The effects of consumption of functional foods consisting of high oleic canola oil, docosahexaenoic acid and barley β-glucan on plasma lipids

by

Anjalika Susan Abraham

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Abstract

Functional foods containing high oleic canola oil, omega-3 oils and barley β-glucan have been individually shown to improve blood lipid profiles. However, this is the first study to test the intake of a combination of these ingredients incorporated into a single food product in a human trial. The objective was to test the synergistic impact of consuming a combination of novel foods containing one or more functional ingredient on blood lipid profile and the Framingham risk score. The secondary objective was to predict the mechanism of action of the cholesterol lowering effected by these ingredients. A randomized single-blinded free-living crossover study design was used. This study consisted of thirty-five participants, consisting of four intervention phases of four weeks each. Each phase was separated by a four-week washout period. The participants were randomly assigned to treatments which consisted of one test muffin and two test cookies per day. At endpoint, the high oleic canola docosahexaenoic acid treatment showed a significant increase ($P< 0.05$) in high-density lipoprotein levels (HDL-C) and a significant decrease ($P< 0.05$) in triglyceride levels (TG) when compared to control. We also observed a significant decrease ($P< 0.05$) in very low-density lipoprotein levels (VLDL-C), total cholesterol to high-density lipoprotein ratio (TC: HDL-C) and diastolic blood pressure (DBP) when compared to the control. The combination of high oleic canola oil, omega-3 oils and barley β-glucan showed a significant increase ($P< 0.05$) in high-density lipoprotein levels (HDL-C) and a significant decrease ($P< 0.05$) in triglyceride levels (TG) ($P< 0.05$) when compared to control. We also observed a significant decrease ($P< 0.05$) in very low-density lipoprotein levels (VLDL-C), total cholesterol to high-density lipoprotein ratio (TC: HDL-C) and diastolic blood pressure

(DBP) at endpoint when compared to the control. No significant differences were observed for total cholesterol (TC) ($P< 0.05$), low-density lipoprotein level (LDL-C) ($P< 0.05$), glucose level and systolic blood pressure (SBP) ($P< 0.05$). At endpoint when comparing the combined high oleic canola docosahexaenoic acid and high molecular weight barley β-glucan treatment to control the Framingham risk score, decreased ($P< 0.05$) for both males and females. For the non-cholesterol sterols the high oleic canola docosahexaenoic acid treatment showed a significant increase ($P< 0.05$) in sitosterol when compared to control. The combination of high oleic canola oil, omega-3 oils and barley β-glucan showed a significant increase ($P< 0.05$) in sitosterol when compared to control. However the findings showed no change in cholesterol synthesis. We conclude that when compared to their individual treatments alone the combination of high oleic canola oil, omega-3 oil and barley β-glucan as a single food product do have additional positive outcomes on blood lipid profiles and the Framingham risk score.

Keywords: high oleic canola oil, barley β-glucan, DHA, cholesterol, blood lipid profiles.

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Dedication

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I dedicate this thesis to my family.

To my parents Thomas Ajit and Naila Abraham

And to my sister Dr Nikita Sarah Abraham.

I would not have been able to do this without you

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Abbreviations

AHA	American Heart Association
ALA	Alpha linolenic acid
AMPK	Amp-dependent kinase
ANOVA	Analysis of variance
Apo B	Apolipoprotein B
Apo E	Apolipoprotein E
BBG	Barley beta-glucan
β	Beta
BMI	Body mass index
CHD	Coronary heart disease
CHMS	Canadian Health Measures Survey
COMIT	Canola oil multicentre intervention trial
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
FDA	Food & Drug Administration
FPG	Fasting plasma glucose
FRS	Framingham 10-year CHD risk score

GI	Gastrointestinal tract
HDL-C	High-density lipoprotein cholesterol
HMW	High molecular weight
IDF	International Diabetes Federation
LA	Linoleic acid
LDL-C	Low-density lipoprotein cholesterol
LMW	Low molecular weight
MetS	Metabolic syndrome
MUFA	Monounsaturated fatty acid
NCEP	National Cholesterol Education Program
NCS	Non-cholesterol sterols
PS	Plant sterol
PUFA	Polyunsaturated fatty acid
RCFFN	Richardson Centre for Functional Foods and Nutraceuticals
SBP	Systolic blood pressure
SFA	Saturated fatty acid
SCFA	Short chain fatty acid
TC	Total cholesterol
TG	Triglyceride

TMS	Trimethylsilyl
VLDL-C	Very low-density lipoprotein cholesterol
WHO	World Health Organization

Introduction

Metabolic syndrome (MetS) influences nearly one quarter of North Americans, and is a major health concern due to its link to cardiovascular disease (CVD). Studies predict that over the next 5-10 years the risk of developing CVD will be higher for those with MetS than those without the disease (Calton et al., 2014; Hosseini et al., 2016). MetS results from a combination of CVD risk factors. The International Diabetes Federation (IDF) defines a person with MetS as having central obesity with a waist circumference ≥ 94 cm for men and ≥ 80 cm for women with specific values for ethnic groups. In addition any two of the following factors must be present: a) triglyceride (TG) level ≥150 mg/dL (1.7 mmol/L), b) high-density lipoprotein level cholesterol (HDL-C) < 40 mg/dL (1.03 mmol/L) in males and < 50 mg/dL (1.29 mmol/L) in females, c) low-density lipoprotein level cholesterol (LDL-C) > 2.7 mmol/, d) systolic blood pressure (SBP) ≥ 130 or diastolic blood pressure (DBP) ≥ 85 mmHg, or treatment of previously diagnosed hypertension, and e) fasting plasma glucose (FPG) ≥ 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes (T2DM) (The International Diabetes Federation, 2006). Unlike pharmacological treatment of statins, the exact etiology of the pathogenesis of MetS is unknown. This knowledge gap renders the development of nutritional therapeutic agents to target this disease difficult.

Adiponectin is a key factor counteracting MetS because of its anti-atherogenic and insulinomimetic properties as well as its ability to facilitate lipid oxidation. Mohammad et al., (2015) investigated the relation between adiponectin and the individual risk markers of MetS. The findings indicated a negative association between

adiponectin with fasting insulin, glucose and TG, and an indirect correlation with the risk of diabetes mellitus, hypertension, dyslipidemia or coronary heart disease (CHD). Adiponectin modulates its effect on plasma TG and HDL-C via a catabolic effect of modulating chylomicrons while upregulating lipoprotein lipase and apolipoprotein E (Apo E) activity (Castro et al., 2007). Adiponectin also plays a significant role in the control of blood pressure (BP) in both healthy and T2DM populations (Furuhashi et al., 2003). Chow et al., (2007) demonstrated that low adiponectin levels had an inverse relation with the occurrence of hypertension. Through the activation of AMP-dependent kinase (AMPK), adiponectin maintains its effect, thereby directly suppressing hepatic gluconeogenesis, rate of endogenous glucose production and stimulating fatty acid oxidation in the liver (Wagner et al., 2009; Calton et al., 2013). This probable mechanism may explain the relation between hypoadiponectinemia and hypertension. Higher levels of adiponectin protect against the development of T2DM, thus leading to a more favorable CVD outcome.

As per data from World Health Organisation (WHO) CVD is the number one cause of death globally. The number of deaths as per statistical data is estimated to 17.3 million people (2008) which represents 30% of all global deaths from CVD annually, than from any other diseases. Evidence suggests that by the year 2030 the number of deaths from CVD will reach 23.3 million. (WHO, 2014; WHO 2011). Statistical analysis from 2009-2013 by the Canadian Health Measures Survey (CHMS) showed that 21% of Canadian adults aged 18-79 years suffer from MetS (Statistics Canada, 2015). The survey also showed that 38% of Canadian adults aged 18-79 years suffer

from dyslipidemia, however, in most cases they were unaware of their condition (Statistics Canada, 2015). The guidelines by the American Heart Association (AHA) suggest that consuming a Mediterranean style diet could decrease the risk and incidence of CVD (Alberti et al., 2009). The Lyon Heart Trial showed significant reductions in CVD death, with changes in major and minor secondary end-points, using a Mediterranean type diet compared to a western diet (Etherton et al., 2001). The Mediterranean diet includes components apart from monounsaturated fatty acids (MUFA) that have been shown to improve insulin resistance and reduce inflammation, oxidative stress, and pathogenic factors in obesity, diabetes and MetS. Current dietary recommendations by The Adult Treatment Panel III (ATP III) (National Cholesterol Education Program, 2002), American Heart Association (AHA) (Lichtenstein et al., 2006) for populations with high risk for MetS aim specifically at increasing the consumption of Mediterranean dietary components, such as long chain n-3 fatty acids (Lopez, 2012), carbohydrates (<60%) (Lichtenstein et al., 2006) and dietary fibre (Health Canada, 2012), due to their innate bioactive structure. The intake of these nutritional components have reportedly shown remarkable positive effects in the management of MetS. Several cross-sectional studies have shown an inverse relationship between the intakes of a Mediterranean diet against the incidence of MetS (Salas et al., 2008; Jenkins et al., 2010; Kastorini et al., 2011; Mozumdar, & Liguori, 2011; Kesse et al., 2013).

Understandably, high blood cholesterol concentrations are a modifiable and important risk factor for CVD. A number of meta-analyses and reviews have underlined

the need to look to evidence for a better understanding of contributing factors. Reduction of occurrence of CVD by 2-3% is in direct response with 1% of total blood cholesterol lowering (Astrupet al., 2011). Findings from metabolic studies have shown that diet modifications reduced total blood cholesterol concentration by 10-15% (Gylling et al., 2001) and have changed some or all mechanisms of total blood cholesterol lowering (Tang et al., 1998; Chowdhury et al., 2014). Results from previous human clinical trials have shown the positive effects of individual dietary components, high oleic canola oil (HOCO), docosahexaenoic acid (DHA) and high molecular weight barley β-glucan (HMW-BBG) individually in reducing CVD risk factors. Results from the COMIT study conducted by (Senanayake et al., 2014) showed that a diet consisting of a combination of MUFA and polyunsaturated fatty acid (PUFA) rich diet resulted in modified lipid biomarkers, thereby lowering the cholesterol risk factors associated with CVD (Jones et al., 2014). The results also indicated a decline in the ten-year Framingham risk score (FRS) with the consumption of HOCO-DHA when contrasted with conventional canola oil or the control (Jones et al., 2014). The Omni-Heart Trial compared a diet with energy from MUFA (21%) and PUFA (10%) against a higher carbohydrate diet and showed a decrease in BP and CHD risk (Swain et al., 2008). Recommendations by the ATP III dietary guidelines advise up to 20% of calories from MUFAs and up to 10% from n3:n6 PUFAs (National Cholesterol Education Program, 2002). Hunter et al., (2010) reported a significant decrease in LDL-C of 7.8% after a diet including 18% of energy intake as MUFAs, when compared with a saturated fatty acid (SFA) diet. Omega-3 PUFA’s are inversely associated with risk of CVD. Chiuve et al., (2012) studied the effect of substituting SFA with a higher proportion of dietary fat

of PUFAs and found that PUFAs lowered the risk of CVD in women. PUFAs may play a role in protecting against ventricular arrhythmia's by influencing voltage- gated $Na^{(+)}$ and L-type $Ca^{(2+)}$ cardiac ion channels, β-adrenergic receptors, cell-signaling pathways, gap junction communication, and membrane fluidity (Chiuve et al., 2012). Rathnayake et al., (2016) developed a food exchange model (DIVAS2) to study the effects of replacing SFAs with MUFAs or PUFAs on CVD risk factors. The study was successful in showing a decrease in DBP after consumption of test fat meal when compared to control ($P<0.05$) in postmenopausal women. Vafeidou et al., (2015) their findings from the (DIVAS) study, substituted SFA with MUFA and PUFA in the daily diet with exchangeable fats and showed significant reductions in TC, LDL-C, TC: HDL-C ratio in a population defined with CVD risk.

Linear mixed linkage (1→3) (1→4) β-D-glucans (β-glucan) are active soluble dietary fibre components in oats/barley known to reduce serum TC levels by 5–10% (Ripsin et al., 1992; McIntosh et al., 1995). Oats contain 4% by weight β-glucan, whereas barley contains 5–10% by weight of β-glucan (Ripsin et al., 1992; McIntosh et al., 1995) and both of the these soluble fibres shown to have similar effects on lipid lowering. The cholesterol lowering effect of mixed linkage (1→3) (1→4) β-D-glucans (β-glucan) are mediated through reductions in serum lipids (Keogh et al., 2003; Aman, 2006). Studies indicate that the cholesterol lowering effect of β-glucan may be dependent on the physicochemical properties rather than their dose alone. The physicochemical characteristics of β-glucan, more specifically concentration, viscosity and molecular weight, have shown a positive correlation with its physiological benefits

(Wolever et al., 2010; Vuksan et al., 2011). The reduction in the pancreatic amylase activity and movement of released sugars to the gut wall is as a result of an increase in luminal viscosity, which then impairs the rate of digestion and absorption of glucose (Dunaif et al., 1981). Keenan et al., (2007) studied the effect of 3 g/d HMW/ LMW or 5 g/d HMW/ LMW barley β-glucan vs. 0 g/d β-glucan on blood lipids. The trial was successful in showing reduced TC, LDL-C and TC: HDL-C in all treatments. Rondanelli et al., (2011), who demonstrated that 5.99 g/d HMW-BBG reduced TC and LDL-C significantly, also observed similar findings. Regand et al., (2011) showed a lowered glucose response with an increase in-vitro extract viscosity of β-glucan and decrease in the in-vitro starch digestibility. Solubility is a function of molecular weight and the total amount of β-glucan available in a sample (Regand et al., 2011). β-glucan solubility depends on several factors such as cereal source, food formulation, processing and storage conditions (Pidhainy et al., 2007; Tosh et al., 2010). Wang et al., (2016) showed that a 3 g/d intake of HMW-BBG intake was sufficient to reduce blood cholesterol levels by -0.12 mmol/L and demonstrated that varying doses of BBG with different molecular weights resulted in differences in body weight, waist circumference and blood lipid profiles. Importantly, consuming 3 g/d β-glucan with high viscosity with HMW showed a greater efficacy in lowering TC than the control diet (Wang et al., 2016). The claim that a daily intake of a minimum of 3 g/d β-glucan soluble fibre from barley/oat grain products can reduce blood cholesterol concentrations are approved by food regulatory agencies worldwide such as the U.S. Food and Drug Administration, 1997, European Food Safety Authority (EFSA), 2011 and Health Canada, 2012.

Incorporation of dietary interventions or the use of statin therapy are specifically aimed at decreasing cholesterol levels, thereby reducing the risk factors associated with CVD. Phytosterols, present in esterified or free form are known to lower cholesterol levels, with the former having a higher efficacy rate (Jessup et al., 2008).The delivery of phytosterols to the intestine is a dependent factor that explain its effectiveness, which then further competes with cholesterol for absorption (Jessup et al., 2008). Non-cholesterol sterols (NCS) are known for their cholesterol lowering effect, however their function is not limited to modifying lipid profiles; they also serve as surrogate markers of cholesterol absorption and synthesis (Miettinen et al., 2011; Mackay et al., 2012). Miettinen et al., (2011) evaluated the measurement of cholesterol absorption and synthesis rate using relative and absolute variables, identifying NCS as valid surrogate markers of cholesterol absorption and synthesis. Studies suggest that the validity of NCS should not be based on only one surrogate markers but have atleast 2 or more markers, providing a better understanding of cholesterol synthesis and absorption rate with the goal of providing therapeutic agents aimed at targeting hyperlipidemia (Miettinen et al., 2003; Nissinen et al., 2008). Taking into consideration the above-mentioned positive factors, the current study focuses on testing the effects of a combination of HOCO, DHA and HMW-BBG on blood lipid profile and FRS.

