Associations Between Dietary and Circulating Phytosterols and Cardiovascular Disease Risk Biomarkers in a Manitoba Adult Cohort

By

Ramandeep Kaur

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Department of Food & Human Nutritional Sciences
University of Manitoba
Winnipeg, Manitoba

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Abstract

Cardiovascular disease (CVD) is a leading cause of death worldwide. Elevated serum lipid levels and increased arterial stiffness are some of the risk factors responsible for the development of CVD. Phytosterols are well known for their cholesterol-lowering ability. Besides cholesterol-lowering, whether dietary phytosterols are beneficial for vascular function is not completely known yet. This thesis aimed to investigate the association between dietary and circulating phytosterols with serum lipid levels and arterial stiffness biomarkers, including blood pressure (BP), augmentation index (AIx), and pulse wave velocity (PWV). The secondary objective was to investigate the relationship of the Mediterranean-style dietary pattern scoring (MSDPS) with dietary phytosterols and its impact on CVD risk biomarkers. This cohort study on Manitoban adults included 157 men and 120 women, aged 30-46 years old. This study measured dietary and circulating phytosterols using 24-hour dietary recall and gas chromatography, respectively, and the MSDPS was used to evaluate the dietary pattern using the diet history questionnaire. Data were statistically analyzed using analysis of covariance, multiple linear regression and correlation. After adjustment for age, body mass index (BMI), total energy intake, fats as a percentage of energy intake, and dietary fiber, this study found that participants with high phytosterol intake (392.7±108.8 mg/d) in the highest quartile had 0.52 mmol/L (-10.2%) lower serum total cholesterol (TC) (P<0.05) and 0.47 mmol/L (-14.4%) lower (P<0.05) serum low-density lipoprotein cholesterol (LDL-C) than did the participants in the lowest quartile (97.7±36.5 mg/d). Phytosterol intake in the highest quartile showed a significant reduction in systolic BP (-5.3%, P<0.01), diastolic BP (-4.5%, P<0.01), and PWV (-3.4%, P<0.05) than those in the lowest quartile. The increased sitosterol-to-cholesterol ratio correlated with reduced serum TC, LDL-C, triglyceride, and diastolic BP, PWV (all P<0.001) and systolic BP (P<0.01) and increased high-density lipoprotein (P<0.01). A lower lathosterol-to-cholesterol ratio was correlated with decreased PWV (P<0.0001). No significant associations were found between MSDPS and the dietary phytosterols. The study results concluded that dietary phytosterol intake is associated with cholesterol-lowering and reduced arterial stiffness. Therefore, the present study suggests that increased intake of dietary phytosterols could help to reduce the risk of CVD.
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I would like to express my deepest gratitude to my supervisor, Dr. Semone Myrie for her continuous support, valuable guidance, motivation and immense knowledge I received throughout my master’s research and I will carry these skills with me for the rest of my life. I have learned so much from the incredible opportunity she provided me to work on this project and the publication we have completed together. I would also like to thank the rest of my thesis committee members, Dr. Dylan Mackay, Dr. Michael Czubryt, and Dr. Jennifer Protudjer for providing their insightful comments, encouragement and suggestions during my research which challenged me to think outside the box. I would like to thank Dr. James House for permitting me to use equipment in the Duff Roblin lab. I would also like to express my sincere gratitude to Mark and Dennis Joseph for providing me with their consistent support and technical assistance throughout my lab work and all my lab members and colleagues Brianne, Trevor, Yongbo, Ravinder, Afra, Jyoti, Maryam, Nooshin, Maryam Shamloo, and Ruchira for their kind cooperation. A special thanks to my friends Hardeep, Parneet, Puneet, Khush, Gursharan, Anuroop, Himani, Thilakam, and Sam Sidhu for supporting me, encouraging me, providing me ever willing help and giving me beautiful memories which will always remain with me for the rest of my life. I owe my special thanks to Dr. Jitendra Paliwal, Associate Dean in the Faculty of Agricultural and Food Sciences for providing me financial support and extending the University of Manitoba Graduate Fellowship for 6 months. I would like to acknowledge Research Manitoba for funding the TMPLR project. Lastly, I would like to extend my appreciation to the International Graduate Student Entrance Scholarship, Manitoba Graduate Scholarship, the Faculty of Graduate Studies Research Completion Scholarship, and the Bursaries I got for financial support.
Dedications

I dedicate my thesis to my family.

Especially to my father Mr. Ranjit Singh, Mother Mrs. Rajbir Kaur and my siblings Aman and Joban and thank them for their unconditional love, care, endless support in all ups and downs and all the sacrifices they have made to make me reach where I am today.

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<tbody>
<tr>
<td>ACAT2</td>
<td>Acyl-CoA cholesterol acyltransferase-2</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ABCG 5/8</td>
<td>ATP binding cassette transporter genes</td>
</tr>
<tr>
<td>ASA24-hr</td>
<td>Automated self-administered 24-hour dietary recall</td>
</tr>
<tr>
<td>AIx</td>
<td>Augmentation index</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CNF</td>
<td>Canadian nutrient file</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>cSys BP</td>
<td>Central systolic blood pressure</td>
</tr>
<tr>
<td>cDia BP</td>
<td>Central diastolic blood pressure</td>
</tr>
<tr>
<td>DASH</td>
<td>Dietary approaches to stop hypertension</td>
</tr>
<tr>
<td>DHQ</td>
<td>Diet history questionnaire</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated Dilation</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatography- flame ionization detector</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>MDS</td>
<td>Mediterranean diet score</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSDPS</td>
<td>Mediterranean style dietary pattern score</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acids</td>
</tr>
<tr>
<td>NCEP ATP</td>
<td>National Cholesterol Education Program Adult Treatment Panel</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1 Like 1</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse wave velocity</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acids</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
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<td>TMPLR</td>
<td>The Manitoba Personalized Lifestyle Research</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Chapter 1: Introduction

1.1 Introduction

Cardiovascular disease (CVD) is the primary cause of death in the world (WHO, 2017). High serum levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are lipid components that play a significant role in the development of CVD. Diet is one of the modifiable factors that play an essential role in altering the serum lipid levels (WHO, 2011). Epidemiological and clinical studies have shown that modifications in dietary habits and the inclusion of more plant-based foods in one's daily diet might act as a preventative factor to help reduce the risk of developing CVD (Lacroix et al., 2017, Simonen et al., 2017). Mechanistically, studies have shown that a plant-based diet can be a practical approach for reducing cardiovascular risk by increasing cholesterol excretion and decreasing intestinal cholesterol absorption (Jenkins et al., 2003). Some of the lipid-lowering effects of plant-based diets may be due to the phytosterol component of the diet. Dietary phytosterols are natural components that are structurally similar to cholesterol present in plant-based foods such as fruits, vegetables, cereal grains, soy, nuts, and vegetable oils (Klingberg et al., 2008a; Mach et al., 2019). Although the human body cannot synthesize phytosterols, they are capable of influencing cholesterol metabolism and lowering serum LDL-C concentrations (Lambert et al., 2017; Ras, Geleijnse, & Trautwein, 2014). A 2 g/d intake of phytosterols has been shown to reduce serum LDL-C concentration by 10-12% (Gylling et al., 2014). Health Canada has also approved the use of up to 2 g of phytosterols per day for supplementation in the diet (Health Canada, 2010); however, the typical western diet provides only about 0.3 mg/d (Cabral & Klein, 2017). Many observational studies have found an inverse association of dietary phytosterols from the habitual diet with serum LDL-C (Andersson et al., 2004; Klingberg et al., 2008b; Klingberg et al., 2013; Ras et al., 2015a; Wang et al., 2012).

Phytosterols are bioactive components found in plant-based foods (Moreau, Whitaker, & Hicks, 2002; Racette et al., 2015). The traditional Mediterranean diet is characterized by a high intake of fruits, vegetables, olive oil; moderate intake of fish and alcohol; and a low intake of dairy products, meat, and sweets (Willett et al., 1995). Thus, this heavily plant-based dietary approach is known as the Mediterranean dietary pattern, which includes fewer servings of non-vegetarian foods compare to other dietary patterns (Stewart et al., 2016). These Mediterranean dietary patterns are a well-recognized approach to reduce hypertension, and hypercholesterolemia,
and CVD mortality (Lynch et al., 2018). Numerous studies on Mediterranean dietary patterns have highlighted the benefits of dietary phytosterols as a bioactive component in the Mediterranean diet (Estruch et al., 2018; Schwingshackl et al., 2020). However, whether the dietary phytosterols are one of the factors responsible for reducing CVD risk and mortality by the Mediterranean diet is still inconclusive; therefore, more research is needed in this area.

Besides the effect of dietary phytosterols on lowering serum cholesterol levels, numerous studies were interested in exploring the effects of dietary phytosterols on vascular function when evaluating the efficacy of interventions on vascular health. Thus far, observational studies that examined the effects of dietary phytosterols on vascular health biomarkers, such as arterial stiffness biomarkers were found to be equivocal (Gylling et al., 2013, Klingberg et al., 2013; Ras et al., 2015a). Arterial stiffness biomarkers are shown as an important determinant to predict the risk of CVD (Cavalcante et al., 2011). Numerous factors such as physiological, lifestyle factors and diseases, including age, smoking, obesity, hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease (CAD) and chronic heart failure, and stroke are related to arterial stiffness and its hemodynamic consequences (Laurent et al., 2006). Pulse wave velocity (PWV) and augmentation index (AIx) are the most reliable measures and non-invasive parameters used to assess arterial stiffness and predict future cardiovascular events (Vlachopoulos et al., 2012; Oliver & Webb, 2003). PWV is known as the gold standard method to evaluate arterial stiffness (Hamilton et al., 2007). PWV is defined as the speed at which the arterial pressure waves, expelled by systolic contraction is propagated through the arterial system (Pereira et al., 2015). PWV depends on the vessel and also changes as the aortic pressure changes along with the forward pressure wave to the periphery (Wilmer et al., 2005). It has been shown that a 1 meter/second (m/s) increase in PWV is associated with a 39% increase in CVD events. The studies evaluating the effects of dietary phytosterols on vascular function reported neither beneficial nor harmful effects of dietary phytosterols on vascular health biomarkers in the general population (Gylling et al., 2013, Klingberg et al., 2013; Ras et al., 2015a).

Chapters II and III comprise reviews of the literature on the areas covered within this thesis. Chapter II provides a comprehensive literature review about the physiology and metabolism of phytosterols and their mechanisms of action for cholesterol-lowering, effects of supplementary and dietary phytosterols on serum lipid levels, and vascular health biomarkers.
Since diet plays a prominent role concerning the occurrence or prevention of chronic diseases, thus, to measure foods and nutrients from daily dietary intake, appropriate methods of dietary assessment are required. Therefore, chapter III summarizes current knowledge about dietary assessment methods such as dietary record, 24-hour dietary recall, diet history, and food frequency questionnaire, to better estimate the dietary intake. This review will also look at the strengths and limitations of the various methods. These methods can be used to assess dietary intake patterns and to calculate macronutrients, micronutrients, and dietary phytosterols. This chapter also summarizes the current literature about dietary patterns and their use in epidemiological studies. In summary, in both chapters II and III, the existing literature is reviewed to summarize the information available about the association of dietary and circulating phytosterols with CVD risk biomarkers.
The following chapter contains a manuscript that provides a comprehensive review of the association of dietary phytosterols with CVD risk biomarkers in humans. The central focus is on dietary phytosterols from the habitual diet and their role as a bioactive component in the diet for reducing serum cholesterol concentrations and improving vascular health. Moreover, the role of phytosterols incorporated in the food products on cholesterol-lowering and vascular health biomarkers are also reviewed. Ramandeep Kaur was the principal manuscript author, and Semone Myrie contributed to the construction of the manuscript.
Chapter 2: Literature Review 1: Manuscript

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Association of Dietary Phytosterols with Cardiovascular Disease Biomarkers in Humans

Ramandeep Kaur¹, Semone B. Myrie¹

¹ Department of Food and Human Nutritional Sciences, Richardson Centre for Functional Food and Nutraceuticals, University of Manitoba, 196 Innovation Drive, Winnipeg, MB, Canada, R3T 2N2.

Corresponding author: Semone B. Myrie, Department of Food and Human Nutritional Sciences, Richardson Centre for Functional Food and Nutraceuticals, The University of Manitoba, 196 Innovation Drive, Winnipeg, MB R3T 2E1, MB, Canada, Email: semone.myrie@umanitoba.ca, Tel No. (204) 474-7290
2.1 Abstract

Cardiovascular disease (CVD) is a leading cause of death worldwide. Elevated concentrations of serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are major lipid biomarkers that contribute to the risk of CVD. Phytosterol well known for their cholesterol-lowering ability, are non-nutritive compounds that are naturally found in plant-based foods and can be classified into plant sterols and plant stanols. Numerous clinical trials demonstrated that 2g phytosterols per day have LDL-C lowering efficacy ranges of 8%-10%. Some observational studies also showed an inverse association between phytosterols and LDL-C. Beyond the cholesterol-lowering beneficial effects of phytosterols, the association of phytosterols with CVD risk events such as coronary artery disease and premature atherosclerosis in sitosterolemia patients have also been reported. Furthermore, there is an increasing demand to determine the association of circulating phytosterols with vascular health biomarkers such as arterial stiffness. Therefore, this review aims to examine the benefits of phytosterols for CVD risk prevention by reviewing the current data that looks at the association between dietary phytosterol intake and serum lipid biomarkers, and the impact of circulating phytosterol levels on vascular health biomarkers. The clinical studies in which the impact of phytosterol on vascular function is investigated show minor but beneficial phytosterol effects over vascular health. The aforementioned vascular health biomarkers are pulse wave velocity, augmentation index, and arterial blood pressure. The current review will begin to address the research gap that exists between the association of dietary phytosterols with CVD risk biomarkers.

**Keywords:** Phytosterols · Cardiovascular disease · Cholesterol · Low-density lipoproteins · Vascular health.
2.2 Introduction

Cardiovascular disease (CVD) is a leading cause of death worldwide, especially in western societies (World Health Organization, 2017). In 2016, the World Health Organization (WHO) reported an estimated 17.9 million or 31% of global deaths occurred due to CVD (World Health Organization, 2017). CVD is a leading cause of mortality and morbidity in North America and represents a big economic burden on the direct and indirect cost of the healthcare system (Statistics Canada, 2018). Statistics from the American Heart Association stated that CVD accounted for 840,678 deaths in the US in 2016 (American Heart Association, 2018).

CVD is an arterial disease that leads to an irregular supply of blood to the heart and/or brain or some peripheral regions of the body (Liepa, 2006). Obesity, sedentary lifestyle, hypertension, cigarette smoking, glucose intolerance, and high serum lipid profile are traditional risk factors for CVD (Expert Panel on Detection, 2001). Elevated serum concentrations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and low concentration of high-density lipoprotein cholesterol (HDL-C) are major lipid risk biomarkers that contribute to the development of CVD (Katzke et al., 2017; Libby, 2012). Based on the wide range of data available, it has been confirmed that there is a direct association between the risk of CVD and elevated serum TC and LDL-C levels (Anderson et al., 2016; Expert Panel on Detection, 2001).

Diet and lifestyle factors are recognized as important factors affecting CVD risks. Although it has been reported that 80% of serum cholesterol levels are determined by a person’s genotype, the remaining 20% is determined by dietary practices and lifestyle habits (Silverman et al., 2016). Numerous preventive measures could reduce the risk of CVD, such as the use of pharmacological treatment, dietary treatment, and adopting a healthy lifestyle, including changes in dietary patterns and dietary habits, lifestyle habits, and use of lipid-lowering drugs (NCEP, 2002; Rippe, 2019). Moreover, the US National Cholesterol Education Program (NCEP) also focuses on therapeutic lifestyle changes, including exercise, diet, smoking cessation, and weight loss (NCEP, 2002).

Focusing on dietary practices, various dietary strategies can be used to reduce CVD risk (Expert Panel on Detection, 2001; Trautwein et al., 2018a). In ancient times, our distant ancestor’s eating pattern was mostly based on plant-derived foods, which were typically low in cholesterol (Jenkins et al., 2003). A plant-based diet is considered a healthy diet because many plant foods are recognized to increase cholesterol excretion and lower intestinal cholesterol absorption (Jenkins et
The most typical nutrients achieved from these plant-based diets are mono and polyunsaturated fatty acids (MUFA and PUFA), dietary fibers, and phytonutrients such as phytosterols, thought to reduce serum cholesterol levels (Schwab et al., 2014).

Several dietary guidelines also emphasize the benefits of a high intake of plant-based foods such as fruits, vegetables, pulses, soy, and nuts (Jellinger et al., 2017; Mach et al., 2019; Piepoli et al., 2016). Based on dietary guidelines, healthy dietary habits, including consumption of dietary phytosterols that are found in foods of plant origin, are important among individuals who may be at increased risks of developing CVD. Moreover, scientific evidence has shown that also for individuals suffering from CVD and treated with drug therapy, modification in dietary habits might be a preventative factor to help reduce mortality (Lacroix et al., 2017; Mozaffarian and Ludwig, 2010).

In the early 1990s, the food industries were focused on developing new functional food products, for example, phytosterols, based on the new insights showing that esterification of phytosterols with fatty acids could effectively improve their handling and physical properties (Mattson et al., 1982). To optimize the hypolipidemic effects of phytosterols, they have to be made soluble in food formats, e.g. with esterification. Plant sterol has been shown to have a great solubility with fat. In 1995, plant stanols (the saturated derivatives of plant sterols) were esterified with dietary fatty acids, resulting in an increase in their fat solubility and their efficacy to be incorporated with fat-rich foods, such as margarine (Miettinen et al., 1995). Fatty acids are well known to play an important role in altering serum lipid levels. Numerous studies on fatty acids have reported that saturated fatty acids increase whereas polyunsaturated fatty acids reduce the blood plasma cholesterol (DiNicolantonio and O’Keefe, 2018).

After evaluating the efficacy and safety of food products, phytosterols were recommended to be used as a nonpharmacological therapeutic approach for lowering cholesterol levels (Ras et al., 2014). Intake of phytosterol enriched foods or supplements is considered a good dietary treatment to reduce the risk of CVD (Trautwein et al., 2018b). Many clinical trials have reported that a daily dose of 2 g phytosterols could significantly lower serum LDL-C concentration by an average of 8–10% (0.31–0.34 mmol/L) regardless of sex, age, ethnic background, or bodyweight (Cabral and Klein, 2017; Gylling et al., 2014). Several different dietary guidelines and consensus available throughout the world recommend the intake of phytosterols in the diet to prevent
hypercholesterolemia or reduce the risk of CVD (Gylling et al., 2014; Jellinger et al., 2017; Piepoli et al., 2016). For example, the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP) and the American Heart Association (AHA) guidelines promote less intake of saturated fatty acids and dietary cholesterol, and more consumption of fruits, vegetables, whole grains, and low-fat dairy products for primary prevention of CVD (Arnett et al., 2019; NCEP, 2002). Moreover, the NCEP ATP III guidelines recommend 2 g/day intake of phytosterols as a therapeutic option for the prevention of CVD (Expert Panel on Detection, 2001; NCEP, 2002). A 2014 consensus panel paper from the European Atherosclerosis Society (EAS) concluded that 2–3 g per day of phytosterols lowers serum LDL-C concentrations by up to 12% (Gylling et al., 2014).

It has been reported that higher intake of dietary phytosterols (i.e., non-supplementary phytosterols) from the daily habitual diet (449 mg/2000 kcal) reduced cholesterol absorption and increased cholesterol excretion into feces compared with low phytosterol intake (Lin et al., 2010). Since high cholesterol absorption efficiency is associated with increased risk of coronary artery disease (Myocardial Infarction Genetics Consortium, 2014), therefore, an alteration toward an improvement in cholesterol metabolic profile after phytosterol consumption may have cardioprotective effects. Two large prospective population-based studies examined the effects of dietary phytosterols on vascular health, and the results found neither cardioprotection nor increased risk of CVD (Gylling et al., 2014; Klingberg et al., 2013). Many observational studies have also found an inverse association between dietary phytosterols taken from the daily habitual diet and LDL-C reduction (Andersson et al., 2004; Klingberg et al., 2008b, 2013; Ostlund Jr et al., 2002; Ras et al., 2015b; Wang et al., 2012); however, significant evidence supporting the association of dietary phytosterols with a reduction in other CVD risk biomarkers such as vascular health biomarkers is still lacking (Gylling et al., 2014; Klingberg et al., 2013; Ras et al., 2015a). Therefore, this review aims to examine the ability of phytosterols to reduce CVD risk by reviewing the recent findings for an association of dietary phytosterols intake with serum lipid biomarkers, and the impact of circulating phytosterol level on vascular health biomarkers. This review will address the research gap that exists between the association of dietary phytosterols with CVD risk factors.
2.3 Phytosterols: Definition, Classification, and Natural Food Sources

Plant sterols and their saturated derivatives, plant stanols, are collectively referred to as phytosterols (Weihrauch and Gardner, 1978). Phytosterols have similar functions in plants as that of cholesterol in humans (Katan et al., 2003; Ostlund Jr., 2002). Phytosterols are also structurally similar to cholesterol except having a difference in their carbon side chains (Weihrauch and Gardner, 1978). Plant sterols are unsaturated sterol compounds with a double bond at the C-5 position in the sterol ring (Fig. 2.1), whereas plant stanols do not contain any double bond in the sterol ring (Fig. 2.1) (Weihrauch and Gardner, 1978). There are numerous plant sterols (Christie, 2011; Wasowicz, 2002), however, sitosterol, campesterol, and stigmasterol are the three most abundant phytosterols, which contribute 65%, 30%, and 3% of total dietary sterol, respectively (Valitova et al., 2016). Campestanol and sitostanol are the two most common 5α-saturated stanol derivatives, structurally similar to sitosterol and campesterol, except for the absence of a double bond in the carbon chain. Moreover, they only account for about 2% of the total phytosterols (Gylling and Simonen, 2015). Phytosterols can be found in the free or bound form attached to fatty acids, known as esterified phytosterols, or to carbohydrates, known as glycosides (Ogbe et al., 2015). Over 250 phytosterols have been identified in the free and esterified form (Cohn et al., 2010; Moreau et al., 2002).

Phytosterols are non-nutritive bioactive components found in plants which must be obtained from the diet as they are not synthesized in the human body (Racette et al., 2015). The highest amounts of phytosterol found in edible oils, nuts, legumes, and cereals, whereas the lowest amounts are found in tubers, fruits, and vegetables (Ostlund Jr., 2002). Among vegetable oils, the highest amounts of phytosterols are found in rice oil (1230.9 mg/100 g) followed by corn oil (700–900 mg/g), rapeseed oil (878.6 mg/100 g) and sesame oil (652.9 mg/100 g). Comparatively, other oils contain a lower amount of phytosterols such as sunflower, soybean, and olive oils with 411 mg/100 g, 320 mg/100 g, and 300 mg/100 g, respectively (Wang et al., 2018). Phytosterols are also found to be higher in legumes (129–275 mg/100 g), followed by nuts (18.9–255 mg/100 g) and cereals (11–94 mg/100 g) (Gupta et al., 2011; Wang et al., 2018). Although nuts are usually eaten in a small amount, they still contribute significantly to the total dietary intake of phytosterols (Valsta et al., 2004). On the other hand, fruits and vegetables, which contain a lower amount of phytosterols in comparison to other foods, are large and important components of daily diet making
them an important source of phytosterols (Gupta et al., 2011; Wang et al., 2018). The content of phytosterols in fruits and vegetables varies widely, ranging from 20–40 mg/100 g in different types. Vegetables including broccoli, brussels sprouts, cauliflower, olives, and fruits such as passion fruit, figs, orange, and pineapple are good sources of phytosterols (Ellegård et al., 2007).

![Chemical structure of cholesterol and phytosterols](image)

**Figure 2.1** The chemical structure of cholesterol; unsaturated plant sterols: sitosterol, campesterol, stigmasterol; and saturated plant sterols: sitostanol, campestanol

The total estimated phytosterol intake reported in the typical western or regular diet was approximately 300 mg/d (Klingberg et al., 2008a; Sioen et al., 2011), whereas up to 300–600 mg/d of phytosterols is achieved with plant-based or vegetarian diets (Jaceldo-Siegl et al., 2017; Jenkins et al., 2001; Vuoristo and Miettinen, 1994). In 2010, based on available evidence showing that 2 g phytosterols could significantly reduce serum LDL-C concentrations, Health Canada approved the use of up to 2 g phytosterol enrichment in foods as a cholesterol-lowering strategy (Health Canada, 2010). In 2010, the FDA also approved a phytosterols health claim, which stated that daily total intake of at least 1.3 g plant sterols and 3.4 g plant stanols as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease (Department of Health and Human Services, Food and Drug Administration, 2010). Therefore, to meet at least a requirement of 1 g phytosterols per
day, a person would need to include at least 1 kg of grains and 2 kg of vegetables in their daily diet (Moreau et al., 2002).

**2.4 Metabolism of Phytosterols and Mechanism of Action of Cholesterol-Lowering**

Despite the similarity between the chemical structure of phytosterols and cholesterol (Fig. 2.1), the most remarkable differences are in their de novo synthesis, intestinal uptake, and biliary secretion in human metabolism (Gylling and Simonen, 2015). Reduction of intestinal cholesterol absorption is the primary mechanism of phytosterols which ultimately leads to a reduction in serum cholesterol levels. The potential role of phytosterols in intestinal cholesterol absorption reduction (30–50%) has been extensively studied (AbuMweis et al., 2014; Cohn et al., 2010). Figure 2.2 shows the mechanism of action of phytosterols in cholesterol reduction, which starts with the consumption of phytosterols and cholesterol from the diet, and cholesterol secreted from bile. When phytosterols enter into the intestinal lumen, they interfere with the dietary and biliary uptake of cholesterol into micelles and, consequently, their absorption into the intestinal epithelium (Smet et al., 2012). In the intestinal lumen, free phytosterols compete with cholesterol for solubilization into the mixed micelles. It has been shown that high intestinal plant stanol concentrations increases the hydrolysis of various phytosterols above that of cholesterol and decreases the micellar solubility of cholesterol, resulting in decreased intestinal absorption of cholesterol (Nissinen et al., 2002). Moreover, it was also reported that several mechanisms are responsible for the inhibitory effects of phytosterols on cholesterol absorption such as modification in the expression of genes encoding sterol transporter proteins including (1) down-regulate the activity of Niemann-Pick C1 Like 1 (NPC1L1), by reducing the transport of cholesterol into the enterocytes or (2) promoting the activity of ATP binding cassette ABCG5 and ABCG8 transporter promoting cholesterol efflux from enterocytes back into the intestinal lumen, by reducing the rate of cholesterol esterification in the enterocytes, or by increasing cholesterol removal from the body via trans intestinal cholesterol excretion (Gylling and Simonen, 2015). As ezetimibe (a cholesterol-lowering drug) is well known to inhibit the activity of NPC1L1 (Betters and Yu, 2010), similarly, sitosterol has shown to downregulate the activity of NPC1L1, which could partly play a role in cholesterol absorption reduction (Jesch et al., 2009). The dietary cholesterol is re-esterified to a fatty acid by acyl-CoA cholesterol acyltransferase-2 (ACAT2) in the enterocytes, then incorporated with chylomicrons (Gylling et al., 2006) and transported further for lymph circulation. Excess dietary and endogenous cholesterol and most of the absorbed phytosterols enter back into the intestinal
lumen from enterocytes by ABCG5/ABCG8 transporters and from the liver into bile and excrete them from the body (Smet et al., 2012). Moreover, ABCG5/ABCG8 transporters are known to play a major role in trans intestinal cholesterol excretion (Nakano, Inoue & Murakoshi, 2019). Normén et al. (2000) reported a 30–40% decrease in cholesterol absorption in humans after consumption of 1.5–1.8 g/day phytosterols.

