Effect of consuming conventional and high oleic canola oil on cholesterol metabolism and bile acid synthesis in subjects at risk for metabolic syndrome

By

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Abstract

The prevalence of metabolic syndrome (MetS) estimated by the International Diabetes Federation is approximately a quarter of the world’s population. Current clinical research reveals that replacement of saturated fatty acid (SFA) with monounsaturated fatty acid (MUFA) helps to reduce the risk of MetS. The main objective of this thesis was to investigate the effect of consuming conventional and high oleic canola oil on cholesterol metabolism and bile acid synthesis in subjects at risk for metabolic syndrome. A secondary objective was to assess how cholesterol metabolism contributes to the regulation of plasma cholesterol levels after the consumption of canola oil. The Canola Oil Multicenter Intervention Trial II (COMIT II) was a randomized, double-blind, controlled feeding, crossover, clinical trial that consisted of three, 6-week feeding phases separated by ≥4-week washout periods. The clinical trial was conducted from 2014-2016 at the following institutes: the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg, the Institute of Nutrition and Functional Foods (INAF) at Laval University in Québec City, the Canadian Centre for Agri-Food Research in Health and Medicine (CCARM) at St. Boniface Hospital Albrechtsen Research Center (SBRC) in Winnipeg, and the Department of Nutritional Sciences at the Pennsylvania State University (PSU) in University Park, Pennsylvania. One hundred and seventy-four participants were recruited and met the criteria of the study. Finally, one hundred and twenty-five people completed the study. Statistical analyses were performed using the SAS mixed-model ANOVA for the endpoint intertreatment comparison. The endpoint cholestanol to cholesterol ratio, a surrogate marker of cholesterol absorption, was lower (P<0.05) in the two canola oil
treatments compared to the control oil treatment. The other two cholesterol absorption surrogate markers, campesterol and sitosterol, failed to show the same influence, likely due to the fact that the treatment canola oil was rich in phytosterols. According to the lathosterol ratio and fractional synthesis rate of free cholesterol (FSR-FC) data, the replacement of a Western diet oil (WDO) with canola oil had no influence on cholesterol synthesis. Moreover, the bile acid synthesis biomarker, 7α-hydroxy-4-cholesten-3-one (7αC4), in the MetS patients after consuming high oleic canola oil (HOCO), was higher (P<0.05) compared to those consuming the WDO. In conclusion, the consumption of HOCO has the potential to improve MetS patients’ lipid profile by reducing cholesterol absorption and promoting bile acid synthesis.
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To Cynthia Srigley, I appreciate your expertise in phytosterol analysis in the collaborative study. Thank Canola Oil Multicenter Intervention Trial II (COMIT II) program for funding the study. I appreciate the collaboration from the Institute of Nutrition and Functional Foods at Laval University in Québec City, the Canadian Center for Agri-Food Research at Health and Medicine at St. Boniface Hospital Albrechtsen Research Center in Winnipeg, and the Department of Nutritional Sciences at the Pennsylvania State University in University Park, Pennsylvania. Last but not least, I would like to thank my family for their support, love and sacrifice.
Foreword

This thesis is written to satisfy the graduation requirements of the master program at the Department of Food and Human Nutritional Sciences at the University of Manitoba. The study “Effect of consuming conventional and high oleic canola oil on cholesterol metabolism and bile acid synthesis in subjects at risk for metabolic syndrome” is funded by Canola Oil Multicenter Intervention Trial II (COMIT II) program. This thesis is written in manuscript format and it comprises one literature review, two individual manuscripts and one general discussion. Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a Western fatty acid profile in adults with central adiposity has been published in The Journal of Nutrition. As the coauthor, I introduced the COMIT II plasma lipid profile data from this published article in my thesis. My duty in the COMIT II study was to analyse how a diet containing conventional and high oleic canola oil influenced the cholesterol metabolism in subjects at risk for metabolic syndrome.
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## Abbreviations

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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ACAT2</td>
<td>Acyl-CoA:Cholesterol Acyltransferase 2</td>
</tr>
<tr>
<td>ALA</td>
<td>α-Linolenic Acid</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
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<tr>
<td>7αC4</td>
<td>7α-Hydroxy-4-Cholesten-3-One</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl Ester Transporting Protein</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>COMIT</td>
<td>Canola Oil Multicenter Intervention Trial</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Cytochrome P450 7A1</td>
</tr>
<tr>
<td>FSR-FC</td>
<td>Fractional Synthesis Rate of Free Cholesterol</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas Chromatography with Flame Ionization Detector</td>
</tr>
<tr>
<td>GC-IRMS</td>
<td>Gas Chromatography with Isotope-Ratio Mass Spectrometry</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High-Density Lipoprotein Cholesterol</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-Methylglutaryl-CoA</td>
</tr>
<tr>
<td>HOCO</td>
<td>High Oleic Canola Oil</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic Acid</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low-Density Lipoprotein Cholesterol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated Fatty Acid</td>
</tr>
<tr>
<td>NCSs</td>
<td>Non-Cholesterol Sterols</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1 like 1</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic Acid</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>RCO</td>
<td>Regular Canola Oil</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Clinical Trials</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated Fatty Acid</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TC</td>
<td>Total Cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>Ultra Performance Liquid Chromatography with Tandem Mass Spectrometer</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>Very Low-Density Lipoproteins Cholesterol</td>
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<tr>
<td>WDO</td>
<td>Western Diet Oil</td>
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Chapter I

Literature review

1.1 Introduction

Metabolic syndrome (MetS) is a group of health problems that increases the risk of developing cardiovascular diseases (CVD) and diabetes. The prevalence of MetS in Canada is 19.1%, and 34.3% for the American population (Riediger & Clara, 2011). In the first decade of the 21st century, MetS became a global issue due to the rapidly increasing MetS population worldwide (Saklayen, 2018). Patients with MetS have a 2- to 5-fold increase in the risk of developing CVD, type 2 diabetes mellitus (T2DM), and stroke (Alberti et al., 2009). MetS is diagnosed if three or more of the MetS criteria are met. The criteria for MetS include abdominal obesity (waist circumference > 102 cm for males or > 88 cm for females), elevated plasma triglyceride (TG) level (≥ 1.7 mmol/L), decreased high-density lipoprotein cholesterol (HDL-C) level (< 1.03 mmol/L for men and < 1.30 mmol/L for women), elevated blood pressure (BP) (≥ 130/85 mm Hg), or elevated fasting glucose level (≥ 6.1 mmol/L) (Riediger & Clara, 2011).

Therapeutic approaches for MetS include lifestyle modification, pharmaceutical therapy, and surgery. For instance, statins and ezetimibe are the medications commonly used for the treatment of dyslipidemia, which subsequently helps to improve MetS condition. The lifestyle modification method involves adopting a healthy diet and increased physical activity (Cornier et al., 2008). According to Canada’s Food Guide (2019), a healthy diet includes consuming vegetables, fruit, whole grains, and protein foods regularly, replacing foods that contain mostly saturated fat with foods that contain...
mostly unsaturated fat, choosing water as the beverage to support health and promote hydration. Fat is one of the important macronutrients in the human diet and the quality of fat refers to the composition of fatty acids in the edible oil. Monounsaturated fatty acid (MUFA) has been widely recommended due to its potential to reduce CVD risks, such as the reduction in plasma low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) levels (Baxheinrich et al., 2012; Harland, 2009; Lopez-Huertas, 2010). Canola oil and olive oil are good sources of dietary MUFA (Harland, 2009), which makes them a healthier alternative to high-saturated fatty acid (SFA) oil sources in the Western diet (Senanayake et al., 2014).

In 1979, a new rapeseed variety, named “canola”, was developed in Canada to be low in erucic acid and glucosinolates (Mag, 1983). Similar to olive oil, canola oil is high in MUFA, but canola oil has enhanced functional properties, particularly in relation to its use as a cooking oil. The better thermal stability and oxidative stability of canola oil allows it to be heated to a higher temperature during cooking and also have a longer shelf life (Duarte et al., 2018; Santos et al., 2002). Unlike olive oil, canola oil contains minimal flavour, potentially satisfying those who do not like the fruity or grassy taste of olive oil (Lin et al., 2013). Currently, canola ranks as the third-largest edible oil crop in the world, playing an important role in the Canadian economy (Smyth & Phillips, 2016). A study released by the Canola Council of Canada (2017) shows that Canadian-grown canola contributes $26.7 billion to the Canadian economy every year. To extend the market of canola oil globally, the focus shifted to canola oil’s nutrient value and potential health benefits. Canola oil contains 7% SFA, abundant MUFA and polyunsaturated fatty acid (PUFA), including 61% oleic acid (OA), 21% linoleic acid (LA), and 11% α-linolenic
acid (ALA) (Lin et al., 2013). Compared to conventional canola oil, high-oleic canola oil (HOCO) is higher in OA (70%) with similar SFA content (7%). The elevated OA percentage in HOCO gives its better thermal stability and longer shelf life compared to the conventional canola oil (Goulson & Warthesen, 1999). Moreover, the consumption of regular canola oil or HOCO has potential to increase plasma fatty acid percentage of MUFA (Raatz et al., 2018), which subsequently down-regulates the plasma LDL-C levels (Dietschy et al., 1993). Additionally, canola oil has been found to be a rich source of phytosterols, including campesterol (1.84 mg/g), β-sitosterol (3.60 mg/g), and brassicasterol (0.49 mg/g) (Vlahakis & Hazebroek, 2000). Numerous clinical studies have provided evidence that the intake of approximately 2g of phytosterols per day is closely associated with reductions in plasma LDL-C of 8-10% (Cabral & Klein, 2017).

Recent clinical trials have also provided evidence for health benefits related to the consumption of canola oil. Ten clinical studies, including two randomized clinical trials (RCT) and eight intervention studies, were reviewed to assess whether the intake of MUFA in rapeseed/canola oil improved plasma lipid profiles (Harland, 2009). Eight of the 10 studies yielded significant reductions in both plasma LDL-C and TC when dietary SFA was substituted for MUFA. Baxheinrich (2012) also found that a high intake of rapeseed/canola oil enriched with ALA reduced plasma LDL-C and TC, as well as body weight in MetS patients. However, the diets in the two intervention groups were both low in energy density, which meant that the treatment oil was not the only variable factor in this study. A previous study, known as the Canola Oil Multicenter Intervention Trial (COMIT) program, was a crossover RCT aimed to investigate the relative effects of five different oil blends on plasma lipid, lipoprotein, and apolipoprotein concentrations in
patients with MetS (Jones et al., 2014). The five treatment oils were conventional canola oil (n-9 rich), HOCO with docosahexaenoic acid (DHA) (n-9 and n-3 rich), a blend of corn and safflower oils (n-6 rich), a blend of flax and safflower oils (n-6 and short-chain n-3 rich) and HOCO (extremely n-9 rich). Canola oil showed its potential to decrease plasma lipid levels in the study, but the lack of a Western dietary control treatment became the main limitation of the study. The studies described above provided evidence that the intake of MUFA (or Canola oil) helped to reduce plasma LDL-C and TC. However, the mechanistic role of the MUFA dietary intervention in modulating cholesterol metabolism was not fully understood.

1.2 Canola oil and MUFA

Because of its potential to prevent the development of CVD, diabetes, obesity, cancers, asthma, depression, and cognitive decline, the Mediterranean diet has been well-studied (Sofi et al., 2008). The typical Mediterranean diet includes MUFA from olive oil, fruits and vegetables, whole-grain foods high in fibre, nuts and legumes, fish, and moderate consumption of alcohol (Widmer et al., 2015). The prevalence of MetS in the U.S. is approximately two times higher than that in Mediterranean countries (Gillingham, et al., 2011). Previous research has shown strong associations between the Mediterranean diet and MetS risk (Estruch et al., 2004; Kesse-Guyot et al., 2013). For instance, Estruch (2004) performed an RCT to compare the effect of the Mediterranean diet versus a low-fat diet on CVD risk. The authors concluded that the Mediterranean diet decreased plasma glucose levels (-0.39 mmol/L), systolic BP (-5.9 mm Hg), and TC/HDL-C ratio (-0.38) after the three-month intervention, with more significant changes in the risk factors compared with the low-fat diet.
As one of the important components in the Mediterranean diet, the potential health effects of MUFA have been subjected to study via clinical intervention trials. Canola oil is one of the most commonly consumed edible oil sources of MUFA, and its consumption has been recommended as a good replacement of SFA in the Western diet (Senanayake et al., 2014). The U.S. Food and Drug Administration, in 2006, also authorized a qualified health claim that an intake of approximately 1.5 tablespoons (19 grams) of canola oil, which is to replace the same amount of SFA with equivalent calories, may reduce the risk of coronary heart disease (Food and Drug Administration, 2009). Even though the health effects of canola oil consumption on CVD risk factors have been identified through various studies, the association between the intake of canola oil and the changes in each risk factor for individuals with MetS are still not clear.

1.3 Canola oil and MetS risk factors

As mentioned above, MetS is characterized by elevations in fasting serum levels of glucose, TG, TC, and LDL-C; hypertension; a reduction in serum HDL-C levels; and the presence of abdominal obesity (Riediger & Clara, 2011). Although numerous clinical studies have examined the health effects of canola oil consumption in MetS patients, the influence on individual risk factors varies. Currently, most of the previous studies have used high SFA oil as the control oil to evaluate whether the intake of canola oil impacts circulating serum cholesterol levels. This approach has been used due to evidence linking a high-SFA diet to elevations in plasma TC and LDL-C levels, which then increase the risk of developing CVD (Gebauer et al., 2006).

Hodson and others (2001) conducted an RCT to examine the effect of substituting a SFA-rich (17.7% energy) oil with canola oil in the daily diet on plasma lipid profile. In
the canola intervention group, the OA levels in the plasma fatty acid pool increased significantly from 37.3% to 39.5%. The plasma levels of TC, LDL-C, and HDL-C decreased by 12%, 15%, and 4%, respectively, during the substitution of the canola oil (Hodson et al., 2001). Matheson and others (1996) also carried out a crossover RCT to examine how plasma lipids were influenced by the intervention of canola margarine and canola oil. Compared to the baseline control (high SFA margarine) period, the plasma TC and LDL-C dropped by 7.0% and 10.0%, respectively, after the intervention of canola margarine (Matheson et al., 1996). Noakes and Clifton (1998) conducted a similar RCT as well, but changed one treatment to a margarine blended with PUFA. These authors also found that the substitution of SFA with MUFA in margarine resulted in a reduction in plasma TC (6.6%) and LDL-C (7.8%). There were no significant changes in either plasma TC or LDL-C between the interventions of the margarine with MUFA and the margarine with PUFA (Noakes & Clifton, 1998).

In a more recent study, thirty-seven men with MetS were randomized into two groups: one consuming an original butter spread, and the other consuming canola oil. The participants’ serum lipids, oxidized LDL and arterial elasticity were examined at the end of each phase. The researchers found that the replacement of butter with canola oil reduced circulating serum TC, LDL-C and oxidized LDL, reflective of changes associated with a lower risk of CVD development (Palomäki et al., 2010). Based on the above mentioned studies, evidence supports the position that the replacement of SFA with MUFA in the diet has the potential to improve plasma TC and LDL-C profiles while maintaining HDL-C levels.
With respect to fat composition, TG is the main component of fat existing in both edible oil sources and plasma lipids. Excessive intakes of both fat and carbohydrates contribute to increases in circulating TG levels in serum. Hence, strategies to reduce circulating TG levels includes decreasing the total fat and energy intake or replacing the SFA with MUFA or PUFA in the diet (Lin et al., 2013). Many of the previous studies were unable to show a statistically significant shift in circulating TG levels between the canola oil diet and the high-SFA diet (Iggman et al., 2011). As such, the evidence to support a claim of TG reduction from the consumption of canola oil is limited.

