Nutrigenetic Investigations into Vitamin C and Plant Sterols

by

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Abstract

This thesis focuses on the development and conduct of nutrigenetic studies: beginning with a literature review of ascorbic acid from a nutrigenetic perspective; to the protocol of an observational cohort study that could be utilized to find nutrigenetic associations; and finally to results of clinical trial investigating the responsiveness of LDL cholesterol to the consumption of plant sterols within a pre-defined genoset previously associated with plant sterol response. The convergence of large datasets pertaining to variations in genetics, nutrient status, and health status have resulted in an enormous number of putative associations between these variables. Findings from the literature review of the nutrigenetics of ascorbic acid in human health and disease found inconsistent effects of genetic associations related to ascorbic acid status. The likely source of this issue is that these hypotheses of these genetic associations are often developed and tested from *post hoc* analyses of observational cohort studies. A solution to these inconsistencies may be to progress nutrigenetics research into clinical intervention trials. As an example a randomized two-period cross-over clinical trial using *a priori* genetic recruitment of participants to determine if genotypes were predictive of a biological response to nutrients, a was conducted. In this trial participants consumed 2 g/day of plant sterols per day or placebo for 28 days and low density lipoprotein cholesterol response was measured. The hypothesis was that the combined *APOE*-(ε3/ε3) and *CYP7A1*-(T/T) genoset would predict non-response of LDL cholesterol to plant sterol consumption. 42 participants of 23 to 68 years of age completed the trial. Reductions in LDL cholesterol were consistent across all genoset groups indicating that the *APOE*-(ε3/ε3) and *CYP7A1*-(T/T) genoset is not predictive of non-response of LDL cholesterol to plant sterol consumption. Recruitment of participants that were genotyped *a priori* into a randomized clinical trial was unable to validate previous observations with respect to these genotypes, likely indicating that the initial associations were spurious.

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Dedications

I dedicate this thesis to my family.

To Ming for putting up with my incessant complaining and your support.

To my parents Betty and Norman,

thank you for your unconditional love and support.

Contributions of Authors

Manuscript 1

Dietary Vitamin C in Human Health

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Manuscript 2

The Manitoba Personalized Lifestyle Research (TMPLR) study protocol: a multicentre bidirectional observational cohort study with administrative health record linkage investigating the interactions between lifestyle and health in Manitoba, Canada

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Dylan MacKay and Rebecca Mollard developed the original concept of the study for the original grant application with input from co-investigators. Dylan MacKay prepared the drafts of the study protocol manuscript and compiled feedback and changes from other authors. Rebecca Mollard and Matthew Granger assisted in the preparation of the study protocol manuscript. PF developed the branding and logo for TMPLR study, and the manuscript figures and tables. NCH prepared the data model and was involved in the public engagement. SB (project lead, indigenous health), HB (project lead, nutrition), JC, TAD (project lead, physical activity), PKE (project lead, genetics), EK (project lead, gut microbiome), LML (project lead, biostatistics), DEM (project lead, sleep), SBM, AR, NT, MBA (project lead, developmental origins of chronic disease), and PJJ (Director) are study co-investigators, and were all involved in writing the original grant application. All authors have carefully read, contributed to, and approved the final version of the study protocol manuscript.

Manuscript 3

Genetic basis for prediction of non-responders to dietary plant sterol intervention (GenePredict-PS): a study protocol for a double-blind, placebo controlled, randomized two-period crossover study

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Maryam Shamloo sought ethical approval and prepared the drafts of the manuscript and compiled feedback and changes from other authors. Matthew Granger assisted with the draft manuscript preparation and feedback. Dylan MacKay designed the study protocol and sought funding and ethical approval. James House contributed to the development of the study protocol, ethical approval, and manuscript. Dylan MacKay designed the selection criteria of the participants. All authors contributed to and made critical revisions to the final manuscript before submission. All authors read and approved the final manuscript.

