EFFECTS OF WEIGHT LOSS AND PHENOTYPE TRAITS ON CHANGES IN BODY COMPOSITION AND CHOLESTEROL METABOLISM IN OVERWEIGHT INDIVIDUALS

by

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Global obesity is linked to chronic diseases including hypercholesterolemia, a cardiovascular disease risk factor, thus weight reduction in obesity is a key priority for combatting obesity. The cholesterol transporters ABCG5, ABCG8 and NPC1L1 mediate cholesterol trafficking across the intestinal wall, thus are important in regulating cholesterol metabolism and circulating levels. The objective of this study was to examine if single nucleotide polymorphisms (SNP) of cholesterol transporters ABCG5, ABCG8 and NPC1L1 are associated with changes in cholesterol synthesis and absorption and lipid parameters (LP) subsequent to weight loss (WtL) in overweight individuals. Eighty-nine individuals from two WtL trials (Trial A (n = 54) and Trial B (n = 35)) completed a 20-wk WtL period. After 10% WtL, lipid parameters excluding LDL-C were improved in Trial A, while all lipid parameters were ameliorated after 12% of WtL when Trial A and B were combined. Post-WtL, cholesterol synthesis (CS) was reduced; however, cholesterol absorption was not changed in either Trial A or the combined trials. Polymorphisms in ABCG8 V632A were associated with changes in TC and TG levels after WtL in both trial A and the combined data. SNPs in ABCG5 Q604E, ABCG8 T400K, were associated with changes in CS because of WtL in Trial A; however, the association is no longer seen in combined analysis. In conclusion, cardio-protective changes in LP due to weight loss were mediated by reductions in CS. Additionally, polymorphisms in ABCG8 were associated with amelioration in LP after WtL. Thus, the benefits in CVD risk subsequent to weight loss vary across individuals due to genetic factors associated with cholesterol trafficking.

ACKNOWLEDGEMENTS

I would like to show my deepest gratitude to my supervisor, Dr. Peter J.H. Jones for his mentorship which provided me such a great adventure from initial to final. His encouragement, guidance and support enabled me to develop an understanding of the subject. Thank you very much for everything Dr. Jones!

I would like to thank my committee members, Dr. Harold Aukema, Dr. Peter Zahradka and Dr. Jonathan McGavock for your intriguing comments and ample suggestions.

I highly appreciated the help from Dr. Amira Kassis who has been a great tutor from me in establishing and conducting Trial A. Thanks very much Amira ;) Special thanks to Daryl Hurrie who volunteered to design the physical activity guidelines and being the exercise physiologist of Trial A. I would also like to thank Darren Speziale to help in the PA part of Trial A, and for all his support, knowledge in exercise and jokes which lighten the day. I would like to thank Christopher Marinangeli for his help in the trial. I would like to thank Jusin Penner for accompanying me as a clinical coordinator of Trial A.

I would like to show my gratitude to Dr. Sylvia Santosa who provided the data and samples from Trial B, as well as, Isabelle Demonty, Dr. Alice H. Lichtenstein, and Dr. Jose M. Ordovas for their help in Trial B.

Thank you to Khatima Khalloufi, Yen Ming Chan, Dr. Vanu Ramprasath, Dylan McKay, Dennis Labossiere, Dr. Todd Rideout and Dr. Scott Harding for your help and guidance in the laboratory.

Very special thanks to Dr. Peter Eck and Dr. Natasha Yurkova who provided guidance for DNA and SNPs analysis.

I would like to thank Andrea Klymasz, Dylan MacKay, Lynn Grant, Debbie Macgregor and Jin Ya Chen for their help in proof reading my initial draft of my thesis.

To all Dr. Jones' lab members and clinical coordinators, past and present, and Richardson Centre for Functional Food and Nutraceutical staff members who have supported me along the way.

I am grateful for Manitoba Graduate Scholarship and Canadian Institute Health Research for their funding support.

Very exceptional thanks to Asadilan Indrabudi. Thank you for always being there for me.

Finally, I would like to thank my parents, Siswadi Mintarno and Titin S. Linggadihardja, and my sister Monica for their extraordinary support and encouragement which enabled me to achieve this experience.

DEDICATION

This thesis is dedicated to my God, Jesus Christ, my mother, Titin Sumarni Linggadihardja, my father, Siswadi Mintarno, my sister, Monica Mintarno and my boyfriend, Asadilan Indrabudi. Without their encouragement, support and endless love the completion of the work would not be possible.

CONTRIBUTION OF KNOWLEDGE

The candidate was responsible for writing both manuscripts found in this thesis. The candidate conducted and coordinated the clinical weight loss trial at the RCFFN (Trial A). As a clinical co-coordinator, the candidate was responsible for recruiting and monitoring of Trial A study participants. The candidate was also responsible for executing the Trial A study protocol, as well as, collecting and analyzing the data. The candidate also analyzed all trial A DEXA images, blood lipid profiles. The candidate analyzed cholesterol absorption and synthesis measurements, and genetic polymorphisms in both Trial A and Trial B conducted in McGill (Santosa *et al*, 2007).

Dr. Peter Jones, the candidate's supervisor, edited all the manuscripts included in this thesis. Dr. Jones was the principal investigator, developing the initial protocol of both Trial A and Trial B. Dr. Jones also conducted regular weekly meetings with the candidate to monitor progress and provide any necessary guidance.

Dr. Amira Kassis developed the initial protocols for trial A and helped in beginning of the Trial A.

Dr. Peter Eck and Dr. Natasha Yurkova provided guidelines on DNA extraction, DNA concentration and SNPs determinations.

Dr. Sylvia Santosa conducted Trial B and provided the raw data of Trial B participants' lipid profile, FM and FFM, as well as body weights.

Dr. Harold Aukema, Dr. Peter Zahradkha, and Dr. Jon Mcgavock are the candidate's committee members who edited the final draft of the thesis.

TABLE OF CONTENTS

Abstract ……..…….…... ii

Acknowledgements…… iii

Dedication……..…… v

Contribution of Knowledge……..……..vi

Table of Contents……... viii

List of Tables…….…xi

List of Figures…… xiii

List of Abbreviations……..xiv

List of Copyrighted Material for which Permission was Obtained……... xvi

Chapter I: Introduction ……..…. 1

1.1 Study Objective…… 4

1.2 Hypothesis…… 4

Chapter 2: Literature Review …… 5

2.1 Introduction ……. 5

2.2 Obesity and Cardiovascular risk……..…….5

2.3 Weight loss and Alterations in Body Composition on Lipid Levels……7

2.4 Effects of Weight Loss on Cholesterol Metabolism…… 9

2.5 The Effect of Genetic Polymorphisms in Cholesterol Transporters on Cholesterol Metabolism……... 13

2.6 Summary and Conclusion…… 19

Chapter 3: Manuscript 1……. 20

Genetic Basis for Heterogeneity in Response of Serum Lipids and Cholesterol Absorption and Synthesis to Weight Loss in Overweight Individuals

3.1 Abstract…… 21

3.2 Introduction…….. 23

3.3 Materials and Methods……. 25

3.4 Results…….. 35

3.5 Discussion…… 48

3.6 Conclusion……54

3.7 Literature Cited ……..…..55

Chapter 4: Bridge ……62

Chapter 5: Manuscript 2 …… 63

Polymorphism of V632A in ABCG8 is Associated with Reduced Serum Lipid Profiles Due to Weight Loss in Overweight Individuals

5.1 Abstract…… 64

5.2 Introduction…….. 66

5.3 Materials and Methods……. 68

5.4 Results…….. 75

5.5 Discussion……..... 86

5.6 Conclusion……...90

5.7 Literature Cited …….... 90

Chapter 6: General Discussion and Conclusion ……... 97

6.1 Summary and Discussion……...……. 97

6.2 Future Directions……...…….. 99

6.3 Final Conclusions……..……. 100

Chapter 7: References……..101

Appendix…… 114

1. Subject Consent Forms of Research Protocol…….. 114
2. Ethics Approval Letters…….……...126
3. Participant Screening Form…….……..… 128
4. Dietary Guidelines…….……...130
5. Sample Three Day Food Record ……. 132
6. Food Groups for Exchanges…… 134
7. Sample Dietary Regimen……. 135
8. Subject Weight Loss Graph – Actual and Target ……... 136
9. Weekly Exercise Journal…….. 138
10. Sterol Derivatization Method …….…. 139
11. Additional Figures…… 140

LIST OF TABLES

Table 2-1. BMI classification and risk level developing health problem……..... 6

Table 3.1. Missense mutation of SNPs, reference SNP number, allele and amino acid mutation change……..…….…….…….…….…….…….…… 34

Table 3.2. Baseline characteristics of weight loss study participants (n=54)……... 36

Table 3.3. Change and percent change in body weight and compartmental masses before and after 20-wk weight loss intervention in overweight and obese individuals (n=53). ……..…….. 39

Table 3.4. Correlations of change in cholesterol precursors and plant sterols with change in serum total and lipoprotein cholesterol levels before and after 20 wk weight loss intervention in overweight and obese individuals (n=54). ….……..... 43

Table 3.5. Genotype distribution and frequency of missense SNPs of ABCG5 and ABCG8 (n = 54). ……..…….. 44

Table 3.6. Change in lipid profiles, surrogates of synthesis and absorption stratified based on ABCG5 SNPs (n=54)..…….…….…….. 46

Table 3.7. Change in cholesterol synthesis and absorption surrogates marker and lipid profiles stratified based on ABCG8 SNPs (n=54).…….……..... 47

Table 5.1. Missense mutation of 8 SNPs, reference SNP number, allele as well as amino acid change…….…….…….…….…….…….…… 74

Table 5.2. Baseline characteristics of weight loss study participants in combined analysis (n = 89). ……... 76

Table 5.3. Change and percent change in body weight and composition after a 20 wk weight intervention, according to DEXA (n = 53) and MRI (n = 35) in overweight and obese individuals in the combined analysis (n = 88).……..78

Table 5.4. Correlations of change in cholesterol precursors and plant sterols with change in serum total and lipoprotein cholesterol levels before and after 20 weeks weight loss intervention in overweight and obese individuals in the combined analysis (n = 89)…….…….... 81

Table 5.5. Genotype distribution and frequency of missense SNPs of ABCG5 and ABCG8 in overweight and obese individuals in the combined analysis (n = 89).

……...…….**.....** 82

Table 5.6. Change in surrogates of synthesis and absorption and serum lipid profiles stratified based on ABCG5 SNPs in the combined analysis (n = 89)…….**.....** 84

Table 5.7. Change in surrogates of cholesterol synthesis and absorption and lipid profiles stratified based on ABCG8 SNPs in the combined analysis (n=89).…… 85

LIST OF FIGURES

Figure 2.1 In vivo model of ABCG8, ABCG5 and NPC1L1 in regulating cholesterol and plant sterol.…….…….…….…….…….. 14

Figure 3.1. Change in individual body weights (kg) before and after 20 wk of the weight loss period in overweight and obese individuals (n = 54)…….…… 38

Figure 3.2. Lipid parameters before and after weight loss in overweight and obese individuals (n=54) …….…… 41

Figure 3.3. Response of synthesis and absorption across study in overweight and obese individuals (n=54).…….…….. 42

Figure 5.1. Change in individual body weights (kg) before and after 20 wk of weight loss in obese and overweight individuals in the combined analysis (n = 89)… 77

Figure 5.2. Lipid parameters before and after weight loss in overweight and obese individuals in the combined analysis (n = 89).……..... 79

Figure 5.3. Response of synthesis and absorption across study in overweight and obese individuals in the combined analysis (n = 89).…… 80

LIST OF ABBREVIATIONS

AA	African American
ABCG5	ATP Binding Cassette G5
ABCG8	ATP Binding Cassette G8
ANOVA	Analysis of Variance
BMI	Body Mass Index
CAS	Cholesterol Absorption and Synthesis
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DEXA	Dual Energy X-ray Absorptiometry
DNA	Deoxyribonucleic Acid
FFM	Fat Free Mass
FM	Fat Mass
HDL-C	High Density lipoprotein cholesterol
IDEA	International Day for Evaluation of Abdominal obesity
LDL-C	Low Density Lipoprotein Cholesterol
MS	Metabolic Syndrome
METS	Metabolic Equivalent of Task
NPC1L1	Neiman Pick Cell 1L1
NS	Non-synonymous
NCS	Non-cholesterol Sterols
PA	Physical Activity

RT-PCR	Real Time Polymerase Chain Reaction
rs	Reference SNP
PS	Plant Sterol
RBC	Red Blood Cells
RMR	Resting Metabolic Rate
SEM	Standard Error of Means
SNP	Single Nucleotide Polymorphism
TG	Triglycerides
TC	Total Cholesterol
TEE	Total Energy Expenditure
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

In 2008, the World Health Organization (WHO) estimated that 1.46 billion adults (age 20+) were overweight, while 500 million adults were obese (Finucane *et al.*, 2011). Consistent with WHO statistics, the 2004 Canadian Community Health survey indicated that nearly a quarter (23.1 %) or 5.5 million of Canadians aged 18 years or older are obese while another 8.6 million (36.1 %) are overweight; thus, 59.1 % of Canadians aged 18 years or older are overweight and obese (Tjepkema, 2006). A quarter century ago (1978/1979), 49.2 % of the Canadian population was considered overweight or obese; since this time, the prevalence of overweight and obesity has risen an additional 20 % (Tjepkema, 2006). If the current trend continues, the WHO projects that in 2015, 2.5 billion adults will be overweight and more than 700 million will be clinically obese. The necessity to avert this upward trend is urgent because clinical diagnoses of overweight and obesity are followed by a number of health consequences. The prevalence of cardiovascular disease (CVD), hypertension, type 2 diabetes and kidney disease increases with obesity (Flegal *et al.*, 2007; Field *et al.*, 2001; Prospective Studies Collaboration *et al.*, 2009). Newgard and coworkers (Newgard *et al.*, 2009) found a significantly elevated risk of CVD indicated by higher level of low density lipoprotein cholesterol (LDL-C), triglycerides (TG) and lower level of high density lipoprotein cholesterol (HDL-C), in 73 obese compared with 67 lean individuals (Newgard *et al.*, 2009). Since obesity increases the risk of chronic diseases and morbidity, this rising obesity prevalence will need to be reduced.

Negative energy balance, occurring when energy expenditure exceeds energy intake for a defined period, is required to reduce body weight (Catenacci & Wyatt, 2007), hence reduce obesity. Physical activity combined with dietary changes increases the likelihood of successful weight loss (Goldberg & King, 2007) and is suggested as being the best strategy to combat obesity (Donnelly & Smith, 2005). Additionally, supervised structured weight loss programs have been proven to be more effective than self monitored weight loss (Andersen *et al.*, 1999; Evans *et al.*, 1999; Janssen *et al.*, 2002; Jeffery *et al.*, 2003; Miller *et al.*, 2002; Volek *et al.*, 2002)

An important aim of weight loss is to lose fat mass (FM) while maintaining fat free mass (FFM), in order to maintain an obese individual's metabolic and physical capacities (Verdich *et al.*, 2011). Body composition is a vital measure in determining health benefits consequent of weight loss (Santosa *et al.*, 2007a). Beneficial alterations in lipid levels were attributed to a decrease in body fat percentages as shown in previous trials (Frige' *et al.*, 2009; Ross & Janiszewski, 2008). Since excess adipose tissue contribute to cardiovascular disease risk (Allende-Vigo, 2010a), then obesity therapies including weight loss should be focusing on fat mass reduction (Allende-Vigo, 2010b).

The mechanism of cardio-protective changes from weight loss remains unclear. The level of cholesterol in the body is maintained by its absorption, synthesis and fecal excretion. Research has found that the rate of cholesterol synthesis is synonymous with weight loss (Santosa *et al.*, 2007b; Di Buono *et al.*, 1999; Raeini-Sarjaz *et al.*, 2001). However,

cholesterol absorption, which was observed to have a reciprocal relation with synthesis, (Gylling & Miettinen, 2002; Miettinen *et al.*, 2003) did not change post weight loss (Santosa *et al.*, 2007a) and thus needs to be explored further.

The ATP binding cassette G5 (ABCG5) and ABCG8, located in intestinal brush border membrane, are responsible for the excretion of cholesterol and plant sterols (PS) both from hepatocytes and enterocytes (Klett *et al.*, 2004). Additionally, Neiman Pick Cell 1L1 (NPC1L1) is a protein which facilitates the intestinal and hepatic absorption of cholesterol (Altmann *et al.*, 2004). Polymorphism in these transporters might be a detrimental factor affecting cholesterol biosynthesis and absorption. In 2007, Santosa et al. showed that in 35 hypercholesterolemic women, post weight reduction cholesterol metabolism was altered. Interestingly, these changes involved single nucleotide polymorphisms in ABCG5 and ABCG8 cholesterol transporters (Santosa *et al.*, 2007b). Therefore, the effect of weight loss in both males and females on cholesterol metabolism and the SNPs of these transporters need to be analyzed further.

1.1. Study Objective

- To evaluate the mechanisms related to cholesterol trafficking that underlie alterations in circulating lipoprotein profile due to weight loss and changes in body composition in overweight individuals.
- To examine if changes in cholesterol metabolism, due to weight loss in overweight individuals, are associated with genotypic traits of key cholesterol transporters ABCG5/G8 and NPC1L1.

1.2. Null Hypotheses

- Changes in circulating lipoprotein profiles in overweight and obese individuals secondary to weight loss are not a result of shifts in cholesterol synthesis and/or absorption
- Single nucleotide polymorphisms in ABCG5, ABCG8 and/or NPC1L1 do not explain the variability in response of cholesterol metabolism to intentional weight loss

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

This literature review explores the impact of obesity on coronary heart disease (CHD); evaluates the effect of weight loss and subsequent alterations in body composition on blood lipid levels as risk factors of CVD; examines changes in cholesterol absorption and synthesis (CAS) due to weight loss; as well as analyzes the association of genotypic trait in ABCG5, ABCG8 and NPC1L1 cholesterol transporters on changes in CAS rates due to weight loss.

2.2. Obesity and Cardiovascular risk

Overweight and obesity are determined by body mass index (BMI). Based on the World Health Organization, BMI is calculated by dividing body weight (kg) with the square of height (m). BMI between 25 kg/m^2 and 30 kg/m^2 is categorized as overweight while BMI above 30 kg/m^2 is classified as obese (**Table 2.1**). The greater the BMI of an individual, the higher the risk of developing health problems.

Obesity exists as an independent risk factor for CVD (Klein *et al.*, 2004). Cardiovascular diseases as a category are comprised of CHD, myocardial infarction, angina pectoris, congestive heart failure, stroke, hypertension and atrial fibrillation.

Table 2.1. BMI Classification and Risk Level Developing Health Problem

	BMI range (kg/m^2)	Risk of developing health problems
Underweight	<18.5	Increased
Normal weight	18.5 to 24.9	Least
Overweight	25.0 to 29.9	Increased
Obese Class I	30.0 to 34.9	High
Obese Class II	35.0 to 39.9	Very high
Obese Class III	≥ 40.0	Extremely high

According new Canadian guidelines, aligned with those of the World Health Organization

BMI=weight (kg) / height $(m)^2$

A number of studies have investigated the relationship of obesity in CVD and development of CVD. The Framingham Heart Study was one of the first to show that high LDL-C levels, as well as low HDL-C levels, increases the mortality risk due to CHD (Castelli, 1988; Wilson *et al.*, 1988). In the 2748 Framingham Heart Study participants after 12 years follow up, it was found that individuals with low levels of HDL-C were associated with increased mortality (Wilson *et al.*, 1988). In a 44 year follow up, overweight individuals were associated with increased relative risk of hypertension and hypercholesterolemia (Wilson *et al.*, 2002). In the Framingham offspring study of 1566 men and 1679 women, BMI was found to be significantly linearly associated with systolic blood pressure, glucose levels, total cholesterol (TC), VLDL and LDL-C cholesterol while inversely associated with HDL-C labels (Lamon-Fava *et al.*, 1996).

Similarly, the International Day for the Evaluation of Abdominal Obesity (IDEA) study evaluated 168,000 primary care patients in 63 countries and identified 24 % of men and 27 % of women as obese. The study investigators found strong correlations between BMI and waist circumference with the risk of CVD and diabetes (Balkau *et al.*, 2007). All of the studies above support the notion that overweight and obesity adversely affect dyslipidemia as CVD risk factors.

2.3. Weight Loss and Alterations in Body Composition on Lipid Levels

Major health organizations, including WHO (1998) and Canadian obesity guidelines (2006), advised individuals with BMI > 25.0 kg/m^2 to have a goal of 5 % to 10 % of weight loss within 6 months to achieve beneficial health outcomes, regardless of age and sex. Over the last 60 years, weight loss has been found to improve CVD risk factors including decreasing plasma cholesterol levels (Walker & Wier, 1951). From then many trials have studied the effects of weight loss on lipid parameters (Miller *et al.*, 2005; Nordmann *et al.*, 2006). A meta-analysis of 70 studies indicated that weight loss was associated with levels of plasma TC, LDL-C, VLDL and TG. These investigators also found that for every kilogram decrease in body weight TC, LDL-C and TG decreased by 0.05 mmol/l, 0.02 mmol/l, and 0.015 mmol/l, respectively (Dattilo & Kris-Etherton, 1992). Another meta-analysis by Proobalan et al. found that for every 10 kg of weight loss a decline of 0.23 mmol/l of cholesterol was observed in obese individuals (Poobalan *et al.*, 2004).

One disadvantage of using body weight to height square ratio (BMI) as an indicator of obesity is that this index does not provide information regarding the actual fat mass of individuals. Obese individuals have an elevated level of fat-free-mass (FFM) and fat-mass (FM). A review of PUBMED English publications between 1994 and 2010 found that fat or adipose tissue accumulation and dysfunction contributed to most cardiometabolic risk factors including insulin resistance, atherogenic dyslipidemia, inflammation and others (Allende-Vigo, 2010b). During weight loss, maintaining FFM while reducing FM is an important aim. Excessive loss of FFM is undesirable since it is responsible for maintaining resting metabolic rate, regulating body temperature, preserving skeletal integrity and maintaining function and quality of life (Marks & Rippe, 1996). A systematic review of 26 cohorts indicates that the proportion of FFM loss is influenced by the extent of caloric restriction and physical activity (Chaston *et al.*, 2007). Thus, body composition assessment, to measure changes in FM and FFM, should be a standard measure to ensure safe weight loss outcome (Chaston *et al.*, 2007). Additionally, even with minimal loss of body weight, changes in body shape or composition by increasing physical activity could improve cardio respiratory fitness (Ross & Janiszewski, 2008). In 2003, Okura et al. studied 90 obese women, divided to three groups: diet only, diet plus walking, and diet plus aerobic dance groups (Okura *et al.*, 2003). Diet and aerobic dance group has significantly greater reduction of LDL-C compared with the other two groups (Okura *et al.*, 2003). A recent trial also has shown that reductions of cholesterol and TG proportionally correlate with decreases in FM but not FFM (Frige' *et al.*, 2009).

An excess in visceral adipose tissue or abdominal fat, also known as central obesity, explains mortality and morbidity risk beyond BMI in the IDEA study which analyzed 91,246 ambulatory patients in 27 European countries (Fox *et al.*, 2009). Another study by Lapidus et al. showed that women with the highest waist to hip ratio have an increased mortality risk from CVD (Lapidus *et al.*, 1984).

Therefore, the importance of body composition measurement after weight loss is to ensure a safe weight loss outcome and to serve as an independent factor associated with CVD risk.

2.4. Effects of Weight Loss on Cholesterol Metabolism

The level of whole body pool cholesterol *in vivo* is maintained by the summation of cholesterol absorption, synthesis and excretion. *In vivo* TC pool sizes are comprised of endogenously and exogenously sourced cholesterol (Dietschy & Siperstein, 1967; Spady & Dietschy, 1983). Human body, hepatic or extra-hepatic/peripheral tissues synthesize about 50 - 60 % of endogenous cholesterol daily, whereas about 35 % to 70 % is absorbed from dietary cholesterol in the intestine (Calpe-Berdiel *et al.*, 2009). In healthy adults, 50 % to 60 % of cholesterol and less than 5 % of non-cholesterol sterols, including PS as well as precursors of cholesterol, are absorbed via the intestine daily (Calpe-Berdiel *et al.*, 2009; Patel & Thompson, 2006).

Some non-cholesterol sterols have been used as surrogate markers of cholesterol synthesis and absorption (Miettinen *et al.*, 1990; Gylling & Miettinen, 1988). The ratio of desmosterol and lathosterol, both precursors of cholesterol in its biosynthesis pathway, to cholesterol has been used quite extensively as an indirect marker of cholesterol synthesis (Kempen *et al.*, 1988; Pfohl *et al.*, 1999). Likewise, the ratio of PSs, that is, sterols originated from plant based materials including campesterol and β-sitosterol, to cholesterol, has been used as a surrogate marker of cholesterol absorption (Miettinen *et al.*, 1990). This ratio method of measuring CAS has been widely used due to its inexpensiveness and efficiency for use in large population based studies, compared with direct methods including the stable isotopes ratio method (Santosa *et al.*, 2007b).

A comparison of 74 metabolic syndrome (MS) participants with their sex and age-matched controls, showed that participants with MS possessed elevated cholesterol synthesis surrogate markers ratios, including desmosterol, lathosterol and squalene ratio to cholesterol compared with a control group (Gylling *et al.*, 2007). Moreover, the MS group had lower absorption marker ratios than their controls (Gylling *et al.*, 2007). The above results indicate that higher synthesis and lower absorption might be associated with higher CVD risk in individuals, and weight loss might ameliorate any perturbations in cholesterol metabolism.

Limited studies have analyzed the effects of weight loss and cholesterol kinetics. One of the first studies which demonstrated the relationship between weight loss and cholesterol biosynthesis was conducted by Di Buono et al (1999). After 6.8 ± 2.6% weight reduction

within a six month period in six mild-hypercholesterolemic-obese men, cholesterol fractional synthesis rates were reduced significantly from 8.42 ± 3.90%/d to 3.04 ± 1.90%/d , while the absolute synthesis rates were decreased from 1.66 ± 0.84 g/(kg.d) to 0.59 ± 0.38 g/(kg.d) (Di Buono *et al.*, 1999) . Moreover, Raeini-Sarjaz et al. also obtained the same conclusion in a weight loss group prescribed low energy diets and low energy-low fat diets, resulting in FSR decreases of 27.7 % and 25.5 %, respectively (Raeini-Sarjaz *et al.*, 2001). Another study by Santosa et al demonstrated similar results in 35 hypercholesterolemic women, where the fractional synthesis rate of cholesterol was decreased by 3.86 % after 14.5 % weight loss over a 20 week period, although cholesterol absorption rate was not changed (Santosa *et al.*, 2007a).

Conversely, a trial by Griffin et al. (1998) and Simonen et al. (2000) observed no significant change in cholesterol synthesis after weight loss. However, the latter trial observed an increase in cholesterol absorption from both dual isotopes ratio methods 29.5 % to 37.6 %, as well as serum PS ratio to cholesterol (campesterol from 162 ± 14 × 10^2 mmol/mol cholesterol to 197 ± 14 × 10^2mmol/mol cholesterol and β-sitosterol from 87 ± 5 × 10^2 mmol/mol cholesterol to 103 ± 8 × 10^2 mmol/mol cholesterol) in 16 type 2 diabetic participants undergoing 3 months of weight loss followed by 21 months of a weight maintenance diet resulting in 6 ± 1 kg of weight loss (Simonen *et al.*, 2000).

Another trial by the same group, observed 10 obese-type 2 diabetic patients consuming a very-low-energy-free of cholesterol, cholestanol and PS diet for 3 months. The intervention significantly reduced body weight by 15.5 ± 1.7 kg compared with patients'

initial body weights (Simonen *et al.*, 2002). The desmosterol and lathosterol to cholesterol ratios were decreased by 20 % indicating suppression of cholesterol synthesis, while PS ratio to cholesterol tended to decrease, but not significantly (Simonen *et al.*, 2002).

Wilund et al. examined the effect of endurance exercise on surrogate markers of CAS (Wilund *et al.*, 2009). After 6 months of endurance exercise training, 65 sedentary participants with at least one metabolic syndrome risk factor, increased their PS levels by 10 % even with no change in lathosterol level (Wilund *et al.*, 2009). This finding indicated that the alteration of cholesterol absorption rate is observable, even without a change in cholesterol synthesis.

Although results are inconclusive, the majority of these trials observed a decline in cholesterol synthesis. However, the absorption of cholesterol, which was observed to have a reciprocal relation with synthesis (Gylling & Miettinen, 2002; Miettinen *et al.*, 2003), did not change post weight loss (Santosa *et al.*, 2007b). Thus, the shift in CAS, post weight loss, still needs to be explored. Moreover, the majority of trials have only focussed on diet-induced weight loss in obese individuals and had small numbers of participants. Also, a combination effect of diet and endurance exercise, to induce weight loss and subsequent changes in body composition, was examined only in women. Therefore, the effects of weight loss on CAS, in overweight and obese healthy men and women, needs to be explored further.

