 g body weight, IP injection) and blood was collected by cardiac puncture into EDTA tubes. Blood was centrifuged at 3000 g for 20 min at 4°C and plasma was collected. Plasma, liver and brain were immediately flash frozen in liquid nitrogen for storage at -80°C until later analysis.

**TABLE 10. Composition of Experimental Diets used in Aim 3, Chapter 5**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control</th>
<th>HH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Methionine (g/kg)</td>
<td>4.0</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Cysteine (g/kg)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline (g/kg)</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Folic acid (mg/kg)</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyridoxine (mg/kg)</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Cobalamin (µg/kg)</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Riboflavin (mg/kg)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

The HH diet contained double the amount of methionine, minimal concentrations of folic acid and 5.0 g/kg succinyl sulfathiazole to prevent intestinal bacterial growth. Choline is supplied as
choline bitartrate. The HH and control diet contained no cholesterol and supplied 14% and 17%, respectively, of total energy from fat (soybean oil). Except for folic acid in the HH diet, diets contained adequate concentrations of nutrients as recommended for mice by the NRC.

**BIOCHEMICAL ANALYSES**

Total homocysteine and methionine concentrations in plasma were quantified by HPLC with fluorescence detection, as described previously in Chapters 3 and 4. AdoMet and AdoHcy concentrations in liver and brain were quantified by HPLC using ultraviolet detection, as previously described (30). Tissue samples were deproteinized in ice-cold 0.4 mol/L perchloric acid, homogenized, and centrifuged. The supernatant fraction was immediately frozen and stored at -80°C for later analysis of AdoMet and AdoHcy concentrations. Quantification of total homocysteine, methionine, AdoMet and AdoHcy were conducted in the Metabolomics core of the Nutrition and Metabolism Research Program (directed by Dr. S. Innis) at the CFRI.

**BISULFITE PYROSEQUENCING**

Genomic DNA was extracted from liver and brain using the DNeasy Blood & Tissue kit (Qiagen) with RNase I treatment to remove RNA. DNA samples (0.5 μg) were bisulfite-treated (converts non-methylated cytosines into uracil, while the methylated cytosines remain unchanged) using the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer’s suggested protocol and stored at -80°C until further analysis. The sequence for each mouse gene, to identify CpG rich regions, was obtained using the UCSC Genome Browser.
PCR and sequencing primers were designed using the PyroMark Assay Design Software and PCR reactions used HotStart Taq DNA Polymerase (Qiagen). The percent methylation of each CpG dinucleotide was quantified separately using the PyroMark CpG Software (Qiagen) and all samples were run in duplicates (79).

Specifics for the H19 assay: a CpG rich region within the H19 DMD was selected (227 bp fragment) and amplified by PCR from the bisulfite-treated liver and brain DNA samples using the following primers (Integrated DNA Technologies): forward, 5’-GGG TAG GAT ATA TGT ATT TTT TAG GT-3’; and reverse, 5’-CCT AAC CTC AAA AAC CCA TAA CTA-3’, containing a biotin label at the 5’ end. The PCR products were sequenced using the following sequencing primers (Integrated DNA Technologies): MC57BL/6J, 5’-GGA TTT TTA AAT TAA TAA GG-3’, to detect the maternal (C57BL/6J) allele; and PCAST/EiJ, 5’-GGA TTT TTA AAT TAA TAA GA-3’, to detect the paternal (Cast) allele. Each sample was sequenced twice, first to detect the maternal allele and second to detect the paternal allele.

REAL-TIME PCR

Similar materials and methods were used as previously described in Aim 1 (Chapter 3)

STATISTICAL ANALYSIS

A one-way ANOVA, followed by the least significant difference test for multiple comparisons, was used to compare findings between groups: Cbs +/- control mice, Cbs +/- HH mice and Cbs
+/- HH mice. These analyses were accomplished by SPSS for Windows version 16.0 (SPSS Inc.).

5.3 RESULTS

DIET-INDUCED HHCY AND TISSUE METHYLATION CAPACITY

Hybrid [C57BL/6J x CAST/EiJ] Cbs +/+ and Cbs +/- offspring mice were fed either a control diet or a high methionine/low folate diet (HH) for 3 weeks to induce HHcy and reduce tissue methylation capacity. To assure there was no effect of the HH diet on body weight, final body weights were recorded. No effect the HH diet on body weight was observed (Table 11). To confirm diet-induced HHcy, total homocysteine concentrations in plasma from all groups were quantified. As expected, Cbs +/- mice fed the HH diet had higher ($P < 0.001$) total homocysteine concentrations in plasma compared to Cbs +/+ mice fed the control and HH diet (Table 11). Similarly, Cbs +/- mice fed the HH diet had higher ($P < 0.001$) methionine concentrations in plasma compared to Cbs +/+ mice fed the control and HH diet (Table 11).

Methylation capacity was assessed by quantifying AdoMet and AdoHcy concentrations in liver and brain (Table 11). In liver, no effect of HH diet on AdoMet concentrations was observed. In liver, Cbs +/- mice fed the HH diet had higher ($P < 0.05$) AdoHcy concentrations and a lower ($P < 0.05$) AdoMet/AdoHcy ratio in liver compared to Cbs +/+ mice fed the control and HH diet (Table 11). In brain, no effect of the HH diet on AdoMet and AdoHcy concentrations or AdoMet/AdoHcy ratio was observed (Table 11). These results suggest that the relationship
between altered methyl metabolism (i.e. HHcy) and AdoMet and AdoHcy concentrations are tissue-specific.