**Figure 2.2** Mechanism of action of phytosterols for cholesterol-lowering.

*Phytosterols and cholesterol are taken from the diet and cholesterol is secreted from bile. The first important step to reduce cholesterol absorption is that phytosterols replace micellar cholesterol, thus less cholesterol is transported into the enterocytes. NPC1L1 is a cholesterol transporter protein, located in the cell membrane of enterocytes in the small intestine and responsible for the transportation of cholesterol from the intestinal lumen to enterocytes. Plant stanols down-regulate the activity of NPC1L1 protein. In enterocytes, phytosterols inhibit the activity of acyl CoA, cholesterol acyltransferase (ACAT2), by which...*
fewer cholesterol esters transported are into the chylomicrons (CM). Consequently, less cholesterol is circulated from enterocytes to the lymph. In the end, excess cholesterol and phytosterols are pumped back into the intestinal lumen by ATP binding cassette (ABC) transporter proteins, ABCG5 and ABCG8.

The mechanism showed that a decrease in cholesterol absorption increased the expression of LDL-receptors, resulting in a reduction of circulating LDL-C concentration. The intestinal absorption efficiency of plant sterols (<2%) and stanols (<0.2%) is many folds lower than cholesterol (approximately 50%) (Gylling and Simonen, 2015). Phytosterols alter LDL-C levels by reducing the intestinal absorption of cholesterol by 30–50% (AbuMweis et al., 2014). However, some studies also showed that phytosterols may also lower serum triacylglycerol concentrations in individuals with elevated baseline concentrations (Blom et al., 2019; Penchalaraju et al., 2018).

2.5 Phytosterols as a Dietary Strategy to Reduce Cardiovascular Disease Risk

Phytosterols and Serum Cholesterol Lowering

Increased serum LDL-C level is one of the recognized risk factors for CVD events such as atherosclerosis and coronary artery disease (CAD) (Malakar et al., 2019; Silbernagel et al., 2019). Thus, a reduction in LDL-C concentration is expected to prevent the development of atherosclerosis and CAD (NCEP, 2002). Since 1950, numerous studies have reported that foods supplemented with phytosterols reduce serum concentrations of LDL-C (Demonty et al., 2009; Katan et al., 2003; Ras et al., 2014). In 1951, Peterson tested the effects of plant sterols on cholesterol-lowering in chicks by adding soybean sterol to their cholesterol-rich cottonseed oil feed. The comparison between soybean sterol and cholesterol-rich feed showed that the feed having soybean sterols helped in the reduction of high plasma cholesterol levels (Peterson, 1951). A year later, another study using chicks reported that enrichment of soybean sterols in cholesterol-containing feed significantly reduced the incidence of atherosclerosis (Peterson et al., 1952). In the following year, the effects of sitosterol on total blood cholesterol were tested out in humans, and the results confirmed that sitosterol significantly decreased plasma TC levels by 28% in 26 healthy subjects fed plant sterol doses ranging from 5.6-10 g/day for 2 weeks (Pollak, 1953). The aforementioned findings have shown that plant sterol could significantly reduce serum cholesterol concentrations.

Several meta-analyses have been conducted to validate the efficacy of phytosterols in LDL-C lowering by summarizing the results of numerous randomized, placebo-controlled clinical trials
that examined the effects of phytosterol-enriched foods at different dose ranges in hypercholesterolemia subjects (Abumweis et al., 2008; Demonty et al., 2009; Katan et al., 2003; Musa-Veloso et al., 2011; Ras et al., 2014; Wu et al., 2009). The meta-analysis by Katan and coworkers in 2003 analyzed 41 randomized clinical trials and reported that the consumption of fatty foods enriched with phytosterols consistently decreased LDL-C levels by 10% (Katan et al., 2003). This meta-analysis summarized that enrichment of 2 g/day phytosterols in different food products and supplements could significantly lower LDL-C levels by 10% regardless of age, sex, body weight, and background diet.

Another meta-analysis of 124 clinical trials reported a clear dose-response relationship between phytosterols and cholesterol reduction. It showed that an increased intake of phytosterols from 0.6 to 3.3 g/day significantly lowered LDL-C levels by 6–12% (Ras et al., 2014). However, phytosterols did not alter HDL-C concentrations but showed a little reduction in serum triacylglycerol levels only when baseline levels were elevated (Ras et al., 2014). A meta-analysis by Demonty et al. (2009) showed that approximately 2.15 g/day supplementation of phytosterols in different food fat matrices reduced TC and LDL-C concentration by 8.8% and 9.3%, respectively. Additionally, several factors such as the frequency of intake, food format, the food matrix including, type of fat present in the food, the form of supplement used (tablet or capsule), and free or esterified phytosterols used collectively might also affect the efficacy of phytosterols for cholesterol-lowering (Jones et al., 2018). Numerous human studies were focused on the formulation and composition of phytosterol enriched foods and supplements (Davidson et al., 2001; Gylling et al., 2010). A recently published meta-analysis observed that sitosterol and sitostanol produce beneficial effects on serum cholesterol levels and reported that these two phytosterols increase the hypocholesterolemic efficacy of phytosterol enriched foods (Ying et al., 2019). This meta-analysis examined 51 randomized controlled trials and reported that the group with high sitosterol and sitostanol reduced LDL-C levels significantly (p < 0.00001) as compared to the low sitosterol and sitostanol group (p = 0.002). Overall, the net result is a significant reduction in serum LDL-C levels. Health Canada reviewed 84 randomized controlled trials involving phytosterol supplementation from 1994 to 2007 and found that an average intake of 2 g/day phytosterols significantly reduced serum LDL-C levels by 8.8% (Health Canada, 2010). Health Canada concluded that there is enough scientific evidence available to support the association between consumption of phytosterol enriched foods and cholesterol-lowering. In 2010, Health
Canada approved a health claim to use 2 g/day of phytosterols in food products and supplements (Health Canada, 2010).

The replacement or reduced reliance on pharmacological treatment with dietary therapies such as the idea of enrichment of phytosterols in food and dietary supplements for safely and effectively lowering cholesterol has drawn huge interest. To date, many food products enriched with phytosterols have been developed and introduced in the market. The meta-analyses discussed above have examined the cholesterol-lowering effects of phytosterol enriched food products in individuals with primary hypercholesterolemia, hyperlipidemia, familial hypercholesterolemia, type 1 and type 2 diabetes, coronary artery disease, and metabolic syndrome (Demonty et al., 2009; Katan et al., 2003; Ras et al., 2014). Moreover, the safe and effective use of phytosterol supplementations in individuals with primary hypercholesterolemia, normolipidemia, and familial hypercholesterolemia has been studied (Gylling et al., 2014).

Based on available findings, it is reported that 2 g/day of supplementation of phytosterols in food products led to a reduction of LDL-C concentrations by 8–10%, which could be beneficial for contributing to a reduction in CVD risks and improvement in vascular events of those already presenting with CVD once drug treatments are first addressed. For example, a meta-analysis by Silverman et al. (2016) reported that each 1 mmol/L reduction in serum LDL-C was associated with a 23% relative reduction in the risk of major vascular events. Overall, this meta-analysis concluded that reduction in serum LDL-C concentrations achieved with pharmacological and nonpharmacological therapies together were associated with lower rates of major vascular events. Therefore, a reduction in LDL-C levels through the incorporation of healthy dietary strategies that include phytosterols might be considered as one approach to help improve cardiovascular health.

2.6 Role of Dietary Phytosterols in Lowering Cholesterol Absorption and Serum Cholesterol Levels

To date, many interventional clinical trials have been performed based on supplementation of the habitual diet with phytosterol-enriched food products or with phytosterol supplements without controlling the actual phytosterols amount already present in the background diet. Evidence showed that the reproducible LDL-C lowering effects of phytosterols were attained at a minimum dose of 800 mg/d but optimum effects were achieved at 2000 mg/d (Demonty et al., 2009; Ras et al., 2014; Statistics Canada, 2012; Trautwein et al., 2018b). However, the level of phytosterols
achieved through the natural dietary intake was very low as compared to the level achieved with phytosterol-enriched products (Andersson et al., 2004; Klingberg et al., 2008b; Wang et al., 2012). It has been assumed that natural intrinsic phytosterols might not have sufficient and significant effects on reducing CVD risk due to low levels present in natural foods and daily dietary intake. There is also some evidence from observational studies which showed that dietary phytosterols might have appreciable physiological effects (Andersson et al., 2004; Klingberg et al., 2008b, 2013; Li et al., 2018; Ras et al., 2015b; Wang et al., 2012). Little information is available on the effects of dietary phytosterols intake on CVD risk factors in the general population as compared to the data available on the effects of phytosterols enriched foods on cardiovascular events.

Some feeding trials in the general population have demonstrated the physiological effects of dietary phytosterols on whole-body cholesterol metabolism, absorption efficiency, biosynthesis, and excretion (Ostlund Jr et al., 2002; Phillips et al., 2002). For instance, Phillips et al. demonstrated the effects of different phytosterol-rich edible oils on cholesterol absorption (Phillips et al., 2002). The most commonly consumed edible oils such as soybean, corn, peanut, and olive oil have phytosterol content ranging from 200–600 mg/100 g. Moreover, all these oils contain a large amount of sitosterol compared with sitostanol and campestanol (<5 mg/100 g), while corn oil is the richest source of sitostanol among other oils (14–16 mg/100 g), which effectively reduce cholesterol absorption (Phillips et al., 2002).

However, the types of fatty acids (SFA, MUFA, and PUFA) naturally present in the diet also affect the cholesterol-lowering efficacy of phytosterols. For example, a controlled feeding intervention study tested the cholesterol-lowering efficacy of phytosterols using three different experimental diets: a diet containing corn oil (rich in phytosterols and PUFA), or olive oil (low in phytosterols and high in MUFA), or olive oil supplemented with phytosterols (Howell et al., 1998). The two diets containing olive oil with natural phytosterols showed higher plasma concentrations of LDL-C and TG (p < 0.05) whereas corn oil showed a significant reduction in these two biomarkers. However, the addition of phytosterol in olive oil decreased LDL-C and TG levels significantly and suppressed the significant differences that occurred in LDL-C and TG concentrations between corn and olive oil. Moreover, a diet with corn oil and phytosterol-enriched olive oil showed an increase in cholesterol biosynthesis due to their high phytosterols contents. Overall this study concluded that the phytosterol amount present in both PUFA and MUFA rich
oils is partly responsible for significant differences observed in serum lipid levels and synthesis (Howell et al., 1998). Likewise, another study developed three diets containing 0, 400, and 2000 mg/d phytosterols by controlling fiber, saturated fat and trans-fatty acids, and extracted phytosterols from all foods containing high phytosterols in the background diet. Moderate (459 mg/d) and high (2059 mg/d) phytosterol diets were found to increase cholesterol excretion significantly by 36 ± 6% and 74 ± 10%; and cholesterol biosynthesis by 31 ± 6% and 50 ± 7%, respectively. Moderate and high phytosterol doses also exhibit decreased intestinal cholesterol absorption efficiency as compared to the phytosterols deficient diet. Furthermore, LDL-C decreased significantly (-8.9 ± 2.3%; P<0.01) with high phytosterol dose only, however, LDL/HDL cholesterol ratio decreased significantly (P<0.05, P<0.01) with moderate and high phytosterols. This study suggested that if the mechanism of increased cholesterol excretion and altering cholesterol efflux by phytosterols could be found, then it would be expected to see its effect in lowering the atherosclerotic risk (Racette et al., 2010). It has also been demonstrated that a moderate amount of phytosterols present in daily dietary intake could be beneficial to reduce cholesterol absorption; however, elimination of naturally existing phytosterols from foods would attenuate their cholesterol-lowering effects (Ostlund Jr et al., 2002). Ostlund Jr et al. (2002) showed that even a modest amount of intrinsic phytosterols found in natural food matrices lowered cholesterol absorption and promoted cholesterol excretion. A purified corn oil with removed sterols was used to prepare breakfast meals, which in turn increased cholesterol absorption by 38% as compared to unprocessed original corn oil-based breakfast meal. Eventually, the addition of 150 and 300 mg phytosterols back into the purified corn oil decreased cholesterol absorption by 12% and 27%, respectively (Ostlund Jr et al., 2002). In cereals, wheat and rye are the richest sources of phytosterols (Jiménez-Escurrig et al., 2006; Nyström et al., 2008) and consumption of these foods could also improve the cholesterol metabolism (Ostlund Jr et al., 2003). The intake of original wheat germ containing 328 mg phytosterols also reduced cholesterol absorption by 42.8% as compared to phytosterol-free wheat germ (Ostlund Jr et al., 2003). Altogether, these findings suggest that dietary phytosterols exert beneficial effects in the cholesterogenesis process (cholesterol synthesis and absorption) (Howell et al., 1998; Ostlund Jr et al., 2002, 2003).

Interestingly, numerous studies are discussed in Table 2.1 showing the inverse association of dietary phytosterol intake with serum cholesterol-lowering, mainly LDL-C levels (Andersson et al., 2004; Klingberg et al., 2008b; Li et al., 2018; Wang et al., 2012).
In the general population, high phytosterol intake (300–500 mg/d) through the habitual diet was found to be more effective than low phytosterol intake (<200 mg/d) in the reduction of serum TC and LDL-C levels (Andersson et al., 2004; Klingberg et al., 2008b). In 2004, the European Prospective Investigation into Cancer and Nutrition (EPIC) population study was the first cross-sectional study that found an inverse association of dietary phytosterols with serum cholesterol concentrations in a free-living population (Andersson et al., 2004). This study of 22,256 subjects (Andersson et al., 2004) found around 289 and 281 mg/d increase in dietary phytosterols intake (lower quartile vs. higher quartile) in men and women was inversely correlated to 0.25 and 0.15 mmol/L reduction in serum TC and 0.14 and 0.13 mmol/L reduction in LDL-C concentrations, respectively, after adjusting for potential confounders such as age, body mass index (BMI), and total energy intake (Andersson et al., 2004). Higher phytosterol intake was found to be positively associated with higher total energy intake, total fat, saturated, mono polyunsaturated fatty acids, total dietary cholesterol, and dietary fiber intake. This finding suggested that an average increase of 200 mg daily phytosterol intake from natural foods could lower serum LDL-C significantly (Andersson et al., 2004). In northern Sweden, a study of 78,000 subjects examined dietary phytosterols density (in mg/MJ) with serum cholesterol concentrations. A high-density phytosterol intake across increasing quintiles (mg/MJ) was found to be inversely associated with serum TC levels in both men and women by 2.6–3.5% reduction and LDL-C (3.5%) only in women (Klingberg et al., 2008b). Even though phytosterols’ role in cholesterol-lowering is well established, however, the same physiological effect of dietary phytosterols has always been questioned because of its low amount present in natural foods. Thus, the abovementioned studies were two of the first and foremost studies to find the inverse association of dietary phytosterols with serum cholesterol.

Another feeding trial demonstrated the effects of phytosterols on blood lipid levels using foods rich in phytosterols and supported the association of increasing phytosterol intake and LDL-C lowering. This feeding trial randomized high CVD risk subjects to two Mediterranean diets supplemented with either virgin olive oil or mixed nuts, and the subjects were advised to be on a low-fat diet for 1 year (Escurriol et al., 2009). A Mediterranean diet with virgin olive oil or mixed nuts significantly reduced LDL-C by 4.2% and 6.8%, respectively, from the baseline values, and the LDL-C/HDL-C ratio was reduced by 6.1% and 9.5%, respectively. Moreover, a diet supplemented with mixed nuts also increased HDL-C by 5.2%. High phytosterol content in nuts
might impact its higher efficacy in lowering LDL and increasing HDL-C. Besides, no changes occurred in other blood lipid biomarkers such as triacylglycerols, triacylglycerols/HDL-C ratios, lathosterol, or campesterol (Escurriol et al., 2009). Overall, this study suggested that the Mediterranean diet with increased consumption of phytosterols could beneficially improve the serum lipid profile (Escurriol et al., 2009).

Observational studies have shown that phytosterol-rich foods have enough potency to lower cholesterol absorption than phytosterol enriched foods products or supplements. Some factors that might explain the lower efficacy of phytosterols in clinical trials are a great amount (2g) of phytosterol supplementation in a diet without controlling the actual phytosterols amount present in the diet, higher baseline serum lipid levels, short duration of the trial, and other co-existing dietary components such as fiber or unsaturated fatty acids. Therefore, uncontrolled phytosterol content in clinical trials might be responsible for their actions in higher cholesterol reduction. A randomized and crossover study (Table 2.1) used two diets differing in their phytosterol content: phytosterol deficient (126 mg/2000 kcal) and phytosterols abundant diet (449 mg/2000 kcal) along with the presence of similar content of other nutrients present in the diet. The results reported that the phytosterol abundant diet has a substantial reduction in fractional cholesterol absorption (26%) and a significant increase in cholesterol excretion (79%) as compared to increase in cholesterol absorption (73.2 ± 1.3%) with the poor phytosterol diet which was relatively higher than mean cholesterol absorption reported (60%) (Lin et al., 2010).

It has been shown that some confounding factors might affect the cholesterol-lowering efficacy of phytosterols. For instance, a cross-sectional study of 3940 Chinese adults adjusted some confounders, including age, BMI, menopausal status (in women) and dietary intake of energy, cholesterol, saturated fatty acids, and fiber (Wang et al., 2012). This study, which reported an increase in phytosterols by 241 and 264 mg/d in the diet of women and men diet was inversely associated with 5.3%, 6.7%, and 6.9% decreases in serum TC, LDL-C, and non-HDL-C of women (p < 0.001); and 3.6%, 4.2%, and 3.3% in men (p > 0.05), respectively. Additionally, increased phytosterols intake in the highest quartile also showed a small but significant (p < 0.05) reduction in left internal intima-media thickness both in men (7.6%) and women (5.1%) compared to the lowest quartile. Overall, even a small increase in moderate intake of phytosterol might be favorable to prevent the development of atherosclerosis or CVD (Wang et al., 2012). Similar results were found by another cross-sectional study comparing the quartiles of dietary phytosterol intake (Li et
Lifestyle factors, including physical activity and dietary practices, also play an important role in altering the body weight and other CVD risk markers along with serum lipid profile. This study examined 913 healthy individuals from the general population and reported a dose-dependent significant inverse association (all P<0.001) between highest quartile of total dietary phytosterols intake ($383 \pm 54.4$ mg/d) and BMI, waist circumference, systolic blood pressure, diastolic blood pressure, TC, and LDL-C. Moreover, increases in energy, fat, protein, fiber, and cholesterol intake were also observed throughout the increasing quartiles of phytosterol intake. Also, overweight/obesity and abdominal obesity were found to be less prevalent among those individuals with a higher intake of phytosterols (Li et al., 2018). Interestingly, reduction in blood pressure has been shown as beneficial for lowering cardiovascular morbidity risk (Patel et al., 2016). Therefore, a moderate intake of phytosterols from the daily habitual diet might be effective to control blood pressure and manage overweight/obesity (Li et al., 2018).

Furthermore, Klingberg et al. (2013) also reported the cardioprotective effects of phytosterols from daily dietary intake. This study reported that phytosterol intake across increasing quartiles ($340$ mg/d) decreased myocardial infarction risk by 29% for men than lower quartile intake ($150$ mg/d), and showed a significant inverse relationship between high natural phytosterol intake and risk of myocardial infarction (Klingberg et al., 2013). All these findings suggest that a high intake of phytosterols from a natural diet might help to reduce the risk of CVD and could also improve cholesterol metabolism (Sanclemente et al., 2012).

It seems that observational studies on natural dietary phytosterols show greater effects than randomized controlled trials on phytosterol supplementation. Thus, the average daily intake of phytosterols from natural plant-based foods might be considered a good primary strategy to prevent the risk of CVD.
Table 2.1 Phytosterol Effects on Cholesterol Absorption and Serum Cholesterol Levels

<table>
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<th>References</th>
<th>Study design</th>
<th>Subjects and age</th>
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<th>Diet type and amount of food</th>
<th>Changes in cholesterol absorption and blood lipid levels</th>
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<td><strong>Supplementary phytosterols and cholesterol absorption</strong></td>
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<tr>
<td>Lin et al. (2010)</td>
<td>Randomized, crossover</td>
<td>N = 20, Healthy, Aged 57.5 ± 3 y</td>
<td>4 weeks for each diet 1 week washout</td>
<td>Phytosterol poor diet (126 mg/2000 kcal) &lt;br&gt;Soluble fiber: 6.7 g/2000 kcal &lt;br&gt;Insoluble fiber: 20.4 g/2000 kcal</td>
<td>Phytosterol abundant diet &lt;br&gt;Lower cholesterol absorption by 26%; 54.2 ± 2.2 % vs. 73.2 ± 1.3% (PS poor diet), P&lt;0.0001 &lt;br&gt;↑ cholesterol excretion 97%; 1322 ± 112 vs. 739 ± 97 mg/d (Phytosterols poor diet), P&lt;0.0001</td>
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<td>Racette et al. (2009)</td>
<td>Placebo-controlled, randomized, crossover</td>
<td>N = 18, Healthy, Aged 63 ±3 y</td>
<td>4 weeks for each diet 1 week washout</td>
<td>Phytosterol deficient diet (50 mg/2000 kcal) &lt;br&gt;Fiber: 17 natural + 9 g soluble fiber added &lt;br&gt;Beverages supplemented Phytosterol (0, 400, 2000 mg/d) &lt;br&gt;Fiber: 16-18 g natural + 9g soluble fiber)</td>
<td>Reduced cholesterol absorption &lt;br&gt;10 % (459 mg/d) and 25% (2059 mg/d) &lt;br&gt;Increased cholesterol excretion &lt;br&gt;459 mg /d Phytosterols (36 ± 6%) P&lt;0.01 &lt;br&gt;2059 mg/d Phytosterols (74 ± 10%), P&lt;0.01; &lt;br&gt;↓LDL 8.9 ± 2.3 % (2059 mg/d Phytosterols).</td>
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<td>Study Authors and Year</td>
<td>Design and Description</td>
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<tr>
<td>Li et al. (2018)</td>
<td>Cross-sectional, retrospective</td>
<td>N = 913</td>
<td>Aged 18 - 60 y</td>
<td>Daily dietary intake of phytosterol from natural foods (measured in quintiles)</td>
<td>High PS 383 ± 54.4 mg/d associated with ↓ BMI, WC, SBP, DBP, TC, LDL-C (all P&lt; 0.05).</td>
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<td>Wang et al. (2012)</td>
<td>Community-based cross-sectional study</td>
<td>N = 3940</td>
<td>Healthy, Aged 31 – 75 y</td>
<td>241 mg/d phytosterol increase in women diet, 264 mg/d increase in men diet</td>
<td>↓ TC, LDL-C, and non-HDL-C by 5.3%, 6.7%, and 6.9% in women (p&lt;0.01), ↓ TC, LDL-C, and non-HDL-C by 3.6%, 4.2%, 3.3% in men (P&gt;0.05)</td>
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<td>Escurriol et al. (2009)</td>
<td>Parallel group, multicentre, randomized and controlled</td>
<td>N = 106</td>
<td>Hypercholesterolemic, MED diet with VOO (n=35), aged 66.1 ± 6.2 y</td>
<td>2 Mediterranean diets</td>
<td>Mediterranean diet with nuts ↓ 8.3 % LDL and ↓ 11.5% LDL-C/HDL-C ratio (P&lt;0.05)</td>
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<td>1) MED diet supplemented with VOO (50 ml/d) or</td>
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<td>2) MED diet supplemented with nuts (30 g/d, as 15 g walnuts, 7.5 g almonds, and 7.5 g hazelnuts)</td>
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<td>3) Low-fat diet</td>
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<td>Study</td>
<td>Design</td>
<td>N</td>
<td>Duration</td>
<td>Intervention</td>
<td>Phytosterol density measured from natural diet</td>
</tr>
<tr>
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<tr>
<td>Klingberg et al. (2008b)</td>
<td>Cross-sectional</td>
<td>N = 2,60,000</td>
<td>10 years</td>
<td>MED diet with nuts (n=37), aged 65.1 ± 6.3 y</td>
<td>Higher phytosterol density- 326</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aged 30-60 y</td>
<td></td>
<td></td>
<td>Lower phytosterol density- 185</td>
</tr>
<tr>
<td>Andersson et al. (2004)</td>
<td>Cross-sectional</td>
<td>N = 22000</td>
<td>4 years</td>
<td>Low fat diet (n=34), aged 67.6 ± 6.6y</td>
<td>Daily dietary intake of phytosterol from natural foods (measured in quintiles)</td>
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<tr>
<td></td>
<td></td>
<td>aged 39-79 y</td>
<td></td>
<td></td>
<td>Highest quintiles-463 mg/d phytosterols</td>
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<td>Lowest quintile- 178 mg/d phytosterols</td>
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</tbody>
</table>

TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; BMI: body mass index; WC: waist circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure
2.7 Phytosterols and Vascular Function

Besides the role of phytosterols in cholesterol-lowering, some studies have also shown its significant benefits to improve vascular health, including endothelial function and arterial stiffness (Simonen et al., 2017). Some studies showed neither improved nor impaired effects of phytosterols on vascular health (Simonen et al., 2017). Numerous intervention trials have examined the effects of foods supplemented with phytosterols and showed the beneficial effects of phytosterols on serum lipid profile (Devaraj et al., 2006; Gylling et al., 2013; Hallikainen et al., 2006; Raitakari et al., 2008; Ras et al., 2015a). Besides lipid profile, a few of these studies also examined vascular functions, with two studies showing beneficial effects of phytosterols on vascular function (Gylling et al., 2013; Hallikainen et al., 2006; Raitakari et al., 2008). A randomized, double-blind, crossover trial on plant sterols, or plant stanol esters (2 g/day) found a significant reduction in serum LDL-C levels by 9–12% with no significant effects on endothelial function (Hallikainen et al., 2006). No significant change has been found on variables of endothelial markers such as inflammation, C-reactive protein (CRP) after phytosterol supplementation, however, sterol esters reduced brachial artery diameter compared to stanol esters (Hallikainen et al., 2006). Interestingly, a decrease in vascular diameter has been shown to alter the balance between vasodilation and vasoconstriction and produce more effect on vasoconstriction leading to an unfavorable response. Cholesterol synthesis marker, desmosterol, was positively correlated with brachial artery diameter. Overall, brachial artery diameter was unchanged during plant stanol intervention in the control group, whereas it decreased only after sterol ester intervention (Hallikainen et al., 2006). After this study, Raitakari et al. found that 2 g/day of plant stanols supplementation in different oils including camelina, rapeseed, or sunflower oil for 3 months reduced serum TC and LDL-C levels (9%) significantly as compared to controls (p < 0.001) but did not alter the endothelial function and elasticity of the carotid artery (Raitakari et al., 2008). Plant stanols decreased the absorption of cholesterol and phytosterols and increased cholesterol synthesis, which in turn inhibited the improvement in vascular function (Raitakari et al., 2008). Moreover, this study did not report any difference in blood pressure, which is one of the major risk markers of vascular dysfunction following phytosterol supplementation. Recently, another study investigated the effects of 3 g/day added plant sterols on vascular function and found a 7% reduction in serum LDL-C levels after 12 weeks, whereas it neither improved nor worsened the endothelial function measured by brachial artery flow-mediated dilation (Ras et al., 2015a). Based on the results of previous studies, it has
been speculated that at least a 10–12% reduction in LDL-C might improve vascular function (Jones et al., 2018).