Rationale

Based on the understanding of their individual mechanisms in lowering cholesterol concentrations, combining HOCO, DHA and HMW-BBG will create an overall synergistic effect on serum lipid profiles and FRS. Such an approach to a clinical trial anticipates providing knowledge on dietary approaches in the management of MetS and CVD. Results from the COMIT study indicated a decline in the FRS with consumption of HOCO-DHA when contrasted with conventional canola oil or the control. The findings are also a clear indication of decrease in lipid profiles with significant reductions for TG, VLDL-C, SBP and significant increase in HDL-C. Regulatory bodies support the qualified health claim that a 3 g/d intake of BBG is sufficient to reduce blood cholesterol levels. A randomized, single-blinded, crossover trial consisting of four treatment phases for thirty-five days each, using 3 g/d HMW-BBG was conducted and was successful in lowering cholesterol in response to the dietary treatment provided. Taken together, we envision that combining HOCO, DHA and HMW-BBG will provide a synergistic effect and consequently lower blood lipid profiles thereby reducing the risk of MetS and CVD**.** This is the first study to test the effects of combined HOCO, DHA and HMW-BBG products on CVD risk. CVD is a multifactorial disease, and a blend of various bioactives with improved advantages to health are observed in various cross-sectional studies. Research strategies are aimed at examining the synergetic impacts of combining at least two or more bioactive components into the daily diet to achieve additional beneficial effects on CVD. Investigating the combination of HOCO, DHA and HMW-BBG in relation to its responsiveness of lowering cholesterol is highly warranted.

Hypotheses

1. Consumption of a combination of HOCO-DHA and HMW-BBG for four weeks will lead to health benefits such as improved blood lipids, and improve the ten-year Framingham risk score compared to their consumption individually.
2. Non-cholesterol sterols will help predict the changes in cholesterol synthesis and absorption with a diet consisting of a combination of HOCO, DHA and HMW-BBG.

Objective

1. The objective was to test the synergistic impact of consuming a combination of novel foods containing HOCO, DHA and HMW-BBG on blood lipid profile and the Framingham risk score.
2. The secondary objective was to predict the mechanism of action of the cholesterol lowering effected by these ingredients.

Thesis Layout

Literature review of this thesis comprises a narrative review of scientific evidence established as seen in Introduction. This review comprises of randomized control trials analyzing the physiological effect of HOCO, DHA and HMW-BBG on cholesterol lowering in a population with MetS and CVD. These findings of individual effect are, in essence, the basis that leads to the research initiative and project data presented in Materials and methods and Results and Discussion. Materials and Methods represents a comprehensive information of the study design and and various analytical methods used. Results and Discussion introduces the primary and secondary outcomes within the entire

intervention study. In addition, as the preliminary results of analyses, the post-treatment blood lipid profiles, FRS of all complete participants are explained to reflect the efficacy of the combined treatment across all dietary phase. Overall Conclusion, summarizes the entire study with implications and future directions for this research.

Literature Review

Several clinical studies and meta-analyses, have examined the individual impact of HOCO, DHA and HMW-BBG on circulating cholesterol concentrations. Elevated cholesterol levels and systemic inflammation are well known examples of these biomarkers in the progression of MetS and CVD. Although nutritional strategies are effective in modulating lipids, inter-individual variability exists. Various factors such as gene-diet interactions, gut microbiome, environmental factors play a role in regulating the effects on cholesterol metabolism. The purpose of the following sections is to provide a brief summary of the current state of knowledge surrounding the impacts of HOCO, DHA and HMW-BBG intake on cholesterol lowering in epidemiological and human intervention trials.

Effect of high oleic canola oil on CVD risk

Investigations continue to find suitable dietary oil replacement for lowering circulating cholesterol concentrations associated with CVD. Findings from clinical studies indicate that the low prevalence of CVD risk factors are associated with substituting SFA with MUFA and PUFA intakes (Berglund et al., 2007). Investigations on the favourable effects of dietary portfolios with the inclusion of Mediterranean diet stimulates research into identifying the distinct health attributes of MUFA and PUFA. Evidence from clinical trials show that HDL-C levels are maintained, through comparatively less hypolipidemic effects of MUFA to PUFA (Etherton., 1999; Iggman et al., 2011; Schwingshackl et al., 2014). Jenkins et al., (2010) studied the effect of replacing 13% of calories from carbohydrates with MUFA. The findings showed a 12.5%

increase in HDL-C without altering LDL-C levels. Unlike other oils HOCO has a nutrition profile of approximately 70% MUFA, 23% PUFA (3% omega-3) and (20% omega-6) and 7% SFA and a high smoke point (475° F/246℃) higher than canola oil and with oleic acid (61%), linoleic acid (21%), alpha-linolenic acid (ALA) (11%), plant sterols (0.53-0.97%) and tocopherols (700-1,200 ppm) (Warner et al., 1997). The increased levels of the MUFA oleic acid in HOCO displaces a small percent of PUFA found in classic canola oil. The stability of the oil has a direct relation with the degree of unsaturation (Warner et al., 1997). With an enhanced oxidative sustenance, a higher oleic acid content and a higher heat resistance than canola oil, HOCO stands as a suitable replacement to high SFA and high trans lipid products that are currently available in the market (Liu et al., 2012).

In the year 2006, US FDA approved a qualified health claim stating that intake of canola oil (19 g) can help reduce the risk of CVD (US FDA, 2006). Observational data from studies conducted by Iggman et al., (1994), Baudet et al., (1988), Hodson et al., (2008) Stricker et al., (2008) & Kruse et al., (2015) examined the effect of canola oil on lipid profile. Their findings indicated a decrease in TC and LDL-C when compared to the control (olive oil/sunflower oil) ($P<0.05$). However, some studies failed to show significant effects of susceptibility of LDL-C to oxidation with canola oil. (Kratz et al., 2002, Nielsen et al., 2002). The dosage of canola oil varied in each of these studies conducted. With the aim to improve the effects of canola oil on CVD risk reduction, the pioneering work of Stephansson led to the development of canola oil enhanced with high oleic acid.

According to a randomized single blinded hybrid study by Gillingham et al., (2011), consumption of HOCO for twenty-eight days reduced circulating LDL-C levels (7.4%), TC (3.5%) and decreased LDL-C: HDL-C ratio when compared to a western diet. The HOCO treatment showed no change for inflammatory markers when compared to a western diet. The lipid lowering effects of HOCO are based on the utilization of the oil alone or when combined, thus exhibiting a cardio-protective role. The findings from the COMIT study supported this effect of HOCO, with lowered LDL-C levels (−7.0 ± 2.8%) over the four-week period when contrasted with diets rich in linoleic acid, from corn and safflower oil (Jones et al., 2014; Senanayake et al., 2014).

Effect of DHA on CVD risk

Studies show that omega-3 fatty acids have a variety of cardioprotective effects. The beneficial effects on CVD risk reduction include modifying lipid profile, reducing heart rate, reducing BP levels, improved endothelial function, improved arterial compliance and alleviation of inflammatory responses (Egert et al., 2009; Ray et al., 2011; Bradberry et al., 2013). The fall in plasma TG concentrations reflects the control of hepatic VLDL-C production and secretion. Conversely, the increase in LDL-C concentration from diminished precursor lipoprotein production suggests the independent secretion of small TG Apo B particles form LDL-C, thus increasing its concentration (O'Keefe et al., 2008.).

Studies have shown that intakes as low as 1.5 g/d of DHA have variable effects on TG, LDL-C, HDL-C (Balk et al., 2006). Theobald et al., (2004) showed that 1 g/d of DHA intake increased LDL-C by 7.1%. Findings from the study conducted by Milte, et

al., (2008) showed that 1 g/d of DHA intake increased HDL-C by 4.4%. Howe et al., (2009) conducted a meta-analysis of 47 clinical studies and demonstrated the TG lowering effects of DHA on subjects with CVD when compared to the placebo group. However, no significant changes were observed for serum TC, LDL-C or HDL-C levels. Using a parallel study design Kelley et al., (2007), tested the effect of 3 g/d DHA on moderately hyperlipidemic population, results showed a decrease in TG and VLDL-C. However, it elevated LDL-C concentration (12.6%) when compared to an olive oil diet. Skulas-Ray et al., (2011) compared the effect of high dose (3.4g) vs. low dose (0.85 g) EPA + DHA. The higher dose lowered TG levels compared with the placebo.

Increased intake of omega-3 fatty acids and reduced morbidity and mortality from CVD are related with an impact on atherothrombosis, and cardiac arrhythmias. Effects on atherothrombosis include the modulation of endothelial leukocyte adhesion molecules, inflammatory cytokines and cyclooxygenase (COX)-2) and the hepatic synthesis of VLDL-C. The modulatory effects are slow in onset, requiring inclusion into cell membrane phospholipids and the doses in humans are usually in the order of 3 g/day or higher (Bradberry et al., 2013). Effects on cardiac arrhythmias involve Na, K+ and Ca ion channels complex interactions, that require the presence of free fatty acids in extracellular fluids and occurring with lower doses of around 1 g/d of nutritional or pharmacological intake (Massaro et al., 2008; Etherton et al., 2003; Delgado et al., 2012). Epidemiological and clinical studies suggest that consumption of omega-3 contributes CVD risk reduction through modulation of cellular metabolic functions, gene expression and beneficial effects on lipid profile or BP (Cabo et al., 2012). Miller et al., (2014) through a meta-

analysis of seventy randomized trials showed the effect of DHA on lowering SBP and DBP. Lahoz C et al., (1997) conducted a study with 42 healthy subjects undergoing four consecutive diets rich in saturated fat, MUFA, omega-6 PUFA and omega-3 PUFA. The findings showed a significant reduction in BP. The DHA rich phase in the COMIT trial showed a significant reduction in SBP when compared to other diets (Jones et al., 2014; Senanayake et al., 2014)

In 2004, the US FDA approved a qualified health claim for DHA and EPA omega-3 fatty acids in the reduction of CVD (US FDA, 2004). Wei et al., (2011) conducted a meta-analysis wherein 13 out of 15 studies after DHA treatment showed significant reductions in TG concentrations from 0.03 to 0.64 mmol/L, compared with the placebo. The beneficial effects of DHA on CVD include particularly lowering circulating TG levels and the TG: HDL-C ratio observed in the absence of any blood cholesterol lowering (Hansen et al., 2007). The AHA recommendation of DHA and EPA for those with CHD is 1 g/d and for those with hypertriglyceridemia is 2-4 g/d (Miller et al., 2011). The n−3 fatty acids DHA and EPA are supplemented to improve CHD risk by affecting metabolic variables such as blood TG concentrations and through independent mechanisms related to antiarrhythmic effects (Miller et al., 2011). Large scale epidemiological studies support the 2000 AHA dietary recommendations to include at least two servings of fish per week (Houlob, 2009; Huertas et al., 2012). The beneficial effects of DHA on CVD risk factors are observed in almost all studies independent of blood cholesterol lowering.

Effect of barley β-glucan on CVD risk

β-glucan, a water-soluble fibre predominant in oats and barley, show an extensive variety of health benefits including an impact on lipid metabolism, and has received considerable interest recently in nutritional research due to its functional and bioactive status (Health Canada, 2012, European Food Safety Authority, 2011 and U.S. Food and Drug Administration, 1997)**Error! Bookmark not defined.**. Meta-analysis of eleven clinical trials support the health claim that an intake of a minimum of 3 g/d β-glucan soluble fibre from barley/oat grain products can reduce blood cholesterol concentrations (AbuMweis et al., 2010). Researchers recognize the physicochemical properties, of molecular weight and viscosity, may play a role in the cholesterol-lowering effect of β-glucan (Behall et al., 2004; Pidhainy et al., 2007; Vuksan et al., 2011).

Evidence suggest that the consumption of β-glucan in high enough doses slows gastric emptying, digestion, and absorption, reducing circulating cholesterol levels through the following mechanisms: The first mechanism suggests that β-glucan increases intestinal viscosity and raises the thickness of upper wall of the lumen and results in decrease in intestinal cholesterol absorption (Dikeman et al., 2007). Dietary fibre mediates its effect through colonic fermentation and forms a highly viscous solution in the human gut leading to a decrease in the rate of diffusion. Viscous dietary fibre may thus be able to alter the cholesterol absorption (Dikeman et al., 2007; Rideout et al., 2008). The second mechanism that may explain the lipid lowering effect of β–glucan is by interrupting the enterohepatic circulation of bile acids through a similar mechanism as seen in bile acids. By inhibiting bile acid reabsorption and up-regulating bile acid

synthesis from hepatic cholesterol, β-glucan further removes cholesterol from circulation (Charlton et al., 2008; Chiang, 2009).Water-soluble β-glucan has the ability to produce a high intestinal viscosity dependent on its concentration and molecular weight, which prevents the bile acids from combining with cholesterol, thus lowering plasma cholesterol levels. Small amounts of bile acids escape reabsorption and are excreted via fecal matter. Interrupting the enterohepatic circulation of bile acids that leads to depletion of liver cholesterol for replenishing the loss of fecal bile acids is one of the strategies for lowering cholesterol levels (Cohen, 2008; Martinez et al., 2008). Cholesterol 7-α-hydroxylase pathway is also known as the bile acid pathway. Bile acid synthesis has a large influence on cholesterol homeostasis (Fuschs, 2003). The CYP7A1 gene codes for the cholesterol 7-α -hydroxylase, the enzyme responsible for the rate limiting step in the bile acid synthesis (Axelson et al., 1991; Sauter et al., 1996).

A third mechanism suggests that β-glucan inhibits cholesterol biosynthesis through short chain fatty acids (SCFA) produced as a result of β-glucan fermentation by gut microbiota. The human body itself cannot produce glucanases for breaking down β-glucan. Similar to other non-digestible carbohydrates, the degradation of β-glucan in humans relies on carbohydrate active enzymes produced by resident gut microorganisms (Cantarel et al., 2012). Therefore, the entry of β-glucan to the human gastrointestinal tract can possibly influence the microbial population. On the other hand, by-products generated from β-glucan degradation such as SCFA have shown to affect cholesterol metabolism (Flint et al., 2012; Wang et al., 2016).

In 1997, the US FDA approved a health claim for β-glucan soluble fibre from oats for reducing plasma cholesterol levels and risk of heart disease (US FDA, 1997). Talati, et al., (2009) conducted a meta-analysis of eight randomized trials. Their findings indicated that the subjects consuming BBG showed a significant reduction of 12 mg/dL in TC when compared with a control group. AbuMweis et al., (2010) conducted a meta-analysis of eleven randomized trials to determine the effects of dietary consumption of BBG on serum cholesterol concentrations. Findings of the study indicated a 0.30 mmol/L reduction in LDL-C after consuming BBG for four-weeks when compared to control. The lipid-lowering effect of BBG suggested that an intake of 3 g/day of BBG for four-eleven weeks reduces serum blood concentrations of total and LDL-C levels. Following the positive outcome Health Canada in 2012 issued a health claim for BBG indicating that 3 g/d intake reduces blood cholesterol (Health Canada, 2012).