Manuscript 4

Genosets for APOE and CYP7A1-rs3808607 variants do not predict low-density lipoprotein cholesterol lowering upon intervention with plant sterols – results of the Genetic Basis for Prediction of Non-responders to Dietary Plant Sterol Intervention (GenePredict-PS) a double-blind, placebo-controlled, randomized two-period crossover trial

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The authors' responsibilities were as follow: Dylan MacKay, James D. House, and Elke A. Trautwein designed the study protocol, sought funding, and submitted ethics for approval; Itzel Vazquez-Vidal, Matthew J. Granger, and Maryam Shamloo conducted the clinical trial and collected the data; Maryam Shamloo and Matthew J. Granger analyzed all the samples; Itzel Vazquez-Vidal and Matthew J. Granger conducted the statistical analysis and wrote the paper; James D. House and Dylan MacKay share responsibility for the final content of this manuscript. All the authors read and approved the final paper.

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Abbreviations

ACC	American College of Cardiology
AHA	American Heart Association
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
APOE	Apolipoprotein E
ASA24	Automated Self-Administered 24 hour Dietary Assessment Tool
AST	Aspartate Aminotransferase
BREB	Biomedical Research Ethics Board
BUN	Blood Urea Nitrogen
BMI	Body Mass Index
CAD	Canadian dollars
CHD	Coronary heart disease
CKD	Chronic Kidney Disease
CVD	Cardiovascular Disease
CYP7A1	Cholesterol 7 Alpha-Hydroxylase
DHQ	Diet History Questionnaire
DNA	Deoxyribonucleic Acid
D2O	Deuterium Water
DSM	Diagnostic Services Manitoba
DXA	Dual-energy X-ray absorptiometry
eGFR	Estimate glomerular filtration rate
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
GC-FID	Gas chromatography with flame ionisation detection
GGT	Gamma-Glutamyl Transferase
GLO	L-gulonolactone oxidase
GST	Glutathione S-transferase
GSTM1	Glutathione S-transferase Mu 1
GSTO	Glutathione S-transferase Omega
GSTT1	Glutathione S-transferase Theta 1

HbA1c	Hemoglobin A1c
HDL	High Density Lipoprotein
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPV16	Human papillomavirus 16
Hp	Haptoglobin
KOH	Potassium hydroxide
LDL-C	Low Density Lipoprotein Cholesterol
MCHP	Manitoba Centre for Health Policy
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
NCS	Non-cholesterol sterols
NFH	Nutritional Fundamentals of Health
PHIN	Personal Health Identification Number
PS	Plant Sterols
RCFFN	Richardson Centre for Functional Foods and Nutraceuticals
RDA	Recommended dietary allowance
rRNA	Ribosomal ribonucleic acid
SAS	Statistical Analysis Software
Se	Selenium
SLC23A1	Solute carrier family 23 member 1
SLC23A2	Solute carrier family 23 member 2
SOGH	Seven Oaks General Hospital
SNPs	Single-Nucleotide Polymorphisms
T2D	Type 2 diabetes
TC	Total Cholesterol
TG	Triglycerides
TMPLR	The Manitoba Personalized Lifestyle Research study
TMS	Tri-Methylsylation
TV	Television
VLDL	Very Low-Density Lipoprotein

Zn	Zinc

Chapter 1
Overall Introduction

1.1 Introduction

Since the completion of the Human Genome Project in 2003 advancements in DNA sequencing and other technologies have enabled researchers to begin asking very specific questions regarding what our genome means. In the field of human nutrition, this has led to the creation of two primary fields connecting human genetics, nutrition, and health. The first is nutrigenomics which is the study of how nutrients affect the expression of our genome; the second is nutrigenetics which investigates the role that genetic variations related to nutrient metabolism affect health and disease.[1] The combined goal of these fields is to allow the creation of a diet or nutrient prescription for an individual based on their personal genome in hopes of optimizing their health status, this is called precision nutrition.[2] The two nutrients that will be the primary focus of this thesis are ascorbic acid (AA), also known as vitamin C, and compounds which are collectively known plant sterols or stanols (PS).