Interestingly, Gylling et al. found significant effects of plant stanol consumption on arterial stiffness and endothelial function. In hypercholesterolemia subjects provided with 3 g/day of plant stanol ester, significantly reduced serum TC, LDL-C and non-HDL-C levels by 6.6%, 10.2%, and 10.6%, respectively, were observed within 6 months as compared to controls (Gylling et al., 2013). A significant increase in arterial stiffness of small (augmentation index; AI) and large arteries (cardio-ankle vascular index; CAVI) was observed in controls but not in those with plant stanol supplementation. Cardio-ankle vascular index is an indicator of arterial stiffness and arteriosclerosis in thoracic, abdominal, iliac, and femoral arteries independent of arterial blood pressure (Shirai et al., 2011). The augmentation index measures arterial stiffness in small arteries, arterioles and quantifies the reflected wave at the aorta (Nichols and Singh, 2002; Shirai et al., 2011). Thus, no change after plant stanol supplementation in the study discussed above showed that stanol might prevent the progression of arterial stiffness in large and small arteries (Gylling et al., 2013). Serum sitostanol concentration was also increased by 96%, which played a role in improving the serum cholesterol profile and endothelial function, thereby reducing atherosclerosis risk (Gylling et al., 2013).

Beyond lowering serum cholesterol concentrations, some studies found that phytosterols could also reduce CRP concentration, a prototypic marker of inflammation. For instance, phytosterol-enriched beverage (orange juice) was found to significantly reduce serum TC and LDL-C concentrations by 5% and 9.4%, and CRP concentrations significantly by 12% (Devaraj et al., 2006). However, the mechanism behind this is not known. Therefore, more studies are needed to delineate the mechanism. Table 2.2 presents the overall characteristics of the findings discussed above, including study design, population, and the results of the change in vascular function and serum cholesterol concentration by phytosterols.

However, based on the current data available from intervention studies, phytosterols supplementation does not show any consistent evidence for beneficial changes in the surrogate markers of cardiovascular risk events including intima-media thickness, flow-mediated dilation, and arterial stiffness, besides its cholesterol-lowering effects.
It is well documented that the intake of phytosterol enriched foods can reduce LDL-C by 10%, as shown in numerous studies above. However, lowering LDL in these studies might not reach the intensity that is required for influencing vascular health parameters (Devaraj et al., 2006; Gylling et al., 2013; Hallikainen et al., 2006; Raitakari et al., 2008; Ras et al., 2015a). Different observational studies that examined the association of phytosterols with CVD risk found conflicting results (Gylling et al., 2014; Klingberg et al., 2013; Ras et al., 2015a). Thus, more observational and clinical trials are needed to find the actual association of circulating phytosterols with cardiovascular health.

2.8 Summary

This review presented an overview of the physiology of phytosterols, food sources of phytosterols, the use of phytosterols as a dietary therapy for lowering serum cholesterol levels and prevention of CVD risk in the general population. Beyond cholesterol-lowering, the impact of circulating phytosterols on vascular health markers was also discussed. Phytosterols are bioactive compounds that have remarkable effects on whole-body cholesterol metabolism, absorption, and excretion. Several health experts and authorities, including Health Canada (Health Canada, 2010), U.S. Food and Drug Administration (FDA) (Food and Drug Administration, 2011), European Food and Safety Authority (EFSA) and Food Standards Australia New Zealand (FSANZ) (AbuMweis et al., 2014) recommended the safe use of phytosterols in natural foods and industrial food products to reduce serum cholesterol and CVD risk. Moreover, several dietary guidelines which focused on the treatment of hypercholesterolemia, dyslipidemia, and other CVD risk events, recommended approximately 2 g/day intake of phytosterols which helps lower serum LDL-C concentrations by 10% along with changes in dietary practices and lifestyle habits (Gylling et al., 2014; Jellinger et al., 2017; Piepoli et al., 2016). Numerous cross-sectional studies have shown a consistent inverse association of increasing daily dietary intake of phytosterols from natural food sources with serum LDL-C levels reduction. Moreover, even a low intake of phytosterols from the daily plant-based diet was reported to have enough efficacy to lower cholesterol absorption and serum cholesterol levels, compared to the recommended intake of phytosterols. However, most of the data published showed that the dietary phytosterols from natural food sources play a beneficial role in altering blood lipid concentrations, but it still needs to be determined whether or not dietary phytosterols have beneficial effects on cardiovascular events. Thus, more research needs to be done to see the
impact of circulating phytosterols on non-sitosterolemic individuals. To conclude, there is further research needed to understand whether the intake of dietary phytosterols is a preventive measure to reduce the risk of CVD and improve other CVD biomarkers along with serum lipid levels such as vascular health biomarkers.
<table>
<thead>
<tr>
<th>References</th>
<th>Study design</th>
<th>Subjects and age</th>
<th>Study duration</th>
<th>Plant sterol/plant stanol dose (g/d)</th>
<th>Vascular function assessment and results</th>
<th>Changes in serum lipid concentrations (P-value different from baseline or/and controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hallikainen et al. (2006)</td>
<td>Randomized, double-blind, crossover (only Intervention group)</td>
<td>Hypercholesterolemia (n=80) 21-72 y</td>
<td>10 weeks each</td>
<td>1.98 g STAEST enriched spread (25g) 1.93g - enriched STEEST spread (25 g)</td>
<td>FMD (NS)</td>
<td>STAES: ↓ LDL-C 4-6%, 6-9% (P&lt; 0.05; baseline &amp; control) STEES: 6-9%, 9-12% (P&lt; 0.001, P&lt;0.05); baseline, controls</td>
</tr>
<tr>
<td>Raitakari et al. (2008)</td>
<td>Randomized, double-blind, parallel</td>
<td>Hypercholesterolemia (n=190) 20-50 y</td>
<td>12 weeks each</td>
<td>2 g plant stanol in 25 g vegetable spread</td>
<td>FMD, coronary artery compliance (all NS)</td>
<td>↓ LDL-C 6% (P&lt;0.001, baseline) 9.3% (P&lt;0.05, controls)</td>
</tr>
<tr>
<td>Ras et al. (2015a)</td>
<td>Randomized, double-blind, placebo-controlled, parallel</td>
<td>Hypercholesterolemia (n=232) 40-65 y</td>
<td>12 weeks</td>
<td>3 g plant sterols in 20 g spread</td>
<td>FMD, PWV, AI (all NS)</td>
<td>↓ TC 4.5 % (P&lt;0.05, controls) ↓LDL-C 6.7 %, P&lt;0.05, controls</td>
</tr>
<tr>
<td>Gylling et al. (2013)</td>
<td>Randomized, placebo-controlled, double-blind, parallel</td>
<td>Hypercholesterolemia (n=92) 25-66 y</td>
<td>6 months</td>
<td>3 g plant stanol in 20 g spread</td>
<td>CAVI, AI, RHI (P=0.046, P=0.023, NS),</td>
<td>↓TC 6.6 ± 1.9% ↓LDL-C 10.2 ± 2.7% (P&lt;0.001 for both, controls)</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Participants</td>
<td>Intervention</td>
<td>Outcome Measures</td>
<td>Results</td>
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</table>
| Devaraj et al. (2006) | Randomized, double-blind, placebo-controlled | Healthy (n=77), 19-74 y | 2 g plant sterol in 240 ml Orange juice beverage | CRP (12%; P<0.005) | ▼TC 5% (p<0.01)  
▼LDL-C 9.4 % (P< 0.001)  
↑HDL-C 6% (P<0.02, baseline)  
▼Non-HDL-C 8.8 % (P<0.02, baseline & controls) |

TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; FMD: Flow-mediated dilation; AI: augmentation index; PWV: pulse wave velocity; CAVI: cardio-ankle vascular index; RHI: reactive hyperemia index; CRP: C-reactive protein; NS: nonsignificant.
Bridge to Chapter 3

In the previous chapter, we discussed the association of dietary phytosterols with CVD risk biomarkers. Therefore, to examine the effects of phytosterols on various CVD biomarkers, the first important step is to calculate the phytosterol amount from the dietary intake using dietary assessment tools. The next chapter comprises a comprehensive overview of various dietary assessment tools, that are most widely used to assess the dietary intake of individuals. This review will also look at the strengths and limitations of the various methods. These methods could be used to assess dietary intake patterns and calculate macronutrients, micronutrients, and dietary phytosterols.
Chapter 3: Literature Review 2

Dietary Assessment Tools and Diet Quality Indices

3.1 Introduction

Diet is well known to have a tremendous influence on health, including obesity, hypertension, and other risks related to CVD (WHO, 2003). For many health conditions, specific diet pattern or nutrients have been directly linked with specific health biomarkers, such as the link between saturated fat intake and serum LDL-C levels (Mensink et al., 2003), sodium intake with blood pressure (MacGregor, 1999), and energy balance with obesity (Hu, 2008). Moreover, to examine such links, various factors in diet intake and dietary patterns, including variability in the frequency of food intake, type of food, and the amount of food consumed need to be considered (Thompson & Subar, 2017). Therefore, dietary assessment methods are needed to make a more accurate assessment of diet, which takes all the factors mentioned earlier (variability in the frequency of food intake, type of food, and the amount of food consumed) into account. Dietary assessment involves recording the dietary intake of an individual and evaluating the diet quality by analyzing the food consumed to dietary reference intake values to determine if any nutrient deficiency or excess is likely to occur (Agostoni et al., 2010). Several dietary assessment methods, including food or dietary records, 24-hour food recall, and food frequency questionnaires, are most commonly used to assess dietary intake (Thompson & Subar, 2017). These methods varied from each other by the duration for collection of dietary intake information and by the measures used to quantify portion sizes (Thompson & Subar, 2017).

Besides providing the estimates for food and nutrient intake, dietary assessment tools are also used to estimate the dietary patterns of interest and their relationship with human health (Shim et al., 2014). Dietary patterns that account for inter-relations of food choices and illustrate accumulative exposure to different diet components have been evaluated in many studies and found them explicitly linked with health (Zampelas & Magriplis, 2020; Reedy et al., 2018). Evaluation of dietary patterns, along with the estimation of nutrient intake, provides a more appropriate estimation of healthy or unhealthy dietary habits (Reedy et al., 2018). Several dietary indices such as the Mediterranean Diet Score (MDS), the Dietary Approaches to Stop Hypertension (DASH) score, and the Healthy Eating Index have been developed based on the
dietary patterns and recommended dietary guidelines for the general population to assess the diet quality of dietary patterns that includes almost all food groups in the calculation of total score (Martínez-González et al., 2014; Salas-Salvadó et al., 2018). Mediterranean dietary patterns are the most studied and well-known dietary patterns (Nettleton et al., 2009; Stewart et al., 2016). The traditional Mediterranean diet was first proposed by Ancel Keys, an American scientist who launched a seven countries study and observed the relationship of eating habits and lower incidence of mortality from CVD in communities of Mediterranean nations such as Greece and Italy (Keys et al., 1986, Keys, 1980). Observation studies have shown that westernized dietary patterns, including higher consumption of refined carbohydrates, and red and processed meat, are directly associated with increased CVD risk (Bauer et al., 2013; Stewart et al., 2016). However, evidence suggests that the Mediterranean dietary patterns high in olive oils, fruits, vegetables, legumes, whole grains, and nuts are associated with reduced CVD risk (Keys, 1980; Stewart et al., 2016). Adherence to the Mediterranean dietary pattern has been shown to reduce serum lipid levels, blood pressure, arterial stiffness, and oxidative stress along with minimizing the incidences of atherosclerosis and overall CVD risk (Salas-Salvadó et al., 2018). It has been shown that consumption of a lower amount of saturated fats and higher amounts of MUFA (Fernández-Real et al., 2012) and fiber (King, 2005) from the Mediterranean diet were responsible for lower serum lipid levels and reduced mortality for CVD. In 2009, Saura-Calixto and Goñi proposed that various components in the diet, such as MUFA-to-saturated lipid ratio, intake of dietary fiber, the antioxidant capacity of the whole diet, and the intake of phytosterols plays essential roles in the Mediterranean diet. Numerous studies have highlighted the beneficial role of phytosterols as a bioactive component in the Mediterranean diet (Escurriol et al., 2009; Estruch et al., 2018; Schwingshackl et al., 2020). However, the role of increased consumption of phytosterols as a part of the Mediterranean diet contribution to CVD risk reduction needs more research.

Various diet quality indices have been validated to estimate nutrient intake and to evaluate the dietary patterns. To comprehend many of the indices being used in today's research, it is essential to understand how the various indices were validated. Many dietary scoring patterns have been developed and validated to assess the quality of diet patterns and their associations with CVD mortality. The purpose of this review is to describe and compare the various methods used to assess nutrient intake, including phytosterols. This review will also describe the benefits of
Mediterranean dietary patterns on CVD health and compare different Mediterranean dietary scoring indices.

3.2 Dietary Assessment Tools

The term dietary assessment defines a broad spectrum of methods used for analyzing food intake within a population or an individual. It includes the production and supply of food at the national level, food purchased for consumption at the household level, and an accurate assessment of the dietary intake and patterns of foods consumed at the individual level (Thompson & Subar, 2017). Individual-level dietary assessment methods will be discussed in this review because they are most widely used in research studies to estimate the frequency of food and nutrient intake, to identify the trends in food consumption, dietary patterns, and to examine diet-disease relationships (Thompson & Subar, 2017; Smiciklas-Wright et al., 2001). Individual-level dietary assessment methods are classified into two methods, namely: 1) prospective methods that assess food intakes at the time of being consumed, using techniques such as food/dietary records and duplicate meal method, and 2) retrospective methods that measure the past dietary intake, using techniques such as diet histories, food frequency questionnaire (FFQ), single or multiple 24-hour dietary recalls (Naska, Lagiou, & Lagiou, 2017; Thompson & Subar, 2017). The diet histories, FFQ, single or multiple 24-hour recalls, and food records are the most commonly used methods in clinical trials and epidemiological studies for assessing diet and disease associations (Naska, Lagiou, & Lagiou, 2017; Shim et al., 2014). These dietary assessment methods involve the collection of information about the frequency of food intakes and portion size of food and beverages consumed over a specified time. These methods also play a significant role in the coding and processing of the collected data to compute the intake of energy, nutrients, and other dietary constituents using food composition tables (Bates et al., 2017). The retrospective dietary assessment methods are entirely reliant on the respondents’ memory to remember all the foods and portion sizes they ate during that particular time (Thompson & Subar, 2017). Respondents are asked to report the portion size of food consumed using pictures of foods, and by using standard household measures such as cups and spoons (Smiciklas-Wright et al., 2001; Shim et al., 2014; Thompson & Subar, 2017). However, the researcher needs to understand how these dietary assessment methods were validated, before implementing them in research. The various dietary assessment methods are discussed below, along with their advantages and limitations. These dietary assessment methods provide an
overview of practical considerations for collecting, processing, analyzing, interpreting, and reporting dietary data.

3.3 Dietary/Food Record

Dietary record collects detailed information about food and beverages consumed over a specified recording period, which can vary from one to several consecutive or non-consecutive days or in 3-7 consecutive days, and is commonly used to better estimate the usual intake (Thompson & Subar, 2017; Buzzard, 1998; FAO, 2018). Ideally, participants are asked to report the portion size of each food item they consumed either by weighed diary methods or using estimation (non-weighted diary method), using food models, photographs, or standard household measures (Johnson, 2002). Weighing food increases the accuracy of recorded portions; however, it also increases the burden on respondents, while the estimation of portion sizes needs certified staff (e.g. dietitian) to estimate the portion of each food item consumed (Thompson & Subar, 2017; Berdanier et al., 2007). Respondents should be instructed to maintain their usual eating patterns and clear instructions should also be given to respondents as to how to fill out the form. Some people do maintain their usual eating habits and track their daily diet, however, some people do not. Therefore, prior to the start of the dietary assessment, clear instructions should be provided to all respondents on how to maintain and report their usual eating patterns (Thompson & Subar, 2017; Berdanier et al., 2007). Because self-reporting of dietary intake has been shown to misreport the intake of energy and other nutrients, therefore, the chances of serious measurement error are high. However, the use of statistical approaches to adjust for underreporting or overreporting of nutrients, mainly energy intake, while analyzing the diet and disease relationship has been shown to reduce the effects of misreporting (Ravelli and Schoeller, 2020).

Moreover, the demands of record-keeping in this method can lead to a lack of interest in respondents, therefore, comprehensive instructions for respondents are important for improving record-keeping (Ortega et al., 2015). After completion of records, they should be appropriately checked to ensure all the sections or necessary information has been provided. Reviewing food records with respondents is much better for clarifying entries and to probe missing information (Thompson & Subar, 2017; Berdanier et al., 2007).

The dietary record method has some advantages, including providing quantitatively accurate information on food consumed during the recording period (Table 3.1). By recording
food intake at the time of consumption, there are always fewer chances of memory lapse that would result in the omission of food items eaten, and the food details are usually fully provided. Moreover, the reported amount of foods consumed provides a more accurate portion size estimation than recalling the portion size of foods eaten in the past (Thompson & Subar, 2017). Multiple 7-day records are considered as a "gold standard", used to validate other methods, e.g. FFQ (Hu, 2008). A 7-day food recording is burdensome to most respondents; therefore, some improvements have also been made in the context of technological innovation to minimize the burden on respondents. For example, the Technology Assisted Dietary Assessment (TADA) tool was developed to collect food images before and after eating occasions. This tool also provides a scale to estimate the type and amount of foods consumed (Khanna et al., 2010).

The limitations (Table 3.1) of using the food record method are that it is more prone to bias both while choosing the type of format and upon the completion of the number of days of diet records. However, the dietary records highly depend on the respondent's memory as the number of days of a recording period increases, and it decreases the validity of information collected in the earlier days (Ortega et al., 2015). Dietary records can also lead to underestimation of dietary intake. It has been reported that incomplete and inaccurate recording of data and recording of the diet can impact the dietary choice, which can lead to undereating (Gibson, 2005; Thompson & Subar, 2017). Respondents should be guided to maintain their usual eating patterns. The respondents alter their eating habits while monitoring their intake which may lead to reactivity bias, for instance, overeaters may underreport and undereaters may overreport their dietary intake (Ravelli and Schoeller, 2020). This study reports that underreporting of energy intake varies as a function of BMI. For instance, individuals with higher BMI have been found to do the highest level of underreporting as compared to individuals with normal BMI (Thompson & Subar, 2017). Another limitation is that recording data on paper every day can be burdensome for respondents and also for researchers to code, process, and can cause high personnel costs (Thompson & Subar, 2017). This method demands more economic resources, therefore, it is not a practical method to be used in large population studies (Ortega et al., 2015).

Overall, several approaches are needed to minimize underreporting in the dietary record. Therefore, the approaches should include proper training of respondents and asking psychological questions from respondents to reduce the effect of underreporting. Even though, this method
provides comparatively accurate information versus other dietary methods, however, hiring personnel to train respondents can lead to high costs. Moreover, computer-based tools are not widely practiced in extensive cohort studies yet. This tool has more likely similar limitations as paper-based dietary records such as reactivity bias which is problematic for capturing usual dietary exposure in epidemiological research (Marcinow et al., 2018).

### 3.4 24-hour Recall Method

A 24-hour recall is an entirely open-ended survey that captures a variety of information about dietary intake. The recall information is usually collected by nutritionists or dietitians, who have been well trained in interview tactics (FAO, 2018). The interviewer plays a very crucial role in collecting the recall data; therefore, the interviewer should know about the foods available in the market, types of food including regional or ethnic foods, and food preparation practices (Thompson and Subar, 2017). The interview can be conducted face to face or by telephone, or it could be computer-assisted (Thompson & Subar, 2017). In the interview, the respondents are asked to report detailed information on food and beverages consumed, amount, the brand name of commercial products, preparation, cooking methods, and ingredients used in mixed dishes (Robertson et al., 2005; Subar et al., 2012). For the portion size estimation of food eaten, different reference measures such as household standard size containers, e.g. bowls, cups, and glasses, standard measuring cups, and spoons, a three-dimensional food model are used. In telephone-administered interviews, two-dimensional aids such as photographs can be provided to the respondents before asking them questions on their diet (FAO, 2018). The collected information on portion size can help to calculate the amount of energy and nutrients. The 24-hour recalls can be collected as a single recall, or multiple recalls on non-consecutive days. A single recall is useful only when the study aims to look at the mean intake of the population (Thompson & Subar et al., 2017; Shim et al., 2014). It has been reported that a single 24-hour dietary recall does not provide an accurate estimation of individuals' usual intake (Buzzard, 1998; FAO, 2018). Therefore, multiple non-consecutive recalls are required when the study aims to estimate individuals' usual intake or to examine the correlations of individuals' usual intake (Albar et al., 2016; Carter et al., 2015). Moreover, multiple 24-hour recalls are also used as a reference to validate FFQ (FAO, 2018).
Currently, new innovative technologies are emphasizing more on minimizing the respondents' burden, making the dietary recall less time consuming, improving their accuracy, and self-administering of multiple recalls (Thompson & Subar, 2017). The innovative computer-based technologies developed a comprehensive online system for data collection, automated coding, entry, and calculation of intakes (Moshfegh et al., 2008; Slimani et al., 2011; Thompson et al., 2010, Timon et al., 2016). Currently, multiple-pass 24-h food recall methods are considered as the standardized approach and found to be more useful in epidemiological research studies (FAO, 2018; Shim et al., 2014). The most widely used 24-h food recall approach in the United States is the United States Department of Agriculture's (USDA) computerized Automated Multiple-Pass Method (AMPM) (Blanton et al., 2006; FAO, 2018). This method is used to collect dietary intake information in the National Health and Nutrition Examination Survey (NHANES). The USDA 5-pass method approach consists of five steps. The first step is a 'quick list' of foods eaten over the past 24 hours. The second step is 'forgotten foods list', which involves probes of possibly forgotten foods. The third step is 'time and occasion', wherein the respondent is asked to report the time and occasion for each food they consumed. The fourth step is 'detail cycle', which involves a detailed description of the amount, preparation methods, ingredients, and portion sizes. The last step is 'final review' in which the respondent is asked to review the recalled information and to probe for anything else consumed in the last 24 hours (FAO, 2018; Thompson & Subar, 2017).

Similarly, a menu-driven standardized 24-hour program that was used in the European Prospective Investigation into Cancer and Nutrition study called the EPIC-Soft (Slimani et al., 2011). These computer-based approaches reduce bias in the data collected and allow researchers to collect and identify dietary information online in a standardized manner. It may also enhance the accuracy of data reported even if the collected data is from diversified population groups (Shim et al., 2014). The automated tools are recognized as a more suitable method to collect high-quality dietary data with minimal bias in large-scale population studies (Shim et al., 2014). Therefore, using many segments of the AMPM interview method developed by USDA, the Automated Self-Administered 24-hour dietary recall (ASA24-hr) was developed at the National Cancer Institute (NCI) (Thompson & Subar, 2017). The ASA24-hr is a freely available, web-based tool that has been adapted to reflect the difference in Canada and US food fortification practices. A large-scale population study on 1,083 adults compared the estimated food, and nutrient intake data from
AMPM and ASA24-hr recalls and found that more than 70% of the participants favored the ASA24-hr recalls over the AMPM method (Thompson et al., 2015).

There are some advantages to using 24-hour recalls (Table 3.1). If the recall is interviewer-administered, there will be less burden on respondents. Moreover, the literacy and numeracy skills of the respondent are not required if the recall is interviewer-administered (Satija et al., 2015; Willett & Lenart, 2013). However, in the self-administered method, accurate estimate entirely relies on respondents’ literacy to provide detailed information about food and to report portion size (FAO, 2018; Thompson & Subar, 2017). Therefore, new computer-based tools need to provide a quick-start guide to minimize literacy issues or help participants to overcome technological and other issues. The NCI has developed a quick start guide on their website that can be used by different populations, which minimize the bias due to literacy. The web-based self-administered 24-hour recalls have an automated coding system that helps to save a significant amount of time and eliminate the need for an interviewer and coder which is time-consuming, expensive, and laborious (Thompson & Subar, 2017).

There are some limitations to using self-administered and interviewer-administered 24-hour dietary recalls. The major limitation of using self-reported 24-hour recall is that respondent may not disclose their actual intake due to various reasons such as knowledge or literacy (Marcinow et al., 2018). Interviewer-administered recalls are expensive in terms of providing training to the interviewers for data entry, coding, and processing. Another limitation is that an estimation of food intake completely relies on a respondent's memory (Satija et al., 2015).

Overall, this method is less time consuming, less expensive, and less prone to reactivity bias as compared to dietary records. The use of automated 24-hour recall is increasing because they are cost-effective and convenient. The automated web-based 24-hour dietary recall is becoming a more feasible tool of choice in nutritional epidemiology because of its accuracy and precise estimation of nutrients (Carroll et al., 2010). It provides comprehensive details on dietary intake and measures nutrient and food intake with less bias than FFQs (Marcinow et al., 2018). The automated 24-hour recall is less prone to bias than interview-based recalls as respondents can fill the recalls on unannounced days which limits the reactivity bias. The automated-24-hour recall reduces the social desirability bias as respondents filled their recall on their own compared to the interview administered recalls. Moreover, the inclusion of a quick start guide and inclusion of
forgotten foods list and comprehensive description of foods eaten can contribute to minimizing underreporting, which can help to reduce memory bias (Marcinow et al., 2018). Therefore, automated 24-hour recall is considered as a tool of choice to use in large cohort studies.