Numerous studies support the health claim that an intake of a minimum of 3 g/d β-glucan soluble fibre from barley grain products can reduce blood cholesterol concentrations. Behall et al., (2004) investigated the effect of 0, 3, and 6 g BBG for 5 weeks after a 2-week adaptation period. TC and LDL-C decreased significantly with 3 and 6 g/d BBG when compared to control. Keenan et al., (2007) studied the effect of 3 g/d HMW-BBG and LMW-BBG or 5 g/d HMW-BBG and LMW-BBG or control BBG on lipid profile in hypercholesterolaemic population. After 6 weeks, TC and LDL-C decreased with treatments however, the higher dose of 5 g/d HMW-BBG showed a greater effect in cholesterol lowering. Ikegami et al., (1996), Li et al., (2003), Hinata et al., (2007) and Shimzu et al., (2008) fed a barley diet and examined its effect on Japanese

population. The individual studies improved TC and LDL-C, with findings from Hinata et al., (2007) focused on improving the effects on FPG. In a four-week crossover trial Rondenelli et al., (2011), tested BBG versus rice bran foods. Significant reductions were seen in TC, LDL-C, TC: HDL-C, LDL-C: HDL-C and glucose. Lowered post-prandial glucose response with diets consisting of BBG, have been reported in studies conducted by Liljeberg et al., (1996), Casiraghi et al., (2006), Thondre et al., (2009) and Ames et al., (2015). However, it is important to note that the dose as well as study design varies across all intervention trials. Wang et al., (2016) used a controlled four-phase crossover trial, in mildly hypercholesterolemic subjects with 5 g low molecular weight (LMW), 3 g HMW-BBG, 3 g LMW-BBG or a control, each for five weeks. Their study showed a significant reduction in serum TC and LDL-C levels with 3 g/d HMW-BBG. However, studies conducted by Biörklund et al., (2005) and Ibru¨gger et al. (2013) compared the effect of BBG with oat fibre diet and showed no significant lowering effect. Similar results were also observed in studies conducted Keogh et al., (2003) and Smith et al., (2008) when 6 g/d and 10 g/d HMW-BBG respectively was compared to the control, suggesting that the mechanisms may be responsible for the benefits of β-glucan rather than dose alone in improving risk factors associated with CVD.

Combinations of foods on CVD risk

Large groups of studies with combination of treatments have shown to be effective in lowering cholesterol concentrations when compared to a single food component. Dietary components combination in short-term studies are equally effective as statins in reducing serum cholesterol concentrations (Jenkins et al., 2011). However

there are studies which have looked at different combinations but have failed to observe significant effects. Golay et al., (2000) tested 2 diets a 25% protein, 47% carbohydrates and 25% lipids (a combination diet) against a 25% protein, 42% carbohydrates and 31% lipids (balanced diet) and reported no significant difference between the groups for weight loss. The blood lipid parameters decreased in both groups but were not significantly different. Braaten et al., (1994) reported a significant effect of oat β-glucan on serum LDL-C concentrations when mixed into juice, however the effect was not statistically significant when used in bread. Biörklund et al., (2005) reported no significant effects of β-glucan from barley when mixed with10 g/d juice on serum LDL-C concentrations. Lovegrove et al., (2000) reported no significant effects of cereals mixed with low-fat yogurt or low-fat milk given for 8 weeks with 20 g/d of oat bran concentrate, which consisted of 3 g/d of β-glucan on fasting plasma concentrations of TC, LDL-C and TG however, they observed a decrease in HDL-C concentrations. Jenkins et al., (1999) reported no significant effects on serum lipids and lipoproteins with a daily consumption of breakfast cereals containing 36 g of soy protein combined with 168 mg of isoflavones for 3 weeks. Beck et al., (2010) reported no significant changes on plasma cholesterol levels with incorporating different doses of β-glucan from oats (5–6 g/d or 8–9 g/d) into cereal, porridge, and muesli bars in health overweight subjects.

With reference to the HOCO, DHA and HMW-BBG study here below are example of the use of effective food combinations in reducing CVD risk factors. Natale et al., (2012) studied the effects of consumption of a combination of baked products enriched with n-3 fatty acids, folates, β-glucan and tocopherols and reported lowered

serum cholesterol levels in patients with hyperlipidemia. In an another example, Baril et al., (2015) studied intake of a combination of canola oil and DHA in the diet and the results showed an increase in plasma adiponectin concentrations and reduced interleukin (IL)1B levels. Jenkins et al., (2011) used a portfolio diet consisting of a combination of plant sterols, soy protein, nuts and dietary fibre and reported a 13% reduction in LDL-C. Theuwissen et al., (2009) studied the effect of combining β-glucan with plant stanol and reported a 9.6% decrease in LDL-C when compared to the control. Van Horn et al., (2001) showed a decrease in TC and LDL-C with a combination of oats and soy protein when compared to the wheat with milk or oats with milk.

Effect of high oleic canola oil and DHA on CVD risk

According to studies examining effects of oleic acid, the intake in the diet is considered beneficial due its cardio-protective role (Kruse et al., 2015). To enhance the benefits of HOCO on CVD, a double-blinded randomized controlled crossover study (COMIT) was conducted using 131 participants with CVD. DHA enriched HOCO in the ratio of 15:85 was studied. Findings show that the treatment enhanced HDL-C and reduced TG, TC and LDL-C levels when compared from baseline to endpoint. The trial consisted of a four-week treatment period along with a four-week washout period. The primary goals of this study were to determine the impacts of a western diet with the incorporation of HOCO-DHA on blood lipid profiles and FRS. Results demonstrated that consumption of HOCO-DHA reduced levels of serum TC and LDL-C when compared to a western diet. Significant increases in HDL-C levels (3.5%) as well as reduced TG levels

(20%), BP (3.3%) and a reduced FRS were observed, thereby decreasing the risk of CVD (Jones et al., 2015, Jones et al., 2014).

Other factors

Gene-diet interactions

Genetic heterogeneity has a huge effect on the synthesis and absorption of cholesterol metabolism (Philips, 2013). Given that our study is based on the positive outcomes of previously conducted trials, certain evidence focuses towards genetic markers as a proposed factor associated with cholesterol lowering. Mackay et al., (2014), Abdullah et al., (2015), and Wang et al., (2016) in each of their studies demonstrated that gene-by-diet interactions were observed for CYP7A1 SNPs rs3808607 in the TC and LDL-C lowering response to diets with the CYP7A1 SNPs rs3808607-G allele as a dominant factor. However there are other variants that have been identified such as ABCG5 (SNP rs6720173), DHCR7 (SNP rs760241), CETP, ABCA1, ABCG1, ADIPOQ (Abdullah et al., 2015). The cholesterol 7-alpha-hydroxylase (CYP7A1) stands out, with its proposed G-allele higher transcriptional activity located in the promoter region of the CYP7A1 gene and as the rate-limiting enzyme for bile acid synthesis in the liver, the characteristic mechanism for cholesterol elimination (Mackay et al., (2014), Abdullah et al., (2015) & Wang et al., (2016). This enhanced gene expression, thereby leading to a higher association in TC and LDL-C concentrations may play an important role in cholesterol metabolism.

Gut microbiome

New evidence suggests the use of gut microbiota in prevention of CVD by improving the proportion of beneficial members of the microbial group. There is limited evidence supporting the functioning of β-glucan in the upper & lower gastrointestinal (GI) tract. The degradation of β-glucan is dependent on the microbes residing in the lower GI tract, as humans lack the enzymes for breaking down dietary fibre (Hughes et al., 2008; Mitsou et al., 2010). Of noteworthy importance are SCFA production, they modulate various physiological functions and are fundamental in the cholesterol-genesis pathways (Flint et al., 2012). Further research is required in understanding the role of SCFA in enhancing dietary fibre substrates and their effect on cholesterol homeostasis. Wang et al., (2016) reported the shifts in gut microbiota was associated with reduced CVD risk factors which was in response to a molecular weight-dependent BBG consumption. More specifically the consumption of 3g/d HMW-BBG resulted in elevated bacteroidetes and decreased firmicutes and several of their respective bacterial taxa.

Environmental factors

Compelling evidence suggests that lifestyle factors also strongly influence the incidence of CVD. Data from the cohort studies have identified unhealthy diet, smoking, obesity, seasonal variations, physical inactivity, and unhealthy sleep patterns, as important determinants of CVD risk (Ockene et al., 2004; Yu et al., 2016). Modifying one or all factors may reduce the risk of CVD. However, there is minimal control over some of these causes. Therefore identifying innovative strategies, through further research, is a key step to facilitate the prevention and treatment of CVD.

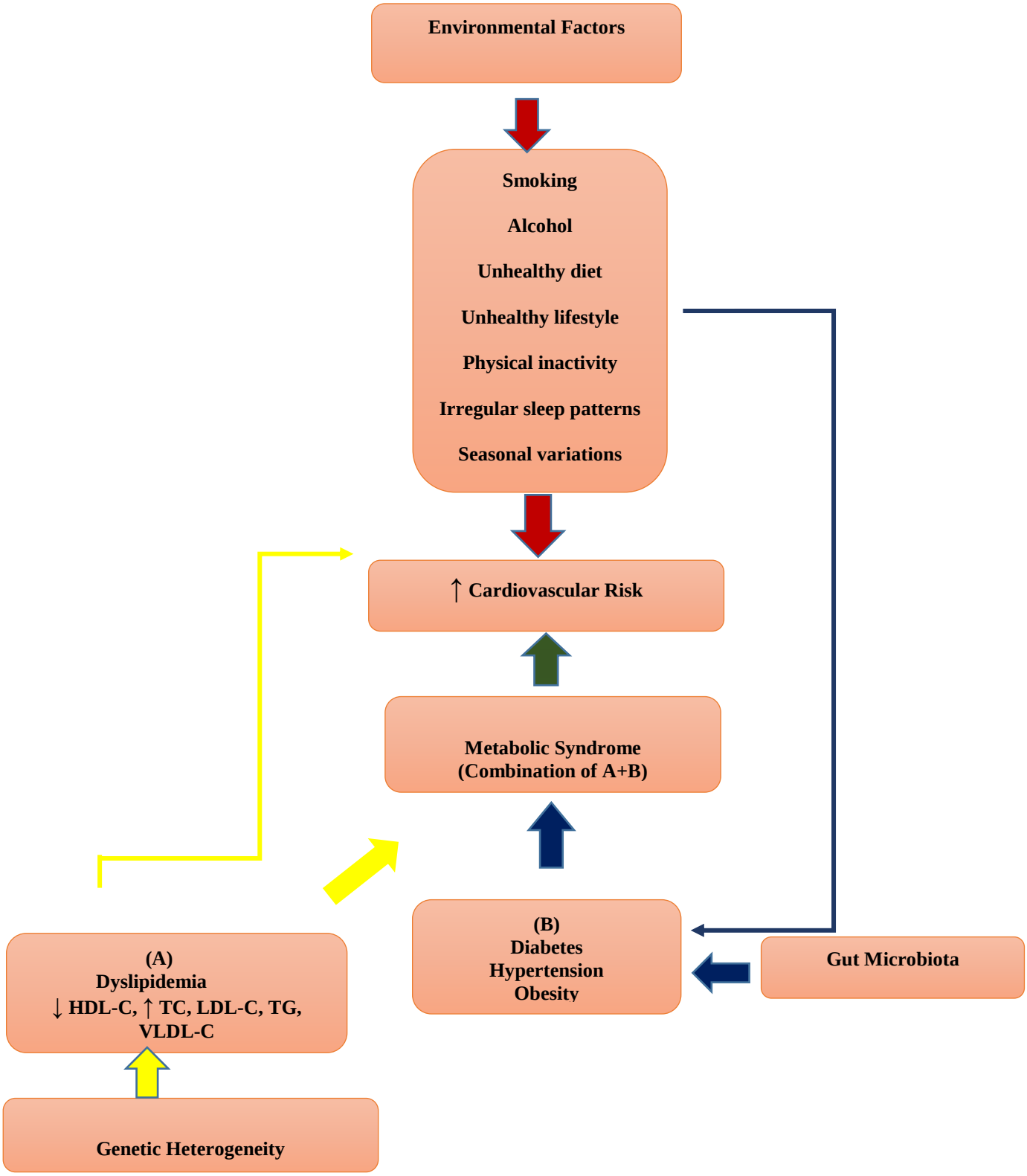


Fig 1. Diagrammatical representation of risk factors associated to CVD as explained in the literature review. Differently colour coded to illustrate their individual associations to CVD.

Additional Biomarkers

Framingham risk score and CVD risk

The development of CVD in most cases results from a combination of risk factors. With the aim of primary prevention or initiating lipid lowering therapy, there is a need to base an assessment on the individual's total risk of CVD rather than focusing only on one particular risk factor. The practical use of FRS in primary prevention of CVD have been well elucidated in studies conducted by Conroy et al., (2003) and Ferket et al., (2014) and illustrating FRS as the superior predictor of CVD. Goya et al., (2005), studied 5128 men (40-59 years) for 20 years with no history of CVD or T2DM and showed that FRS was a better predictor of CVD and stroke in comparison to MetS. Their findings showed the area under the curve for FRS when compared to the number of metabolic abnormalities were 0.68 vs. 0.59 for CHD, 0.60 vs. 0.70 for DM2, and 0.66 vs. 0.55 for stroke ($P<.001$ for all). Along with these findings, recent studies suggest the addition of risk markers such as CT coronary artery calcium score, high sensitivity C-reactive protein, the ankle-brachial index and measurement of the thickness of the carotid intima media to provide a more accurate FRS for predicting CVD outcomes (US Preventive Task Force, 2009). With the addition of these risk markers the novel markers should be then reclassified into clinically relevant risk categories. For e.g: <10% (low risk), 10–19% (intermediate risk) and ≥ 20% (high risk) (Setayeshgar et al., 2013).

Non-cholesterol sterols

Increased serum cholesterol concentrations are modifiable risk factors that are associated with the development of CVD. Early diagnosis is critical in providing for

intervention in lifestyle, nutritional, statin therapy, cholesterol lowering medications, PCSK9 inhibitors or even intestinal bypass surgery to help lower the impact of CVD risk (Health Canada, 2010). The findings of Miettinen et al., (2011), identify NCS as valid markers of cholesterol synthesis and absorption. NCS, are defined as markers of cholesterol intestinal absorption and hepatic synthesis, prompting a finer knowledge of cholesterol balance in the human population (Lupattelli et al., 2012). Quantification of small amounts of NCS in human blood through appropriate chromatographic methods allow the investigation of cholesterol absorption and synthesis. Sitosterol and campesterol are viewed as a surrogate marker for cholesterol absorption, while lathosterol, precursor of cholesterol, is considered a surrogate marker of synthesis (Lupattelli et al., 2012). Elevated levels of lathosterol identify patients with increased risk of CVD. Studies show that 80% of cholesterol synthesized goes through lathosterol while only 20% to desmosterol, indicating that lathosterol is a valid marker for cholesterol production (Mackay et al., 2015). Individuals with increased sitosterol levels have higher rate of cholesterol absorption in the intestine, which is a direct measure of cholesterol absorption. Decreased values, which reflect low cholesterol absorption, are optimal. The sitosterol reduces cholesterol absorption, by blocking the transportation of cholesterol from the plasma membrane to the endoplasmic reticulum. This blockage reduces the absorption of cholesterol, thereby reducing cholesterol synthesis by inhibiting the liver enzyme HMG-CoA reductase (Nissinen et al., 2008). NCS are expressed as ratios to cholesterol and have been validated against absolute measures of cholesterol metabolism. In normolipidemic humans, plasma levels of campesterol and sitosterol are usually within a range of 7-14 and 4-8 umol/L, respectively (Berger et al., 2005). Mackay et al., (2015)

showed that the lathosterol to cholesterol ratio was an effective predictor of cholesterol lowering. Similarly, findings from other clinical trials have also shown sitosterol as a marker of cholesterol lowering (Nissinen et al., 2008; Mackay et al., 2012). Therefore, using these surrogate markers would aid in the prevention of CVD risk factors.