Ascorbic acid is a water soluble vitamin found in fruits and vegetables including peppers, acerola, rose hips, guava, currants, kiwifruit, citrus fruits, mustard spinach, kale, and broccoli.[3,4] While many eukaryotes have retained the ability to synthesize AA, many species have lost this ability, namely homo sapiens.[5] The inability to synthesize AA is due to the loss of seven of the twelve exons due to the accumulation of numerous mutations in the L-gulonolactone oxidase (*GLO*) gene that is responsible for catalyzing the synthesis of L-gulono-1,4-lactone to L-ascorbic acid.[6] Physiologically, AA acts as an electron donor and is known to be a cofactor for at least 15 different enzymes, including prolyl hydroxylase, lysyl hydroxylase, and prolyl 4-hydroxlyase which are involved in collagen synthesis.[7,8,9,10,11] Nutritionally, AA deficiency can result in scurvy, which can often present with non-specific symptoms such as weakness, reduced ambulation, depression, joint pain, and halitosis.[12] Eventually, due to the role of AA in collagen synthesis, deficiency of AA can results in the development of rashes on the skin and hair including petechiae, perifollicular hemorrhage, follicular hyperkeratosis, curled hairs, purpura, ecchymoses, gingivitis, anemia, and loss of teeth.[13,14,15,16] The mechanisms of transport of AA across the apical intestinal epithelia occurs via SLC23A1.[17] Transport of AA by SLC23A1 and SLC23A2 is sodium-dependent.[18] Nutrigenetically, AA is of great interest due to genetic variations in these transporters that have been associated with a range of diseases. Variations in SLC23A1 have been

implicated in follicular lymphoma and chronic lymphocytic leukemia (rs6596473), and Crohn's disease (rs10063949), while variations in SLC23A2 have been associated with non-Hodgkin lymphoma (rs6133175, rs1715385, rs1776948, rs6139587), gastric and bladder cancer (rs12479919) primary open-angle glaucoma (rs1279683).[19,20,21,22,23] Of these variations, rs6596473, rs12479919, rs1279683 have been associated with lower concentrations of plasma AA.[24] Based on this evidence, there is great research interest dedicated to elucidating these putative nutrigenetic disease associations of AA.

Given the size of the human genome, approximately 3 billion base pairs, it would be a near impossible task to test the effects of each nucleotide variation, which are estimated to number over 150 million, let alone all the possible combinations of these variations.[25,26] A single nucleotide polymorphism (SNP) is a variation in a single nucleotide, adenine, guanine, thiamine or cytosine. Depending on the frequency of the variation, these variations are classified as either SNPs if they are found in 1% or more of the population; if the variation is found in less than 1% of the population, it is referred to as a single nucleotide variation (SNV). The use of rapid genomic sequencing technology and cross-sectional, observational studies have been utilized to circumvent this problem.[27] Genome-wide association studies (GWAS) involving mass data gathering of genomic and health information have created an abundance of associations to investigate. User-friendly databases such as the GWAS catalog (https://www.ebi.ac.uk/gwas/home) have been created to facilitate the examination of publications that have reported gene-nutrient associations.[28]

One shortcoming of these bulk data collection studies is that they often omit the collection of dietary information altogether. This has contributed to the replication crisis in nutrigenetics since biomarkers related to a gene-nutrient interaction may not be detected outside of a deficiency or intervention of that nutrient to create a sufficient signal that can be measured. The other issue when the collection of dietary intake has been included in a study, is that unreliable tools such as self-reported 24-hour dietary recalls or food frequency questionnaires are often utilized and have led to widespread misreporting and implausible results of dietary and nutrient intakes.[29] The utilization of metabolomic technologies to develop food signatures from blood biomarkers to provide accurate nutrient intake data is one proposed solution that is currently in development.[30,31]