**ALLELE-SPECIFIC H19 METHYLATION ASSAY DESIGN AND VALIDATION**

As a first step, the mouse *H19/Igf2* imprinting region sequence was obtained from the University of California, Santa Cruz (UCSC) Genome Browser (149). A CpG rich region (6 CpG sites) was located in the *H19* differentially methylated domain (DMD) between -4000 and -2000, relative to the *H19* transcriptional start site at +1, and selected to quantify DNA methylation status (Figure 28A). Hybrid offspring from two strains of mice (C57BL/6J x CAST/EiJ) were studied to enable allele-specific quantification of DNA methylation. A strain-specific variant was identified, G (C57BL/6J allele) → A (Cast allele) at nucleotide -4437, and used to distinguish parental alleles (Figure 28A). To utilize the strain-specific variant, two separate sequencing primers were designed: one to detect the maternal (C57BL/6J) allele and the other to detect the paternal (CAST/EiJ) allele.

To validate that the assay design could detect differences in *H19* methylation status amongst the parental alleles, a standard curve was created. Each standard contained a known volume of the C57BL/6J maternal allele and CAST/EiJ paternal allele (Standards = 100/0, 75/25, 50/50, 25/75, 0/100). Percent methylation in these standards was quantified and the mean methylation was analyzed on 5 different days. This experiment demonstrated that *H19* percent methylation increased as the volume of the paternal allele (relative to the maternal allele) in each standard increased (Figure 28B).
TISSUE-SPECIFIC *H19* DMD METHYLATION STATUS

In liver, allele-specific *H19* methylation in each CpG site and the mean methylation were quantified and are shown in Table 12 and Figure 29A-B. On the maternal allele, *Cbs* +/- mice fed the HH diet had lower (*P* < 0.05) methylation in CpG sites 1, 2, 4, 5 and mean methylation, compared to *Cbs* +/- mice fed the control diet (Table 12, Figure 29A). On the paternal allele, no effect of the HH diet on *H19* methylation status in liver was observed (Table 12 and Figure 29B). These results suggest that in the liver, reduced methylation capacity is associated with allele-specific hypomethylation of *H19*.

In brain, allele-specific *H19* methylation in each CpG site and the mean methylation were quantified and are shown in Table 13 and Figure 29C-D. On the maternal allele, *Cbs* +/- mice fed the HH diet had higher (*P* < 0.05) methylation in CpG sites 4, 5, 6 and mean methylation, compared to *Cbs* +/- mice fed the control and HH diet (Table 13, Figure 29C). On the paternal allele, *Cbs* +/- mice fed the HH diet also had higher (*P* < 0.05) methylation, but only in CpG site 2 compared to *Cbs* +/- mice fed the control and HH diet (Table 13). No effect of the HH diet on *H19* mean methylation in brain was observed on the paternal allele (Figure 29D). These results suggest that in brain, allele-specific hypermethylation of *H19* is independent of the methylation capacity marker. Overall, these results suggest that both allele-specific and tissue-specific differences *H19* DMD methylation status are associated with methylation capacity.
TISSUE-SPECIFIC H19 AND IGF2 EXPRESSION

To determine whether the allele-specific and tissue-specific changes in H19 methylation status were associated with changes in gene expression, mRNA expression of H19 and Igf2 was quantified in liver and brain (Figure 30). In liver, Cbs +/- mice fed the HH diet had lower ($P < 0.05$) mRNA expression of Igf2 compared to Cbs +/+ mice fed the control and HH diets (Figure 30A). However, mRNA expression of H19 in liver was only lower ($P < 0.05$) in Cbs +/+ mice fed the HH diet compared to Cbs +/+ mice fed the control diet (Figure 30B). In brain, no effect of the HH diet on mRNA expression of Igf2 was observed (Figure 30C). Cbs +/- mice fed the HH diet had lower ($P < 0.05$) mRNA expression of H19 in brain compared to Cbs +/+ mice fed the control diet (Figure 30D). These results also suggest tissue-specific expression of H19 and Igf2 in Cbs +/- mice with HHcy.
### TABLE 11. Body Weights, and Plasma, Hepatic, and Brain Metabolites

<table>
<thead>
<tr>
<th></th>
<th>Cbs +/- Control Diet</th>
<th>Cbs +/- HH Diet</th>
<th>Cbs +/- HH Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>18.10 ± 1.49</td>
<td>20.61 ± 0.74</td>
<td>18.94 ± 0.92</td>
</tr>
<tr>
<td><strong>Plasma (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Homocysteine</td>
<td>2.24 ± 0.19</td>
<td>5.07 ± 0.80</td>
<td>10.36 ± 1.59*</td>
</tr>
<tr>
<td>Methionine</td>
<td>42.04 ± 6.37</td>
<td>68.27 ± 11.38</td>
<td>88.14 ± 20.47*</td>
</tr>
<tr>
<td><strong>Liver (nmol/g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdoMet</td>
<td>41.58 ± 3.62</td>
<td>47.54 ± 2.10</td>
<td>38.75 ± 4.54</td>
</tr>
<tr>
<td>AdoHcy</td>
<td>17.14 ± 2.93</td>
<td>29.51 ± 5.06</td>
<td>50.80 ± 8.92*</td>
</tr>
<tr>
<td>AdoMet/AdoHcy</td>
<td>3.04 ± 0.60</td>
<td>2.30 ± 0.40</td>
<td>0.91 ± 0.15*</td>
</tr>
<tr>
<td><strong>Brain (nmol/g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdoMet</td>
<td>14.05 ± 1.24</td>
<td>16.71 ± 1.01</td>
<td>16.05 ± 0.64</td>
</tr>
<tr>
<td>AdoHcy</td>
<td>1.09 ± 0.20</td>
<td>1.06 ± 0.14</td>
<td>1.25 ± 0.15</td>
</tr>
<tr>
<td>AdoMet/AdoHcy</td>
<td>14.96 ± 2.23</td>
<td>16.11 ± 1.73</td>
<td>12.31 ± 1.68</td>
</tr>
</tbody>
</table>

Values are means ± SEM. (n = 5-11 mice/group) *Effect vs. Cbs +/- mice fed control diet, P < 0.05; **Effect vs. Cbs +/- mice fed HH diet, P < 0.05.
FIGURE 27. Allele-Specific H19 DMD Methylation Assay Design and Validation

A. Region of the H19 DMD sequences analyzed for methylation status. The CpG sites are in bold. Numbering of the sequence is relative to the transcriptional start site (+1). *Location of the species-specific variant, G (C57BL/6J allele) → A (CAST/EiJ allele) at nucleotide -4437, used to quantify alleles separately. 