Conclusion

In light of the above evidence, the individual components of HOCO, DHA and HMW-BBG are positively associated with their impact on the biomarkers of overall CVD health. This is the first study to test this combination of HOCO, DHA and HMW-BBG and determine its efficacy on a population with MetS. Dietary interventions consistent with guidelines for healthy eating have the potential to produce substantial health benefits**.** Food combinations with cholesterol lowering properties have proven to be highly effective. Despite this, inter-individual variability exists rendering it difficult to standardize these diets. The work presented in this thesis aims to provide evidence in regards to impacts of combining these functional foods HOCO, DHA and HMW-BBG based on their recommended intake in Canada on traditional and emerging biomarkers of cardiovascular health.

Materials and Methods

Study design

The HOCO, DHA and HMW-BBG (CONFIDENCE) study was a randomized single blinded free-living crossover trial at the RCFFN. This was a seven-month study with four phases each. Each phase consisted of twenty-eight days per phase with an interval of a four-week washout period. Participants consumed one muffin along with the breakfast provided, and the two cookies packed for consumption during the latter part of the day. Institutional ethics boards reviewed and approved the study protocol. Trial registration can be viewed at www.clinicaltrials.gov with ID NCT-02091583 PROTOCOL NO: B2014:029. All participants provided written informed consent as prescribed by the research ethics board.

Eligibility criteria and subject selection

Study advertisements used local media including newspapers, the official website of RCFFN, university campus and our pre-existing database of participants. The eligibility criteria included age : 18-70 years, Body mass index : >25kg/m^2, Waist Circumference: Men: >94 cm, Women > 80cm. Individuals with at least two of the following criteria were accepted into the study: i) TG >1.7 mmol/L; ii) HDL-C <1 mmol/L for male and <1.3 mmol/L for female; iii) fasting glucose >5.6 mmol/L iv) LDL -C >2.7 mmol/L and v) blood pressure >130/100. The exclusion criteria included: (i) participants consuming lipid lowering medications or nutritional supplements known to affect blood lipids, or having any dietary restrictions that would prevent them from consuming the study diet for 28 days, (ii) participants with current or history of any diseases and disorders that could interfere with fat absorption, (iii) participants planning

to conceive during the study period, (iv) smoking or people consuming more than one alcoholic drink per day or history of alcoholism or drug dependence, or (v) people having consumed any experimental medication within one month before screening or as concomitant medications. Twelve-hour fasting blood samples collected on days 1, 2, 29 and 30 to test the lipid profiles. The main outcome measures were serum lipid variables, including TC, LDL-C, HDL-C, TG,VLDL-C. Blood samples were centrifuged at 3000 rpm for 20 minutes at 4 °C, and were separated to yield serum, plasma, and RBC, and then stored at -80 °C until analysis. Monitoring of blood pressure, body weight and waist circumference was done at the beginning and at the end of each treatment period. Three measurements were taken and the average of the three was considered. Whole body DEXA scan (Lunar Prodigy Advance, Lunar Corp., Madison WI) was conducted to determine changes in body fat composition at the end of each treatment phase.

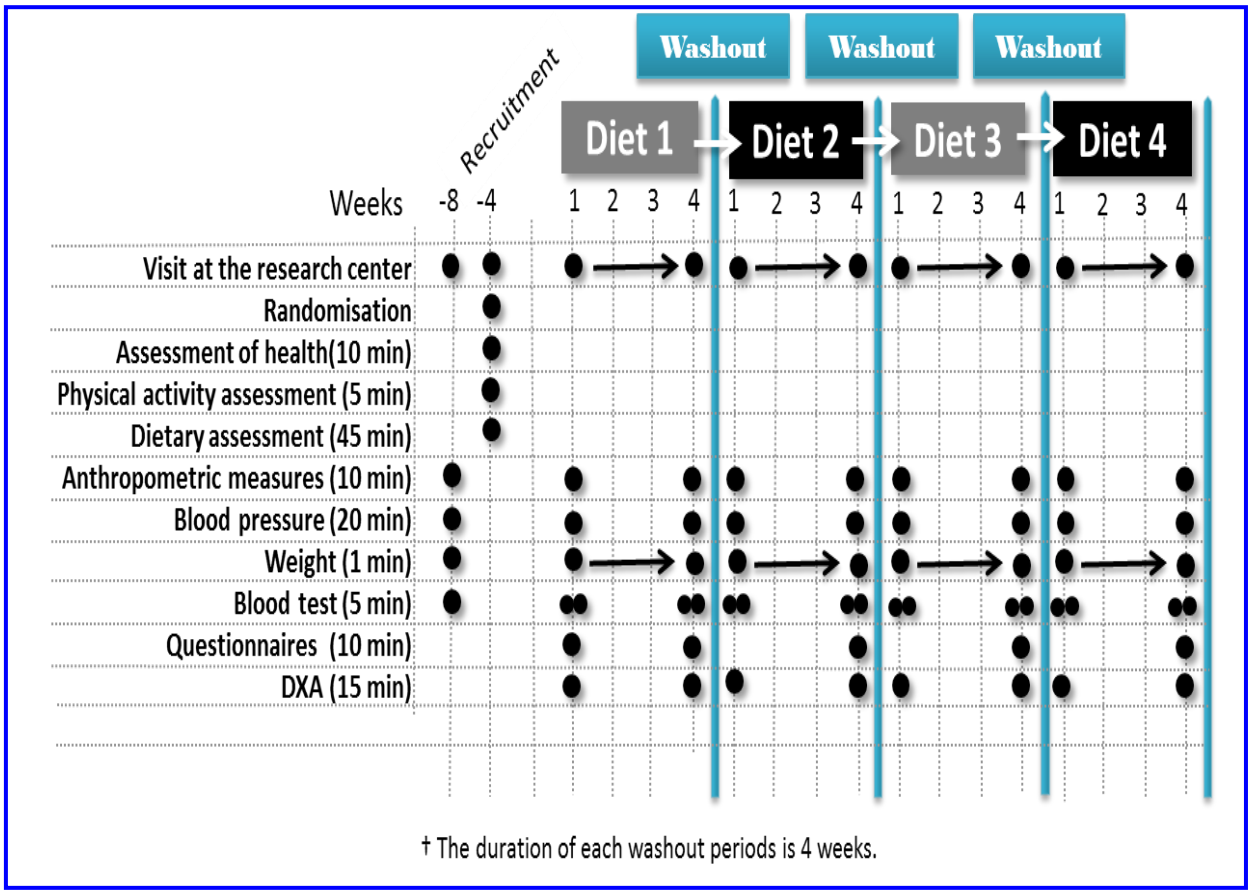


Figure 2. Schematic representation of the study design, and study measurements for the study at the RCFFN**.** Taken from the proposal, published in Bio-Med Central.

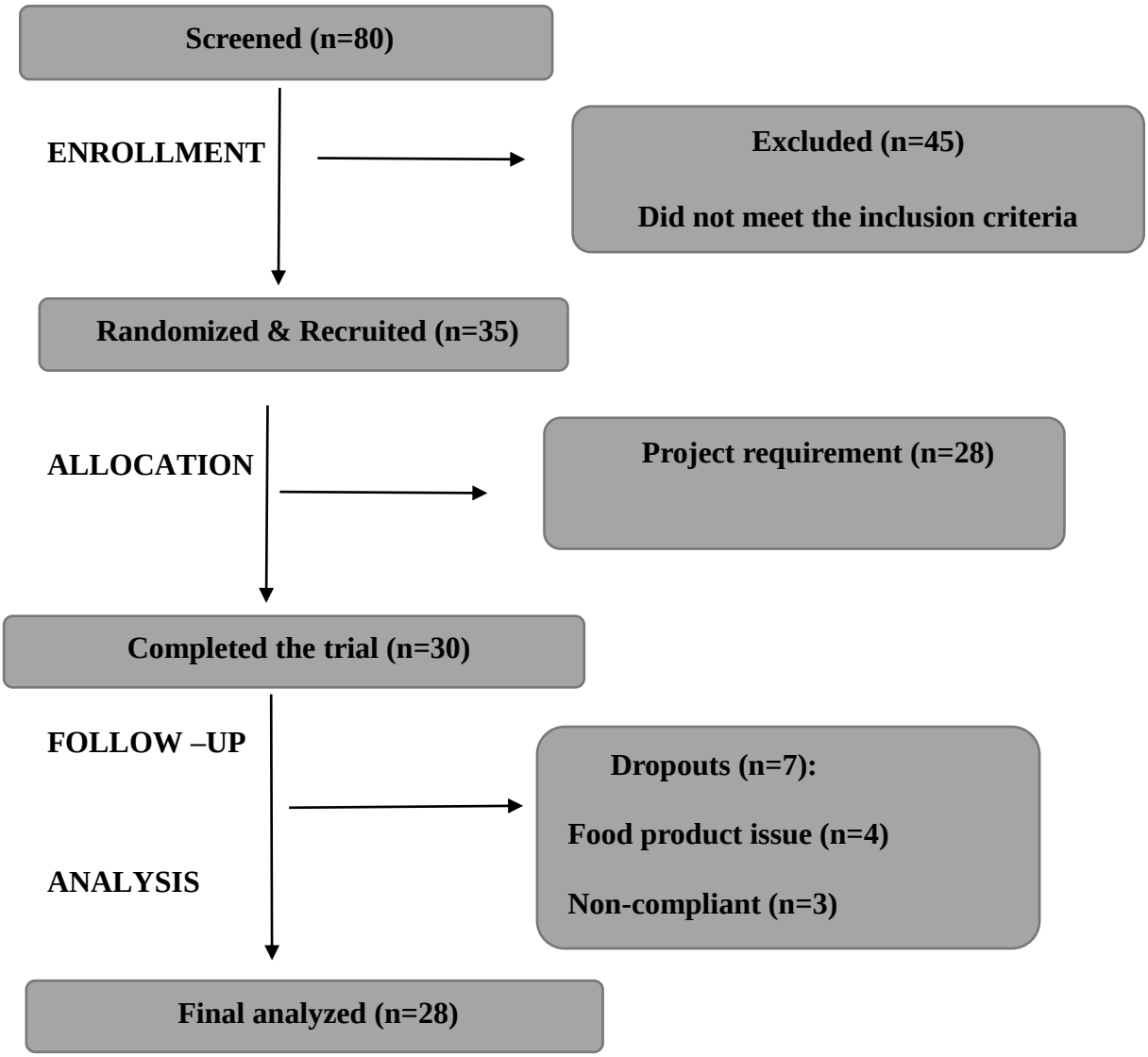


Fig 3. The flow of participants in the CONFIDENCE study. The participant flow through each step of the recruitment, screening and study protocol process at the RCFFN.

Study diets

A randomized, single-blinded crossover trial was conducted at the RCFFN, University of Manitoba. This was a seven-month study and consisted of four phases, each with twenty-eight days per phase and with an interval of a four-week washout period. Prior to the commencement of the study as per protocol, each participant was required to be approved by the physician. Participants consumed one muffin along with the breakfast at the centre and the two cookies were packed for consumption later in the day. There were two flavours of muffins (pumpkin spice and vanilla) and three flavours of cookies (cocoa, lemon and ginger). The flavours of the muffins and cookies were provided in rotation with equal numbers of days per flavour for each treatment period. For the weekend, the muffin and cookies were packed and given on Friday. There was minimal control over the background diets of the participants. The dietitian provided individualized dietary recommendations based on the Canada's Food Guide according to his/her energy requirements before the commencement of the study. Almost 50% of the treatments consumed were under supervision to ensure optimal compliance. Participants were instructed to restrict their intake of fish or seafood products. A 3-day food record was collected at the beginning and end of each treatment phase. Participants were strongly advised to maintain consistency in their dietary intake, physical activity patterns as well as body weight during the experimental period. Recruited participants were randomly assigned to one of the treatment sequences using a Latin square design. The nutrient compositions of the muffins and cookies for the different treatments of the study are listed in **Tables 1 and 2** respectively. Calculations for the nutritional composition of study muffins and study cookies are based on 1g CHO- 4 kcal, 1g of Protein - 4 kcal, 1g

of Fat- 4 kcal, 1g of Fibre - 2 kcal (Health Canada, Canada Food Guide). The nutritional composition tables 1 and 2 are also published in Biomed Central as part of the protocol submission (Ramprasath et al., 2015). The four treatments were as follows: (1) Muffins and cookies containing all-purpose flour and 50 g/day of a blend of sunflower oil, safflower oil, and butter as a control oil. (2) Barley flour with 4.36 g/day of HMW-BBG and 50 g/day of a blend of sunflower oil, safflower oil, and butter as a control oil. (3) All-purpose flour and 50 g/day HOCO-DHA (85:15 DHA oil consists of 40 % DHA) (DHA dosage 3 g/day) and (4) Combination of barley flour with 4.36 g/day, HMW-BBG and 50 g/day of HOCO-DHA. The control oil used in the study represented a typical Western dietary fat intake. The control treatment was composed largely of saturated fat with substantial levels of n-6 linoleic acid as seen commonly in North American diets. Dosages for HOCO-DHA and HMW-BBG were determined based on previous clinical studies done by our research group. The most common procedure of analysis is to perform a separate calculation based on target effect sizes for each of the interventions compared with their respective controls.

The set of four treatments were analyzed using a 2x2 factorial design, which represent two factors (Oil and Flour) each at two levels. The two levels of Oil are Control oil and HOCO-DHA, and the two levels of Flour are Control flour and HMW-BBG **(see Figure 4)**. The effects investigated by this design are the two main effects of the two factors and their interaction effect. A factorial design is an efficient way for exploring the effects of oil and flour in the treatments we used in our study. This trial design is useful to detect a link between factors, while reducing the possibility of experimental error or

confounding variables. It also highlights the relationships between factors, allowing the effects of manipulating a single factor to be isolated and analyzed singly. The purposes of a factorial design are (i) to acquire data from a single experiment by measuring the average effects of all the factors of a sample size (ii) to expand the basis of inferences on one factor by testing it under varied conditions of others and (iii) to analyze the way in which the effects of factors show an interaction with each other. Neufingerl et al., (2013) for example used a 2x2 factor experiment to study the effects of two factors theobromine and cocoa on blood lipids. Their analysis showed a significant main effect of theobromine on HDL-C ($P<0.05$) but not cocoa, and no significant interaction effect. Yusuf et al., (2016) using a 2x2 factorial trial showed the significant effect of the rosvastatin group on lowering risk of CVD events ($P<0.05$) but not candesartan + hydrochlorothiazide and no interaction effect was seen. These two examples of factorial analysis illustrate how the effects of individual factors and their interaction can be studied.