2.5. The Effect of Genetic Polymorphisms in Cholesterol Transporters on Cholesterol Metabolism

Through the rare human genetic disorder termed β-sitosterolemia and via studies that examine the effect of ezetimibe, a cholesterol absorbent blocker, three key proteins, ATP binding cassette subfamily G5 (ABCG5), G8 and Neiman Pick C1-like 1 (NPC1L1) were discovered (Altmann *et al.*, 2004; Berge *et al.*, 2000; Lee *et al.*, 2001). A genetic mutation in ABCG5 and ABCG8 results in β-sitosterolemia, a disease where patients have abnormal elevated levels of PS in their body, similar to hypercholesterolaemia without the high cholesterol observed which results in premature coronary artery disease (Salen *et al.*, 2002). In β-sitosterolemic patients, cholesterol absorption is increased while cholesterol removal is delayed because of mutations in cholesterol transporter ABCG5 and ABCG8 which are responsible for cholesterol efflux into the intestine or biliary secretion in faecal excretion (Salen *et al.*, 2002; Kidambi & Patel, 2008). NPC1L1 is a cholesterol transporter which is primarily found in the intestinal brush border membrane as well as in hepatocytes (Altmann *et al.*, 2004; Deluis *et al.*, 2010). NPC1L1 is responsible for absorbing cholesterol by transporting cholesterol into the enterocyte or hepatocyte (Altmann *et al.*, 2004; Davis & Altmann, 2009; Betters & Yu, 2010). A summary concerning the mechanism of ABCG5/ABCG8 and NPC1L1 action in regulating cholesterol and PS trafficking across intestinal and hepatic membrane can be seen in **Figure 2.1.**

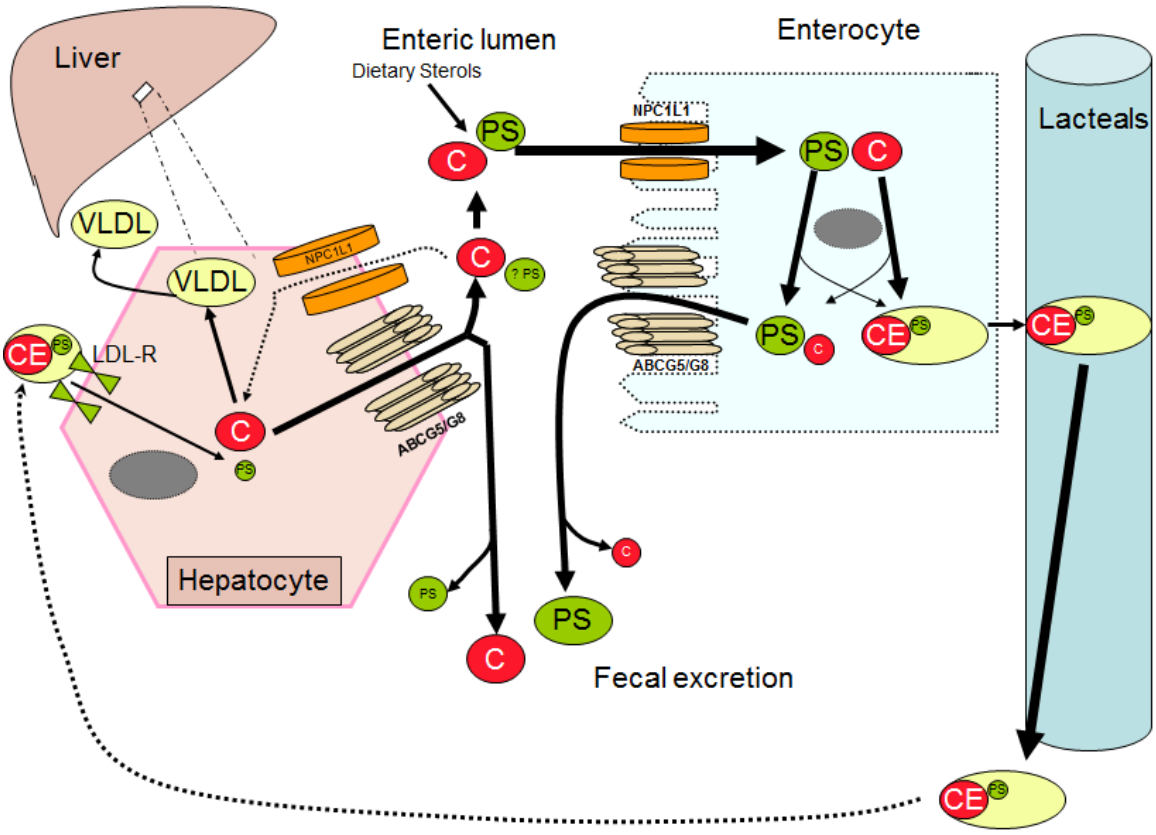

Figure 2.1 In vivo model of ABCG8, ABCG5 and NPC1L1 in regulating cholesterol and PS (Adapted from Kidambi & Patel, 2008).

In the lumen of the small intestine, dietary cholesterol and biliary cholesterol are incorporated into micelles consisting of bile salts, phospholipids and sterols. These micelles are absorbed into the enterocyte, dependent on NPC1L1 activity. Free cholesterol is esterified to form cholesterol esters and packed into chylomicrons and secreted at the basolateral surface. Because of the high affinity of PS to ABCG8/ABCG5 transporters, they are then transported back with small amount of cholesterol to enteric lumen. In the circulation, chylomicrons travel through the body and return the sterols to the liver via receptor-mediated uptake through the LDL receptor. In the hepatocyte, cholesterol can enter metabolic pathways and be re-secreted as VLDL, while cholesterol,

originated from both endogenous/synthesized and exogenous/absorbed, and PS are pumped into biliary circulation by ABCG5/ABCG8 and travel back to the intestine. A small quantity of cholesterol can be reabsorbed by NPC1L1 back to the hepatocyte from bile duct. (Kidambi & Patel, 2008)

Genetic polymorphisms on ABCG5/ABCG8 have been shown to be associated with plasma cholesterol and non-cholesterol sterol levels (Hubacek *et al.*, 2004; Weggemans *et al.*, 2002; Berge *et al.*, 2002; Chen *et al.*, 2008), along with a number of cholesterol-lowering mechanisms such as diet interventions (Weggemans *et al.*, 2002; Herron *et al.*, 2006), diet and PA induced weight loss (Santosa *et al.*, 2007b), PS supplementation (Zhao *et al.*, 2008; Plat *et al.*, 2005) and treatment with statin (Kajinami *et al.*, 2004).

The influence of SNPs of ABCG5/ABCG8 on movement of cholesterol kinetics due to weight loss was explored by Santosa et al. in 35 hypercholesterolemic women (Santosa *et al.*, 2007b). After 20 weeks of weight loss, participants' body weights were reduced by 11.7 ± 2.5 kg. Those individuals with Q carriers in ABCG5 (Q604E) had a greater decline in cholesterol absorption and larger increases in cholesterol synthesis compared with 604E carriers (Santosa *et al.*, 2007b). Moreover, in ABCG8, C54Y, participants with the C54 variant showed a smaller decline in cholesterol synthesis (Santosa *et al.*, 2007b). Some of the limitations of this trial were that only women were studied and the sample size used was not sufficiently large for haplotype analysis to be conducted.

A meta-analysis by Jakulj et al. (2010) showed no significant association between 5 SNPs (Q604E, D19H, Y54C, T400K and A632V) in ABCG5/8 and baseline indirect markers of

cholesterol metabolism in a group of 245 hypercholesterolemic individuals. Subsequently, these researchers evaluated 3,364 subjects from 16 studies, and found that individuals who carried the 632V allele showed correlations with lower LDL-C concentrations in contrast with homozygous 632A variants ($p < 0.01$), while other SNPs were not associated with lipid levels (Jakulj *et al.*, 2010). Moreover, in D19H, the presence of the H allele was significantly associated with lower campesterol to TC ratio (n = 83), β-sitosterol/TC, and cholestanol/TC, and higher lathosterol/TC ratios compared with homozygous19D allele carriers (n = 591) (Jakulj *et al.*, 2010). These results indicated that cholesterol absorption declined and cholesterol synthesis increased in 19H variants without discernable changes in lipid parameters.

To date, no trials have investigated the relationship of weight loss and subsequent changes in cholesterol kinetics to genetic polymorphism in the NPC1L1. Nevertheless, genetic polymorphisms in NPC1L1 gene have been found to affect sterol absorption and plasma LDL-C levels (Cohen *et al.*, 2006). Cohen and colleagues stratified 256 individuals with highest and lowest campesterol to lathosterol ratios (high and low absorber) obtained from 1,043 European-Caucasians and 1,832 African-Americans (AA) from the Dallas Heart study subjects (Cohen *et al.*, 2006). The majority of non-synonymous (NS) sequence variants were found in AA, 19 NS were observed in low absorbers (n = 26) while 5 NS sequence were uniquely found in high absorbers (Cohen *et al.*, 2006). Since the polymorphisms found have very low allele frequency (0.03 - 0.6 %), the findings were insufficient for meaningful statistical analysis at the individual level (Cohen *et al.*, 2006). However, cumulatively AA, with at least one NS identified 66

women and 39 men, were significantly associated with reduction in campesterol to lathosterol ratios and plasma LDL-C concentration compared with AA individuals (926 women and 674 men) who did not have these unique alleles (Cohen *et al.*, 2006).

Polymorphisms in NPC1L1 have been found to be associated with lower LDL-C levels in individuals undergoing ezetimibe treatment, although no such relationship was found before treatment was given (Hegele *et al.*, 2005; Simon *et al.*, 2005; Wang *et al.*, 2005). After haplotype analysis in NPC1L1 SNPS, Hegele et al found significant association in the response of LDL-C to 12 weeks ezitimibe treatment in 101 dyslipidemic subjects (Hegele *et al.*, 2005). Participants without common NPC1L1 haplotype 1735C-25342A-27677T, had a significantly higher reduction in LDL-C with ezetimibe than participants with at least one copy of this haplotype (Hegele *et al.*, 2005). Simon and colleagues, sequenced 376 healthy individuals and genotyped hypercholesterolemic patients from clinical trial cohorts, and found no association with baseline, but a significant association of the change in LDL-C levels in patients treated with ezetimibe (Simon *et al.*, 2005). These findings demonstrate that genetic variability in NPC1L1 can lead to a better understanding of the inter-individual drug response.

Several studies have been conducted to evaluate effects of SNPs in NPC1L1, ABCG8 and ABCG5 on the cholesterol lowering efficacy of PS treatment. SNPs in NPC1L1, ABCG8 and ABCG5 have been found to effect changes in lipid parameters subsequent to 2 g / d PS treatment in 82 hypercholesterolemic men divided into high vs. low basal plasma PS consuming spreads with PS or control (Zhao *et al.*, 2008). In ABCG8, T400K,

the level of LDL-C in A allele carriers with high basal plasma PS was 3.9-fold lower than their counterparts with low basal plasma PS after PS treatment (Zhao *et al.*, 2008). Zhao and colleagues also found that C allele carriers had higher plasma concentration of campesterol (12.2 ± 0.8 μmol/l vs. 9.7 ± 0.9 μmol/l), β-sitosterol (6.5 ± 0.4 μmol/l vs. 5.1 ± 0.5 μmol/l) and sum of campesterol and β-sitosterol (18.7 ± 1.2 μmol/l vs. 14.8 ± 1.4 μmol/l) (Zhao *et al.*, 2008). Additionally, in subjects with the NPC1L1 872 C > G (L272L) and 3929 G > A (Y1291Y) substitutions, a 2.4-fold greater reduction in LDL-C was found in individuals with mutant alleles versus their wild type (Zhao *et al.*, 2008). Moreover, Rudkowska et al. (2008) found no common SNPs in ABCG5/8 and NPC1L1 in the top 3 PS responders as well as 3 subjects who did not respond to PS treatment, although they found one patient carrying a rare SNP in NPC1L1who did not respond to PS treatment (Rudkowska *et al.*, 2008).

Variability in NPC1L1 is also associated with basal circulating cholesterol levels (Maeda *et al.*, 2010; Chen *et al.*, 2009; Polisecki *et al.*, 2010). A study of 42 Japanese volunteers found an SNP in NPC1l1 (1732 C/G) affecting basal serum campesterol level in homozygous G/G to be significantly higher than C carriers, indicating higher absorption rate in G allele compared to C allele carriers. Nevertheless, no significant differences in basal cholesterol synthesis surrogate markers, or lathosterol levels, were found (Maeda *et al.*, 2010). Moreover, another SNP in NPC1l1 (762 T > C) was shown to affect the lipid profiles and promoter activity of 762 T <C in 50 Chinese individuals (Chen *et al.*, 2009). Individuals with the -762C allele were shown to have significantly higher serum TC and LDL-C levels than those with the T allele (Chen *et al.*, 2009). Furthermore, four SNPs of

NPC1L1 (-18 A > C, L272L, V1296V, and U3_28650 A > G, minor allele frequencies 0.15 - 0.33) were genotyped in 5,804 elderly people from the PROSPER study, randomized to pravastatin or a control treatment (Polisecki *et al.*, 2010). These four SNPs were responsible for the variability in LDL-C levels at baseline, while one SNP (-133 A > G) was associated with a LDL-C lowering effect from statins (Polisecki *et al.*, 2010).

2.6. Summary and Conclusions

In summary, to our knowledge, there is only one study by Santosa et al. (Santosa *et al.*, 2007b) that has examined the relationship between the genetic polymorphism in ABCG5/ABCG8 and weight loss. This lack of information highlights the importance of studying this association further. Furthermore, many trials have studied the association between cholesterol and SNPs in NPC1L1; however, an absence exists of any trials investigating the effect of NPC1L1 polymorphism on changes in cholesterol metabolism consequent to weight loss. Therefore, the influence of weight loss and subsequent changes in body composition on lipid profile, cholesterol kinetics, ABCG5/ABCG8 and NPC1L1phenotype traits in obese and overweight individuals, needs to be explored further.

CHAPTER 3

MANUSCRIPT 1

Genetic Basis for Heterogeneity in Response of Serum Lipids and Cholesterol Absorption and Synthesis to Weight Loss in Overweight Individuals

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3.1 Abstract

Background: The global increase in obesity leads to development of chronic disorders including hypercholesterolemia. The ABCG5 and ABCG8 transporters mediate intestinal and hepatic excretion of cholesterol and plant sterols (PS), while the NPC1L1 transporter facilitates intestinal and hepatic influx of cholesterol and PS. **Objective:** The objective of this study was to examine if single nucleotide polymorphisms (SNP) of ABCG5, ABCG8 and NPC1L1 were associated with changes in cholesterol absorption and synthesis (CAS) subsequent to weight loss in overweight and obese individuals. **Methods:** Fifty-four healthy males and females (age, 49.5 ± 1.7 yr; BMI, 32.5 ± 0.5 kg/m^2) completed a 21 wk weight loss intervention study. Weight reduction was achieved by one wk of stabilization followed by 20 wk of self-directed caloric restriction and imposition of a physical activity program. Ratios of non-cholesterol sterols to total cholesterol (TC) measured using gas-liquid chromatography were used to estimate CAS. SNPs were determined using TaQMan genotyping assay. **Results and Discussion:** After weight reduction of 10.5 % (9.36 ± 0.57 kg, $p < 0.0001$), desmosterol and lathosterol to cholesterol ratios were reduced ($p < 0.001$) by -0.09 ± 0.02 µmol/mmol (11.0%) and -0.21 ± 0.03 µmol/mmol (14.6%), respectively. However, campesterol/TC and β-sitosterol/TC ratios were not altered. The ABCG8 V632A SNP was associated with changes in serum TC and triglyceride levels after weight loss. Homozygous C/C carriers in V632A showed a greater reduction ($p < 0$ .05) in TC (-0.41 ± 0.10 mmol/l) and TG (-0.59 ± 0.10mmol/l) compared with changes in TC (-0.03 ± 0.14mmol/l) and TG (-0.20 ± 0.11mmol/l) in heterozygous C/T carriers across the weight loss period. In ABCG8 Q604E, the homozygous G/G was associated with greater ($p < 0.05$) declines in

desmosterol/TC ratio than either heterozygous C/G or the combination of C/C and C/G. In ABCG8 T400K, the lathosterol/TC ratio in participants carrying homozygous A/A (-0.57 ± 0.05 µmol/mmol) showed smaller ($p < 0.05$) decreases compared to homozygous C/C (-0.19 ± 0.04 µmol/mmol) carriers. The present study is the first to demonstrate that changes in TC and triglyceride levels post weight loss associate with polymorphisms at V632A in ABCG8. **Conclusion**: In conclusion, weight loss of 10 % initial body weight induced cardio-protective changes in blood lipids, potentially mediated by reduced cholesterol biosynthesis. Additionally, SNPs in ABCG5 and ABCG8 were responsible for inter-individual variation in the alteration of cholesterol synthesis post weight loss. (Supported by Canadian Institutes of Health Research).

Keywords: weight loss, BMI, DEXA, body composition, fat mass, fat free mass, cholesterol absorption, cholesterol synthesis, HDL-C, LDL-C, TC, TG, SNP, ABCG5, ABCG8, NPC1L1.

3.2 Introduction

The World Health Organization (WHO) estimated that 1.5 billion adults (age 20+) were overweight, while half a billion adults were obese in 2008 (Finucane *et al.*, 2011). In 1978 / 1979, 49.2 % of the Canadian population was overweight or obese. Twenty-five years later, the prevalence of overweight and obesity has risen an additional 20 % to 59.1 % (Tjepkema, 2006). If the current trend continues, the WHO projects that in 2015, 2.5 billions adults will be overweight and more than 700 million clinically obese. Clinical diagnoses of overweight and obesity are followed by a number of health consequences. The prevalence of cardiovascular disease, hypertension, type-2 diabetes and kidney disease increases with obesity (Field *et al.*, 2001; Flegal *et al.*, 2007; Prospective Studies Collaboration *et al.*, 2009).Thus, it is necessary to avert this upward obesity trend.

Weight loss through dietary changes and an increase in physical activity is suggested to be the best recommendation to combat obesity (Donnelly & Smith, 2005). In order to maintain an obese individual's metabolic and physical capacities, losing FM while maintaining FFM is an essential weight loss aim (Verdich *et al.*, 2011). Beneficial alterations in lipid levels contribute to decrease body fat percentages as shown in previous trials (Janssen *et al.*, 2002; Nieman *et al.*, 2002).

Cholesterol levels in the human body are maintained by the rate of cholesterol absorption, synthesis and fecal excretion. The rate of cholesterol synthesis was found to decrease after weight loss (Di Buono *et al.*, 1999; Raeini-Sarjaz *et al.*, 2001; Santosa *et al.*, 2007a; Simonen *et al.*, 2002). Conversely, cholesterol absorption, which was observed to have a

reciprocal relation with synthesis (Gylling & Miettinen, 2002; Miettinen *et al.*, 2003) was not altered after weight loss (Santosa *et al.*, 2007a). However, trials by Griffin et al. (1998) and Simonen et al. (2000) observed no significant changes in cholesterol synthesis after weight loss, although the latter trial observed an increase in cholesterol absorption. Most studies examining weight loss and CAS only employed diet-induced weight loss in obese individuals with a small number of subject participants, while a combination effect of diet and physical activity to induce weight loss was only examined in women. Thus, the effects of weight loss on CAS in healthy men and women in overweight and obese individuals need to be further explored.

Three key proteins, ATP binding cassette subfamily G5 (ABCG5), G8 and Neiman Pick C1-like 1 (NPC1L1), are responsible for transporting cholesterol across the intestinal and hepatic membranes (Salen *et al.*, 2002; Kidambi & Patel, 2008; Altmann *et al.*, 2004; Deluis *et al.*, 2010). ABCG5 and ABCG8 are responsible for cholesterol efflux into intestine or biliary secretion for faecal excretion (Salen *et al.*, 2002; Kidambi & Patel, 2008). NPC1L1 is responsible for absorbing cholesterol by transporting cholesterol into the enterocyte or hepatocyte (Altmann *et al.*, 2004; Davis & Altmann, 2009; Betters & Yu, 2010). The SNPs of these three transporters might be essential in explaining inter-individual variability response to change in CAS post weight loss. To date, only one study has analysed the influenced of SNPs of ABCG5/ABCG8 on the movement of cholesterol kinetics due to weight loss. Santosa et al. explored this in 35 hypercholesterolemic women (Santosa *et al.*, 2007b). No previous intervention had

examined the association of SNPs in NPC1L1 and change in cholesterol metabolism after weight loss.

Subsequently, the aims of the present study were to evaluate mechanisms underlying alterations in circulating lipoprotein profile due to weight loss and subsequent changes in body composition in overweight individuals, and to examine if changes in cholesterol metabolism due to weight loss in overweight and obese individuals are associated with genotypic traits of ABCG5/G8 and NPC1L1.

3.3 Material and Methods

Study Design

Participant Characteristics

Seventy-eight healthy males and non-pregnant non-lactating females meeting inclusion criteria of 18 - 70 years of age and BMI of 28 - 37 kg/m^2 were recruited using posters, word of mouth and newspaper advertisements in and around Winnipeg, MB, Canada. Participants consuming stable dosage of thyroid and hypertension medication throughout the study duration were also included. As seen in the participant screening form in the Appendix 3, participants were non-smokers, non-diabetics and were excluded if they have anemia and/or had less than three mo or chronic use of oral hypolipidemic therapy, including consumption of > 4 g/d of fish oil or probucol within the previous six mo; a history of chronic alcohol use > 2 drinks/d; systemic antibodies, corticosteroids, androgens or phenytoin use; myocardial infarction, coronary artery bypass or other major surgical procedures within last six mo; recent onset of angina, congestive heart failure,

inflammatory bowel disease, pancreatitis, diabetes and hypothyroidism; gastrointestinal, renal, pulmonary, hepatic or biliary disease within previous three mo; cancers or evidence of active lesions, chemotherapy or surgery in the past year; chronic use of fibre or stimulant laxatives greater than 2 doses/wk, or reported history of eating disorders or binging.

A total of 54 participants completed the study, while two participants were requested to leave the program because of non-compliance issues. Some could not continue due to time (11), family emergency (four), health (three), and others (four). All participants gave their written informed consent at the beginning of the study; consent form used is attached in Appendix 1.

Human Ethics Considerations

The study protocol was approved by the University of Manitoba Biomedical Research Ethics Board, with ethics file number: B2007:198 (HS10435), under Dr. Peter J.H. Jones as the principal investigator. (See Appendix 2)

Study Protocol

The 21 wk longitudinal clinical trial was conducted from May – December 2008 at the Richardson Centre for Functional Food and Nutraceuticals, Winnipeg, MB, Canada. A one wk stabilization period was followed by 20 wks of weight loss. Body weight, height and other initial data collections including three day food records were gathered on the first day of stabilization period and participants were asked to keep their regular diet and

physical activity for the stabilization period. During the weight loss period, participants were weighed weekly during the first and last month, while biweekly weights were collected in between. If participants were having difficulties losing weight then they would be asked to come weekly until the weight loss goal was achieved. Two consecutive blood samples were collected by a registered nurse at the beginning (d 1 and 2) and end (d 140 and 141) of the weight loss period. Some participants had a two wk extension from their 20 wk of weight loss due to difficulties in achieving weight loss goals and for them another two consecutive days (d 161 and d 162) of fasting blood samples were taken. Participants were asked to fast, and consume no food or drink except water, for 12 hr prior to every blood collection. In addition, at the beginning and end of weight loss period, whole body composition was measured by using dual energy x-ray absorptiometry (DEXA) to determine percent lean and fat mass.

Weight Loss Protocol

After one wk of stabilization, 20 wk of weight loss was undertaken. Participants' baseline total energy expenditure (TEE) was calculated based on multiplication of resting metabolic rate (RMR) with 1.65 activity factor indicating moderate activity level. The RMR was calculated by Mifflin's equation (Mifflin *et al.*, 1990) using the participant's gender, height, weight and age. Body weight, height and age used in the equation were taken from their first visit at stabilization period (d -7).

Mifflin-St Jeor equations for measuring RMR in men and women:

(1) For men: RMR = (9.99 × weight (kg)) + (6.25 × height (cm)) - (4.92 × age) + 5

(2) For women: RMR = (9.99 × weight (kg)) + (6.25 × height (cm)) - (4.92 × age) – 161

(3) TEE = RMR × 1.65 (activity factor)

The target weight loss of 0.75 kg/wk was monitored weekly resulting in 15 kg of weight loss over the 20 wk period. The minimum weight loss goal for participants was 0.5 kg/wk for a total of 10 kg of weight loss in 20 wk (see Appendix 8 for target weight loss's visual aid given to participants). On the first day of the weight loss period (d 1), participants were given dietary guidelines with a reduction in energy intake by 500 kcal/d from calculated TEE. The physical activity requirements were introduced on day 8 of the weight loss period. Participants' energy needs were also adjusted to their three day dietary records collected on their first visit (d -7). For some participants with very low initial energy intakes, another 100 kcal - 200 kcal was reduced from the prescribed dietary guidelines.

Energy Restriction from Diet

Group sessions were held at the beginning of the study to educate participants on dietary guidelines set by a registered dietician who trained investigators and clinical coordinators (see Appendix 4 and 6). Afterwards, individual dietary counselling was performed when required throughout the study. Participants were taught to use an exchange system which will provide 50 % of energy from carbohydrates, 20 % of energy from proteins and 30% of energy from fats. To monitor compliance and understanding of dietary principles,

participants were asked to keep three day food records weekly (sample attached in Appendix 5). Pamphlets quantifying amount of calories ingested into exercise as well as sample recipes and menus were given to participants (see Appendix 7 for examples).

Energy Expenditure from Physical Activity

Participants were given a list of suggested exercises including walking, running on a treadmill, jogging intervals and sets of flexibility/muscle building training. A personal trainer demonstrated and taught proper exercise techniques and routines. Exercise was done independently by participants.

Depending on each participant's ability to exercise, they were separated into four exercise groups which burned10 % (PA1), 20 % (PA2), 30 % (PA3) and 40 % (PA4) of the 750 kcal energy deficit through exercise with the remaining calories compensated by reductions in calories ingested. The four groups were able to exercise fully with access to sport equipments (PA4); able to exercise fully without access to equipments and/or exercise facilities (PA3); some limitations in ability to exercise with access to exercise facilities or having sport equipment (PA2); and some limitations in ability to exercise without access to any sport facilities or equipment (PA1).

Energy burned through exercise was measured by the following equation:

(4) Energy expenditure (Kcal) = (METs × 3.5 × weight (kg)) / 200 × exercise duration (minutes)

With METS = metabolic equivalent of task; conventionally '1 MET' is considered as the RMR obtained during quiet sitting.

Participants were given a handout with METs values for different activities.

Subject Compliance with the Study Protocol

Compliance was monitored by weight changes at weekly weigh-ins, three day food records, daily exchange checklists, and using a weekly exercise journal (sample attached in Appendix 9). These materials were reviewed when participants failed to meet minimal weight loss weekly target required. Participants were encouraged through the use of visual graphs which included the plotted projected and actual body weight changes (attached in Appendix 8).

Analytical Methods

All analyses described were performed at the Richardson Centre for Functional Food and Nutraceuticals, University of Manitoba. Body weight was measured to the nearest 0.1 kg and height to the nearest 0.1 cm with participants wearing only light clothing. BMI (kg/m^2) was calculated from weight and height recorded at the beginning and end of weight loss period.

Blood Sample Separation

Twelve hr fasting blood was drawn for two consecutive days at the beginning and at the end of the study (d 1, 2, 140 141 and for some d 161, 162). Collected blood was centrifuged using a Sorvall Legend RT refrigerated table top centrifuge equipped with

swing-out rotor 4 place (Thermo Electric Company, Inc, West Chester, Pennsylvania) at 3000 rpm, 4 °C for 20 min to separate red blood cells (RBC) and plasma/serum. Separated plasma EDTA, plasma heparin, their respective RBC's and buffy coats as well as serum were transferred to labelled cryovials and stored in -80 ºC for future use.

Lipid Profile Analysis

Serum lipid profile was obtained using enzymatic reaction on an auto-analyzer - VITROS® 350 chemistry system (Ortho-Clinical Diagnostics, Rochester, NY, USA). TC, TG, and HDL-C were measured directly by the auto-analyzer. LDL-C was measured indirectly using the Friedewald equation (Friedewald *et al.,* 1972).

(5) LDL-C = TC - (HDL-C + VLDL)

(6) VLDL = TG / 2.2 or 0.456 × TG

Body Composition Analysis

The assessment of body composition and regional fat masses was carried out using whole body DEXA (GE Lunar BX-1 L-8743, General Electric Healthcare) at the RCFFN in order to obtain complete and accurate images of the full body composition. Before scanning, participants were asked to remove all metal and jewellery, and then instructed to lie down with open palms. Participants' ankles were strapped together to hold them in rest position. Participants were to remain still during the scan. Daily calibration was performed to maintain the measurement quality. Each scan set was carried out by a trained technician for a period of 7 min. Each set of images was integrated to yield three dimensional body compartment volume for tissues including lean mass, bone mass and

adipose mass across various regional sites, analyzed using Software - Encore 2005 (GE Healthcare).

Cholesterol Absorption and Synthesis Determination

The ratios of PSs (campesterol and β-sitosterol) to cholesterol were used as surrogate markers of cholesterol absorption while the ratios of cholesterol precursors (desmosterol and lathosterol) to cholesterol were utilized as indirect markers of cholesterol synthesis (Miettinen *et al.*, 2003; Miettinen *et al.*, 1990; Gylling *et al.*, 2007;Miettinen, 1982). Plasma PSs and cholesterol precursors were extracted from 500 µl of plasma EDTA and then quantified by gas liquid chromatography-flamed ionization detector using a previously described method (Ntanios & Jones, 1998). Briefly, 100 µl [0.1 mg/ml] of internal standard 5-alpha-cholestane (Sigma-Aldrich Canada Ltd) was added to plasma samples which were then saponified with 8 ml of freshly prepared 0.5 M methanolic-KOH. Sterols were extracted twice from the mixture with 4 ml of petroleum ether. Extracted sterols were derivatized with TMS reagent (pyridine:hexamethyldisilazan: trimethylchlorosilane 9:3:1) (Sylon^{TM} HTP, Sigma-Aldrich Canada Ltd) using a modified derivatization method seen in the Appendix 10. Samples were then evaporated under nitrogen (TECHNE sample concentrator) and re-suspended in hexane to be injected into a gas-liquid chromatography equipped with a flame ionization detector (6890N GC system, Agilent Technology) and separated on a 30-m SAC-5 capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm (Supelco). After some method development, the flow rate of carrier gas (helium) was set to 1.0 ml/min. Samples were injected at 300 °C. The detector was set at 310 °C. The oven temperature remained at 50

°C for 1 min after injection, was increased to 245 °C at a rate of 45 °C/min, and then was kept constant for 4 min, after which it rose to 280 °C at a rate of 15 °C/min and was kept constant at 280 °C for 4 min. Thereafter, oven temperature was raised to 300 °C at a rate of 2 °C/min and held for 2 min, then raised to 315 °C at a rate of 45 °C/min and maintained for 5 min. The total run time was 33 min. Sterol concentrations were determined in duplicate by identifying the peak sizes and expressing them relative to an internal standard. Desmosterol, lathosterol, campesterol and β-sitosterol levels were identified using authentic standards (Sigma-Aldrich Canada Ltd).