B. Concentrations of methylation in samples containing known amounts of the maternal allele (C57BL/6J) and the paternal allele (CAST/EiJ). Results shown are the mean ± SD.
TABLE 12. Hepatic Allele-Specific *H19* DMD Methylation Status

<table>
<thead>
<tr>
<th>CpG</th>
<th>Cbs +/+ Control Diet</th>
<th>Cbs +/+ HH Diet</th>
<th>Cbs +/- HH Diet</th>
<th>Cbs +/+ Control Diet</th>
<th>Cbs +/+ HH Diet</th>
<th>Cbs +/- HH Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.42 ± 5.72</td>
<td>30.73 ± 5.18</td>
<td>15.37 ± 1.96</td>
<td>**90.15 ± 2.75</td>
<td>89.15 ± 3.56</td>
<td>90.71 ± 3.90</td>
</tr>
<tr>
<td>2</td>
<td>29.88 ± 3.60</td>
<td>24.20 ± 3.02</td>
<td>15.46 ± 1.75</td>
<td>**71.71 ± 2.71</td>
<td>71.35 ± 3.83</td>
<td>74.84 ± 4.61</td>
</tr>
<tr>
<td>3</td>
<td>16.23 ± 4.85</td>
<td>9.98 ± 1.73</td>
<td>10.04 ± 1.23</td>
<td>55.12 ± 4.88</td>
<td>55.72 ± 3.14</td>
<td>52.41 ± 5.69</td>
</tr>
<tr>
<td>4</td>
<td>24.19 ± 4.43</td>
<td>16.88 ± 2.92</td>
<td>12.37 ± 1.59</td>
<td>**67.34 ± 3.23</td>
<td>67.00 ± 3.44</td>
<td>66.32 ± 5.18</td>
</tr>
<tr>
<td>5</td>
<td>40.58 ± 6.49</td>
<td>29.25 ± 4.31</td>
<td>21.05 ± 2.67</td>
<td>**86.11 ± 4.13</td>
<td>84.62 ± 3.58</td>
<td>83.92 ± 4.44</td>
</tr>
<tr>
<td>6</td>
<td>20.01 ± 4.97</td>
<td>12.74 ± 2.04</td>
<td>12.56 ± 1.34</td>
<td>**65.84 ± 6.50</td>
<td>67.61 ± 4.20</td>
<td>60.79 ± 5.55</td>
</tr>
</tbody>
</table>

Values are means ± SEM, (*n* = 5-8 mice/group). *P* < 0.05, vs. Cbs +/+ mice fed the control diet. **P* < 0.05, vs. Cbs +/+ mice fed the HH diet.
TABLE 13. Brain Allele-Specific *H19* DMD Methylation Status

<table>
<thead>
<tr>
<th>CpG</th>
<th>Cbs +/+ Control Diet</th>
<th>Cbs +/+ HH Diet</th>
<th>Cbs +/− HH Diet</th>
<th>Cbs +/+ Control Diet</th>
<th>Cbs +/+ HH Diet</th>
<th>Cbs +/− HH Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG 1</td>
<td>18.37 ± 3.22</td>
<td>20.47 ± 2.73</td>
<td>22.90 ± 4.59</td>
<td>97.33 ± 0.93</td>
<td>96.54 ± 0.89</td>
<td>96.88 ± 0.58</td>
</tr>
<tr>
<td>CpG 2</td>
<td>25.27 ± 1.98</td>
<td>26.43 ± 1.50</td>
<td>29.68 ± 1.89</td>
<td>79.03 ± 1.52</td>
<td>77.49 ± 1.84</td>
<td>83.94 ± 1.25**</td>
</tr>
<tr>
<td>CpG 3</td>
<td>8.88 ± 0.83</td>
<td>8.91 ± 0.46</td>
<td>12.86 ± 2.39</td>
<td>57.26 ± 3.84</td>
<td>57.67 ± 2.14</td>
<td>64.04 ± 2.77</td>
</tr>
<tr>
<td>CpG 4</td>
<td>11.56 ± 1.07</td>
<td>12.63 ± 0.82</td>
<td>17.48 ± 2.67**</td>
<td>70.79 ± 2.39</td>
<td>71.24 ± 1.68</td>
<td>73.73 ± 2.49</td>
</tr>
<tr>
<td>CpG 5</td>
<td>14.53 ± 1.74</td>
<td>16.24 ± 1.66</td>
<td>25.06 ± 2.81**</td>
<td>82.76 ± 1.51</td>
<td>82.31 ± 1.50</td>
<td>85.98 ± 1.59</td>
</tr>
<tr>
<td>CpG 6</td>
<td>8.21 ± 1.59</td>
<td>9.64 ± 1.42</td>
<td>17.72 ± 2.96**</td>
<td>75.76 ± 3.15</td>
<td>74.81 ± 2.20</td>
<td>78.53 ± 2.04</td>
</tr>
</tbody>
</table>

Values are means ± SEM, (*n* = 5-7 mice/group). *P* < 0.05, vs. Cbs +/+ mice fed control diet. **P* < 0.05 vs. Cbs +/+ mice fed HH diet.
FIGURE 28. Tissue-Specific H19 DMD Mean Methylation Status

Hepatic H19 mean percent methylation of the A. maternal and B. paternal allele. Brain H19 mean percent methylation of the C. maternal and D. paternal allele. Data presented as mean ± SEM. (n = 5-6 mice per group). *Effect vs. Cbs +/+ mice fed the control diet, P < 0.05. **Effect vs. Cbs +/+ mice fed the HH diet, P < 0.05.
FIGURE 29. Tissue-Specific \textit{H19} and \textit{Igf2} mRNA Expression

Hepatic mRNA expression of \textbf{A}, \textit{Igf2} and \textbf{B}, \textit{H19}. Brain mRNA expression of \textbf{C}, \textit{Igf2} and \textbf{D}, \textit{H19}. Data presented as mean ± SEM. \((n = 5-6\) mice per group). *Effect vs. \textit{Cbs} +/- mice fed the control diet, \(P < 0.05\). **Effect vs. \textit{Cbs} +/- mice fed the HH diet, \(P < 0.05\).