Muffin and cookies recipes were developed and prepared in the Agriculture and Agri-Food Canada laboratories and at the RCFFN metabolic kitchen. Alberta Barley Commission provided the barley grain (cultivar CDC Rattan) and milled into whole grain flour by the Canadian International Grains Institute, Winnipeg. The cultivar CDC Rattan was selected based on the high beta-glucan content and because CDC Rattan is a popular food barley variety in Western Canada. All-purpose flour (Robin Hood) was purchased from the local supermarket. Richardson Oilseed Limited provided the high oleic canola oil. Life's DHA oil was purchased from DSM Nutritional Products (Ayr, ON, Canada). Macronutrients, including protein, carbohydrates, and fibre content of the foods, were

analyzed at the Agriculture and Agri-Food Canada laboratories, RCFFN, Winnipeg, Canada. Total fat and fatty acid profiles of the foods were measured at the RCFFN laboratories (Winnipeg, MB, Canada). The macronutrient contents of treatment foods were similar between different treatments and there were no differences in nutritional values between different flavours of muffins or cookies.

		FLOUR
	Control flour	**Barley β-glucan**
OIL **Control Oil**	**Control oil * Control flour**	**Control oil * Barley β-glucan**
HOCO-DHA	**Control flour * HOCODHA**	**HOCO-DHA* Barley β-glucan**

***Figure 4*.** The 2x2 factorial set of treatments each at two levels. Butter, sunflower & safflower oils (55:20:25) (control oil; 50g/d) & all-purpose flour. Barley β- glucan (4.3g/d) & butter sunflower & safflower oils (55:20:25) (control oil; 50g/d). High Oleic Canola DHA (85:15) (50g/d, 3g/d) & butter sunflower & safflower oils (55:20:25) (control oil; 50g/d) & all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β- glucan (4.3g/d).

Sample collection and analysis

Twelve-hour fasting blood samples were collected on days 1, 2, 29, and 30 of each intervention phase. Participants were advised to be fast for twelve hours and not consume any alcoholic beverages for at least forty-eight hours before blood collection. Participants were instructed to refrain from engaging in intense physical activity including aerobic, spinning or running for at least twenty-four hours before each visit. To determine participant compliance, on the days of blood collection, a medical questionnaire was filled to record the twelve-hour diet, 48 hours alcohol consumption, and 24 hours physical activity. Blood samples were centrifuged at 3000 rpm for 20 minutes at 4 °C. Aliquots of samples yielded serum, plasma EDTA and heparin, and red blood cells (RBC) EDTA and heparin. The samples were then stored at −80 °C until analysis.

Table 1

Nutritional composition of study muffins

Nutritional component measurements	Control oil + Control flour	Control oil + HMW BBG	HOCO-DHA + Control flour	HOCO-DHA + HMW BBG
Weight (g)	112	108	113	109
Energy (kcal/kJ)[a]	379	383	379	383
Total carbohydrate (g)[a]	32.7	36.6	33	36.59
Total protein (g)[a]	8.34	8.79	8	9
Total fibre (g)[a]	0.36	5.77	0.36	5.77
β-glucan (g)[a]	0	2.18	0	2.18
Total fat (% weight)	22.5	22.9	24	23
SFA (%)	34.08	34.24	13.2	14.77
MUFA (%)	27.93	27.16	56.71	52.44
PUFA (%)	37.96	38.16	30.04	33.79
n-3 PUFA (%)	0.86	1.01	5.59	5.64
n-6 PUFA (%)	37.11	37.6	24.93	27.15

Note: DHA docosahexaenoic acid, HOCO high-oleic acid canola oil, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid, SFA saturated fatty acid.[a]Values presented are based on values of the ingredients. Muffins were made with two different flavours: vanilla and spice. Values presented are the average of both the flavours, and there were no major differences between flavours for the measurement. Butter, sunflower and safflower oils (55:20:25) (control oil; 50 g/d) and all-purpose flour. Barley β-glucan (4.3 g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50 g/d). High Oleic Canola DHA (85:15) (50 g/d, 3 g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β- glucan (4.3g/d)

.

Table 2

Nutritional composition of study cookies

Nutritional component measurements	Control oil + Control flour	Control oil + HMW BBG	HOCO-DHA + Control flour	HOCO-DHA + HMW BBG
Weight (g)	53	50	52	52
Energy (kcal/kJ)[a]	213	221	213	221
Total carbohydrates (g)[a]	23	25	23	25
Total proteins (g)[a]	4	4	4	4
Total fibre (g)[a]	0.46	3.17	0.46	3.17
β-glucan (g)[a]	0	1.09	0	1.09
Total fat (% weight)	24	24	24	23
SFA (%)	33.75	35.4	12.5	12.6
MUFA (%)	31.08	29.0	63.4	64.23
PUFA (%)	35.2	35.7	24.13	23.20
n-3 PUFA (%)	0.48	0.61	6.44	6.28
n-6 PUFA (%)	34.7	34.9	17.5	16.9

Note: DHA docosahexaenoic acid, HOCO high-oleic acid canola oil, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid, SFA saturated fatty acid. [a]Values presented are based on values of the ingredients. Cookies were made with three different flavours: cocoa, lemon and ginger. Values presented are the average of both the flavours, and there were no major differences between flavours for the measurement. Butter, sunflower and safflower oils (55:20:25) (control oil; 50 g/d) and all-purpose flour. Barley β-glucan (4.3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50 g/d). High Oleic Canola DHA (85:15) (50 g/d, 3 g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50 g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50 g/d, 3 g/d) & barley β-glucan (4.3 g/d).

Lipid profile and Framingham risk score analysis

To obtain the plasma lipid profiles, average value of days 1 and 2 at baseline and average value of days 29 and 30 at endpoint were considered of each treatment phase. Analysis of plasma samples for lipid profiles including TC, HDL-C, TG, LDL-C, VLDL-C and glucose levels utilizing robotized enzymatic routines on Vitros 350 Chemistry System (Ortho-Clinical and Diagnostics, Johnson and Johnson). For the analyses, 0.5 ml of plasma heparin sample was used to measure the lipid profile. The system was initially calibrated for each parameter and then measured using Calibrator Kit 1 (glucose), Calibrator Kit 2 (TC, TG, LDL-C) and Calibrator Kit 25 (HDL-C). This was followed by using Performance Verifier 1 and 2 to check for the value of each parameter in the given ranges as prescribed online. The Performance Verifier was run every 8 hours to ensure consistency in results. Serum LDL-C concentrations were estimated using the Friedewald formula (LDL-C = TC - HDL-C - TG/5) (Friedewald et al., 1972). FRS ten year CHD risk score were estimated by FRS online calculator based on age, sex, SBP, TC, and HDL-C values of each participant at the end of each dietary phase (D'Agostino et al., 2008). Blood pressure was measured using a digital sphygmomanometer. 3 readings were taken at 10 minutes, 13 minutes and 16 minutes. The average of the 3 readings was considered as the final BP measurement. Any error in reading was considered a faulty measurement and the measurement was redone.

Non-cholesterol sterol analysis

The surrogate marker measurement analysis was done using a method adopted by Lütjohann et al. (2002) and Teunissen et al., (2001), with 5 a-cholestane (Sigma-Aldrich,

Oakville, ON, Canada) as the internal standard. Non-cholesterol sterols and cholesterol were determined using the GC-FID (Agilent Technologies). Serum samples (500 ul) were added with an internal standard, 5-α-cholestane, the samples were then saponified with methanolic KOH. Sterols were extracted twice from the mixture with petroleum ether and evaporated till the tubes were completely dry under nitrogen gas. The samples were further resuspended in 400ul hexane and derivatized with trimethylsilyl (TMS), with the addition of 100ul HMDS+TMCS+Pyridine (3:1:9) (Supelco). NCS and cholesterol were run by the GC-FID with the injector temperature set at 280°C and the detector temperature set at 300°C conducted in duplicates of (1µl) injections. The carrier gas used was helium and the flow rate was 1.0 ml/min with the inlet splitter set at 22.9:1. Cholestanol, desmosterol, lathosterol, campesterol, and sitosterol was measured separately in each samples and expressed as ratios in umol/mmol of cholesterol. Individual peaks of sterols and stanols were identified using authenticated standards (Sigma-Aldrich Canada Ltd., Oakville, ON).

Statistical analysis

Statistical analysis of the effects of the treatments on the lipid profile, Framingham risk and effects of surrogate markers (lathsterol, β-sitosterol, campesterol, cholestanol, desmosterol) on serum cholesterol concentration was tested using SAS (version 9.4; SAS Institute, Inc., Cary, NC, USA). A power calculation indicated that a minimum of 35 participants were necessary to detect an interaction effect in LDL-C concentrations of 0.43 mMol/L with a standard deviation of 0.13 mMol/L in a 2x2 factorial full design, 2 sided testing with alpha = 0.05 and power = 0.8. To allow for a dropout rate of 20%, the desired sample size was 28. The mixed model procedures in

SAS (Proc mixed in version 9.4) was used with treatment, sequence, phase and gender considered as fixed effects, whereas subject/participant was added as a random effect in the model. Treatment effects in the model were analyzed as a factorial set with the two factors, Oil type and Flour type and their interaction. The purpose of analyzing treatment effects as a factorial is to dissect the factorial effects of oil with two levels (Control and HOCO-DHA), flour with two levels (Control and BBG) and their interaction. The normality of distribution of data was determined by a Shapiro-Wilk test, and the non-normal variables were normalized by identifying the outliers from the studentized residuals before comparisons with other treatments. Results for treatments in the text and figures are expressed as least-squares means and their standard errors from the model described above, unless stated otherwise. Multiple comparison of treatment means was carried out using Tukey's test with a type I error of 0.05.

Results

Baseline characteristics

Thirty-five participants were randomly assigned to each of the four treatments from January 2015-November 2015. No statistically significant differences were observed for subjects, age, BMI, TC, glucose, LDL-C, HDL-C, TG, body weight, waist circumference, SBP and DBP at baseline as seen in Table 3. Thirty subjects completed all four-treatment periods. Seven participants were excluded from the study. Four dropped out during the study due to low tolerance to the test products. One was non-compliant during the study and the remaining two individuals were considered non compliant after lab and statistical analysis and were excluded. Twenty-eight participants were finally statistically analyzed.

Table 3

Baseline characteristics of study participants

	Control oil + Control flour	**Control oil + HMW BBG**	**HOCO-DHA + Control flour**	**HOCO-DHA + HMW BBG**
MALE[a] (n)	12	12	12	12
FEMALE[a] (n)	17	17	17	17
AGE[a] (years)	50 ± 2	50 ± 2	50 ± 2	50 ± 2
BMI[a] (kg/m^2)	31.6 ± 5.2	31.6 ± 5.2	31.5 ± 5.2	31.4 ± 5.1
Body weight(kg)	89.3 ± 17.1	89.1 ± 17.2	88.8 ± 16.7	88.5 ± 17.1
TC[b] (mmol/L)	5.30± 0.7	5.32 ± 0.72	5.38 ± 0.72	5.36 ± 0.8
LDL-C[b] (mmol/L)	3.31 ± 0.7	3.23 ± 0.6	3.33 ±0.6	3.34 ± 0.7
HDL-C[b] (mmol/L)	1.19 ± 0.21	1.17 ±0.22	1.21 ± 0.22	1.17 ± 0.2
VLDL [b] (mmol/L)	29.9 ± 13.0	34.7 ± 17.0	31.37 ± 12.0	32.3 ± 14.9
TG[b] (mmol/L)	1.69 ± 0.74	1.96 ± 1.0	1.76 ± 1.0	1.82 ± 0.84
GLUCOSE[b] (mmol/L)	5.52 ± 0.6	5.43 ± 0.72	5.47 ± 0.72	5.60 ± 0.7
SBP[b] (mmHg)	119 ± 12.4	120 ± 14.0	118 ± 13.9	120 ± 15.4
DBP[b] (mmHg)	79.6 ± 8.1	81.1 ± 8.6	80.0 ± 8.6	79.7 ± 7.1

Note: Values are representative of subsample of 29 participants (RCFFN) for individual treatment phases with means and their standard deviations. [a]Anthropometric measurements. [b]Lipid profile.TC-Total Cholesterol, LDL-C- Low-density lipoprotein cholesterol, DHDL- Direct High-density lipoprotein, TG-Triglycerides, SBP- Systolic blood pressure, DBP- Diastolic blood pressure.

Effect of treatment on lipid profile, blood pressure and FRS

At endpoint, the HOCO, DHA treatment showed a significant increase ($P< 0.05$) in HDL-C and a significant decrease ($P< 0.05$) in TG when compared to control. We also observed a decrease ($P< 0.05$) in VLDL-C, TC: HDL-C and DBP when compared to the control. The combination of HOCO, DHA and HMW-BBG showed a significant increase ($P< 0.05$) in HDL-C and a significant decrease ($P< 0.05$) in TG when compared to control. The combination of HOCO, DHA and HMW-BBG also showed a significant decrease ($P< 0.05$) in VLDL-C, TC: HDL-C and DBP at endpoint when compared to the control. No significant differences were observed for TC, LDL-C, glucose level and SBP **(Table 4)**. Between treatments, the HOCO-DHA treatment was significantly different ($P<0.05$) when compared to HMW-BBG treatment for TG, HDL-C, VLDL-C, TC: HDL-C and DBP. Similarly the combined HOCO, DHA and HMW-BBG treatment was significantly different ($P<0.05$) when compared to HMW-BBG treatment for TG, HDL-C, VLDL-C, TC: HDL-C and DBP. At endpoint, in the 2x2 factor analysis, there was a significant main effect ($P< 0.05$) on the HDL-C, VLDL-C, TC: HDL-C, TG and DBP seen for HOCO DHA oil factor but not for the HMW-BBG factor. There was no significant interaction effect between HOCO, DHA and HMW-BBG on any of the lipid parameters ($P>0.05$) **(Table 5)**.

At endpoint the combination of HOCO, DHA and HMW-BBG was also able to show an overall significant decrease ($P< 0.05$) in cholesterol FRS for both males and females when compared to the control **(Figure 5)**. Individual treatments HOCO-DHA and HMW-BBG did not show any significant difference ($P>0.05$) when compared to control. Between treatments there were no significant differences ($P>0.05$).