Single Nucleotide Polymorphism Determinations

Single nucleotide polymorphisms were analyzed using the TaqMan SNP Genotyping method. Genomic DNA was extracted from white blood cells obtained from 200 µl EDTA buffy coat using QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, Calif.) from the 54 participants. EDTA treated buffy coat was used because heparin has been shown to inhibit real time polymerase chain reaction (RT-PCR).

SNPs in NPC1L1, ABCG5 and ABCG8 (as listed in the **Table 3.1**) were amplified by a Step One plus RT-PCR (Applied Biosystem, Foster City, California), using TaqMan® SNP Genotyping Assays (Applied Biosystem, Foster City, California). Half µl of TaqMan® Genotyping assays and 4.5 µl of [2 ng/µl] of human DNA were mixed with 5 µl of TaqMan® GTXpress™ Master Mix (Applied Biosystem, Foster City, California) to yield a in 10 µl PCR reaction volume. Each reaction mixture was subjected to one holding stage to activate DNA polymerase at 95 °C for 20 sec, followed by 40 cycles

consisting of denaturing for 3 sec at 95 °C and annealing and elongation for 20 sec at 60 °C. Each SNP determination was conducted using triplicate measurements as differentiated with Step One™ Software v2.1 (Applied Biosystems).

Table 3.1. Missense mutation of SNPs, reference SNP number, allele and amino acid mutation change.

SNPs	rs number	Ref Allele	mRNA Allele change	Amino Acid Change
ABCG5				
Q604E	rs6720173	C/G	CAA ⇒ GAA	Q [Gln] ⇒ E [Glu]
R50C	rs6756629	A/G	CGC ⇒ TGC	R [Arg] ⇒ C [Cys]
ABCG8				
Y54C	rs4148211	A/G	TAC⇒TGC	Y [Tyr] ⇒ C [Cys]
T400K	rs4148217	A/C	ACG ⇒ AAG	T [Thr] ⇒ K [Lys]
A632V	rs6544718	C/T	GTC ⇒ GCC	V [Val] ⇒ A [Ala]
NPC1L1				
A310S	rs79803700	A/C	GCC ⇒ TCC	A [Ala] ⇒ S [Ser]
I1206N	rs52815063	A/T	ATC ⇒ AAC	I [Ile] ⇒ N [Asn]
D1114H D1087H	rs79519744	C/G	GAC ⇒ CAC	D [Asp] ⇒ H [His]

Statistical Analysis

Data are presented as mean ± standard error of means (SEMs). Paired student t-tests (2-tailed) were conducted to test the significance of results comparing beginning to endpoint body weight, body composition, lipid parameters, as well as CAS surrogate markers. Moreover, analysis of variance (ANOVA) with post hoc Scheffe multiple comparisons was used to test the difference in cholesterol kinetics and lipid profiles changes within an

SNP. Homozygous variant groups with less than five individuals were automatically collapsed into the heterozygous group. A two-sample Student's t-test was then applied to the regrouped data. Pairwise correlations between changes in CAS with LDL-C or TC were calculated using Pearson's correlation. A p value < 0.05 was considered to be significant. All statistical analyses were performed using SPSS software for Windows version 17.0 (SPSS Inc, Chicago, IL).

3.4 Results

A total of 54 participants with average age of 49.5 ± 1.7 yr and initial BMI of 32.4 ± 0.5 kg/m^2 completed the 20 wk weight loss trial. Participants' baseline concentrations of blood lipid profiles, non-cholesterol sterols and body compositions are listed in **Table 3.2**.

Changes in Body Composition Subsequent to Weight Loss

The changes in individual's body weights before and after the 20 wk weight loss period are described in **Figure 3.1**. As seen in **Table 3.3**, participants' mean weights were reduced from 89.9 ± 1.9 kg to 80.5 ± 1.8 kg, resulting in a reduction (p < 0.001) of 9.4 ± 0.6 kg or -10.5 ± 0.6 % after the 20 wk weight loss period. Participants' legs, trunk and total body fat declined significantly by -2.0 ± 0.3 kg, -5.0 ± 0.4 kg, -7.4 ± 0.5 kg, respectively.

Table 3.2. Baseline characteristics of weight loss study participants (n = 54).

Baseline Characteristics	Mean ± SEM
Age (yr)	49.5 ± 1.7
Female (n (%))	41(75.9)
Initial weight (kg)	89.9 ± 1.9
Height (m)	1.66 ± 0.01
BMI (kg/m^2)	32.4 ± 0.5
Lipid profile (mmol/l)	
Total cholesterol	5.53 ± 0.14
LDL-cholesterol	3.27 ± 0.12
HDL-cholesterol	1.82 ± 0.14
Triglycerides	1.43 ± 0.05
Non-cholesterol sterols (µmol/mmol)	
Desmosterol/TC	0.73 ± 0.04
Lathosterol/TC	1.23 ± 0.08
Campesterol/TC	1.45 ± 0.11
Beta-sitosterols/TC	0.88 ± 0.07
Body composition	
Fat tissue (kg)	37.9 ± 0.1
Fat tissue (%)	44.1 ± 0.8
Lean tissue (kg)	48.3 ± 1.4
Lean tissue (%)	55.9 ± 0.8

Data are presented as means ± SEM.

Abbreviations: BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein cholesterol; TC, total cholesterol.

Also, the total percentage of fat in the legs and trunk declined significantly ($p < 0.0001$). Reduction of the trunk's lean tissue was found to be significant (-1.7 ± 0.5 kg, $p < 0.01$)

but not in the leg's lean tissue (-0.4 ± 0.2 kg). Notably, the lean tissue percentage was increased ($p < 0.0001$) by 4.7 ± 0.5 % after weight loss. The bone mineral density declined ($p < 0.01$) by -0.83 ± 0.22 %; however, total bone mineral content did not change significantly (-0.90 ± 0.62 %). Decline in fat percentage in android (upper body part or stomach area) were greater (-5.4 ± 0.7 %) than in gynoid (lower body part or hip and high thigh area) (-3.0 ± 0.5 %) resulting in significant decline in A to G ratio by -4.62 ± 1.04 % ($p < 0.0001$).

Changes in Body Composition Subsequent to Weight Loss

The changes in individual's body weights before and after the 20 wk weight loss period are described in **Figure 3.1**. As seen in **Table 3.3**, participants' mean weights were reduced from 89.9 ± 1.9 kg to 80.5 ± 1.8 kg, resulting in a reduction ($p < 0.001$) of 9.4 ± 0.6 kg or -10.5 ± 0.6 % after the 20 wk weight loss period. Participants' legs, trunk and total body fat declined significantly by -2.0 ± 0.3 kg, -5.0 ± 0.4 kg, -7.4 ± 0.5 kg, respectively. Also, the total percentage of fat in the legs and trunk declined significantly ($p < 0.0001$). Reduction of the trunk's lean tissue was found to be significant (-1.7 ± 0.5 kg, $p < 0.01$) but not in the leg's lean tissue (-0.4 ± 0.2 kg). Notably, the lean tissue percentage was increased ($p < 0.0001$) by 4.7 ± 0.5 % after weight loss. The bone mineral density declined ($p < 0.01$) by -0.83 ± 0.22 %; however, total bone mineral content did not change significantly (-0.90 ± 0.62 %). Decline in fat percentage in android (upper body part or stomach area) were greater (-5.4 ± 0.7 %) than in gynoid (lower body part or hip and high thigh area) (-3.0 ± 0.5 %) resulting in significant decline in A to G ratio by -4.62 ± 1.04 % ($p < 0.0001$).

Figure 3.1. Change in individual body weights (kg) before and after 20 wk of the weight loss period in overweight and obese individuals (n = 54).

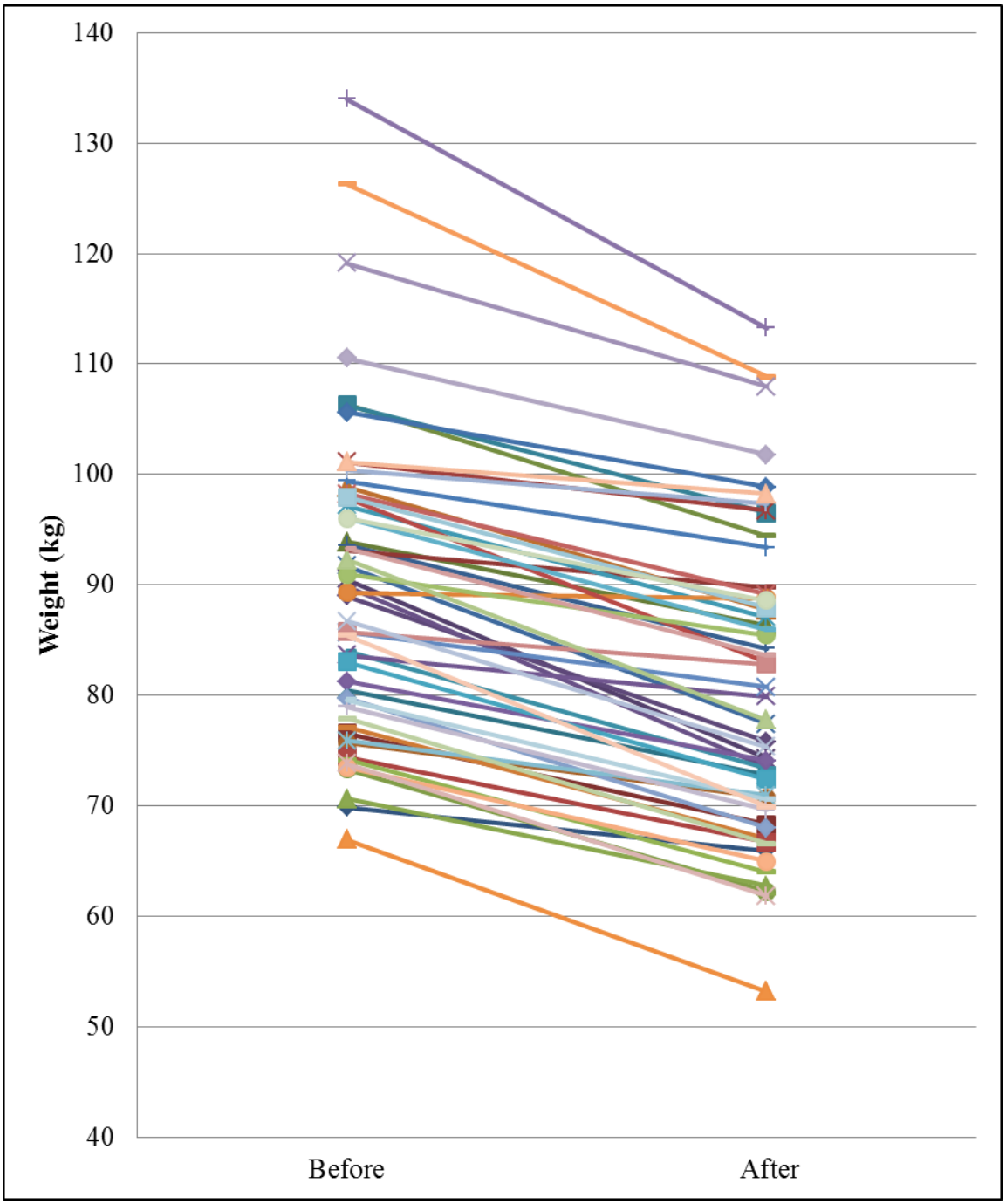


Table 3.3. Change and percent change in body weight and compartmental masses before and after 20-wk weight loss intervention in overweight and obese individuals (n = 53).

Variables	Before	After	Difference	% Difference
Wt. scale. (kg)***	89.9 ± 1.9	80.5 ± 1.8	-9.4 ± 0.6	-10.5 ± 0.6
BMI scale (kg/m^2)***	32.4 ± 0.5	29.0 ± 0.5	-3.4 ± 0.2	-10.5 ± 0.6
Mass DEXA (kg)***	89.0 ± 1.9	79.9 ± 1.8	-9.1 ± 0.6	-10.2 ± 0.6
BMI DEXA***	32.1 ± 0.5	28.8 ± 0.5	-3.3 ± 0.2	-10.2 ± 0.6
Fat legs (kg) ***	11.8 ± 0.4	9.9 ± 0.5	-2.0 ± 0.3	-16.6 ± 2.2
Fat trunk (kg) ***	21.7 ± 0.7	16.6 ± 0.7	-5.0 ± 0.4	-23.2 ± 1.9
Fat total (kg) ***	37.9 ± 0.1	30.5 ± 1.1	-7.4 ± 0.5	-19.9 ± 1.6
Fat legs (%) ***	43.4 ± 1.3	39.3 ± 1.3	-4.0 ± 0.5	-9.5 ± 1.3
Fat trunk (%) ***	46.5 ± 0.7	41.1 ± 1.0	-5.4 ± 0.7	-11.6 ± 1.5
Fat total (%) ***	44.1 ± 0.8	39.4 ± 1.0	-4.7 ± 0.5	-10.8 ± 1.3
Lean legs (kg)	15.5 ± 0.5	15.1 ± 0.5	-0.4 ± 0.2	-2.1 ± 1.4
Lean trunk (kg) **	25.0 ± 0.9	23.3 ± 0.7	-1.7 ± 0.5	-5.1 ± 1.4
Lean total (kg) ***	48.3 ± 1.4	46.6 ± 1.4	-1.7 ± 0.3	-3.4 ± 0.6
Lean tissue (%) ***	55.9 ± 0.8	60.6 ± 1.0	4.7 ± 0.5	8.2 ± 1.0
BMD (g/cm2) **	1.23 ± 0.02	1.22 ± 0.02	-0.01 ± 0.00	-0.83 ± 0.22
BMC legs (kg)	1.06 ± 0.03	1.05 ± 0.03	-0.01 ± 0.01	-1.20 ± 0.75
BMC trunk(kg)*	0.99 ± 0.03	0.94 ± 0.03	-0.04 ± 0.02	-3.60 ± 1.83
BMC (kg)	2.86 ± 0.07	2.84 ± 0.07	-0.03 ± 0.02	-0.90 ± 0.62
Region (% fat)***	42.7 ± 0.8	38.0 ± 1.0	-4.7 ± 0.5	-11.4 ± 1.4
Tissue (kg) ***	86.2 ± 1.9	77.1 ± 1.8	-9.1 ± 0.6	-10.7 ± 0.6
Android (%Fat)***	51.2 ± 0.7	45.9 ± 1.1	-5.4 ± 0.7	-10.7 ± 1.6
Gynoid (%Fat)***	45.9 ± 1.1	42.9 ± 1.2	-3.0 ± 0.5	-6.51 ± 1.1
A/G Ratio***	1.15 ± 0.03	1.09 ± 0.02	-0.06 ± 0.01	-4.62 ± 1.04

Abbreviations: BMI, body mass index, which is calculated as weight in kilograms divided by height in meters squared; DEXA, dual-energy X-ray absorptiometry; BMC, bone mineral content; BMD, bone mineral density; A/G, android to gynoid.

Data are presented as means ± SEM, with *** = p <0.0001; ** = p <0.01; * = p <0.05, statistical differences presented are between before and after weight loss.

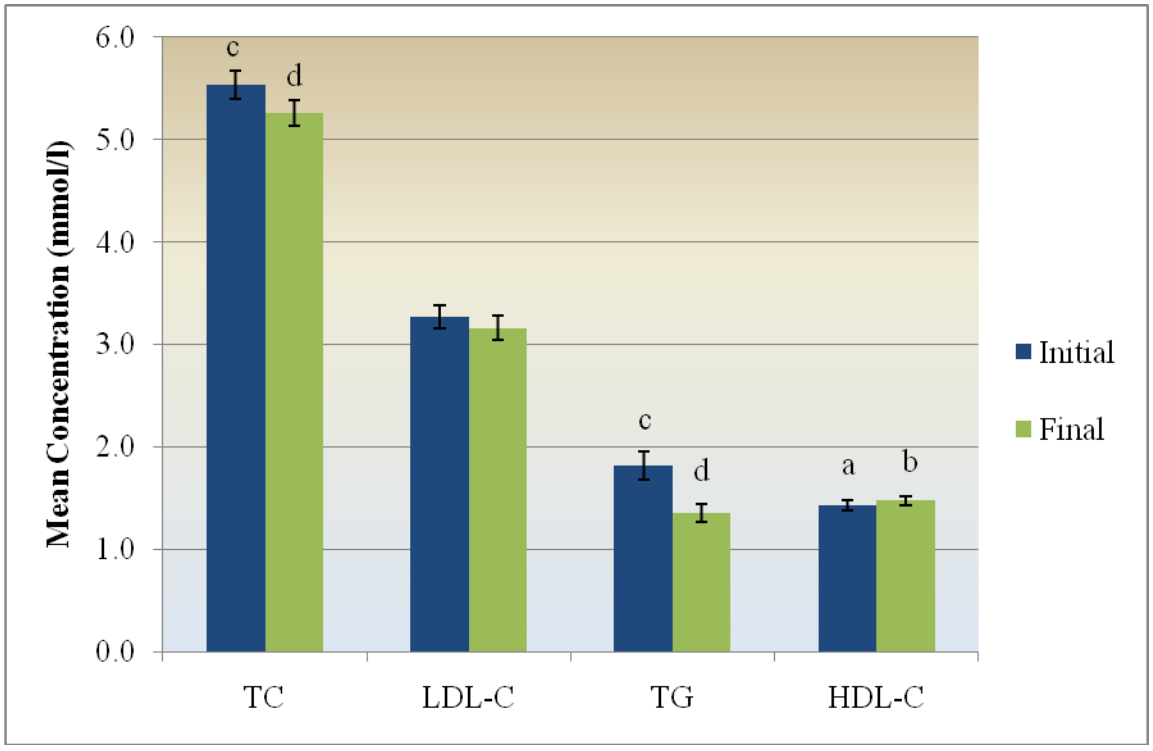
(7) Tissue = total mass - BMC

(8) Regional means = tissue plus BMC of that part or total mass of that area
i.e. Region % fat = fat/ total mass

Changes in Blood Lipid Profiles and Cholesterol Absorption and Synthesis

Total cholesterol and TG declined ($p < 0.001$) by -0.28 ± 0.09 mmol/l (-4.38 %) and -0.46 ± 0.08 mmol/l (-18.25 %), respectively. HDL-C levels increased ($p < 0.05$) by 0.05 ± 0.02 mmol/l (4.84 %) after 20 wk of weight loss (**Figure 3.2**). The ratio of desmosterol and lathosterol to cholesterol was reduced ($p < 0.001$) by -0.09 ± 0.02 µmol/mmol (-11.02 %) and -0.21 ± 0.03 µmol/mmol (14.60 %), respectively. However, the ratio of campesterol to sitosterol was not reduced significantly, -0.08 ± 0.07 µmol/mmol (-0.41 %) and -0.02 ± 0.05 µmol/mmol (7.19 %), respectively (**Figure 3.3**). No significant correlations were observed between the changes in serum lipids profiles, including TC, LDL-C, TG and HDL-C, and the changes in the ratios of surrogate markers of cholesterol absorption (campesterol and β-sitosterol) and synthesis (desmosterol and lathosterol) to TC (**Table 3.4**). The ratio of PSs, however, were positively correlated with each other ($r = 0.798$, $p < 0.001$), and the ratio of precursors were moderately correlated with each other ($r = 0.508$, $p < 0.01$). The ratio of desmosterol to cholesterol was also correlated with campesterol to TC ratio ($r = 0.351$, $p < 0.01$) and β-sitosterol to TC ratio ($r = 0.277$, $p < 0.05$).

Figure 3.2. Lipid parameters before and after weight loss in overweight and obese individuals (n = 54).

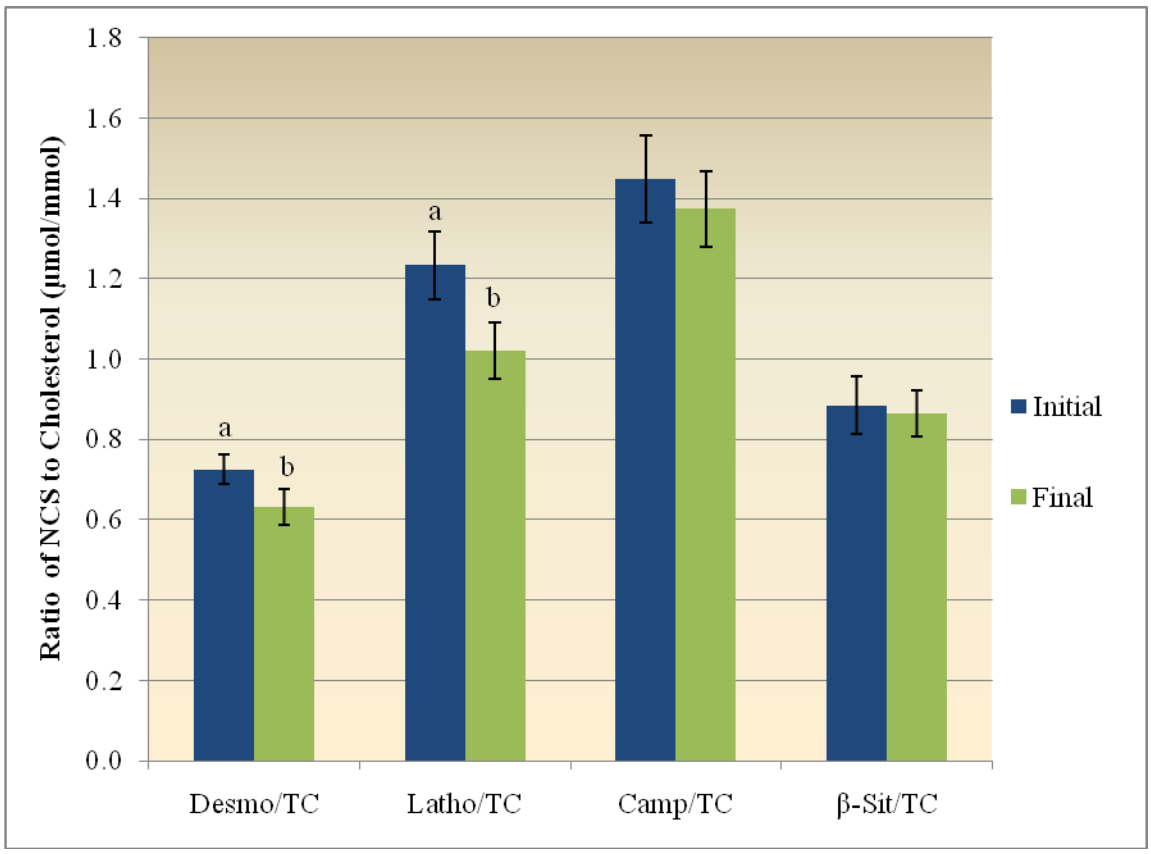


Data are presented as means ± SEM.

Letters indicates significant differences between before and after 20 wk of weight loss trial with a and b indicate $p < 0.05$, while c and d indicate $p < 0.001$

Abbreviations: TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol.

Figure 3.3. Response of synthesis and absorption across study in overweight and obese individuals (n = 54).



Data are presented as means ± SEM.

a and b indicate significant differences between initial and final of weight loss period with p <0.001

Abbreviations: NCS, non-cholesterol sterols; desm, desmosterol; latho, lathosterol; camp; campesterol; β-Sit, β-sitosterol; TC, total cholesterol

Table 3.4. Correlations of change in cholesterol precursors and plant sterols with change in serum total and lipoprotein cholesterol levels before and after a 20 wk weight loss intervention in overweight and obese individuals (n = 54).

Parameters	Desmosterol/TC	Lathosterol/TC	Campesterol/TC	β-Sitosterol/TC
Lipid profile (mmol/l)				
Total cholesterol	0.068	-0.078	0.167	0.056
LDL cholesterol	0.021	-0.106	0.136	0.043
HDL cholesterol	-0.103	-0.227	0.251	0.190
Triglycerides	0.161	0.160	-0.019	-0.077
Non-cholesterol sterols (µmol/mmol)				
Desmosterol/TC	1.000	0.508**	0.351**	0.277*
Lathosterol/TC	0.508**	1.000	-0.003	0.011
Campesterol/TC	0.351**	-0.003	1.000	0.798**
β-Sitosterol/TC	0.277*	0.011	0.798**	1.000

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Cholesterol precursors and plant sterols are reported in µmol/mmol of cholesterol

All values reported are the difference between before and after 20 wk of weight loss trial.

Abbreviations: LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol.

Genotype Distribution and Frequency of Single Nucleotide Polymorphisms

The distribution and frequency of SNPs are explained in **Table 3.5**. Participants' age and BMI were not significantly different among all the allele groups. Due to the very low frequency of heterogeneity found in SNPs in NPC1L1, their relationships with lipid profiles, CAS markers could not be analyzed statistically.

Table 3.5. Genotype distribution and frequency of missense SNPs of ABCG5 and ABCG8 (n = 54).

SNPs	Allele 1/2	Homozygous 1/1			Heterozygous 1/2			Homozygous 2/2		
		N (%)	Age (yr)	BMI	N (%)	Age (yr)	BMI	N (%)	Age (yr)	BMI
ABCG5										
Q604E	C/G	**2 (3.7)**	50.7 ± 9.2	31.6 ± 0.0	11 (20.4)	47.7 ± 3.9	32.3 ± 0.1	41 (75.9)	50.5 ± 1.9	32.5 ± 0.5
R50C	A/G	0			7 (13.0)	48.5 ± 6	32.4 ± 1.3	47 (87.0)	50.2 ± 1.7	32.4 ± 0.5
ABCG8										
C54Y	A/G	18 (33.3)	50.0 ± 2.6	32.6 ± 0.9	23 (42.6)	48.8 ± 2.6	33.0 ± 0.7	13 (24.1)	52.0 ± 3.6	31.2 ± 0.8
T400K	A/C	**3 (5.6)**	55.2 ± 5.9	34.9 ± 2.5	14 (25.9)	47.4 ± 3.8	33.0 ± 0.8	37 (68.5)	50.5 ± 1.9	32.0 ± 0.6
V632A	C/T	36 (66.7)	48.7 ± 2.2	32.2 ± 0.6	18 (33.3)	52.4 ± 2.2	32.8 ± 0.7	0		
NPC1L1										
A310S	A/C	0			0			54 (100)	49.5 ± 1.7	32.4 ± 0.5
I1206N	A/T	0			**1 (1.9)**	61	36.3	53 (98.1)	49.3 ± 1.7	32.4 ± 0.5
D1114H D1087H	C/G	54 (100)	49.5 ± 1.7	32.4 ± 0.5	0			0		

No significant differences were found among all groups ($p < 0.05$).

Data are presented as means ± SEM, BMI values reported are in kg/m^2

Homozygous groups in bold which are lower than 10% or 6 are considered to be collapsed to its heterozygous group.

Abbreviations: BMI, body mass index, SNP, single nucleotide polymorphism.

Effect of Single Nucleotide Polymorphisms on Lipid Parameters

The two ABCG5 SNPs analyzed in the present trial were not significantly associated with changes in lipid profiles after weight loss period (**Table 3.6**). However, the V632A SNP of ABCG8 was associated with changes in TC and TG after weight loss. Thirty six participants carrying homozygous C/C in V632A had larger declines ($p < 0.05$) in TC (-0.41 ± 0.10 mmol/l) and TG (-0.59 ± 0.10 mmol/l) compared with changes in TC (-0.03 ± 0.14 mmol/l) and TG (-0.20 ± 0.11 mmol/l) in18 heterozygous C/T carriers (**Table 3.7**). The rest of the ABCG8 SNPs analyzed in the present study were not significantly associated with lipid parameters including TG, TC, HDL-C and LDL-C (**Table 3.7**).

Effects of Polymorphisms on Cholesterol Absorption and Synthesis Markers

The changes in ratio of desmosterol to cholesterol, as surrogate markers of cholesterol synthesis, were related to Q604E SNPs in ABCG5 (**Table 3.6**). Homozygous carriers of the allele G/G of Q604E were found to have a greater decline ($p < 0.05$) in desmosterol to cholesterol ratio than both heterozygous C/G carriers and the combination of C/C and C/G carriers. In subjects possessing the ABCG8, T400K, the ratio of lathosterol to cholesterol in participants carrying homozygous A/A (-0.57 ± 0.05 µmol/mmol of cholesterol) had smaller decreases ($p < 0.05$) than those who were homozygous C/C (-0.19 ± 0.04 µmol/mmol) after weight loss (**Table 3.7**). Other SNPs measured showed no associations with changes in the ratio of desmosterol, lathosterol, campesterol and β-sitosterol to TC post weight loss.

Table 3.6. Change in lipid profiles, surrogates of synthesis and absorption stratified based on ABCG5 SNPs (n = 54).