5.5 DISCUSSION

The relationship between greater tissue AdoHcy concentrations and a lower AdoMet/AdoHcy ratio ‘methylation capacity’ with DNA methylation is still unclear. This study was designed to
determine a tissue-specific relationship between reduced methylation capacity in Cbs +/- mice with HHcy and allele-specific H19 methylation and H19 and Igf2 expression. Two main findings were revealed from this study. The first is that changes in methyl metabolism (Cbs +/- genotype and diet-induced HHcy) are associated with gene-specific changes in DNA methylation and are tissue-specific. The relationship between reduced methyl capacity and changes in DNA methylation is also tissue-specific. Collectively, this ‘proof-of-concept’ study suggests that disturbances in methyl metabolism are associated with tissue-specific changes in gene expression and DNA methylation, and can occur with or without changes in AdoMet and AdoHcy.

My findings of HHcy associated with higher AdoHcy concentrations and a lower AdoMet/AdoHcy ratio in liver are similar to previous studies reported in mice (51; 59; 70-72). I surprisingly observed no effect of the HH diet on AdoMet and AdoHcy concentrations in brain. This is in contrast to a previous finding by Devlin et al., which reported an association between HHcy in Cbs +/- mice, and higher AdoHcy concentrations and lower AdoMet/AdoHcy ratios in brain.(71). In the current study, I assessed diet-induced HHcy in mice that are a hybrid of two different mouse strains (CAST/EiJ and C57BL/6J) and noticed the scale of HHcy was 2-5-fold lower compared to what Devlin et al. and others have reported for Cbs +/- mice on a C57BL/6J background (59; 71). As such, the extent of the elevation in plasma total homocysteine that I observed in the hybrid Cbs +/- mice may have not been large enough to affect brain AdoHcy concentrations. These findings suggest that brain is less sensitive to elevations in plasma total homocysteine concentrations as it pertains to AdoMet and AdoHcy.
As mentioned earlier, the relationship between increased intracellular AdoHcy concentrations and a lower AdoMet/AdoHcy ratio with DNA methylation is unclear. Conflicting reports in the literature suggest that the effects of AdoHcy and AdoMet/AdoHcy ratio on DNA methylation are tissue-specific. To test this, I chose to target \textit{H19} to assess the effect of HHcy and changes in tissue AdoMet and AdoHcy concentrations on gene-specific DNA methylation in liver and brain tissue from \textit{Cbs +/-}mice. In addition, to quantify allele-specific DNA methylation, the \textit{Cbs +/-} mice assessed were hybrid offspring from two strains of mice that had a strain-specific genetic variant in the \textit{H19 DMD} region I selected to study. In liver, \textit{Cbs +/-} mice with HHcy had lower maternal allele \textit{H19} methylation, accompanied by higher AdoHcy concentrations and lower AdoMet/AdoHcy ratios. Interestingly, in brain \textit{Cbs +/-} mice with HHcy had higher maternal allele \textit{H19} methylation, despite no effect of the HH diet on AdoHcy concentrations and AdoMet/AdoHcy ratios. These findings suggest that in \textit{Cbs +/-} mice with diet-induced HHcy, changes in DNA methylation can occur in the brain without accompanying changes in AdoMet and AdoHcy concentrations. These data further suggest a tissue-specific relationship between diet-induced HHcy, tissue AdoMet and AdoHcy concentrations, and \textit{H19 DNA methylation}.

Initially, it was predicted that HHcy and changes in tissue AdoHcy concentrations would affect paternal allele methylation, because this is the allele reported to be methylated, and therefore silenced (86; 260; 261). As expected, I did find a high percentage of methylation on the paternal allele. I did detect some methylation (4-40%) on the maternal allele in liver and brain from all groups of mice. This was surprising, as it demonstrates that the maternal allele isn’t completely unmethylated. This finding is novel and can be attributed to the fact that bisulfite pyrosequencing was used to quantify allele-specific \textit{H19} methylation, which is more quantitative than traditional
bisulfite sequencing or restriction enzyme analyses. To my knowledge, these findings are the first to report an effect of diet-induced HHcy on maternal allele $H19$ methylation.

I speculated that HHcy in $Cbs +/ -$ mice would be associated with changes in $H19$ and $Igf2$ expression because of the observed changes in $H19$ paternal allele methylation. This was based on the proposed imprinting model, whereby paternal allele methylation at the $H19$ DMD blocks enhancer access to $H19$ which results in enhancer-related activation of $Igf2$ transcription. This phenomenon results in expression of $Igf2$, but not $H19$ on the paternal allele (134). Despite the fact that no effect of HHcy was observed on methylation status for the paternal allele in liver and brain, I did observe a tissue-specific pattern of $H19$ and $Igf2$ mRNA expression associated with methylation in the $H19$ maternal allele. In $Cbs +/ -$ mice with HHcy, lower maternal allele methylation in liver was associated with lower $Igf2$ expression; whereas in brain, higher $H19$ maternal allele methylation was associated with lower $H19$ mRNA. Together these findings demonstrate an association between tissue-specific and allele-specific changes in $H19$ methylation and $H19$ and $Igf2$ expression.
PART 2

5.6 INTRODUCTION

I propose to determine if gene-specific changes in DNA methylation and expression are associated with changes in AdoMet and AdoHcy concentrations in heart, and contribute to the obesity-related cardiac remodeling observed in Cbs +/- mice.