In the past two decades, there have been numerous studies performed to evaluate the influence of dietary fat on BP. For instance, Ferrara and others (2000) conducted a crossover RCT with hypertensive patients to compare diets with high MUFA (extra-virgin olive oil) versus those with high PUFA (sunflower oil) inclusion on resting BP. They found that the resting BP at the end of the MUFA treatment was significantly lower than that of the PUFA treatment (Ferrara et al., 2000). In a comprehensive review of the literature, consistent evidence was positioned regarding the impact of consuming olive oil in the Mediterranean diet and subsequent reductions of BP (López-Miranda et al., 2010). Moreover, the OA found in olive oil was recognized to be independently associated with the reduction of BP (Terés et al., 2008). However, not all studies have shown that the consumption of MUFA or olive oil had a hypotensive effect on BP (Aro et al., 1998; Mutanen et al., 1992). Thus, it is controversial to state that the consumption of MUFA has the potential to reduce BP.

Excessive dietary SFA (17.6% total energy) consumption does not only increase the plasma cholesterol level but can also impact glycemic control and insulin sensitivity.
(Vessby, 2000). Hence, substituting dietary SFA with MUFA may contribute to more optimal glycemic control and insulin sensitivity. An RCT was conducted to investigate whether the type of fat influenced insulin action in humans. One hundred and sixty-two healthy subjects were randomly distributed into two groups: One consuming a high MUFA (21.2% of total energy) diet and the other consuming a high SFA (17.6% of total energy) diet. The investigators found a significant impairment in insulin sensitivity in the SFA group compared with the MUFA group, but no effect on insulin secretion (Vessby et al., 2001). Another crossover RCT was carried out using a similar treatment design, and the authors found that the replacement of SFA with MUFA improved insulin sensitivity and β-cell function (López et al., 2008). There were very few studies to report the influence of canola oil intake on serum glucose levels and insulin action compared with the other types of edible oil, leading to a lack of evidence to support the role of canola oil on blood glucose regulation (Lin et al., 2013).

Currently, weight reduction is one of the best options to improve the health condition of patients with abdominal obesity or MetS (Grundy et al., 2005). The consumption of MUFA was investigated in several studies to evaluate whether it has the potential to reduce body weight and android fat mass. These studies were conducted because there was an association between a high-MUFA diet, and elevated energy expenditure and an oxidation rate (Liu et al., 2016). Liu and others (2016) conducted a crossover controlled feeding RCT to investigate how the intake of a high-MUFA (19.3% of total energy) diet influenced body composition in MetS patients. The study revealed that the high-MUFA diet reduced central obesity compared with a high-PUFA diet. Gillingham and others (2012) compared a HOCO and flaxseed oil with a control oil that
contained an identical fatty acid composition to that observed within a typical Western diet. The study concluded that the replacement of typical Western dietary oil (rich in SFA) with HOCO or flaxseed oil has no significant influence on energy expenditure, substrate oxidation or body composition in patients with hypercholesterolemia (Gillingham et al., 2012). It is therefore controversial to conclude that canola oil has the potential to reduce body weight and improve the health condition of patients with abdominal obesity.

1.4 Circulating cholesterol and cholesterol metabolism

LDL and HDL are the two types of lipoprotein that transfer cholesterol within the bloodstream, between liver and extrahepatic tissues. LDL helps transfer cholesterol from the liver to the peripheral cells while HDL brings cholesterol from the blood vessels back to the liver. Increasing LDL stimulates cholesterol accumulation in blood vessels, which then contributes to the development of CVD. However, HDL makes a positive effect on cholesterol clearance in the blood (Packard & Shepherd, 1982).

A model built in Figure 1.1, based on Dietschy’s (1993) study, explains how the regulation of plasma LDL-C is influenced by the consumption of a high MUFA oil. Cholesterol is synthesized from acetyl-CoA in the liver, small intestine and other extrahepatic tissues, but the liver plays a predominant role in the regulation of LDL-C. The newly synthesized cholesterol in extrahepatic tissues is carried to the liver by HDL-C, while the cholesterol from both synthesis and absorption in the small intestine is delivered to the liver in the chylomicron particle (CM). The very low-density lipoproteins (VLDL) transport not only TG, but also the free and esterified cholesterol. Then, the LDL-C is formed by the removal of the TG core from VLDL-C. The LDL receptor
located in the liver is responsible for the clearance of the LDL-C and VLDL-C remnants. When the LDL receptor activity is suppressed, less of the LDL-C and VLDL-C remnants are cleared in the liver, subsequently increasing LDL-C production. Both the decrease of LDL-C removal by the LDL receptor and the increase of LDL-C production contribute to the accumulation of LDL-C in the blood. When there is a net inflow of cholesterol into the liver, LDL receptor activity is suppressed due to the increased size of the putative regulatory pool of cholesterol. Because MUFA is a good substrate for cholesteryl ester formation, the high MUFA levels promote the formation of cholesteryl esters, which reduce the size of the putative regulatory pool. As a consequence, the suppression of LDL receptors reduces to a lower magnitude and then down-regulates the plasma LDL-C levels (Dietschy et al., 1993). Moreover, the decreased size of the putative regulatory pool stimulates hepatic cholesterol synthesis, with no changes in extrahepatic cholesterol synthesis (Woollett et al., 1992). However, if MUFA is replaced by SFA, both plasma LDL-C level and cholesterol synthesis appear to shift inversely.

The circulating cholesterol balance is maintained by the endogenous pathway, including cholesterol synthesis in the liver and extrahepatic tissues, cholesterol catabolism, and cholesterol secretion into bile; and the exogenous pathway, consisting of cholesterol and biliary source absorption in the intestine (Shepherd, 2001). Therefore, cholesterol synthesis and cholesterol absorption play important roles in maintaining circulating cholesterol levels. The main function of bile acid in the gastrointestinal system is to facilitate the emulsification and the absorption of lipids (Lefebvre et al., 2009). Bile acid biosynthesis is the predominant pathway in cholesterol catabolism as well because it takes up approximately half of the daily bioconversion of cholesterol.
Almost 5% of the bile acid is not absorbed in the intestinal tract, with subsequent excretion via the faeces. As such, the stimulation of bile acid biosynthesis may contribute to the reduction of circulating cholesterol (Packard & Shepherd, 1982). Therefore, to analyse cholesterol synthesis, cholesterol absorption and bile acid biosynthesis help to explain the mechanisms behind the change in plasma lipid levels in intervention studies.

Figure 1.1 The relation between cholesterol metabolism and the regulation of the plasma LDL-C concentration. The circulating cholesterol balance is maintained by the endogenous pathway and exogenous pathway. Endogenous pathway includes cholesterol synthesis in the liver and extrahepatic tissues, cholesterol catabolism, and cholesterol secretion into bile. Exogenous pathway consists of cholesterol and biliary source absorption in the intestine. LDL receptor plays an important role in regulating LDL uptake by the liver. For instance, the suppressed LDL receptor activity will reduce the LDL uptake by the liver, which subsequently elevate the plasma LDL-C concentration.
Cholesterol synthesis, cholesterol absorption and bile acid biosynthesis are important pathways in cholesterol metabolism (Shepherd, 2001). Cholesterol de novo synthesis pathway is shown in Figure 1.2. Cholesterol de novo synthesis starts from acetyl-CoA and the process is regulated by the critical rate-limiting enzyme HMG-CoA reductase (Thongtang et al., 2012). The cholesterol synthesis precursors, including desmosterol and lathosterol, are surrogate markers of cholesterol synthesis. However, desmosterol and lathosterol diverge during the last steps of cholesterol synthesis. Desmosterol converts to cholesterol through the Bloch pathway, while lathosterol undergoes the Kandutsch-Russell pathway (Myant, 2014). The direct cholesterol synthesis measurement, deuterium incorporation method, regarded as the “gold standard” in measuring cholesterol synthesis, is always used to assess the validity of other measurements (Jones et al., 1998). This method uses deuterated water as a tracer to assess how fast the incorporated deuterium water participates in the de novo cholesterol synthesis. Both the isotope incorporation method and the surrogate marker method are credible to measure cholesterol synthesis (MacKay et al., 2014). Dietary and biliary cholesterols are absorbed in the intestine (Shepherd, 2001). Phytosterols, such as campesterol, β-sitosterol and cholestanol, have a similar chemical structure to that of cholesterol and compete with cholesterol absorption. These phytosterols are surrogate markers of cholesterol absorption as well (Mackay et al., 2014). Moreover, the double isotope method is a more credible method to access cholesterol absorption but time-consuming. The participants consume $[^{14}\text{C}]$ cholesterol and $[^{3}\text{H}]$ β-sitosterol for more than 10 days during the test. $^{14}\text{C}/^{3}\text{H}$ in both dietary and faecal samples are then measured to calculate the cholesterol absorption rate (Crouse & Grundy, 1978).
Both pharmaceutical and nutraceutical therapies are used to treat dyslipidemia in MetS patients. For instance, statins and ezetimibe are the common medications for the treatment of dyslipidemia (Cornier et al., 2008). The mode of action of cholesterol-lowering pharmaceuticals and nutraceuticals include intestinal Niemann-pick C1 like 1 (NPC1L1) competitors, acyl-CoA:cholesterol acyltransferase 2 (ACAT2) inhibitors, 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase inhibitors, bile acids sequestrants, cytochrome P450 7A1 (CYP7A1) activators, and cholesteryl ester transporting protein (CETP) inhibitors (Chen et al., 2011). Biliary cholesterol and dietary cholesterol are transported into enterocytes via NPC1L1, then ACAT2 esterifies these free cholesterols for further transportation. The esterified cholesterol is packed into CM and transferred into the blood through the lymphatic system. Both the NPC1L1 competitors and ACAT2
inhibitors lower plasma cholesterol by decreasing intestinal cholesterol absorption. Dietary phytosterols and phytostanols are structurally similar to cholesterol, which makes them able to compete with cholesterol for NPC1L1 (Plat & Mensink, 2005). HMG-CoA reductase is the critical rate-limiting enzyme of cholesterol synthesis. The HMG-CoA reductase inhibitors, primarily statins, lower plasma cholesterol by suppressing cholesterol synthesis (Thongtang et al., 2012). The function of the LDL receptor is explained in the Dietschy’s (1993) model (Figure 1.1) previously positioned, and the LDL receptor activators can promote the removal of LDL-C from the blood. Bile acid sequestrants, also known as bile acid absorption inhibitors, can bind bile acids in the intestine and form insoluble compounds. Bile acid reabsorption is then inhibited by the sequestrants, which also promotes bile acid synthesis from cholesterol, thus decreasing hepatic cholesterol levels. The subsequent change in hepatic cholesterol concentrations then up-regulates the expression of LDL receptors and promotes the removal of LDL-C from the bloodstream. Fibres, like β-glucan, are typical bile acids sequestrants (Kim & White, 2009). CYP7A1 is the rate-limiting enzyme in bile acid synthesis and also plays an important role in regulating plasma cholesterol (Chiang, 2009). The up-regulation of CYP7A1 also decreases hepatic cholesterol levels and promotes the expression of LDL receptors. CEPT modulates the distribution of LDL-C and HDL-C in plasma. The CETP inhibitors lower CETP activity, which increases the HDL-C level and decreases the LDL-C level (Lam et al., 2008). The effect of canola oil intake on cholesterol metabolism in MetS patients is not clearly explained. Further studies are required to test which of the above mechanisms may be linked to diets containing canola oil.
Many studies have reported that patients with MetS have elevated cholesterol synthesis and reduced cholesterol absorption (Gylling et al., 2007). Simonen and others (2000) conducted a study to investigate the effect of weight reduction after a low-energy diet on cholesterol metabolism in MetS people. This study found that weight reduction increased cholesterol absorption and improved glucose metabolism. This result suggested that high cholesterol synthesis rates and low rates of cholesterol absorption are hallmarks of MetS and insulin resistance syndrome (Simonen et al., 2000). Most of the clinical studies showed the potential to improve the MetS condition by modulating cholesterol mechanism (Gylling et al., 2007; Rideout et al., 2010; Simonen et al., 2000), but few of them used high-MUFA (or -canola) diets to test how the intake of MUFA influences cholesterol mechanism (Fernandez & West, 2005).
**Research gap**

Canola oil is an oleic acid-rich edible oil, that also contains an abundant amount of phytosterols, including campesterol, β-sitosterol and brassicasterol. The consumption of different fatty acids in the diet subsequently has differential influences on plasma lipid profiles. Canola oil in the diet has the potential to increase the plasma fatty acid percentage of MUFA, which then decrease the suppression of LDL receptor activity. It is widely proven that the intake of a high-SFA diet results in elevated TC and LDL-C, which increases the risk of CVD. The replacement of SFA with MUFA or PUFA is recommended in a typical Western diet to reduce the risk of CVD. Most previous studies used a high-SFA diet as a control to assess the health benefits of canola oil, but few included the Western diet as a comparable treatment. As discussed above, the consumption of MUFA has the potential to reduce circulating cholesterol levels and to stimulate cholesterol synthesis. The phytosterol content of canola oil is relatively high, compared to most other edible oils, especially compared to animal oils. The consumption of phytosterol in canola oil may compete with cholesterol absorption in human beings, which decreases the circulating cholesterol level. But the daily intake of phytosterol from a typical canola oil-enriched diet is less than the amount recommended by health claim. Whether the content of phytosterol in canola is enough to influence cholesterol metabolism is still required to test. Currently, the reversed change of cholesterol synthesis and cholesterol absorption is widely found in many cholesterol-control pharmaceutical studies and nutritional clinical trials. For instance, cholesterol synthesis decreases after the statin therapy, while cholesterol absorption increases. The hypothesis of this study is that a high intake of canola oil reduces cholesterol absorption and increases cholesterol...
synthesis. As the main cholesterol conversion pathway, the biosynthesis of bile acid plays an important role in the removal of cholesterol by the liver. It is therefore worthy to set the measurement of bile acid synthesis as another target in the study.

**Objectives**

1. To investigate how the consumption of canola oil influences cholesterol synthesis, cholesterol absorption, and bile acid biosynthesis in subjects at risk for metabolic syndrome.

2. To assess how cholesterol metabolism contributes to the regulation of plasma cholesterol levels after the consumption of canola oil.

**Hypotheses**

1. Replacing SFA with MUFA from conventional and high-oleic acid canola oils will reduce cholesterol absorption and increase cholesterol synthesis and bile acid biosynthesis in subjects at risk for metabolic syndrome.

2. The endpoint plasma TC and LDL-C will negatively correlated with cholesterol synthesis surrogate markers and positively correlated with cholesterol absorption surrogate markers.
Chapter II

Manuscript

Effect of consuming canola oil on cholesterol synthesis, cholesterol absorption, and bile acid biosynthesis

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\textsuperscript{2}Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB, Canada
\textsuperscript{3}Department of Nutritional Science, The Pennsylvania State University, University Park, PA, U.S.
2.1 Introduction

Metabolic syndrome (MetS) is a group of different metabolic abnormalities, including abdominal obesity, hypertension, elevated levels of triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C), and decreased levels of high-density lipoprotein cholesterol (HDL-C) (Al Dhaheri et al., 2016). The prevalence of MetS estimated by the International Diabetes Federation is approximately a quarter of the world’s population (O’neill & O'driscoll, 2015). Many epidemiological and observational studies have stated that the quality of edible oil (or fatty acid composition) is closely associated with the development of MetS (Kris-Etherton et al., 2007). High intake of saturated fatty acid (SFA) is generally related to MetS risk factors (Zhao et al., 2018). Thus, the substitution of SFA with monounsaturated fatty acid (MUFA) or polyunsaturated fatty acid (PUFA) in the typical Western diet is recommended (Siri-Tarino et al., 2010).

Results from clinical studies have provided evidence that the consumption of MUFA reduces the risk factors for MetS, especially via an improvement in plasma lipid profiles (Gillingham et al., 2011). A literature review, including ten intervention studies, summarized findings regarding the replacement of SFA with MUFA in the diet, with results indicating significantly decreased serum TC and LDL-C levels (Harland, 2009). However, the influence of dietary fatty acids on HDL-C and TG levels was not clear.