Table 4

Between treatment comparisons of blood lipid profile, blood pressure levels

	Control oil + Control flour	**Control oil + HMW BBG**	**HOCO-DHA + Control flour**	**HOCO-DHA + HMW BBG**
TC[b] (mmol/L)	5.40± 0.14	5.26 ± 0.00	5.39 ± 0.13	5.20 ± 0.15
LDL-C[b] (mmol/L)	3.35 ± 0.12	3.25 ± 0.13	3.35 ±0.13	3.19 ± 0.13
HDL-C[b] (mmol/L)	1.20 ± 0.03[a]	1.18 ±0.04[a]	1.30 ± 0.05[b]	1.31 ± 0.05[bc]
TG[b] (mmol/L)	1.79 ± 0.13[a]	1.84 ± 0.13 [a]	1.58 ± 0.16[b]	1.56 ± 0.13[bc]
VLDL[b] (mmol/L)	32.4 ± 2.5[a]	30.7 ± 2.1[a]	28.5 ± 2.8[b]	27.3 ± 2.3[bc]
TC: HDL-C[b] (mmol/L)	4.54 ±0.48 [a]	4.53± 0.15 [a]	4.23 ± 0.15[b]	4.09 ± 0.14[bc]
GLUCOSE[b] (mmol/L)	5.49 ± 0.10	5.40 ± 0.11	5.71 ± 0.17	5.63 ± 0.15
SBP[b] (mmHg)	122 ± 3.55	118 ± 2.75	119 ± 3.13	120 ± 2.70
DBP[b] (mmHg)	81.0 ± 1.81[a]	80.0± 1.50 [a]	78.2 ± 1.80[b]	78.1 ± 1.70[bc]
FRS*	7.77±0.49 [a]	7.17± 0.40 [a]	7.04± 0.49 [a]	6.87± 0.50 [bc]

Note: Values are representative of subsample of 28 participants (RCFFN) for individual treatment phases with means and their standard errors at endpoint. [b] Lipid profile. TC-Total Cholesterol, LDL-C- Low-density lipoprotein cholesterol, DHDL- Direct High-density lipoprotein, TG- Triglycerides, SBP- Systolic blood pressure, DBP- Diastolic blood pressure. Means in a column for a given factor or interaction which do not show a common letter are significantly different ($P<0.05$). A mixed-model ANOVA and post hoc Tukey's test was used. Butter, sunflower and safflower oils (55:20:25) (control oil; 50gm/d) and all-purpose flour. Barley β-glucan (4.3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d). High Oleic Canola DHA (85:15) (50g/d, 3g/d) & Butter sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β-glucan (4.3g/d). *For FRS the HOCODHA & HMW-BBG treatment is only significantly different from the control oil+ control flour treatment.

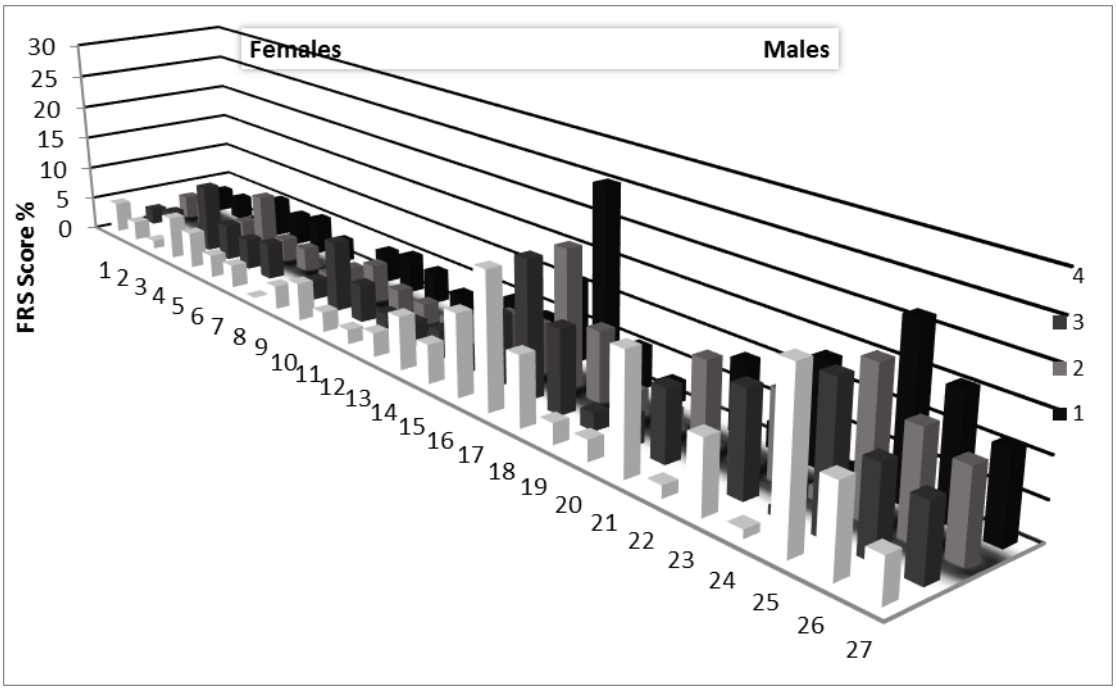


Fig 5: Individual changes for both males and females in Framingham risk score at endpoint. Numbers 1-27 indicate individual participant changes for all four treatments. FRS is calculated for ages 30-100 years, as one participant is below 30 years total number of participants is 27. Females are 1-16 and Males are from 17-27. The legends in numbered format indicate: Treatment 1: Butter, sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all- purpose flour. Treatment 2: Barley β-glucan (4.3gm/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d). Treatment 3: High Oleic Canola DHA (85:15) (50g/d, 3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50gm/d) and all purpose flour. Treatment 4: High Oleic Canola DHA (85:15) (50g/d, 3g/d) & Barley β-glucan (4.3g/d).

Table 5

Factorial effects and their significance for blood lipid profiles, blood pressure levels (P values)

	HMW BBG	HOCO-DHA	HOCO-DHA + HMW BBG
TC[b] (mmol/L)	0.09	0.90	0.29
LDL-C[b] (mmol/L)	0.06	0.63	0.61
HDL-C[b] (mmol/L)	0.5	<0.001**	0.55
TG[b] (mmol/L)	0.48	0.01**	0.81
VLDL[b] (mmol/L)	0.38	0.00**	0.82
TC:HDL[b] (mmol/L)	0.22	<0.001**	0.26
Glucose[b] (mmol/L)	0.91	0.10	0.60
SBP[b] (mmHg)	0.27	0.80	0.17
DBP[b] (mmHg)	0.61	0.04**	0.72

Note: Values are representative of subsample of 28 participants (RCFFN) for individual treatment phases at endpoint. [b]Lipid profile. TC-Total Cholesterol, LDL-C-Low-density lipoprotein cholesterol, DHDL-Direct High-density lipoprotein, TG-Triglycerides, SBP-Systolic blood pressure, DBP-Diastolic blood pressure. Means in a column for a given factor or interaction which show** are significant (P<0.05). A mixed-model ANOVA and post hoc Tukey's test was used. Butter, sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. Barley β-glucan (4.3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d). High Oleic Canola DHA (85:15) (50g/d, 3g/d) & Butter sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β-glucan (4.3g/d)

Non-cholesterol sterols as markers on lipid lowering

At endpoint the high oleic canola docosahexaenoic acid treatment showed a significant increase ($P < 0.05$) in sitosterol when compared to control. Between treatments, the high oleic canola docosahexaenoic acid treatment was significantly different ($P<0.05$) when compared to HMW-BBG treatment for sitosterol. Similarly the combined high oleic canola docosahexaenoic acid and high molecular weight barley β-glucan treatment was significantly different ($P<0.05$) when compared to high molecular weight barley β-glucan treatment for sitosterol. The combination of high oleic canola oil, omega-3 oils and barley β-glucan showed a significant increase ($P < 0.05$) for sitosterol when compared to control. There were no significant changes in the other surrogate markers identified for analysis **(Table 6)**. At endpoint, in a 2x2 factor analysis, there was a significant main effect ($P < 0.05$) in the sitosterol to cholesterol ratio value seen for the HOCO DHA oil factor but not for the HMW-BBG factor. There was no significant interaction effect between HOCO, DHA and HMW-BBG on any of the parameters measured ($P>0.05$) **(Table 7).**

Table 6

Non-cholesterol sterol analysis

	Control oil + Control flour	**Control oil + HMW BBG**	**HOCO-DHA + Control flour**	**HOCO-DHA + HMW BBG**
Lathosterol[a] (umol/mmol)	0.89 ± 0.08	1.03 ± 0.13	1.17 ± 0.12	1.16 ± 0.11
Desmosterol[a] (umol/mmol)	1.39 ± 0.21	2.44 ± 0.14	1.45 ± 0.16	1.13 ± 0.12
Sitosterol[a] (umol/mmol)	1.36 ± 0.14[a]	1.77 ± 0.22 [a]	2.74 ± 0.49[b]	2.08 ± 0.26[bc]
Campesterol[a] (umol/mmol)	3.10± 1.02	2.90± 0.62	3.01± 0.37	2.56± 0.33
Cholestanol[a] (umol/mmol)	0.72 ± 0.14	0.75 ± 0.11	0.80 ± 0.15	0.89 ± 0.12

Note: Values are representative of subsample of 28 participants (RCFFN) for individual treatment phases with means and their standard errors at endpoint. [a] Non-cholesterol sterols. Means in a column for a given factor or interaction which do not show a common letter are significantly different ($P<0.05$). A mixed-model ANOVA and post hoc Tukey's test was used. Butter, sunflower and safflower oils (55:20:25) (control oil; 50gm/d) and all-purpose flour. Barley β-glucan (4.3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d). High Oleic Canola DHA (85:15) (50g/d, 3g/d) & Butter sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β-glucan (4.3g/d).

Table 7

Factorial effects and their significance for non-cholesterol sterols (P values)

	HMW BBG	HOCO-DHA	HOCO-DHA + HMW BBG
Lathosterol (umol/mmol)	0.96	0.06	0.65
Desmosterol (umol/mmol)	0.57	0.62	0.48
Sitosterol (umol/mmol)	0.91	0.00**	0.22
Campesterol (umol/mmol)	0.75	0.06	0.38
Cholestanol (umol/mmol)	0.66	0.69	0.24

Note: Values are representative of subsample of 28 participants (RCFFN) for individual treatment phases at endpoint. Means in a column for a given factor or interaction which show** are significant (P<0.05). A mixed-model ANOVA and post hoc Tukey's test was used. A mixed-model ANOVA and post hoc Tukey's test was used. Butter, sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. Barley β-glucan (4.3g/d) & butter sunflower and safflower oils (55:20:25) (control oil; 50g/d). High Oleic Canola DHA (85:15) (50g/d, 3g/d) & Butter sunflower and safflower oils (55:20:25) (control oil; 50g/d) and all-purpose flour. High Oleic Canola DHA (85:15) (50g/d, 3g/d) & barley β-glucan (4.3g/d).

Discussion

The purpose of this study was to evaluate the effect of combining HOCO, DHA and HMW-BBG on blood lipid profiles and FRS in a population with MetS. After a four-week treatment period the combination of HOCO, DHA and HMW-BBG improved HDL-C, lowered TC: HDL-C ratio, TG, VLDL-C and DBP as well as showed an overall decrease in cholesterol FRS for both males and females when compared to the control. The overall significant decrease in cholesterol FRS for the combined treatment observed in this study was mainly as a result of increased HDL-C concentrations and a decrease in blood pressure. However, cholesterol FRS changes in other and between treatments were not significantly different. Decrease in blood pressure based on previous literature are identified to be related to changes in vascular function, hence blood pressure measurements were conducted in a consistent manner. Understandably, the HOCO-DHA treatment also showed an increase in HDL-C, decrease in TC: HDL-C ratio, TG, VLDL-C and DBP when compared to control. Some of the results of the study are consistent with the COMIT trial, where the HOCO-DHA was the only diet that increased HDL-C, produced greatest reduction in TG and lowered SBP and DBP when compared with the other diets (Jones et al., 2014). The TC: HDL-C ratio is also a strong predictor of CVD risk. Owing to the disproportion between the atherogenic cholesterol carrier and the protective lipoproteins cholesterol carrier, individuals with a higher TC: HDL-C ratio have greater CVD risk (Genest et al., 2003; Millán et al., 2009). The HMW-BBG individual treatment showed no significant effects when compared to control. The AHA guidelines recommend 2-4 g/d fish oil supplement daily for individuals at risk for CVD (Etherton, et al., 2003). Effect of dose response studies showed that 3 g/d of DHA was

capable of lowering TG levels whereas dosages as low as 0.85 g/d did not. The dose of DHA (3 g/d) used in the current study was based on the recommended range for TG lowering, to evaluate individuals with CVD risk factors. Serum LDL-C tended to decrease after the consumption of the combination of HOCO, DHA and HMW-BBG, however, this reduction was not statistically significant. Omega-3 fatty acid studies have shown TG lowering effect with an increase in LDL-C concentration (Delgado et al., 2012). The theory is that the intake of DHA based on previous literature could have resulted in an increase in LDL-C while lowering TG. At the same time the effect of HMW-BBG could have resulted in lowering of LDL-C. The increase in LDL- C could have blunted the effect of HMW-BBG, as seen in previously conducted trials. As a result of this push-pull mechanism, it may have led to no change observed for LDL-C.

The main objective of this study was to determine the efficacy of functional ingredients of HOCO, DHA and HMW-BBG combined into a single food format and observe whether a synergistic effect exists on blood lipids and FRS. Our findings show that the significant effects of the combined HOCO, DHA and HMW-BBG treatment seen in the lipid profile and FRS were not over and above the individual treatment effect sizes, indicating that the positive outcomes of combining HOCO, DHA and HMW-BBG had no overall synergistic impacts.

The factorial analysis for the blood lipid profile and NCS showed the main effect of HOCO DHA oil improved blood lipid concentrations and showed an increase in sitosterol. HMW-BBG flour given as per the health claim dosage requirement, led to a non-significant effect on blood lipid profile and no change in sterols markers. There was no significant interaction effect between HOCO DHA oil and HMW-BBG on blood lipid

profile and NCS. This finding suggests that HOCO DHA oil is the major active component, and maybe responsible for the significant effect seen.

Individuals with a higher rate of cholesterol absorption in the intestine have elevated plasma levels of sitosterol, which is a direct measure of cholesterol absorption (Miettinen et al., 2011). The main findings from the NCS analysis show an increase in sitosterol when the HOCO, DHA and HMW-BBG treatment was compared to control. No significant changes were observed in the other markers analyzed.

The cholesterol lowering ability of sitosterol has been extensively studied. Absorption and synthesis are inversely related (Miettinen et al., 1997; Alphonse et al., 2016). Decreased values, which reflect low cholesterol absorption, are optimal. The mechanism, by which sitosterol reduces cholesterol absorption is, by blocking the transportation of cholesterol from the plasma membrane to the endoplasmic reticulum. This blockage reduces the absorption of cholesterol, reducing cholesterol synthesis through inhibition of the liver enzyme HMG-CoA reductase (Miettinen et al., 1990; Mackay et al., 2014). Sitosterols reduce the pool of absorbable cholesterol by displacing cholesterol from intestinal micelles, as well as are rapidly taken up by enterocytes and increase the expression of the adenosine triphosphate-binding cassette A1 sterol transporter. This reabsorption and limited control over the background diet may explain the increase in sitosterol levels. In addition to lowering cholesterol absorption, plant sterols and stanols are reported to induce a compensatory rise in cholesterol synthesis. (Jones et al., 2000). The HOCO, DHA and HMW-BBG combination treatment, HOCO-DHA treatment and HMW–BBG treatment also show an increase lathosterol marker when compared to control, though not statistically significant. Our findings with HMW-

BBG treatment correlate with the findings of Wang et al., (2016) wherein the cholesterol fractional synthesis rate was not affected by β-glucan ingestion. These findings suggested that increasing bile acid synthesis rather than inhibiting cholesterol synthesis or absorption is the mechanism responsible for the cholesterol-lowering effect of β-glucan. Since β-glucan interferes with cholesterol synthesis and DHA decreases synthesis by increases absorption, there may be a push and pull mechanism, resulting in an over-compensatory effect in the cholesterol synthesis, increasing lathsoterol levels. As a result, we do not observe a TC or LDL-C lowering in the lipid values analyzed. Previous literature identify decrease in VLDL-C and an increase in HDL-C levels to be correlated with cholesterol absorption, while decrease in TC and increase in LDL-C correlates with synthesis markers (Miettinen et al., 2010; Lupattelli et al., 2012 & Dayspring et al., 2015).