RATIONALE

In Part 1, I determined that disturbances in methyl metabolism contribute to a tissue-specific relationship between AdoMet and AdoHcy concentrations, DNA methylation, and gene expression in Cbs +/- mice with HHcy. Studies conducted in chapters 3 and 4 demonstrated that Cbs +/- mice are a good model to investigate the role of methyl metabolism in the pathogenesis of obesity-related cardiac remodeling. The underlying molecular mechanisms responsible for the observed obesity-related cardiac remodeling in Cbs +/- mice are unknown, but may involve the epigenetic process, DNA methylation. Therefore, this study was designed to determine if gene-specific changes in DNA methylation and expression are associated with changes in AdoMet and AdoHcy concentrations in the heart, and contribute to the obesity-related cardiac remodeling observed in Cbs +/- mice.
To delineate a role for DNA methylation in the augmented cardiac remodeling observed in *Cbs* +/− mice with diet-induced obesity, a gene-targeted approach will be used. The *Ppara* gene was targeted for two reasons. First, in Chapter 4, greater *Ppara* expression in heart was associated with altered cardiac fatty acid metabolism in *Cbs* +/− mice with obesity-related cardiac remodeling. Secondly, expression of *Ppara* gene is known to be regulated by DNA methylation in the heart (171; 239). Hearts from mice studied in Aim 2, Chapter 4 were used to quantify the methylation status of the *Ppara* promoter and AdoMet and AdoHcy concentrations.

### 5.7 METHODS

#### ANIMALS AND DIETS

For Part 2, the same set of mice used in Aim 2, Chapter 4 was used in this study. Heart samples were acquired from the subset of mice used for the isolated working heart experiments.

#### BIOCHEMICAL ANALYSES

AdoMet and AdoHcy concentrations in heart were quantified by HPLC using UV detection, as previously described in Part 1 (30). Quantification of AdoMet and AdoHcy in Part 2, were conducted in Dr. J. Miller’s laboratory at Rutgers, The State University of New Jersey.
BISULFITE PYROSEQUENCING

Similar materials and methods were used as previously described in Part 1.

Specifics for the Ppara assay: a CpG rich region within the Ppara promoter sequence was selected (107 bp fragment) and amplified by PCR from bisulfite-treated heart DNA samples using the following primers (Integrated DNA Technologies): forward, 5’-GAT TTG TAG GAG GAG TGT AGT TTT AG-3’; and reverse, 5’-CCT ATA CAC CCA CCT CAC TAT-3’, containing a biotin label at the 5’ end (Integrated DNA Technologies). The PCR products were sequenced using the following sequencing primer: 5’-CCA CCC ACC TCA CTA T-3’.

REAL-TIME PCR

Similar materials and methods were used as previously described in Aim 1 (Chapter 3)

STATISTICAL ANALYSIS

Statistical analysis for Part 2 is identical to what was used in Chapters 3 and 4.
5.8 RESULTS

CARDIAC PPARA PROMOTER DNA METHYLATION STATUS

In Aim 2 (Chapter 4) I observed that Cbs +/- mice had alterations in cardiac fatty acid metabolism associated with a higher Ppara mRNA expression in left ventricle compared to Cbs +/- mice. Building from this data, I sought to determine if the greater Ppara mRNA expression is a consequence of alterations of Ppara promoter methylation status and/or changes in AdoMet and AdoHcy concentrations in the left ventricle. A CpG rich region in the Ppara promoter was identified between -300 and +1, relative to the Ppara transcriptional start site the UCSC Genome Browser and Methprimer (170). The region contains 6 CpG sites (Figure 30).

Percent methylation of each CpG site and the mean methylation of all 6 sites within the Ppara promoter region were quantified in left ventricle and shown in Table 14. No effect of diet or genotype on percent methylation in CpG sites 1, 5 and 6 in left ventricle was observed. In mice fed the HFD, percent methylation was lower ($P < 0.05$) in CpG site 4 and for mean methylation in left ventricle compared to mice fed control diet (Table 14). An interaction ($P < 0.05$) between diet and genotype on percent methylation in CpG sites 2 and 3 was detected (Table 14). In mice fed the HFD, Cbs +/- mice had lower ($P < 0.05$) percent methylation in sites 2 and 3 in left ventricle compared to Cbs +/- mice. Although differences were statistically significant, percent methylation was very low amongst all groups and therefore any changes may not have biological relevance. Therefore, these findings must be interpreted with caution and the biological relevance remains to be determined.
CARDIAC ADOMET AND ADOHCY CONCENTRATIONS

To determine if alterations in cardiac DNA methylation status is associated with reduced methylation capacity in left ventricle, AdoMet and AdoHcy concentrations were quantified. I observed no effect of diet or genotype on AdoMet, AdoHcy or the AdoMet/AdoHcy ratio in left ventricle (Figure 31A-C).

5.9 FIGURES AND TABLES

FIGURE 30. Ppara Promoter Methylation Assay Design

Region of the Ppara promoter sequence selected to quantify methylation status. Diagram is not to scale. The arrow corresponds to the Ppara transcriptional start site and nucleotide +1, at exon 1. UCSC Genome Browser revealed a CpG rich region located between -300 and +1, relative to the Ppara transcriptional start site. The 6 CpG sites selected for analysis are in bold. Figure adapted from Genebank accession NM_011144, mouse chromosome 15.
TABLE 14. Cardiac Ppara Promoter Methylation Status

<table>
<thead>
<tr>
<th>% Methylation</th>
<th>Control Cbs +/+</th>
<th>Control Cbs +/-</th>
<th>HFD Cbs +/+</th>
<th>HFD Cbs +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG 1</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>CpG 2†</td>
<td>5.8 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>5.6 ± 0.4</td>
<td>5.2 ± 0.4**</td>
</tr>
<tr>
<td>CpG 3†</td>
<td>3.4 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>3.0 ± 0.3**</td>
</tr>
<tr>
<td>CpG 4</td>
<td>2.4 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>1.8 ± 0.1**</td>
<td>2.1 ± 0.2**</td>
</tr>
<tr>
<td>CpG 5</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>CpG 6</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Mean Methylation</td>
<td>2.9 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>2.8 ± 0.1**</td>
<td>2.7 ± 0.1**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 5-6 mice per diet/genotype group). †Diet x genotype interaction, P < 0.05; *Effect of diet, P < 0.05; **Effect of genotype, P < 0.05.