Canola oil is the abbreviation of Canadian Oil Low Acid, which is characterized by its low content of erucic acid and low in glucosinolates (Mag, 1983). Canola oil is not only one of the richest sources of dietary MUFA, but also contains abundant phytosterols, which can contribute to improvements in plasma lipid profiles (Kostik et al., 2013).
Phytosterols are structurally similar to cholesterol (Moreau et al., 2002), and compete with cholesterol in the absorption process (Ostlund Jr, 2007). The consumption of phytosterol in the diet reduces cholesterol absorption and increase cholesterol synthesis (Jones et al., 2000). According to the cholesterol metabolism model built in Figure 1.1, based on Dietschy’s (1993) study, the substitution of SFA with MUFA subsequently increased the MUFA content in plasma fatty acid composition, which reduced the suppression of LDL-C receptor activity. With the recovered activity of the LDL-C receptor and the reduced activity of LDL-C production, the circulating LDL-C level was down-regulated (Dietschy et al., 1993). As a consequence, hepatic cholesterol synthesis was promoted with no change in extrahepatic cholesterol synthesis (Woollett et al., 1992).

Cholesterol synthesis, cholesterol absorption, cholesterol catabolism, and the urinary or faecal removal of bile acid are the main pathways which balance the circulating cholesterol level (Gylling & Miettinen, 1992). Non-cholesterol sterols (NCSs) are verified surrogate markers to assess cholesterol metabolism. Cholesterol precursors, such as lathosterol and desmosterol, are used as markers of endogenous cholesterol synthesis, while plant sterols, such as campesterol, sitosterol and cholestanol, are regarded as markers of cholesterol absorption (MacKay et al., 2014). Bile acid biosynthesis is the predominant pathway in cholesterol catabolism because it takes up approximately half of the daily bioconversion of cholesterol (Insull Jr, 2006). Almost 5% of the bile acid is not absorbed in the intestinal tract, with subsequent excretion via the faeces. As such, the stimulation of bile acid biosynthesis might contribute to the reduction of circulating cholesterol levels (Packard & Shepherd, 1982). 7α-hydroxy-4-
cholesten-3-one (7αC4) is reported as a biomarker that reflects bile acid synthesis and the activity of 7α-hydroxylase (Bertolotti et al., 2008). As the rate-limiting enzyme in bile acids synthesis, the activity of 7α-hydroxylase indicates how fast cholesterol converts to bile acids in the human body (Chiang, 2009).

A previous study, known as Canola Oil Multicenter Intervention Trial (COMIT) was a crossover randomized clinical trial (RCT) study including five treatment diets: 1) conventional canola oil (n-9 rich); 2) high-oleic canola oil (HOCO) with docosahexaenoic acid (DHA) (n-9 and n-3 rich); 3) a blend of corn and safflower oils (n-6 rich); 4) a blend of flax and safflower oils (n-6 and short-chain n-3 rich); 5) HOCO (extremely n-9 rich). This study showed that all the treatments significantly decreased the Framingham Risk Score for metabolic syndrome during the intervention. All the treatment diets significantly reduced serum levels of TC and LDL-C. The consumption of DHA enriched canola oil significantly increased serum levels of HDL-C and reduced serum TG levels. Canola oil showed its potential to promote the serum lipid profile, but the lack of a Western control oil became the main limitation of the study (Jones et al., 2014). Compared to conventional canola oil, HOCO is higher in OA (70%) with similar SFA content (7%). The consumption of regular canola oil or HOCO has potential to increase plasma fatty acid percentage of MUFA (Raatz et al., 2018), which subsequently down-regulates the plasma LDL-C levels (Dietschy et al., 1993). The objective for the current study includes assessing the impact of oleic acid intake, through comparing regular canola oil (RCO) and HOCO with typical Western diet oil (WDO), on serum lipid profile and cholesterol metabolism.
2.2 Experimental methods

2.2.1 Study design and population

This study was conducted within the framework of COMIT II. COMIT II was a randomized, double-blind, controlled feeding, crossover, clinical trial that consisted of three, 6-week feeding phases separated by ≥4-week washout periods. The clinical trial was conducted from 2014-2016 at the following institutes: the Richardson Center for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg, the Institute of Nutrition and Functional Foods (INAF) at Laval University in Québec City, the Canadian Centre for Agri-Food Research in Health and Medicine (CCARM) at St. Boniface Hospital Albrechtsen Research Center (SBRC) in Winnipeg, and the Department of Nutritional Sciences at the Pennsylvania State University (PSU) in University Park. The study protocol and related documents were approved by the research ethics boards in all collaborative institutes. The trial was registered at clinicalTrials.gov as NCT02029833.

Adult women and men from 20 to 65 years with MetS risk factors were recruited at each collaborative institute. MetS risk was defined as an increased waist circumference (men ≥94 cm, women ≥80 cm, according to International Diabetes Federation(IDF) cut points) in addition to at least one of the following inclusion criteria: elevated fasting blood glucose (≥5.6 mmol/L), TG (≥1.7 mmol/L), systolic blood pressure (≥130 mmHg), diastolic blood pressure (≥85 mmHg); and/or decreased high-density lipoprotein cholesterol (HDL-C; men <1 mmol/L, women <1.3 mmol/L) (Riediger & Clara, 2011). Exclusion criteria included smokers; consumption of >14 alcoholic beverages per week; use of prescription lipid-modifying medications in the last three months or chronic anti-
inflammatory medications; kidney disease, liver disease, diabetes, or uncontrolled thyroid
disease; and pregnant or lactating women. Participants were required to provide written
informed consent upon enrollment. Threes phases were randomized using
randomization.com to generate the treatment sequences. COMIT II researchers were not
allowed to access to the sequence code.

The previous study, COMIT I, was a randomized, double-blind, controlled-
feeding, crossover, clinical trial that consisted of five, 4-week feeding phases. COMIT I
data showed that the intake of oleic acid has the potential to improve serum levels of
HDL-C, decrease triglycerides, and reduce blood pressure (Jones et al., 2014). However,
the lack of a control diet and the relatively short intervention period became the
limitations of the study. The duration of the intervention should be no less than 14 days to
reach a steady state in lipid levels, whereas the assessment of cholesterol metabolism
biomarkers requires a longer duration for measurable change (MacKay et al., 2017). The
COMIT II study involved a Western control diet and prolonged the intervention phase to
assesses whether the intake of canola oil for a longer period still shows the same health
benefit in MetS participants. The COMIT II program also made it possible to explore
which mechanism drives the shift of blood lipid profile after the intervention of the high-
oleic acid diet.

2.2.2 Controlled diets and oil interventions

COMIT II study contained three 6-week treatment phases separated by two ≥4-
week washout periods (Figure 2.1). During the intervention phase, participants
consumed an iso-caloric, full-feeding diet with one of the following oils: 1) regular
canola oil (Canola Harvest Canola Oil, Richardson International, Winnipeg, Canada), 2)
high-oleic acid canola oil (Canola Harvest Canola Oil, Richardson International, Winnipeg, Canada), or 3) control oil blend, which consisted of ghee oil (Verka, New Delhi, Delhi, India), safflower oil (eSutras, Illinois, Chicago, USA), coconut oil (eSutras, Illinois, Chicago, USA), and flaxseed oil (Shape Foods, Brandon, Canada). The regular canola oil contained 6.6% SFA, 65.3% MUFA, 19.6% n-6 PUFA, and 8.5% n-3 PUFA α-linolenic acid, and the high-oleic canola oil contained 6.7% SFA, 75.9% MUFA, 14.8% n-6 PUFA, and 2.6% n-3 PUFA α-linolenic acid. The control oil blend with fixed source composition (49% ghee oil, 29% safflower oil, 14% flaxseed oil, and 8% coconut oil) contained 40.2% SFA, 22.0% MUFA, 29.6% n-6 PUFA, and 8.2% n-3 PUFA α-linolenic acid. The percentages of energy from the macronutrients were identical (35% fat, 50%carbohydrate, and 15% protein of total energy), whereas the percentages of energy from MUFA, PUFA, and SFA (Table 2.1) were different due to the lipid profile of the intervention oils.

![Study design of the crossover clinical trial](image)

**Figure 2.1** Study design of the crossover clinical trial
Table 2.1

Macronutrient composition of the three experimental diets containing the oils 1,2

<table>
<thead>
<tr>
<th></th>
<th>Canola oil diet</th>
<th>HOCO diet</th>
<th>Control oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>15.87</td>
<td>15.87</td>
<td>15.71</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>50.79</td>
<td>50.79</td>
<td>50.75</td>
</tr>
<tr>
<td>Fat</td>
<td>35.26</td>
<td>35.26</td>
<td>35.21</td>
</tr>
<tr>
<td>MUFA</td>
<td>17.45</td>
<td>19.11</td>
<td>10.50</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>15.55</td>
<td>17.86</td>
<td>5.92</td>
</tr>
<tr>
<td>PUFA</td>
<td>9.21</td>
<td>7.02</td>
<td>9.96</td>
</tr>
<tr>
<td>α-linolenic acid</td>
<td>2.10</td>
<td>0.76</td>
<td>1.73</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>6.42</td>
<td>5.56</td>
<td>7.28</td>
</tr>
<tr>
<td>SFA</td>
<td>6.56</td>
<td>6.43</td>
<td>12.26</td>
</tr>
</tbody>
</table>

1 This data has been published in the previous COMIT II study (Bowen et al., 2019).
2 The average macronutrient composition from the 7-day rotating menu, estimated at the 3000 kcal level, using Food Processor Nutrition Analysis Software (ESHA Research, Salem OR). Nutrients are presented as a percentage of total energy. HOCO, high-oleic acid canola oil.

The treatment oils were incorporated into a smoothie beverage and divided equally into two portions. Participants consumed one portion at breakfast and the other portion at supper to avoid gastrointestinal distress from the fat load and the strong taste from oil. A seven-day rotating menu cycle was designed to serve participants with various combinations of foods. The treatment meals were prepared in the clinical kitchen of the individual institute, and the sequence code of meals was blinded to both researchers and participants. Participants took their daily meal in the morning every weekday. At the same time, a clinical coordinator measured the participants’ body weight and waist circumference during the visit. The waist circumference was measured by the measuring tape, which started from the hip bone, went all the way around the body, and levelled with the belly button.
The participant was also asked to complete a questionnaire every week to ensure they follow clinical instructions. During the weekends and holidays, the treatment meal packed in a cooler bag was delivered to the participants’ residence or handed out to them. A checklist that included all the foods in the pack was provided to participants as well, to verify the treatment meal consumption. Participants were advised to consume no less than 90% of the smoothies served at each phase to ensure compliance. Moreover, participants were asked to finish one smoothie under the supervision of a clinical coordinator when they picked up their daily meal in the morning. During the washout period, participants were informed to keep their habitual diets and maintain their normal physical activity level. Clinical coordinators were accessible to the participants at both the intervention phase and the washout phase to maximize compliance.

### 2.2.3 Blood sample collections

On days 1, 2, 41 and 42 of each dietary treatment, 20 ml fasting blood samples were collected in blood collection tubes (Hematology tubes, Becton Dickinson, Franklin Lakes, NJ, US). After the centrifugation at 3000 rpm for 20 minutes at 4°C, the serum fraction was separated as supernatant in the vacutainer. The serum samples were then stored at -80°C for further analysis.

### 2.2.4 Serum lipid and lipoprotein levels

The endpoint measurement of serum TC, HDL-C and TG were performed on an autoanalyzer (Roche/Hitachi cobas c 501, Roche Diagnostics, Switzerland) by the Department of Nutritional Sciences at the Pennsylvania State University (PSU) in University Park,
Pennsylvania. LDL-C was estimated according to the Friedewald equation (Friedewald et al., 1972):

\[
\text{VLDL-C} = \frac{\text{TG}}{5}
\]

\[
\text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{VLDL-C})
\]

### 2.2.5 Surrogate markers of cholesterol synthesis and cholesterol absorption

The measurements of the surrogate markers of cholesterol synthesis and cholesterol absorption were performed based on previously published methods (Mackay et al., 2014), with minor modifications. A 0.5 ml sample of serum was mixed with 0.1 ml of 1mg/ml internal standard, 5-α-cholestane (Sigma-Aldrich Ltd., Oakville, ON, Canada), and underwent hydrolysis with an alkaline agent (methanol-KOH) to convert sterol-fatty acid esters to free sterols. Petroleum ether was added to the mixture as an extracting solvent after the tube had cooled to room temperature, the samples mixed, and the ether phase transferred to a new tube. The organic solvent extraction was repeated, and the combined ether phases containing the extracted lipid fractions were dried under nitrogen gas. The dried residue was resuspended in 0.4 ml of heptane, and mixed with 0.1 ml of 1,1,1,3,3,3-hexamethyldisilane (HMDS) + Trimethylchlorosilane (TMCS) + Pyridine (3:1:9) (Sigma-Aldrich Ltd., Oakville, ON, Canada), and placed under moderate heating conditions (55°C) overnight to complete the derivatization process.

The derivatized lipid extracts were injected into a GC-FID (Agilent Technologies) equipped with a SAC-5 column (30m ×0.25mm, Sigma-Aldrich Ltd., Oakville, ON, Canada) to separate sterols from the solvent. The initial temperature for the injector and detector was set to 280 and 300 °C, respectively. The column temperature was initially
held at 160 °C for 0.5 minutes. Then the oven temperature ramped to 270 °C at 30 °C/min and held for 10 minutes, followed by a ramp to 290 °C at 10 °C/min and held for 9 minutes, then finally increased to 320 °C at 40 °C/min and held for 5 minutes. The flow rate of the carrier gas (helium) was 1.3 ml/min, and the split ratio was set at 12.0:1. The elution time of individual sterol was measured by using authenticated standards (Sigma-Aldrich Canada Ltd., Oakville, ON), which made it possible to identify the sterols in the chromatograph. The peaks for cholesterol, cholestanol, desmosterol, lathosterol, campesterol, and β-sitosterol are displayed in a chromatogram shown in Figure 2.2.

![Figure 2.2 Peaks of cholesterol, cholestanol, desmosterol, lathosterol, campesterol, and β-sitosterol located on the GC-FID chromatograph](image)

2.2.6 Surrogate marker of bile acid synthesis

Bile acid biosynthesis was analysed by measuring the serum concentration of 7α-hydroxy-4-cholesten-3-one (7αC4) via an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; ACQUITY UPLC System, Waters, Mississauga, ON, Canada) system adapted from Steiner’s method (Steiner et al., 2010). The 7αC4 was extracted from the serum sample by using solid phase extraction (SPE). A
volume of 0.5 ml of serum sample was mixed with 0.1 ml of double distilled water, 0.2 ml of methanol and 0.05 ml of 200 ng/ml 7α-hydroxy-4-cholesten-3-one-d7 (7αC4-d7; Medical Isotopes. Inc. Pelham, NH, USA) at the initial step. SPE cartridges (HF Bond Elut-C18, 200 mg, 3ml, Agilent Technologies, Mississauga, ON, Canada) were preconditioned with 2 ml methanol, followed by 2 ml double distilled water. The mixed sample prepared at the initial step was loaded onto the conditioned SPE cartridge with a light vacuum. Subsequently, the cartridge was washed twice with 2 ml distilled water, followed by flash drying with nitrogen gas. The retained compounds were eluted with 2 ml methanol twice and collected in a new tube. The extracted solution was evaporated under nitrogen gas at 30°C and redissolved in 0.08 ml methanol. The final purification step was performed by passing the sample through a 4 mm syringe filter (Phenex 0.2 μm PTFE membrane; Phenomenex, Torrance, CA, US) prior to injection into the UPLC system. A Kinetix XB-C18 column (2.1 x 100mm, particle size 1.7 μm; Phenomenex, Torrance, CA, US) with an in-line filter (KrudKatcher ULTRA HPLC In-Line Filter, 0.5μm Depth Filter x 0.004in; Phenomenex, Torrance, CA, US) was applied for the separation. The gradient system was obtained by using mobile phase A (0.1% formic acid in ddH2O) and mobile phase B (0.1% formic acid in acetonitrile). The gradient system was initialized at 10% of phase A and 90% of phase B for 6 min. Then phase B increased linearly to 100% for 4 min and subsequently held for 4 min. Finally, the system returned to initial settings for 4 min. Quantification of 7αHC was performed using multiple reaction monitoring (MRM) mode using peak areas. The MRM transitions for 7αC4 were 401.3 > 383.3 m/z and for 7αC4-d7 were 408.3> 309.3.
2.2.7 Statistical method

Statistical analyses were performed using the SAS mixed-model ANOVA procedure (v9.4, Cary, NC). All results were expressed as least square means ± standard error of the mean. Normality of data was observed and checked visually based on the plot of residuals. Treatment, the sequence of treatment, age of participant, and sex were considered as fixed factors, whereas participant and test centre were considered as random factors. Tukey-adjustment was used to account for multiple comparisons. Statistical difference was set at P < 0.05 for all analyses. Participants with a weight change of >5% during any diet were removed from the analyses to eliminate the confounding effects of substantial weight change on the outcomes.