In conclusion, the present study demonstrated that the combination of HOCO, DHA and HMW-BBG had no significant synergistic impacts on circulating cholesterol levels and FRS during 28 day intervention period. Whether optimizing the ratio of the oils or preliminary screening of the right genotype could provide better clinical outcomes, this requires further investigation.

Overall Conclusions

Summary and implications

Despite the overall positive outcome of the study, there are underlying factors that need to be addressed and considered for future research. The combination of HOCO, DHA and HMW-BBG have potential benefits and the impact of this study is a step towards achieving a better understanding of evaluating the impacts of dietary combinations on CVD. The focus is streamlined at examining genetic factors that may contribute to the existing picture of evidence. It may therefore be advisable to stratify based on their responsiveness to achieve better outcomes for clinical trials in future.

Limitations and future directions

Our study was not able to show a decrease in LDL-C, TC, glucose and SBP levels. Given that, the study was a free-living trial, we based our outcomes only on compliance achieved through the foods provided. There was minimal control of the background diet, which is a limitation of our study. Randomized clinical trials with control over the background diet offer better insights into the potential influences of the intake of each treatment, providing more outcomes that are effective (Sorensen et al., 2006). We can only assume that the participants followed the dietary advice provided by the dietitian.

The lowering of circulating cholesterol levels and prevention of CVD are generally associated with dietary interventions. However, recent findings suggest that some individuals are hyper-responders to certain dietary interventions while others are hypo-responders. This inter-individual variability suggests an existence of genetic heterogeneity. Statistically, genetic factors account for approximately 40-60% of the

inter-individual variability in CVD risk apart from diet, both directly and through influences on lipid biomarkers (Abdullah et al., 2015). Reviewed studies indicate that carriers of certain genotypes within cholesterol related genes show a higher response to a given dietary intervention (Philips, 2003; Agrawal et al., 2014). This has been seen in investigations of lipoprotein responsiveness to dietary manipulation and ApoE phenotype, suggesting that there is a direct relationship between serum cholesterol and phenotype (Mackay et al., 2015). Wang et al., (2016) suggested that the cholesterol lowering effect of BBG might be due to genetic characteristics, with the results of their study showing a gene diet interaction. Individuals with the CYP7A1 rs3808607 gene had higher responses to 3 g HMW-BBG/d in lowering TC than TT carriers ($P<0.05$). Bile acid synthesis has a large influence on cholesterol homeostasis. The CYP7A1 gene codes for the cholesterol 7-a-hydroxylase, is the enzyme responsible for the rate limiting first step in the bile acid synthesis (Meaney, 2014). We are assuming our subjects consisted of only TT carriers or that individually the effect on some participants for a lowered TC or LDL-C may have been significant. However, the overall difference was not large enough to show a significance at ($P<0.05$).

The health claim of BBG is based on a high fibre and a low fat background diet, demonstrated to be effective in lowering circulating cholesterol levels. Optimizing the ratio of the oils in the test foods, to test the effects of a combination of HOCO, DHA and HMW-BBG on plasma lipids would be advisable. Considering these limitations, future dietary intervention studies with a fully randomized controlled design need to adopt a prior selection and recruitment process of a large sample size of carriers of genotypes known to be associated with their specific responsiveness. Such studies would enable

thorough understanding of genetic variability in response to plasma lipid and cholesterol metabolism.

In addition to the above-mentioned directions, analyzing certain other markers could aid in a better understanding of cholesterol lowering in the management of CVD. Some of them include, measuring the LDL-C and HDL-C particle size in estimating the individuals CVD risk. Occurrence of small, dense LDL-C particles, are usually related with increased concentrations of TG and VLDL particles, along-with reduced concentrations of HDL-C and large HDL-C particles. Examining these lipoprotein subclasses will help understand the effectiveness of the interventions aimed at targeting CVD. Another marker that can be analyzed are the inflammatory markers. The NCEP ATP III guidelines identify these markers as emerging risk factors, such as high-sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA), white blood cell (WBC) count and fibrinogen which could be used to estimate the absolute risk against standard risk factors.

Although a 4-week treatment period is widely accepted, with beneficial outcomes seen in numerous clinical trials, the duration may have not been sufficient in this study to observe a change in TC or LDL-C lowering. Increasing the duration to 6 or even 8 weeks, with the addition of a run in period or a full intervention trial that could serve as a valuable tool in screening out ineligible or non-compliant individuals.

Final conclusion

CVD remains the leading cause of morbidity, mortality and increased health care related costs in Canada. Cholesterol lowering is the primary prevention target for CVD

risk reduction through dietary modification, lifestyle changes and physical activity (Ramprasath et al., 2010). The work presented in this thesis is a step towards understanding the impact of combination of functional foods on CVD risk. Additionally this study helped us understand whether a combination is better than single functional food on cholesterol lowering. The combination of HOCO, DHA and HMW-BBG does improve blood lipid profiles and FRS when compared to control. However, the evidence clearly outlines the need to find an acceptable dosage of oil to match with 3 g/d HMW-BBG in order to achieve the targeted goal. The research also sheds light on inter-individual variability as a coherent factor in lowering CVD risk. Specifically, suggesting common genetic variants in the cholesterol metabolic pathways, including those related to bile acid flux, are involved in determining responses of serum cholesterol, as well as cholesterol absorption and biosynthesis rates in humans (Fuchs, 2003). The plasma levels of the biomarkers and their relative distribution heavily depends on the reciprocal interaction between endogenous cholesterol absorption and synthesis, and the manner in which each mechanism drives one another. In future, measuring blood lipid profiles with the use of genetic markers for risk assessment in responders to dietary cholesterol, along-with control over the background diet may lead to better clinical outcomes (AbuMweis et al., 2010). Thus providing a better understanding of the pathophysiological processes concerning CVD. In the long-run, the findings from this research may help improve the overall cardiovascular health and increase the commercial availability of canola-barley products worldwide.

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Appendix A

Ethics approval for the study

UNIVERSITY OF MANITOBA | BANNATYNE CAMPUS
Research Ethics Board

P126 - 770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Telephone 204-789-3255
Fax 204-789-3414

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR AMENDMENTS AND ADDENDUMS

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. Peter Jones	U of M/Richardson Centre for Functional Foods and Nutrceuticals	B2014:029
BREB MEETING DATE (If applicable):	**APPROVAL DATE:** February 6, 2015	
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable): N/A		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
Appl#312802; NUT	Appl#312802; NUT Developing and Evaluating a Novel Food Supplement, CONFIDENCE (Canola Oil and Fibre with DHA Enhanced) Aiming at the Management of CVD Risk in a Population with Metabolic Syndrome - formerly Developing and Evaluating a Novel Supplement Beverage, CONFIDENCE (Canola Oil and Fibre with DHA Enhanced) Aiming at the Management of CVD Risk in a Population with Metabolic Syndrome

SPONSORING AGENCIES AND/OR COORDINATING GROUPS:
CIHR

REMINDER: THE CURRENT BREB APPROVAL FOR THIS STUDY EXPIRES: March 24, 2015

REVIEW CATEGORY OF AMENDMENT:	Full Board Review ☐	Delegated Review ☒
Submission Date of Investigator Documents: January 23, 2015	BREB receipt date of Documents: January 23, 2015	

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol: Amendment per submission form		January 23, 2015
Consent and Assent Form(s): Research Participant Information and Consent Form		23-Jan-2015
Other:		

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the amendment to the research study/project named on this ***Certificate of Approval*** as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. The amendment and documents listed above were 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 regulation of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in

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. This amendment 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 approvals 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,

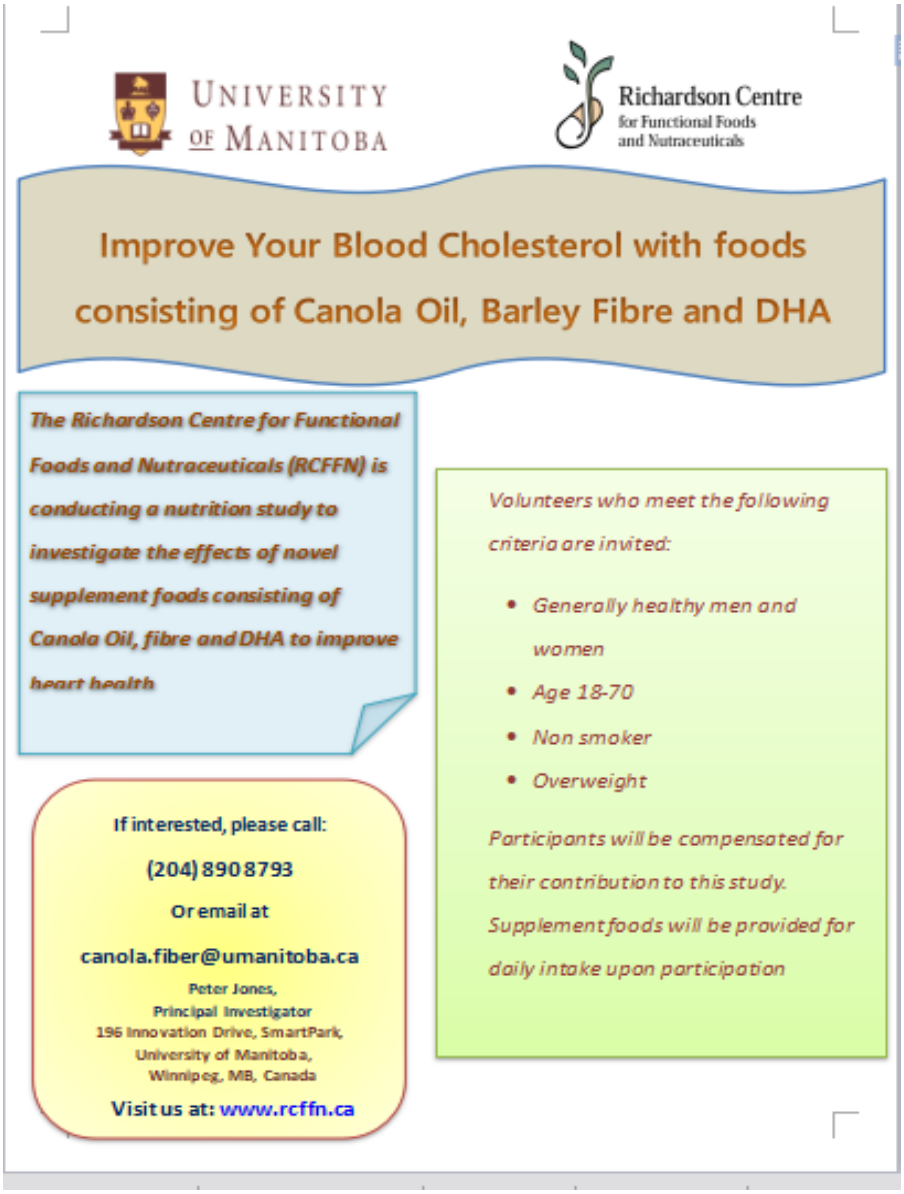
[redacted]

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

Appendix B

Forms corresponding to the study

Study advertisement – Poster



UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

Screening Consent

I have expressed an interest in participating in the above named study. I have been invited to have my health assessed to determine if I meet the requirements of the study.

Depending on my cholesterol level and other health indicators, and my availability over the next 7 months, I will be offered the opportunity to participate in this study.

To allow the necessary information to be obtained, I agree to provide fasting blood samples (approximately 10 ml or 2 teaspoons) for the measurement of blood cholesterol and other biochemical and hematological tests. I also agree to have a physical assessment done (blood pressure, weight, height, waist and hip measurement etc.), to complete a health history form.

Risks and Discomforts

As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part in the study. 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. Shahrokh Nejad Ghaffar, will be available to contact at any time. Dr. Shahrokh Nejad Ghaffar can be reached at [redacted]

Confidentiality

Information gathered during screning study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the

Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned patient number (or code).

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals. With your permission your Family Physician (GP) will be notified about your participation in this study.

Prior to taking part in the study, I shall be given the specific study consent form to read and sign if I am still interested in participating.

I understand I can withdraw from this process at any time at my discretion.

_____ _____

Participant's Signature **Participant's Name (please print)**

Date

_____ _____

Investigator's Signature **Investigator's Name (please print)**

Date

(or signature of person who (position)

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

You are being asked for permission to be contacted in the future for participation in research studies. Please take your time to review this consent form and discuss any questions you may have. You are free to discuss this form with your friends, family and others before you make your decision.

If you agree to be contacted in the future for research purposes, information about you will be entered into an electronic database. The database will be maintained by Dr. Peter Jones, (Principal Investigator) and Julia Rempel (Clinical Coordinator) at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN). You are only agreeing to be contacted about participating. For each study you will be given additional information including a consent form specific to that study.

The Database will have the following information about you: name, phone number, mailing address and email address.

Confidentiality of your information will be maintained in the following manner: Only the Principal Investigator and Clinical Coordinators at the RCFFN will have access to the electronic database which is on a password protected computer in a locked office. No outside clinics or institutions will have access to your information. Your contact information will be kept for 10 years. After 10 years your information will destroyed.

This consent form and the information in the database may be inspected by a University of Manitoba Research Ethics Board to ensure that your information is being collected and maintained in an ethical manner.

Your decision to allow your information to be in the database is completely voluntary. While there may be no benefit to you, your information will help researchers to find individuals that may be interested in participating in a research study. If you change your mind after agreeing to this, your information can be removed from the database. You will not be penalized in any way if you refuse to participate, or if change your mind and ask that your information be removed.

If you have any questions about this database, please contact:

Dr. Peter Jones at 204-474-9787 or Julia Rempel at [redacted]

If you no longer want to be part of the research database, please contact:

Julia Rempel at [redacted]

If you have questions about your rights as a research participant, you may contact The University of Manitoba, Bannatyne Campus Research Ethics Board Office at 204-789-3255.

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

Statement of Consent

I have read this consent form. I have had the opportunity to ask questions and discuss what is involved. I understand that my personal information will be kept confidential. By signing this consent form, I have not waived any of my legal rights.

Participant signature_____Date _____

Participant printed name: _____

I, the undersigned, have fully explained the relevant details of this research to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____ Date _____

Signature: _____

Role: _____

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Developing and Evaluating Novel Food Supplements, Consisting of Canola Oil, Fibre and DHA, Aiming at the Management of CVD Risk in a Population with Metabolic Syndrome.

Principal Investigator: Peter Jones, PhD

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba
196 Innovation Drive,
Winnipeg, Manitoba R3T 2N2
Phone: (204) 474-9787

Co-Investigators: Nancy Ames, PhD

Agriculture and Agri-Food Canada,

196 Innovation Drive

Winnipeg, Manitoba R3T 2N2
Phone: 204-474-7187

Vanu R Ramprasath, PhD

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba

196 Innovation Drive,
Winnipeg, Manitoba R3T 2N2
Phone: 204 272 8383

Sijo Joseph, PhD

Agriculture and Agri-Food Canada,

196 Innovation Drive

Winnipeg, Manitoba R3T 2N2
Phone: 204 272 8383

Sponsors: Canadian Institute of Health Research
Room 97, 160 Elgin Steet,
Address locator: 4809A, Ottawa, (Ontario)
K1A 0W9 Tel.: (613) 941-2672

You are being asked to participate in a Clinical Trial (a human research study). Please take your time to review this 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 before you make your decision. 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 or/ institution) is (are) receiving professional fees and financial support to conduct this study.