Q604E	C/C	C/C+C/G	C/G	G/G	Total
N	2	13	11	41	54
Desm/TC	-0.03 ± 0.06	0.01 ± 0.06[a]	0.02 ± 0.07[a]	-0.13 ± 0.02[b]	-0.09 ± 0.02
Latho/TC	-0.15 ± 0.04	-0.15 ± 0.06	-0.15 ± 0.07	-0.23 ± 0.04	-0.21 ± 0.03
Camp/TC	0.02 ± 0.15	-0.06 ± 0.07	-0.07 ± 0.09	-0.08 ± 0.09	-0.08 ± 0.07
β-sito /TC	0.16 ± 0.03	-0.03 ± 0.12	-0.07 ± 0.14	-0.01 ± 0.06	-0.02 ± 0.05
TC	0.18 ± 0.58	0.04 ± 0.05	-0.29 ± 0.17	-0.30 ± 0.10	-0.28 ± 0.09
LDL-C	-0.02 ± 0.37	-0.22 ± 0.16	-0.12 ± 0.16	-0.11 ± 0.08	-0.10 ± 0.07
HDL-C	0.13 ± 0.08	-0.10 ± 0.14	0.02 ± 0.06	0.05 ± 0.03	0.05 ± 0.02
TG	0.15 ± 0.25	-0.31 ± 0.17	-0.40 ± 0.19	-0.51 ± 0.09	-0.46 ± 0.08
R50C	A/A		A/G	G/G	Total
N	0	-	7	47	54
Desm/TC			0.01 ± 0.11	-0.11 ± 0.02	-0.09 ± 0.02
Latho/TC			-0.13 ± 0.06	-0.23 ± 0.04	-0.21 ± 0.03
Camp/TC			-0.05 ± 0.05	-0.08 ± 0.08	-0.08 ± 0.07
β-sito /TC			0.11 ± 0.15	-0.04 ± 0.05	-0.02 ± 0.05
TC			-0.27 ± 0.28	-0.28 ± 0.09	-0.28 ± 0.09
LDL-C			0.02 ± 0.24	-0.12 ± 0.07	-0.10 ± 0.07
HDL-C			0.05 ± 0.05	0.05 ± 0.03	0.05 ± 0.02
TG			-0.71 ± 0.28	-0.42 ± 0.08	-0.46 ± 0.08

Data are presented as means ± SEM.

[a b] indicates significant differences with $p < 0.05$ among different alleles

Abbreviations: SNP, single nucleotide polymorphism; desm, desmosterol; latho, lathosterol; camp; campesterol; β-sito, beta sitosterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol.

Cholesterol precursors and plant sterols are reported in μmol/mmol of cholesterol while lipid parameters are reported in mmol/l.

All values reported are the difference between before and after 20 wk of weight loss trial.

Table 3.7. Change in cholesterol synthesis and absorption surrogates marker and lipid profiles stratified based on ABCG8 SNPs (n = 54).

C54Y	A/A	A/A+A/G	A/G	G/G	Total
N	18	-	23	13	54
Desm/TC	-0.11 ± 0.03		-0.08 ± 0.04	-0.10 ± 0.06	-0.09 ± 0.02
Latho/TC	-0.21 ± 0.05		-0.25 ± 0.06	-0.16 ± 0.06	-0.21 ± 0.03
Camp/TC	-0.01 ± 0.07		-0.15 ± 0.14	-0.05 ± 0.13	-0.08 ± 0.07
β-sito /TC	0.04 ± 0.07		-0.07 ± 0.07	0.00 ± 0.13	-0.02 ± 0.05
TC	-0.31 ± 0.18		-0.26 ± 0.11	-0.27 ± 0.18	-0.28 ± 0.09
LDL-C	-0.18 ± 0.13		-0.04 ± 0.10	-0.12 ± 0.16	-0.10 ± 0.07
HDL-C	0.08 ± 0.04		0.05 ± 0.03	0.00 ± 0.05	0.05 ± 0.02
TG	-0.44 ± 0.14		-0.56 ± 0.14	-0.32 ± 0.13	-0.46 ± 0.08
T400K	A/A	A/A+A/C	A/C	C/C	Total
N	3	17	14	37	54
Desm/TC	-0.25 ± 0.08	-0.12 ± 0.03	-0.10 ± 0.03	-0.08 ± 0.03	-0.09 ± 0.02
Latho/TC	-0.57 ± 0.05[a]	-0.27 ± 0.06	-0.21 ± 0.06	-0.19 ± 0.04[b]	-0.21 ± 0.03
Camp/TC	-0.04 ± 0.35	-0.10 ± 0.07	-0.11 ± 0.06	-0.06 ± 0.10	-0.08 ± 0.07
β-sito /TC	0.07 ± 0.19	0.03 ± 0.07	0.02 ± 0.08	-0.04 ± 0.07	-0.02 ± 0.05
TC	-0.87 ± 0.54	0.04 ± 0.04	-0.29 ± 0.18	-0.23 ± 0.10	-0.28 ± 0.09
LDL-C	-0.43 ± 0.46	-0.39 ± 0.18	-0.10 ± 0.12	-0.08 ± 0.09	-0.10 ± 0.07
HDL-C	0.08 ± 0.07	-0.15 ± 0.13	0.03 ± 0.04	0.05 ± 0.03	0.05 ± 0.02
TG	-1.06 ± 0.46	-0.55 ± 0.15	-0.45 ± 0.15	-0.42 ± 0.10	-0.46 ± 0.08
V632A	C/C	C/T+T/T	C/T	T/T	Total
N	36	0	18	0	54
Desm/TC	-0.10 ± 0.03		-0.07 ± 0.03		-0.09 ± 0.02
Latho/TC	-0.22 ± 0.04		-0.19 ± 0.07		-0.21 ± 0.03
Camp/TC	-0.08 ± 0.10		-0.07 ± 0.08		-0.08 ± 0.07
β-sito /TC	-0.03 ± 0.07		0.01 ± 0.05		-0.02 ± 0.05
TC	-0.41 ± 0.10[a]		-0.03 ± 0.14[b]		-0.28 ± 0.09
LDL-C	-0.16 ± 0.09		0.00 ± 0.11		-0.10 ± 0.07
HDL-C	0.03 ± 0.03		0.08 ± 0.03		0.05 ± 0.02
TG	-0.59 ± 0.10[a]		-0.20 ± 0.11[b]		-0.46 ± 0.08

[a] and [b] indicates $p < 0.05$ among different alleles

Data are presented as means ± SEM.

Abbreviations: SNP, single nucleotide polymorphism; desm, desmosterol; latho, lathosterol; camp; campesterol; β-sito, beta sitosterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol.

Cholesterol precursors and plant sterols are reported in μmol/mmol of cholesterol.

Values reported are the difference between before and after 20 wk of weight loss trial.

3.5 Discussion

The present study includes a number of novel findings. An alteration in circulating lipid parameters, subsequent to 10 % weight loss and further improvement of body composition, was shown in overweight and obese individuals. The reduction of lipid parameters was accompanied by a decrease in whole body cholesterol biosynthesis. Moreover, the present study represents the first investigation into the associations of genotypic traits of sterol transport genes, ABCG5- R50C, ABCG8-A632V and NPC1L1, with their response to CAS, as well as serum cholesterol levels to weight loss intervention in overweight and obese men and women. Polymorphisms in ABCG5 Q604E and ABCG8 T400K were significantly associated with variability responses of cholesterol synthesis due to weight loss. Additionally, polymorphism in ABCG8 A632V showed that allele determination explained the individual variability response of blood lipid parameters subsequent to weight loss.

Body composition changes, subsequent to weight loss, are essential to morbidity and mortality risk. This weight loss study shows a greater reduction of fat mass and a minimal loss of lean mass. These data explain the overall increase in percentage of lean tissue after weight loss, even though the actual lean mass had decreased. Declines in the lean tissue percentages, shown in this study, are desirable since excessive loss of FFM is responsible for maintaining a resting metabolic rate, regulating body temperature, preserving skeletal integrity and maintaining the function and quality of life (Marks & Rippe, 1996). Additionally, even with a minimal loss of body weight, changes in body shape or composition by way of an increase in physical activity could improve cardio

respiratory fitness (Ross & Janiszewski, 2008). Moreover, the reduction in the android (upper or stomach area) to gynoid (hip and high thigh area) ratio observed in this study could serve as one of the factors in ameliorating serum lipid profiles since previous studies have shown that women with the highest waist to hip ratio have an increased mortality risk from CVD (Lapidus *et al.*, 1984).

Weight loss resulted in favorable changes in blood lipid profiles suggesting a decline in CVD risk. The reduction of the TC levels observed was in line with previous studies (Poobalan *et al.*, 2004; Dattilo & Kris-Etherton, 1992). A meta-analysis of 70 studies indicates that weight loss is associated and correlated with TC, LDL-C, VLDL and TG. They also found that for every kilogram decrease in body weight TC, LDL-C and TG is decreased by 0.05 mmol/l, 0.02 mmol/l, and 0.015 mmol/l, respectively (Dattilo & Kris-Etherton, 1992). Another meta-analysis by Proobalan et al. found that for every 10 kg of weight loss, a decline of 0.23 mmol/l of cholesterol is observed in obese individuals (Poobalan *et al.*, 2004). However, in the present study, after weight loss, LDL-C showed a trend in reduction but it did not reach statistical significance. The reason might be attributed to the indirect methodology used to measure LDL-C in this study. The free living study design used presently might be another possible explanation. Dietary guidelines were given to the participants as weight loss tools, while 3 day food records served as a compliance tool for the participants. However, the absence of a fully controlled diet in this study could contribute to the non-significant reduction and/or variability in results in changes of LDL-C observed after weight loss.

Several studies compared and validated the Friedewald equation used in the present study to measure LDL-C concentration (Cordova *et al.,* 2004; Fukuyama *et al.,* 2008; Nauck *et al.*, 1996). The Friedewald equation was known to underestimate the LDL-C level when the triglyceride level of the individual was higher than 4.5 mmol/l (Nauck *et al.*, 1996) and at low LDL-C concentrations (Scharnagl *et al.*, 2001). Jun et al. (2008) compared 38243 Koreans (TG < 4.52mmol/l) and found the Friedewald calculated LDL-C value was lower than direct LDL-C measurements in 96.3 % of participants and the percentage difference of both methods was more than ± 5 % in 75.4 % of participants (Jun *et al.*, 2008). Moreover, this review has shown significant (25 % to 40 %) errors with individuals having TG level between 2.27 mmol/l and 4.52 mmol/l (Sniderman *et al.*, 2003). Furthermore, equal values of TC and HDL-C the reduction of TG affected calculated LDL-C to automatically rise while the actual LDL-C concentrations remain unknown (Lane, 1997). Additionally, a recent study by Fukuyama et al., found that the calorie content of the last meal prior to blood collection, affected LDL-C values calculated by the Friedewald equation compared to direct LDL-C measurement using N-geneous assay (Fukuyama *et al.*, 2008). A lower energy meal (658 kcal) eaten prior to blood collection resulted in higher calculated LDL-C values compared with direct LDL-C values while a higher energy meal (1011 kcal) consumed prior to blood collection had significantly lower indirect LDL-C values compared to direct LDL-C (Fukuyama *et al.*, 2008). Thus, the significant reduction in TG post weight loss observed in this study might have resulted in an increase in calculated LDL-C post weight loss and the energy level of a participant's last meal prior to blood withdrawal which might have influenced the calculated LDL-C values. For future work, instead of using the Friedewald equation,

LDL-C concentrations would need to be measured by direct methods such as ultracentrifugation or lipoprotein electrophoresis.

The present study is the first to discover that the changes in TC and TG post weight loss are associated with polymorphisms in V632A ABCG8. Our results have demonstrated that individuals with homozygous C/C in A632V have better cardio-protective changes than do subjects with the heterozygous C/T post weight loss. Previously, SNP on A632V have been shown to be associated with the baseline TC (Berge *et al.*, 2002). A meta-analysis by Jakulj and coworkers in 2010 found an association between polymorphism in V632A and LDL-C baseline concentrations, where individuals carried minor allele 632V (n = 367) was associated with reductions (p = 0.01) of baseline LDL-C in comparison to individuals with homozygous 632A (n = 614) (Jakulj *et al.*, 2010). Individual studies included in the meta-analysis did not show the associations found (Jakulj *et al.*, 2010). To our best knowledge, no other study has successfully linked the SNP in A632V and hypercholesterolemia and response to interventions.

The level of cholesterol in vivo is maintained by the regulation of cholesterol absorption, synthesis and excretion. Cholesterol synthesis was significantly reduced after weight loss even in the absence of changes in cholesterol absorption. The current study result was in line with previous works (Di Buono *et al.*, 1999; Santosa *et al.*, 2007a; Raeini-Sarjaz *et al.*, 2001). After a 6.8 ± 2.6 % weight reduction, within a six month period and in six mildly-hypercholesterolemic-obese men, cholesterol fractional synthesis rates were reduced significantly from 8.42 ± 3.90 %/d to 3.04 ± 1.90 %/d , while their absolute

synthesis rates were decreased from 1.66 ± 0.84 g/(kg.d) to 0.59 ± 0.38 g/(kg.d) (Di Buono *et al.*, 1999). Raeini-Sarjaz et al. (2001) also obtained the same conclusions from weight loss groups prescribed low energy diets and low energy-low fat diets, resulting in FSR decreases of 27.7 % and 25.5 %, respectively (Raeini-Sarjaz *et al.*, 2001). Furthermore, Santosa et al (2007) demonstrated similar results in 35 hypercholesterolemic women, where the fractional synthesis rate of cholesterol was decreased by 3.86 % after 14.5 % weight loss over a 20 wk period although the cholesterol absorption rate was not changed (Santosa *et al.*, 2007a). Present results supported the notion that weight loss is a potent inhibitor to cholesterol synthesis which is not compensated by the alteration of cholesterol absorption.

The present study is the second one to prove that polymorphisms in ABCG5 and ABCG8 are associated with cholesterol synthesis post weight loss. However, in contrast with previous findings, participants carrying the major allele (G/G) in Q604E ABCG5 (n = 41) were found to have had a greater decrease ($p < 0.05$) in cholesterol synthesis post weight loss compared with heterozygous C/G carrier (n = 11) and a combination of minor allele (C/C) and heterozygous C/G carrier (n = 13). Previous trial has shown that individuals possessing the heterozygous allele C/G (n = 13) showed a decrease (-7.39 ± 9.36 %/d) in fractional synthesis rate ($p < 0.05$) while the minor allele (n = 3) had an increase (1.69 ± 10.0 %/d) in FSR (Santosa *et al.*, 2007b). The trial by Santosa et al. also found other associations including Q604E with cholesterol absorption and C54Y SNP in ABCG8 with cholesterol synthesis which were not found in the present trial (Santosa *et al.*, 2007b). Another novel finding from the present trial is the association of polymorphisms

in T400K ABCG8 and an indirect marker of cholesterol synthesis, the lathosterol/TC ratio. Participants with the minor allele (A/A) exhibited a lower cholesterol synthesis decline than those with major allele C/C. Previous trial did not find any differences in CAS post weight loss in this SNP (Santosa *et al.*, 2007b). The present disparity could be a result of different methods used in measuring the cholesterol synthesis as well as participant's characteristic variability, including gender differences, and level of baseline cholesterol. The present study used an indirect CAS measurement approach using NCS ratio to TC, which employed 54 men and women with normal to high cholesterol levels while the previous weight loss trial used stable isotopes methods to measure FSR and cholesterol absorption in 35 hypercholesterolemic women.

The remaining SNP's measured were not associated with serum lipid levels or non-cholesterol sterol ratios. A meta-analysis comprising of 3364 subjects from 16 studies found no associations between SNPs in ABCG5/G8- Q604E, C54Y, D19H, T400K and A632V with baseline levels of lipid profiles and NCS ratios to cholesterol (Jakulj *et al.*, 2010), even though individual studies have shown associations with baseline markers of cholesterol metabolism.

The alteration of CAS subsequent to weight loss has never previously been associated with SNPs in NPC1L1. Unfortunately, due to very low heterogeneity found in NPC1L1 SNPs studied, no meaningful statistical analysis can be performed. Nevertheless, one individual with a rare mutation of NPC1L1 was found in this study. Overall, more studies need to be conducted in order to confirm inter-individual variations in cholesterol

metabolism response after weight loss since there are 34 missense SNPs of NPC1L1 known according to the National Center for Biotechnology Information database (dbSNP).

Limitations of this study include the number of missense SNPs studied (8SNPs) whereas according to the National Center for Biotechnology Information database, so far, there are 22 missense SNPs in ABCG5, 30 missense SNPs in ABCG8 and 34 missense SNPs in NPC1L1 that have been identified in the human genome. Moreover, the combinations of common polymorphisms of these transporters might be needed to fully explain the inter-individual variations of cholesterol metabolism. Other limitations include the gender proportion in the present study which was not equal since the women outnumbered the men, and the methodology used in the present study included indirect CAS and calculated LDL-C measurements.

3.6 Conclusion

In conclusion, weight loss of 10% of initial body weight induced cardio-protective changes in blood lipid profiles, which might be explained by the reduction in cholesterol biosynthesis. Single nucleotide polymorphisms in ABCG5 Q604E and ABCG8 T400K were associated with inter-individual variations of changes in CAS after weight loss. Individuals with homozygous allele C/C in ABCG8 A632V had a better response in improving the CVD risk factors including TC and TG compared with its heterozygous allele C/T.

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CHAPTER 4

BRIDGE

The previous chapter showed that weight loss ameliorated blood lipid profiles as a result of a reduction in cholesterol biosynthesis. The results in Chapter 3 (Trial A) revealed a different association of ABCG8 and ABCG5 polymorphisms with changes in cholesterol absorption and synthesis post weight loss compared with Santosa et al. (2007b) (Trial B). The difference in methodology might explain the variability between the two trials. Due to the similarity of the weight loss study design, the Trial B blood samples were reanalyzed using the ratio of non-cholesterol sterols to cholesterol as in Trial A. Additionally, since Trial B only measured 3 out of the 8 SNPs analyzed in Trial A, eight SNPs determined in Trial A would be examined in Trial B's participants. A bigger sample size enabled us to enhance statistical power as well as to reconfirm our findings. Therefore, the second manuscript combined and analyzed the samples of both Trial A conducted by the candidate (as seen in Chapter 3) and Trial B conducted by Santosa et al. (2007a; 2007b). The purpose of the second manuscript was to reconfirm the association between polymorphisms of ABCG5, ABCG8 and NPC1L1 genes and the change in cholesterol absorption and synthesis due to weight loss previously seen in the Santosa et al study (2007b).

CHAPTER 5

MANUSCRIPT 2

Polymorphism of V632A in ABCG8 is Associated with Reduced Serum Lipid Profiles Due to Weight Loss in Overweight Individuals

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5.1 Abstract

Global obesity has increased dramatically and is linked to chronic diseases including cardiovascular disease (CVD). The ABCG5 and ABCG8 proteins are responsible for the intestinal and hepatic excretion of cholesterol and plant sterols (PS), while the NPC1L1 is a protein which facilitates intestinal and hepatic influx of cholesterol. Single nucleotide polymorphisms (SNP) in these transporters might affect cholesterol biosynthesis and absorption (CAS). The objective of this study was to examine if single nucleotide polymorphisms (SNP) of ABCG5, ABCG8 and NPC1L1 were associated with changes in CAS subsequent to weight loss in overweight and obese individuals. Eighty-nine healthy males and females (age, 49.4 ± 1.1 yr; BMI, 32.0 ± 0.3 kg/m^2) completed a 20 wk weight loss period. Weight reduction was achieved by self-directed caloric restriction and physical activity. Ratios of non-cholesterol sterols (NCS) to total cholesterol (TC) measured using gas-liquid chromatography were used to estimate CAS. SNPs were determined using TaQMan genotyping assay. After weight reduction of 12.1% (10.3 ± 0.4 kg, $p < 0.0001$), serum TC, LDL-C and triglycerides (TG) were reduced ($p < 0.01$) while HDL-C was increased ($p < 0.01$). The ratio of desmosterol and lathosterol to TC were reduced ($p < 0.001$) by -0.08 ± 0.02 µmol/mmol (-11.49 %) and -0.18 ± 0.05 µmol/mmol (-9.52 %), respectively. However, campesterol/TC and sitosterol/TC were not altered. The SNP of ABCG8 V632A was associated with changes in TC and TG levels after weight loss. Participants carrying homozygous C/C in V632A showed a greater reduction ($p < 0.05$) in serum TC (-0.56 ± 0.08 mmol/l) and TG (-0.56 ± 0.07 mmol/l) compared with changes in TC (-0.23 ± 0.11mmol/l) and TG (-0.27 ± 0.08 mmol/l) in heterozygous C/T carriers. The present study is the first to demonstrate that

changes in TC and TG levels post weight loss associate with polymorphism at ABCG8 V632A. SNPs in cholesterol transporter, ABCG5, ABCG8, did not associate with CAS subsequent to weight loss. In conclusion, weight loss of 12.1 % initial body weight induced cardio-protective changes in blood lipids, potentially mediated by reduced cholesterol biosynthesis. Additionally, participants carrying homozygous allele C/C in ABCG8 V632A had greater improvement in CVD risk factors by reducing TC and TG compared with its heterozygous allele C/T. (Supported by Canadian Institutes of Health Research).

Keywords: weight loss, BMI, DEXA, body composition, fat mass, fat free mass, cholesterol absorption, cholesterol synthesis, HDL-C, LDL-C, TC, TG, SNP, ABCG5, ABCG8, NPC1L1.

5.2 Introduction

The prevalence of obesity in North America has increased dramatically over the past two decades. Nearly a third or 59.1 % of Canadians aged 18 years or older are overweight and obese (Tjepkema, 2006). Obesity serves as an independent risk factor for CVD (Klein *et al.*, 2004). WHO (1998) and Canadian obesity guidelines (2006) advise people with BMI > 25.0 kg/m^2 to have a goal of 5 % to 10 % of weight loss within 6 months to achieve beneficial health outcomes regardless of age and sex. As early as 60 years ago, weight loss was found to improve CVD risk factors including plasma cholesterol levels (Walker & Wier, 1951).

The delicate balance of cholesterol in the body, a key CVD risk marker, is maintained by rate of cholesterol absorption, synthesis and faecal excretion (Santosa *et al.*, 2007c). The impact of weight loss on CAS rates has been mixed. A decrease in cholesterol synthesis due to weight loss had been shown in several studies (Di Buono *et al.*, 1999; Santosa *et al.*, 2007a; Raeini-Sarjaz *et al.*, 2001); conversely, some studies have shown no significant change in cholesterol synthesis post weight loss (Griffin *et al.*, 1998; Simonen *et al.*, 2000). Simonen et al. observed an increase in cholesterol absorption (Simonen *et al.*, 2000), while Mintarno et al (Chapter 3) and Santosa et al. observed no change in absorption post weight loss (Santosa *et al.*, 2007a; Simonen *et al.*, 2000).

Through the rare human genetic disorder termed β-sitosterolemia and via studies that examine the effect of ezetimibe, a cholesterol absorption blocker, three proteins key to the cholesterol absorption were discovered: ATP binding cassette subfamily G5

(ABCG5), G8 and Neiman Pick C1-like 1 (NPC1L1) (Berge *et al.*, 2000; Lee *et al.*, 2001; Altmann *et al.*, 2004). Genetic variability in ABCG5/G8 and NPC1L1, which are responsible for cholesterol efflux and influx from the liver and the enterocyte (Altmann *et al.*, 2004; Salen *et al.*, 2002; Kidambi & Patel, 2008; Davis & Altmann, 2009; Betters & Yu, 2010), might explain the variability seen in the impact of weight loss on cholesterol metabolism.

The previous results showed that weight loss ameliorated blood lipid profiles as a result of a reduction in cholesterol biosynthesis (Chapter 3 and (Santosa *et al.*, 2007a). Moreover, the results from Chapter 3 (Trial A) showed a different association of ABCG8 and ABCG5 polymorphisms with changes in CAS post weight loss compared with Santosa et al. (2007a; 2007b) (Trial B). The difference in methodology is hypothesized to explain the variability between the two trials. Due to the similarity of the weight loss study design, blood samples from Trial B were reanalyzed using ratio of non-cholesterol sterols to cholesterol as Trial A did. Additionally, since Trial B only measured 3 out of the 8 SNPs analyzed in Trial A, all 8 SNPs determined in Trial A would be conducted in Trial B's participants.

Therefore, the aim of this study was to reconfirm the association between polymorphisms of ABCG5, ABCG8 and NPC1L1 genes and the change in CAS due to weight loss.

5.3 Materials and Methods

Two weight loss trials conducted by our group were pooled on the basis of study design similarity. Trial A was carried out at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Food and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg, Canada. Trial B was performed at the Mary Emily Clinical Nutrition Research Unit of McGill University, Montreal, Canada. Specific details on Trial A study design and protocols were explained in Chapter 3 of Mintarno's thesis while Trial B's study design and protocol have been published previously (Santosa *et al.*, 2007a; Santosa *et al.*, 2007b).

Study Design

Participant

A total of 89 participants with BMI between 28 and 39 kg/m^2 were pooled from both trials. Both trials excluded participant who were treated with oral hyperlipidemic therapy within the 3 months before starting the study; had any history of chronic diseases; or had a previous history of an eating disorder. All participants gave their written informed consent at the beginning of the study. The participant characteristics were different between Trial A and B. Trial A included 54 men and women aged between 18-70 years with normal to hypercholesterolemic individuals, while Trial B include hypercholesterolemic women at 35-60 years of age.

Human Ethics Considerations

Trial A experimental protocol was approved by the University of Manitoba Biomedical Research Ethics Board, with ethics file number: B2007:198 (HS10435). Trial B experimental protocol was approved by the Faculty of Medicine Ethics Review Board at McGill University and the Human Investigation Review Committee of Tufts University. Dr. Peter J.H. Jones was the principal investigator for both trials.

Study Protocol

Both longitudinal clinical trials employed a 20 wk weight loss period. Trial A had one week of stabilization time prior to the weight loss period, while Trial B had two weeks of stabilization time at the beginning and end of weight loss period. Blood samples were collected by a registered nurse at the beginning and end of the weight loss period. Participants were asked to fast, and consume no food or drink except water, for 12 h prior to every blood collection. In addition, at the beginning and end of the weight loss period, whole body composition was measured using dual energy x-ray absorptiometry (DEXA) in Trial A and magnetic resonance imaging for Trial B to determine percent lean and fat mass.

Weight Loss Protocol

A 20 wk weight loss period was undertaken in a free-living environment. Participants' baseline total energy expenditure (TEE) was calculated by multiplying Mifflin's equation (Mifflin *et al.*, 1990) by 1.65 activity factor in Trial A. Energy expenditure was estimated using the Harris–Benedict equation (Harris & Benedict, 1919) multiplied by an activity

factor in Trial B. Weight loss was achieved by reducing energy intake and increasing energy expenditure through exercise. Participants were taught to use the US exchange system consisting of grains, fruits and vegetables, meat and alternatives, milk, and fat food groups which will provide 50 % of energy from carbohydrates, 20 % of energy from proteins and 30 % of energy from fats. Compliance was monitored by weight changes at weekly weigh-ins with no shoes and light clothing. Participants were encouraged to lose weight through the use of visual graphs which included the plotted projected and actual body weight changes.

Analytical Methods

Body weight was measured to the nearest 0.1 kg and height to the nearest 0.1 cm with participants wearing only light clothing. BMI (kg/m^2) was calculated from weight and height recorded at the beginning and end of weight loss period. Both Trial A and B participants were combined in the analysis of cholesterol synthesis and absorption as well as genotyping determination, while their body composition as well as blood lipid values were measured and reported previously (Santosa *et al.*, 2007a; Santosa *et al.*, 2007b).

Blood Sample Separation

Collected blood was centrifuged at 3000 rpm, 4 °C for 20 min to separate red blood cells (RBC) and plasma/serum and stored in -80 ºC for future use for Trial A. For Trial B, blood samples were centrifuged at 1500 rpm for 15 min to separate RBC and plasma within 30 min of phlebotomy and immediately stored at –20 °C.

Lipid Profile Analysis

Enzymatic reactions were used to measure TC, TG, and HDL-C for both trials. Low density lipoprotein cholesterol was measured indirectly using the Friedewald equation (Friedewald *et al.*, 1972) in Trial A and the dextran/magnesium sulphate method (Rifai *et al.*, 1998) in Trial B.

Body Composition Analysis

The assessment of fat and fat free masses was carried out using whole body dual energy x-ray absorptiometry (GE Lunar BX-1 L-8743, General Electric Healthcare) in Trial A and magnetic resonance images were obtained using a Siemens 1.5. Tesla MRI scanner (Siemens, Mississauga, Canada) using a T-1 weighted spin-echo sequence with a 322 ms repetition time and a 12ms echo time was used in Trial B.

Cholesterol Absorption and Synthesis Determination

For all participants, the ratios of PSs (campesterol and β-sitosterol) to cholesterol were used as surrogate markers of cholesterol absorption while the ratio of cholesterol precursors (desmosterol and lathosterol) to cholesterol were utilized as an indirect marker of cholesterol synthesis (Miettinen, 1982). Plasma PS and cholesterol precursors were extracted from 500 µl of plasma EDTA and then quantified by gas liquid chromatography-flamed ionization detector using modified described method (Ntanios & Jones, 1998). Briefly, 100 µl [0.1 mg/ml] of internal standard 5-alpha-cholestane (Sigma-Aldrich Canada Ltd) was added to plasma samples which were then saponified with 8 ml

of freshly prepared 0.5 M methanolic-KOH. Sterols were extracted twice from the mixture with 4 ml of petroleum ether. Extracted sterols were derivatized with TMS reagent (pyridine:hexamethyldisilazan:trimethylchlorosilane 9:3:1) (Sylon^{TM} HTP, Sigma-Aldrich Canada Ltd) using a modified derivatization method. Samples were then evaporated under nitrogen (TECHNE sample concentrator) and re-suspended in hexane to be injected into a gas-liquid chromatography equipped with a flame ionization detector (6890N GC system, Agilent Technology) and separated on a 30 m SAC-5 capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm (Supelco). After some method development, the flow rate of carrier gas (helium) was set to 1.0 ml/min. Samples were injected at 300 °C. The detector was set at 310 °C. The oven temperature remained at 50 °C for 1 min after injection, was increased to 245 °C at a rate of 45 °C/min, and then was kept constant for 4 min, after which it rose to 280 °C at a rate of 15 °C/min and was kept constant at 280 °C for 4 min. Thereafter, oven temperature was raised to 300 °C at a rate of 2 °C/min and held for 2 min, then raised to 315 °C at a rate of 45 °C/min and maintained for 5 min. The total run time was 33 min. Sterol concentrations were determined in duplicate by identifying the peak sizes and expressing them relative to an internal standard. Desmosterol, lathosterol, campesterol and β-sitosterol levels were identified using authentic standards (Sigma-Aldrich Canada Ltd). Two consecutive days measurement at the beginning and end was analyzed in Trial A. However, due to limited sample availability, only a single day measurements at the beginning and end were analyzed in Trial B.