3.5 Diet History

The diet history method was first developed by Burke in 1947 (Burke, 1947) to assess the long-term usual dietary intake of individuals and their eating patterns. The diet history method consists of three parts: in-depth face-to-face interviews to evaluate the usual food intake and dietary pattern, a food checklist, and a 3-day diet record. The diet history method requires a well-trained nutritionist or dietitian with enough experience in dietary assessment to interview the respondents to collect the information on their usual food intake and eating patterns during the meal and in between the mealtimes (Burke, 1947). The interviewer asks the respondent to complete a 24-hour recall, a 3-day food record, and a checklist of foods they consumed over the previous month along with their likes and dislikes in foods (Burke, 1947). The respondents were also asked to report the portion size of food consumed using the real size of food e.g. small, medium, or large apple, or by using household measures (bowls, spoons, cups). In the end, the reported frequency and quantity of food were cross-checked with the list of food groups (Burke, 1947). However, this original diet history method was considered inappropriate for use because of high labor-intensity and being time-consuming for the individual; it demands the trained interviewer to perform the interviews, to collect and enter the data, and code and process the data. For example, the Western Electric Study conducted an interview followed by another interview a year later (Orencia et al., 1996). Nutritionists used standardized interviews and questionnaires based on Burke's diet method. Using these questionnaires, they collected data on usual eating patterns, diets, and changes in dietary habits. They provided 195-item cross-check food-frequency list to determine the quantity of food consumed in the past 28 days. Portion sizes were estimated using wax models of foods and dishes which are commonly consumed (Orencia et al., 1996). However, many modified versions of diet history were developed after Burke's version (Liu et al., 1994; Mensink et al., 2001). Few prospective studies have been using diet history methods. For example, in 1994, Liu and colleagues examined the reliability and validity of the Coronary Artery Risk Development in Young and Adults (CARDIA) diet history in 128 young adults from the United States. This study was interviewer-administered and collected reliable quantitative data on dietary intake. This method
was validated with 7-day 24-hour dietary recalls and the mean nutrient intakes of both methods were found to be correlated; with a coefficient of correlation above 0.50 (Liu et al., 1994). However, the finding above also stated that there are some limitations of using the diet history method such as it is time-consuming, expensive, requires trained interviewers, and cannot be self-administered (Liu et al., 1994). Later, computer-based diet history questionnaires were modeled after the CARDIA study. In 2001, the Dietary Interview Software for Health Examination Studies (DISHES 98), a computerized diet history questionnaire, was designed to assess the dietary intake of adults and an elderly German population (Mensink et al., 2001). The DISHES 98 software was validated by comparing the results of this method with results from a 3-day weighed dietary record and 24-hour recall. The mean intake of most of the nutrients from DISHES 98 was lower than the intake recorded by 3-day dietary record and with 24-hour recall with a 0.51 and 0.46 average coefficient of correlation, respectively. This study concluded that DISHES 98 dietary history method is considered comparatively reliable to assess dietary intake compare to the aforementioned diet history methods (Mensink et al., 2001). However, Burke's method and modified versions of Burke's methods used in the Western Electric study and CARDIA did not produce an appropriate level of accuracy about the foods and nutrients eaten by subjects and did not show expected results while testing the hypothesis of interest. Therefore, fewer studies support the diet history method as it is generally used as a reference to endorse other dietary assessment methods such as 24-hour recall and FFQ (Thompson et al., 2015).

There are some advantages and limitations of using a diet history tool (Table 3.1). The advantages are that respondents need not be literate, detailed information on individual foods are obtained, foods consumed less regularly, are also taken into account, and intake of energy and most nutrients can be estimated (FAO, 2018). However, the limitations are that this method is more time-consuming and expensive because it requires well-trained staff to perform interviews, for processing and analyzing food and nutrient intake data. Moreover, this method is also highly susceptible to recall bias, the same as the other dietary prospective methods, because all these methods rely on individuals' memory to recall all the foods they consumed in the past (Hu, 2008). Overall, no further advancements have been seen in this method in the field of nutritional epidemiology, thus this method is rarely practiced in epidemiological nutritional studies involving thousands of participants (Shim et al., 2014; FAO, 2018).
3.6 Food Frequency Questionnaire

The framework of FFQ is based on the diet history method developed by Burke in 1947. The questionnaires are designed according to study objectives and period that needs to be covered by the FFQ such as daily, weekly, monthly, or yearly (FAO, 2018). The questionnaire can be self-administered or interview administered by well-trained personnel. FFQ captures the frequency in which foods and beverages are consumed over a specific period, yearly, or in a shorter period (Thompson & Subar, 2017). The FFQ contains a food list and frequency categories, which are usually closed-ended multiple-choice format. It has been reported that the food list of FFQ needs to be comprehensive because it plays a vital role in capturing total dietary intake. However, a finite list cannot correctly estimate many different foods, brands, and preparation practices from an individual's reported intake. The food list may range from 20 food items (may be used for screening purposes only) to a comprehensive food list, including 160 to 180 food items to assess total dietary intake (Cade et al., 2002). The number of foods listed in the questionnaire may vary with the study purpose and population. Respondents are asked to answer the frequency of food eaten based on the response categories, which range from the number of times per day to several times a year or never (Hu, 2008; Thompson & Subar, 2017)). The FFQs that collect portion size information are known as semiquantitative questionnaires. The respondents are asked to report portion size using individual and standard household measures, such as bowls, cups, and spoons, and portion size-frequency is reported based on how often that specified portion size was consumed (Cade et al., 2002). Including portion sizes in the questionnaire allows one to calculate the estimated quantity of food and nutrient intake. For instance, Subar et al. (2001) reported that providing information on portion sizes and frequency of portion size intake could be useful for estimating the absolute daily intake of macronutrients. If two foods were eaten together, respondents are asked to report the frequency of those particular foods both alone and in mixtures or to report the frequency of each food separately (Thompson and Subar, 2008; Shim et al., 2014; Willett & Hu, 2007). For example, in the FFQ developed by Willett, portion sizes were asked in the same question where the food item was listed (Willett & Lenart, 2013), whereas in the NCI Diet History Questionnaire (DHQ), the portion sizes for each food consumed were asked in a separate question (Subar et al., 2001). To gauge the quantity of portion size of foods, a well-constructed food database is needed (Welch et al., 2005; Mulligan et al., 2014).
To construct such a food database, several approaches are needed. Those approaches include information on the dietary intake from the group targeted to estimate the nutrient density of a specific category of food (Thompson & Subar, 2017; Shim et al., 2014). All the individual food codes mentioned in a population survey for each food group can be used to estimate the mean or median composition of nutrients. Furthermore, the nutrient intake estimates for each respondent can be calculated using dietary analysis software, specific to each FFQ (Willett & Lenart, 2013; Thompson & Subar, 2017). Finally, analytical decisions are major issues in the processing of food frequency data. Some questionnaires that are not automated cannot check for the quality of data to ensure if all the questions are answered or not. Therefore, analytical actions about how to deal with the missing information are needed (Lamb et al., 2017; Steinemann et al., 2017). Particularly, in self-administered questionnaires, respondents skip answering many questions about foods that they never like or eat. Therefore, two approaches have mostly been used to deal with the missing information. The first approach is to allot zero values to the unanswered questions, and the second approach is the imputation of frequency values to the invalid answers (Parr et al., 2008). In the imputation process, the missing data is replaced with the nearest lowest or highest probable value based on other available values (Ichikawa et al., 2019). However, whether imputation is an effective approach in frequency analysis or not is currently unclear. Therefore, the use of paper-based administration has been replaced by electronic administration, in which the automated system minimizes the risk of missing data (Parr et al., 2008; Thompson & Subar, 2017).

There are many strengths in the FFQ method (Table 3.1). This method is relatively simple and inexpensive to administer and process than other methods (i.e. 24-hour recall and dietary records) and puts less burden on respondents. This method captures a range of foods, specific nutrients, or specific food groups and assesses the usual intake over a long period (Satija et al., 2015). FFQs can collect detailed information on portion size, food preparation methods (FAO, 2018). Moreover, most FFQs, if completed using the paper version of the document, can be optically scanned, and coding and analysis are calculated automatically using analysis software that makes it feasible to calculate estimated nutrient intake (Satija et al., 2015; Pan et al., 2018; Thompson & Subar, 2017).

On the other side, there are some limitations of using FFQ (Table 3.1) such as they can be administered only to literate populations and those that have good numeracy skills (FAO, 2018;
Satija et al., 2015; Willett & Lenart, 2013). FFQs can be cognitively complex in terms of recalling long-term food and beverage intake or recalling the frequency of foods consumed. Another limitation is that by using self-administered FFQs, respondents may omit the food items they do not understand or may misinterpret the questions which leads to creating a systematic error in the data (Pan et al., 2018). To overcome the systematic errors, the nutrients are adjusted for total energy intake while performing statistical analysis. Because nutrients and total energy are derived from the same food, therefore the measurement errors in the food and nutrients (underreporting or overreporting) are likely to be correlated. Therefore, it has been suggested that adjustment for total energy intake can reduce the correlated errors. (Satija et al., 2015; Willett & Lenart, 2013).

Overall, FFQs are made up of a standard set of questions, and food items with portion size, processing, and coding of data are simply straightforward. However, some cohort studies have reported that the FFQ data is more prone to bias as compared to 24-hour recall and other dietary methods (Shim et al., 2014; Thompson and Subar, 2017). It has been suggested that the estimates of nutrient intake obtained from FFQs should be considered as a crude approximation. It is reported that FFQs may be able to provide a better estimate of food groups which can be more feasible to assess dietary patterns (Thompson and Subar, 2013). This will be discussed in the later sections of the review.

**Table 3.1 Strengths and Limitations of Dietary Assessment Methods**

<table>
<thead>
<tr>
<th>Dietary Assessment Instruments</th>
<th>Strengths</th>
<th>Limitations</th>
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<tbody>
<tr>
<td><strong>Dietary Records</strong> (Frank, 2008; Thompson &amp; Subar, 2017; Ortega et al., 2015)</td>
<td>• Provide quantitatively accurate information on food consumed&lt;br&gt;• Open-ended questionnaires are not limited to specific eating patterns&lt;br&gt;• Provides information on eating patterns&lt;br&gt;• Less cognitive pressure on respondents to recall foods consumed, since the data is</td>
<td>• Need to train respondents about when and how to fill the form&lt;br&gt;• Time-consuming, labor extensive&lt;br&gt;• High administration and data analysis cost&lt;br&gt;• Need well educated and trained interviewers&lt;br&gt;• Increase the burden on respondents</td>
</tr>
<tr>
<td>24-hour Dietary Recall</td>
<td>recorded at the time of consumption</td>
<td>The reliability of collected information decreases over time as the number of record days increases</td>
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</table>
| (Satija et al., 2015; Willett & Lenart, 2013; FAO, 2018; Thompson & Subar, 2017) | • Provides an accurate estimate of nutrient intake  
• Relatively less burden on respondents  
• Collects information on eating habits, methods used for food preparation, and place of eating.  
• Participants generally filled their recalls on unannounced days, limits the reactivity bias.  
• The open-ended format is suitable for diversified eating patterns  
• Less burden on the respondent’s memory to recall foods consumed, therefore, provides better accuracy and response rate | • Expensive  
• Time-consuming  
• Inherent bias related to self-report; respondents’ might underreport their intake or might alter their intake if the recall date is known in advance  
• Possible recall bias  
• Trained interviewer required; possible interviewer bias  
• Multiple days required to estimate actual food and nutrient intake |
### Diet History

(Hu, 2008; Shim et al., 2014; Pan et al., 2018)

- Provides information about eating patterns
- Provides quantitative estimation of energy and nutrient intakes over an extended period
- Does not depend on the respondent’s literacy
- Does not affect people’s normal eating habits

- Depends on the respondent’s memory, can produce recall bias
- High cost
- Time-consuming
- Longer interviews are needed to collect more detailed information, creates a high burden on the respondent
- Requires a trained interviewer with detailed knowledge of foods and dietary patterns.

### Food Frequency Questionnaire

(Satija et al., 2015; Pan et al., 2018; Thompson & Subar, 2017; FAO, 2018; Willett & Lenart, 2013)

- Cost-effective, time saving
- Can obtain a range of foods, specific nutrient(s) (quantitative FFQ) or a specific food group
- Quite straightforward to deliver and cost-effective when compared with other assessment methods (i.e. 24-hour recall, dietary records)
- Most suitable for epidemiological studies; it can be administered using a machine-scannable format, reducing data-entry errors
- Could use for ranking individuals based on their usual intake

- Lack of details and specificity of diet intakes
- May not provide an accurate estimate of nutrient intake
- Relies completely on individuals’ memory; in Self-administered FFQs, the respondent might misinterpret the questions, and it can result in errors while reporting a frequency and portion size estimation
- Not feasible to use in a diversified population with distinctive dietary patterns.

FFQ, food frequency questionnaire
3.7 Food Frequency Questionnaire in Epidemiological Studies

Epidemiological studies usually demand a comprehensive assessment of dietary intake to examine the diet-disease relationship (Naska, Lagiou, & Lagiou, 2017; Shim et al., 2014). FFQ is recognized as the most economical and practical method for the collection of dietary data in epidemiological research (Shim et al., 2014).

Many FFQ's have been developed and adapted for different populations, and depending on the research objectives, the FFQ requires validation before using it as a part of a dietary assessment tool for research (Willett et al., 1985, Block et al., 1986; Subar et al., 2001). In North America, the most commonly used FFQs are the Block Questionnaire (Block et al., 1986), the Harvard University Food Frequency Questionnaires/Willett Questionnaires (Willett et al., 1985), the Fred Hutchinson Cancer Research Center Food Frequency Questionnaire (Patterson et al., 1999), and the NCI's DHQ. Dietary analysis software is commercially available for the Willett, Block, and Fred Hutchinson FFQs, and freely available for the NCI DHQ (Thompson & Subar, 2017).

In 1985, Willett and colleagues validated a 61-item semiquantitative FFQ against dietary records using a large prospective study among women only. During the survey, four one-week dietary records and two FFQs, at an interval of one year, were administered to the participants. This study reported that the difference between the dietary methods was small (1620 kcal +/- 323 kcal, 1418 kcal +/- 496 kcal, and 1371 kcal +/- 482 kcal, respectively). In summary, it was reported that semiquantitative FFQ is a straightforward and inexpensive method that can appropriately measure the usual intake of a variety of nutrients. Additionally, this study also stated that this questionnaire was only validated using data of a group of American women. Therefore, this method would need to be re-validated to make it more useful among diversified populations (Willett et al., 1985). A year later, Block and his colleagues developed another self-administered DHQ for use in epidemiological research and intervention studies (Block et al., 1986). Both the foods and the nutrients list were developed using the dietary intake data of 11,658 adult participants from the Second NHANES (McDowell, 1981). The number of food items (147 foods) was selected and ranked based on their contribution (in percentage) to all the 17 nutrients and energy intake. Moreover, the frequency and portion size of foods consumed were also taken into account. The developed food list and the nutrient database were applied to a diet record to calculate nutrient values, resulting in a strong correlation of r > 0.70. The estimated mean values of nutrients were
similar to the values of the national data (NHANES) (Block et al., 1986). In 1990, this questionnaire was validated using multiple diet records with correlation ranges from 0.5-0.6 (Block et al., 1990). Later, considering the need for improvement in the measurement of usual dietary intake, NCI developed a new cognitive-based FFQ, called DHQ (Subar et al., 2001). The modifications were made into the Block and Willets' methods to develop a new DHQ. This DHQ was improved in three major areas. First, 75 respondents aged 50-70 years with varied incomes, education, and ethnicity were cognitively interviewed. Based on individuals' responses in FFQ, various issues such as the order of food items, intake of seasonal foods, average intake from multiple food items, and format were addressed in the new DHQ. Second, the list of foods and portion size ranges were developed using dietary data from the USDA's 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII) (USDA/ARS, 2000). Third, the analytical approach was improved to convert the frequency responses into daily nutrient intake estimates. The Block FFQ was used to derive both nutrient and portion size values. It has been reported that the newly designed format allows for more flexibility in terms of asking nonstandard questions in a better way (Siou et al., 2017). It was reported that cognitive-based changes made in the DHQ enhanced the validity of frequency estimates (Siou et al., 2017). Overall, the DHQ is considered more reliable than the other previously developed Willett and Block questionnaires (Siou et al., 2017; Subar et al., 2001). Later, another study tested if cognitive-based changes in FFQ improves its accuracy. This study addressed four major design issues while testing accuracy. These four design issues were: 1) groupings: asked about food mixtures in single or multiple separate questions; 2) different forms of food: asked about the frequency and portion size of leading food (e.g., milk) vs frequency and portion size of different forms of food (e.g., whole, low fat, non-fat milk); 3) additions: asked about the addition of one food to another food (e.g., sugar into coffee) independently vs asked about additions along with leading food (sugar and coffee); 4) units: asked about the frequency and portion size (e.g., small, medium, large) vs frequency of units, i.e. cups of coffee. The study results reported that greater accuracy was found using separate questions about food mixture, asking the frequency and portion size of the main food (e.g. milk), and in both units' approaches, i.e. frequency and portion size and frequency of units (Thompson et al., 2002).

Moreover, advancements in innovative technology in the context of FFQs are limited to the online versions. For example, web-based versions are available for the DHQ-III developed by NCI, and the Canadian version (CDHQ-II) (Csizmadi et al., 2016; National Cancer Institute,
2020). Overall, it is concluded that FFQs should be evaluated for accuracy before using them as a dietary assessment tool in research. It has also been reported that a coefficient correlation ranging from 0.5 – 0.7 is considered moderate in validation studies (Shim et al., 2014). Concluding the above findings, C-DHQ is a comparatively more reliable tool of choice to use in epidemiological research.

3.8 Evaluation of Dietary Pattern Using Dietary Assessment Methods

3.8.1 Approaches to Define Dietary Patterns

A dietary pattern is a set of dietary habits related to the intake of food and beverages. Research in nutritional epidemiology has reported on various distinct dietary patterns such as the Mediterranean, the Asian, the Western, and the prudent diet (Panagiotakos, 2008; Sanches Machado d’Almeida et al., 2018; Steffen & Hootman, 2016). Some of these diets have been shown to have excellent or adverse effects on human health. Multiple different approaches have been used to examine the relationship between CVD and diet, specific nutrients, food groups, or dietary patterns. Over the last few years, dietary pattern evaluation has evolved as complementary research in nutritional epidemiology to recognize the role of diet in disease risk reduction (Kant, 2010; Rodríguez-Monforte et al., 2015). Dietary patterns can be defined in two different ways, either a posteriori or a priori (Tapsell et al., 2019). The 'a posteriori' approach refers to the construction of dietary patterns based on the consumption patterns of the population (Tapsell et al., 2019). The a posteriori approach is constructed by applying 'principal components analysis', 'factor analysis', and 'cluster analysis' to the dietary information collected by FFQ, 24-hour recall, and dietary records to identify the foods that correlated to each other for the construction of a dietary pattern (Hu, 2002). This approach is used to identify common dietary patterns such as the prudent diet, which is known to reduce the risk of CVD, diabetes mellitus, and metabolic syndrome, whereas the Western diet tends to be associated with an increased risk of CVD (Tapsell et al., 2019). The a priori approach defines dietary patterns based on the existing knowledge about relationships between food, nutrients, and disease (Tapsell et al., 2019). This approach derives dietary patterns based on the established dietary indices or scores such as Healthy Eating Index (Krebs-Smith et al., 2018), the Alternate Healthy Eating Index (AHEI) (Chiuve et al., 2012), the Diet Quality Index (DQI) (Patterson et al., 1994), DASH score (Fung et al., 2008), and MDS (Trichopoulou et al., 2003). In particular, the a priori approach measures the quality of dietary patterns that have already
been recommended as 'healthy'. In 2015, Liese et al. used the four most common dietary indices, the Healthy Eating Index, AHEI, DASH, and Alternate Mediterranean Diet (Fung et al., 2009) score to identify the dietary patterns. The results showed that all the dietary indices were consistent with each other while classifying the quality of an individual’s diet. Higher diet quality from all the indices was found to be associated with a significant decrease in mortality (Liese et al., 2015). Overall, these dietary scores, and so the associated dietary patterns, are evaluated against the health outcome such as CVD and other chronic diseases (Liese et al., 2015). For the past two decades, evaluation of dietary patterns has been found to be useful for studying the effects of diet on health because it accounts for both the complexity and synergistic effects of the food that make up a diet (Reedy et al., 2017).

The usual dietary intake of individuals is important to capture using dietary assessment tools such as FFQ validated for the target population with 24-hour recall on non-consecutive days or dietary records (Kant, 2010) before examining the diet-disease relationship. As reviewed above, each dietary assessment tool has its strengths and limitations, and differences in these dietary assessment tools have shown some inconsistencies in the identified dietary patterns (Cespedes & Hu, 2015). FFQ is considered valuable to assess dietary intake because it captures a wide range of foods and food groups in the diet. It is considered a useful tool to evaluate dietary patterns (Aoun et al., 2019).

In the context of a priori dietary patterns, the Mediterranean diet is one specific dietary pattern that attracted much interest in nutritional epidemiology and research over the past few decades because this dietary pattern has been known to confer a cardioprotective effect in observational studies and RCTs (Martinez-Gonzalez & Bes-Rastrollo, 2014; Sofi et al., 2010).

3.9 Mediterranean Dietary Pattern

The Mediterranean dietary pattern is a traditional dietary pattern of Mediterranean countries such as Italy, Greece, and Spain (Willett et al., 1995). The Mediterranean dietary pattern is characterized by high consumption of plant-based foods such as fruits, vegetables, cereals, legumes, nuts, and seeds; olive oil as a culinary fat; moderate consumption of dairy products (mainly cheese and yogurt), poultry, fish, seafood, eggs; low consumption of red and processed meat, and moderate intake of wine, usually with meals (Bach et al., 2006; Schwingshackl et al., 2020). Extensive evidence demonstrated an inverse association between adherence to Mediterranean dietary
patterns and a reduction in CVD mortality (Martinez-Gonzalez & Bes-Rastrollo, 2014; Schwingshackl et al., 2020). The Spanish landmark Prevención con DIeta Mediterránea trial (PREDIMED) was the first extensive randomized study that showed that adherence to the Mediterranean diet reduces the risk of CVD clinical events in primary prevention (Estruch et al., 2018). This trial was conducted in 7,447 men and women aged 55 to 80 years, who were at high CVD risk but with no CVD events. All the participants were randomly assigned into one of three different diets: a Mediterranean Diet rich in mixed nuts, a Mediterranean Diet rich in extra virgin olive oil, and a control group on a low-fat diet. The participants who were allocated to the Mediterranean diets showed a 30% risk reduction in cardiovascular endpoints such as myocardial infarction, stroke, or cardiovascular death (Estruch et al., 2018). Another Spanish EPIC Cohort Study on 41,078 participants, aged 29-69 years, reported that a high Mediterranean dietary score was associated with a 40% decline in coronary heart disease risk after a mean follow-up of 10.4 years (Buckland et al., 2009). The data from the follow-up of the Seven Countries Study have also shown an inverse association between Mediterranean dietary pattern and CVD or its risk factors (Menotti et al., 1999).

3.10 Mediterranean Dietary Scores

Several diet scores have been used to evaluate the adherence to the Mediterranean diet of the population (Bach et al., 2006). The most often used Mediterranean diet score, the Mediterranean diet scale (MDScale), was proposed by Trichopoulou and colleagues in 1995 and revised in 2003 (Trichopoulou et al., 1995; 2003). The MDScale was sample-specific and used sex-specific median values to set a cut-off point for each diet component. The MDScale was comprised of 9 dietary components such as vegetables, legumes, fruits and nuts, dairy products, cereals, meat and poultry, fish, the ratio of monounsaturated to saturated fatty acids, and alcohol. The total MDScale score ranged from 0 to 9, as each dietary component is assigned a value of 0 indicated minimum adherence to the Mediterranean diet or 1 for maximum Mediterranean adherence. For the food with beneficial effects (vegetables, legumes, fruits and nuts, cereals, and fish), a value of 1 was assigned to those whose consumption was at or above the sex-specific median values, whereas, those whose consumption was below the sex-specific median values were assigned a value of 0. However, for individuals consuming food components with fewer health benefits such as dairy products, meat, and poultry, a value of 1 was assigned to those whose consumption was below the
median values, and a value of 0 was assigned to those whose consumption was at or above the median values. For alcohol consumption, a value of 1 was assigned for men and women who consumed 10-50 g per day and 5g-25g of alcohol per day, respectively (Trichopoulou et al., 2003). In 2009, Leighton et al. developed another Mediterranean dietary score (MDS). The MDS focused on 14 dietary components, including ten beneficial (vegetables, legumes and nuts, fruits, whole-grain cereals, lean meat, fish and shellfish, low-fat and fermented dairy products, vegetable oils, olive and canola oils, avocado and moderate wine consumption ideally with meals) and four harmful components (full-fat dairy products not fermented; fatty meat and processed meat; sugar; and excessive or no wine consumption). Every single dietary component was assigned with a value of 0, 0.5, or 1 based on the frequency of consumption and nutritional quality. In 2015, a HAPIEE (Health, Alcohol and Psychosocial factors In Eastern Europe) study in Eastern Europe reported that each one standard deviation (SD) increase in MDS was inversely associated with death from CVD (0.90, 0.81–0.99) and all other causes (HR, 95 % CI 0.93, 0.88–0.98) (Stefler et al., 2017).

To conclude, maximum adherence to the Mediterranean diet was found to decrease the risk of CVD mortality (Stefler et al., 2017). In 2009, Fung et al. developed the alternate Mediterranean Diet index (aMedDiet) based on the original score by Trichopoulou et al. (1995) (Fung et al., 2009). However, the authors performed some modification in food groups while developing this dietary score and tried not to include some food groups which were presumed to be associated with chronic disease risk. Therefore, fruits and nuts were separated into two individual groups, excluded potato products from the vegetable group, the dairy group was excluded, only whole grains from the cereal grains group were included, and only red and processed meat from the meat group were included. The score values were assigned following the MDscale (Trichopoulou et al., 1995; 2003). A cohort Nurses' Health Study in the United States on 11,793 participants analyzed the association of an aMedDiet score with subsequent CVD risk. This study reported that in the first 4-year follow-up, increasing diet score lead to a 9% (95% CI) reduction in CVD risk (Sotos-Prieto et al., 2015).

Considering the positive effects of the Mediterranean diet, it was proposed to apply the Mediterranean diet score to assess non-Mediterranean dietary patterns. However, due to some variations in the dietary and lifestyle habits among Mediterranean and non-Mediterranean populations, it is unfeasible to implement original Mediterranean Diet scores (Hoffman & Gerber,
2013). In 2009, Rumawas and colleagues developed the Mediterranean-Style Dietary Pattern Score (MSDPS) to examine the compliance of an individual's diet with a traditional Mediterranean-style diet. The authors observed that some studies from the past constructed Mediterranean scores based on the actual intake of the study population rather than applying the amount of intake recommended by the Mediterranean diet pyramid. However, some dietary scores were developed based on the Mediterranean diet pyramid food groups but without considering the recommended intake assigned to each food group. Therefore, MSDPS was developed based on the recommended intake of 13 food groups defined by the Mediterranean diet pyramid, i.e. whole-grain cereals, fruits, vegetables, dairy, wine, fish, poultry, olives-legumes-nuts, potatoes, eggs, sweets, meats, and olive oil. A score was ranged from 0 to 10 for each dietary component, except olive oil, based on the degree of equivalence with recommendations (Rumawas et al., 2009). The total score was standardized to a 0-100 scale. The MSDPS was implemented to the dietary data collected in the Framingham Offspring Cohort (Feinleib et al., 1975), to examine the content validity of specific nutrients that are reported to be associated with the Mediterranean-style dietary pattern. The study reported a positive association between high MSDPS and nutrients (dietary fiber, omega three fatty acids) known to reduce the risk of CVD and an inverse association of MSDPS with high consumption of detrimental nutrients known to increase the CVD risk (Rumawas et al., 2009).

Overall, the review of the above studies shows that the Mediterranean diet scores is a feasible tool to enhance the understanding of the role of Mediterranean dietary patterns in CVD risk prevention and can be used for both Mediterranean and non-Mediterranean populations (Rumawas et al., 2009; Sotos-Prieto et al., 2015; Stefler et al., 2017).