Unfortunately for nutrigenetic research, “correlation is not causation” remains true regardless of the subject being referenced. The initiation of research projects based on the false premises detailed above has resulted in nutrigenetic research that is fraught with inconsistent findings when it comes to gene-nutrient-disease associations. The common pattern has been the discovery of a gene-nutrient-biomarker association, followed by one or more publications not observing that same association under similar conditions and within similar populations. With respect to the contents relevant to this thesis, this pattern has been observed with the following variants related to LDL-C response to plant sterol (PS) consumption: rs3808607, rs4148217, rs5882, rs2072183.[32,33,34,35]

The ability of PS to reduce cholesterol in humans has been known since the 1950’s, since then, research has sought to further elucidate the dose of PS required to elicit an effective reduction in blood cholesterol, the mechanisms by which PS affects blood cholesterol, as well as the genes and their variations that may affect this response.[36] In Canada it is estimated that up to 2.4 million people have coronary heart disease (CHD) creating a cost of $22.2 billion per year when factoring in physician services, hospitalizations, lost wages, and decreased productivity.[37,38] Given the role of elevated low-density lipoprotein cholesterol (LDL-C) in CHD risk, and the association of a reduction in LDL-C with reduced risk of CHD and cardiovascular mortality, it is imperative that interventions are applied to curtail the burden of CHD.[39,40] While statins are the recommended treatment for dyslipidemia in Canada, some patients experience musculoskeletal and possible diabetogenic side effects, creating an interest in alternative and adjunctive treatments.[41,42,43,44] Plant sterols (PS) have been assessed to be an economically feasible solution to address the healthcare costs of cardiovascular disease in Canada and the literature supports the efficacy of PS in conjunction with statin therapy.[45,46] Consumption of 2 g/day of plant sterols/stanols (PS), have not been shown to reduce CHD mortality, but have been shown to safely reduce LDL-C by 5 to 15%.[47,48] In 2010, due to the safety track record and consistent efficacy of PS in reducing LDL-C Health Canada approved a health claim for PS.[49] The mechanism that PS reduce LDL-C is through inhibiting the absorption of cholesterol in the intestine.[50] *In vitro* and animal studies of PS have also observed the induction of the ATP-binding cassette A1 (ABCA1) transporter resulting in the efflux of cholesterol from intestinal enterocytes into the lumen.[51,52,53,54] The effect of PS consumption on blood cholesterol concentrations is dose-responsive.[55] However, there is an upper limit to the safety of plant

sterols where fat soluble nutrients can become reduced, as well as a ceiling effect on blood cholesterol lowering beyond a dose of 3 g/day of PS.[56]

Despite the proven effectiveness of PS acting in a dose-response fashion to reducing blood cholesterol, there exists numerous reports of a highly variable responsiveness to PS consumption, from no response to increasing LDL-C.[32,57,58] One potential explanation for the variation in the response to PS is interindividual variability due to an individual's genetics. Dietary cholesterol is transported across the enterocyte apical membrane in the small intestine via the Niemann–Pick C1-Like 1 (NPC1L1) membrane protein.[59] From the enterocyte cholesterol is either packaged into chylomicrons, very low-density lipoproteins, or high-density lipoproteins for basolateral release into the bloodstream and lymph.[60] Previous studies investigating single nucleotide polymorphisms (SNPs) in *CYP7A1* (cholesterol 7 alpha- hydroxylase, rs3808607) and *APOE* (Apolipoprotein E, rs7412 and rs429358) have observed variability in the responsiveness of cholesterol to PS.[32,61,62,63] CYP7A1 encodes the protein that is the rate-limiting step of bile acid synthesis, while APOE encodes a glycoprotein involved in cholesterol transport signaling in LDL-C.[64,65] A previous trial investigating the effects of PS in hypercholesterolemic individuals found an association between *CYP7A1*-rs3808607-T/T and *APOE*-ε3/ε3 homozygotes with cholesterol non-responsiveness to PS.[32] For the study in this thesis (Chapters 4 and 5), we chose to investigate these genosets in an *a priori* fashion to determine if the association with non-responsiveness can be replicated in a randomized clinical trial.