The four treatments are listed as follows:

i) *Muffins and cookies made with all purpose flour and containing 50g/day of a blend of high oleic acid canola oil (HOCO) and DHA.*
ii) *Muffins and cookies made with all purpose flour and containing 50g/day of a blend of sunflower oil, safflower oil and butter as a control oil.*
iii) *Muffins and cookies made with barley flour and containing 50g/day of a blend of high oleic acid canola oil (HOCO) and DHA.*
iv) *Muffins and cookies made with barley flour and containing 50g/day of a blend of sunflower oil, safflower oil and butter as a control oil.*

Control oil comprised largely of saturated fat with substantial levels of n-6 linoleic acid, common to current North American intakes. Treatments will be incorporated into muffins and cookies and consumed in equal parts at breakfast, snacks and supper. No known risk of short term deficiency or excess in energy, protein, fat or carbohydrate is present with the experimental diets. This study is with a double blinded design which means neither you nor the clinical staff will know which diets you will be receiving. In an emergency, this information will be made available. You will receive all 4 diets.

Description of tests

Overall health status for eligibility: During recruitment, an overall assessment of your health will be done to ensure your eligibility for the study. A first screening visit of 30 minutes will be done to assess your eligibility. During this visit, we will check blood pressure, anthropometric measurements and a blood test. At a second visit, we will review your medical history and the medications you take and we will clearly explain the instructions of the study. These visits will be conducted by a nutritionist, under the supervision of our medical research team.

Resting blood pressure: Blood pressures at rest (following a 10-minute rest) will be taken early in the morning after a 12 hours fasting and will be repeated twice at an interval of three minutes. These measures will be taken at the first screening visit and at the beginning and at the end of each 4-weeks period.

Anthropometric measurement: Measures of your weight, your height (once), your waist and your hip circumference will be done at the screening visit and at the beginning and at the end of each 4-week period. Taking waist and hip circumference is done using a measuring tape and involves no pain. You will be weighed every day of the week during each 4-weeks period to monitor changes in your weight.

Body composition measurement (DXA): Body composition (fat mass and lean mass) will be measured with a procedure called dual energy x-ray absorptiometry (DXA). The examination takes place in a room specially designed for this test. A radiology technician or other person authorized to operate the device will perform the test. The test does not require any special preparation for the subject. You will be asked not to wear anything metal. The procedure takes about 15 minutes and the dose of radiation is very low (0.037 to 0.074 mrem according to the exposure time required to obtain results of high precision). The total exposure in this project with 5 measures of body composition with DXA will be a maximum of 0.185 mrem, which is 54 times less than a dental x-ray exposure which is estimated at 10 mrem. Exposure to cosmic rays during a 6 hours flight from Montreal to Vancouver is estimated at 3 mrem. The exposure in this project level is therefore quite safe.

Stable isotope tracer intake: To assess cholesterol synthesis, you will be asked to consume D_2O (heavy water) at the end of each phase. On day 29, 0.7 g of D_2O /kg estimated body water will be given orally prior to breakfast as a tracer to measure fractional cholesterol synthesis rate over 24 hours. Fasting blood will be obtained on days 29 and 30.

Microbial analysis: In addition, we would also study the influence of these diets on gastrointestinal microbial diversity in the current study population. For this analysis, you will be asked to provide your stool samples (4 or 5 scoops - 4g) at the beginning and end (days 2 and 30) of each phase of the trial. Stool sample collection kits including containers will be provided to collect stool samples. However, it is optional for you to provide the stool sample. It will not affect your study participation even if you do not select the option to provide stool samples.

Blood samples: At the screening visit, fasting blood sample of 2 teaspoons will be collected to check your blood counts, your lipid profile and to ensure the proper functioning of your liver, your kidneys and your thyroid gland. During days 1 and 30 of each phase fasting blood samples approximately 2 tablespoons will be taken on each blood draw day. On days 2 and 29 of each phase fasting blood samples approximately 1 tablespoon of blood will be taken on each blood draw day. The total amount of blood collected for all samples in the study will be about 600 ml (about 2.25 cups) spread over a period of about seven months. Generally a person can give up to 450 ml of blood every 2 months safely. The amount of blood collected in the context of this project is lower than that. However, you must refrain from donating blood during the study and for a

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. Shahrokh Nejad Ghaffar, will be available to contact at any time. Dr. Shahrokh Nejad Ghaffar can be reached at (204) 805-1811.

Benefits

By participating in this study, you will be providing information to the study doctors that will show the effects of novel beverage supplement on heart health. There may or may not be direct medical benefit to you from participating in this study. We hope the information learned from this study will benefit other participants with increased risk for cardiovascular disease in the future. In addition to the above, you will also receive your results when they become available.

Costs

All clinic and professional fees, diagnostic and laboratory tests which 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. The study cost and honorariums will be covered by Canadian Institutes of Health Research, the study sponsor.

Payment for participation

You will receive up to a maximum of $1000 at completion of this study for your time and inconvenience of the study schedule. This amount will be divided into 4 portions. You will receive $200 after the completion of each phase until the end of phase 3 and $400 after the completion of phase 4. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives

You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study.

confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned patient code number.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals. With your permission your Family Physician (GP) will be notified about your participation in this study.

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 not to participate or to withdraw from the study will not affect your other medical care at this site. If your study doctor feels that it is in your best interest to withdraw you from the study, your study doctor will remove you without your consent. We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study.

If you decide to participate, you will agree to co-operate fully with the study visit schedule, and will follow the study staff's instructions. If you are an employee of University of Manitoba, be sure that your performance evaluation will not be affected by your decision not to participate. 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 the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Shahrokh Nejad Ghaffar at [redacted] 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 or releasing the investigator(s) or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Shahrokh Nejad Ghaffar 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 participant. 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 listed in page 1. For questions about your rights as a research participant, you may contact The University of Manitoba Biomedical Research Ethics Board at (204) 789-3389. Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with Peter Jones and/or his study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by the Food and Drug Administration, the Health Protection Branch, government agencies in other countries, and The University of Manitoba Biomedical Research Ethics Board. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to being contacted in relation to this study. Yes ☐ No ☐

I agree to my family physician being notified of my participation in this study. Yes ☐ No ☐

I agree to being contacted for future studies at the RCFFN. Yes ☐ No ☐

Participant printed name: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____Date _____(day/month/year)

Signature: _____

Role in the study: _____

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Medical Questionnaire D1/D29

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐

BLOOD PRESSURE AT REST (AFTER 10 MINUTES OF REST)

ARM : ☐ **LEFT** ☐ **RIGHT**

TYPE OF DEVICE : ☐ **Mercury** ☐ **Automatic**

10 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

13 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

16 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

Avg. Systolic BP (mmHg) = _____ Avg. Diastolic BP (mmHg) =_____

Heart rate / min = HR(1):_____ HR(2): _____ HR(3): _____ HR(mean):_____

Side effects questionnaires completed: Yes ☐ No ☐

ANTHROPOMETRIC MEASURMENTS

Body weight (kg): _____

Waist girth (cm) : WG(1): _____ WG(2): _____ WG(3): _____

Hip girth (cm) : HG(1): _____ HG(2): _____ HG(3): _____

Completed by: _____

Date of your last periods (1st day) : _____

Not applicable: ☐

BLOOD SAMPLES: CHECKLIST

Did you eat or drink anything except water in the last 12 hours?

☐ Yes ☐ NO

Have you consumed alcohol or a product containing alcohol in the last 48 hours?

☐ Yes ☐ NO

In the last 24 hours, have you done intensive exercise?

☐ Yes ☐ NO

BLOOD SAMPLES: ☐ LEFT ARM ☐ RIGHT ARM

BLOOD SAMPLES: ☐ Yes ☐ NO

EXTRA BLOOD SAMPLE (Keep frozen) ☐ Yes ☐ NO

Nurse's initials: _____

For visit D1 of diet 2, diet 3, diet 4:

Washout duration before this diet: _____ days

☐ Not applicable (D1 of the first diet)

☐ Not applicable (visit D27)

COMMENTS

_____

_____

_____

_____

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WEEK	DATE OF OBSERVATION			# SUBJECT(CODE)	
	DAY	MONTH	YEAR		

Medical Questionnaire D2/D30

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐

ANTHROPOMETRIC MEASURMENTS

Body weight (kg): _____

BLOOD SAMPLES: CHECKLIST

Did you eat or drink anything except water in the last 12 hours?

☐ Yes ☐ NO

Have you consumed alcohol or a product containing alcohol in the last 48 hours?

☐ Yes ☐ NO

In the last 24 hours, have you done intensive exercise?

☐ Yes ☐ NO

BLOOD SAMPLES: ☐ LEFT ARM ☐ RIGHT ARM

BLOOD SAMPLES: ☐ Yes ☐ NO

EXTRA BLOOD SAMPLE (Keep frozen) ☐ Yes ☐ NO

Nurse's initials: _____

For visit D30 of all diets:

Number of days on the diet: _____ **days** ☐ **Not applicable (visit D2)**

3-Day food records collected ☐ Yes ☐ NO

Fecal samples collected ☐ Yes ☐ NO

FISH INTAKE

Did you consume one serving of fish or seafood products during the last month?

☐ Yes ☐ NO

If yes;

7.2 When? _____

7.3 What kind? _____ 7.4 Amount: _____

CHANGE IN MEDICATION Yes ☐ No ☐

Name of drug	Dose	Freq.	Indication	Start	Stop
_____	_____	_____	_____	__/__/____	__/__/__
_____	_____	_____	_____	__/__/____	__/__/__

CHANGE IN NATURAL HEALTH PRODUCTS Yes ❑ No ❑

Name of drug	Dose	Freq.	Indication	Start	Stop
_____	_____	_____	_____	__/__/__	__/__/__
_____	_____	_____	_____	__/__/__	__/__/__
_____	_____	_____	_____	__/__/__	__/__/__
_____	_____	_____	_____	__/__/__	__/__/__

PHYSICAL ACTIVITY

During the last month, has your level of physical activity changed?

❑ Yes, my level increased

❑ Yes, my level decreased

❑ Yes, I didn't exercise

❑ No

COMMENTS

____

Side effects questionnaire- Frequency



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Canola Oil, Fibre and DHA Trial 2014

DATE OF OBSERVATION			# SUBJECT(CODE)	
DAY	MONTH	YEAR		

QUESTIONNAIRE ON SIDE EFFECTS-FREQUENCY

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐

Day 1 ☐ Day 28 ☐

Indicate if you experienced side effects listed below over the **last four weeks** and if so, how hard was the intensity.

Side effects	Frequency			
	Never[0]	Rarely[1]	Sometimes[2]	Often[3]
1. Headache	☐	☐	☐	☐
2. Anxiety	☐	☐	☐	☐
3. Fatigue / exhaustion	☐	☐	☐	☐
4. Lack of energy	☐	☐	☐	☐
5. Tend to become exhausted quickly	☐	☐	☐	☐
6. Decreased appetite	☐	☐	☐	☐
7. Increased appetite	☐	☐	☐	☐
8. Hiccup	☐	☐	☐	☐
9. Nausea	☐	☐	☐	☐
10. Vomiting	☐	☐	☐	☐
11. Indigestion	☐	☐	☐	☐
12. Stomach or abdominal pain	☐	☐	☐	☐
13. Constipation	☐	☐	☐	☐
14. Diarrhea	☐	☐	☐	☐
15. Flatulence	☐	☐	☐	☐
16. Abdominal bloating	☐	☐	☐	☐
17. Palpitations	☐	☐	☐	☐
18. Balance disorders	☐	☐	☐	☐
19. Decreased ability to concentrate	☐	☐	☐	☐
20. Flushing	☐	☐	☐	☐
21. Feeling cold	☐	☐	☐	☐
22. Joint or members pain	☐	☐	☐	☐
23. Numbness, burning or itching (feet/hands)	☐	☐	☐	☐
24. Dark or depressing thoughts	☐	☐	☐	☐
25. Other	☐	☐	☐	☐

Side effects questionnaire- Intensity



UNIVERSITY OF MANITOBA

Canola Oil, Fibre and DHA Trial 2014

DATE OF OBSERVATION			# SUBJECT(CODE)	
DAY	MONTH	YEAR		

QUESTIONNAIRE ON SIDE EFFECTS-INTENSITY

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

Day 1 ☐ Day 28 ☐

Indicate if you experienced side effects listed below over the **last four weeks** and if so, how hard was the intensity.

Side effects	Intensity			
	None[0]	Mild[1]	Moderate[2]	Severe[3]
1. Headache	☐	☐	☐	☐
2. Anxiety	☐	☐	☐	☐
3. Fatigue / exhaustion	☐	☐	☐	☐
4. Lack of energy	☐	☐	☐	☐
5. Tend to become exhausted quickly	☐	☐	☐	☐
6. Decreased appetite	☐	☐	☐	☐
7. Increased appetite	☐	☐	☐	☐
8. Hiccup	☐	☐	☐	☐
9. Nausea	☐	☐	☐	☐
10. Vomiting	☐	☐	☐	☐
11. Indigestion	☐	☐	☐	☐
12. Stomach or abdominal pain	☐	☐	☐	☐
13. Constipation	☐	☐	☐	☐
14. Diarrhea	☐	☐	☐	☐
15. Flatulence	☐	☐	☐	☐
16. Abdominal bloating	☐	☐	☐	☐
17. Palpitations	☐	☐	☐	☐
18. Balance disorders	☐	☐	☐	☐
19. Decreased ability to concentrate	☐	☐	☐	☐
20. Flushing	☐	☐	☐	☐
21. Feeling cold	☐	☐	☐	☐
22. Joint or members pain	☐	☐	☐	☐
23. Numbness, burning or itching (feet/hands)	☐	☐	☐	☐
24. Dark or depressing thoughts	☐	☐	☐	☐
25. Other	☐	☐	☐	☐

Weight checklist

HIGH OLEIC CANOLA OIL, DHA , BARLEY FIBER STUDY

Subject ID: _____

WEIGHT CHECKLIST

Week	Day	Date	Weight (kg)
	1		
	2		
1	3		Saturday
	4		Sunday
	5		
	6		
	7		
	8		
	9		
2	10		Saturday
	11		Sunday
	12		
	13		
	14		
	15		
	16		
3	17		Saturday
	18		Sunday
	19		
	20		
	21		
	22		
	23		Saturday
4	24		Sunday
	25		
	26		
	27		
	28		

Food checklist

PARTICIPANT FOOD CHECKLIST

PHASE : **PARTICIPANT CODE:**

Week	Day	Date	Participant initial		Student Initial
			Muffin	Cookies	
	1	8/10/2015			
1	2	9/10/2015			
	3	10/10/2015			
	4	11/10/2015			
	5	12/10/2015			
	6	13/10/2015			
	7	14/10/2015			
	8	15/10/2015			
2	9	16/10/2015			
	10	17/10/2015			
	11	18/10/2015			
	12	19/10/2015			
	13	20/10/2015			
	14	21/10/2015			
	15	22/10/2015			
3	16	23/10/2015			
	17	24/10/2015			
	18	25/10/2015			
	19	26/10/2015			
	20	28/10/2015			
	21	29/10/2015			
	22	30/10/2015			
4	23	31/10/2015			
	24	1/11/2015			
	25	2/11/2015			
	26	3/11/2015			
	27	4/11/2015			
	28	5/11/2015			