3.11 Phytosterol as a Contributing Factor for CVD Risk Reduction in the Mediterranean Dietary Pattern

It has been reported that plant-based foods largely contribute to the Mediterranean Diet to reduce the risk of CVD (Salas-Salvado et al., 2018). It is reported that MUFA-to-SFA ratio, dietary fiber, the antioxidant capacity of the whole diet, and some bioactive components such as phytosterols, are vital components that play essential roles in the health effects of the Mediterranean diet (Saura-Calixto & Goni, 2009). For example, existing evidence reported that the Mediterranean dietary pattern is linked with a reduction in serum cholesterol levels (Tosti et al., 2018). Some recent studies suggested that phytosterols present in natural food matrices might
be playing an influential role in CVD risk reduction by the Mediterranean dietary pattern (Gylling & Simonen, 2016). A meta-analysis has also reported that high consumption of phytosterols from nuts, seeds, whole grains, vegetables, and fruits may play a prominent role in serum cholesterol reduction (Abumweis et al., 2008). A large PREDIMED study reported that the Mediterranean diet supplemented with virgin olive oil or nuts showed improved serum lipid profile (decrease LDL-C by 8.3% and LDL/HDL-C ratio by 11.5%) as compared to a low-fat diet (Escurriol et al., 2009). This study concluded that phytosterols might be a part of a significant reduction in the lipid profile by the Mediterranean diet due to its increased consumption. For instance, traditional Mediterranean foods such as olive oil, nuts, and legumes are also rich in phytosterols. Also, phytosterols are highly bioavailable in olive oils (Escurriol et al., 2009; Estruch et al., 2018). In 2011, Athyros and colleagues compared the effects of the Mediterranean diet and plant stanol esters on vascular risk factors and estimated CVD risk. This study reported the comparative effects of both the Mediterranean diet and phytosterols. The results showed that a 2 point increase in the 10-point scale of the Mediterranean diet was associated with favorable changes in TC (P<0.002), LDL-C (P <0.01), TGs (P <0.002), and HDL-C (P <0.01). They observed that a 2-point increase in the 10-point scale is closely linked with a 30% decrease in estimated CVD risk. On the other side, plant stanol esters (2g/d) gradually induced a significant reduction in TC (P <0.002), LDL-C (P <0.002) and led to a 25-30% reduction in estimated CVD risk similar to that achieved by the Mediterranean diet scale mentioned above (Athyros et al., 2011). Considering the favorable effects of phytosterols from natural food matrices or phytosterol supplementation in CVD risk reduction, it can be concluded that dietary phytosterols might be one of the contributing factors responsible for lowering CVD mortality in Mediterranean countries (Jiménez-Escrig et al., 2006; Schwingshackl et al., 2020). Overall, more research is needed to find the role of phytosterols as a bioactive in the Mediterranean diet and their effects on CVD risk reduction in free-living populations.
Chapter 4: Associations Between Dietary and Circulating Phytosterols and Cardiovascular Disease Risk Biomarkers in a Manitoba Adult Cohort

4.1 Rationale

Several factors, including dietary habits, hypertension, high serum lipid concentrations, and arterial stiffness are influencing risk factors for the development of CVD (Silverman et al., 2016). Various dietary strategies are used to reduce the risks of developing CVD (Trautwein et al., 2018a). Phytosterol intake has long been known as a non-pharmaceutical treatment to lower serum TC and LDL-C levels by decreasing intestinal cholesterol absorption (Simonen et al., 2017). Research indicates that there is an inverse association between phytosterols and serum lipid biomarkers (Andersson et al., 2004; Klingberg et al., 2008b; Wang et al., 2012; Li et al., 2018). Besides the cholesterol-lowering efficacy of phytosterols, there is an increasing demand to include assessments of vascular function when evaluating the efficacy of interventions on cardiovascular health. There have been suggestions that the use of phytosterols might improve vascular function, however, most of the studies showed neither beneficial nor detrimental effects of phytosterols on vascular health, including endothelial function, blood pressure and arterial stiffness (Klingberg et al., 2013; Ras et al., 2015a). In the general population, the results of epidemiological studies regarding the associations between dietary phytosterol intake and vascular function are still inconclusive. Given the current state of knowledge, it could be hypothesized that dietary phytosterols may have varying effects on CVD risk biomarkers. It has been reported that cholesterol absorption is closely associated with vascular function. In general, it has been shown that decreasing intestinal cholesterol absorption improves serum cholesterol levels, which may also help to improve vascular function in healthy individuals (Ishibashi et al., 2018). Circulating phytosterol-to-cholesterol ratios are well-known predictors to examine cholesterol absorption (Mackay et al., 2015; Hallikainen et al., 2014). Therefore, this research will examine the impact of dietary and circulating phytosterols on serum lipid profile and arterial stiffness biomarkers, including BP, AIx, and PWV. Moreover, dietary intake pattern is also recognized as one of the influencing factors for CVD risk. Mediterranean dietary patterns are the most studied and well-known dietary patterns associated with CVD risk reduction (Nettleton et al., 2009; Stewart et al., 2016). Adherence to a Mediterranean dietary pattern has been shown to reduce serum lipid levels, blood pressure, arterial stiffness, and oxidative stress along with minimizing the incidence of atherosclerosis and overall
CVD risk (Salas-Salvadó et al., 2018). The beneficial role of phytosterols as bioactive components in the Mediterranean diet has been highlighted in numerous studies (Escurriol et al., 2009; Estruch et al., 2018; Schwingshackl et al., 2020). Therefore, this research will also investigate the food groups in the Mediterranean diet using the MSDPS to find their association with dietary phytosterol intake and CVD risk biomarkers in the general population. Overall, the outputs of this research will be to enhance the understanding regarding the effects of dietary phytosterols from the habitual diet on CVD risk biomarkers.

4.2 Overall Objective

The overall objective of The Manitoba Personalized Lifestyle Research (TMPLR) cross-sectional observational study was to investigate the relationships between diet, nutritional status, lifestyle, and their association with additional risk factors for cardiovascular diseases prevalent in a Manitoban cohort of adults, aged 30-46 years. Using the TMPLR study platform, the primary research objective for this thesis was to investigate the associations between dietary and circulating phytosterols and CVD risk, as assessed using various biomarkers, including serum lipid levels, and arterial stiffness biomarkers, including blood pressure, PWV and AIx. The secondary objective was to evaluate the dietary pattern using Mediterranean-style dietary pattern scoring and investigate its relationship with dietary phytosterols and impact on CVD risk biomarkers.

4.3 Hypotheses

This study hypothesized that:

1) Higher daily dietary phytosterol intake will help to improve serum lipid profile and vascular health, as measured using arterial stiffness biomarkers.

2) High Mediterranean style dietary pattern score (MSDPS) will be correlated with high phytosterol intake and reduction in CVD risk biomarkers.

4.4 Material and Methods

4.4.1 Ethics and Dissemination

The study was approved by the Health Research Ethics Boards at the University of Manitoba and the Saint Boniface Hospital. Explicit informed consent was obtained from each individual before participation in the study. Eligible participants were verbally informed by trained research
personnel regarding the nature and purpose of the study, were given time to decide whether or not to participate, and had any questions or concerns answered before consent and at any point throughout the study. All participants were informed that they may withdraw from the study at any time without penalty and were remunerated for the portion of the study that they have completed up to that point (Mackay et al., 2019).

4.4.2 Study Population

The Manitoba Personalized Lifestyle Research (TMPLR) is a prospective population study that involves 455 Manitobans, consisting of 257 males and 198 females, aged 30-46 years, stratified by BMI, geography (urban and rural), and sex. The study participants were recruited through random sampling from the general population between 2016-2018. Women who were pregnant and lactating were not allowed to participate in the study. This study was focused on recruiting participants who must have been living in Manitoba for a minimum of the last five years (Mackay et al., 2019).

4.4.3 Study Design

On two consecutive days, participants came to either the urban TMPLR study site at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba or TMPLR's mobile research unit, which traveled to other areas of Winnipeg and southern Manitoba. TMPLR's mobile research unit was a custom-built 12m mobile lab that was equipped with a phlebotomy area, a dual-energy X-ray absorptiometer (DXA), and a bicycle ergometer with a metabolic cart. During this visit, participants completed questionnaires, underwent various health assessments, and fasting blood samples were taken. The same protocols were followed at both sites (Mackay et al., 2019). The measurements were taken on two consecutive days. Fasting blood samples were collected, approximately 122 ml total for analysis of numerous established and emerging health biomarkers. Participants were asked to fill out questionnaires such as 24-hour dietary recall and diet history questionnaire through a secure online portal or on paper, within seven days.

4.4.3.1 Linkage to administrative health data

At enrollment, TMPLR participants were asked to provide their personal health information number (PHIN) and grant permission to link their study data with administrative health records (including hospital discharge abstracts, physician billing claims, and prescription records). These
data were accessed through the Manitoba Centre for Health Policy (MCHP) Population Research Data Repository and linkage was achieved using the PHIN, following the standard procedures established by the MCHP and the Manitoba Health Information Privacy Committee. The data linkage was used to capture retrospective information on early life as well as prospective information on numerous health outcomes, including a diagnosis of hypertension or CVD (Mackay et al., 2019).

4.4.4 Anthropometric Measurements

Anthropometric measurements, including height and body weight, were collected on the first day. Weight was measured after participants changed into lightweight scrub tops and bottoms, with shoes removed, to the nearest 0.1kg using a digital calibrated floor scale (7562EF, Taylor Precision Products, Oak Brook, Illinois, USA). Height was measured, without shoes, to the nearest 0.1cm using a wall-mounted stadiometer with a movable headpiece (Model 206, SECA North America, Chino, California, USA). BMI was calculated as weight in kilograms divided by square of height in meters (kg/m²) (Mackay et al., 2019).

4.4.5 Dietary Assessment

4.4.5.1 Dietary Assessment using 24-hour Dietary Recall

In this study, a web-based dietary assessment tool was used to collect 24-hour dietary recall information. The Automated Self-Administered 24-hour (ASA24-hr, NCI, Rockville, Maryland, USA) dietary assessment tool is a web-based tool that enables multiple, automatically coded, self-administered 24-hours recall (Kirkpatrick et al., 2017). Participants enrolled from March 2016 to February 2017 used the ASA24-hr-Canada-2014 edition; those enrolled after February 2017 used the updated version of ASA24-hr-Canada-2016 edition. Both ASA24-hr-Canada-2014 and ASA24-hr-Canada-2016 use the same nutrient databases. The ASA24-hr-Canada-2016 which is available in both English and French, had some new features added, including 1) allowed respondents to complete single or multi-day foods record and 24 hour recalls 2) allowed respondents to report supplement intake data on the same module where the other food and beverages are reported, and 3) images of portion size were also added. Foods and beverages reported in the ASA24-hr are automatically coded and linked to the USDA and Food and Nutrient database for dietary studies (FNDDS) to acquire the nutrient values of the foods reported (Kirkpatrick et al., 2014; Subar et al., 2012). This dietary assessment tool is less expensive for
obtaining detailed dietary data and allows a participant to complete a 24-hour recall without an interviewer. The ASA24-hr system consists of two websites including one for the respondents to record their dietary intake, and another for a researcher to manage the respondents' collected data logistics and to obtain food and nutrient analysis data files. In the recall, respondents were asked to report their eating occasion and time of consumption and also asked to provide the details about the type of food, preparation technique, and portion size to assign a food code to a particular food item. Moreover, ASA24-hour also contains images to assist the respondent in reporting portion size. Data analysis of ASA24-hour provides data on energy, 64 nutrients, and 37 USDA Food Patterns components, including total energy (kcals), daily servings of fruits and vegetables, grains and grain products, legumes, and cooking oils (Suabr et al., 2012). Unfortunately, the 64 nutrient data analysis automatically generated from the ASA24-hr system does not include the estimation of phytosterols intake.

4.4.5.2 Estimation of Phytosterol Intake

The content of phytosterols in food items was analyzed by the chromatography method and obtained primarily from the USDA National Nutrient Database and Canadian Nutrient File (CNF) by Health Canada as Standard References (United States Department of Agriculture, 2017; Health Canada, 2015). The USDA and the CNF databases provide nutrient content information on β-sitosterol, campesterol, stigmasterol, and total phytosterol content in 595 foods. Foods reported by study participants were carefully matched to those in the USDA and the CNF databases. For some foods, we could not find a match in the USDA or the CNF databases. In that case, we extracted phytosterol content information of those foods from individual published articles (Awad et al., 2000; Phillips et al., 2005; Jimnez-Escrig et al., 2006; Kalogeropoulos et al., 2010; Normén et al., 2000; 2002; Phillips et al., 2002; Piironen et al., 2002, Piironen et al., 2003; Ryan et al., 2007).

4.4.5.3 Dietary Pattern Assessment Tool

The Canadian Diet history questionnaire II (C-DHQ II), a freely available online comprehensive food frequency questionnaire (FFQ), was used in the TMPLR study (Subar et al., 2001). The C-DHQ I and C-DHQ II were originally adapted from the American National Cancer Institute's DHQ using Canadian databases to reflect the Canadian diet. Specifically, the C-DHQ II food list was established based on the foods available in the Canadian Market and foods reported in the 24-hour dietary data in the national Canadian Community Health Survey Cycle 2.2, Nutrition (2004)
(Csizmadi et al., 2007; 2016; National Cancer Institute, 2020). Some modifications were made to the original American DHQ II to reflect the Canadian diets better considering Canadian-specific dietary differences and food availability and fortification practices (Csizmadi et al., 2016). The detailed methods for modification and evaluation of the C-DHQ have been published earlier (Csizmadi et al., 2016). The C-DHQ II consists of 165 questions: 153 of them are food questions, ten questions are about supplemental nutrients (vitamins, minerals, and herbal supplements), one question queries the cooking of meat and another one asks about the cooking of a vegetarian diet. The most common foods were grouped into the following categories in the DHQ: fruit beverages, alcoholic beverages, cereals, legumes, fruits, vegetables, milk and milk products, pork, beef, and cooking oils. The respondents were asked to report the frequency of foods consumed over the past 12 months based on one of the nine categories as: never, one time per month or less; 2–3 times/month; 1–2 times/week; 3–4 times/week; 5–6 times/week; once/day; 2–3 times/day; 4–5 times/day; 6 or more times per day. The completed C-DHQ collected from participants were then optically scanned and sent to Alberta Health Services. Alberta Health Services used Diet*Calc software to generate frequency estimates of nutrient and food group intake (National Cancer Institute, 2018). Estimated nutrient intake from the C-DHQ was determined using the Canadian Nutrient File (CNF) modified nutrient database (Csizmadi et al., 2007).

The C-DHQ was used to assess adherence to the Mediterranean dietary pattern. The dietary pattern was assessed based on the reported frequency of food group intake. Some previously published studies reported the beneficial effects of Mediterranean dietary patterns on CVD risk reduction (Martínez-González et al., 2015; Tong et al., 2016; Salas-Salvado et al., 2018). Moreover, most of the food groups in Mediterranean dietary patterns are also considered as good sources of phytosterols. Therefore, to examine the correlation between estimated phytosterol intake from 24-hour dietary recall and adherence to the dietary pattern, the MSDPS system was used (Rumawas et al., 2009).

The MSDPS was developed in 2009, based on the Mediterranean diet pyramid (Ministry of Health and Welfare), to assess an individual's diet compliance with the traditional-style Mediterranean dietary pattern (Rumawas et al., 2009). The MSDPS score was constructed based on 13 components corresponding to the recommended intakes of 13 food groups of the Mediterranean diet pyramid, i.e. whole-grains, fruits, vegetables, dairy, wine, fish, poultry, olives-
legumes, nuts, potatoes, eggs, sweets, meats, and olive oil (Rumawas et al., 2009). Each food group was assigned a score from 0 to 10 depending on the degree of correspondence with the number of servings recommended in the pyramid (e.g., if a subject consumed 60% of the recommended amount for that food group, the score would be 6). In case of exceeding the recommendations, a penalty was assigned by subtracting a score proportionally to the number of servings consumed that exceeded the recommended intake (e.g., consumed 60% more than the recommended serving would result in a score of 4). The score defaulted to zero when the total or component of the MSDPS was negative due to the overconsumption penalty. Olive oil was assigned a score of 10 based on its exclusive use, 5 if the olive oil was used along with other vegetable oils, and 0 for no use of olive oil. The total MSDPS was calculated using the equation (Formula 1) below, that is, the value was obtained by summing up the scores of all 13 components and dividing the calculated sum by a theoretical maximum of 120 and multiplying by 100, and calculated values were standardized to 0-100 scale (Rumawas et al., 2009). This score was developed for a non-Mediterranean population, but an individual's diet might include some of the foods which are not part of the Mediterranean diet, i.e., a mixture of the Mediterranean and non-Mediterranean foods. Therefore, to account for the non-Mediterranean foods, the standardized sum of the 13 components was weighted using a continuous factor ranging from 0 to 1, based on the proportion of energy intake obtained from foods that were not a part of the MSDPS (e.g., consumed 35% of energy from food not included in the pyramid, would result in a calculated weighting factor of 0.65) (Rumawas et al., 2009).

4.4.6 Serum Lipid Profile

On day 1 and day 2 of the study, fasting blood samples were collected. Serum was separated from whole blood samples within an hour after collecting the samples by centrifugation method at 3000 rpm for 20 minutes and stored immediately at -80° C until further analysis. Circulating serum TC, LDL-C, HDL-C, and TG were analyzed by using the Cobas c111 autoanalyzer system (Cobas C111, Roche Diagnostics Laval, Quebec).

4.4.7 Plasma Phytosterol Analysis

Plasma phytosterol concentrations, including sitosterol, campesterol, stigmasterol, avenasterol, brassicasterol, sitostanol, and campestanol concentrations were quantified using a gas chromatography flame ionization detector (GC-FID) method that was previously reported
In summary, an internal standard, 5-α-cholestane, was added to 300 µL of plasma samples and then alkaline hydrolysis was performed using 4 mL methanolic potassium hydroxide for 1 hour at 100° C temperature. Sterols were extracted twice from the mixture using 4 mL petroleum ether and then dried under nitrogen. The dried sterols were resuspended in 1000 µL of hexane and dried under nitrogen again at a temperature of 75° C. The dried mixture was again resuspended in 400 µL of heptane and 100 µL trimethylsilyl (TMS) derivatizing agent and the samples incubated overnight at 75° C. After derivatization, the samples were run on a GC-FID for sterol separation and detection (Mackay et al., 2014). The sterols were separated on a nonpolar phenyl arylene polymer DB5MS 30M column (Agilent Technologies, USA), have slightly polar stationary phases such as dimethylpolysiloxane or methylphenyl-siloxane in a Bruker 450 gas chromatography (GC-FID, Bruker-450; Agilent Technologies, USA) after splitless injection at 280°C. Hydrogen was used as a carrier gas with a total gas flow rate of 1 mL/min. The oven temperature was first kept at 200°C for 1 min, followed by an increased rate of 5°C/min to a temperature of 270°C for 1 min, and then raised by 20°C to a final temperature of 310°C for 10 min. The retention times and peaks of all standards were detected using authentic standards such as 5α-cholestane, sitosterol, campesterol, stigmasterol, sitostanol, campestanol, cholesterol, lathosterol, desmosterol, and cholestanol (Sigma-Aldrich Canada Ltd). The internal standard, 5α-cholestane (100µg), was used to calculate the concentrations of other sterols. The area amount of each individual standard was divided by the area of 5α-cholestane and multiplied by the amount of 5α-cholestane used in the sample.

4.4.8 Non-Invasive Assessment of Arterial Stiffness Biomarkers

Arterial stiffness biomarkers, including arterial blood pressure, PWV, and AIx at heart rate 75 (AIx@75) were measured using a validated Mobil-O-Graph device (Sarafidis et al., 2014; Wassertheurer et al., 2010; Wei et al., 2010; Franssen & Imholz, 2010). Mobil-O-Graph (IEM GmbH, Stolberg, Germany) is a cuff-based electronic oscillometric ambulatory blood pressure monitor device that allows for pulse wave analysis (PWA), including PWV and AIx. PWV provides information about wall resistance and arterial elasticity and constitutes the "gold standard" for arterial stiffness (Blacher et al., 1999; Ben-Shlomo et al., 2014). The Mobil-O-Graph is a non-invasive device and only requires participants to wear a cuff that is inflated, similar to a sphygmomanometer. This device performs a brachial oscillometric BP measurement and records
the pulse waves immediately at the level of the brachial artery. The measurement was taken on the left arm. For the blood pressure measurement, the cuff was inflated to maximum systolic pressure, then slowly deflated to detect and capture the subjects' blood pressure. After the first reading, a 30sec pause occurred, and second inflation and deflation occurred. For the pulse wave analysis, once the minimum mmHg was acquired in the second deflation, the cuff remained inflated at brachial diastolic pressure for 10 sec. Then, the cuff began to re-inflate slightly (~15-25 mmHg) until a sufficient pulse pressure was detected. Once a pulse pressure was detected, the cuff inflated more, and the PWA was performed (5-15 seconds).

For calibration of the brachial pulse waveforms, the system uses the oscillometric brachial systolic and diastolic BPs. The aortic pulse waveform is generated by applying a transfer function using the ARC Solver algorithm that is implemented in the Mobil-O-Graph monitor. With the Mobil-O-Graph software (HMS), wave separation analysis is performed by decomposing the aortic pulse waveform into forward and reflected pulse waves using uncalibrated triangular aortic flow waveform. The system software calculates central pressures and AIx@75 and provides an indirect estimate of PWV through mathematical models.

4.4.9 Statistical Analysis

The sample size for TMPLR study was selected based on the consideration of the feasibility of recruitment, costs, and logistics. However, a power analysis was performed using the established values from other sources and estimated that our study will have an 80% power at a 5% significance level (two-sided) to detect a minimum body fat difference of 2.5% for rare exposures (ie, experienced by 10% of participants, such as smoking) and 1.7% for more common exposures (experienced by 25% of participants, such meeting the Canadian recommended 150 min of moderate-to-vigorous physical activity). However, due to various reasons, the sample size was diminished, which could reduce the statistical power of the remaining data (Mackay et al., 2019).

Further, statistical analyses were performed using the statistical package IBM SPSS (v.26). For the prospective data analysis, the following exclusion criteria were applied: missing (n=54) and incomplete (n=80) ASA 24-hour dietary recall data, have extremely low or high reported energy intakes (n=31), phytosterols intake after extrusion (n=5). After the exclusion of subjects with incomplete information and missing data on DHQ (n=84). Out of 455 total participants, phytosterol intake analyses included 277 individuals. Dietary phytosterol intake was the primary
focus of this study report, and all regression analyses of CVD risk biomarkers such as serum lipid levels and arterial stiffness were thoroughly dependent on the dietary phytosterol intake. Therefore, the participants with missing or incomplete dietary information were excluded from further statistical analysis.

Sex-specified analyses were performed in the present study. We were able to see significant sex differences, only when linear regression analysis was performed on dietary factors across the quartiles of phytosterol intake. However, no significant differences were seen when regression analyses were performed to examine the association between dietary phytosterol intake and CVD risk biomarkers across quartiles of phytosterol intake, which was a primary objective of the present study. Therefore, we decided to combine both sexes throughout all the analyses performed further. Simple plotting (box plots) was performed to check the approximate normality. A logarithmic transformation was applied to the non-normal variables before analysis. The data were reported as mean ± SD and covariate-adjusted mean ± SE. The estimated phytosterols intake of individual subject per recall day was calculated by using the following formula:

Formula 1:

Estimated Phytosterol Intake = Σ Cn × Pn

where C refers to the reported grams of food n consumed and P refers to the milligrams of phytosterol content per 100 grams of food n.

After calculating phytosterol intake from three ASA24-hr recalls, an average of the three intakes was calculated to obtain a mean daily intake estimate. Phytosterol intake was distributed in quartiles using data for men and women combined. For descriptive statistics, means and SDs (or frequencies and percentages) for demographic characteristics and dietary factors were calculated across quartiles of dietary phytosterols and compared using post hoc one-way analysis of variance. We carried out a test for trend for continuous variables by fitting the quartile group number (1–4) as a variable in linear regression. Differences in dietary factors and cardiovascular risk factors such as serum lipids and arterial stiffness biomarkers by quartiles of dietary phytosterol intake were determined using post hoc with ANOVA. A multiple linear regression model was used to analyze associations between dietary phytosterol intake and serum lipids, arterial stiffness biomarkers, plasma phytosterols, and MSDPS in combined sex. Serum lipids and arterial stiffness
Biomarkers were adjusted for various confounders such as age, BMI, total energy intake, saturated fat (percentage of energy intake), monosaturated fat (percentage of energy intake), polyunsaturated fat (percentage of energy intake), dietary fiber, and heart rate. Bivariate correlation (without variants adjusted) and partial correlation analysis (adjusted for energy intake) was also performed to assess the correlation of dietary phytosterols with serum lipids, arterial stiffness biomarkers, and plasma phytosterols and the MSDPS with dietary phytosterols, serum lipids, and arterial stiffness biomarkers. Descriptive frequencies were performed to determine the intake distributions within each food group and component score. Statistical significance was set at P<0.05 for all analyses.

4.5 Results

4.5.1 Demographic Characteristics and Dietary Intakes

The general demographic characteristics of the study population are shown in Table 4.1, which shows that of the 277 study participants, 43.3% were women and 56.7% were men. Sex-specific analyses were performed to determine if there were any significant differences in outcomes parameters related to sex throughout all the analyses. We found significant sex differences only when linear regression analysis was performed on dietary factors across the quartile of phytosterol intake (Table 4.3). Conversely, no sex differences were found when we examined the association between dietary phytosterol intake and CVD risk biomarkers across quartiles of phytosterol intake. Therefore, both sexes were combined throughout all the further analyses performed.

Participants with the highest phytosterol intake (4th quartile) had the lowest BMI (P<0.05) (Table 4.2). Mean intakes of sitosterol, campesterol, stigmasterol, and total phytosterols, are shown as 105.0 ± 77.1 mg/d, 28.8 ± 21.3 mg/d, 13.0 ± 13.4 mg/d, and 226.8 ± 125.1 mg/d, respectively, for combined sexes. Men had a mean phytosterol intake of 235.1 ± 130.2 mg/d whereas women had 215.6 ± 118.3 mg/d. Increasing intake of dietary phytosterols through the quartiles was associated with a higher intake of total energy, fats including total fat, saturated fat, monounsaturated fat, polyunsaturated fat, and dietary fiber (Table 4.2).