2.3 Results

2.3.1 Baseline characteristics
One hundred and seventy-four participants were recruited and met the criteria of the study, and finally, one hundred and twenty-five people completed the study, resulting in a dropout rate of approximately 28%. Six of the participants were removed from the analysis process because of a large weight change (> 5%) during the intervention phase. The baseline characteristics of the participants in COMIT II is shown in Table 2.2., highlighting that more females participated in the study, and that most people were mid-aged.

2.3.2 Serum lipid profile
The serum lipid and lipoprotein levels at the endpoint test across three treatments are shown in Table 2.3. The TC, LDL-C, as well as non-HDL-C levels after the canola
oil diet treatment or HOCO diet, were significantly ($P \leq 0.05$) lower than the value after the control oil diet. The measures for TC, LDL-C and non-HDL-C levels were not significantly different between the two canola oil treatments. Consumption of the HOCO diet resulted in a higher TC: HDL-C ratio compared to the control oil diet ($P \leq 0.05$), but no statistical significance observed compared to the canola oil diet.

Table 2.2
Baseline characteristics of the COMIT II participants $^{1,2}$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value $^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>75 (63%)</td>
</tr>
<tr>
<td>Male</td>
<td>44 (37%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44 ± 13 (22 – 65)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.3 ± 18.7 (60.4 – 146.4)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>31.7 ± 5.3 (22.6 – 52.6)</td>
</tr>
<tr>
<td>MetS criteria</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>105 ± 13 (80 – 151)</td>
</tr>
<tr>
<td>Male</td>
<td>109 ± 13 (94–151)</td>
</tr>
<tr>
<td>Female</td>
<td>103 ± 12 (80–131)</td>
</tr>
<tr>
<td>TG (mmol/L) $^4$</td>
<td>1.60 ± 0.73 (0.33 – 3.67)</td>
</tr>
<tr>
<td>HDL-C (mmol/L) $^4$</td>
<td>1.33 ± 0.35 (0.67 – 2.49)</td>
</tr>
<tr>
<td>Female (n=75)</td>
<td>1.41 ± 0.35 (0.87 – 2.49)</td>
</tr>
<tr>
<td>Male (n=43)</td>
<td>1.20 ± 0.31 (0.67 – 1.97)</td>
</tr>
<tr>
<td>Glucose (mmol/L) $^4$</td>
<td>5.30 ± 0.59 (4.16 – 8.00)</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>120 ± 14 (88 – 164)</td>
</tr>
<tr>
<td>Diastolic blood pressure $^4$</td>
<td>79 ± 11 (54 – 100)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L) $^4$</td>
<td>5.17 ± 0.90 (3.38 – 7.36)</td>
</tr>
<tr>
<td>LDL-C (mmol/L) $^4$</td>
<td>3.11 ± 0.75 (1.04 – 5.33)</td>
</tr>
</tbody>
</table>

$^1$This data has been published in the previous COMIT II study (Bowen et al., 2019).
$^2$Values are means ± SDs (minimum-maximum) or frequency (%), n=119. BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MetS, metabolic syndrome; TG, triglyceride.
$^3$Data collected on days 1 and 2 of diet period 1. Fasting lipids, lipoproteins, and glucose were assessed in serum.
$^4$n =118 due to missing values.
Table 2.3
Fasting serum lipid and lipoprotein levels at endpoint test across the three treatments

<table>
<thead>
<tr>
<th></th>
<th>Canola oil diet</th>
<th>HOCO diet</th>
<th>Control oil diet</th>
<th>P for diet effect</th>
<th>P for time effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>4.54 ± 0.04</td>
<td>4.58 ± 0.04</td>
<td>4.74 ± 0.04</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>TGs (mmol/L)</td>
<td>1.45 ± 0.04</td>
<td>1.44 ± 0.04</td>
<td>1.40 ± 0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.25 ± 0.01</td>
<td>1.28 ± 0.01</td>
<td>1.26 ± 0.01</td>
<td>NS</td>
<td>0.005</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.64 ± 0.04</td>
<td>2.67 ± 0.04</td>
<td>2.83 ± 0.04</td>
<td>&lt;0.0001</td>
<td>0.02</td>
</tr>
<tr>
<td>TC: HDL-C ratio</td>
<td>3.82 ± 0.04</td>
<td>3.77 ± 0.04</td>
<td>3.92 ± 0.04</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Non-HDL-C (mmol/L)</td>
<td>3.30 ± 0.05</td>
<td>3.31 ± 0.05</td>
<td>3.45 ± 0.05</td>
<td>0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 This data has been published in the previous COMIT II study (Bowen et al., 2019).
2 Values are least squares means ± SEMs, n=119. Labeled means in a row without a common superscript letter differ, P≤0.05. A repeated measures mixed model was used to assess the effects of diet, time, sex, center, and sequence, and the interactions diet-by-time, diet-by-sex, diet-by-center, and diet-by-sequence. The diet specific baseline value used as a covariate. Final models included diet and only significant terms. Pairwise comparisons were assessed using the Tukey-Kramer method when there was a significant effect of diet. HDL-C, high-density lipoprotein-cholesterol; high-oleic acid canola oil, HOCO; LDL-C, low-density lipoprotein-cholesterol; MetS, metabolic syndrome; NS, P > 0.05; TC, total cholesterol.

2.3.3 Surrogate markers for cholesterol metabolism and bile acid biosynthesis

The elution times of cholesterol, cholestanol, desmosterol, lathosterol, campesterol, as well as β-sitosterol (Table 2.4) were identified as 15.761, 16.047, 16.425, 16.699, 17.509, and 19.128 minutes, respectively. The absolute values of serum NCSs levels at the endpoint of each treatment are shown in Table 2.5. The consumption of both the HOCO diet and the canola oil diet resulted in lower serum cholesterol levels compared to the control diet (P ≤ 0.05). The endpoint absolute values of desmosterol, campesterol, and β-sitosterol after the canola oil diet and HOCO diet were significantly (P ≤ 0.05) higher than the values observed after the control oil diet treatment. There were no significant differences between the two canola oil treatments for these measurements.
Table 2.4
The elution time of NCSs and cholesterol in serum sample measured by GC-FID

<table>
<thead>
<tr>
<th></th>
<th>cholesterol</th>
<th>cholestanol</th>
<th>desmosterol</th>
<th>lathosterol</th>
<th>campesterol</th>
<th>β-sitosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution time (min)</td>
<td>15.761</td>
<td>16.047</td>
<td>16.425</td>
<td>16.699</td>
<td>17.509</td>
<td>19.128</td>
</tr>
</tbody>
</table>

Table 2.5
Serum cholesterol precursors and phytosterol concentrations at endpoint tests across the three treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HOCO diet</th>
<th>Canola oil diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.54 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.48 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.72 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholestanol (µmol/L)</td>
<td>7.64 ± 0.30</td>
<td>7.53 ± 0.10</td>
<td>7.85 ± 0.10</td>
</tr>
<tr>
<td>Lathosterol (µmol/L)</td>
<td>7.10 ± 0.28</td>
<td>7.17 ± 0.28</td>
<td>7.51 ± 0.28</td>
</tr>
<tr>
<td>Desmosterol (µmol/L)</td>
<td>5.37 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.18 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.92 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Campesterol (µmol/L)</td>
<td>12.38 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.69 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.37 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Sitosterol (µmol/L)</td>
<td>5.05 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.05 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.55 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>All values are least-squares means ± SEMs; n = 111. Mean values within a row with different superscript letters are significantly different between treatments, P < 0.05 (mixed-model ANOVA and post hoc Tukey’s test). The dependent variables used for data analysis were the mean of the day 41 and day 42 values at the end of each dietary phase. HOCO, high-oleic acid canola oil.

The values of cholesterol metabolism surrogate markers at the endpoint of each treatment are shown in Table 2.6. The consumption of both HOCO diet and canola oil diet resulted in a lower cholestanol to cholesterol ratio, lathosterol to campesterol ratio, and lathosterol to β-sitosterol ratio compared to the control diet (P ≤ 0.05). The endpoint values of desmosterol to cholesterol ratio, campesterol to cholesterol ratio, and β-sitosterol to cholesterol ratio after the canola oil diet and HOCO diet were significantly (P ≤ 0.05) higher than the value after the control oil diet. There were no significant differences between the two canola oil treatments for these measurements.
Table 2.6

Cholesterol synthesis and cholesterol absorption surrogate markers at endpoint test across the three treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HOCO diet</th>
<th>Canola oil diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholestanol/Cholesterol ($\frac{\mu\text{mol/L}}{\text{mmol/L}}$)</td>
<td>1.64 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lathosterol/Cholesterol ($\frac{\mu\text{mol/L}}{\text{mmol/L}}$)</td>
<td>1.60 ± 0.07</td>
<td>1.61 ± 0.07</td>
<td>1.58 ± 0.07</td>
</tr>
<tr>
<td>Desmosterol/Cholesterol ($\frac{\mu\text{mol/L}}{\text{mmol/L}}$)</td>
<td>1.21 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Campesterol/Cholesterol ($\frac{\mu\text{mol/L}}{\text{mmol/L}}$)</td>
<td>2.75 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.86 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.99 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Sitosterol/Cholesterol ($\frac{\mu\text{mol/L}}{\text{mmol/L}}$)</td>
<td>1.12 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.97 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lathosterol/Campesterol ($\frac{\text{mmol/L}}{\text{mmol/L}}$)</td>
<td>0.66 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.04 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lathosterol/Sitosterol ($\frac{\text{mmol/L}}{\text{mmol/L}}$)</td>
<td>1.71 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.72 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lathosterol/Cholestanol ($\frac{\text{mmol/L}}{\text{mmol/L}}$)</td>
<td>1.00 ± 0.05</td>
<td>1.04 ± 0.05</td>
<td>1.03 ± 0.05</td>
</tr>
</tbody>
</table>

All values are least-squares means ± SEMs; n = 118. Mean values within a row with different superscript letters are significantly different between treatments, P < 0.05 (mixed-model ANOVA and post hoc Tukey’s test). The dependent variables used for data analysis were the mean of the day 41 and day 42 values at the end of each dietary phase. HOCO, high-oleic acid canola oil.

The serum 7αC4 level at the endpoint of each treatment is shown in Figure 2.3.

The consumption of the HOCO diet resulted in a higher serum 7αC4 level compared to the control diet (P ≤ 0.05). There were no significant differences between the two canola oil treatments, or between the canola oil diet and the control diet.
Figure 2.3 Measurement of 7α-hydroxy-4-cholesten-3-one concentration in serum at endpoint test across the three treatment groups

<table>
<thead>
<tr>
<th></th>
<th>HOCO diet</th>
<th>Canola oil diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-hydroxy-4-cholesten-3-one (ng/ml)</td>
<td>24</td>
<td>22</td>
<td>21</td>
</tr>
</tbody>
</table>

All values are least-squares means ± SEMs; n = 112. Mean values within a row with different superscript letters are significantly different between treatments, P < 0.05 (mixed-model ANOVA and post hoc Tukey’s test). The dependent variables used for data analysis were the mean of the day 41 and day 42 values at the end of each dietary phase. 7αC4, 7α-hydroxy-4-cholesten-3-one; HOCO, high-oleic acid canola oil.
2.4 Discussion

The circulating cholesterol levels dropped significantly after the consumption of both regular canola oil and HOCO, which agrees with the previous MUFA studies (Hodson et al., 2001; Matheson et al., 1996; Noakes & Clifton, 1998; Palomäki et al., 2010). However, the efficiency of substitution of SFA (or WDO) with MUFA (or canola oil) on blood cholesterol levels varied in many studies. The LDL-C and TC levels were reduced by an average of 17% (11.1 – 25.2%) and 12.2% (6.7 - 20.1 %), respectively, when WDO was replaced by canola oil in the diet according to others’ studies (Gillingham et al., 2011; Gustafsson et al., 1994; Lichtenstein et al., 1993; McDonald et al., 1989; Wardlaw & Snook, 1990). The diversity of the efficiency may be due to differences in the treatment matrix (i.e. oil supplement or margarine) and/or the variability in baseline plasma lipid levels (Lin et al., 2013).

In the current study, the consumption of canola oil and HOCO resulted in a reduction of circulating cholesterol levels, however, the underlying mechanism(s) behind this reduction remains to be defined. The consumption of the high-MUFA oil diet was associated with an elevation in oleic acid, as a percentage of the total fatty acid composition in the blood (Ruiz-Gutiérrez et al., 1996; Samieri et al., 2011). Because MUFAs are a good substrate for cholesteryl esters, the existence of a high MUFA environment promotes the formation of cholesteryl esters, which reduce the size of the putative regulatory pool that negatively regulates the LDL-C receptor. Because of the des-suppression of the LDL-C receptor, more LDL-C and VLDL-C are cleared in the liver, which subsequently increases the activity of LDL-C production. With the combination of elevated LDL-C receptor activity and reduced LDL-C production activity, the plasma
lipid profile presents with a reduced circulating LDL-C level (Dietschy et al., 1993). Moreover, the decreased size of the putative regulatory pool stimulates hepatic cholesterol synthesis, with no changes in extrahepatic cholesterol synthesis (Woollett et al., 1992). But if MUFA is replaced by SFA, both plasma LDL-C level and cholesterol synthesis appear to shift in an inverse manner.

Canola oil is also rich in phytosterols, including campesterol (1.84 mg/g), β-sitosterol (3.60 mg/g), and brassicasterol (0.49 mg/g) (Cabral & Klein, 2017). As one of the cholesterol absorption inhibitors, the intake of phytosterols reduces the plasma cholesterol level by competing with cholesterol for incorporation into mixed micelles (Jesch & Carr, 2017). Cholesterol absorption is generally inversely correlated with cholesterol synthesis in many studies (Cohen, 2008; Hirose et al., 1991; Pihlajamäki et al., 2004; Tilvis & Miettinen, 1986).