Single Nucleotide Polymorphism Determinations

Single nucleotide polymorphisms were analyzed using the TaqMan SNP Genotyping method. Genomic DNA was extracted from white blood cells obtained from 200 µl EDTA buffy coat using QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, Calif.) from the 54 participants of Trial A. EDTA treated buffy coat was used because heparin has been shown to inhibit real time polymerase chain reaction (RT-PCR). The white blood cell residues, found in the red blood cell fraction from the incomplete separation of buffy coat and red blood cells after centrifugation of whole blood, were used in 21 of 35 individuals from Trial B instead of buffy coat due to sample availability.

Eight SNPs in NPC1L1, ABCG5 and ABCG8 (as listed in the **Table 5.1**) were amplified by a Step One plus RT-PCR (Applied Biosystem, Foster City, California), using TaqMan® SNP Genotyping Assays (Applied Biosystem, Foster City, California). Half µl of TaqMan® Genotyping assays and 4.5 µl of [2 ng/µl] of human DNA was mixed with 5 µl of TaqMan® GTXpress™ Master Mix (Applied Biosystem, Foster City, California) resulting in 10 µl PCR reaction volume. Each reaction mixture was subjected to one holding stage to activate DNA polymerase at 95 ˚C for 20 sec, followed by 40 cycles consisting of denaturing for 3 sec at 95 ˚C and annealing and elongation for 20 sec at 60 ˚C. Each SNPs determination was conducted using triplicate measurements as differentiated with Step One™ Software v.2.1 (Applied Biosystems).

Table 5.1. Missense mutation of 8 SNPs, reference SNP number, allele as well as amino acid change.

SNPs	rs number	Ref Allele	mRNA Allele change	Amino Acid Change
ABCG5				
Q604E	Rs6720173	C/G	CAA ⇒ GAA	Q [Gln] ⇒ E [Glu]
R50C	Rs6756629	A/G	CGC ⇒ TGC	R [Arg] ⇒ C [Cys]
ABCG8				
Y54C	Rs4148211	A/G	TAC⇒TGC	Y [Tyr] ⇒ C [Cys]
T400K	Rs4148217	A/C	ACG ⇒ AAG	T [Thr] ⇒ K [Lys]
A632V	Rs6544718	C/T	GTC ⇒ GCC	V [Val] ⇒ A [Ala]
NPC1L1				
A310S	rs79803700	A/C	GCC ⇒ TCC	A [Ala] ⇒ S [Ser]
I1206N	rs52815063	A/T	ATC ⇒ AAC	I [Ile] ⇒ N [Asn]
D1114H D1087H	rs79519744	C/G	GAC ⇒ CAC	D [Asp] ⇒ H [His]

Statistical Analysis

Data is presented as mean ± standard error of means (SEMs). Paired student t-tests (2-tailed) were conducted to test the significance of the results comparing beginning to endpoint body weight, body composition, lipid parameters, as well as CAS surrogate markers. Moreover, analysis of variance (ANOVA) with post hoc Scheffe multiple comparisons was used to test the difference in cholesterol kinetics and lipid profiles changes within a SNP. Homozygous variant groups with less than five individuals were automatically collapsed into the heterozygous group. A two-sample Student's t-test was then applied to the regrouped data. Pairwise correlations between changes in CAS with

LDL-C or TC were calculated using Pearson's correlation. A p value < 0.05 was considered to be significant. All statistical analyses were performed using SPSS software for Windows version 17.0 (SPSS Inc, Chicago, IL).

5.4 Results

A total of 89 participants (49.4 ± 1.1 yr; initial BMI of 32.4 ± 0.5 kg/m^2) completed a 20 wk weight loss trial and were included in our analyses. Participants' baseline concentrations of blood lipid profiles, non-cholesterol sterols and body compositions are listed in **Table 5.2**.

Body Composition Changes Subsequent to Weight Loss

Pooled participant body weight changes before and after the 20 week weight loss period are described in **Figure 5.1**. Participants' mean weights were reduced from 86.6 ± 1.4 kg to 76.3 ± 1.4 kg, resulting in reduction of -10.3 ± 0.4 kg or -12.1 ± 0.5 % (p < 0.0001) post weight loss period (**Table 5.3**). Participants' fat masses (FM) declined (p < 0.0001) by 8.4 ± 0.4 kg or -23.6 ± 1.3 % and the percentage of FM declined significantly (p < 0.0001) by 5.7 ± 0.4 %. Participants' fat free masses (FFM) were decreased (p < 0.0001) by 1.7 ± 0.2 kg or 3.5 ± 0.4 %. However, the overall percentage of FFM was increased by 5.7 ± 0.4 % (p < 0.0001) after weight loss.

Table 5.2. Baseline characteristics of weight loss study participant in combined analysis (n = 89).

Baseline Characteristics	Mean ± SEM
Age (years)	49.4 ± 1.1
Female (n (%))	76 (85.4)
Initial weight (kg)	86.6 ± 1.4
Height (m)	1.64 ± 0.01
BMI (kg/m^2)	32.0 ± 0.3
Lipid profiles (mmol/l)	
Total cholesterol	5.60 ± 0.14
LDL-cholesterol	3.44 ± 0.08
HDL-cholesterol	1.32 ± 0.04
Triglycerides	1.84 ± 0.10
Non-cholesterol sterols (µmol/mmol)	
Desmosterol/TC	0.59 ± 0.03
Lathosterol/TC	1.30 ± 0.07
Campesterol/TC	1.24 ± 0.08
β-sitosterol/TC	0.72 ± 0.05
Body composition	
FM (kg)	36.6 ± 0.8
FM (%)	44.2 ± 0.6
FFM (kg)	48.1 ± 1.0
FFM (%)	56.7 ± 0.6

Data are presented as means ± SEM.

Abbreviations: BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein cholesterol; TC, total cholesterol.; FM, fat mass; FFM, fat free mass.

Figure 5.1. Change in individual body weights (kg) before and after 20 wk of the weight loss period in obese and overweight individuals in the combined analysis (n = 89).

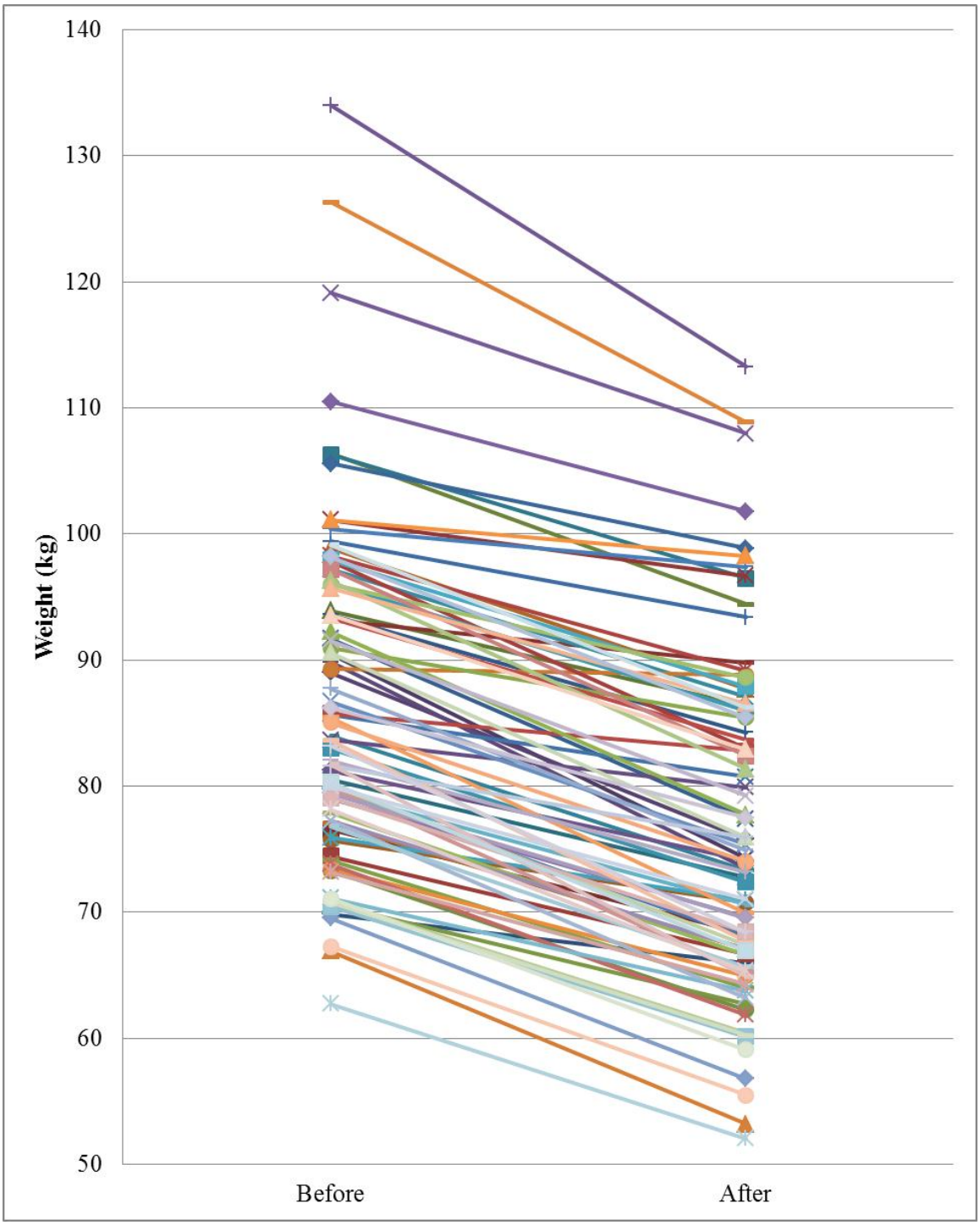


Table 5.3. Change and percent change in body weight and composition after a 20 wk weight intervention, according to DEXA (n = 53) and MRI (n = 35) in overweight and obese individuals in the combined analysis (n = 88).

Variables	Before	After	Difference	% Difference
Weight-scale (kg) *	86.6 ± 1.4	76.3 ± 1.4	-10.3 ± 0.4	-12.1 ± 0.5
FM (kg)*	36.6 ± 0.8	28.2 ± 0.9	-8.4 ± 0.4	-23.6 ± 1.3
FM (%) *	44.2 ± 0.6	38.4 ± 0.7	-5.7 ± 0.4	-13.3 ± 1.0
FFM (kg)*	48.1 ± 1.0	46.3 ± 1.0	-1.7 ± 0.2	-3.5 ± 0.4
FFM (%) *	56.7 ± 0.6	62.4 ± 0.7	5.7 ± 0.4	10.2 ± 0.8

Data are presented as means ± SEM. Abbreviations: DEXA, dual-energy X-ray absorptiometry (n = 53); MRI, Magnetic Resonance imaging (n = 35); FFM, fat free mass and FM, fat mass.

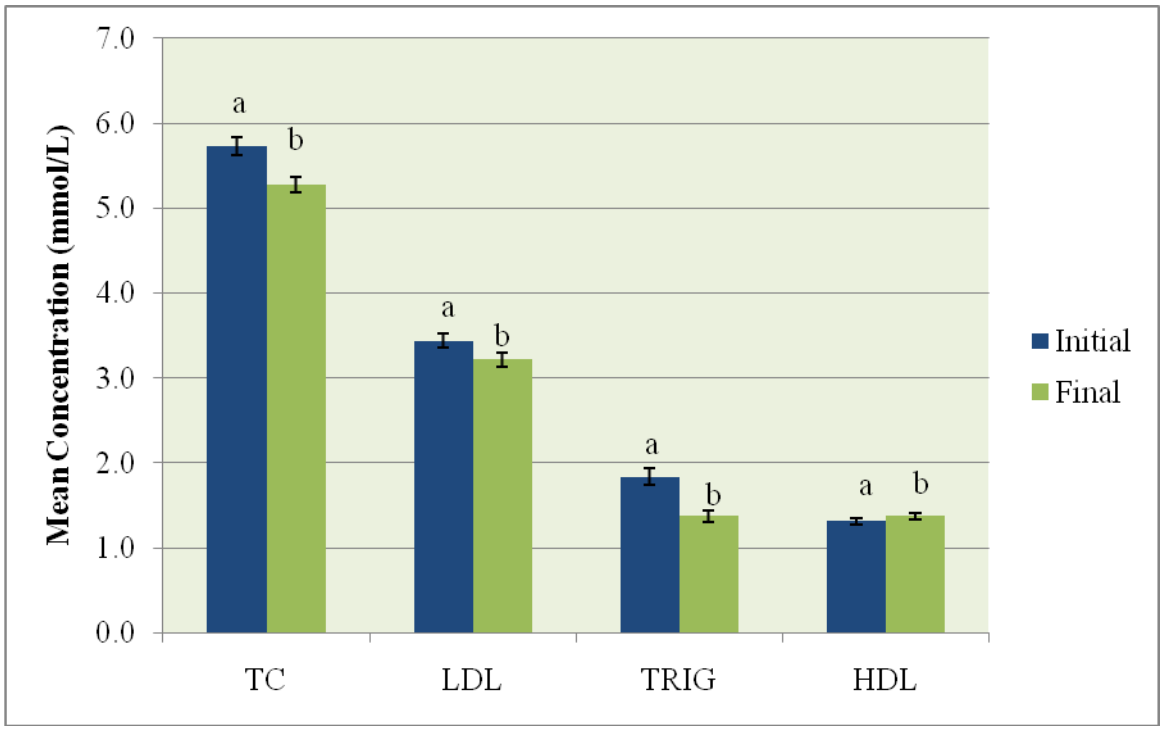
* $p < 0.0001$ of changes before and after weight loss

Changes in Blood Lipid Profile, Cholesterol Absorption and Synthesis

Total cholesterol, LDL-C and TG declined ($p < 0.01$) by -0.45 ± 0.06 mmol/l (-7.27 %), -0.22 ± 0.05 mmol/l (-5.52 %), and -0.47 ± 0.06 mmol/l (20.59 %), respectively. HDL-C levels increased ($p < 0.05$) by 0.06 ± 0.02mmol/l (5.52%) after 20 wk of weight loss (**Figure 5.2**). The ratio of desmosterol and lathosterol to cholesterol was reduced ($p < 0.001$) by -0.08 ± 0.02 µmol/mmol (-11.49 %) and -0.18 ± 0.05 µmol/mmol (-9.52 %), respectively. However, the ratio of campesterol and β-sitosterol to cholesterol was not changed significantly, -0.02 ± 0.05 µmol/mmol and 0.02 ± 0.03 µmol/mmol, respectively (**Figure 5.3**). No significant correlations were observed between changes in TC, LDL-C

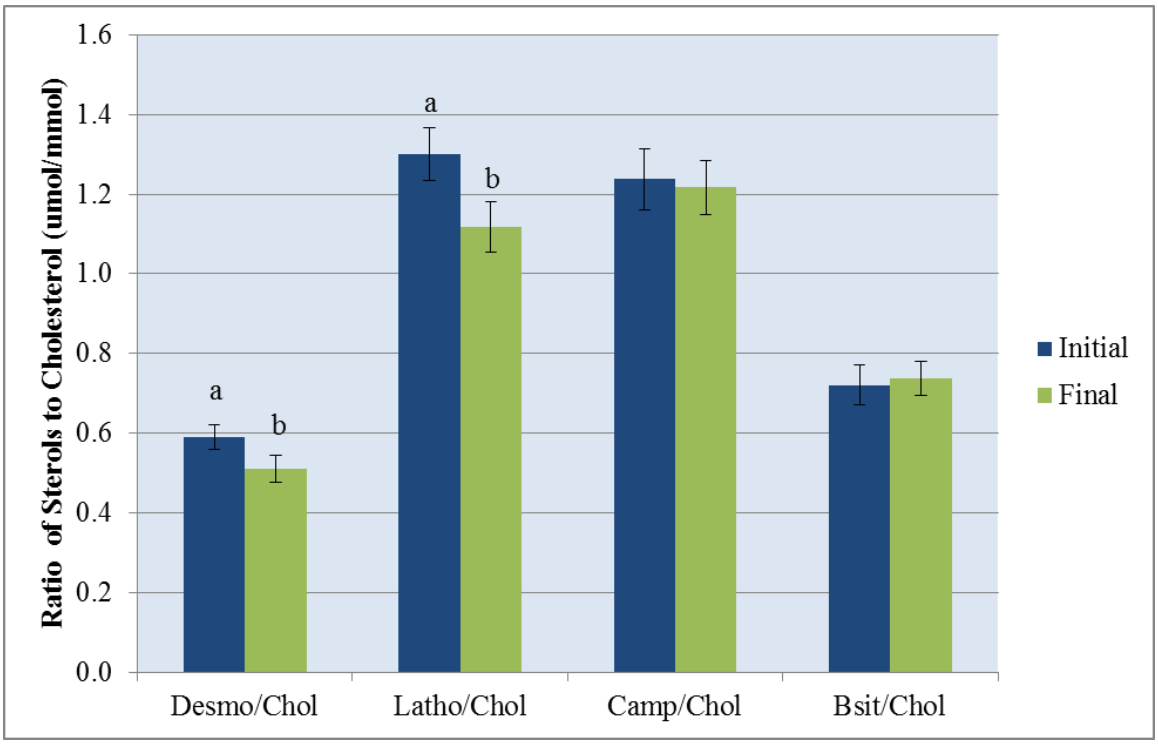
and TG with changes in the ratios of surrogate markers of cholesterol absorption (campesterol and β-sitosterol) and synthesis (desmosterol and lathosterol) to cholesterol (**Table 5.4**). HDL-C was positively correlated ($p < 0.05$) with campesterol/TC but the Pearson correlation value was low (r = 0.252). The ratios of PSs were strongly positively correlated with each other (r = 0.756, $p < 0.001$); however, the ratios of precursors were not correlated with each other (r = 0.131). The ratio of desmosterol to cholesterol was also correlated with campesterol to TC ratio (r = 0.345, $p < 0.01$) and β-sitosterol to TC ratio (r = 0.317, $p < 0.05$).

Figure 5.2. Lipid parameters before and after weight loss in overweight and obese individuals in the combined analysis (n = 89).



Data are presented as means ± SEM.
a and b indicates significant differences with $p < 0.01$
Abbreviations: TC, total cholesterol; LDL, low density lipoprotein cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol.

Figure 5.3. Response of synthesis and absorption across study in overweight and obese individuals in the combined analysis (n = 89).



Data are presented as means ± SEM.

a and b indicates significant differences with p <0.001

Abbreviations: Desm, desmosterol; latho, lathosterol; camp; campesterol; BSit, β-sitosterol; Chol, cholesterol

Table 5.4. Correlations of change in cholesterol precursors and PSs with change in serum total and lipoprotein cholesterol levels before and after a 20 wk weight loss intervention in overweight and obese individuals in the combined analysis (n=89).

Parameters	Desmosterol/TC	Lathosterol/TC	Campesterol/TC	β-Sitosterol/TC
Serum lipids (mmol/l)				
Total cholesterol	0.062	0.002	0.109	-0.006
LDL cholesterol	0.022	0.019	0.094	-0.016
HDL cholesterol	-0.015	-0.028	0.252*	0.187
Triglycerides	0.139	0.002	-0.040	-0.059
Ratio of non-cholesterol sterols to total cholesterol (µmol/mmol)				
Desmosterol/TC	1.000	0.131	0.345**	0.317**
Lathosterol/TC	0.131	1.000	0.067	0.006
Campesterol/TC	0.345**	0.067	1.000	0.756***
β-Sitosterol/TC	0.317**	0.006	0.756***	1.000

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Cholesterol precursors and PSs is reported in µmol/mmol of cholesterol

All values reported are the difference between before and after 20 wk of weight loss trial.

Abbreviations: LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol.

Genotype Distribution and Frequency of Single Nucleotide Polymorphisms

The distribution and frequency of SNPs are explained in **Table 5.5**. Participants' age and BMI were not significantly different among all the allele groups. Due to a very low frequency of heterogeneity found in SNPs in NPC1L1, their relationships with lipid profiles, CAS markers could not be analyzed statistically.

Table 5.5. Genotype distribution and frequency of missense SNPs of ABCG5 and ABCG8 in overweight and obese individuals in the combined analysis (n = 89).

SNPs	Homozygous Allele 1/1			Heterozygous Allele 1/2			Homozygous Allele 2/2		
	N (%)	Age (yr)	BMI	N (%)	Age (yr)	BMI	N (%)	Age (yr)	BMI
ABCG5									
Q604E	5 (5.6)	50.6 ± 3.5	30.96 ± 0.82	23 (25.84)	49.1 ± 2.1	32.04 ± 0.63	61 (68.54)	49.5 ± 1.4	32.05 ± 0.43
R50C	0			16 (17.98)	49.6 ± 2.8	31.19 ± 0.72	73 (82.02)	49.4 ± 1.2	32.16 ± 0.38
ABCG8									
C54Y	34 (38.2)	50.1 ± 1.6	31.7 ± 0.58	35 (39.33)	47.8 ± 1.8	32.5 ± 0.5	20 (22.47)	51.1 ± 2.6	31.59 ± 0.71
T400K	5 (5.6)	53.2 ± 3.5	33.37 ± 1.87	24 (26.97)	47 ± 2.4	32.41 ± 0.6	60 (67.42)	50.1 ± 1.3	31.71 ± 0.41
V632A	60 (67.4)	48.9 ± 1.4	32.01 ± 0.42	28 (31.46)	51 ± 1.7	32 ± 0.6	1 (1.12)	39.	30.5

No significant differences were found among all groups ($p > 0.05$).

Data are presented as means ± SEM, BMI values reported are in kg/m^2

Homozygous groups in bold which are lower than 10 % or 6 are considered to be collapsed to its heterozygous group.

Abbreviations: BMI, body mass index; SNP, single nucleotide polymorphism.

Effect of Single Nucleotide Polymorphisms on Lipid Parameters

The presence of V632A SNP in ABCG8 was associated with the extent of change in TC and TG after weight loss. Participants carrying homozygous C/C in V632A had a larger decline ($p < 0.05$) in TC (-0.56 ± 0.08 mmol/l) and TG (-0.56 ± 0.07 mmol/l) compared with changes in TC (-0.23 ± 0.11 mmol/l) and TG (-0.27 ± 0.08 mmol/l) in heterozygous C/T and combination of C/T and T/T carriers (TC: -0.22 ± 0.11 mmol/l; TG: -0.28 ± 0.08 mmol/l) post weight loss (**Table 5.7**). The rest of the SNPs analyzed in the present study were not significantly associated with serum lipid parameters including TG, TC, HDL-C and LDL-C (**Table 5.6 and 5.7**)

Effects of Polymorphisms on Cholesterol Absorption and Synthesis Markers

None of the SNPs measured showed associations with changes in the ratio of desmosterol, lathosterol, campesterol and β-sitosterol to TC post weight loss (**Table 5.6 and 5.7**). Two individuals with a rare genetic mutation in NPC1L1 were found. When stratified based on SNPs in ABCG5 and ABCG8, no significant correlations were found after correlating percent differences in LDL-C or TC with percent change in surrogate markers of cholesterol synthesis and absorption after weight loss (See Appendix 11).

Table 5.6. Change in surrogates of synthesis and absorption and serum lipid profiles stratified based on ABCG5 SNPs in the combined analysis (n = 89).

Q604E	C/C	C/C+C/G	C/G	G/G	Total
N	5	28	23	61	89
Desm/TC	-0.07 ± 0.03	-0.03 ± 0.04	-0.03 ± 0.04	-0.10 ± 0.02	-0.08 ± 0.02
Latho/TC	-0.30 ± 0.10	-0.09 ± 0.11	-0.04 ± 0.13	-0.22 ± 0.05	-0.18 ± 0.05
Camp/TC	0.03 ± 0.08	0.03 ± 0.05	0.03 ± 0.06	-0.04 ± 0.07	-0.02 ± 0.05
β-sito/TC	0.08 ± 0.05	0.01 ± 0.06	0.00 ± 0.07	0.02 ± 0.04	0.02 ± 0.03
TC	-0.32 ± 0.28	-0.48 ± 0.11	-0.51 ± 0.12	-0.44 ± 0.08	-0.45 ± 0.06
LDL-C	-0.22 ± 0.15	-0.25 ± 0.08	-0.26 ± 0.09	-0.21 ± 0.07	-0.22 ± 0.05
HDL-C	0.07 ± 0.05	0.06 ± 0.03	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.02
TG	-0.13 ± 0.17	-0.39 ± 0.10	-0.45 ± 0.11	-0.50 ± 0.07	-0.47 ± 0.06
R50C	A/A		A/G	G/G	Total
N	0	-	16	73	89
Desm/TC			-0.01 ± 0.06	-0.10 ± 0.02	-0.08 ± 0.02
Latho/TC			-0.18 ± 0.12	-0.18 ± 0.05	-0.18 ± 0.05
Camp/TC			-0.01 ± 0.06	-0.02 ± 0.06	-0.02 ± 0.05
β-sito/TC			0.08 ± 0.07	0.00 ± 0.04	0.02 ± 0.03
TC			-0.51 ± 0.15	-0.44 ± 0.07	-0.45 ± 0.06
LDL-C			-0.20 ± 0.12	-0.23 ± 0.06	-0.22 ± 0.05
HDL-C			0.06 ± 0.03	0.06 ± 0.02	0.06 ± 0.02
TG			-0.62 ± 0.16	-0.43 ± 0.06	-0.47 ± 0.06

Data are presented as means ± SEM.

No significant difference was found among all groups ($p > 0.05$).

Abbreviations: SNP, single nucleotide polymorphism; desm, desmosterol; latho, lathosterol; camp; campesterol; β-sito, beta sitosterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; Trig, triglycerides; HDL-C, high density lipoprotein cholesterol.

Cholesterol precursors and PSs are reported in μmol/mmol of cholesterol while lipid parameters are reported in mmol/l. All values reported are the difference between before and after 20 weeks of weight loss trial.

Table 5.7. Change in surrogates of cholesterol synthesis and absorption and lipid profiles stratified based on ABCG8 SNPs in the combined analysis (n=89).

C54Y	A/A	A/A+A/G	A/G	G/G	Total
N	34	-	35	20	89
Desm/TC	-0.09 ± 0.03		-0.07 ± 0.03	-0.09 ± 0.04	-0.08 ± 0.02
Latho/TC	-0.11 ± 0.09		-0.25 ± 0.07	-0.17 ± 0.08	-0.18 ± 0.05
Camp/TC	0.01 ± 0.05		-0.05 ± 0.10	-0.03 ± 0.11	-0.02 ± 0.05
β-sito/TC	0.06 ± 0.04		-0.02 ± 0.05	0.02 ± 0.09	0.02 ± 0.03
TC	-0.52 ± 0.11		-0.42 ± 0.09	-0.39 ± 0.14	-0.45 ± 0.06
LDL-C	-0.28 ± 0.08		-0.18 ± 0.09	-0.21 ± 0.11	-0.22 ± 0.05
HDL-C	0.07 ± 0.03		0.06 ± 0.03	0.03 ± 0.04	0.06 ± 0.02
TG	-0.48 ± 0.09		-0.54 ± 0.10	-0.31 ± 0.09	-0.47 ± 0.06
T400K	A/A	A/A+A/C	A/C	C/C	Total
N	5	29	24	60	89
Desm/TC	-0.17 ± 0.07	-0.12 ± 0.03	-0.11 ± 0.03	-0.06 ± 0.02	-0.08 ± 0.02
Latho/TC	-0.43 ± 0.09	-0.09 ± 0.09	-0.02 ± 0.10	-0.23 ± 0.05	-0.18 ± 0.05
Camp/TC	-0.04 ± 0.19	-0.02 ± 0.06	-0.02 ± 0.06	-0.02 ± 0.07	-0.02 ± 0.05
β-sito/TC	0.09 ± 0.11	0.04 ± 0.05	0.03 ± 0.05	0.01 ± 0.04	0.02 ± 0.03
TC	-0.91 ± 0.31	-0.59 ± 0.13	-0.52 ± 0.14	-0.38 ± 0.07	-0.45 ± 0.06
LDL-C	-0.52 ± 0.28	-0.31 ± 0.09	-0.27 ± 0.10	-0.18 ± 0.06	-0.22 ± 0.05
HDL-C	0.05 ± 0.05	0.03 ± 0.03	0.03 ± 0.03	0.07 ± 0.02	0.06 ± 0.02
TG	-0.75 ± 0.32	-0.53 ± 0.11	-0.48 ± 0.11	-0.44 ± 0.07	-0.47 ± 0.06
V632A	C/C	C/T+T/T	C/T	T/T	Total
N	60	29	28	1	89
Desm/TC	-0.09 ± 0.02	-0.06 ± 0.02	-0.07 ± 0.02	0.25	-0.08 ± 0.02
Latho/TC	-0.18 ± 0.06	-0.19 ± 0.07	-0.21 ± 0.07	0.37	-0.18 ± 0.05
Camp/TC	-0.03 ± 0.06	0.00 ± 0.08	-0.01 ± 0.08	0.30	-0.02 ± 0.05
β-sito/TC	0.00 ± 0.04	0.04 ± 0.04	0.03 ± 0.04	0.34	0.02 ± 0.03
TC	-0.56 ± 0.08[a]	-0.23 ± 0.11[b]	-0.22 ± 0.11[b]	-0.59	-0.45 ± 0.06
LDL-C	-0.27 ± 0.07	-0.12 ± 0.09	-0.11 ± 0.09	-0.56	-0.22 ± 0.05
HDL-C	0.04 ± 0.02	0.1 ± 0.03	0.10 ± 0.03	0.16	0.06 ± 0.02
TG	-0.56 ± 0.07[a]	-0.27 ± 0.08[b]	-0.28 ± 0.08[b]	-0.03	-0.47 ± 0.06

[a] and [b] $p < 0.05$

Data are presented as means ± SEM.