Table 4.1 Demographic Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>277</td>
<td>157</td>
<td>120</td>
</tr>
<tr>
<td>Age (y)</td>
<td>37.8 ± 4.7</td>
<td>37.1 ± 4.7</td>
<td>38.6 ± 4.5</td>
</tr>
</tbody>
</table>
**BMI (kg/m²)** | 27.0 ± 6.3 | 26.9 ± 5.5 | 27.2 ± 7.2  
---|---|---|---
Underweight (<18.5) | 3 | 2 | 1  
Normal weight (18.5-24.9) | 103 | 47 | 56  
Overweight (25.0-29.9) | 106 | 74 | 32  
Obese (>30) | 65 | 34 | 31  

**Geography within Manitoba**

<table>
<thead>
<tr>
<th></th>
<th>Urban</th>
<th>Rural</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight (&lt;18.5)</td>
<td>188</td>
<td>89</td>
</tr>
<tr>
<td>Normal weight (18.5-24.9)</td>
<td>119</td>
<td>38</td>
</tr>
<tr>
<td>Overweight (25.0-29.9)</td>
<td>69</td>
<td>51</td>
</tr>
</tbody>
</table>

BMI: body mass index; N: number of participants. Age and BMI data as mean ± S.D
Table 4.2 Distribution of Characteristics and Dietary Components of the Overall Study Population by Quartiles of Total Dietary Phytosterol Intake

<table>
<thead>
<tr>
<th></th>
<th>Overall Mean</th>
<th>Q1 20.2-&lt;143 mg/d</th>
<th>Q2 143-&lt;206 mg/d</th>
<th>Q3 206-&lt;296 mg/d</th>
<th>Q4 296-810 mg/d</th>
<th>P¹ ANOVA</th>
<th>P² Linear trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>N³</td>
<td>277</td>
<td>69</td>
<td>69</td>
<td>69</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>37.8 (4.7)</td>
<td>38.1 (4.7)</td>
<td>37.6 (4.5)</td>
<td>37.8 (4.3)</td>
<td>37.7 (5.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0 (6.3)</td>
<td>27.2 (5.8)</td>
<td>28.9 (6.0)</td>
<td>26.4 (6.9)</td>
<td>25.7 (6.1)*</td>
<td>0.021</td>
<td>0.042</td>
</tr>
<tr>
<td>Total phytosterols (mg/d)</td>
<td>226.8 (125.1)</td>
<td>97.7 (36.5)</td>
<td>165.8 (17.1)*</td>
<td>248.1 (25.4)*</td>
<td>392.7 (108.8)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Sitosterol (mg/d)</td>
<td>105.0 (77.1)</td>
<td>47.4 (29.7)</td>
<td>74.5 (27.3)*</td>
<td>114.6 (46.9)*</td>
<td>186.2 (94.7)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Campesterol (mg/d)</td>
<td>28.8 (21.3)</td>
<td>15.7 (29.7)</td>
<td>21.4 (9.2)</td>
<td>31.9 (14.0)*</td>
<td>45.9 (29.0)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Stigmasterol (mg/d)</td>
<td>13.0 (13.4)</td>
<td>4.2 (5.0)</td>
<td>10.5 (7.8)*</td>
<td>14.2 (10.4)*</td>
<td>22.8 (18.7)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Energy (Kcal/d)</td>
<td>2351.8 (934.5)</td>
<td>1914.1 (766.6)</td>
<td>2221.5 (913.8)</td>
<td>2447.2 (988.9)</td>
<td>2817.8 (830.4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>218.8 (120.9)</td>
<td>213.6 (166.3)</td>
<td>214.4 (122.0)</td>
<td>212.1 (87.7)</td>
<td>234.9 (92.6)</td>
<td>0.605</td>
<td>0.341</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>79.9 (41.4)</td>
<td>78.4 (45.9)</td>
<td>76.9 (46.7)</td>
<td>78.5 (35.3)</td>
<td>85.6 (36.7)</td>
<td>0.645</td>
<td>0.296</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>99.5 (49.1)</td>
<td>78.3 (41.9)</td>
<td>89.6 (37.1)</td>
<td>102.7 (52.2)*</td>
<td>127.1 (50.3)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Total fat (% energy intake)</td>
<td>37.7 (8.5)</td>
<td>35.6 (7.9)</td>
<td>36.9 (9.3)</td>
<td>37.7 (6.9)</td>
<td>40.5 (9.2)*</td>
<td>0.006</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Saturated fatty acids (g/d)</td>
<td>31.6 (16.7)</td>
<td>27.6 (17.9)</td>
<td>30.4 (15.0)</td>
<td>31.3 (17.2)</td>
<td>36.9 (15.7)*</td>
<td>0.01</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Saturated fatty acids (% of energy intake)</td>
<td>11.9 (3.7)</td>
<td>12.2 (3.9)</td>
<td>12.5 (4.8)</td>
<td>11.3 (2.6)</td>
<td>11.7 (3.2)</td>
<td>0.256</td>
<td>0.822</td>
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<tr>
<td>Monounsaturated fat (g/d)</td>
<td>37.2 (20.5)</td>
<td>28.3 (15.1)</td>
<td>32.4 (13.6)</td>
<td>39.1 (23.2)*</td>
<td>49.1 (22.1)*</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Monounsaturated fat (% of energy intake)</td>
<td>Q1 20.2-&lt;143 mg/d</td>
<td>Q2 143-&lt;206 mg/d</td>
<td>Q3 206-&lt;296 mg/d</td>
<td>Q4 296-810 mg/d</td>
<td>P1 ANOVA</td>
<td>P2 Linear trend</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
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<tr>
<td>N</td>
<td>37</td>
<td>25</td>
<td>32</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>39.9 (4.3)</td>
<td>37.6 (4.1)</td>
<td>38.3 (4.5)</td>
<td>38.1 (4.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.1 (5.4)</td>
<td>30.2 (8.1)</td>
<td>24.8 (7.7)</td>
<td>27.3 (7.2)</td>
<td>0.047*</td>
<td>0.462</td>
<td></td>
</tr>
<tr>
<td>Total phytosterols (mg/d)</td>
<td>98.1 (40.7)</td>
<td>168.3 (14.1)</td>
<td>249.3 (29.1)</td>
<td>386.9 (91.32)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001†††</td>
<td></td>
</tr>
</tbody>
</table>

BMI: body mass index;
N³: number of participants
All data values reported as Mean (S.D.)

*P < 0.05; ** P < 0.01 for difference in the quartiles compared to the 1st quartile by analysis of variance;
† P < 0.001 value for linear trend by linear regression analysis

Table 4.3 Distribution of Characteristics and Dietary Intakes of women and men Separately by Quartiles of Total Dietary Phytosterol Intake
<p>| | | | | | | |</p>
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<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitosterol (mg/d)</td>
<td>45.0 (29.8)</td>
<td>67.0 (36.4)</td>
<td>117.5 (59.6)</td>
<td>176.0 (100.3)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Campesterol (mg/d)</td>
<td>13.7 (10.6)</td>
<td>18.5 (9.7)</td>
<td>28.3 (14.7)</td>
<td>45.3 (31.1)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Stigmasterol (mg/d)</td>
<td>4.4 (5.4)</td>
<td>7.9 (7.5)</td>
<td>17.4 (13.8)</td>
<td>20.9 (12.5)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Energy (Kcal/d)</td>
<td>1719.9 (693.3)</td>
<td>1885.0 (567.0)</td>
<td>2153.8 (578.0)</td>
<td>2517.6 (805.7)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>182.8 (91.5)</td>
<td>189.4 (120.6)</td>
<td>166.5 (53.7)</td>
<td>210.8 (85.2)</td>
<td>0.311</td>
<td>0.479</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>74.0 (40.9)</td>
<td>63.4 (32.2)</td>
<td>65.4 (24.4)</td>
<td>77.3 (37.2)</td>
<td>0.370</td>
<td>0.865</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>72.7 (34.5)</td>
<td>80.8 (32.2)</td>
<td>90.9 (24.9)</td>
<td>109.5 (33.8)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Total fat (% energy intake)</td>
<td>37.1 (7.2)</td>
<td>38.5 (9.8)</td>
<td>38.7 (7.6)</td>
<td>39.9 (7.7)</td>
<td>0.581</td>
<td>0.173</td>
</tr>
<tr>
<td>Saturated fatty acids (g/d)</td>
<td>25.1 (15.3)</td>
<td>29.2 (16.4)</td>
<td>26.5 (9.8)</td>
<td>31.1 (10.7)</td>
<td>0.294</td>
<td>0.148</td>
</tr>
<tr>
<td>Saturated fatty acids (% of energy intake)</td>
<td>12.2 (3.7)</td>
<td>13.7 (6.3)</td>
<td>11.1 (2.5)</td>
<td>11.4 (2.8)</td>
<td>0.070</td>
<td>0.162</td>
</tr>
<tr>
<td>Monounsaturated fat (g/d)</td>
<td>26.6 (13.2)</td>
<td>28.7 (13.3)</td>
<td>34.2 (9.8)</td>
<td>40.7 (12.0)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Monounsaturated fat (% of energy intake)</td>
<td>13.6 (3.5)</td>
<td>13.5 (3.5)</td>
<td>14.8 (4.5)</td>
<td>15.0 (3.3)</td>
<td>0.354</td>
<td>0.097</td>
</tr>
<tr>
<td>Polyunsaturated fat (g/d)</td>
<td>14.1 (5.6)</td>
<td>15.5 (5.3)</td>
<td>22.2 (7.2)</td>
<td>28.0 (12.5)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Poly unsaturated fat (% of energy intake)</td>
<td>7.7 (2.2)</td>
<td>7.6 (2.5)</td>
<td>9.4 (2.4)</td>
<td>10.0 (3.6)</td>
<td>0.001**</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Fiber (g/d)</td>
<td>13.7 (5.3)</td>
<td>16.7 (4.4)</td>
<td>22.7 (6.3)</td>
<td>26.4 (10.0)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>328.2 (175.8)</td>
<td>289.4 (167.3)</td>
<td>306.8 (154.6)</td>
<td>391.0 (252.2)</td>
<td>0.231</td>
<td>0.273</td>
</tr>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>32.0</td>
<td>44.0</td>
<td>37.0</td>
<td>44.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>35.9 (4.3)</td>
<td>37.5 (4.7)</td>
<td>37.4 (4.2)</td>
<td>37.4 (5.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3 (6.3)</td>
<td>28.1 (4.3)</td>
<td>27.7 (6.0)</td>
<td>24.8 (5.3)</td>
<td>0.024*</td>
<td>0.026†</td>
</tr>
<tr>
<td>Nutrient</td>
<td>Quartile 1</td>
<td>Quartile 2</td>
<td>Quartile 3</td>
<td>Quartile 4</td>
<td>P-value</td>
<td>P-value</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Total phytosterols (mg/d)</td>
<td>97.2 (31.7)</td>
<td>164.4 (18.7)</td>
<td>247.1 (22.1)</td>
<td>396.1 (118.8)</td>
<td>0.000***</td>
<td>0.000***</td>
</tr>
<tr>
<td>Sitosterol (mg/d)</td>
<td>50.2 (29.9)</td>
<td>78.8 (19.6)</td>
<td>112.1 (33.0)</td>
<td>192.3 (92.0)</td>
<td>0.000***</td>
<td>0.000***</td>
</tr>
<tr>
<td>Campesterol (mg/d)</td>
<td>17.9 (15.1)</td>
<td>23.0 (8.5)</td>
<td>35.1 (12.8)</td>
<td>46.4 (28.0)</td>
<td>0.000***</td>
<td>0.000***</td>
</tr>
<tr>
<td>Stigmasterol (mg/d)</td>
<td>4.0 (4.6)</td>
<td>12.1 (7.6)</td>
<td>11.3 (5.3)</td>
<td>23.9 (21.1)</td>
<td>0.000***</td>
<td>0.000***</td>
</tr>
<tr>
<td>Energy (Kcal/d)</td>
<td>2138.6 (796.0)</td>
<td>2412.8 (1018.8)</td>
<td>2701.0 (1190.3)</td>
<td>2995.2 (801.5)</td>
<td>0.000**</td>
<td>0.000***</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>249.3 (220.3)</td>
<td>228.6 (121.9)</td>
<td>251.5 (92.8)</td>
<td>249.2 (94.8)</td>
<td>0.853</td>
<td>0.763</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>83.5 (51.3)</td>
<td>84.6 (52.0)</td>
<td>89.8 (39.5)</td>
<td>90.5 (36.0)</td>
<td>0.866</td>
<td>0.417</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>84.8 (48.9)</td>
<td>94.6 (39.1)</td>
<td>113.0 (66.2)</td>
<td>137.4 (55.6)</td>
<td>0.000***</td>
<td>0.000***</td>
</tr>
<tr>
<td>Total fat (% energy intake)</td>
<td>34.0 (8.4)</td>
<td>36.1 (9.0)</td>
<td>36.9 (6.2)</td>
<td>40.9 (10.1)</td>
<td>0.005**</td>
<td>0.001††</td>
</tr>
<tr>
<td>Saturated fatty acids (g/d)</td>
<td>30.7 (20.3)</td>
<td>31.0 (14.3)</td>
<td>35.5 (20.9)</td>
<td>40.3 (17.3)</td>
<td>0.056</td>
<td>0.008††</td>
</tr>
<tr>
<td>Saturated fatty acids (% of energy intake)</td>
<td>12.1 (4.1)</td>
<td>11.7 (3.6)</td>
<td>11.4 (2.6)</td>
<td>12.0 (3.4)</td>
<td>0.829</td>
<td>0.87</td>
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<tr>
<td>Monounsaturated fat (g/d)</td>
<td>30.3 (17.1)</td>
<td>34.5 (13.5)</td>
<td>43.3 (29.9)</td>
<td>54.0 (25.1)</td>
<td>0.001***</td>
<td>0.001***</td>
</tr>
<tr>
<td>Monounsaturated fat (% of energy intake)</td>
<td>12.3 (3.3)</td>
<td>13.2 (3.4)</td>
<td>14.0 (3.0)</td>
<td>16.0 (5.2)</td>
<td>0.001***</td>
<td>0.001***</td>
</tr>
<tr>
<td>Polyunsaturated fat (g/d)</td>
<td>16.2 (9.7)</td>
<td>21.1 (11.5)</td>
<td>24.8 (15.0)</td>
<td>31.2 (15.2)</td>
<td>0.001***</td>
<td>0.001***</td>
</tr>
<tr>
<td>Polyunsaturated fat (% of energy intake)</td>
<td>6.5 (2.3)</td>
<td>8.0 (3.0)</td>
<td>8.2 (2.4)</td>
<td>9.3 (3.2)</td>
<td>0.001**</td>
<td>0.001***</td>
</tr>
<tr>
<td>Fiber (g/d)</td>
<td>13.4 (6.2)</td>
<td>22.7 (30.9)</td>
<td>23.9 (7.2)</td>
<td>33.5 (11.6)</td>
<td>0.001***</td>
<td>0.001***</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>398.2 (297.1)</td>
<td>392.6 (243.3)</td>
<td>460.9 (372.6)</td>
<td>447.1 (250.1)</td>
<td>0.653</td>
<td>0.304</td>
</tr>
</tbody>
</table>

N*: number of participants; BMI: body mass index;

All data values reported as Mean (S.D.)

* P < 0.05; ** P < 0.01 for difference in the quartiles compared to the 1st quartile by analysis of variance;
† P < 0.05; †† P < 0.01; ††† P < 0.001 value for linear trend by linear regression analysis.
4.5.2 Phytosterol Intake and Serum Lipid Levels

The serum lipid levels distributed by quartiles of phytosterols intake are shown in Table 4.4. There was a negative linear trend (P<0.01) found between dietary phytosterol intake and serum TC, LDL-C, and TG concentrations using linear regression. Cofactors were adjusted in two different models using multiple linear regression, wherein model 1 was adjusted for BMI, and model 2 was adjusted for BMI, total energy intake, saturated fat as a percentage of energy intake, monounsaturated fat as a percentage of energy intake, polyunsaturated fat as a percentage of energy intake, and dietary fiber. Results in Table 4.4 show the regression coefficients, which represent the change in lipid concentrations to one mg increase in dietary phytosterols consumption. We ran two regression models; one adjusting for BMI only, and another one adjusting for BMI, total energy intake, saturated fat as a percentage of energy intake, monounsaturated fat as a percentage of energy intake, polyunsaturated fat as a percentage of energy intake, dietary fiber. After adjusting for BMI, participants with highest phytosterol intake (392.7±108.8 mg/d) showed 0.52 mmol/L (10.16%) lower serum TC concentration (P<0.01), 0.44 mmol/L (13.5%) lower LDL-C (P<0.01), and 0.26 mmol/L (20.6%) lower TG (P<0.01) than the participants with lowest phytosterol intake (97.7±36.5 mg/d). Further adjustments for the intake of total fat, saturated fat, monounsaturated fat, polyunsaturated fat, and dietary fiber, showed 10.2% and 14.4% decreased concentrations of serum TC (P<0.05) and LDL-C (P<0.05) among participants in the highest quartile of phytosterol intake than the lowest quartile. In the second adjusted model, the association between phytosterol intake and TG was no more statistically significant. Significant differences were found for serum TC (P < 0.01), LDL-C (P<0.05), and TG (P<0.05) concentrations across the quartiles of phytosterol intake. No significant difference or change was observed in serum HDL concentration in both adjusted models (P>0.05) (Table 4.4).
Table 4.4 Distribution of Means and Covariate-Adjusted Means of Serum Lipid Concentrations and Multiple Linear Regression of Serum Lipid Concentrations Based on the Quartiles of Dietary Phytosterol Intake

<table>
<thead>
<tr>
<th></th>
<th>β coefficient* ± S.E.</th>
<th>Q1 20.2-&lt;143 mg/d</th>
<th>Q2 143-&lt;206 mg/d</th>
<th>Q3 206-&lt;296 mg/d</th>
<th>Q4 296-810 mg/d</th>
<th>P² for linear trend</th>
<th>% Difference</th>
<th>P³ for ANOVA³, ANCOVA⁴ difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>68</td>
<td>67</td>
<td>69</td>
<td>70</td>
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</tr>
<tr>
<td>TC (mmol/L)</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-0.174±0.050</td>
<td>5.13(0.97)c</td>
<td>4.96(0.98)</td>
<td>4.82 (0.89)</td>
<td>4.59 (0.88)</td>
<td>0.001**</td>
<td>-10.5</td>
<td>0.007†</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>-0.164±0.050</td>
<td>5.13±0.11d</td>
<td>4.94±0.11</td>
<td>4.85±0.11</td>
<td>4.60±0.11</td>
<td>0.001**</td>
<td>-10.2</td>
<td>0.014†</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>-0.141±0.057</td>
<td>5.12±0.12</td>
<td>4.94±0.11</td>
<td>4.85±0.11</td>
<td>4.60±0.12</td>
<td>0.010*</td>
<td>-10.2</td>
<td>0.04†</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-0.152±0.046</td>
<td>3.25(0.88)</td>
<td>3.17 (0.92)</td>
<td>3.03 (0.79)</td>
<td>2.79 (0.80)</td>
<td>0.001**</td>
<td>-14.2</td>
<td>0.010†</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>-0.140±0.046</td>
<td>3.25±0.10</td>
<td>3.14±0.10</td>
<td>3.10±0.10</td>
<td>2.81±0.10</td>
<td>0.002**</td>
<td>-13.5</td>
<td>0.020†</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>-0.126±0.052</td>
<td>3.26±0.11</td>
<td>3.13±0.10</td>
<td>3.06±0.10</td>
<td>2.79±0.11</td>
<td>0.016*</td>
<td>-14.4</td>
<td>0.034†</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>0.047±0.024</td>
<td>1.44 (0.44)</td>
<td>1.39 (0.45)</td>
<td>1.5 (0.46)</td>
<td>1.56 (0.44)</td>
<td>0.055</td>
<td>8.3</td>
<td>0.135</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>0.290±0.023</td>
<td>1.45±0.06</td>
<td>1.43±0.05</td>
<td>1.49±0.05</td>
<td>1.53±0.05</td>
<td>0.201</td>
<td>5.5</td>
<td>0.543</td>
</tr>
<tr>
<td>Adjusted²</td>
<td>0.012±0.026</td>
<td>1.46±0.05</td>
<td>1.44±0.05</td>
<td>1.48±0.05</td>
<td>1.51±0.05</td>
<td>0.658</td>
<td>1.0</td>
<td>0.555</td>
</tr>
<tr>
<td>Log TG* (mmol/L)</td>
<td>Unadjusted</td>
<td>Adjusteda</td>
<td>Adjustedb</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>-0.043±0.013</td>
<td>1.34 (0.79)</td>
<td>1.33±0.08</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1.26 (0.70)</td>
<td>1.19±0.08</td>
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<td></td>
<td></td>
<td>1.06 (0.71)</td>
<td>1.09±0.08</td>
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<td></td>
<td></td>
<td>1.02 (0.57)</td>
<td>1.07±0.08</td>
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<td></td>
<td></td>
<td>0.001**</td>
<td>0.002**</td>
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<tr>
<td></td>
<td></td>
<td>-23.8</td>
<td>-20.6</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.018†</td>
<td>0.074</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adjustedb</td>
<td>-0.012±0.013</td>
<td>1.28±0.08</td>
<td>1.20±0.08</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1.09±0.08</td>
<td>1.11±0.08</td>
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<tr>
<td></td>
<td></td>
<td>0.369</td>
<td>0.369</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>-13.3</td>
<td>0.396</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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

1 β coefficient represents a change in serum lipid concentrations by an increase in dietary phytosterols intake
2 P values for linear trend calculated by using the significance of coefficient for quartile numbers (1–4) fitted as a variable in linear regression; * P < 0.05; ** P < 0.01
3 P values for differences in serum lipid levels across the quartiles of dietary phytosterols intake
4 P values for differences in serum lipid levels across the quartiles of dietary phytosterols intake after adjusting for cofactors
† P < 0.05; †† P<0.01
a Model adjusted for BMI
b Model adjusted for BMI, total energy intake, saturated fat as a percentage of energy intake, monounsaturated fat as a percentage of energy intake, polyunsaturated fat as a percentage of energy intake, dietary fiber
c Values log-transformed prior to analysis

Values reported as actual Mean (SD) in adjusted a
Values reported as estimated marginal mean ± SE adjusted b
4.5.3 Phytosterol Intake and Arterial Stiffness Biomarkers

Higher intake of dietary phytosterol was found to be inversely associated with cSys, cDia, and AIx (P<0.05). Results in Table 4.5 show the regression coefficients, which represent the change in arterial stiffness biomarkers to one mg increase in dietary phytosterol consumption. The results indicate that increasing phytosterol intake was associated with reduced cSys and AIx (P<0.05) after adjusting for age, and lower cSys and PWV (P<0.05) after adjusting for age, BMI, and total energy intake. The age-adjusted mean values of cSys blood pressure (BP), cDia BP, and AIx were significantly decreased by 4.4% (P<0.05), 4.1% (P<0.05), and 38% (P<0.05), respectively. In the highest quartile of phytosterol intake, adjustments for age, BMI, and total energy intake decreased mean values of cSys BP, cDia BP, and PWV by 5.3% (P<0.01), 4.5% (P<0.05), and 3.4% (P<0.05), respectively, as compared to the lowest quartile of phytosterol intake. The fully adjusted model shows 1.61 mmHg, 1.27 mmHg, and 0.046 m/s decrease in cSys BP (P<0.01), cDia BP (P<0.01), and PWV (P<0.05) after every unit increase in total phytosterol consumption (Table 4.5).
Table 4.5 Distribution of Means and Covariate-Adjusted Means and Multiple Linear Regression of Arterial Stiffness Biomarkers Based on the Quartiles Of Dietary Phytosterol Intake

<table>
<thead>
<tr>
<th></th>
<th>β coefficient¹ ± S.E.</th>
<th>Q1 20.2-&lt;143 mg/d</th>
<th>Q2 143-&lt;206 mg/d</th>
<th>Q3 206-&lt;296 mg/d</th>
<th>Q4 296-810 mg/d</th>
<th>P² for linear trend</th>
<th>% Difference</th>
<th>P¹ for ANOVA³, ANCOVA⁴ difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>66</td>
<td>68</td>
<td>69</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cSys BP (mmHg)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Unadjusted</td>
<td>-1.618±0.660</td>
<td>111.3 (12.1)</td>
<td>110.1 (15.5)</td>
<td>108.0 (12.0)</td>
<td>106.7 (12.9)</td>
<td>0.014*</td>
<td>-4.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Adjustedª</td>
<td>-1.609±0.660</td>
<td>111.32±1.63</td>
<td>110.15±1.60</td>
<td>107.98±1.59</td>
<td>106.75±1.59</td>
<td>0.021*</td>
<td>-4.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Adjustedᵇ</td>
<td>-2.157±0.633</td>
<td>112.47±1.50</td>
<td>109.19±1.45</td>
<td>108.03±1.42</td>
<td>106.54±1.50</td>
<td>0.001**</td>
<td>-5.3</td>
<td>0.03†</td>
</tr>
<tr>
<td>cDia BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-1.292±0.510</td>
<td>78.4 (9.9)</td>
<td>78.3 (10.5)</td>
<td>75.6 (8.5)</td>
<td>74.9 (9.1)</td>
<td>0.013*</td>
<td>-4.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Adjustedª</td>
<td>-1.271±0.507</td>
<td>78.31±1.14</td>
<td>78.12±1.12</td>
<td>75.53±1.11</td>
<td>74.94±1.11</td>
<td>0.010*</td>
<td>-4.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Adjustedᵇ</td>
<td>-1.529±0.536</td>
<td>78.59±1.13</td>
<td>77.70±1.10</td>
<td>75.48±1.08</td>
<td>75.14±1.13</td>
<td>0.005**</td>
<td>-4.5</td>
<td>0.11</td>
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<tr>
<td>AIx</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-1.357±0.551</td>
<td>10.8 (9.7)</td>
<td>11.9 (10.1)</td>
<td>10.9 (9.7)</td>
<td>6.6 (8.9)</td>
<td>0.011*</td>
<td>-38.8</td>
<td>0.01†</td>
</tr>
<tr>
<td>Adjustedª</td>
<td>-1.349±0.551</td>
<td>10.77±1.24</td>
<td>11.87±1.22</td>
<td>10.90±1.21</td>
<td>6.65±1.21</td>
<td>0.014*</td>
<td>-38.0</td>
<td>0.23</td>
</tr>
<tr>
<td>Adjustedᵇ</td>
<td>-0.797±0.580</td>
<td>9.55±1.10</td>
<td>10.55±1.07</td>
<td>10.99±1.05</td>
<td>9.03±1.10</td>
<td>0.170</td>
<td>-5.3</td>
<td>0.54</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>-0.047±0.030</td>
<td>5.9 (0.6)</td>
<td>5.9 (0.5)</td>
<td>5.8 (0.5)</td>
<td>5.7 (0.6)</td>
<td>0.126</td>
<td>-3.4</td>
<td>0.47</td>
</tr>
<tr>
<td>Adjusted(^a)</td>
<td>-0.040±0.023</td>
<td>5.85±0.05</td>
<td>5.87±0.05</td>
<td>5.80±0.05</td>
<td>5.74±0.05</td>
<td>0.080</td>
<td>-1.7</td>
<td>0.27</td>
</tr>
<tr>
<td>Adjusted(^b)</td>
<td>-0.057±0.022</td>
<td>5.88±0.05</td>
<td>5.84±0.05</td>
<td>5.80±0.04</td>
<td>5.74±0.05</td>
<td>0.010(^*)</td>
<td>-3.4</td>
<td>0.072</td>
</tr>
</tbody>
</table>

cSys BP: central systolic blood pressure; cDia BP: central diastolic blood pressure; AIx: augmentation index; PWV: pulse wave velocity
\(^1\) \(\beta\) coefficient represents a change in arterial stiffness biomarkers by an increase in dietary phytosterols intake
\(^2\) P values for linear trend calculated by using the significance of coefficient for quartile numbers (1–4) fitted as a variable in a linear regression; \(^*\) \(P < 0.05\); \(\text{**} P < 0.01\)
\(^3\) P values for differences in arterial stiffness biomarkers across the quartiles of dietary phytosterols intake
\(^4\) P values for differences in arterial stiffness biomarkers across the quartiles of dietary phytosterols intake after adjusting for cofactors
\(^\dagger\) \(P < 0.05\); \(\text{††} P<0.01\)
\(^a\) Model adjusted for age
\(^b\) Model adjusted for age, BMI, total energy intake
Values reported as actual Mean (SD) in adjusted \(^a\)
Values reported as estimated marginal mean ± SE adjusted \(^b\)
4.5.4 Correlation Between Dietary Phytosterol Intake and CVD Risk Biomarkers and Plasma Phytosterols

As shown in Table 4.6, dietary phytosterol intakes were found to be inversely correlated with BMI, TC, LDL-C, HDL-C, TG, cSys BP, cDia BP, AIx, lathosterol and ratio of sitosterol-to-cholesterol (P<0.001 for TC, P<0.01 for BMI, LDL-C, TG, cSys BP, and sitosterol-to-cholesterol ratio, P<0.05 for HDL-C, cDia BP, AIx, and lathosterol). After adjusting for total energy intake, the associations between dietary phytosterol intake and CVD risk biomarkers were stronger than unadjusted (P<0.001 for TC, LDL-C and cSys BP, P<0.01 for TG, cDia BP, lathosterol, lathosterol-to-cholesterol ratio, sitosterol-to-cholesterol ratio, P<0.05 BMI, HDL-C) (Table 4.6).

| Table 4.6 Correlation Analysis of Dietary Total Phytosterols versus Serum Lipid Levels, Arterial Stiffness Biomarkers and Plasma Phytosterols |
|-----------------------------------|------------------|------------------|------------------|------------------|
|                                    | Unadjusted      | Adjusted for energy intake |
|                                    | r     | P     | r     | P     |
| Overall                            |       |       |       |       |
| BMI                                | -0.194 | 0.001** | -0.154 | 0.011* |
| TC (mmol/L)                        | -0.213 | <0.001*** | -0.224 | <0.001*** |
| LDL-C (mmol/L)                     | -0.198 | 0.001** | -0.225 | <0.001*** |
| HDL-C (mmol/L)                     | 0.122  | 0.043*  | 0.132  | 0.030*  |
| Log TG (mmol/L)                    | -0.187 | 0.002** | -0.210 | 0.001** |
| cSys BP (mmHg)                     | -0.167 | 0.006** | -0.243 | <0.001*** |
| cDia BP (mmHg)                     | -0.141 | 0.020*  | -0.176 | 0.004** |
| AIX@75 (%)                         | -0.140 | 0.021*  | -0.079 | 0.200  |
| PWV (m/s)                          | -0.106 | 0.080  | -0.116 | 0.058  |
| Desmossterol (µmol/L)             | -0.059 | 0.331  | -0.041 | 0.506  |
| Lathosterol (µmol/L)              | -0.136 | 0.025*  | -0.173 | 0.004** |
| Campesterol (µmol/L)              | -0.017 | 0.778  | -0.002 | 0.980  |
### Sitosterol (µmol/L)

<table>
<thead>
<tr>
<th></th>
<th>0.071</th>
<th>0.242</th>
<th>0.075</th>
<th>0.219</th>
</tr>
</thead>
</table>

### Lathosterol:cholesterol (µmol/mmol)

<table>
<thead>
<tr>
<th></th>
<th>-0.116</th>
<th>0.056</th>
<th>-0.173</th>
<th>0.004**</th>
</tr>
</thead>
</table>

### Campesterol:cholesterol (µmol/mmol)

<table>
<thead>
<tr>
<th></th>
<th>0.079</th>
<th>0.193</th>
<th>0.109</th>
<th>0.074</th>
</tr>
</thead>
</table>

### Sitosterol:cholesterol (µmol/mmol)

<table>
<thead>
<tr>
<th></th>
<th>0.162</th>
<th>0.008**</th>
<th>0.201</th>
<th>0.001**</th>
</tr>
</thead>
</table>

**BMI:** body mass index; **TC:** total cholesterol; **LDL-C:** low-density lipoprotein cholesterol; **HDL-C:** high-density lipoprotein cholesterol; **TG:** triglycerides; **cSys BP:** central systolic blood pressure; **cDia BP:** central diastolic blood pressure; **AIx:** augmentation index; **PWV:** pulse wave velocity

*a* Values log-transformed prior to analysis

*, **, *** represents P<0.05, P<0.01, P<0.001

### 4.5.5 Correlations Between Plasma Sitosterol to Cholesterol Ratio and CVD Risk Biomarkers

Results in Table 4.7 shows the association of plasma sitosterol-to-cholesterol ratio with CVD risk biomarkers. The results show that increased ratio of sitosterol-to-cholesterol was correlated with decreased **TC** (P<0.001), **LDL-C** (P<0.001), **TG** (P< 0.001), **cSys BP** (P<0.01), **cDia BP** (P<0.001), and **PWV** (P<0.001), and increased **HDL-C** (P<0.01). However, no significant association was observed between plasma sitosterol-to-cholesterol ratio and **AIx** (Table 4.7).