In this study, we hypothesized that an elevated cholesterol synthesis, which might be stimulated by the intake of MUFA in cooperation with a reduction in cholesterol absorption caused by competition from phytosterol, will contribute to a reduction in circulating cholesterol levels. As a surrogate biomarker of cholesterol absorption, the cholestanol to cholesterol ratio showed an inverse trend compared with the campesterol to cholesterol ratio and the β-sitosterol to cholesterol ratio. The cholestanol to cholesterol ratio supported our assumption, while the other two biomarkers failed. The paradoxical result might be due to the high content of phytosterols in canola oil. The elevated intake of phytosterols increased their content in plasma, resulting in a higher ratio of phytosterol to cholesterol. It was concluded in Miettinen’s study (2011) that the campesterol and sitosterol to cholesterol ratios failed to indicate cholesterol absorption if they were rich in
the intervention diet, but the cholestenol to cholesterol ratio was still a good marker to assess the cholesterol absorption in clinical trial even if phytosterols were served in the diet (Miettinen et al., 2011). The cholestanol to cholesterol ratio was more sensitive as a cholesterol absorption surrogate marker in our study. The absolute serum campesterol and β-sitosterol levels during the two canola oil diet phases were higher compared with those observed during the control diet phase, which was induced by the intake of high phytosterols in canola treatment oil. The ratio between lathosterol to campesterol or β-sitosterol, which was used to assess the competition between cholesterol synthesis and cholesterol absorption, did not show significant differences in the current study. As cholesterol synthesis biomarkers, the desmosterol to cholesterol ratio proved our assumption that replacement of WDO with canola oil in diet stimulated the cholesterol synthesis in MetS patients, but the lathosterol ratio failed to do so. Desmosterol and lathosterol are both cholesterol precursors, but diverge during the last steps of cholesterol synthesis. Desmosterol converts to cholesterol through the Bloch pathway, while lathosterol undergoes the Kandutsch-Russell pathway (Myant, 2014). The consumption of canola oil may differentially influence the two pathways in cholesterol synthesis. According to the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) database, canola oil contains <700 mg of plant sterols per 100 g of oil. Within the design of the COMIT II study, dietary treatments had 60g of oil per 3,000kcal diet, thus providing <450 mg per day of sterols. However, the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III recommends 2g per day of phytosterols to lower cholesterol, which means the dose of phytosterols in the HOCO diet
and regular canola oil diet may not be sufficient to influence the cholesterol synthesis or cholesterol absorption.

The plasma 7αC4 level after the HOCO diet, canola oil diet, and control oil diet interventions were 24.37 ng/ml, 23.47 ng/ml, and 22.36 ng/ml, respectively. The plasma 7αC4 level in healthy people ranges from 19 ng/ml to 27 ng/ml (Björkhem et al., 1987). As such, the bile acid biosynthesis rate of the participants in the current (COMIT II) study was within the range observed in healthy individuals. Our study showed that the consumption of HOCO stimulated bile acid biosynthesis rates. A previous bile acid-binding resin cholestyramine study reported that the elevation of bile acid synthesis rates increased LDL uptake by the liver and then reduced circulating cholesterol levels (Bertolotti et al., 2008). The plasma TC and LDL-C decreased by 11.9% and 11.8%, respectively, after treatment of bile acid-binding resin cholestyramine (Bertolotti et al., 1991). Thus, the consumption of HOCO has the potential to reduce circulating cholesterol levels by facilitating bile acid synthesis.

The current COMIT II study was a well-controlled, full feeding, cross-over RCT. It was no surprise to find that intake of canola oil and HOCO resulted in reduced TC and LDL-C. Because of the high content of campesterol and β-sitosterol existing in canola oil and HOCO, the campesterol to cholesterol ratio and β-sitosterol to cholesterol ratio failed to reflect changes in cholesterol absorption. However, the cholestanol to cholesterol level reduced significantly after the canola oil diet and HOCO diet, compared to the control oil diet, which indicated that the consumption of canola oil or HOCO suppressed the cholesterol absorption in patients with MetS. The suppressed cholesterol absorption and
enhanced bile acid synthesis may explain why the consumption of HOCO reduced the circulating cholesterol level.
Chapter III

Manuscript

The effect of canola oil enriched diets on the association between cholesterol metabolism and serum lipid profiles in individuals at risk for metabolic syndrome

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\textsuperscript{2}Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, MB, Canada

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3.1 Introduction

Metabolic syndrome (MetS) represents a group of health problems that increases the risk of developing cardiovascular diseases (CVD) and diabetes. Elevated total cholesterol (TC), total triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), blood pressure (BP), and waist circumference, as well as reduced high-density lipoprotein (HDL-C), are all identified as risk factors closely associated with MetS (Riediger & Clara, 2011). Lifestyle modification, pharmaceutical therapy, and surgery are therapeutic approaches for MetS. The quality of dietary oil, referring to the composition of fatty acids in the edible oil, plays an important role in modulating MetS through manipulating the fatty acid composition of serum lipid esters (Warensjö et al., 2006).

As a source of monounsaturated fatty acids (MUFA), canola oil has been suggested to replace saturated fatty acids (SFA) in a typical Western diet. Increased dietary MUFA intake has been linked to reduced plasma cholesterol levels (Hodson et al., 2001; Matheson et al., 1996; Noakes & Clifton, 1998; Palomäki et al., 2010), decreased BP (Ferrara et al., 2000; López-Miranda et al., 2010; Terés et al., 2008), enhanced insulin sensitivity (López et al., 2008), and increased energy expenditure (Liu et al., 2016). Although it has been widely shown that substitution of SFA with MUFA has a protective influence on plasma lipid profiles, including reducing plasma TC and LDL-C, the mechanism behind the health benefit requires exploration.

The circulating cholesterol concentrations are maintained through a balance of the endogenous pathways, including cholesterol synthesis in the liver and extrahepatic tissues, cholesterol catabolism, and cholesterol secretion into bile; and the exogenous pathways, consisting of cholesterol and biliary source absorption in the intestine.
(Shepherd, 2001). Statins (Blumenthal, 2000), ezetimibe (Berthold et al., 2008; Shepherd, 2001), and phytosterols (Jones et al., 2000) have been recommended as good therapeutical methods to reduce serum LDL-C levels. The portfolio diet study was aimed to assess whether the combination of viscous fibres, soy protein, plant sterols and nuts yielded the same magnitude of LDL-C reduction compared with statin therapy (Kendall & Jenkins, 2004). Each component in the portfolio diet contributes differently to regulate plasma cholesterol. For instance, the intake of viscous fibres increases bile acid losses, while the consumption of plant sterols increase faecal cholesterol losses (Jenkins et al., 2002). In the three portfolio diet studies, a diet including viscous fibres, soy protein, plant sterols and nuts, resulted in a 28% to 35% reduction in serum LDL-C compared to baseline (Jenkins et al., 2002; Jenkins et al., 2003). The statin treatment yielded an approximately 34% reduction in serum LDL-C (Ornish et al., 1990), which was close to the efficiency of the portfolio diet. These studies showed the modification of diet may also effectively reduce the LDL-C levels. In chapter II, the data indicated that the consumption of canola oil in the diet has the potential to reduce plasma TC and LDL-C levels. It is worthy to assess whether the reduction of circulating cholesterol level after a canola oil dietary intervention affords similar therapeutic potential as statins, ezetimibe, or phytosterols.

During the past seventy years, many different methods have been developed to measure cholesterol synthesis and cholesterol absorption (MacKay & Jones, 2011). Non-cholesterol sterols (NCSs) are verified surrogate markers to assess cholesterol metabolism. Cholesterol precursors, such as lathosterol and desmosterol, are used as markers of endogenous cholesterol synthesis, while plant sterols, such as campesterol,
sitosterol and cholestanol, are regarded as markers of cholesterol absorption (MacKay et al., 2014). Measurement of these sterols in serum/plasma represents a convenient method for the measurement of both cholesterol synthesis and cholesterol absorption from one sample. The deuterium incorporation method, regarded as the “gold standard” in measuring cholesterol synthesis, is always used to assess the validity of other measurements (Jones et al., 1998). This method uses deuterated water as a tracer to assess how fast the incorporated deuterium water participates in the de novo cholesterol synthesis. Both the deuterium enrichment in plasma water and free cholesterol existing in RBC are required for further calculation. Hence, the isotope method for measuring cholesterol synthesis is more complex and time-consuming compared to the indirect method. In the previous chapter, desmosterol to cholesterol ratio and lathosterol to cholesterol ratio were measured as surrogate markers to indicate cholesterol synthesis. However, desmosterol to cholesterol ratio and lathosterol to cholesterol ratio showed different results in the previous chapter. As such, the deuterium incorporation method was applied in the same subjects with the objective of providing a more definitive assessment of cholesterol metabolism.

Currently, many studies focused on the relation between cholesterol metabolism and glucose metabolism (Gylling et al., 2007; Pihlajamäki et al., 2004; Simonen et al., 2000). The combination of high cholesterol synthesis and low cholesterol absorption rates was closely related to insulin resistance, which subsequently increased the prevalence of MetS (Pihlajamäki et al., 2004; Simonen et al., 2000). However, few previous studies have investigated the correlation between cholesterol metabolism and circulating cholesterol levels, which is also regarded as a criterion of MetS. The current (COMIT II)
study found that the intake of canola oil helped to reduce LDL-C and TC levels. However, whether the drops in circulating cholesterol were associated with cholesterol metabolism is not clear. The objective of this study is was to assess the association between cholesterol metabolism markers and serum lipid profiles from patients enrolled in a randomized clinical trial (RCT) with different dietary lipids included as the treatment arms.

3.2 Experimental methods

3.2.1 Study design and population

Study design and participant enrollment were introduced in Chapter II. Participants consumed a dose of 0.7g deuterium oxide/kg body weight (99%, Cambridge Isotope Laboratories, Inc.) in the morning on day 41 of each phase. Participants were asked to drink the deuterium water under the supervision of coordinator. It was optional to drink more pure water after intake of the deuterium water.

3.2.2 Controlled diets and oil interventions

Controlled diets and oil interventions were introduced in Chapter II.

3.2.3 Blood sample collections

On days 1, 2, 41 and 42 of each dietary treatment, 20 ml fasting blood samples were collected. Blood samples used for stable isotope measurement were collected on the last two days (day 41 and 42) at each phase. Plasma and red blood cell (RBC) fraction was separated from the blood sample and allocated at the bottom of the vacutainer after
the centrifugation (3000 rpm, 20 minutes at 4°C). The supernatant fraction was the serum sample. Both serum and RBC samples were then stored at -80°C for further analysis.

3.2.4 Surrogate markers of cholesterol synthesis and cholesterol absorption

Surrogate markers of cholesterol synthesis and cholesterol absorption were introduced in Chapter II.

3.2.5 Stable isotope intake and analysis

Cholesterol was extracted from the RBC samples through the use of the Folch method (Folch et al., 1957). The sample (0.5g RBC) was added to 8 ml of methanol, the tube capped tightly and mixed on a vortex mixer for 10 seconds. Following mixing, the mixture was heated at 55°C in a shaking water bath for 15 minutes. Tubes were left to cool to room temperature in a fumehood, and then 24 ml of extraction solvent (hexane: chloroform (4:1)) and 2 ml of double-distilled water was added to the mixture. The mixture was mixed on a vortex mixer for 30 seconds, followed by centrifugation at 1500 rpm for 15 minutes at 4°C. Following centrifugation, the supernatant was transferred into a new tube, and the solvent extraction process repeated, and the resultant supernatant fractions pooled. The pooled supernatant fraction was dried under a stream of nitrogen gas, and redissolved in 1ml chloroform. Finally, the samples were stored in sealed vials and stored at -80°C for further analysis.

The extracted solvent was then injected into a GC (Agilent 6890N; Agilent Technologies Canada Inc., Mississauga, ON, Canada)-IRMS (Thermo Finnigan, Bremen, Germany) system to measure the deuterium enrichment. A SAC-5 column (30m ×0.25mm, Sigma-Aldrich Ltd., Oakville, ON, Canada) was used to separate the
cholesterol compounds from the lipid extracts. The isolated cholesterol compound was then directed to the combustion reactor (Combustion Interface III; Bremen, Germany) to release H\textsubscript{2}. The plasma water was purified by passing the plasma sample through a 4 mm syringe filter (Phenex 0.2 μm PTFE membrane; Phenomenex, Torrance, CA, US) prior to injection into the same GC-IRMS system. The deuterium enrichment was measured relative to Standard Mean Ocean Water in the MS unit. The equation used to calculate the fractional synthesis rate of free cholesterol (FSR-FC) is shown below:

\[
\text{FSR - FC} \left( \frac{\text{pools}}{\text{day}} \right) = \left( \frac{\delta \text{ cholesterol} \%_0}{\delta \text{ plasma water} \%_0 \times 0.478} \right)
\]

Where δ cholesterol is the change of deuterium enrichment over the last 24 hours (between day 41 and day 42), δ plasma water is the change of deuterium enrichment over the same duration. The factor 0.478 refers to the ratio of the hydrogen atoms that may be enriched by deuterium to total hydrogen in a cholesterol molecule.

### 3.2.6 Statistical method

#### 3.2.6.1 Endpoint comparison across treatments

Statistical analyses were performed using the SAS MIXED procedure (v9.4, Cary, NC). All results were expressed as least square means ± standard error mean. Normality of data was observed and checked visually based on the plot of residuals. Treatment, the sequence of treatment, age of participant, and sex were considered as fixed factors, whereas participant and test centre were considered as random factors. Tukey-adjustment was used to account for multiple comparisons. Statistical difference was set at P < 0.05 for all analyses.
3.2.6.2 Correlation assessment

Normality of endpoint variables was assessed using the univariate procedure. All variables were not normally distributed; therefore, Spearman rank correlations were assessed. Correlations were reported by treatment to satisfy the assumption of independence.

3.3 Results

The absolute values of serum NCSs concentrations at the endpoint of each treatment are shown in Table 2.5. The value of cholesterol metabolism surrogate markers at the endpoint of each treatment are shown in Table 2.6. The interpretation of these data is shown in Chapter II.

The FSR-FC values at the endpoint of each treatment are shown in Figure 3.1. There were no significant differences in endpoint FSR-FC among the three treatments. All the desmosterol to cholesterol ratio, lathosterol to cholesterol ratio, and FSR-FC from every participant in three treatments were included in Spearman correlation analysis to verify the surrogate marker method in testing cholesterol synthesis. Both desmosterol to cholesterol ratio and lathosterol to cholesterol ratio were significantly associated with FSR-FC (Table 3.1).

Table 3.1 Correlations between FSR-FC and cholesterol synthesis biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Desmosterol/ Cholesterol (µmol/L/mmol/L)</th>
<th>Lathosterol/ Cholesterol (µmol/L/mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSR-FC (pools/day)</td>
<td>0.329</td>
<td>0.873</td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
Figure 3.1 Measurement of FSR-FC in RBC at endpoint test across the three treatment groups. All values are least-squares means ± SEs; n = 111. Mean values within a row with different superscript letters are significantly different between treatments, P < 0.05 (mixed-model ANOVA and post hoc Tukey’s test). FSR-FC, fractional synthesis rate of free cholesterol; HOCO, high-oleic acid canola oil; RBC, red blood cell.

Spearman correlations between endpoint cholesterol metabolism surrogate markers and endpoint serum lipid profiles, as well as lipoproteins in HOCO, canola oil, and control oil diet treatments are shown in Table 3.2, Table 3.2, and Table 3.3, respectively. Campesterol/cholesterol and β-sitosterol/cholesterol were still good cholesterol absorption surrogate markers in WDO treatment due to the negligible phytosterol intake. HDL-C level was negatively correlated with the cholesterol synthesis markers, desmosterol/cholesterol \( r_s = 0.37 \) to \( -0.45 \) and lathosterol/cholesterol \( r_s = -0.33 \).
to -0.36). TG levels were positively correlated with the cholesterol synthesis markers, desmosterol/cholesterol ($r_s=0.27$ to 0.40) and lathosterol/cholesterol ($r_s=0.41$ to 0.47), but negatively correlated with cholesterol absorption marker, $\beta$-sitosterol/cholesterol ($r_s=-0.20$ to -0.31).