Abbreviations: SNP, single nucleotide polymorphism; desm, desmosterol; latho, lathosterol; camp; campesterol; β-sito, beta sitosterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol.

Cholesterol precursors and PSs are reported in μmol/mmol of cholesterol

Values reported refer to the difference between before and after 20 wk of weight loss trial.

5.5 Discussion

The combined data demonstrated a number of findings consistent with those found in the individual trials. Weight loss intervention successfully lowered the initial body weight of 89 overweight and obese participants by 12.1 %. The weight reduction and subsequent improvement in body compositions resulted in cardio-protective changes in serum lipid profiles. The beneficial shift in lipid parameters was also accompanied by a decrease in whole body cholesterol biosynthesis. The SNP in sterol transport gene ABCG8 A632V showed that allele determination significantly associated with the individual variability response of blood lipid parameters including TC and TG subsequent to weight loss. However, contradictory with individual trials, the SNPs of sterols transport genes, ABCG5, ABCG8 and NPC1L1, did not associate with the inter-individual variability in the shift of CAS post weight loss.

Consistent with results from individual trials, pooled results showed a major reduction (23.6 %) of FM and a relatively small decline (3.5 %) in FFM. These changes contributed to the overall increase in percentage of FFM after weight loss, even though the actual FM had decreased. The small reduction of FFM shown in this study is advantageous since FFM is responsible for maintaining resting metabolic rate, regulating body temperature, preserving skeletal integrity and maintaining function and quality of life (Marks & Rippe, 1996).

Weight loss ameliorated blood lipid profiles suggest that weight reduction decreases the risk of CHD. The reductions of pooled cholesterol levels observed were in line with

individual results from Trial A and Trial B. A meta-analysis of 70 studies found that for every kilogram decrease in body weight, TC, LDL-C and TG levels are decreased by 0.05 mmol/l, 0.02 mmol/l, and 0.015 mmol/l, respectively (Dattilo & Kris-Etherton, 1992). Although, the change in LDL-C after weight loss intervention in Trial A was not significant, the reduction was significant in the pooled data. As seen in this study, previous trials also have shown that beneficial alterations in lipid levels also contributed to decreases in percent body fat (Janssen *et al.*, 2002; Nieman *et al.*, 2002).

The bigger sample size in this combined analysis enabled us to enhance statistical power as well as to reconfirm our previously published findings. Along with results from Trial A, the pooled result also showed the homozygous C/C in A632V ABCG8 exhibits a better cardio-protective change than heterozygous C/T post weight loss. No other study has associated A632V SNP in ABCG8 to cholesterol metabolism in response to weight loss intervention. Previously, SNPs in A632V have been shown to be associated with baseline TC values (Berge *et al.*, 2002). Moreover, a recent meta-analysis by Jakulj et al. observed an association between polymorphism in V632A and LDL-C baseline concentration, although the association found was not seen in individual trials included in that analysis (Jakulj *et al.*, 2010).

The beneficial changes in body cholesterol levels subsequent to weight reduction, as observed in present trial, were a result of a shift in cholesterol biosynthesis. Cholesterol synthesis was significantly reduced after weight loss even in the absence of changes in cholesterol absorption. Results of the combined trials showed consistent findings with

individual results from both Trial A and Trial B (Mintarno et al, Chapter 3; Santosa *et al.*, 2007a). Thus, present results strengthen the premise that the significant reduction of cholesterol synthesis observed after weight loss was not compensated through an alteration in cholesterol absorption.

The polymorphisms in ABCG5 and ABCG8 determined in this study were not associated with alterations in cholesterol synthesis or absorption post weight loss. In contrast, when looking at individual trials before pooling, polymorphisms in ABCG5 Q604E were associated with changes in cholesterol synthesis in both Trial A (as seen in Chapter 3) and B (Santosa *et al.*, 2007b), as well as cholesterol absorption in Trial B (Santosa *et al.*, 2007b). Additionally, ABCG8 T400K was associated with cholesterol synthesis after weight loss. Aside from our findings, genetic polymorphisms on ABCG5/ABCG8 have been shown to be associated with plasma cholesterol and non-cholesterol sterol levels (Berge *et al.*, 2002; Hubacek *et al.*, 2004; Weggemans *et al.*, 2002; Chen *et al.*, 2008), along with a number of cholesterol-lowering mechanisms such as diet interventions (Weggemans *et al.*, 2002; Herron *et al.*, 2006), PS supplementation (Zhao *et al.*, 2008; Plat *et al.*, 2005) and treatment with statins (Kajinami *et al.*, 2004).

Some limitations are worth noting in the present study. The number of missense SNPs studied was less than 10, whereas according to National Center for Biotechnology Information database, so far, there are 22 missense ABCG5, 30 missense ABCG8 and 34 missense NPC1L1 SNPs that have been identified in the human genome. Moreover, the combination or haplotype analysis of common polymorphisms of these transporters might

be needed to fully explain the inter-individual variations of cholesterol metabolism. Other limitations are the gender proportion with women outnumbering men; the difference in Trial A and B participant's criteria; and the methodology used including indirect CAS measurements.

The results of this study highlight many more opportunities for further study on this topic. The rest of the missense or nonsense SNPs which were not determined in the present study could potentially be a selection tool in ameliorating CVD mediated by weight loss. Moreover, other phenotypic trait of HMG-CoA- R, APOE, CETP and SR-BI could be useful in explaining the association between shift in lipids as a result of weight loss and cholesterol absorption and/or synthesis rate. A measurement of the whole human genome using gene chip methodology could be a more effective method in looking at polymorphism in humans rather than genotyping numerous SNPs using RT-PCR. Furthermore, using direct methods, such as dual isotope ratio method to determine cholesterol synthesis and deuterium for cholesterol absorption, would be a better tool to reconfirm the findings of Santosa et al. (2007b) which were different from the current results. Lastly, a balanced gender proportion would be ideal to compare gender effect in the associations measured.

5.6 Conclusion

In conclusion, cardio-protective changes in serum lipid parameters as a consequence of weight loss resulted from a reduction in cholesterol biosynthesis. Moreover, SNPs in ABCG5 and ABCG8 failed to be associated with inter-individual variation of changes in CAS after weight loss. Furthermore, current findings strengthen the premise that individuals carrying homozygous allele C/C in ABCG8 A632V, possess a better response in improving CVD risk factors including TC and TG compared with its heterozygous allele C/T.

5.7 Literature cited

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 Summary and Discussion

A moderate weight loss of 10 – 12 % was significant in improving circulating cholesterol levels in this study of overweight and obese males and females. The findings from this research strengthen current knowledge that the improvement in serum lipid parameters subsequent to weight loss was driven by a reduction in cholesterol biosynthesis without altering cholesterol absorption. These results suggest that therapies targeting cholesterol absorption, such as plant sterols, might provide synergistic cardio-protective changes together with a weight loss program comprising of diet and physical activity.

The present research is the second to prove that polymorphisms in ABCG5 and ABCG8 are associated with cholesterol synthesis post weight loss. However, genetic polymorphisms in ABCG5 Q604E were found to associate with cholesterol trafficking in a manner that was different from that observed in our previous study (Santosa *et al.*, 2007b). Another novel finding of this research is that the polymorphism in ABCG8 T400K was associated with inter-individual variations of changes in CAS after weight loss. However, when Trial A was combined with Trial B, the associations between polymorphisms of ABCG5/ABCG8 and the change in cholesterol metabolism due to weight loss, were no longer seen. This discrepancy could have resulted from the difference in methodology used in Trial B and in the current study, and the distinction of subject characteristics in both Trial A and Trial B.

The alteration of CAS subsequent to weight loss has never previously been associated with polymorphisms in NPC1L1. The polymorphisms in NPC1L1 might explain the inter-individual variability responses of cholesterol metabolism after weight loss. Unfortunately, since very low heterogeneity was found in the NPC1L1 SNPs studied, no meaningful statistical analysis could be performed. Nevertheless, two individuals with a rare mutation of NPC1L1 were found.

A bigger sample size enabled us to enhance our statistical power as well as to reconfirm our finding that individuals with homozygous allele C/C in ABCG8 A632V had a better response in improving the CVD risk factors including TC and TG, compared with its heterozygous allele C/T. It is worth noting that the significant association in changes in TC and TG post weight loss, and the polymorphisms in A632V seems to link together, since both TC and TG were observed to exhibit the same trend in both Trial A and the combined trials. In both instances, individuals who are CC homozygous displayed a greater reduction in both TC and TG levels, compared with individuals carrying the heterozygous C/T trait. There is the potential to use A632V as a screening tool to highlight individuals who have better reduction in CVD risk factors after weight loss. Therefore, understanding the effects of weight loss on cholesterol metabolism and genotypic trait will facilitate the advancement of therapies in ameliorating dyslipidemia in overweight and obese individuals.

6.2 Future Directions

The present research highlights many more prospects for further study on this topic. The remaining of the missense or nonsense SNPs in ABCG5, ABCG8 and NPC1L1, listed in National Center for Biotechnology Information database, which were not determined in the present study, could potentially serve as a selection tool in ameliorating CVD mediated by weight loss. The combination or haplotype analysis of common polymorphisms of these transporters could be used to explain the inter-individual variations in cholesterol metabolism. Moreover, other phenotypic traits of HMG-CoA- R, APOE, CETP and SR-BI could enlighten the association between the change in lipids mediated by weight loss and cholesterol absorption and/or synthesis rate. Rather than genotyping numerous SNPs using RT-PCR, a determination of the whole human genome using the gene chip method could be a more effective approach in looking at polymorphism in humans. Furthermore, using more robust methods such as the dual isotope ratio method to determine cholesterol synthesis and deuterium method for cholesterol absorption and direct LDL-C measurement would serve as better tools to reconfirm the findings of Santosa et al. (2007b), which differed from the current results. Finally, a balanced gender proportion would be ideal to compare gender effects in the associations measured.

6.3 Final Conclusions

In conclusion, cardio-protective changes in serum lipid profiles mediated by 10-12% of weight loss and improvement of body compositions were a consequence of the reduction in cholesterol biosynthesis. Moreover, polymorphism in sterol transport genes ABCG5, ABCG8 were associated with inter-individual variation of changes in CAS after weight loss. Furthermore, the present study is the first to demonstrate that individuals carrying homozygous allele C/C, in ABCG8 A632V, have a better response in improving CVD risk factors including TC and TG compared with its heterozygous allele C/T. Therefore, results from the present study represent meaningful advances in unraveling the effect of weight loss and subsequent changes in body composition on cholesterol metabolism and phenotype traits in overweight and obese individuals.

CHAPTER 7.

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APPENDICES

1. SUBJECT CONSENT FORMS OF RESEARCH PROTOCOL

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: **Genetic basis for heterogeneity in response of plasma lipids to weight loss in overweight and obese individuals**

Investigator: Peter Jones, PhD
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Phone: (204) 474-9787

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

The purpose of the study is to examine the genetic differences related to the plasma lipid response to a weight loss program including both dietary energy restriction and exercise.

Study procedures

If you agree to take part of this study, you will be invited to the research center for a first consultation with the dietician where your weight, height, and habitual food intake will be collected. You will need to ensure that you have not consumed any medication and/or natural health supplements affecting fat metabolism such as, cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, HMG CoA reductase inhibitors or statins (Lipitor, zocor etc…) which reduce cholesterol synthesis, as well as high dose dietary supplements, fish oil capsules or plant sterol for at least the last 3 months. You will also need to ensure that you will not be consuming any of these medications/supplements during the study. You will be required to report the use of any new medications and/or natural health products. Subjects using medications including natural health products known to affect lipid metabolism during the trial will be asked to withdraw from the trial. In addition, you have to ensure that you do not smoke or consume large amounts of alcohol (>2drinks/day). You will also need to ensure that you do not currently have diabetes, thyroid, kidney, heart or liver disease or have had any of these diseases at any time during the past three months, in addition any development of diabetes mellitus, thyroid, kidney, heart or liver disease during the trial will lead to exclude from the trial.

Initials of Subject: _____

The study will consist of a 21 week weight loss program including diet and exercise. The study staff will provide you with guidelines of a custom-made weight loss program, including food habits and physical activity. The goal of the program is to incur a steady moderate weight loss and the ensuing cholesterol lowering. On the first two and last two days of the study, you will be scheduled for a blood draw. Each blood test will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 4 tablespoons.

From the blood drawn we would like to measure lipid profile, extract DNA and perform genetic analyses using a laboratory technique that augments and recognizes specific genes to determine why some people decrease their cholesterol levels better than others as a result of weight loss. DNA is a molecule found in the cells of your body that is organized into genes that contain all of the information needed to make the proteins that perform specific biological functions in your body.

Also your body fat composition will be measured at the beginning and at the end of the study through Dual Energy X-ray Absorptiometry (DEXA). The DEXA machine is composed of a bed and a moving arm using radiation of less than $1/20^{th}$ of a standard chest X-ray to determine percent lean and fat mass. For this procedure, you will need to lie in a horizontal position for about 5-10 minutes while the scan arm passes from your head to your feet. The radiation from this test is very low dosage (equivalent to approximately 1 day of natural background radiation). The dosage is 1000 times less than the limit for trivial exposure. You will be asked not to wear anything metal (metal may affect bone density values which will affect body composition calculations).

Please note **PREGNANT WOMEN CANNOT PARTICIPATE IN A DEXA SCAN**; therefore, if you are a female, you will be required to ensure you are not pregnant. Prior to taking part in the scan, you will be asked to provide a urine sample to verify that you are not pregnant. The pregnancy test that we are using can detect pregnancy as early as 4 days before your expected period. No pregnancy test is, however, 100% accurate, and there is always the possibility of a false negative or a false positive result. You may choose not to undergo this test, in which case we will ask you to sign a waiver form releasing the University from any liability related to this decision.

Risks and Discomforts

As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.
Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. The x-ray dose associated with a total body scan using DEXA is very low and safe for repeated measurements. With the exception of pregnant women, there are no known risks associated with a DEXA scan. The potential risks associated with radiation exposure to an unborn fetus are not known, and therefore we ask that you undergo a pregnancy test to verify that you are not pregnant. Having a DEXA scan does not make it unsafe for you to have other x-rays taken in the near future. Also, considering the weight loss nature of the trial you might experience lightheadedness in relation to food restriction. In case you feel any

Initials of Subject: _____

discomfort during the experimental trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at (204) 954-4486.

Benefits

Although you may not benefit from participation in this research, weight loss in overweight individuals is linked to an improvement in health parameters. Moreover, the study should contribute to a better understanding of the cholesterol response to weight loss as a result of individual gene profile, which is involved in blood fat metabolism. You will also receive access to your test results when they become available.

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Compensation

You will receive up to a maximum of $150 at completion of this study for your time and inconvenience of the study schedule

Alternatives

You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study. You should be aware that lipid lowering medications exist as an alternative to lowering blood cholesterol levels.

Confidentiality

Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Study samples will be stored in the freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 3 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Initials of Subject: _____

Confidentiality and safekeeping of DNA samples

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your DNA sample a specific code. This code will link you to your DNA sample and can only be decoded by the principal researcher or an individual authorized by the latter. Samples of your DNA will be kept at the RCFFN under the supervision of Dr. Peter Jones for a 3-year period following the end of the research project. After this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of this research project.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee or a representative from the RCFFN may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the

Initials of Subject: _____

investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204-474-9787
Study Physician	Dr. Edward Kesselman	Tel No.	204-954-4486

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Please circle your answers:

Sex:	M ☐	F ☐
Females: Are you pregnant?	Yes ☐	No ☐
Females: Do you agree to undergo a pregnancy test?	Yes ☐	No ☐
Do you understand the benefits and risks involved in taking part in this test?	Yes ☐	No ☐
Have you had an opportunity to ask questions and discuss testing procedures?	Yes ☐	No ☐
Do you understand that you can stop the DEXA testing at any time and that you do not have to say why?	Yes ☐	No ☐
Do you understand how your information is held confidential?	Yes ☐	No ☐
Do you agree to undergo DEXA scan?	Yes ☐	No ☐

Initials of Subject: _____

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐ No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained.

6. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

7. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Initials of Subject: _____

March 24, 2008

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS

Title of Study: **Effects of weight loss in overweight, hyperlipidemic individuals on cholesterol kinetics**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

NATURE AND DURATION OF PROCEDURE

From the blood drawn during the clinical study entitled "Effects of weight loss in overweight, hyperlipidemic individuals on cholesterol kinetics", we would like to extract DNA and perform genetic analyses using a laboratory technique that augments and recognizes specific genes to determine why some people decrease their cholesterol levels better than others as a result of weight loss. DNA is a molecule found in the cells of your body that is organized into genes that contain all of the information needed to make the proteins that perform specific biological functions in your body.

CONFIDENTIALITY AND SAFEKEEPING OF DNA SAMPLES

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your DNA sample a specific code. This code will link you to your DNA sample and can only be decoded by the principal researcher or an individual authorized by the latter. Samples of your DNA will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 2-year period following the end of the research project. After this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of this research project.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law

Initials of Subject: _____

permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee, or a representative from the Richardson Center for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. Project updates will be mailed at the end of the project. However, we will not communicate any individual results to you.

POTENTIAL RISKS AND/OR BENEFITS

As the DNA will be extracted from blood samples that have already been taken, there is no additional invasive procedure to undergo and no physical risk to you. While there may be no direct benefits to you for taking part in these additional analyses, we hope that these results will provide us with the information on genetic characteristics of people in which weight loss results in an enhanced or inferior cholesterol-lowering capacity.

SIGNATURE OF PARTICIPANT

The content of the procedure and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and procedures involved, and may continue in the future to ask further questions at any time, as it is my right to do so. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained and that my participation in this study is completely voluntary. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. Samples will not be utilized for any additional analyses, nor stored for any prolonged period, nor shared with any other group, other than is indicated in the protocol, without my specific consent.

I, _____, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me.

_____ _____

Signature of Subject Date

_____ _____

Signature of clinical coordinator Date

Initials of Subject: _____

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR TWO WEEKS EXTENTION

Title of Study: **Genetic basis for heterogeneity in response of plasma lipids to weight loss in overweight and obese individuals**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

The purpose of the study is to examine the genetic differences related to the plasma lipid response to a weight loss program including both dietary energy restriction and exercise.

Study procedures

During the 20 week trial, the participant is required to lose a minimum of 10 kg or 10% initial body weight. If you have not met your minimum weight loss goal of 10kg or 10% initial body weight in 20 weeks, we would like to give you an option to prolong the trial for 2 extra weeks in order to meet the targeted weight loss of 10kg.

If you agree to take part of this study, you will be invited to the research center for an additional 2 weeks in addition to your initial 20 weeks weight loss period. You will also need to ensure that you will not be consuming any of these medications/supplements during the study. You will be required to report the use of any new medications and/or natural health products. Subjects using medications including natural health products known to affect lipid metabolism during the trial will be asked to withdraw from the trial. In addition, you have to ensure that you do not smoke or consume large amounts of alcohol (>2drinks/day). You will also need to ensure that you do not currently have diabetes, thyroid, kidney, heart or liver disease or have had any of these diseases at any time during the past three months, in addition any development of diabetes mellitus, thyroid, kidney, heart or liver disease during the trial will lead to exclude from the trial.

Initials of Subject: _____

Additional research subject ICF for two weeks extension for study titled "Genetic basis for heterogeneity in response of plasma lipids to weight loss in overweight and obese individuals"

The study staff will provide you with guidelines of a custom-made weight loss program, including food habits and physical activity. You will meet with the dietitian and the personal trainer once a week for the extra 2 weeks. You will be given more assistance and guidelines on these extra visits. The goal of the program is to incur a steady moderate weight loss and the ensuing cholesterol lowering. On last two days of the study, you will be scheduled for a blood draw. Each blood test will take approximately 5 minutes. The total amount of blood drawn taken each time will be approximately 2 tablespoons. Two additional consecutive days of blood draws and one extra DEXA scan will be required at week 23 for lipids, sterols, hormones and genetics measurements. Therefore, the above mentioned blood draws and DEXA will be performed in both week 21 and 23.

Risks and Discomforts

As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. The x-ray dose associated with a total body scan using DEXA is very low and safe for repeated measurements. With the exception of pregnant women, there are no known risks associated with a DEXA scan. The potential risks associated with radiation exposure to an unborn fetus are not known, and therefore we ask that you undergo a pregnancy test to verify that you are not pregnant. Having a DEXA scan does not make it unsafe for you to have other x-rays taken in the near future. Also, considering the weight loss nature of the trial you might experience lightheadedness in relation to food restriction. In case you feel any discomfort during the experimental trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at (204) 954-4486.

Benefits

Although you may not benefit from participation in this research, weight loss in overweight individuals is linked to an improvement in health parameters. Moreover, the study should contribute to a better understanding of the cholesterol response to weight loss as a result of individual gene profile, which is involved in blood fat metabolism. You will also receive access to your test results when they become available.

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Compensation

There will be no compensation for the two week extensions.

Alternatives

You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study. You should be aware that lipid lowering medications exist as an alternative to lowering blood cholesterol levels.

Initials of Subject: _____

Confidentiality

Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Study samples will be stored in the freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 3 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care. Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions. We will tell you about new information that may affect your health, welfare, or willingness to stay in this study. Should you wish to withdraw your participation from the study, you **must inform** the study coordinators so that your file can be officially close.

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204-474-9787
Study Physician	Dr. Edward Kesselman	Tel No.	204-954-4486

Initials of Subject: _____

Additional research subject ICF for two weeks extension for study titled "Genetic basis for heterogeneity in response of plasma lipids to weight loss in overweight and obese individuals"

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐ No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.
2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.
3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.
4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.
5. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained.
6. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.
7. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Initials of Subject: _____

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

MAR 3 1 2008

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: CIHR

Protocol Reference Number: B2007:198
Date of REB Meeting: December 10, 2007
Date of Approval: March 25, 2008
Date of Expiry: December 10, 2008

Protocol Title: "The Effects of Weight Loss in Overweight, Hyperlipidemic individuals on Cholesterol Kinetics"

The following is/are approved for use:

- **Protocol dated November 24, 2007**
- **Research Participant Information and Consent Form, Version dated November 24, 2007**
- **Research Participant Information and Consent Form – Additional Research Subject Information and Consent form for Genetics Analysis Version 2 dated March 24, 2008**
- **Poster dated November 24, 2007**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letters dated March 11, 2008 and March 24, 2008. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

MAR 3 1 2008

Jennifer - Meridel keeps these on files in her office and copies to Tanya Mira

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: CIHR

Ethics Reference Number: B2007:198
Date of Approval: October 17, 2008

Protocol Title: **Genetic basis for heterogeneity in response of plasma lipids to weight loss in overweight and obese individuals" formerly known as "The Effects of Weight Loss in Overweight, Hyperlipidemic individuals on Cholesterol Kinetics**

The following is/are approved for use:

- **Amendment per letter dated October 10, 2008**
- **Research Participant Information and Consent Form (for extension) dated October 10, 2008**
- **Questionnaire Form Modified September 28, 2008**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee as per your facsimile dated October 10, 2008. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

N R Anthonisen

RECEIVED
OCT 2 1 2008

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax:** (204) 789-3414

3. PARTICIPANT SCREENING FORM

Weight Loss Study (2008-2009)

Subject screening form

DATE (yyyy/mm/dd) _____/_____/_____

Personal Information

Name:

Gender:

Age (18-70): DOB (yyyy/mm/dd):

Telephone: Home ( ) Work ( ) Cell ( )

Weight (Kg/lb): Height (m/ft.ic): BMI (Kg/m^2):
1kg = 2.2 lbs Height:
1 inch = 2.54 cm

Smoking? YES NO

Alcohol consumption > 2 glasses/ day? YES NO

Physical Activity? YES NO If yes please specify:

Medical History

Do you have any history of heart disease, diabetes mellitus, high blood pressure(is it controlled?), kidney disease, thyroid disease (is it controlled?) or liver disease?

Cancer? Yes/No Date treated:

Medications/Supplements

Prescription medication or natural health products? YES NO

Supplements or vitamins? YES NO

Weight loss aids or hunger suppressants? YES NO

If blood pressure thyroid medication used are doses stable? YES NO

If yes for how long?

Fish oil supplements (>4g/day) in the past 2 months?

Plant sterols or plant sterol supplemented foods in the past 6 weeks? YES NO

Cholesterol-lowering medications in the past 6 mo? YES NO

If yes, please specify:

Excluded drugs and natural supplements:

Cholestyramine ,Colestipol, Niacin, Gemfibrozil, Probucol, HMG-CoA reductase inhibitors, Guggul, Lecithin, Evening primrose oil, fish oil, plant sterols, ezetimibe, lipitor, Zocor, Lipentil.

Dietary Habits

Special dietary habits? YES NO

Food allergies? YES NO

If yes, please specify: _____

Available in the next 6 months?

Physical activity:

(add questions) – chest pain, w/ or without exercise

Joint pain?

Doctor prohibited with any type of exercise, describe?

Preliminary eligibility to participate in weight loss study: YES NO

If yes

Subject code:

Subject _____ IS / IS NOT eligible to participate in the weight loss study

Subject _____ will start on: Date (yyyy/mm/dd): _____/_____/_____

Time: ___:___

4. DIETARY GUIDELINES

Richardson Center for Functional Foods and Nutraceuticals

Weight loss study 2008-2010

Dietary plan

General guidelines

- Your diet is based on the exchange system which means you will not be counting calories and food weight but exchanges or servings of each food group.
- Read the attached food exchange list carefully. This list will guide you in your food choices throughout the study.
- Use your exchange list to determine the size and number of daily portions from each food group
- Most serving sizes are measured after cooking
- Read the nutritional information on product labels
- Do not forget your healthy morning and afternoon snacks. If you are a late sleeper, the afternoon snack can be consumed as a night snack instead.
- Choose low-fat, reduced fat, lean, sugar free varieties
- Favor boiling, steaming, grilling and baking over frying
- Meat should be weighed after cooking
- Use your fat exchange allowance for cooking and salad dressings
- Avoid foods that are considered "empty calories" such as candy, sugar-containing soft drinks and replace them by fruits, fruit juices

Food groups

1- Starches

- This food group includes breads, cereals and grains as well as starchy vegetables such as potatoes and corn
- Beans, peas and lentils are part of both the starch and the meat substitute list. One exchange of this group will contain 1 starch and half a meat exchange
- Replace white breads and grains with brown or whole grain options
- Starchy vegetables prepared with fat count as one starch and one fat
- Although the "other carbohydrates" list allows you to account for exchanges in deserts and fatty foods, these food choices should be avoided or consumed rarely during the weigh loss period

2- Meat and substitutes

- This food group includes white (chicken, fish) and red meats as well as eggs and cheese
- Do not consume red meat more than three times a week. Alternate with chicken and fish
- Limit choices from the high fat group to twice a week or less. When you do, remove one fat exchange from your diet
- Choose lean meat varieties, low in fat and cholesterol
- Choose chicken breasts over chicken thighs and wings
- Choose cheeses with less than 5g of fat per ounce such as feta, mozzarella, ricotta and other low fat varieties

3- Milk

- This food group contains milk and yogurt
- Choose low or skimmed varieties
- Choose fruit yogurts with no added sugar

4- Fruits

- This food contains fresh fruits, dried fruits, and fruit juices
- Choose fresh fruits over dried fruits and fruit juices
- Portion sizes for canned fruit exchanges account for the fruit and a small amount of juice

5- Fats and oils

- Use oil and Becel margarine instead of butter and animal fat
- When foods are consumed in restaurants, account for a minimum of one exchange of fat added during cooking

5. SAMPLE THREE DAY FOOD RECORD

Richardson Centre for Functional Food and Nutraceuticals
Weight Loss Study 2008-2010

Food Records

Subject ID: **Date:**

Week:

Meal	Time	Items	Portion	Preparation
Breakfast				
Snack				
Lunch				
Snack				
Dinner				
Snack				

RCFFN-Weight Loss Study 2008-2010 **Food Record Forms**

Subject ID: **Gr:** **Daily exchanges required-1600kcal**
Week: **Date:**

Food group	Number of exchanges	Total daily consumed
Starch	7	
Meat	4	
Milk	2	
Vegetables	6	
Fruits	3	
Fats	4	

Meal	Time	Items	Portion	Preparation	Exchanges
Breakfast					
Snack					
Lunch					
Snack					
Dinner					
Snack					

Melinda Mintarno
Phone: 474-8383 or 298 5483 (msg.)

July 18, 2008

6. FOOD GROUPS FOR EXCHANGES

The exchange lists

Starch

1 starch exchange equals 15g carbohydrates, 3g proteins and 1g fat

Food item	Serving/1exchange
Bread	1 slice (30g)
Pasta	1/2 cup
Rice	1/3 cup
Beans, peas and lentils	1/2 cup
Cereals	1/2 cup

Meat, poultry, eggs and cheese

1 meat exchange equals 7g proteins and up to 5g of fat

Food item	Serving/1 exchange
Beef, chicken, fish	30g/ 1 oz
Cheese	30g/ 1 oz/1 in cube
Soft cheese	1/4 cup
Eggs	1

Dairy

1 milk exchange equals 12g carbohydrates, 8g proteins and up to 5g fat

Food item	Serving/1 exchange
Milk	1 cup/250 ml/8 oz
Yogurt	3/4 cup

Vegetables

1 vegetable exchange equals 5g carbohydrates and 2g proteins

Food item	Serving/1 exchange
Fresh vegetables	1 cup/250 ml
Cooked vegetables	1/2 cup

The vegetable group is considered a "free" group.
Make sure you eat all the vegetable exchanges prescribed in the diet, add more if you like.

Fruits

1 fruit exchange equals 15g carbohydrates

Food item	Serving/1 exchange
Fresh fruit	1 whole
Fruit juice	1/2 cup

Fats and oils

1 fat exchange equals 5g of fat

Food item	Serving/1 exchange
Margarine	1 tsp
Oil	1 tsp
Butter	1 tsp
Low fat Salad dressing	1 tbsp
Regular Salad dressing	1 tsp

7. SAMPLE DIETARY REGIMEN

Daily exchanges required-1600 calories

Food group	Number of exchanges
Starch	7
Meat	4
Milk	2
Vegetables	6
Fruits	3
Fats	4

Note: meal exchanges should be spread throughout the day. It is not recommended to ingest the majority of food in less than 3 meals.