1.2 Rationale

The rationale in the outline and contents of this thesis is a demonstration of the processes involved in nutrigenetic research. The first manuscript (Chapter 2), *Dietary Health and Vitamin C*, is a review of the literature regarding the physiology of AA, dietary and epidemiological data of AA with respect to the Canadian population, and data related to the nutrigenetics of AA from observational and interventional studies including the exploration of genetic associations between AA status and health status. The collection and assessment of information in that review is a necessary step prior to nutrigenetic research to get a grasp of what is and is not known of a particular subject. The second manuscript (Chapter 3) is the protocol paper from The Manitoba Personalized Lifestyle Research Study (TMPLR), which was a cross-sectional observational cohort study involving the gathering of participant data, such as: dietary, early life, lifestyle,

medical, physical activity, socioeconomic, sleep, and stress. The third manuscript (Chapter 4) is the protocol paper from the GenePredict Plant Sterol study (GPS). The presentation of this manuscript subsequent to the observational cohort study is to provide an example of how a nutrigenetic study can be designed and executed to more specifically test a putative nutrigenetic association, with respect to that chapter whether the genoset of the *CYP7A1* SNP rs3808607-(T/T) and *APOE*-ε3/ε3 will predict non-response of LDL-C to plant sterol consumption. The final manuscript (Chapter 5) is the results from GPS study. Since research investigating nutrigenetic associations of LDL-C to PS have been inconsistent, a hypothesis was put forth that perhaps the genoset mentioned above might be able to predict LDL-C response to PS consumption.

1.3 Objectives

The primary objective of this thesis is to describe the methodologies of nutrigenetic research, from the question and hypothesis phase involving the examination of the literature to the design and execution of research studies to gather data for nutrigenetic research and finally to the results of a nutrigenetic study. Within that final study, the objective was to determine whether the genoset of SNP rs3808607-T/T-APOE-ε3/ε3 predicts non-response of LDL-C to plant sterol consumption.

1.4 Hypothesis

The final manuscript in Chapter VI is the only one with a hypothesis: that the genoset of SNP rs3808607-T/T-*APOE*-ε3/ε3 will predict non-response of LDL-C to plant sterol consumption.

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Bridge to Chapter II

The following chapter comprises a manuscript which provides a broad overview of dietary vitamin C in human health with an emphasis of studies investigating the molecular mechanisms of vitamin C maintenance as well as gene–nutrient interactions modifying these relationships. This chapter is an introduction to the nutrigenetics of a well-studied vitamin. Matthew J. Granger and Peter K. Eck co-authored this manuscript.

Chapter II

Manuscript 1

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Dietary Vitamin C in Human Health

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2.1 Abstract

Vitamin C is essential to prevent scurvy in humans, and is implicated in the primary prevention of common and complex diseases such as coronary heart disease, stroke, and cancer. This chapter reviews the latest knowledge about dietary vitamin C in human health with an emphasis on studies of the molecular mechanisms of vitamin C maintenance as well as gene–nutrient interactions modifying these relationships.

Epidemiological evidence indicates 5% prevalence for vitamin C deficiency and 13% prevalence for suboptimal status even in industrialized countries. The daily intake (dose) and the corresponding systemic concentrations (response) are related in a saturable relationship, and low systemic vitamin C concentrations in observational studies are associated with negative health outcomes.

However, there is no evidence that vitamin C supplementation impacts the risks for all-cause mortality, impaired cognitive performance, reduced quality of life, the development of eye diseases, infections, cardiovascular disease, and cancers. This might be related to the fact that prevention would not be realized by supplementation in populations already adequately supplied through dietary sources.

Recent genetic association studies indicate that the dietary intake might not be the sole determinant of systemic concentrations, since variations in genes participating in redox homeostasis and vitamin C transport had been associated with lowered plasma concentrations. However, impact sizes are generally low and these phenomena might only affect individual of suboptimal dietary supply.