FIGURE 31. Cardiac AdoMet, AdoHcy, and AdoMet/AdoHcy Ratio

Cardiac concentrations of A. AdoMet, B. AdoHcy and C. AdoMet/AdoHcy ratio. Open bars, Cbs +/+ mice; closed bars, Cbs +/- mice, (n = 5-9 mice per diet/genotype group).

Data presented as mean ± SEM.
The purpose of this study was to investigate a potential mechanism underlying the findings I reported in Chapters 3 and 4. I proposed to determine if gene-specific changes in DNA methylation and expression are associated with changes in AdoMet and AdoHcy concentrations in heart, and contribute to the obesity-related cardiac remodeling observed in Cbs +/- mice. Two findings were revealed from this study. The first is that no change in \textit{Ppara} promoter methylation status from \textit{Cbs} +/- mice fed HFD was detected in heart, despite greater \textit{Ppara} expression observed in heart from \textit{Cbs} +/- mice. The second is that AdoMet and AdoHcy concentrations in the heart were unaffected by \textit{Cbs} genotype and HFD feeding (obesity). Together, these findings suggest that alterations in cardiac lipid metabolism observed in \textit{Cbs} +/- mice with obesity are associated with changes in epigenetic regulation of the \textit{Ppara} promoter.

A study by Lillycrop et al. investigated the effect of maternal protein-restriction during pregnancy on \textit{Ppara} promoter methylation status in hearts from offspring rats. Specifically, they found that offspring from mother’s fed a protein-restricted diet during pregnancy induced reduced methylation of the \textit{Ppara} promoter and greater \textit{Ppara} mRNA expression in heart (239). This study suggests that \textit{Ppara} expression is regulated via epigenetic mechanisms (DNA methylation) in the heart. Although, this study is limited as DNA methylation was measured using Real-Time PCR, and this method is less sensitive and only semi quantitative compared to more developed methods such as Pyrosequencing.
Findings reported in this thesis are the first to test the direct effects of disturbances in methyl metabolism on lipid metabolism associated with epigenetic changes in DNA methylation. To my knowledge, this study is the first to quantify DNA methylation in mouse heart using bisulfite pyrosequencing which is more quantitative than traditional methods. For all groups of mice, percent methylation within the Ppara promoter was low (1-6 %), suggesting that the Ppara promoter is relatively unmethylated in heart tissue. I did find reduced methylation at CpG sites 2 and 3 within the Ppara promoter in Cbs +/- mice fed the HFD; however, the biological relevance is questionable, as these significant changes in percent methylation were minimal.

Additionally, this is the first animal study to quantify AdoMet and AdoHcy concentrations in heart tissue and furthermore, to investigate relationships between methyl metabolism, DNA methylation, and AdoMet and AdoHcy concentrations in heart. Neither Cbs +/- genotype or HFD had an effect on AdoMet and AdoHcy concentrations in heart. These data, along with the data from Part 1, imply that disturbances in methyl metabolism are associated with tissue-specific changes in DNA methylation and can occur with or without changes in AdoMet and AdoHcy. Moving forward, the AdoMet/AdoHcy ratio is an inadequate maker of ‘methylation capacity’ as it is highly tissue specific.
CHAPTER 6:
CONCLUSION
6.1 SUMMARY

This thesis opens a new area of research addressing the role of methyl metabolism in obesity-related cardiac remodeling, which may contribute to cardiovascular pathology. The contributions and strengths of this PhD research to the fields of methyl metabolism and cardiac remodeling in obesity are summarized below.

AIM 1: These studies are the first to show that disturbances in methyl metabolism enhance cardiac lipotoxicity associated with obesity-related cardiac remodeling.

Methyl metabolism and the transsulfuration pathway are well characterized in the liver and the kidney; however, little is known about their involvement in other tissues such as the heart. My findings are the first to investigate the role of methyl metabolism and the transsulfuration pathway in the heart; and furthermore in the absence and the setting of diet-induced obesity. I am the first to report that Cbs +/- mice are susceptible to diet-induced obesity to a similar degree as Cbs +/- mice, but have greater glucose intolerance. Additionally, findings in this aim are the first to demonstrate that Cbs +/- mice with diet-induced obesity have disturbances in cardiac glutathione homeostasis accompanied by excess triglyceride accumulation, enhanced oxidative stress, and pro-apoptotic signaling in heart. Overall, Cbs +/- mice appear to be more sensitive to cardiac lipotoxicity associated with obesity-related cardiac remodeling. Interestingly, total hepatic glutathione concentrations were unaffected by the Cbs +/- genotype or the HFD. These studies also demonstrate that cysteine in the circulation is lower in Cbs +/- mice with diet-induced obesity, suggesting lower provision of cysteine for cardiac glutathione synthesis. These
findings suggest that CBS and cysteine may play important roles in regulating the pool of glutathione in the heart, especially under conditions of oxidative stress during diet-induced obesity. Taken together, these findings are the first to imply that the protective role of CBS and the transsulfuration pathway may extend to the heart, and are not limited to the liver. These findings also insinuate an important role for CBS in the maintenance of cardiac glutathione and in the prevention of cardiac lipotoxicity associated with obesity-related remodeling.

**AIM 2:** These studies are the first to provide evidence that disturbances in methyl metabolism are associated with alterations in cardiac energy metabolism and function in obesity-related cardiac remodeling.