### Table 3.2 Correlations between endpoint cholesterol metabolism surrogate markers and endpoint serum lipid profile as well as lipoproteins in the HOCO diet treatment

<table>
<thead>
<tr>
<th></th>
<th>TC (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Non-HDL-C (mmol/L)</th>
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<tbody>
<tr>
<td>Cholestanol/ Cholesterol ($\mu$mol/L mmol/L)</td>
<td>-0.00375</td>
<td>0.0801</td>
<td>0.01463</td>
<td>-0.15733</td>
<td>-0.00452</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.9688</td>
<td>0.4033</td>
<td>0.8789</td>
<td>0.0991</td>
<td>0.9625</td>
</tr>
<tr>
<td>Desmosterol/ Cholesterol ($\mu$mol/L mmol/L)</td>
<td>-0.14022</td>
<td>-0.09393</td>
<td>-0.37455</td>
<td>0.28864</td>
<td>0.02495</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.1422</td>
<td>0.3268</td>
<td>&lt;.0001</td>
<td>0.0021</td>
<td>0.7949</td>
</tr>
<tr>
<td>Lathosterol/ Cholesterol ($\mu$mol/L mmol/L)</td>
<td>0.00268</td>
<td>-0.00762</td>
<td>-0.35815</td>
<td>0.46552</td>
<td>0.17042</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.9777</td>
<td>0.9367</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0737</td>
</tr>
<tr>
<td>Campesterol/ Cholesterol ($\mu$mol/L mmol/L)</td>
<td>-0.07075</td>
<td>0.01559</td>
<td>0.12046</td>
<td>-0.29674</td>
<td>-0.10918</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.4606</td>
<td>0.8710</td>
<td>0.2079</td>
<td>0.0016</td>
<td>0.2540</td>
</tr>
<tr>
<td>$\beta$-Sitosterol/ Cholesterol ($\mu$mol/L mmol/L)</td>
<td>-0.15589</td>
<td>-0.12323</td>
<td>0.18068</td>
<td>-0.30504</td>
<td>-0.23018</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.1023</td>
<td>0.1976</td>
<td>0.0577</td>
<td>0.0011</td>
<td>0.0151</td>
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</table>
Table 3.3 Correlations between endpoint cholesterol metabolism surrogate markers and endpoint serum lipid profile as well as lipoproteins in the canola oil diet treatment

<table>
<thead>
<tr>
<th>Surrogate Marker</th>
<th>TC (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Non-HDL-C (mmol/L)</th>
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<tr>
<td>Cholestanol/Cholesterol</td>
<td>0.00726</td>
<td>0.06616</td>
<td>-0.01297</td>
<td>-0.13398</td>
<td>-0.0046</td>
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<tr>
<td></td>
<td>P 0.9397</td>
<td>0.4902</td>
<td>0.8925</td>
<td>0.1609</td>
<td>0.9617</td>
</tr>
<tr>
<td>Desmosterol/Cholesterol</td>
<td>-0.07196</td>
<td>-0.03001</td>
<td>-0.41619</td>
<td>0.26944</td>
<td>0.09384</td>
</tr>
<tr>
<td></td>
<td>P 0.4529</td>
<td>0.7546</td>
<td>&lt;.0001</td>
<td>0.0042</td>
<td>0.3272</td>
</tr>
<tr>
<td>Lathosterol/Cholesterol</td>
<td>-0.03124</td>
<td>-0.05731</td>
<td>-0.34832</td>
<td>0.40674</td>
<td>0.11941</td>
</tr>
<tr>
<td></td>
<td>P 0.7448</td>
<td>0.5502</td>
<td>0.0002</td>
<td>&lt;.0001</td>
<td>0.2119</td>
</tr>
<tr>
<td>Campesterol/Cholesterol</td>
<td>-0.08002</td>
<td>0.00147</td>
<td>0.11809</td>
<td>-0.29353</td>
<td>-0.11283</td>
</tr>
<tr>
<td></td>
<td>P 0.4038</td>
<td>0.9877</td>
<td>0.2171</td>
<td>0.0018</td>
<td>0.2384</td>
</tr>
<tr>
<td>β-Sitosterol/Cholesterol</td>
<td>-0.08478</td>
<td>-0.05653</td>
<td>0.16658</td>
<td>-0.26268</td>
<td>-0.14181</td>
</tr>
<tr>
<td></td>
<td>P 0.3763</td>
<td>0.5556</td>
<td>0.0806</td>
<td>0.0053</td>
<td>0.1376</td>
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</table>
### Table 3.4
Correlations between endpoint cholesterol metabolism surrogate markers and endpoint serum lipid profile as well as lipoproteins in the control oil diet treatment

<table>
<thead>
<tr>
<th></th>
<th>TC (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Non-HDL-C (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholestanol/ Cholesterol</td>
<td>-0.03724</td>
<td>0.05214</td>
<td>-0.07868</td>
<td>-0.03307</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.6980</td>
<td>0.5868</td>
<td>0.4117</td>
<td>0.7304</td>
<td>0.9892</td>
</tr>
<tr>
<td>Desmosterol/ Cholesterol</td>
<td>-0.02807</td>
<td>0.0132</td>
<td>-0.45247</td>
<td>0.40293</td>
<td>0.14864</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.7699</td>
<td>0.8906</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.1195</td>
</tr>
<tr>
<td>Lathosterol/ Cholesterol</td>
<td>0.056</td>
<td>0.03787</td>
<td>-0.32794</td>
<td>0.43226</td>
<td>0.17114</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.5594</td>
<td>0.6931</td>
<td>0.0004</td>
<td>&lt;.0001</td>
<td>0.0725</td>
</tr>
<tr>
<td>Campesterol/ Cholesterol</td>
<td>0.00245</td>
<td>0.03527</td>
<td>0.08377</td>
<td>-0.10356</td>
<td>-0.01379</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.9797</td>
<td>0.7133</td>
<td>0.3821</td>
<td>0.2794</td>
<td>0.8857</td>
</tr>
<tr>
<td>β-Sitosterol/ Cholesterol</td>
<td>-0.15365</td>
<td>-0.12639</td>
<td>0.10138</td>
<td>-0.19606</td>
<td>-0.16869</td>
</tr>
<tr>
<td></td>
<td>$P$ 0.1074</td>
<td>0.1862</td>
<td>0.2897</td>
<td>0.0392</td>
<td>0.0767</td>
</tr>
</tbody>
</table>

### 3.4 Discussion

As the “gold standard” in measuring cholesterol synthesis (Jones et al., 1998), deuterium incorporation method was applied in this study to verify the indirect method used in the previous chapter. The cholesterol synthesis measured by deuterium incorporation method has been shown to correlate well with the value measured by the cholesterol synthesis surrogate method (Matthan et al., 2000). In this method verification study, squalene, lanosterol, desmosterol, and lathosterol to cholesterol ratio were measured from 14 hypercholesterolemic women across 6 treatment diets. These
cholesterol synthesis surrogate markers were all significantly ($P < 0.0001$) correlated with the FSR-FC. Our present study, including 111 participants and 3 treatment diets, provided more abundant data to verify the indirect method. Both the desmosterol to cholesterol ratio and the lathosterol to cholesterol ratio showed significant association with FSR-FC, which meant these two surrogate markers used in this study were credible biomarkers of cholesterol synthesis. There were no significant differences in endpoint FSR-FC among three treatments, which agree with the result of lathosterol to cholesterol ratio.

Accompanied by the result in the previous chapter, the consumption of canola oil and HOCO suppressed the cholesterol absorption with no change in cholesterol synthesis in patients with MetS. The hypothesis of the current study was that the endpoint plasma TC and LDL-C were negatively correlated with cholesterol synthesis surrogate markers and positively correlated with cholesterol absorption surrogate markers. However, the plasma TC and LDL-C were not statistically associated with any cholesterol metabolic surrogate markers. The cholesterol-lowering effect of consuming canola oil was not due to the combination of increased cholesterol synthesis and reduced cholesterol absorption.

Vanu and others (2012) conducted a cross-over clinical trial to investigate whether the decreased plasma TC and LDL-C after high PUFA/SFA (2:1) diets were due to the shift of cholesterol absorption or synthesis. The high PUFA/SFA diets resulted in a lower plasma TC ($P < 0.02$) and LDL-C ($P < 0.02$) level compared with the low PUFA/SFA (1:2) diet. Both the deuterium incorporation method and the surrogate markers method were applied in this study. However, cholesterol metabolism was not influenced by the diet. Thus, the decreased circulating cholesterol level after high PUFA
diets were not related to cholesterol absorption or synthesis (Ramprasath et al., 2012). Accompanied by the findings in our study, the cholesterol-lowering effect of replacing SFA with MUFA or PUFA was not associated with the change of cholesterol metabolism.

Rideout and others (2010) carried out a study to figure out how the basal FCS-FC influenced the therapeutical efficiency of plant sterol on plasma LDL-C. The subjects in this compliance-controlled feeding RCT were asked to consume the isocaloric diet with approximate 2g phytosterol per day during the control phase. The percentage change of LDL-C from the control phase was associated (P = 0.02) with the basal FCS-FC (Rideout et al., 2010). According to this study, patients with elevated cholesterol synthesis may be non-responders to phytosterol therapy. In our present study, we only measured the endpoint cholesterol metabolism surrogate marker. The measurement of baseline cholesterol synthesis or absorption was not included in the study. We were unable to assess how baseline cholesterol metabolism related to the change of TC and LDL-C after the intervention diets.

The lathosterol/cholesterol and desmosterol/cholesterol ratio were negatively related (P < 0.001) to plasma HDL-C level, and positively related (P < 0.001) to plasma TG level. Elevated TG and reduced HDL-C are two of the risk factors of MetS (Riediger & Clara, 2011). Gylling and others (2007) conducted a study to investigate the relationship between cholesterol metabolism and glucose metabolism in MetS patients. They found MetS patients had higher cholesterol synthesis and lower cholesterol absorption compared to healthy subjects (Gylling et al., 2007). According to the present study, MetS patients with elevated cholesterol synthesis may have lower plasma HDL-C
levels and higher TG levels. Consequently, increased cholesterol synthesis may be related to the elevated risk to develop CVD and diabetes. In conclusion, high cholesterol synthesis can be used as another indicator of the presence of MetS, and that people with elevated cholesterol synthesis should monitor their HDL-C and TG.
General discussion and conclusions

4.1 General discussion and implications

In the previous canola oil multicenter intervention trial (COMIT) study, DHA-enriched canola oil showed its potential to enhance cholesterol synthesis, measured by using the lathosterol/cholesterol ratio (Jones et al., 2015). However, the lack of a Western control oil became the main limitation of the study (Jones et al., 2014). COMIT II study compared regular canola oil (RO) and high oleic canola oil (HOCO) with typical Western diet oil (WDO). Both the non-cholesterol sterols (NCSs) method and the deuterium incorporation method were applied to analyse how cholesterol synthesis and absorption were influenced by the diet. Cholesterol precursors, such as lathosterol and desmosterol, are used as markers of endogenous cholesterol synthesis, while plant sterols, such as campesterol, sitosterol and cholestanol, are regarded as markers of cholesterol absorption (MacKay et al., 2014). As the cholesterol absorption surrogate biomarker, the cholestanol to cholesterol ratio showed an inversed trend compared with campesterol to cholesterol ratio and \( \beta \)-sitosterol to cholesterol ratio. The higher cholestanol ratios proved that the intakes of two types of canola oils increase the cholesterol absorption comparing to the WDO, but campesterol and \( \beta \)-sitosterol failed to show the same trend.

In the present study, two methods of plasma TC measurement and two methods of cholesterol synthesis assessment were applied. The plasma TC measured by autoanalyser showed a similar result with the value measured by GC-FID method. The significant reduction of plasma TC after canola oil diet was shown in both methods, as demonstrated in Chapter II. GC-FID method can provide both the plasma TC level and cholesterol metabolism surrogate markers value in one measurement, which is more feasible for the
study that only assesses the relation between total circulating cholesterol and cholesterol synthesis or absorption. In Chapter III, the cholesterol synthesis measured by deuterium incorporation method was correlated ($P < 0.0001$) with lathosterol/cholesterol and desmosterol/cholesterol ratio. Both the isotope incorporation method and the surrogate marker method were credible to measure cholesterol synthesis. The overestimated fear of radioisotopic tracer has been shown to increase the dropout rate in clinical studies (Viteri et al., 2002). Consequently, the surrogate marker method is a more feasible choice of cholesterol synthesis measurement for exploration study in a clinical trial.

The bile acid synthesis biomarker, 7α-hydroxy-4-cholesten-3-one (7αC4), was significantly higher ($P < 0.05$) after the HOCO treatment, as demonstrated in Chapter II. The circulating cholesterol-lowering effect of consuming HOCO was related to the accelerated bile acid conversion. The bile acid de novo synthesis in the liver has been proved to replenish the bile acids lost in the faeces (Chiang, 2013; Cohen, 2008). The elevated 7αC4 in HOCO treatment has the potential to accelerate bile removal in faeces. Moreover, bile acid synthesis plays an important role in regulating the expression of the LDL receptor. The increased bile acid synthesis rate promoted the LDL-C removal by improving LDL receptor expression (Charlton-Menys & Durrington, 2008). In the present study, the effect of consuming HOCO on bile acid removal and the expression of the LDL receptor was not analyzed.

The plasma TC and LDL-C were not statistically associated with any cholesterol metabolism surrogate markers in the COMIT II study. Matthan and others (2003) conducted a study and found that the cholestanol/cholesterol ratio was negatively associated with plasma TC and LDL-C (Matthan et al., 2009). The mechanism of this
statin treatment study is different than our study because statin reduced LDL-C by reduced cholesterol synthesis and increased cholesterol absorption. The hypotheses of the current study were that the endpoint plasma TC and LDL-C was negatively correlated with cholesterol synthesis surrogate markers and positively correlated with cholesterol absorption surrogate markers. However, results from the current in COMIT II study were unable to prove this correlation.

Several studies have shown that cholesterol synthesis was correlated with HDL-C, TG, and BMI (Di Buono et al., 1999; Gylling et al., 2007; Pihlajamäki et al., 2004; Simonen et al., 2000). In the COMIT II study, cholesterol synthesis was negatively related (P < 0.001) to plasma HDL-C level, and positively related (P < 0.001) to plasma TG level. Accompanied by previous studies, people with elevated cholesterol synthesis have a high risk to develop CVD and diabetes. The presence of high cholesterol synthesis was a good indicator to diagnose MetS.

### 4.2 Limitations and future perspectives

The circulating cholesterol level dropped significantly after the consumption of both regular canola oil and HOCO, which agrees with the previous MUFA studies (Hodson et al., 2001; Matheson et al., 1996; Noakes & Clifton, 1998; Palomäki et al., 2010). Based on the cholestanol ratio data, the intake of canola oil decreased the cholesterol absorption in MetS patients. According to the reciprocal relationship between cholesterol absorption and synthesis found in physiological and therapeutical studies (Santosa et al., 2007), the reduced cholesterol absorption may result in elevated cholesterol synthesis to maintain cholesterol homeostasis. But both the lathosterol ratio
and FSR-FC failed to show that the intake of canola oil can increase cholesterol synthesis.

The absence of baseline cholesterol synthesis and absorption data is the predominant limitation of this study. Basal cholesterol synthesis was associated (P = 0.02) with the percentage change of LDL-C after the phytosterol treatment (Rideout et al., 2010). In the future study, baseline surrogate markers for cholesterol synthesis and absorption should also be measured. Whether the change of LDL-C after canola oil treatments is associated with basal cholesterol metabolism information should be evaluated. Moreover, the association between the change of cholesterol synthesis/absorption and change of plasma TC and LDL-C should be assessed.

Lack of plasma fatty acid profile data is another limitation of this study. In this study, the assumption was made that plasma fatty acid is correlated with dietary fatty acid and the intake of canola oil will increase MUFA in plasma. The increased MUFA in plasma down-regulate the LDL-C as demonstrated in Chapter I. At present, it is unclear how canola oil intake reduces the LDL-C without access to full plasma fatty acid profile data.

Canola oil is high in phytosterols while WDO contains a negligible amount of phytosterols. It is a challenge to tell whether the effect of consuming canola oil is due to the intake of MUFA or phytosterols (or both). As such, the phytosterol content in the treatment oil should be evaluated and standardized in future studies. Additionally, campesterol and β-sitosterol are not credible surrogate markers of cholesterol absorption if phytosterols were served in the diet (Miettinen et al., 2011).
The portfolio diet involves varieties of cholesterol-lowering foods and has resulted in comparative therapeutic efficacy with statins. The addition of other cholesterol-lowering agents, such as phytosterols, β-glucan, soybean and fruits, are a good strategy to enhance the health benefit.
References


Gebauer, S. K., Psota, T. L., Harris, W. S., & Kris-Etherton, (2006). n–3 fatty acid dietary recommendations and food sources to achieve essentiality and


on abdominal fat mass in individuals with central obesity. *Obesity*, **24**(11), 2261-2268.


rapeseed oil and butter on serum lipids, oxidized LDL and arterial elasticity in men with metabolic syndrome. *Lipids in Health and Disease, 9*(1), 137.


markers of cholesterol absorption and synthesis and LDL-C lowering in hyperlipidemic patients. *Atherosclerosis*, 225(2), 388-396.