Sample dietary regimen

Breakfast
2 starch (ex: 1 cup of cereals)
1 milk (ex: 1 cup skimmed milk or yogurt)

Snack
1 fruit (ex: 1 apple or ½ cup of orange juice)
1 starch (ex: ¼ cup granola)

Lunch
2 starch (ex: 1 bagel)
2 meat (ex: 30g of low fat cheese and 30g of turkey ham)
2 vegetable (ex: 2 cups of fresh salad greens)
2 fat (2 tbsp of salad dressing)

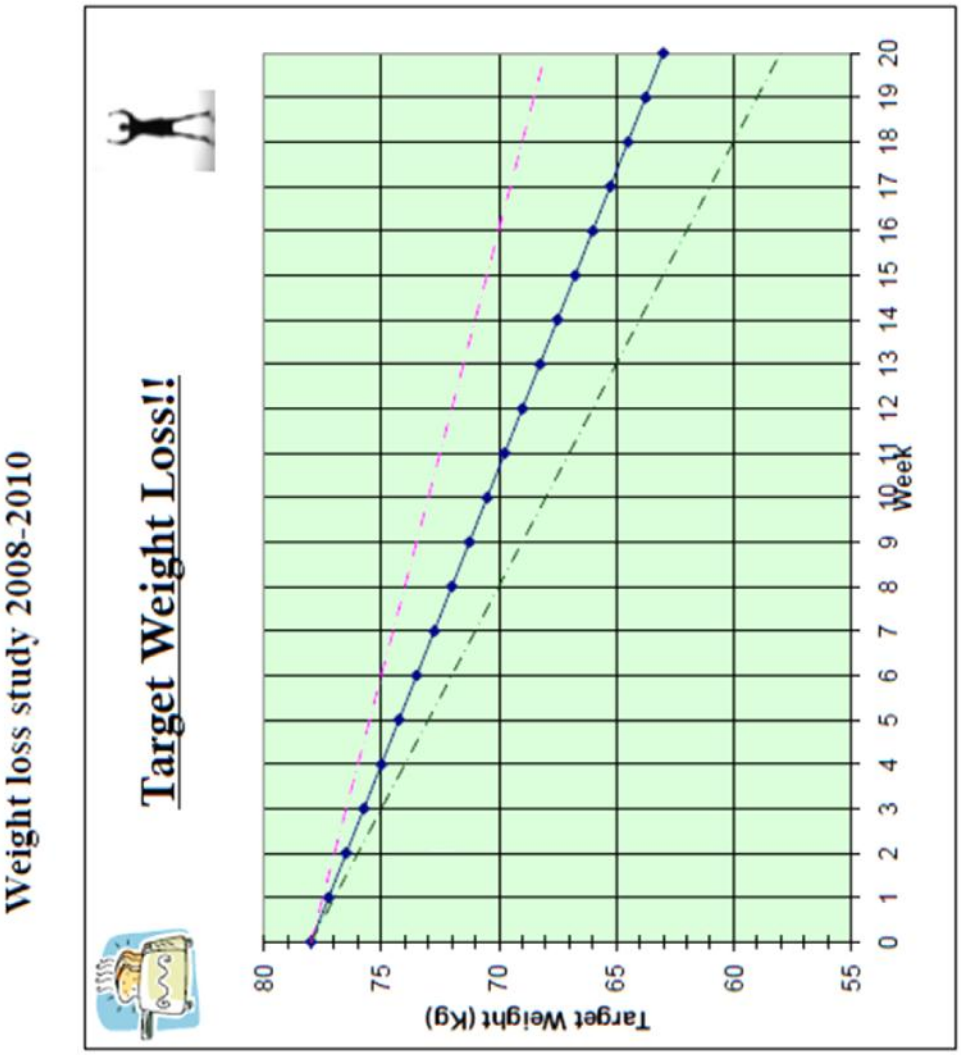
Snack
1 milk (ex: 1 cup of skimmed milk or low-fat yogurt)
1 fruit

Dinner
2 starch (ex: 2/3 cup of rice or 1 cup of pasta)
2 meat (ex: 60g of chicken breast with no skin)
2 fat (ex: 2 tsp of oil)
3 vegetable (ex: 1 cup of cooked vegetables +1 cup of salad)
1 fruit

1 cup = 250 ml 1 tsp =1 teaspoon 1 tbsp = 1 tablespoon

- Use sweeteners instead of sugar in your drinks.
- If only one tablespoon of milk is added to drinks such as coffee or tea, the added milk can be considered free. However, if more than ¼ cup of milk is added (example: Café latte), or if many coffees are consumed in a day, the amount of milk added should be counted within the milk exchanges.

8. SUBJECT WEIGHT LOSS GRAPH – ACTUAL AND TARGET



Subject ID:

weight day 1	78.1
(KG) day 2	77.9
average	78

Weight loss/week				
week	Target 0.75kg	Min 0.5 kg	Max 1kg	
0	78	78	78	
1	77.25	77.5	77	
2	76.5	77	76	
3	75.75	76.5	75	
4	75	76	74	
5	74.25	75.5	73	
6	73.5	75	72	
7	72.75	74.5	71	
8	72	74	70	
9	71.25	73.5	69	
10	70.5	73	68	
11	69.75	72.5	67	
12	69	72	66	
13	68.25	71.5	65	
14	67.5	71	64	
15	66.75	70.5	63	
16	66	70	62	
17	65.25	69.5	61	
18	64.5	69	60	
19	63.75	68.5	59	
20	63	68	58	

RCFFN - Aug 8. 08
Melinda Mintarno

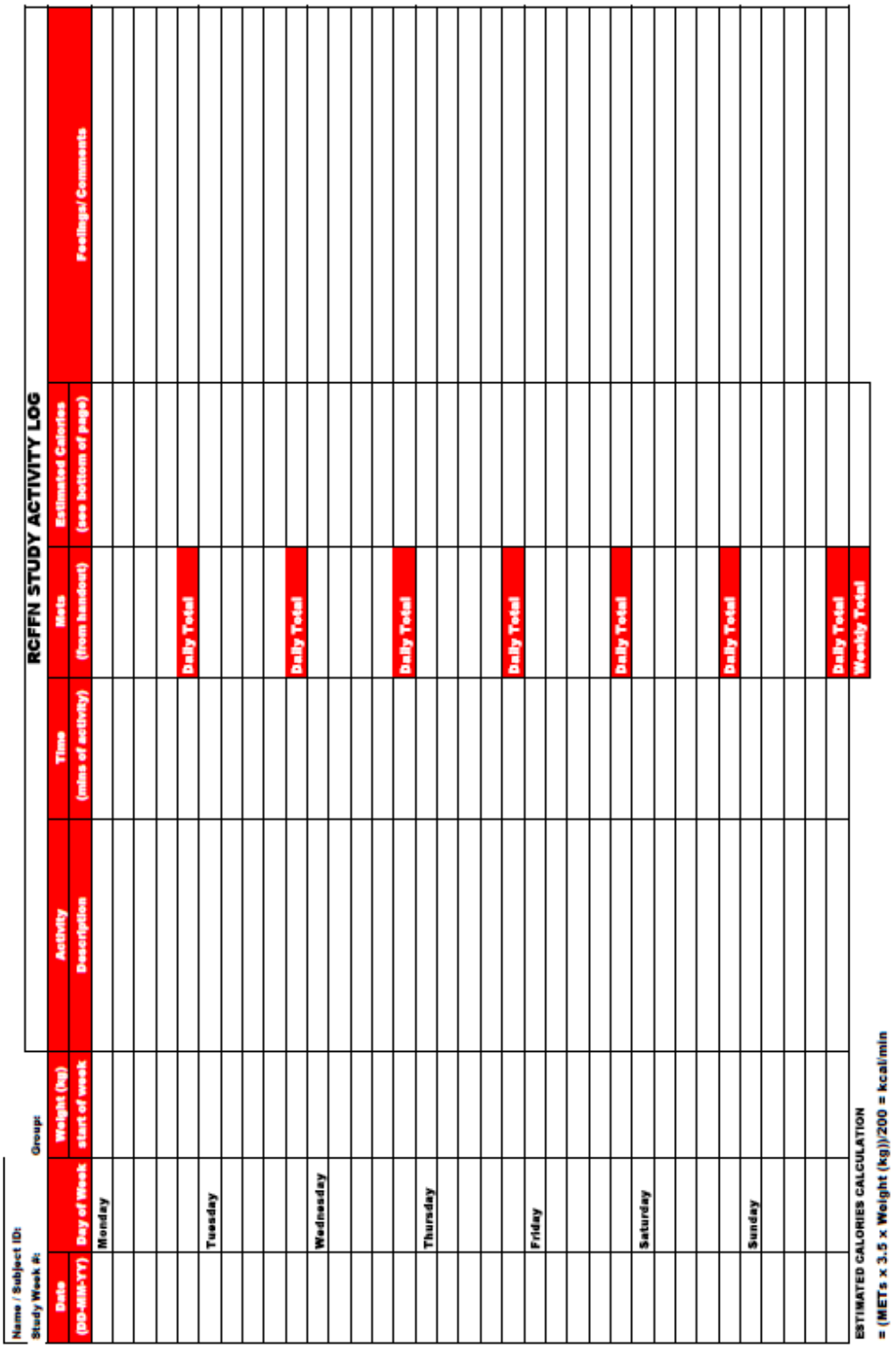
Weight loss study 2008-2010

Weight measurements

Subject ID:

Week	Date	Time	Weight (kg)
Week 0			
Week 0			
Week 1			
Week 2			
Week 3			
Week 4			
Week 5			
Week 6			
Week 7			
Week 8			
Week 9			
Week 10			
Week 11			
Week 12			
Week 13			
Week 14			
Week 15			
Week 16			
Week 17			
Week 18			
Week 19			
Week 20			
Week 20			

9. WEEKLY EXERCISE JOURNAL



Name / Subject ID:
Study Week #:
Group:

RCFFN STUDY ACTIVITY LOG

Date (DD-MM-YY)	Day of Week	Weight (kg) start of week	Activity Description	Time (mins of activity)	Mets (from handout)	Estimated Calories (see bottom of page)	Feelings/ Comments
	Monday						
					Daily Total		
	Tuesday						
					Daily Total		
	Wednesday						
					Daily Total		
	Thursday						
					Daily Total		
	Friday						
					Daily Total		
	Saturday						
					Daily Total		
	Sunday						
					Daily Total		
					Weekly Total		

ESTIMATED CALORIES CALCULATION
= ((METs x 3.5 x Weight (kg))/200 = kcal/min

10. STEROL DERIVATIZATION METHOD

Plant Sterols Derivatization Procedure **Melinda Mintarno**

1. Take out samples (n=24) from freezer; let it **thawed** to room temperature (~30min).

2. In the mean time, **Label** 24 GC vials accordingly and 24 rubber bulb small glass pipette

3. For samples in GC vials without insert: proceed to step#4
   For samples inside an insert: **rinse** insert with **100-200µl of hexane 5 times** and put in respective labelled vial by using disposable rubber-glass pipette.
   For Standards: add appropriate amount of sterols in respective vials

4. **Evaporate** samples till dryness under nitrogen using dry block heater at **55°C (10min)**.
   Make sure to rinse needles with chloroform and use clean needles. Open the main valve of N2 tank, check for flow, lower the metal tube and watch the liquid surface to gage.

5. Once dried, **rinse sides** with **0.5ml of hexane** and **evaporate** again (~10min). Ignore this step if initial sample volume is lower than 0.5ml.

6. Add derivatizing reagent **(TMS) 0.5ml** for each samples (1ml of TMS will derivatized up to 10mg of sample).

7. **Cap, vortex** and **incubate** in dry bath at **55°C for 30min** or longer (45min-1hr)

8. **Evaporate** TMS in dry block heater at **55°C** under nitrogen (45min-1hr). Watch out for white NH4Cl precipitation which will be evaporated; thus, be careful while using nitrogen bath.

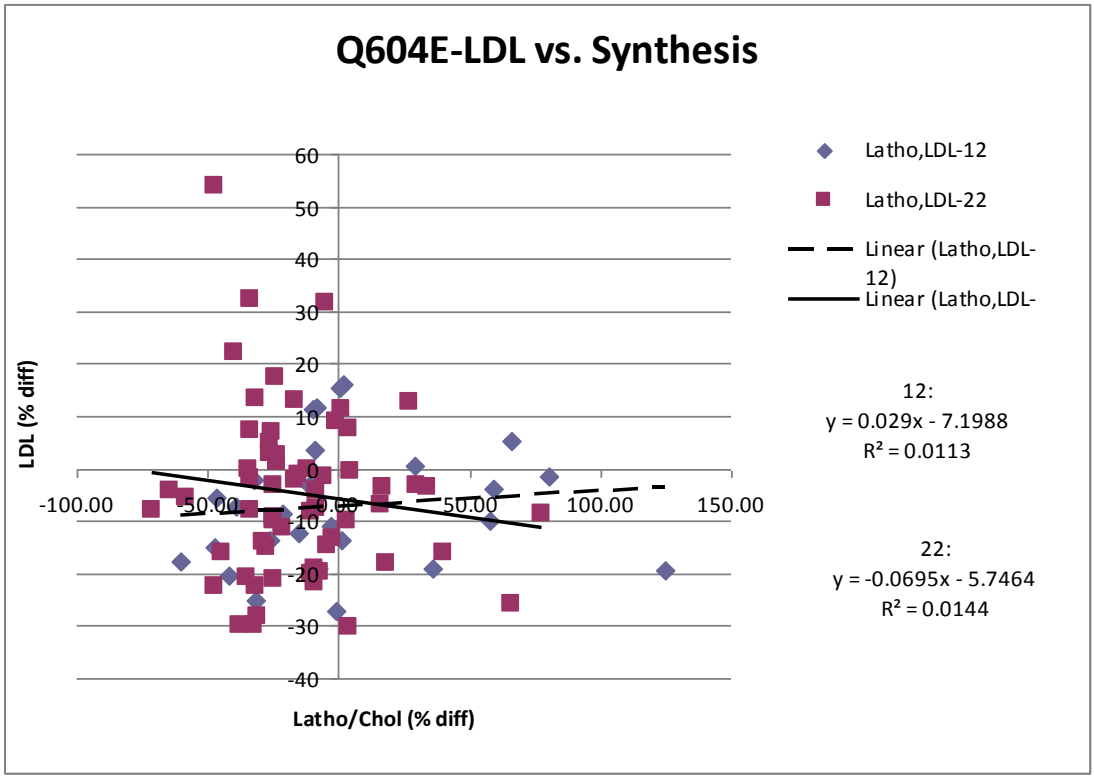
9. Once dried, **rinse sides** with **100µl** of **hexane HPLC** and **transfer** to labelled GC vials using micro pipette.

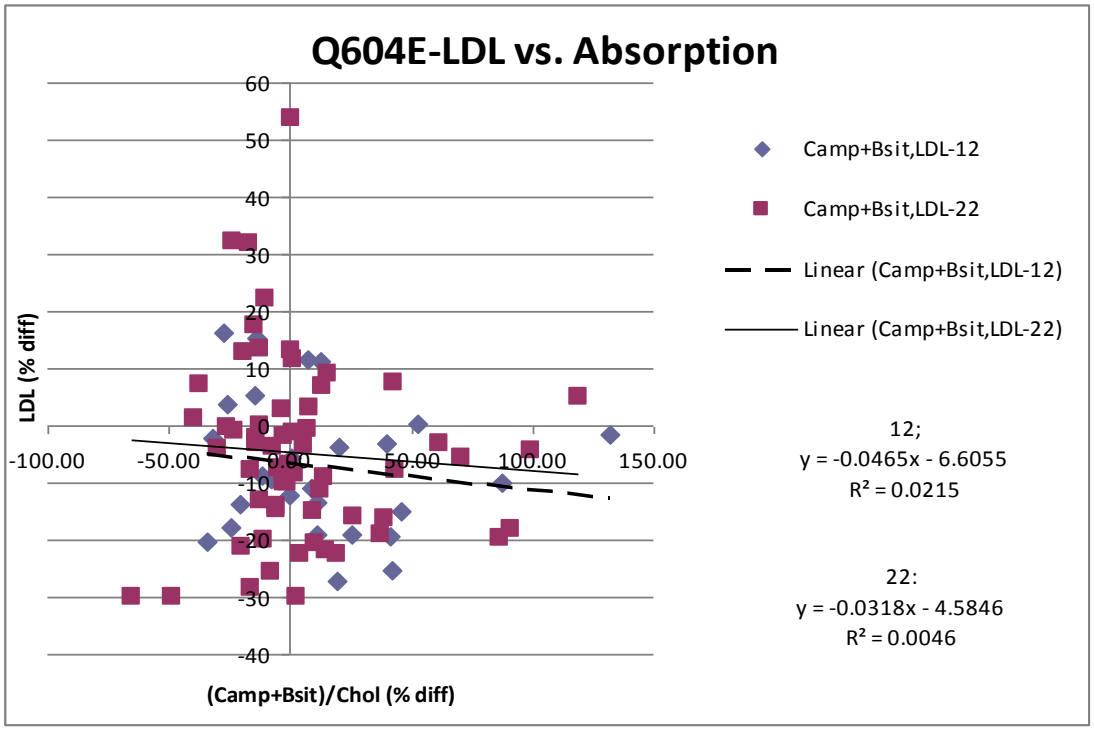
10. Repeat step 9 to make **final concentration of 200µl**.

11. Derivatized sample is ready to be injected to GC or store at 4°C until ready to be injected. Inject samples within 2-3days after being derivatized.

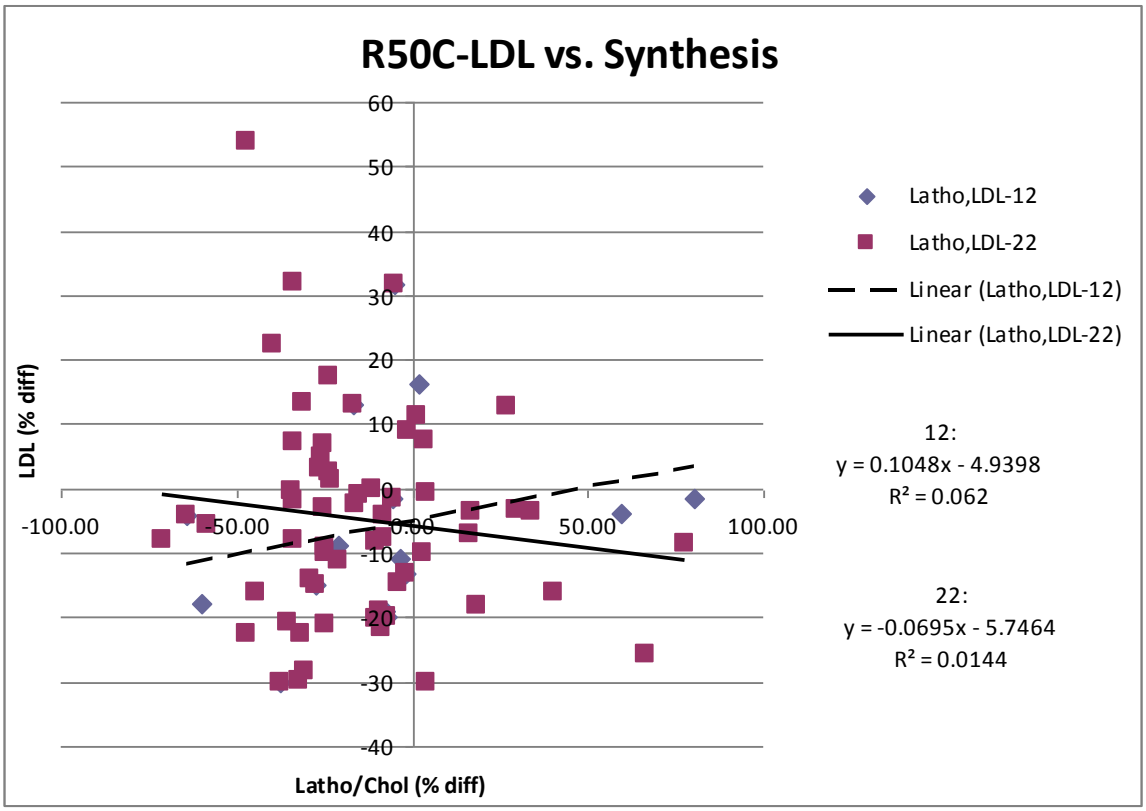
11. ADDITIONAL FIGURES

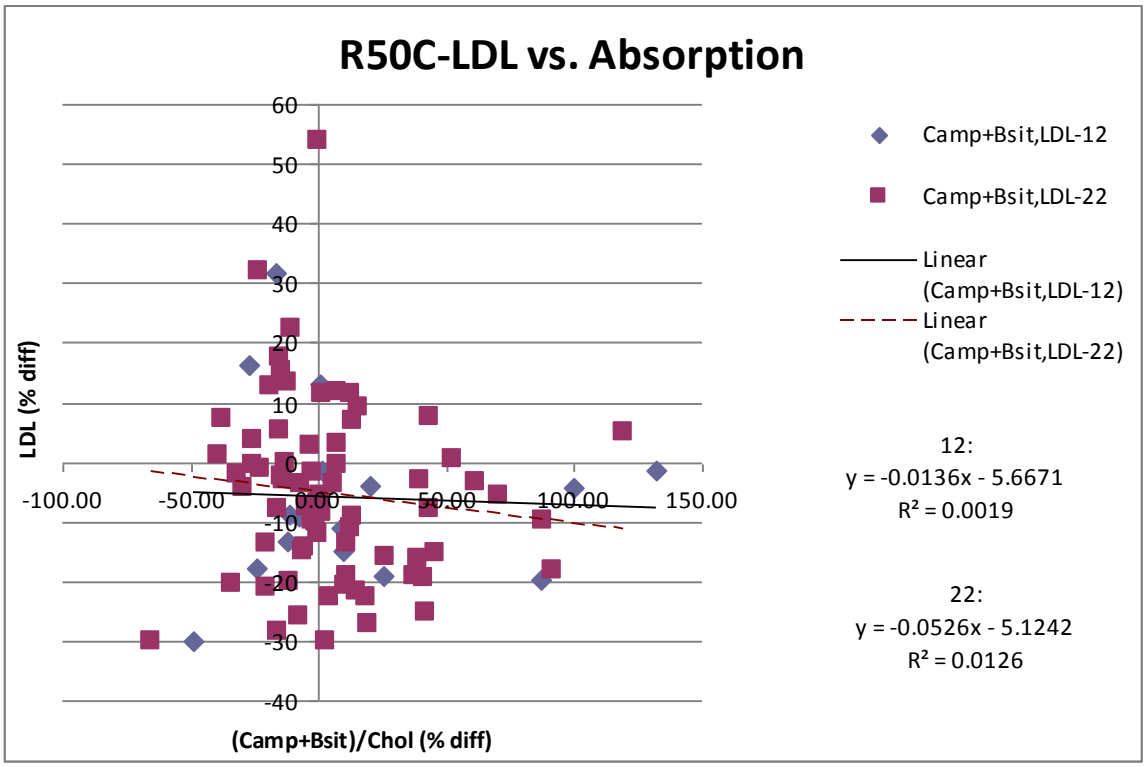
Appendix Figure 1. Relationships of % change in LDL-C and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG5 Q604E in the combined analysis (n = 89).



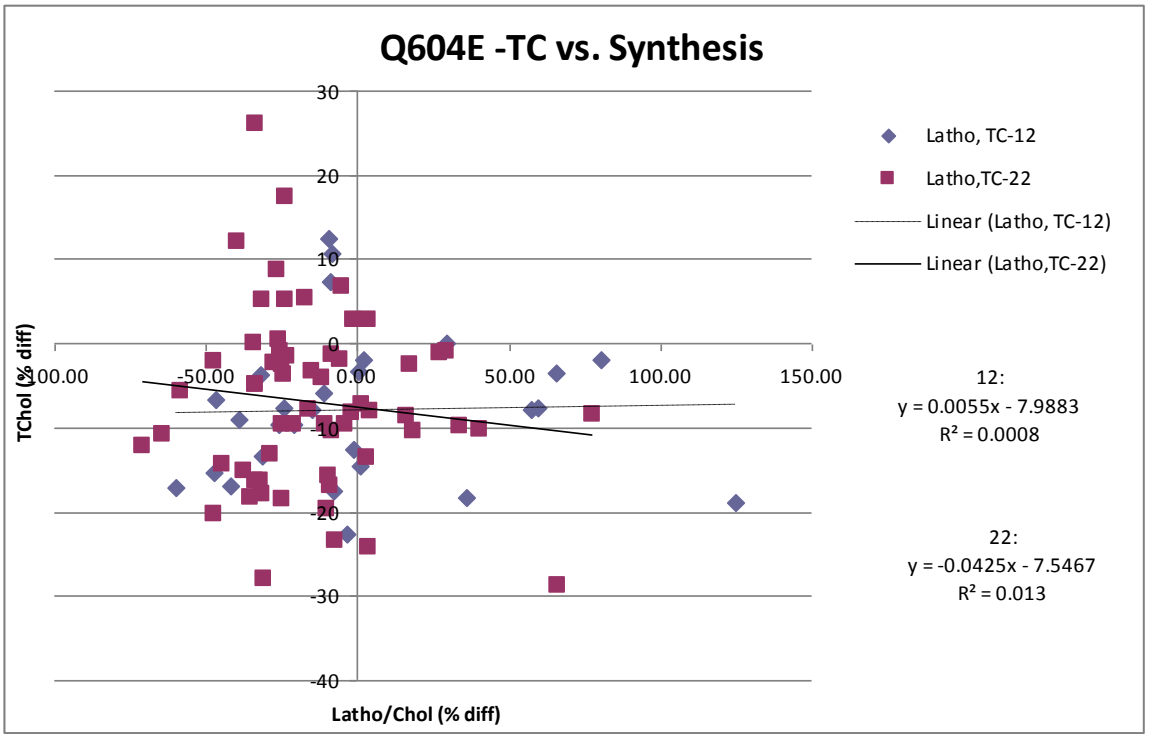


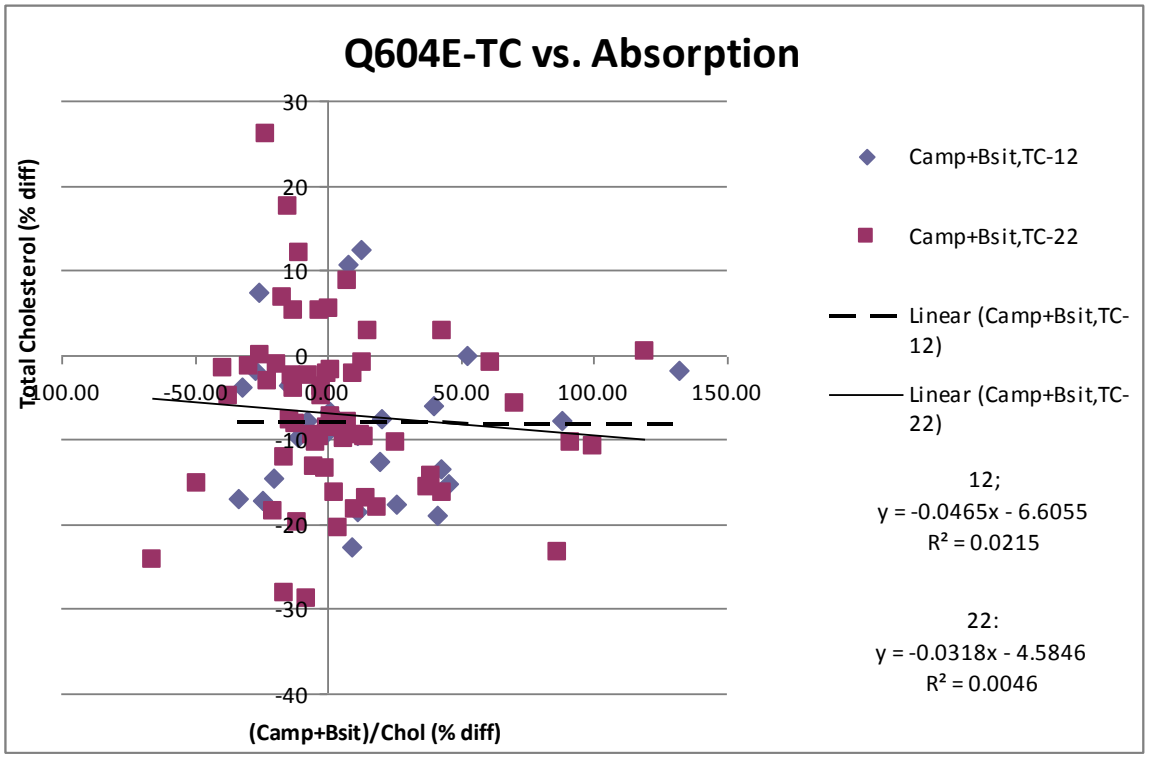
Appendix Figure 2. Relationships of % change in LDL-C and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG5 R50C in the combined analysis (n = 89).