This aim is an extension of the preliminary findings observed in Aim 1. Building from these findings, the objective was to determine functional consequences and portray the underling mechanisms of obesity-related cardiac remodeling in $Cbs^{+/-}$ mice. This study is the first to report that $Cbs^{+/-}$ mice have larger hearts compared to $Cbs^{+/+}$ mice. This phenotype, which suggests cardiac hypertrophy, was observed independent of diet and could be a result of a congenital defect arising from disruption of the CBS gene. These findings exhibit evidence that $Cbs^{+/-}$ mice have changes in ex-vivo cardiac function and fatty acid metabolism compared to $Cbs^{+/+}$ mice, suggesting $Cbs^{+/-}$ mice are more susceptible to cardiac remodeling. Furthermore, these studies are the first to report an association between changes in cardiac long-chain polyunsaturated fatty acids and diet-induced obesity in mice. $Cbs^{+/-}$ mice also have higher total AA and DHA concentrations in heart, evidence to suggest existence of phospholipid remodeling and altered fatty metabolism in the heart. I also revealed that adiponectin circulates
at lower concentrations in Cbs +/- mice, a finding that may lead to less AMPKα stimulation in the heart and may result in the observed changes in cardiac fatty acid metabolism and function. Overall, these studies indicate a unique role for CBS in cardiac fatty acid metabolism, possibly contributing to obesity-related cardiac remodeling. This is an indirect relationship, given that CBS is not expressed or active at any appreciable level in the heart (46; 200). Together, studies in conducted in Aim 1 and Aim 2 reveal that Cbs +/- mice with diet-induced obesity serve as an excellent mouse model to investigate the pathogenesis of cardiac remodeling.

**AIM 3: These studies confirm that disturbances in methyl metabolism contribute to tissue-specific changes in gene expression and DNA methylation.**

The goal of Aim 3 was to investigate potential mechanisms responsible for the studies conducted in Aim 1 and Aim 2. I hypothesized that changes in gene-specific DNA methylation patterns would be a potential mechanism, considering the metabolic relationship between methyl metabolism and DNA methylation. Many have reported that a lower AdoMet/AdoHcy ratio is an indicator of reduced DNA methylation capacity. However, I have questioned its use as an indicator as too many studies have reported inconsistent findings suggesting otherwise, and believe the discrepancies are a result of tissue specificity. Therefore, the studies conducted in **Part 1** were designed to further characterize the relationship between greater AdoHcy concentrations and a lower AdoMet/AdoHcy ratio with DNA methylation in different tissues (liver and brain). These studies clearly confirm that the AdoMet/AdoHcy ratio is not a reliable marker of DNA methylation capacity because these metabolites are highly tissue-specific. This proof-of-concept is summarized below in **Figure 33**.
Figure adapted from: Glier M.B., Green T.J., and Devlin A.M. 2014. Molecular Nutrition and Food Research. 58:172-182.

**Methyl Nutrient Imbalances**

This is a schematic representation illustrating the potential mechanism whereby an imbalance in methyl metabolism, for instance HHcy, can alter gene expression through changes in DNA methylation. Hydrolysis of AdoHcy to homocysteine is a reversible reaction, with synthesis of AdoHcy in favour. Elevated homocysteine concentrations, despite the cause of HHcy, can result in greater AdoHcy concentrations. AdoHcy has been reported to inhibit methyltransferases (MTs) and consequently inhibit DNA methylation reactions. I have shown that the effects of imbalances in methyl metabolism on AdoMet and AdoHcy concentrations and DNA methylation patterns are tissue-specific. This could result in changes to DNA methylation patterns or have no effect at all. Changes to DNA methylation, with or without changes to methyl metabolism, may contribute to a gene expression profile that promotes CVD pathology.
I still questioned whether DNA methylation was an underlying mechanism responsible for the observed obesity-related remodeling in Cbs +/- mice. I did find that greater Ppara mRNA expression in hearts from Cbs +/- mice was associated with lower methylation status of the Ppara promoter in heart from Cbs +/- mice with obesity-related cardiac remodeling. To date, it is difficult to know whether these small changes in DNA methylation status have any biological relevance and remains to be unknown. Besides the fact that I confirmed in Part 1 that the AdoMet/AdoHcy ratio is not a good marker of methylation capacity, I still questioned whether changes to these metabolites occurred in hearts as an effect of CBS genotype and/or diet. To date, I am the first to quantify AdoMet and AdoHcy concentrations in heart tissue and in hearts from Cbs +/- mice with and without obesity-related cardiac remodeling. I did not observe an effect on diet or genotype for either AdoMet or AdoHcy concentrations in the heart. It is important to keep in mind that changes to DNA methylation patterns likely occur, despite no changes in tissue AdoMet and AdoHcy concentrations. These data further highlight that the so-called ‘methylation capacity’ marker (AdoMet/AdoHcy ratio) should not be used to predict tissue methylation status. Numerous steps are involved in the regulation of DNA methylation and gene expression besides the involvement of AdoMet and methyl metabolism and other mechanisms must be at play. Further studies are required to elucidate other mechanisms involved in the regulation of DNA methylation.

In conclusion, each specific aim was designed to build on the previous aim, all together satisfying the overall objective of this thesis. In conclusion, studies conducted in Aims 1-3 provide evidence to confirm that disturbances in methyl metabolism contribute to obesity-related cardiovascular pathology. As such, I believe the studies in this dissertation have addressed the
general hypothesis. Below, **Figure 34** is a schematic diagram to illustrate and summarize the major contributions of this PhD dissertation.

![Figure 34](image)

**FIGURE 33. Thesis Summary**

Cardiac remodeling in obesity is characterized by impaired energy metabolism, lipotoxicity, and structural and functional changes in the heart. Obesity-related cardiac remodeling could lead to CVD complications, and could ultimately lead to heart failure. Work presented in this thesis demonstrates that together, obesity-related cardiac remodeling and an imbalance in methyl metabolism (via *Cbs +/-* mice) are associated with gene-specific changes in DNA methylation and expression in the heart. Importantly, studies in this thesis demonstrate that gene-specific changes in DNA methylation and mRNA expression associated with imbalanced methyl metabolism (via *Cbs +/-* with HHcy) are highly tissue-specific, and occur without changes in
AdoHcy concentrations. This further suggests that tissue-specific AdoHcy levels are well-defended by the body. Changes in DNA methylation and imbalances in methyl metabolism are possible mechanisms that may occur together or independently, both of which may contribute to CVD complications.