### Table 4.7 Correlation Analysis of Plasma Sitosterol-to-Cholesterol Ratio and Lathosterol-to-Cholesterol Ratio versus Serum Lipid Levels and Arterial Stiffness Biomarkers

<table>
<thead>
<tr>
<th></th>
<th>Sitosterol:Cholesterol</th>
<th>Lathosterol:Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r¹</td>
<td>P</td>
</tr>
</tbody>
</table>
| Serum lipid levels
| TC (mmol/L) | -0.209 | 0.001** | 0.012 | 0.845 |
| LDL-C (mmol/L) | -0.212 | <0.001*** | 0.045 | 0.466 |
| HDL-C (mmol/L) | 0.207 | 0.001** | -0.433 | <0.001*** |
| Log TG (mmol/L)* | -0.248 | <0.001*** | 0.443 | <0.001*** |
| Sitosterol and arterial stiffness biomarkers
| cSys (mmHg) | -0.198 | 0.001** | 0.314 | <0.001*** |
| cDia (mmHg) | -0.114 | <0.001*** | 0.288 | <0.001*** |
The correlation between sitosterol/cholesterol ratio (cholesterol absorption biomarker) or lathosterol/cholesterol ratio (cholesterol synthesis biomarker) and PWV are shown in Figures 4.1 and 4.2. Figure 4.1 shows that sitosterol/cholesterol ratio (cholesterol absorption biomarker) is negatively correlated with PWV ($r = -0.285$, $P < 0.001$). Figure 4.2 shows that lathosterol-to-cholesterol ratio (cholesterol synthesis biomarker) was directly correlated with serum TG and cSys BP, cDia BP, PWV ($P < 0.001$ for all) and inversely correlated with HDL-C ($P < 0.001$).

4.5.6 Association Between Dietary Phytosterols and Dietary Scoring

The mean MSDPS in the present study was 21.8 ± 6.07 (range 3.9-39.9) out of the maximum possible score of 100 (Table 4.8). Among the 12 Mediterranean food groups, median component scores were the highest for vegetable consumption (5.31) followed by fruit intake (5.10) and dairy...
consumption (5.04), and the lowest for sweets, fish and meat intake (0.70, 1.33, 1.46). Consumption of Mediterranean food groups accounted for 69.6% of total energy intake. In the correlation analysis between MSDPS and individual component score, the highest correlation coefficient was found in the fruit group (r=0.379; P< 0.01) and lowest in the meat group (r=0.101; P>0.05) (Table 4.8).

Table 4.8 Dietary Intakes, Score Distribution, and Correlation of the Mediterranean-Style Dietary Pattern Score (MSDPS)

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Intake distribution¹</th>
<th>Score distribution¹</th>
<th>Correlation to total MSDPS²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of whole grain servings</td>
<td>0.91 (0.12,2.80)</td>
<td>1.40 (0.15, 3.50)</td>
<td>0.237**</td>
</tr>
<tr>
<td>Fruit servings/d</td>
<td>1.84 (0.349, 5.07)</td>
<td>5.10 (0.56, 9.60)</td>
<td>0.379**</td>
</tr>
<tr>
<td>Vegetable servings/d</td>
<td>4.24 (1.06, 11.91)</td>
<td>5.31 (0.19, 9.59)</td>
<td>0.263**</td>
</tr>
<tr>
<td>Dairy servings/d</td>
<td>1.3 (0.24, 4.51)</td>
<td>5.04 (0.00, 9.65)</td>
<td>0.286**</td>
</tr>
<tr>
<td>Alcohol servings/d</td>
<td>0.24 (0.00, 2.45)</td>
<td>2.09 (0.00, 8.00)</td>
<td>0.334**</td>
</tr>
<tr>
<td>Fish and other seafood servings/wk</td>
<td>0.513 (0.05, 2.54)</td>
<td>1.33 (0.04, 3.94)</td>
<td>0.228**</td>
</tr>
<tr>
<td>Poultry servings/wk</td>
<td>1.61 (0.21, 9.07)</td>
<td>3.75 (0.00, 9.00)</td>
<td>0.187**</td>
</tr>
<tr>
<td>Olives, legumes and nuts servings/wk</td>
<td>1.05 (0.11, 6.89)</td>
<td>3.25 (0.18, 8.51)</td>
<td>0.265**</td>
</tr>
<tr>
<td>Potato and starch servings/wk</td>
<td>2.73 (0.63, 9.89)</td>
<td>4.80 (0.00, 9.40)</td>
<td>0.122*</td>
</tr>
<tr>
<td>Eggs servings/wk</td>
<td>1.26 (0.10, 4.88)</td>
<td>4.01 (0.13, 9.59)</td>
<td>0.143*</td>
</tr>
<tr>
<td>Sweets servings/wk</td>
<td>16.95 (5.13, 52.72)</td>
<td>0.37 (0.00, 3.25)</td>
<td>0.188**</td>
</tr>
<tr>
<td>Meat servings/wk</td>
<td>2.917 (0.64, 10.37)</td>
<td>1.46 (0.00, 8.32)</td>
<td>0.101</td>
</tr>
<tr>
<td>Weighting factor (%)³</td>
<td>69.60 (54.33, 80.01)</td>
<td>0.70 (0.54, 0.80)</td>
<td></td>
</tr>
<tr>
<td>MUFA:SFA ratio</td>
<td>1.30 (0.90, 1.92)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MSDPS: Mediterranean style dietary pattern score; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids

¹ Data are median (5th, 95th percentile), n=277

² Total MSDPS was the sum of 12 components standardized to a 0–100 scale and weighted to the proportion of daily energy intake from Mediterranean diet foods.

³ Total energy intakes are attributed to the consumption of Mediterranean diet foods.
**4.5.7 Correlation Between Dietary Scoring and Dietary Phytosterols, Serum Lipid Levels, and Arterial Stiffness Biomarkers**

As shown in Table 4.9 we found MSDPS negatively correlated with BMI (P<0.01), cSys (P<0.01), and AIx (P<0.05) and positively correlated with HDL-C (P<0.05). However, no significant associations were observed between MSDPS and dietary phytosterols, TC, LDL-C, TG, cDia, and AIx (P>0.05). The ratio of MUFA-to-SFA was inversely correlated with BMI, serum TC, LDL-C, TG, cSys BP, cDia BP, and PWV (all P<0.05) (Table 4.9).

**Table 4.9** Correlation Analysis of MSDPS versus Dietary Phytosterols, Serum Lipid Levels, and Arterial Stiffness Biomarkers

<table>
<thead>
<tr>
<th></th>
<th>MSDPS</th>
<th>MUFA: SFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r¹</td>
<td>P²</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>-0.185</td>
<td>0.002**</td>
</tr>
<tr>
<td>Dietary Phytosterols (mg/d)</td>
<td>0.023</td>
<td>0.700</td>
</tr>
<tr>
<td><strong>Serum lipid levels</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>-0.026</td>
<td>0.668</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>-0.074</td>
<td>0.219</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>0.133</td>
<td>0.028*</td>
</tr>
<tr>
<td>Log TG (mmol/L)</td>
<td>-0.071</td>
<td>0.242</td>
</tr>
<tr>
<td><strong>Arterial stiffness biomarkers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cSys (mmHg)</td>
<td>-0.161</td>
<td>0.008**</td>
</tr>
<tr>
<td>cDia (mmHg)</td>
<td>-0.113</td>
<td>0.064</td>
</tr>
<tr>
<td>AIX@75 (%)</td>
<td>-0.145</td>
<td>0.017*</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>-0.098</td>
<td>0.108</td>
</tr>
</tbody>
</table>

BMI: body mass index; LDL: low-density lipoprotein; HDLc: high-density lipoprotein; TG: triglycerides; cSys BP: central systolic blood pressure; cDia BP: central diastolic blood pressure; AIX: augmentation index; PWV: pulse wave velocity

¹ Correlation coefficient

² P values from correlation analysis

³ Values log-transformed prior to analysis

*, ** represents P<0.05, P<0.01
4.6 Discussion

The basis of our study was to examine the potential effects of dietary and circulating phytosterols on CVD risk biomarkers. The mean intakes of total dietary phytosterols in our study population were 351.1 ± 130.2 mg/d and 215.6 ± 118.3 mg/d for men and women, respectively, which were slightly different than the mean intakes found in previous studies. A European cross-sectional study of 22,256 men and women reported 310 and 303 mg/d, respectively, and a study from Northern Sweden of 37,150 men and 40,502 women reported 252 and 212 mg/d of dietary phytosterols for men and women, respectively (Andersson et al., 2004; Klingberg et al., 2008b). Another study on a northern Chinese population; comprising 509 men and 502 women, reported 267.5 mg/d intake of dietary phytosterols (Li et al., 2018).

In the current study, we found that high phytosterol intake (392.7±108.8 mg/d) in the highest quartile was negatively associated with TC and LDL-C after adjustment for BMI, total energy intake, fats including saturated fat, MUFA, and PUFA as a percentage of energy intake, and dietary fiber than the lowest quartile (97.7±36.5 mg/d). Higher intake of dietary phytosterols was also found to be inversely associated with cSys and PWV after adjustment for age, BMI, and total energy intake. However, it has been shown that potential confounding is a major issue that occurs due to the presence of common causes of exposures and outcomes (Hernán & Robins, 2006; Rothman et al., 2008; Tilaki, 2012). Thus, confounders can cause a misreporting of the observed associations between exposure and outcome (Rothman, 2008; VanderWeele & Ding, 2017). For example, lifestyle and dietary factors such as BMI, fat intake, and fiber intake could affect the management of CVD risk biomarkers. It is well known that foods such as vegetables, grains, and vegetable oils, which are rich in phytosterols, are also good sources of dietary fiber, MUFA and PUFA (Marangoni & Poli, 2010). Numerous studies observed that populations with high phytosterols intake had higher MUFA, PUFA, and fiber intakes, all factors that are well known to reduce serum cholesterol levels, and higher saturated fat intake, which is known to increase serum cholesterol levels (Clifton et al., 2017; DiNicolantonio & O’Keefe, 2018). Although it is difficult to separate the phytosterols from the other dietary components in the diet, adjustment for potential dietary confounders could handle such issues in the best way possible and better examine the relationship between dietary phytosterols and serum lipids. In the current study, the separate
analysis for men and women did not show any significant changes throughout the analyses; therefore, a combined sexes statistical analysis was performed throughout all the data analyzed.

In the current study, after adjusting the potential confounders, such as for BMI, total energy intake, fats including saturated fat, MUFA, and PUFA as a percentage of energy intake, and dietary fiber, the model showed that dietary phytosterols have a substantial impact on serum lipid levels. Our study found that approximately 295 mg/d increase in phytosterol intake from the lowest to the highest quartile was associated with 10.2% and 14.4% decreases (P<0.05) in TC and LDL-C, respectively (Table 4.4). The results of the current study showed a comparatively enhanced reduction in serum TC and LDL-C concentrations than was observed in previously reported observational studies. Our study results are comparable to the results showed in the European Prospective Investigation into Cancer and Nutrition (EPIC-Norfolk) study, in which the researchers showed that increased dietary phytosterol intake in the highest quartile was associated with a reduction in serum TC and LDL-C levels by 4.1% and 3.5%, respectively, in men and by 2.4% and 3.0%, respectively, in women in comparison with the lowest quartile (Andersson et al., 2004). Another study showed a 2.6% and 3.5% reduction in TC and 3.1% and 3.2% in LDL-C levels in men and women, respectively, in the highest quintile compared to the lowest quintile of dietary phytosterol intake (Klingberg et al., 2008b). Moreover, based on the results of the current study, dietary phytosterols from the habitual diet were shown to produce more dynamic effects on serum cholesterol levels than the effects of phytosterol enriched products observed in clinical studies. The clinical studies that have examined the therapeutic benefits of phytosterols did not eliminate or quantify the phytosterols present in the background-controlled diet while examining the exposure-outcome associations (Demonty et al., 2009; Ras, Geleijnse, & Trautwein, 2014). Thus, addressing those challenges, the present study showed an effective improvement in serum cholesterol levels after adjustment for potential confounders. However, after adjustment for confounders, non-significant associations were observed between dietary phytosterols and TG and HDL-C in the highest quartile of phytosterols. A recent observational study found a non-significant (P>0.05) inverse correlation between dietary phytosterols and HDL-C levels (Li et al., 2018). Interestingly, when overall partial correlation analysis adjusting energy intake was performed in the present study, the dietary phytosterol intake was found to be directly correlated with increasing HDL-C (P<0.05) and inversely correlated with TG (P<0.01) (Table 4.6). Although our study population never had high TG baseline levels, increased intake of dietary phytosterols was found
to be associated with reduced TG levels. The consumption of phytosterol has been shown to decrease hepatic very-low-density lipoprotein secretion, which consequently decreases the plasma TG concentrations (Schonewille et al., 2014). Overall, increased phytosterol intake in the highest quartile was found to be significantly inversely associated with serum TC and LDL-C levels compared to the lowest quartile of phytosterol intake.

Phytosterol intake has been shown to reduce cholesterol absorption and thus the serum LDL-C levels. NPCILI and ABCG5/ABCG8 are two important protein transporters involved in the intestinal absorption and efflux of cholesterol and phytosterols in the enterocytes. At the intestinal brush border membrane, phytosterols down-regulate the activity of NPCILI transporter which results in less uptake of cholesterol into the enterocytes and thus leads to reduced cholesterol absorption. Phytosterols act as a competitive inhibitor to reduce cholesterol absorption by diminishing the activity of NPCILI. Compared to 2 g/d supplementations of phytosterols, dietary phytosterols potentially have enough efficacy to lower serum cholesterol levels. Ostlund et al examined the bioactivity of corn oil and showed that chemically removed phytosterols from corn oil resulted in increased cholesterol absorption by 38% and returned to normal when phytosterols were added back to the same oil (Ostlund et al., 2002). Corn oil containing 150 mg of phytosterols supplemented into one meal showed a significant reduction in cholesterol absorption (Ostlund et al., 2002). The results from this study and our study suggest that the inclusion of even low levels of phytosterols is more effective than high doses of supplemented phytosterols. A meta-analysis showed that approximately 700 mg/d consumption of phytosterols resulted in 4.5% of serum LDL-C reduction (Demonty et al., 2009). Numerous clinical studies showed that the same effects of phytosterols for serum cholesterol reduction were achieved at doses of 3g/d and 8-10 g/d (Ras et al., 2014). However, higher phytosterols intake in the control group, higher blood concentrations at baseline ceiling effects might partially responsible for less efficacy of phytosterols seen in clinical studies as compared to the observational studies (Demonty et al., 2009).

Similar to the effects of dietary phytosterols on serum TC and LDL-C, another important finding from the current study showed that dietary phytosterols have an impact on vascular parameters as seen by significant decreases in cSys BP, cDia BP, and PWV values (-5.3%, P<0.01; -4.5%, P<0.01; and -3.4%, P<0.05, respectively) in the highest quartile of phytosterol intake as compared to the lowest quartile (Table 4.5). Arterial stiffness is potentially confounded by factors
such as age and BMI (Mitchell, 2009). Therefore, adjustment for these factors such as BMI, age, and total energy intake in the multivariate model provided a better estimate of arterial stiffness. Fewer observational studies have examined the effects of dietary phytosterols on arterial stiffness biomarkers (Wang et al., 2012), but numerous RCTs have been performed to test this association (Ras et al., 2015a; Gyllng et al., 2013). These studies showed neither beneficial nor detrimental effects of phytosterols on arterial stiffness biomarkers (Ras et al., 2015a; Gyllng et al., 2013). Moreover, the decrease in blood pressure found in the current study is beneficial, because a reduction in blood pressure has been known to reduce the risk of cardiovascular morbidity and mortality (Patel et al., 2016). Furthermore, risk factors such as obesity, lipid disorders (particularly higher TG and lower HDL), and hypertension are associated with the stiffer aorta (Mitchell et al., 2007). In an attempt to explore the effects of dietary phytosteroles on BMI, serum TG, HDL-C, and central blood pressure, weak negative correlations of dietary phytosterols with BMI, TG, and cSys BP and cDia BP were found as -0.154 (P<0.05), -0.210 (P<0.01), -0.243 (P<0.001), -0.176 (P<0.01), respectively.

Cholesterol absorption reduction is known as a possible mechanism for serum LDL-C lowering due to phytosterols. Therefore, to estimate cholesterol metabolism, including cholesterol absorption and synthesis, in our study we assessed circulating phytosterols with cholesterol precursors such as lathosterol or desmosterol, markers for cholesterol synthesis. Sitosterol-to-cholesterol ratio, a cholesterol absorption biomarker and lathosterol-to-cholesterol ratio, a cholesterol synthesis biomarker, were used to investigate the relationship of cholesterol absorption and cholesterol synthesis with CVD risk biomarkers. Our study looked at the effects of dietary phytosterols on cholesterol absorption and cholesterol synthesis biomarkers and observed that increased dietary phytosterols were correlated with increased sitosterol-to-cholesterol ratio (P<0.01) and decreased lathosterol-to-cholesterol ratio (P<0.01) as expected (Table 4.6) (Escurriol et al., 2009; Mackay et al., 2015). Moreover, a similar finding by Racette and coworkers reported that moderate intake of dietary phytosterols (459 mg/d) can significantly reduce the efficiency of intestinal cholesterol absorption (Racette et al., 2010). However, some previous studies also showed that increased absorption of phytosterols can cause the occurrence of phytosterols in atherosclerotic tissues which consequently increases the risk of atherosclerotic cardiovascular disease (John et al., 2007). Therefore, the current study analyzed the relationship between circulating phytosterols and CVD risk biomarkers and found that increasing circulating sitosterol-
to-cholesterol ratio was significantly correlated with decreased TC (P<0.001), LDL-C (P<0.001), TG (P<0.001); cSys BP (P<0.01), cDia BP (P<0.001), and PWV (P<0.001); and increased HDL-C (P<0.01) (Table 4.7). Besides cholesterol absorption biomarkers, our study found that reduction in lathoesterol-to-cholesterol ratio, a cholesterol synthesis biomarker, may also lower the risk of other CVD risk biomarkers (Mackay et al., 2015). As shown in our study results, lathosterol-to-cholesterol ratio was found to be directly correlated with serum TG, cSys BP, cDia BP, PWV (P<0.0001) and inversely correlated with HDL-C (P<0.001) (Table 4.7). The relationship of lathosterol-to-cholesterol ratio with serum HDL-C (r=-0.433) and TG (r=0.443) was stronger compared to the arterial stiffness biomarkers, such as cSys BP (r=0.314), cDia BP (r=0.288), and PWV (r=0.249). Similarly, a study from Rideout and colleagues reported that high cholesterol absorbers who had low cholesterol synthesis showed the most pronounced effects of phytosterols on CVD risk biomarkers (Rideout et al., 2010). Previous studies have shown that increased phytosterol levels may be just a marker that plays a role in cholesterol metabolism but is not individually responsible for causing atherosclerosis (Silbernagel et al., 2010). However, the mechanism of effects of phytosterols on arterial stiffness biomarkers is still not known. Overall, based on the current results with improved CVD risk biomarkers, it can be speculated that reduced cholesterol absorption and low cholesterol synthesis may decrease CVD risk.

In the past few years, advanced research regarding diet and disease relationships have adopted a dietary pattern analysis as a complementary approach to examine the association between diet and risk of various chronic diseases, including CVD. The total MSDPS score found in our study was about 1/5 of the total assigned score. In the current study, there was no association found between MSDPS and dietary phytosterol. However, many studies have shown that phytosterols are one of the bioactive components responsible for lowering LDL-C levels in the population consuming the Mediterranean diet (Escurriol et al., 2009; Zadák et al., 2006). Although, several components of MSDPS such as intake servings of grains, fruits, vegetables, nuts, and legumes were significantly correlated (P<0.05) with dietary phytosterol intake whereas the dietary score of fruits and vegetables was not significantly associated with dietary phytosterols. A study by Escurriol et al. (2009) showed that the Mediterranean diet, enriched with nuts and increased intake of phytosterols reduced serum LDL-C levels significantly by 8.3% (P<0.02). However, due to the negative scores assigned to overconsumption of recommended food group servings in the currently used MSDPS, scores of the same food groups were not found significantly associated
with dietary phytosterols. Several studies from the past decade have suggested that higher intakes of fruits, vegetables, and olive oil, which are rich in MUFA, PUFA, and phytonutrients including phytosterols, can reduce the risk of developing CVD (Key et al., 1986). It has been hypothesized that even though fruits and vegetables contain a lower amount of phytosterols, their larger contribution in the daily diet may contribute largely to increasing the amount of total phytosterol intake per day. The results of our study also found the MSDPS to be inversely associated with BMI (P<0.01), cSys BP (P<0.01), and AIx (P<0.05) and directly correlated with serum HDL-C levels (P<0.05). A recent study reported that the Mediterranean diet reduced systolic BP (-9.2 mm Hg, P<0.05) and AIx (-12.4, P<0.05) with no change in PWV (Jennings et al., 2019). The ratio of MUFA-to-SFA was found to be inversely correlated (P<0.05) with BMI, TC, LDL-C, TG, cSys BP, cDia BP, and PWV in the current study. In 2013, a Greek EPIC cohort study reported a significant inverse association of high Mediterranean score or adherence to the Mediterranean diet with systolic and diastolic BP after controlling for energy intake and expenditure, anthropometric and sociodemographic dietary factors. This study also found olive oil (high ratio of MUFA-to-SFA), vegetables and fruits as major contributing factors responsible for arterial BP-lowering effects conveyed by the Mediterranean diet (Toledo et al., 2013). A sub-analysis of the EVIDENT study consisting of 1,533 subjects (aged 20-80 years) reported that increasing adherence to the Mediterranean diet was inversely associated with reduced PWV, which therefore reduced the overall cardiovascular risk (Van de Laar et al., 2013). Overall, due to the non-Mediterranean dietary pattern followed by the current study population, we found a non-significant association of phytosterols with MDSPS. Otherwise, based on the relationship found between MSDPS and CVD risk biomarkers, following a dietary pattern by the Mediterranean dietary pyramid, which represents a healthy diet, may reduce the risk of CVD.