Appendices

Appendix I: Ethics approval for the study

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES
Full Board Review

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<th>ETHICS #:</th>
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<td>Dr. P. Jones</td>
<td>U of M/RCCFN</td>
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<td>September 3, 2013</td>
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CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on the Certificate of Final Approval at the full board meeting date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.

BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in
Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:
1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. For logistics of performing the study, approval must be sought from the relevant institution(s).
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approval that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the Bannatyne Campus Research Amendment Form.
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the Bannatyne Campus Final Study Status Report.

Sincerely,

[Signature]

Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Human Ethics Number on all correspondence.
Inquiries should be directed to the REB Secretary Telephone: (204) 789-3255/ Fax: (204) 789-3414
BIOMEDICAL RESEARCH ETHICS BOARD (BREB)

CERTIFICATE OF ANNUAL APPROVAL

<table>
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<tr>
<th>PRINCIPAL INVESTIGATOR:</th>
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<th>ETHICS #:</th>
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<tr>
<td>Dr. Peter Jones</td>
<td>U of M and RCFN/Food Sciences</td>
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<td>Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oelic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors (Linked to B2010:052 and B2007:071)</td>
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<tr>
<th>THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:</th>
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<tr>
<td>Document Name(if applicable)</td>
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Annual approval

Annual approval implies that the most recent BREB approved versions of the protocol, Investigator Brochures, advertisements, letters of initial contact or questionnaires, and enrollment methods, etc. are approved.

Consent and Assent Form(s):

CERTIFICATION

The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the annual study status report for the research study/project named on this Certificate of Annual Approval as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. Annual approval was granted by the Chair or Acting Chair, UM BREB, per the response to the conditions of approval outlined during the initial review (full board or delegated) of the annual study status report.

BREB ATTESTATION

The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

umanitoba.ca/research

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QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

<table>
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<th>CONDITIONS OF APPROVAL:</th>
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<tr>
<td>1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. For logistics of performing the study, approval must be sought from the relevant institution(s).</td>
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<tr>
<td>4. This approval is valid until the expiry date noted on this certificate of annual approval. A Bannatyne Campus Annual Study Status Report must be submitted to the REB within 15-30 days of this expiry date.</td>
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Lindsay Nicolle, MD, FRCP
Chair, Bannatyne Research Ethics Board
Bannatyne Campus

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

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study:  
**Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oleic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors**

Investigator: Peter J.H. Jones, PhD  
Richardson Centre for Functional Foods and Nutraceuticals  
University of Manitoba  
196 Innovation Drive  
Winnipeg, Manitoba R3T 6C5  
Phone: 204 474 9787

Sponsor: Canola Council of Canada, 400-10/ Lombard Avenue, Winnipeg, Manitoba, R3B OT6

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 staff to explain any words or information that you do not clearly understand. The study doctor and institution are receiving professional fees and financial support to conduct this study.

**Purpose of study**

Obesity is a contributing factor to most chronic diseases and a burden on human health throughout the world. The ability to limit or even reduce excess weight gain through modification of fatty acid intake would be beneficial in combating the obesity epidemic. The primary objectives of the study are to examine the health benefits of dietary canola oils on body composition and cardiovascular disease risk factors. It is anticipated that consuming these healthy oils will favorably alter body composition, specifically through

May 22, 2015, Version 5  
Participant initials _____
the reduction of android (stomach) fat. Furthermore, we are also examining the effects of dietary canola oils on many other cardiovascular disease risk factors.

Study procedures

Pre-screening procedures
If you agree to take part in this study, you will be asked to give a fasting (nothing to eat or drink 12 hours before the test) blood sample (approximately two teaspoons) to measure your blood lipid levels and additional biochemistry parameters. In addition, we will measure your blood pressure and waist circumference. Prior to beginning the study, you will undergo a physical examination by a physician to ensure that you are in good health. During the physical examination, the physician will measure your vital signs, examine the normality of body systems and ask you some questions regarding your medical history. The study physician and or study staff will review medical history and ask questions to determine whether you are eligible to participate. Any change in your health status at any point during the study needs to be reported to the study investigators.

Study procedures
The study will consist of 3 phases of 42 days (6 weeks) each during which you will consume a fixed composition of a precisely controlled weight-maintaining diet. Each study phase will be followed by a washout period of 6 weeks where you can consume your habitual diets. During each study phase, you will be provided with a balanced, precisely controlled weight-maintaining diet (35% energy from fat, 50% carbohydrate, and 15% protein). Each phase will differ only in the dietary oils provided as part of the controlled diet as follows:

1) Control phase: Dietary fat consumed will provide 35% of total energy and will be comprised of “Western diet” fats in the form of a mixture of common dietary oils.

2) Canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% of total energy from fat from canola oil.

3) High oleic acid canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% of total energy from fat from high oleic acid canola oil. Oleic acid is a specific dietary fatty acid of interest with health benefits.

This study is a double-blind design, which means that neither you nor the study staff will know which oil variety you are receiving in each phase. In the unlikely event of an emergency, this information will be made available.

Study diets will be prepared in the metabolic kitchen of the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN). You will consume at least 1 of 3 daily meals at the RCFFN under supervision. The other meals will be prepared and packed to be taken out. The treatment oils will be provided as a part of the meals, as appropriate for each phase. You will be asked to consume only the food provided by our metabolic kitchen during each study phase. Additionally, we ask that you do limit caffeinated
beverages consumption to one to two beverages a day, alcoholic beverages consumption to 2 beverages a week. No alcoholic beverages are to be consumed within 48 hours prior to blood draws during the study periods. No caffeine beverages consumption within 12 hours prior to blood draws during the study periods

We will measure the amount of fat in your body using a procedure called dual energy x-ray absorptiometry (DXA). These analyses will be performed 6 times in total during the study, once at the beginning of each phase and once at the end of each phase. 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). In addition, you will need to ensure that you will not undergo barium tests/exams, or a nuclear medicine scan or injection with an x-ray dye within two weeks prior to your DXA scan. If you are female and are not post-menopausal you will be asked to take a pregnancy test prior to beginning the study and subsequently before each DXA scan. To measure abdominal subcutaneous and visceral fat, the DXA scan you will undergo will be used to measure this.

From the 28th to 35th day of each dietary period participants will be asked to wear activity monitors around the waist. This activity monitoring period is optional and you may choose not to participate. If you do choose to participate these small devices are about the size of a wrist watch and can be worn on a belt or with special belts that are made for the monitors. These devices measure movement and ambient light and this data will be used to measure 24 hour physical activity, energy expenditures, and sleep/wake measurements. On the 35th day of each period the data stored on the devices will be downloaded and saved under code and the data on the device will be deleted.

During days 1, 2, 41 and 42 of each six-week test diet phase, fasting blood samples will be obtained for assessment of blood fat, fatty acid profile and other cardiovascular diseases (CVD) biomarkers including insulin glucose concentrations and inflammatory markers, oxidative stress markers and markers of adiposity.

Each blood draw will require taking approximately 10 teaspoons of blood and will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 10 tablespoons. The total blood volume required for this trial will be approximately 2 cups.

On day 41, you will be required to consume a small amount of deuterated water (about 2-3 tablespoons). The movement of these tagged materials will permit assessment of the change in fatty acid metabolism of your body in response to your diet. All of the above tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you.
During the last week of each phase, you will also be required to undergo a non-invasive ultrasound test called flow mediated dilatation (FMD), which will help us measure how efficiently your arteries are regulating blood flow. In addition, your arterial health will be further measured through another non-invasive procedure using a Mobil-O-Graph which will only require you to wear a blood pressure cuff which measures blood pressure at the same time as determining your blood vessel elasticity.

**Risks and discomforts**

As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.

The dietary oils contained within the meals at the proposed level has been shown to have no known direct negative side effects on health in several dozen existing animal and human experiments. 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.

Vascular ultrasound test discomfort and risks

There are no known risks associated with ultrasound. Measuring FMD requires the forearms blood pressure cuff to be inflated tightly. This may cause participants' hands and arms below the blood pressure cuff to go numb or feel a tingling or pricking sensation while the cuff is inflated and after it is released; it is similar to the sensation of your hand or arm "falling asleep." During the 5 minutes that the blood pressure cuff is inflated on your forearm, your arm could become numb and we will ask you not to move it. This might be moderately painful. However, any discomfort or numbness should go away within minutes of cuff deflation and there are no known long-term risks associated with this test. There is a possibility for red blotching or mild bruising (petechiae) appearing on the skin above and below the location of the blood pressure cuff. Studies in adults indicate that petechiae is rare (occurring in less than 0.5% of patients), is typically not uncomfortable, and does not require treatment. There are no risks associated with measurement of blood pressure, heart rate, or EKG as long as the participant is not allergic to adhesive tape. Paper and/or foam EKG electrodes are available for individuals with adhesive tape sensitivities. You will be given the opportunity to inspect these electrodes prior to participating so you can determine if you will have a reaction to them. Temporary redness at the site of the electrode placement is possible.

In case you feel any discomfort during the experimental trial a physician, Dr. Erica Luong, will be available to contact at any time. Dr. Erica Luong can be reached at 204 890 3441.

**Benefits**

You may not benefit from participation in this research; however, the study should contribute to a better understanding of the effects of dietary oils on body composition and blood fat levels as well as CVD biomarkers. 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.

Remuneration for participation
You will receive up to a maximum of $750.00 for your time and inconvenience of the study schedule. This amount will be provided as $175.00 remunerated at the end of each of the first two phases. $400.00 will be provided upon completion of the 3rd and final phase. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives
You are not obligated 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.

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 may 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 and National Research Council Research Ethics Board may also review your research-related records for quality assurance purposes. If you are a research participant from the St. Boniface General Hospital, your research related records may be reviewed by the St. Boniface General Hospital for quality assurance purposes. Other agencies that may review your research related records for quality assurance and data analysis include, St. Boniface Research Centre, Manitoba; University of Toronto, Ontario; University of Laval, Quebec; Pennsylvania State University, Pennsylvania. United States; Canola Council of Canada; and Agriculture and Agri-Food Canada. But these agencies will not be able to link your research related data with your personal information. 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 RCFFN.

Study samples will be stored in a locked 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 5 years, nor shared with any other group, other than is indicated in the protocol, without your prior specific consent.

May 22, 2015, Version 5
Participant initials _____
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 closed.

Medical care for injury related to 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. Erica Luong at 204 890 3441 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. Erica Luong 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.

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<tr>
<th>Investigator:</th>
<th>Dr. Peter Jones</th>
<th>Tel No.</th>
<th>204 474 9787</th>
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<td>Coordinator:</td>
<td>Julia Rempel</td>
<td>Tel No.</td>
<td>204 480 1042</td>
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<td>Study Physician:</td>
<td>Dr. Erica Luong</td>
<td>Tel No.</td>
<td>204 890 3441</td>
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For questions about your rights as a research subject, you may contact:

The Biomedical Research Ethics Board, University of Manitoba at 204 789 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.

This study is registered on a publicly available Registry Databank at ClinicalTrials.gov.

ClinicalTrials.gov is a website that provides information about federally and privately

May 22, 2015, Version 5
Participant initials _____
supported clinical trials. A description of this clinical trial will be available on
http://ClinicalTrials.gov. This website will not include information that can identify you.
At most, the website will include a summary of the results. You can search this website
at any time.

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

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

Signature: __________________________ Date: __________________

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: __________

Printed name of above: __________________________ Study role: __________

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

May 22, 2015, Version 5
Participant initials _____
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS

Title of Study: Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oleic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors

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

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B OT6

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.

The study doctor and institution are receiving professional fees and financial support to conduct this study.

Nature and duration of procedure
From the blood drawn during the clinical study as outlined in the Research Subject Information and Consent Form, we would like to extract genetic information from your cells and perform analyses using laboratory techniques that augment and recognize specific genes to determine how your genetic makeup influences the efficiency of your body in converting dietary oil-

May 22, 2015, Version 3
Participant initials _____
additional research subject information and consent form for genetic analysis

derived fatty acids into longer chain fatty acids that are known to have health benefits, and how the intake of these dietary oil-derived fatty acids change your gene expression profile. Genetic information which includes DNA and RNA are molecules found in the cells of your body and are 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 biological samples containing genetic information
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 samples containing genetic information a specific code. This code will link you to your samples containing genetic information and can only be decoded by the principal researcher or an individual authorized by the latter. Samples containing your genetic information will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 5-year period following the end of the research project. After this time, all samples will be destroyed. Your samples containing genetic information 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, a Health Canada representative, or a representative from the Richardson Centre 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
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. In case you feel any discomfort during the experimental trial a physician, Dr. Erica Luong, will be available to contact at any time. Dr. Erica Luong can be reached at 204 890 3441.

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 response to the intake of the treatment oils.

May 22, 2015, Version 3
Participant initials _____
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

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 __________________________

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: __________

May 22, 2015, Version 3
Participant initials _______
# Effects of Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome/Canola Oil Multi-center Intervention Trial 2 (COMIT II)

## Participant Screening Form

To be filled out by participant:

Circle appropriate YES/NO responses

### Participant Information

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### Contact Information

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<tr>
<td>Postal Code:</td>
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Participant Screening Code: __________

August 15, 2014, Version 2
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<tr>
<td>Thyroid disease</td>
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<td>Kidney disease</td>
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<td>Heart disease</td>
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<td>Hypertension</td>
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<tr>
<td>Cholesterol-lowering medication? (in the last 3 months)</td>
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<td>Other medications</td>
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<tr>
<td>Vitamin, mineral supplement</td>
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<tr>
<td>Herbal, food supplement</td>
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<tr>
<td>Laxatives</td>
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<tr>
<td>Fiber</td>
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<tr>
<td>Allergies (food such as peanuts)</td>
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<tr>
<td>Vegetarian</td>
<td></td>
<td></td>
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<tr>
<td>Any metallic bone components</td>
<td></td>
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</tr>
</tbody>
</table>

| Lifestyle                                      |     |         |
| Smoker?                                       |     |         |
| Drink Alcohol?                                |     |         |

Participant Screening Code: __________
August 15, 2014, Version 2
To be filled out by a study coordinator:

<table>
<thead>
<tr>
<th>Screening Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (specify lb or kg)</td>
</tr>
<tr>
<td>Height (specify in or cm)</td>
</tr>
<tr>
<td>BMI (kg/m² or lb/ft²)</td>
</tr>
<tr>
<td>Waist circumference (specify in or cm)</td>
</tr>
<tr>
<td>Blood pressure</td>
</tr>
<tr>
<td>SBP 1:</td>
</tr>
<tr>
<td>Average SBP:</td>
</tr>
<tr>
<td>Screening code (initials:mm/dd/yy)</td>
</tr>
</tbody>
</table>

Is subject fasted for blood sampling?  YES  NO

Fasting blood glucose (mmol/L)  

Triglycerides (mmol/L)  

HDL cholesterol (mmol/L)  

Principal Investigator:

<table>
<thead>
<tr>
<th>Name (printed):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature:</td>
</tr>
<tr>
<td>Date:</td>
</tr>
</tbody>
</table>

Participant Screening Code: 

August 15, 2014, Version 2
SECTION 1: START AND END WEIGHT

Instructions: Ask participants to remove heavy footwear and/or heavy articles of clothing (i.e., jackets, outerwear) before each weight measurement.

Day 1: Weight (lbs) __________ Staff Initials ______
Day 2: Weight (lbs) __________ Staff Initials ______
Day 41: Weight (lbs) __________ Staff Initials ______
Day 42: Weight (lbs) __________ Staff Initials ______

SECTION 2: WAIST CIRCUMFERENCE

Instructions: Waist circumference is measured at either the natural waist, i.e., smallest circumference of the abdomen, or midway between the lowest rib and iliac crest. The measuring tape should be snug around the body, but not pulled so tight that it is constricting.