6.2 FUTURE DIRECTIONS

The experimental investigations conducted in this thesis provide fundamental knowledge pertaining to the role of methyl metabolism in obesity-related cardiac remodeling. This research has generated a few additional questions and has set the stage for new research avenues.

There is still little known pertaining to the involvement of methyl metabolism in cardiomyopathy and heart failure. A further investigation could involve characterizing methyl metabolism pathways in the heart and determining if disturbances in methyl metabolism contribute to obesity-related cardiac remodeling. Since I encountered cardiac tissue limitations throughout my research studies, a subset of $Cbs^+/+$ and $Cbs^{+/-}$ mice fed either the control or HFD for 20 weeks to induce obesity, would be required. Assessment of methyl metabolism pathways in heart would involve quantification of the following metabolites: AdoMet, AdoHcy, homocysteine, cystathionine, and cysteine; the following enzymes: MS, MAT, SAHH, BHMT, PEMT, CBS, and CGL; and the following cofactors: vitamin B6 and vitamin B12. As mentioned previously, obesity is also associated with excess lipid accumulate in other tissues, such as the liver, pancreas and skeletal muscle (33; 68). Similarly, further assessment of methyl metabolism could
involve characterizing the above listed metabolites, enzymes and cofactors in the liver, pancreas and skeletal muscle, using the same mechanisms.

Hydrogen sulfide (H$_2$S) is a signaling gas that has recently gained a lot of attention in the field of cardiovascular research for its physiological functions that may have a protective role against CVD (216; 300). These physiological functions include: pro- and anti-inflammatory properties, resistances to oxidative stress, and pro- and anti-apoptotic activities (156; 301). Of relevance to my thesis, H$_2$S is synthesized endogenously by enzymes CBS and CGL (47; 153). Out of the two, CGL is expressed in the myocardium and vascular smooth muscle cells and the predominant enzyme in the cardiovascular system. Mouse studies have reported evidence to suggest that H$_2$S and CGL may have protective roles in the heart (216; 300). Kondo et al. showed lower concentrations of H$_2$S in plasma and heart from mice following transverse aortic constriction (TAC) to induced cardiac hypertrophy and heart failure (157). They also conveyed that mice lacking the gene for CGL had greater LV cardiac hypertrophy and cardiac dysfunction compared to control mice, following TAC. Whereas, transgenic mice with cardiac-specific overexpression of CGL were able to maintained cardiac structure and function, following TAC (157). Lastly, this study also demonstrated oral H$_2$S therapy (SG-1002, H$_2$S donor) to have a preventative effect during the development of heart failure, after TAC in control mice (157). In my research I discovered that Cbs +/- mice have cardiac hypertrophy and whether this phenotype is physiological or pathogenic remains unknown. Likewise, the role of H$_2$S during the pathogenesis of TAC-induced hypertrophy and heart failure in Cbs +/- mice has never been investigated. Therefore, another further investigation would involve determining if disturbances in methyl metabolism enhance hypertrophy and heart failure induced by TAC and characterizing
the effects of H$_2$S therapy. Again, a subset of $Cbs^{+/+}$ and $Cbs^{+-}$ mice fed the control diet for 20 weeks, would be required. This would include quantifying H$_2$S concentrations in plasma and the heart, evaluation of cardiac structure and function following, and determining the effects of oral H$_2$S therapy (SG-1002, H$_2$S donor), after TAC-induced cardiac hypertrophy and heart failure in $Cbs^{+/+}$ and $Cbs^{+-}$ mice.

The field of epigenetics is still in its infancy, and RNA sequencing (RNA-Seq) technology has become an increasingly popular and is preferred to microarray technology for quantifying gene expression. Recently, RNA-Seq technology has become more affordable and user-friendly data analysis tools are available. RNA-Seq offers a number of advantages compared to microarray technology. For example, it uses an unbiased approach for detection of transcripts, single nucleotide variants, and indels (small insertions and deletions) across the transcriptome. In addition, RNA-Seq offers increased sensitivity and specificity for detection of genes, transcripts, and differential expression compared to microarray technology (75; 133; 188). Moving forward, an additional study could involve determining if gene-specific changes in DNA methylation and expression (using RNA-Seq) contribute to obesity-related cardiac remodeling in $Cbs^{+-}$ mice. Once again, another subset of $Cbs^{+/+}$ and $Cbs^{+-}$ mice fed either the control or HFD for 20 weeks to induce obesity, would be required. This would include validating the list of differentially expressed genes and quantification of DNA methylation status of confirmed genes. This investigation, using RNA-Seq technology, would provide gene-targets likely to be involved in the pathogenesis of obesity-related cardiac remodeling and could lead to further epigenetic studies beyond changes in DNA methylation status.
6.3 SIGNIFICANCE

Findings from this thesis are important because they a baseline understanding of mechanisms underlying modifications that take place during obesity-related cardiac remodeling, such as lipotoxicity and changes to cardiac energy metabolism, function and structure. As summarized above, the overall hypothesis and the three aims used to address my hypothesis have provided new insights to the fields of methyl metabolism and obesity-related cardiovascular pathologies. By exploring and answering the ‘unknown’ findings in the thesis provide knowledge that can foster the development of improved future hypotheses leading to a sound foundation for new discoveries. For example, I discovered that \textit{Cbs +/-} mice with diet-induced obesity serve as an exceptional mouse model to investigate the pathology of cardiac remodeling. This finding highlights a novel role for CBS and disturbances in methyl metabolism in obesity-related cardiovascular pathologies. Studies in the latter half of my thesis provide a proof-of-concept that disturbances in methyl metabolism contribute to changes in tissue-specific gene expression and DNA methylation, associated with obesity-related cardiac remodeling. Further investigations are warranted regarding the role of DNA methylation in the pathogenesis of obesity-related cardiac remodeling in \textit{Cbs +/-} mouse model with diet-induced obesity. Such studies may open doors to new therapies for CVD and possible strategies to prevent heart failure.
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