In summary, the results of our study showed that a small increase (295 mg/d) in dietary phytosterol intake decreased the levels of serum TC, LDL-C and cSys BP, cDia BP, and PWV after adjusting the models for potential confounders. The results of dietary phytosterol intake on serum TC and LDL-C levels were found to be more effective in the present study than in some published studies (Andersson et al., 2004; Klingberg et al., 2008b; Wang et al., 2018). None of the previous studies showed significant effects of dietary phytosterols on vascular health biomarkers (Ras et al., 2015a; Gylling et al., 2014). Due to the ongoing debate and investigation on the atherogenic effects of circulating phytosterols on vascular health, the present study investigated
the relationship between cholesterol metabolism and CVD risk biomarkers. The study results observed that reduced cholesterol absorption was associated with reduced levels of TC, LDL-C, cSys BP, cDia BP, and PWV and increased HDL-C which may reduce the overall CVD risk. The low cholesterol synthesis was correlated with low serum TG, HDL-C, cSys BP, cDia BP, and PWV and increased HDL-C. Our study suggested that reduced levels of arterial stiffness biomarkers by increased dietary phytosterols intake and increased circulating phytosterols might improve vascular health. The present study population did not show good adherence to the Mediterranean diet, therefore no significant associations were found between MSDPS and dietary phytosterols. Overall, this study showed the greater efficacy of dietary phytosterols in improving CVD risk biomarkers.
Chapter 5: Conclusion, Limitations and Future Directions

5.1 Summary and Implications

To summarize, the research on dietary and circulating phytosterols is mainly focused on CVD. The results from the present study indicate that a 295 mg/d increase in dietary phytosterol intake in the highest quartile from the lowest quartile decreased serum TC and LDL-C levels approximately by 10% and 14%, respectively. The reduction in cholesterol levels across increasing quartiles of dietary phytosterol intake in our study falls in the range (10-12%) suggested by RCTs (Ras, Geleijnse, & Trautwein, 2014; Gylling et al., 2014). Several dietary guidelines that focused on the treatment of hypercholesterolemia, dyslipidemia, and other CVD risk events, recommended approximately 2 g/d intake of phytosterols, which helps to lower serum LDL-C concentrations by 10-12% along with changes in dietary practices and lifestyle habits (Gylling et al., 2014; Jellinger et al., 2017; Piepoli et al., 2016). It has been hypothesized that the intake of phytosterols from natural sources is more efficacious in lowering cholesterol than the phytosterol-enriched foods in RCTs (Ras, Geleijnse, & Trautwein, 2014). Our study showed the beneficial impact of dietary phytosterols on pulse wave velocity, which might help to improve vascular function. The impact of circulating phytosterols on CVD risk biomarkers was investigated in our study using surrogate biomarkers of cholesterol metabolism, including sitosterol-to-cholesterol and lathosterol-to-cholesterol ratio. The intake of dietary phytosterols showed a direct association with reduced cholesterol absorption and low cholesterol synthesis. Reduced cholesterol absorption measured using sitosterol-to-cholesterol ratio was found to be associated with improved CVD risk biomarkers, including TC, LDL-C, HDL-C, TG, cSys BP, cDia BP, and PWV. Low cholesterol synthesis was also found to be associated with reduced PWV. Our study suggested that reduced cholesterol absorption and lower cholesterol synthesis are associated with improved arterial stiffness and serum lipid levels. The current study concluded that both dietary and circulating phytosterols might improve arterial stiffness biomarkers, although the results of our study did not show any association between dietary phytosterols and MSDPS. However, based on the observed association between MSDPS and CVD risk biomarkers, it could be hypothesized that following a dietary pattern based on the Mediterranean food pyramid may help to reduce the risk of CVD.

Overall, the results of our study showed that an approximately 300 mg/d increase in dietary phytosterol intake might help to improve the CVD risk biomarkers. In general, to achieve at least 300-350 mg/d dietary phytosterols, one should include more plant-based foods in the daily diet.
The findings of this study suggested that dietary phytosterols are bioactive components and have pronounced effects on cholesterol absorption metabolism and CVD risk biomarkers. Dietary phytosterols have the potency to improve CVD risk biomarkers. However, reduced levels of CVD risk biomarkers, including serum lipid biomarkers and vascular health biomarkers with dietary phytosterol consumption do not assure that dietary phytosterols will reduce the CVD risk. Further studies are needed to examine the causal associations.

5.2 Strengths

Overall, various strengths can be identified from this study, with some of the main factors summarized below:

1. To estimate the phytosterol intake, we used the USDA and CNF nutrient databases. The use of these two databases was beneficial because they are constructed based on the North American dietary pattern and comprised of foods in line with the Canadian Food Guide and Dietary Guidelines for Americans. Based on the databases used by previous studies, we realized that poor estimation of phytosterol intake is mostly affected by incomplete assessment of plant sterol content in foods. Therefore, to address challenges with missing information on the phytosterol content of foods in both of these databases, we compiled the information from several research articles. Overall, all sources together provided a robust food database compared to the databases used in previous studies (Andersson et al., 2004; Klingberg et al., 2008b; Li et al., 2018).

2. Our study used ASA24-hour dietary recall to calculate dietary phytosterol intake, whereas, the past observational studies used FFQ to calculate dietary phytosterols from the diet which we found does not provide an accurate estimation of nutrients based on the literature available (Park et al., 2018). Before the ASA24 hour recall was developed, FFQ was considered as the only cost-effective and feasible method to use in large-scale studies. The 24-hour dietary recalls have been shown to provide a more accurate estimate of food and nutrients than does the FFQ. The literature shows that using FFQ the chances of underreporting of energy intake are 1.5-3 times higher than 24-hour dietary recall (Park et al., 2018). Using a 24-hour dietary recall has also been challenging in large cohort studies due to their high interviewer cost and higher cost for coding of data. However, ASA24 hour dietary recall, a web-based dietary recall, addresses all such challenges by eliminating the cost for interviewer and coders. Additionally, to avoid random measurement error, the
present study collected three repeated measurements of 24-hour recalls rather than recording a single recall. Multiple 24-hour dietary recalls have been shown to produce a better estimate of actual intake than a single recall. In summary, various features of ASA24 hour recall such as providing an accurate and precise estimation of nutrients, less time consuming, economical, and less prone to reactivity bias made this method more useful to calculate phytosterols in the present study.

3. Cardiovascular risk factors such as age, BMI, and dietary factors such as energy and nutrient intake were adjusted in the analysis of data to minimize the bias in dietary intake assessment. A confounding usually occurs when some external factors such as age, BMI, dietary factors are associated with exposure of interest (nutrient of interest) and outcome (disease) which acts as a barrier to test the association of diet with the disease. Therefore, the purpose of adjusting these potential confounders was to examine the effect of external factors that might affect the relationship between exposure and outcome variables. For example, age, BMI, and dietary factors, such as energy intake, saturated and unsaturated fat intake, and fiber are somehow directly or indirectly related to dietary phytosterols in terms of their effects on CVD risk biomarkers. Therefore, adjustments for potential confounders better determine whether a given risk factor is a severe confounder or not after comparing the measured outcome before or after confounding.

5.3 Limitations

This study had several limitations:

1. A limitation of this study was the population size, which affected the ability to observe the sex effect. Although there were 455 participants, 178 participants with missing information on at least one major parameter assessed in this study and therefore were excluded from the statistical analysis. Some of the participants did not provide DHQs because they had trouble with recalling long term food and beverage intake and frequency of foods consumed. Some of these participants also did not provide 24-hour recalls which might be due to less knowledge of foods, literacy, or memory recall-related issues. Therefore, we excluded those from the analysis. The population size used in the current study is quite small compared to many other observational studies that have been performed in the past to examine the association of dietary phytosterols with CVD risk biomarkers (Andersson et al., 2004; Klingberg et al., 2008b; Ras, Geleijnse, & Trautwein, 2014). Nevertheless, the
results of the current study were comparable to studies with larger population sizes. However, we could have alternatively handled the participants with incomplete dietary information. For example, phytosterol intake was the primary focus of the present study and was calculated from 24-hour recall. However, we also excluded the data with missing DHQ which was not the main focus to examine the relationship between diet and disease. Therefore, we could have included the participant with incomplete DHQ’s to increase the strength of the study analysis.

2. Further, the small sample size affected the ability to observe sex differences. Sex stratified analysis is important in population-based studies to avoid confounding bias. However, in the current study, no significant differences were found in sexes after the sex-stratified analysis was performed. Therefore, we decided to perform a combined analysis of sex throughout all the analyses. We assumed that the small sample size might be the reason we have not seen a significant sex difference. Whereas, at the time, if we increase the sample size there is also a possibility that we might still not see a significant difference in sex.

3. A major limitation that our study showed was confounding, a general limitation mostly found in observational studies. Confounding occurs when an individual variable correlates with both the diet and disease (exposure and outcome). Generally, people who consume more plant-based foods get relatively higher phytosterols in their diet. Therefore, we adjusted some potential confounders in our study, including age, BMI, energy, saturated and unsaturated fats, and fiber. Despite these adjustments, some unknown or imprecisely measured variables may still have residual confounding. Therefore, the relationship we observed between exposure and outcome may have been overestimated. At the same time, adjusted confounders might have been overcorrected. Considering these limitations, we may have missed the actual relationship between phytosterols and CVD risk biomarkers.

4. There were some limitations to using 24-hour dietary recalls, particularly when using a self-administered approach. A self-administrated questionnaire requires reading literacy; otherwise, people with low-literacy levels may underreport or overreport their dietary intake. Interviewer-administered recalls work better for the low-literacy population. Moreover, language is another challenge e.g. the ASA24 available in Canada is in two languages only, English and French, therefore, it might be challenging to complete the recalls for people who speak different languages. Interviewer based 24-hour recalls can be
conducted in different languages if resources permit. The respondents may not report actual dietary intake because of various reasons, such as knowledge regarding foods and literacy (Marcinow et al., 2018). There is a random measurement error likely to occur using 24-hour dietary recall. Imprecision occurs if the data is affected by within-person random error. However, repeated measurements of dietary intake were taken in our study, which would reduce a random measurement error.

5. Another limitation of this study was that phytosterol intake was not normalized to energy intake before the quartile distribution. We found that individuals that were in the highest quartile of phytosterol intake had higher energy intake and also had lower BMI as compared to those in the lowest quartile of phytosterol intake. It is likely assumed that individuals with high energy intake but with lower BMI might have high energy expenditure. Physical activity is one of the major factors likely to cause high energy expenditure. Moreover, socioeconomic status and ethnicity are other possible factors associated with dietary intake, which may affect phytosterol intake. For example, people with low socioeconomic status may eat more unhealthy food, including following an unfavorable snacking pattern of diet, where such diets usually have more processed foods, which are not plant-based foods as compared to people with high socioeconomic status who tends to consume more fruits and vegetables. Ethnicity also affects dietary intake, for instance, people from South East Asia, Southern Europe eat more plant-based foods as compared to people from western countries. Therefore, we could have adjusted the dietary intake data for physical activity, socioeconomic status, and ethnicity as confounding factors while examining the relationship between dietary phytosterol intake and CVD risk biomarkers. Smoking is also one of the well-known factors associated with arterial stiffness. Smoking is known to increase blood pressure and the risk of hypertension. Studies have shown that cigarette smoking is one of the prominent causes of CVD and excessive smoking increases arterial stiffness as measured by PWV and AIx (Schmidt et al., 2019). Therefore, smoking should also be considered for adjustment as a confounder. Unfortunately, TMPLR data related to the above-mentioned components, including physical activity, smoking, socioeconomic status, and ethnicity these data were not available at the time for this thesis.
6. There are limitations to our application of the MSDPS. First, we replaced the olive oil group in dietary scoring with the MUFA-to-SFA ratio because Canadian DHQ II does not provide olive oil servings calculated using Diet Calc software. Moreover, olive oil is an important component of the Mediterranean diet; thus, the exclusion of the olive oil group might have reflected the total dietary score. Second, the current study had a non-Mediterranean population who does not follow the Mediterranean dietary pattern; therefore, lack of consumption of the Mediterranean food groups will not provide greater conformity to the Mediterranean dietary pattern. Lastly, the MSDPS developed from the Mediterranean diet pyramid does not determine the serving recommendations based on sex and energy intake, which might cause residual confounding.

5.4 Future Directions

Overall, the findings of this thesis provided useful information regarding the effects of dietary phytosterols on CVD risk biomarkers, including serum lipid levels and arterial stiffness biomarkers. However, there is a lack of evidence available regarding the mechanism of action of dietary phytosterols for improving arterial stiffness biomarkers. Therefore, for a clear understanding of this mechanism, future studies are required. Future studies should take a larger sample size and perform separate analyses for both sexes to get significant results on the effects of dietary phytosterols on CVD risk biomarkers. The results from the present study suggest that dietary phytosterol consumption might help in the reduction of CVD risk biomarkers. The present study only looked at the association not the causation of CVD risk biomarkers, therefore, future studies should conduct intervention trials to see causation. Moreover, there are many factors such as smoking, physical activity, energy expenditure that are related to diet and CVD risk markers that were not adjusted as confounding factors in the present study. Future studies should consider the adjustment for these confounding factors to see if that provides a clear association between phytosterol and CVD risk biomarkers. Future studies should also normalize phytosterol intake to total energy intake and then divide the population into lowest to highest quartiles based on the normalization. Future studies should also investigate the causal association between dietary phytosterols from natural sources and cardiovascular risk. Moreover, considering the limitations mentioned above, future studies may focus on a population with a broader range and higher levels of phytosterols intake from natural dietary sources. To see effective results, future research should
include an increased number of individuals who primarily consume Mediterranean diets or people who are vegan/vegetarians and mainly consume plant-based diets.
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Appendices

Appendix 1: Copyright Licence for Published Material

Chapter II: Manuscript

Association of Dietary Phytosterols with Cardiovascular Disease Biomarkers in Humans

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Sep 10, 2020

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Winnipeg, MB R3T 3X8
Canada
Attn: Ms. Ramandeep Kaur

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Appendix 2: Study Consent form

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

“The Manitoba Personalized Lifestyle Research (TMLR) study”

Principal Investigator: Dr. Peter Jones, University of Manitoba

Co-Investigator:
Dr. Megan Azad    University of Manitoba
Dr. Peter Eck     University of Manitoba
Dr. Eshan Khajipour University of Manitoba
Dr. Lisa Lix      University of Manitoba
Dr. Naveep Tangri University of Manitoba/ Seven Oaks Hospital
Dr. Semone Myrie  University of Manitoba
Dr. Amir Ravandi  University of Manitoba/ St. Boniface Hospital
Dr. Sharon Bruce  University of Manitoba
Dr. Jared Carberg University of Manitoba
Dr. Diana McMillan University of Manitoba
Dr. Heather Blevett St. Boniface Hospital/ CCARM
Dr. Todd Duhamel  University of Manitoba

Sponsor: Research Manitoba, 205-445 Ellice Ave., Winnipeg, Manitoba, R3E 3P5

You are being asked to participate in a 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 study and you may discuss it with your friends, family or (if applicable) your doctor before you make your decision. This consent form may contain words that you do not understand. Please ask
The Manitoba Personalized Lifestyle Research (TMPLR) study

the study staff to explain any words or information that you do not clearly understand.

**Purpose of Study**

Chronic disease is a growing concern among Canadians. In fact, three out of five Canadians over the age of 20 have already developed at least one chronic disease and four out of five are at risk. Research has begun to focus on ways to reduce chronic disease prevalence by approaching it from a variety of different health disciplines. The Manitoba Personalized Lifestyle Research (TMPLR) Program is being conducted to investigate the interaction between lifestyle, genetics, and gut microbiota and their association with additional risk factors for chronic conditions prevalent in Manitobans. Chronic conditions of interest include obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease and kidney disease. Blood samples will undergo analysis for numerous established and emerging health biomarkers (total cholesterol, high density lipoprotein cholesterol (HDL-C), triglycerides, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), insulin, Gastric inhibitory polypeptide (GIP), leptin, adiponectin, c-reactive protein (CRP), fatty acids, hemoglobin A1c (HbA1c), regulatory T-cells (Tregs), creatinine, blood urea nitrogen (BUN), non-cholesterol sterols, adipokines, cytokines, vitamin C, fat soluble vitamins, and lipidomic and metabolomics profiling). This study will include men and women aged 30-46, stratified by Body Mass Index (BMI) and geography.

**Study procedures**

**Pre-screening**

A telephone interview will be performed by research personnel. In this small interview you will be asked about your age, height, weight, ethnicity and residing area. Also, if you are pregnant or lactating, you will be unable to participate in this study.

**Study visits**

After the telephone screening, if qualified, we will ask you to come to the Richardson Centre for Functional Foods and Nutraceuticals at the University of Manitoba to sign a consent form, then come fasted (12 hours) on two consecutive days to undergo measurements.

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Measurements
Measurements in the study will be done over 2-days and will take place at the RCFFN or TMPLR’s mobile research unit. You will then be asked to wear a physical activity monitor for 7 days. Appointments for measurements will last approximately 2 hours each day. Prior to arriving, you will be asked to fast (not eat) 10 to 12 hours before each appointment. No alcoholic beverages are to be consumed within 48 hours prior to blood draws during the study and no caffeinated beverages are to be consumed within 12 hours prior to blood draws during the study’s first 2 days. Anthropometric measurements, weight, height, waist and hip circumference, will be taken after blood draw.

During your first visit, we will request your Personal Health Identification Number (PHIN) to link your information with administrative health records (including hospital discharge abstract, physician billing claims, and prescription record). In order to explore early life exposures we will link your data with maternal pregnancy data such as maternal nutrition, smoking in pregnancy, birth weight and breastfeeding. Additionally, with your PHIN we will be able to link to your administrative health records data in the future to determine if any of the measurements we collect in the study are associated with future health outcomes. Also, we will ask for your authorization to contact your mother. This will allow us to investigate the independent and combined effects of early-life exposures and identify early-life factors, adult lifestyle, genetics and gut microbiome on disease risk.

Day 1
On day 1, study coordinator will interview you on medical history and general health. We will measure your body weight, hip and waist circumference; take your blood pressure in conjunction with pulse wave analysis (PWA) to assess the health of your blood vessels using Mobil-O-Graph. This will only require you to wear a blood pressure cuff, which measures blood pressure at the same time as determining your blood vessel elasticity. This will be taken in triplicate. Following this, approximately, 30 ml (3 tablespoons) of fasting blood sample will be required. Following this, you will be required to consume a small amount of deuterated water, tagged water, (about 2-3
The movement of this tagged water within your body over a 24 period will permit assessment of the change in fatty acid and cholesterol metabolism. The amount of tagged water that is being given is non-radioactive, non-toxic, and do not pose any health risk to you.

You will have the option to come later in the day to complete tests after the blood sample and consumption of deuterated water. The dual energy x-ray absorptiometry (DXA) scan may actually be scheduled within the next 2 weeks if you cannot be scanned today.

After station one and two are completed, we will measure your body composition and bone density using a procedure called dual energy x-ray absorptiometry (DXA). For this procedure, you will need to lie in a horizontal position for about 5-15 minutes while the scan arm passes from your head to your feet. The radiation from this test is very low dosage (equivalent to approximately 1 day of natural background radiation). The dosage is approximately 400 times less than the exposure for a dental bitewing x-ray. You will be asked not to wear anything metal (metal may affect bone density values which will affect body composition calculations). In addition, you will need to ensure that you have not undergone barium tests/exams, or a nuclear medicine scan or injection with an x-ray dye within two weeks prior to your DXA scans. If you are female and are not post-menopausal, you will be asked to take a pregnancy test prior to beginning the study. Subsequently, you will be asked to complete on site a General Health questionnaire with the research personnel. At the end of your appointment, the research personnel will provide a sheet of instructions regarding stool and urine collection. You will be required to bring those samples on your second appointment. Also, you will be given a unique identification code, to log on into the TMPLR secure online portal to answer physical activity, depression, exhaustion and unintentional weight loss questionnaires or these questionnaires will be provided to you on paper to fill out and return.
The Manitoba Personalized Lifestyle Research (TMPLR) study

<table>
<thead>
<tr>
<th>Station</th>
<th>Day 1</th>
<th>Estimated time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Consent process</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Anthropometric Measurements</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>PWArPWV and blood pressure</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Fasting blood samples</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Oral administration of deuterium</td>
<td>5</td>
</tr>
</tbody>
</table>

Participants will have the option of coming later on day 1 to complete tests

<table>
<thead>
<tr>
<th>Snack will be provided</th>
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<tbody>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

| **Total time** | 105 |

Day 2
On your second day of measurements, stool and urine that you collected will be taken by the study staff. The urine samples will undergo analysis for glucose, albumin, creatinine, melatonin, total protein and metabolomics profiling. Your stool sample will be used to analyze your gut microbiota, which is the complex community of bacterial species that live in your digestive tract. Your blood pressure and blood vessels health will be assessed by Mobil-O-Graph, for a second time. This will be taken in triplicate. Following this, approximately 30 ml (3 tablespoons) of fasting blood sample will be required. After this, the Sub-maximal cardiopulmonary fitness test will be performed in the physical activity station. You will be asked to wear a small spirometer while riding a stationary bike. This test will measure the volume of oxygen that is being consumed during the exercise.

Subsequently, you will go to the physical activity assessment station. Physical activity will be assessed with three components: Modified Fried Criteria, questionnaires and an Actigraph (activity monitor). "Modified Fried Criteria", will look into walking speed (5m gait speed), strength (grip strength), and unintentional weight loss, questionnaires will assess depression, cognitive function and level of physical activity. The 5m gait speed, grip strength (muscular strength), and Montreal Cognitive Assessment will be completed.

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onsite. The other questionnaires will be completed online. At the end of your second appointment you will be asked to wear an activity monitor around the waist. These small devices are about the size of a wrist watch and can be worn on a belt or with special belts that are made for the monitors. These devices measure movement and ambient light, and this data will be used to measure 24 hour physical activity, energy expenditures, and sleep/wake measurements. You will be asked to wear the activity monitor for 7 days. After this period of time you will bring it back to the RCFFN. Once you bring the accelerometer back, the data stored on the devices will be downloaded and saved under code, and the data on the device will be deleted.

<table>
<thead>
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<th>Estimated time (min)</th>
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<td>PIWA/PWV and blood pressure (Mobil-O-Graph)</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Fasting blood samples</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Physical capacity (Assessing Frailty using the Modified Fried Criteria)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Sub-maximal cardiorespiratory fitness test (YMCA submaximal cycle ergometer)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Snack can be provided after physical test</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Questionnaires (frail scale, obesity history, mindful eating quest, three factor eating questionnaire, Pittsburgh sleep quality index)</td>
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</tr>
<tr>
<td>5</td>
<td>Instructions for activity monitor</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><strong>Total time</strong></td>
<td><strong>100</strong></td>
</tr>
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</table>

**Risks and Discomforts**

As with any trial, there may be as yet unknown or unforeseen risk of taking part. Some known risks, although rare, are associated with placing needle into the 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 our research personnel will be available at 204-480-1042.

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Benefits
There may or may not be direct benefit to you from participating in this study. We hope the information learned from this study will benefit Manitobans in prevention of obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease and kidney diseases. In addition, the research team will provide you with your information from the tests performed such as the DXA scan, Mobil-O-Graph, physical activity test (Sub-maximal cardiorespiratory fitness test), and muscular strength (hand grip) at the end of the 2nd day. Subsequent test results, from the blood-samples will be provided to you as the analyses are completed, this may take over a year for certain analyses.

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

Payment for participation
You will receive up to a maximum of $100.00 at completion of this study for your time. This amount will be provided after day 7, once activity monitor is handed in and questionnaires completed.

Alternatives
You are not obligated to participate in this study. The study coordinators and principal investigator will answer any questions you have about the experimental group of this study.
The Manitoba Personalized Lifestyle Research (TMPLR) study

Confidentiality
Study records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. The Health Research Ethics Board at the University of Manitoba and the Saint Boniface Hospital may also review your research-related records for quality assurance purposes. 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. Study samples will be stored in a locked freezer at the RCFFN, some samples will be shipped to other specialized laboratories elsewhere in Canada. Only the study staffs and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 10 years after the completion of this study (March 2029), and samples will not be shared with any other group, other than is indicated in the protocol, without your prior specific consent, unless you give consent for longer term storage of samples. All physical records will be kept in a locked secure area and only those persons identified will have access to these records. All records will be coded, your identification linking you to your code will be kept separately from any other records, also in a secure locked area. TMPLR study data that is entered via our secure online portal travels through servers located at FunctionFour Ltd. (141 Bannatyne Ave #101, Winnipeg, MB R3B 0R3), before being saved in a server located at the RCFFN. During the process of turning our paper record into a digital format, TMPLR study data will also leave the RCFFN and travel through servers located at FunctionFour Ltd., during this time any identifying information, such as name or address or PHIN will be encrypted, or scrambled, so that it cannot be identified without the use of a unencrypting key which will be kept at the RCFFN. If you consent to providing your PHIN, the digital TMPLR study data, which will be coded, will leave the RCFFN, via a password protected encrypted digital storage device, to be linked with administrative data at the Manitoba Center for Health Policy (MCHP). At the MCHP TMPLR data will stay on secure server for a period of 7 years and then will be destroyed in a secure fashion in accordance with MCHP policy. Due to the experimental nature of many of the planned analyses, it will not be possible to inform you, or your own doctors, of all the results of any tests.

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including genetics tests on your samples. No information revealing any personal information such as your name, address or telephone number will leave Richardson Centre for Functional Foods and Nutraceuticals except in an encrypted format as outlined above.

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. If the study staff feels that it is in your best interest to withdraw you from the study, they 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.
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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 research personnel. You are not waiving any of your legal rights by signing this consent form or releasing the investigator or the sponsor from their legal and professional responsibilities.

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

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<th>Dr. Peter Jones</th>
<th>Tel No.</th>
<th>204 474 9787</th>
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<tr>
<td>Coordinator:</td>
<td>Dr. Dylan Mackay</td>
<td>Tel No.</td>
<td>204 782 8124</td>
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For questions about your rights as a research subject, you may contact:
The Health Research Ethics Board, University of Manitoba at 204 789 3389
Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent
I have read this consent form. I have had the opportunity to discuss this research study with Dr. 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 statements or implied statements. Any relationship (such as employer, supervisor 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 study 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 any of my records that relate to this study by The Health Research Ethics Board at the
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University of Manitoba and St. Boniface Hospital for quality assurance purposes. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Consent
1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.
2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.
3. I agree to cooperate fully with the study staff and will let them know if I experience any injuries during the study.
4. I am free to withdraw from the study at any time, and for any reason.
5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.
6. By signing and dating this document, I am aware that none of my legal rights are being waived.

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

Mothers contact
Optional
I agree that my mother will be contacted by TMPLR research team to □ Yes □ No take part in the retrospective study section of this study

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**PHIN number access**

*Optional*

I agree to provide my Personal Health Identification Number (PHIN) for retrospective and prospective study section of the TMPLR program  □ Yes  □ No

**Future Contact**

*Optional*

I would like to be contacted after completing my sessions and required questionnaires about potential follow up participation opportunities  □ Yes  □ No

I would like to be contacted about updates on the TMPLR program as well as its overall findings  □ Yes  □ No

**Please send me notifications by:**

□ Email to the following account

__________________________________________

□ Post mail to the following address:

Address: ____________________________________

City: _________________________________________

Postal Code: ________________________________

**Long term storage (questionnaire data)**

We would like you to consider allowing us to store your questionnaire data, including your PHIN for a maximum of 25 years for the purposes of future analyses related to lifestyle and chronic disease risk, other than that currently planned for the TMPLR project. In the study of chronic diseases new analyses are always being developed, some of these analyses may be of interest to TMPLR investigators in the future and...

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could provide new information on chronic disease in Manitoba. Your data will be stored for a maximum of 25 years. There is no risk involved in allowing long-term data storage. It does not require any additional visits. You will not receive any financial compensation for allowing your questionnaire data to be stored, or for any discoveries made using these data.

By accepting this request to allow your data to be held for 25 years, you are making and enormously valuable contribution to a resource health research. In a study such as TMPLR, the samples and information on questionnaires that are collected have the potential to be an invaluable resource for future research. If you choose not to provide consent for longer term storage of your questionnaire data your data that is found in a physical format will be destroyed in a secure manner and your data stored in a digital format will be deleted 10 years after the completion of this study (March 2029).

Optional
I agree that my questionnaire data may be stored for a maximum of 25 years  □ Yes  □ No for future TMPLR analyses,

Participant signature: ___________________________ Date: __________

Printed name of above: ___________________________

Research staff signature: ___________________________ Date: __________

Printed name of above: ___________________________ Study role: __________

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

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Participant Initials: _____

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