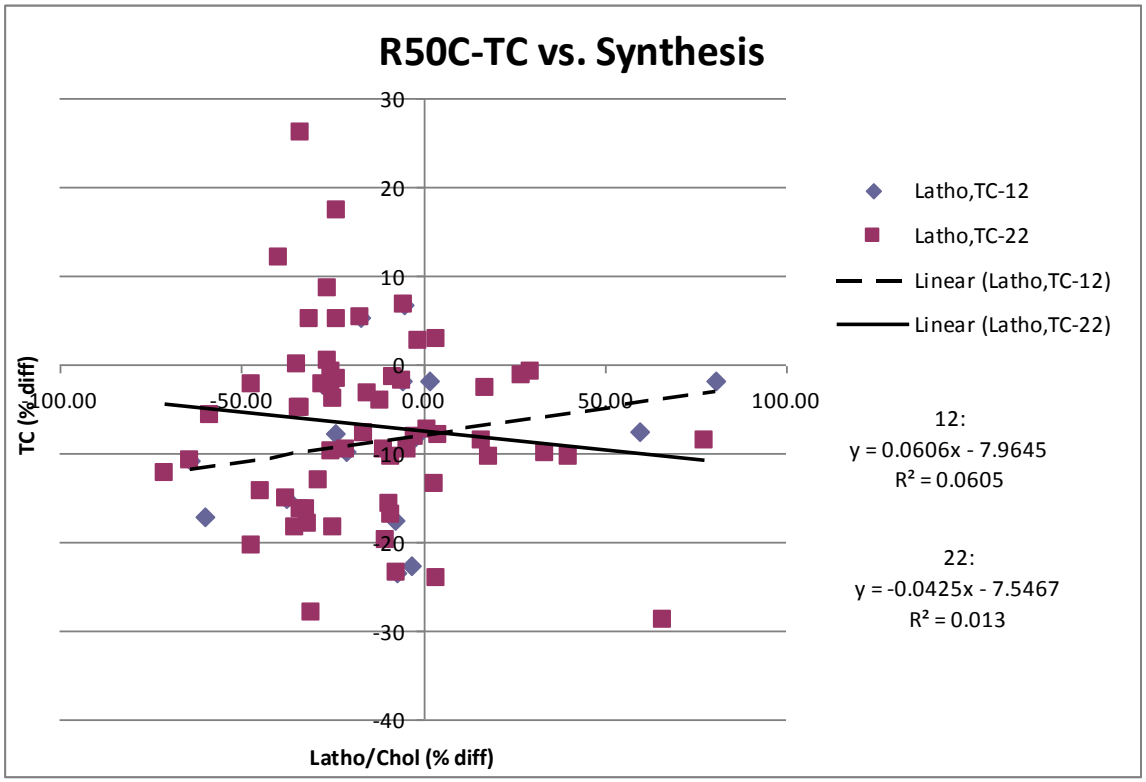


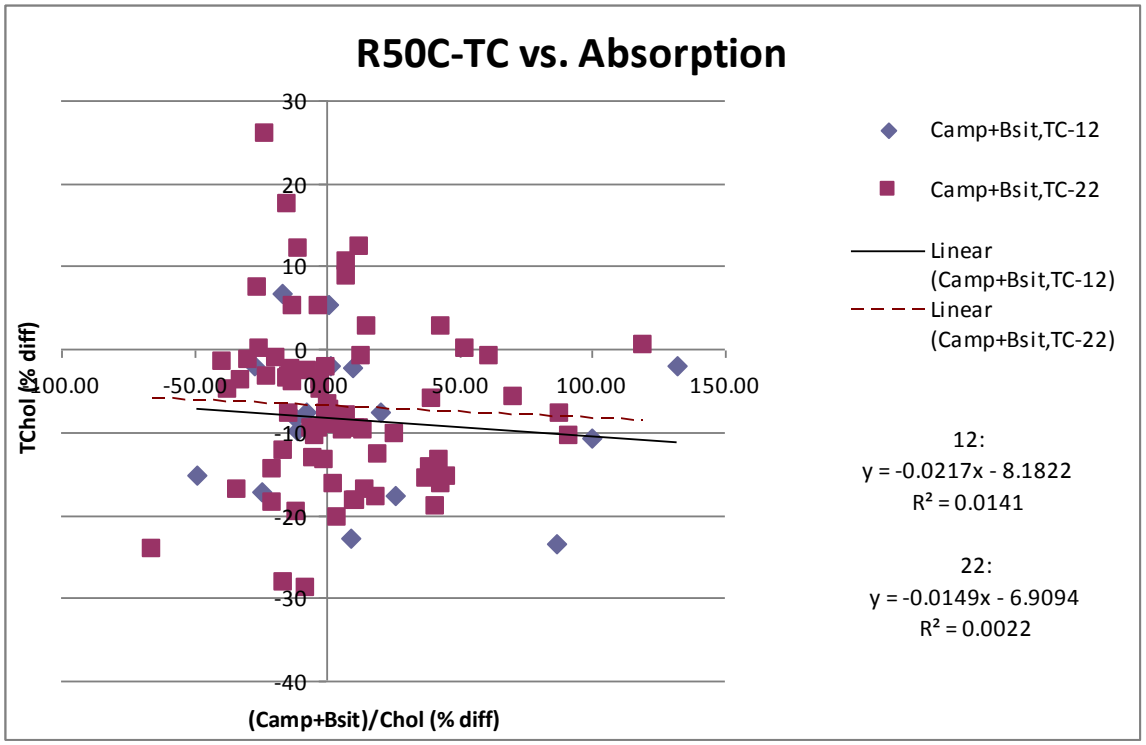
Appendix Figure 3. Relationships of % change in TC and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG5 Q604E in the combined analysis (n = 89).



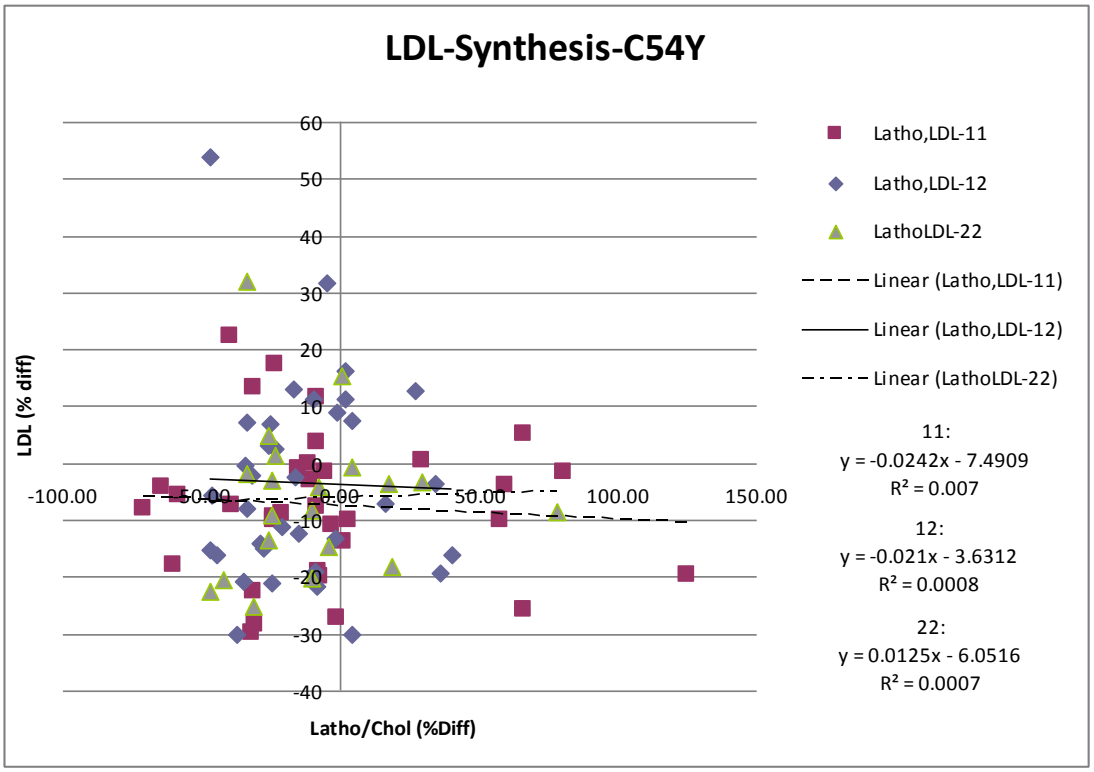


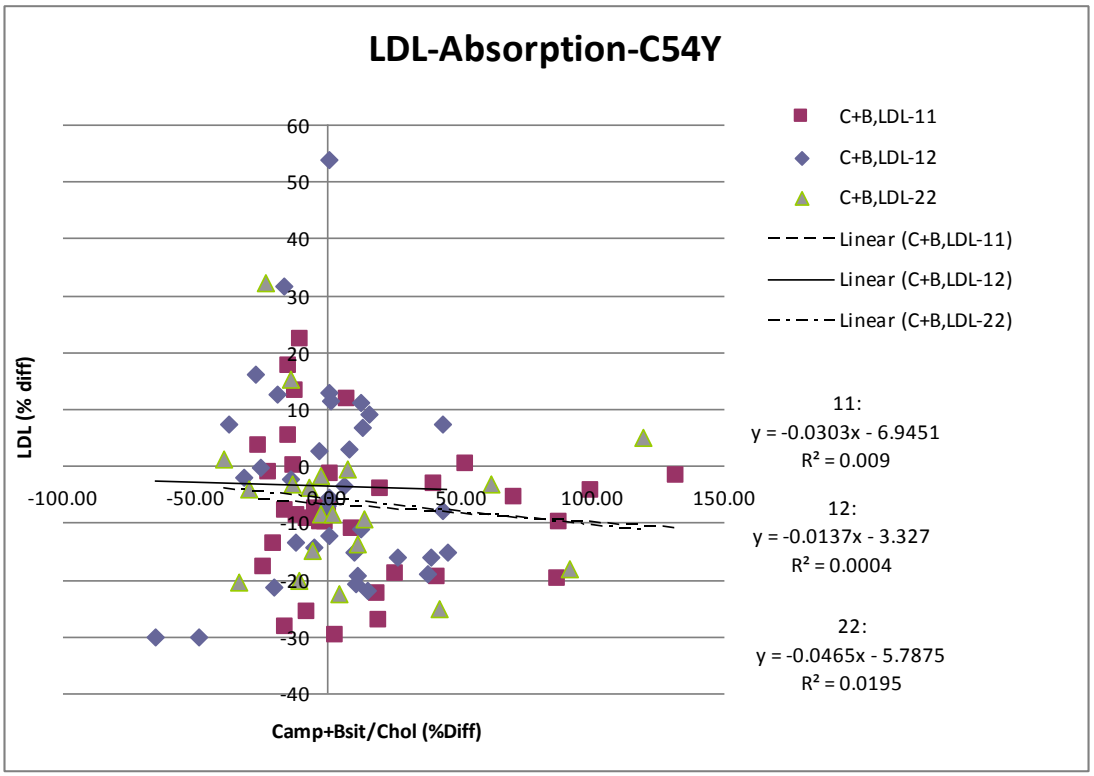
Appendix Figure 4. Relationships of % change in TC and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG5 R50C in the combined analysis (n = 89).



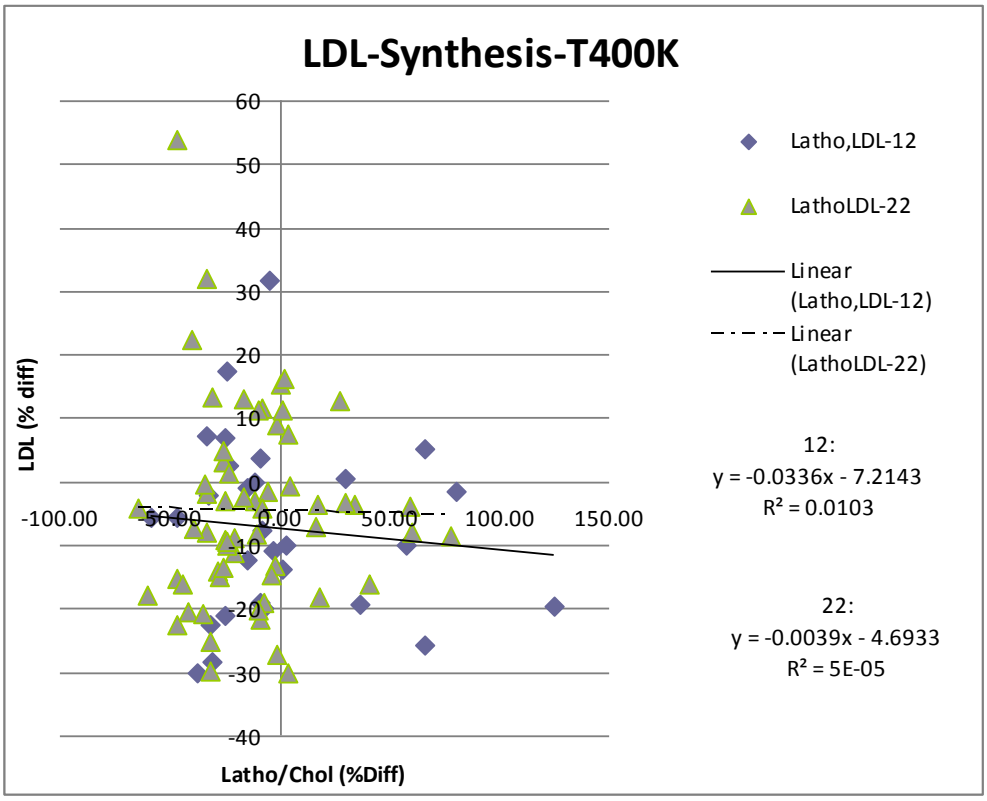


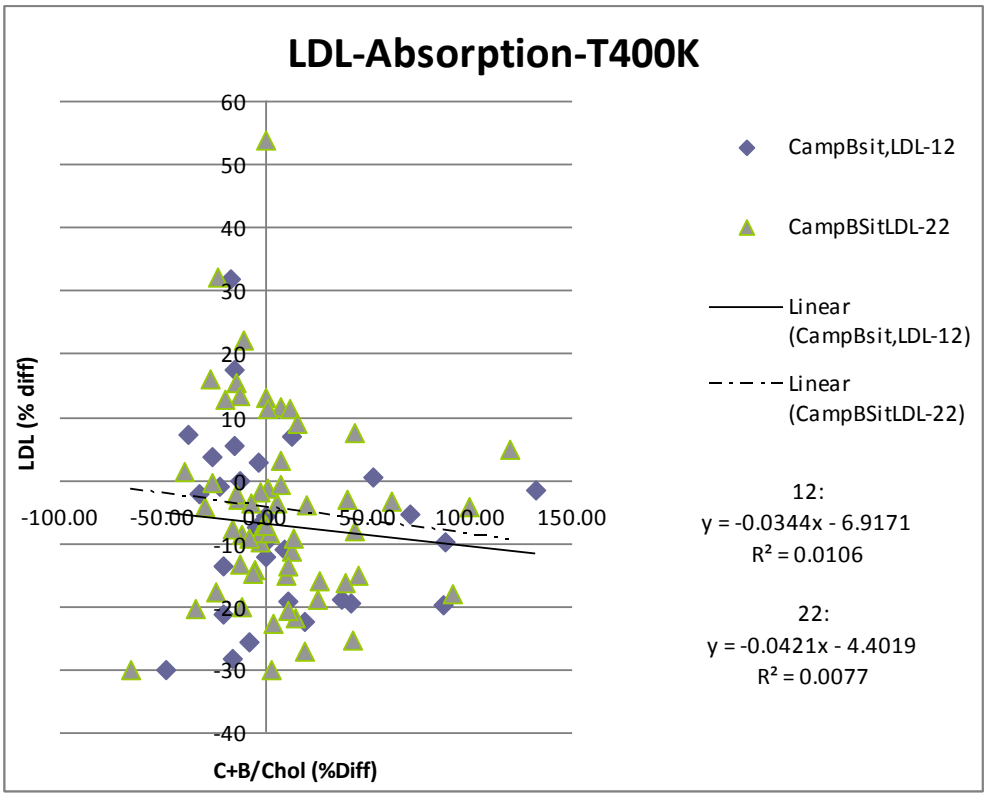
Appendix Figure 5. Relationships of % change in LDL-C and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 C54Y in the combined analysis (n = 89).



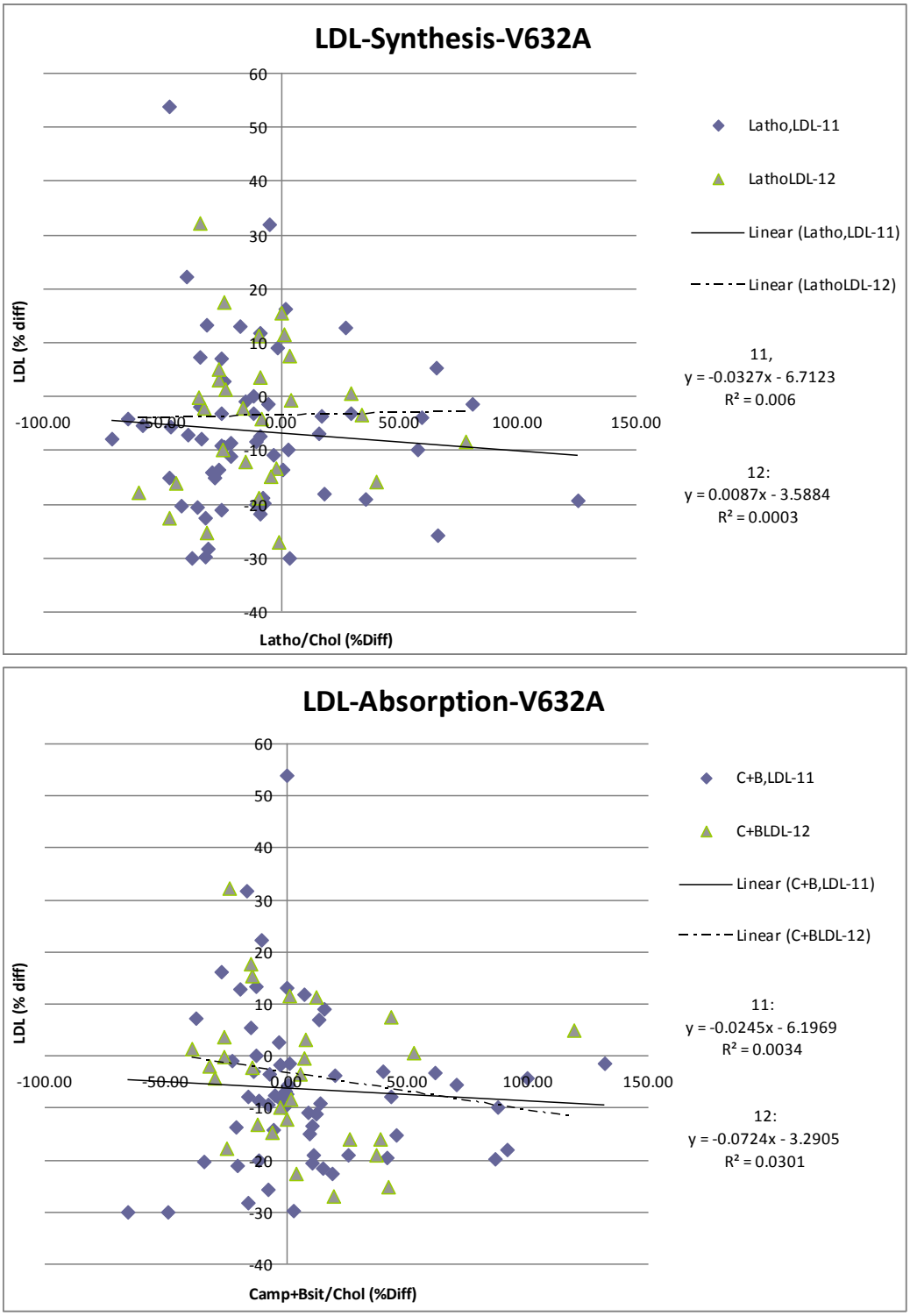


Appendix Figure 6. Relationships of % change in LDL-C and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 T400K in the combined analysis (n = 89).

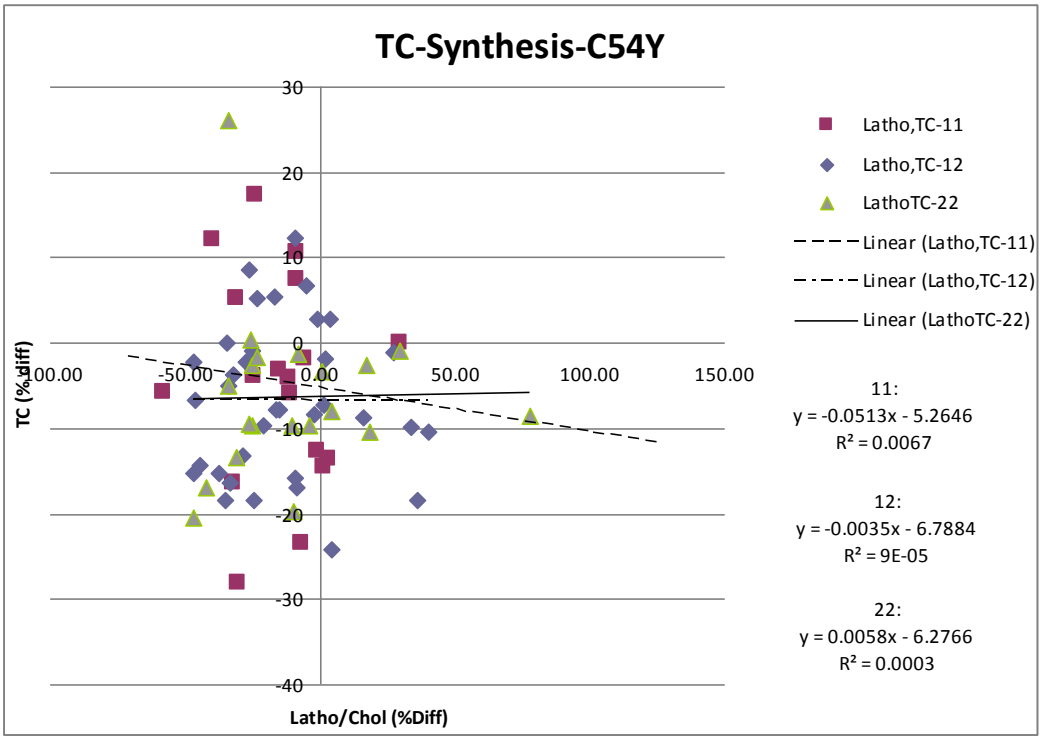


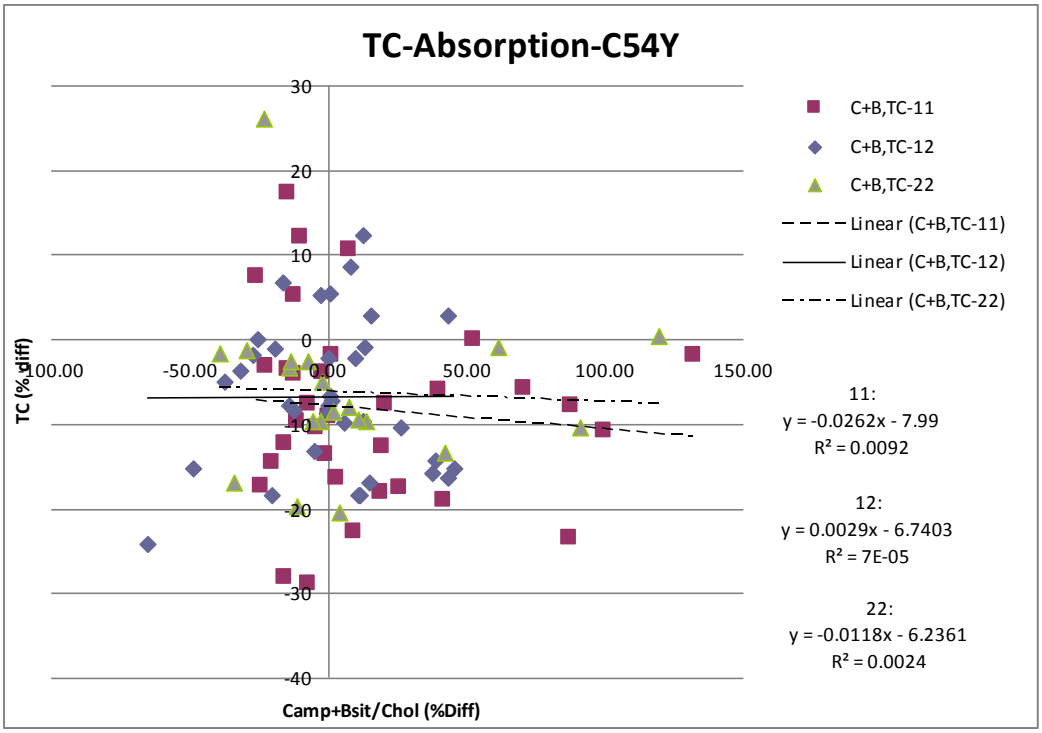


Appendix Figure 7. Relationships of % change in LDL-C and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 V632A in the combined analysis (n = 89).

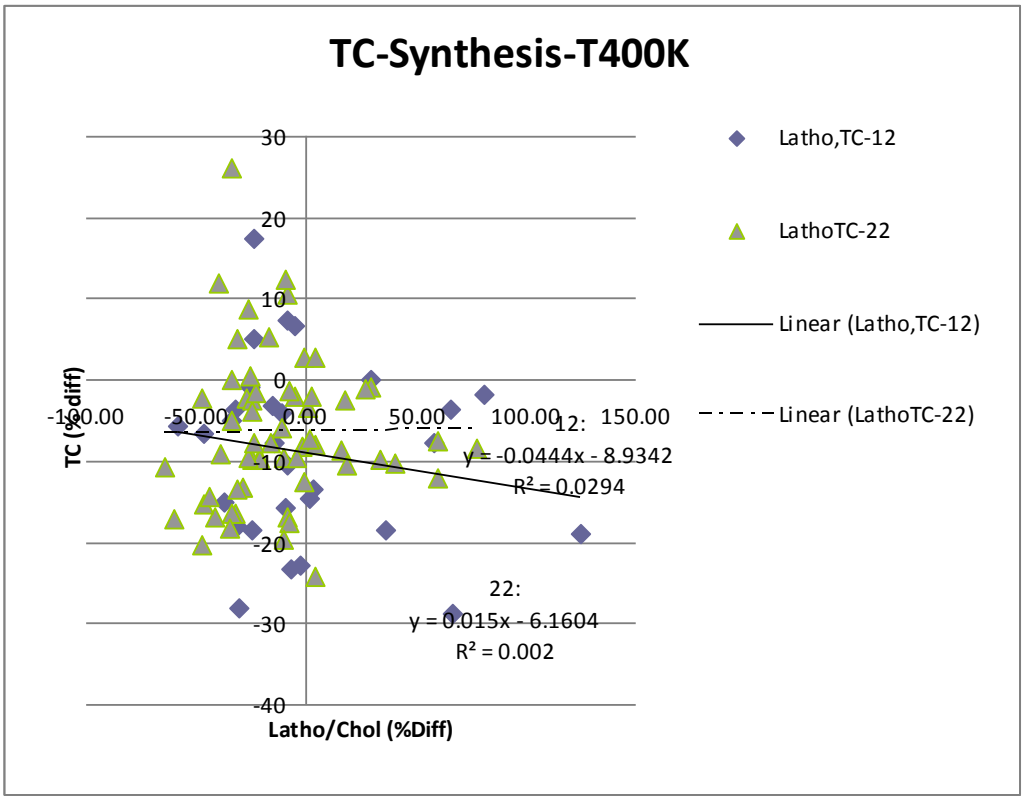


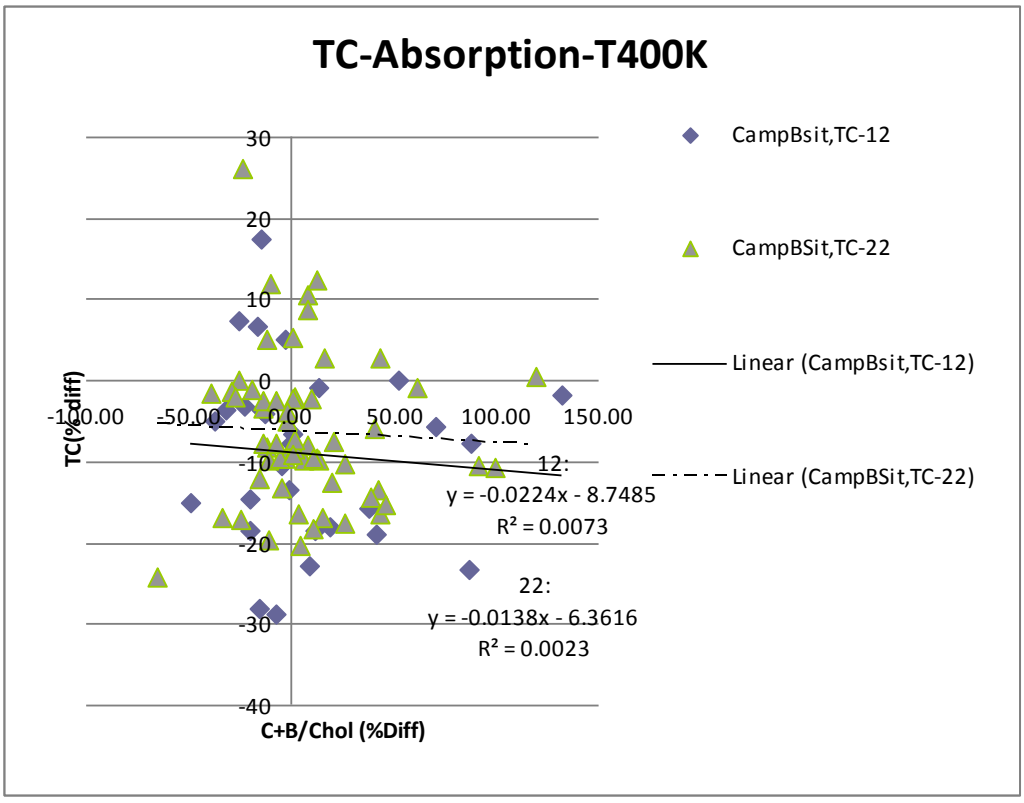
Appendix Figure 8. Relationships of % change in TC and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 C54Y in the combined analysis (n = 89).





Appendix Figure 9. Relationships of % change in TC and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 T400K in the combined analysis (n = 89).





Appendix Figure 10. Relationships of % change in TC and % change in surrogate markers of cholesterol synthesis and absorption stratified based on SNP in ABCG8 V632A in the combined analysis (n = 89).