Day 1 or 2:
a. First measurement (cm): ______
b. Second measurement (cm): ______
c. Average (1st and 2nd) waist circumference measurement: ______
   Staff Initials ______

Day 41 or 42:
a. First measurement (cm): ______
b. Second measurement (cm): ______
c. Average (1st and 2nd) waist circumference measurement: ______
   Staff Initials ______
SECTION 3: SEATED BLOOD PRESSURE

Has the participant consumed caffeine, used any medication or eaten any food in the last 12 hours, and/or exercised in the past 2 hours? □ Yes* □ No

*If yes, participant needs to be rescheduled for a blood pressure measurement.

Instructions: The participant should be instructed to relax as much as possible; ideally, at least 5 minutes should elapse before the first reading is taken. Apply cuff to non-dominant arm. After applying the cuff, the participant must be quiet and remain continuously seated without legs crossed for 5 minutes. Instruct the participant not to talk during the reading. Wait 3 minutes after each reading before taking the next reading.

Day 1 or 2:

a. First blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
b. Second blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
c. Third blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
d. Average (2nd and 3rd) blood pressure measurement: ___ / ___ (SBP/DBP)

Staff Initials ________

Day 41 or 42:

a. First blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
b. Second blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
c. Third blood pressure measurement (mmHg): ___ / ___ (SBP/DBP)
d. Average (2nd and 3rd) blood pressure measurement: ___ / ___ (SBP/DBP)

Staff Initials ________
SECTION 4: BLOOD COLLECTION

DAY 1

Date (Month Day, Year) ________________  Time (HH:MM) ________________

Fasted over last 12 hours?  Yes ___ / No ___

Caffeine in last 12 hours?  Yes ___ / No ___  If Yes, when ________________

Alcohol in last 48 hours?  Yes ___ / No ___  If Yes, when ________________

Arm  Right ___ / Left___

Collect:  
2 x 7.5 ml serum (red/grey SST tube) ___ (day 1, 2, 41, 42)
2 x 4 ml plasma heparin (green top) ___ (day 1, 2, 41, 42)
2 x 4 ml plasma EDTA (purple top) ___ (day 1, 2, 41, 42)

Have all blood samples been obtained?  Yes ___ / No___

** If No, please state reasons why: __________________________________________

________________________________________

Ease of Blood Draw:  Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?):

________________________________________

________________________________________

________________________________________

Name of Phlebotomist: ____________________
SECTION 4: BLOOD COLLECTION (continued)

DAY 2

Date (Month Day, Year) ________________ Time (HH:MM) __________

Fasted over last 12 hours?  Yes ___ / No ___

Caffeine in last 12 hours?  Yes ___ / No ___  If Yes, when ______________

Alcohol in last 48 hours? Yes ___ / No ___  If Yes, when ______________

Arm  Right ___ / Left ___

Collect:  2 X 7.5 ml serum (red/grey SST tube) ___ (day 1, 2, 41, 42)
         2 X 4 ml plasma heparin (green top) ___ (day 1, 2, 41, 42)
         2 X 4 ml plasma EDTA (purple top) ___ (day 1, 2, 41, 42)

Have all blood samples been obtained?  Yes ___ / No** ___

** If No, please state reasons why:________________________________________
________________________________________
________________________________________

Ease of Blood Draw:  Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?):________________________________________
________________________________________
________________________________________

Name of Phlebotomist:________________________
SECTION 4: BLOOD COLLECTION (continued)

D A Y  4 1

Date (Month Day, Year) ________________ Time (HH:MM) __________

Fasted over last 12 hours? Yes ___ / No ___

Caffeine in last 12 hours? Yes ___ / No ___ If Yes, when ________________

Alcohol in last 48 hours? Yes ___ / No ___ If Yes, when ________________

Deuterium water provided?* Yes ___ / No ___ *Only required on day 41 of each phase

Arm Right ___ / Left ___

Collect: 2 X 7.5 ml serum (red/grey SST tube) ___ (day 1, 2, 41, 42)

2 X 4 ml plasma heparin (green top) ___ (day 1, 2, 41, 42)

2 X 4 ml plasma EDTA (purple top) ___ (day 1, 2, 41, 42)

Have all blood samples been obtained? Yes ___ / No*** ___

** If No, please state reasons why: ____________________________________________

________________________________________

________________________________________

Ease of Blood Draw: Excellent ___ Good ___ Difficult ___ Very Difficult ___

Comments (Anything which could impact blood draw results? I.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?):

________________________________________

________________________________________

________________________________________

Name of Phlebotomist: __________________________
SECTION 4: BLOOD COLLECTION (continued)

DAY 42

Date (Month Day, Year) ________________  Time (HH:MM) __________

Fasted over last 12 hours?  Yes ___ / No ___

Caffeine in last 12 hours?  Yes ___ / No ___  If Yes, when ________________

Alcohol in last 48 hours?  Yes ___ / No ___  If Yes, when ________________

Arm  Right ___ / Left ___

Collect:  
2 X 7.5 ml serum (red/grey SST tube) ___ (day 1, 2, 41, 42)
2 X 4 ml plasma heparin (green top) ___ (day 1, 2, 41, 42)
2 X 4 ml plasma EDTA (purple top) ___ (day 1, 2, 41, 42)
1 X 2.5 ml whole blood (PAXgene tube) ___ (day 42)

Have all blood samples been obtained?  Yes ___ / No** ___

** If No, please state reasons why:

☐ No PAXgene tube collected since genetic analysis consent not given

☐ Other (please describe): ________________________________

______________________________

______________________________

Ease of Blood Draw:  Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments (Anything which could impact blood draw results? i.e., Are you feeling stressed? How did you sleep last night? Has anything out of the ordinary happened over the past 48 hours?): ________________________________

______________________________

______________________________

Name of Phlebotomist: ________________
### SECTION 5: DXA

**BASELINE DXA**

**DAY: 1 2 3** *(please circle one)*

Date (Month Day, Year) _______________  Time of scan (HH:MM): _______________

Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)?

Yes __  /  No __

Date & details: ____________________________________________

_________________________________________________________________

**FEMALES OF CHILDBEARING AGE ONLY:**

Possibility of being pregnant  Yes __ / No __

Has a pregnancy test been offered?  Yes __ / No __

Has a test been taken? Yes __ / No*** ___  Test Results (circle): **Negative / Positive**

Result Confirmation:

Participant signature: ______________  Study staff signature: ______________

***If no pregnancy test taken please explain why:

_________________________________________________________________

_________________________________________________________________

Check:

Jewelry removed (including glasses):  Yes __ / No __

Spine straight:  Yes __ / No __

Comments: ____________________________________________

_________________________________________________________________

_________________________________________________________________

Staff Initials: ____________
SECTION 5: DXA (continued)

ENDPOINT DXA
DAY: 40 41 42 (please circle one)

<table>
<thead>
<tr>
<th>Date (Month Day, Year):</th>
<th>Time of scan (HH:MM):</th>
<th>Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>______________</td>
<td>______________</td>
<td>Yes ___ / No ___</td>
</tr>
</tbody>
</table>

Date & details: ____________________________________________________________

<table>
<thead>
<tr>
<th>FEMALES OF CHILDBEARING AGE ONLY:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possibility of being pregnant</td>
</tr>
<tr>
<td>Yes ___ / No ___</td>
</tr>
<tr>
<td>Has a pregnancy test been offered?</td>
</tr>
<tr>
<td>Yes ___ / No ___</td>
</tr>
<tr>
<td>Has a test been taken? Yes ___ / No***</td>
</tr>
<tr>
<td>Test Results (circle): Negative / Positive</td>
</tr>
</tbody>
</table>

Result Confirmation:
Participant signature:_________________ Study staff signature:______________

***If no pregnancy test taken please explain why:
________________________________________________________________________

Check:
Jewelry removed (including glasses): Yes ___ / No ___
Spine straight: Yes ___ / No ___

Comments:__________________________________________________________________

________________________________________________________________________

Staff Initials:______________
SECTION 6: ACTIVITY MONITORS

☐ Consent given to participate in activity monitoring

☐ No consent given to participate in activity monitoring

Contact Dylan Mackay (Dylan.Mackay@umanitoba.ca) for assistance in set-up and programming of the activity monitors.

Instructions
• The ActiGraph GT3X+ activity monitors need to be charged using the USB cables until the green LED light has stopped blinking.
• The monitors then need to be programmed using the ActiLife 6 software. Each monitor will be programmed for a 7 day period of measurement, starting at 12:00 am on day 29 of each dietary period and running until 12:00 am on day 36. With a 30 hz epoch sample rate and triaxial (3 axis enabled) monitoring.
• The monitors will be programmed with the participant code, height, weight, gender, race and date of birth. The serial number of the monitor used for each participant will be recorded in an Excel sheet so that the same monitor can be used by each participant throughout the study.
• The monitors will be given to the participants on day 28 of each study period and collected from the participants on or after day 36.
• The “Instructions for Activity Monitors” sheet should be given and explained to participants each time they are starting to wear the activity monitors.
• After collection the monitor data will be offloaded and saved in the ActiLife data vault, the data files will be named by participant code and the date of day 36 of the dietary period for which the activity data was collected. ie. C212 (2014-02-07).gtx (year-month-day).
COMIT II DATA COLLECTION AND MONITORING FORMS  
Participant Code:_______  Phase:_____

SECTION 7: WEEKLY MONITORING FORMS

WEEK 1

1. In the past week has your exercise level changed? □ Yes □ No
   If Yes, was it:  □ More Active  □ Less Active  □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week? □ Yes □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
<th>End Date</th>
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</table>

3. Have you taken any vitamins, minerals or other supplements in the past week? □ Yes □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
<th>End Date</th>
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</table>

4. Have you had any changes in a medical condition, new illness or injury in the past week? □ Yes □ No
   If Yes, describe: ___________________________________________________________

5. If you were ill in the past week, did your eating change as a result? □ Yes □ No
   If Yes, describe: ___________________________________________________________

6. Have you eaten any foods outside of the study diet? □ Yes □ No
   If Yes, describe: ___________________________________________________________

7. Any specific comments regarding study food:
_________________________________________________________________________
_________________________________________________________________________
Section 7: Weekly Monitoring Forms (continued)

Week 2

1. In the past week has your exercise level changed? □ Yes □ No
   If Yes, was it: □ More Active □ Less Active □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week? □ Yes □ No
   If Yes, specify:

<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
<th>End Date</th>
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</table>

3. Have you taken any vitamins, minerals or other supplements in the past week? □ Yes □ No
   If Yes, specify:

<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
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4. Have you had any changes in a medical condition, new illness or injury in the past week? □ Yes □ No
   If Yes, describe: ____________________________________________________________

5. If you were ill in the past week, did your eating change as a result? □ Yes □ No
   If Yes, describe: ____________________________________________________________

6. Have you eaten any foods outside of the study diet? □ Yes □ No
   If Yes, describe: ____________________________________________________________

7. Any specific comments regarding study food:

   ____________________________________________________________
   ____________________________________________________________
   ____________________________________________________________

   11
SECTION 7: WEEKLY MONITORING FORMS (continued)

WEEK 3

1. In the past week has your exercise level changed? □ Yes □ No
   If Yes, was it: □ More Active □ Less Active □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week? □ Yes □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
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3. Have you taken any vitamins, minerals or other supplements in the past week? □ Yes □ No
   If Yes, specify:
   
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<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
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4. Have you had any changes in a medical condition, new illness or injury in the past week? □ Yes □ No
   If Yes, describe: ____________________________________________

5. If you were ill in the past week, did your eating change as a result? □ Yes □ No
   If Yes, describe: ____________________________________________

6. Have you eaten any foods outside of the study diet? □ Yes □ No
   If Yes, describe: ____________________________________________

7. Any specific comments regarding study food: ____________________________________________
   ____________________________________________
SECTION 7: WEEKLY MONITORING FORMS (continued)

WEEK 4

1. In the past week has your exercise level changed? □ Yes □ No
   If Yes, was it: □ More Active □ Less Active □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week? □ Yes □ No
   If Yes, specify:
   
<table>
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<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
<th>End Date</th>
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3. Have you taken any vitamins, minerals or other supplements in the past week? □ Yes □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
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</table>

4. Have you had any changes in a medical condition, new illness or injury in the past week? □ Yes □ No
   If Yes, describe: _________________________________________________

5. If you were ill in the past week, did your eating change as a result? □ Yes □ No
   If Yes, describe: _________________________________________________

6. Have you eaten any foods outside of the study diet? □ Yes □ No
   If Yes, describe: _________________________________________________

7. Any specific comments regarding study food: _________________________________________________
   _________________________________________________
   _________________________________________________
   _________________________________________________

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SECTION 7: WEEKLY MONITORING FORMS (continued)

WEEK 5

1. In the past week has your exercise level changed?  □ Yes  □ No
   If Yes, was it:  □ More Active  □ Less Active  □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.*

2. Have you taken any prescription or non-prescription drugs in the past week?  □ Yes  □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
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3. Have you taken any vitamins, minerals or other supplements in the past week?  □ Yes  □ No
   If Yes, specify:
   
<table>
<thead>
<tr>
<th>Name</th>
<th>Dosage</th>
<th>Frequency</th>
<th>Reason</th>
<th>Start Date</th>
<th>End Date</th>
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</table>

4. Have you had any changes in a medical condition, new illness or injury in the past week?  □ Yes  □ No
   If Yes, describe: ____________________________________________________________

5. If you were ill in the past week, did your eating change as a result?  □ Yes  □ No
   If Yes, describe: ____________________________________________________________

6. Have you eaten any foods outside of the study diet?  □ Yes  □ No
   If Yes, describe: ____________________________________________________________

7. Any specific comments regarding study food: __________________________________________
   _________________________________________
SECTION 7: WEEKLY MONITORING FORMS (continued)

WEEK 6

1. In the past week has your exercise level changed?  □ Yes  □ No
   If Yes, was it:  □ More Active  □ Less Active  □ No Exercise
   *Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week?  □ Yes  □ No
   If Yes, specify:
   Name  Dosage  Frequency  Reason  Start Date  End Date
   ____________________________________________________________
   ____________________________________________________________
   ____________________________________________________________

3. Have you taken any vitamins, minerals or other supplements in the past week?  □ Yes  □ No
   If Yes, specify:
   Name  Dosage  Frequency  Reason  Start Date  End Date
   ____________________________________________________________
   ____________________________________________________________

4. Have you had any changes in a medical condition, new illness or injury in the past week?  □ Yes  □ No
   If Yes, describe: ____________________________________________

5. If you were ill in the past week, did your eating change as a result?  □ Yes  □ No
   If Yes, describe: ____________________________________________

6. Have you eaten any foods outside of the study diet?  □ Yes  □ No
   If Yes, describe: ____________________________________________

7. Any specific comments regarding study food: ____________________________________________
   ____________________________________________
   ____________________________________________
## ADVERSE EVENT REPORTS

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<th>Start Date</th>
<th>Intensity*</th>
<th>Outcome</th>
<th>Date Ended</th>
<th>Diet Related?</th>
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*Mild: An event that is easily tolerated by the volunteer, causing minimal discomfort and not interfering with everyday activities
Moderate: An event that is sufficiently discomforting to interfere with normal everyday activities
Severe: An event which is incapacitating and prevents normal everyday activities
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<th>Date (Month Day, Year)</th>
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STATUS SUMMARY

☐ Participant completed the study

☐ Participant withdrew from the study

Date of withdrawal: __________

REASON FOR WITHDRAWAL. Tick appropriate box(es):

☐ Informed consent withdrawn by the participant
☐ Failure to comply with study requirements
☐ Investigator decision to withdraw participant

Provide rationale for withdrawal (if applicable):

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

☐ Participant’s final results sent

☐ Statement supplier form completed

Investigator Initials ______