Keywords: ascorbic acid status, complex diseases, supplementation, genetic variation.

2.2 Introduction

Vitamin C, existing in the two main forms of ascorbic and dehydroascorbic acid, is a ubiquitous metabolite in plants and animal. Eukaryotes, plants, fungi, and most animals can synthesize L-ascorbic acid (Drouin et al., 2011). Anthropoid primates, teleost fish, bats, passeriforme birds, and guinea pigs have lost this ability, and for these species it is an essential dietary component (Menniti et al., 1986, Ohta and Nishikimi, 1999). In humans, the ability to synthesize vitamin C was lost due to mutations in the L-gulonolactone oxidase (*GLO*) gene, that is responsible for catalyzing the synthesis of L-ascorbic acid from L-gulono-1,4-lactone, the last step in the ascorbic acid synthesis pathway in mammals (Nishikimi and Yagi, 1991). A profound lack of dietary supply will result in the deficiency disease scurvy, which can be fatal (Padayatty and Levine, 2016).

Due to the presence of scurvy, vitamin C's existence was known before its molecular discovery. In his 1753 work, *Treatise of the Scurvy*, James Lind noted that consumption of citrus fruit prevents scurvy, also known in Latin as scorbutus (Bartholomew, 2002). Hence, ascorbutus was the lack of scorbutus (scurvy), and the molecular name of ascorbic acid originates there (Grzybowski and Pietrzak, 2013). The water soluble L-ascorbic acid was discovered by Albert Szent-Györgyi in 1928, and characterized as the antiscorbutic factor by Szent-Györgyi and King in 1932 (King and Waugh, 1932; Svirbely and Szent-Györgyi, 1932). The chemical structure of ascorbic acid was deduced by William Norman Haworth in 1933 (Carpenter, 2012). Since then vitamin C has been extensively researched with speculations regarding its many beneficial functions in the maintenance of health and the curing of disease (Padayatty et al., 2003; Padayatty and Levine 2016).

This chapter aims to update on the latest knowledge about dietary vitamin C in human health. The many epidemiological or dietary intervention studies scrutinizing the effects of vitamin C consumption and/or supplementation on physiological parameters, biomarkers and clinical end points are not being reviewed in great detail, since they have been reviewed elsewhere. For reviews on technical issues of research on vitamin C, such as adequate sampling and sensitivity of assays refer to Levine et al. (1999), on the role as a physiologic antioxidant refer to Padayatty et al. (2003). The main emphasis is placed on studies of the molecular mechanisms of vitamin C maintenance as well as gene–nutrient interactions modifying these relationships.

2.3 Vitamin C: Basic Physiology

The biological functions of vitamin C revolve around its ability to alter its redox state, which enables it to function as a co-factor for eight known human enzymes and as a water-soluble antioxidant (Padayatty and Levine 2016). At the physiological pH of 7.4**,** vitamin C exists as the ascorbic acid anion (**Figure 2.1**) (Padayatty et al., 2003; Padayatty and Levine 2016).

The reduced ascorbic acid loses electrons sequentially, with the loss of one electron forming the ascorbic acid radical. Compared to other radical species it has a long half-life of many seconds to minutes (Buettner, 1993) and has been measured in blood and extracellular fluid samples (Chen et al., 2007). The oxidized form of vitamin C, dehydroascorbic acid, results from the loss of a second electron, and can be recycled into the reduced form through enzyme mediated or reductive metabolic pathways (Dhariwal et al. 1990, 1991; Wang et al., 1997). Dehydroascorbic acid has a half-life of only minutes, after which it undergoes hydrolytic ring rupture, the resulting 2,3-diketogulonic acid cannot re-form its precursor and is unable to continue its role in vitamin C metabolism. Ascorbic acid and dehydroascorbic acid utilize distinct pathways for cellular entry, while ascorbic acid utilizes sodium dependent membrane transporters, dehydroascorbic acid utilizes facilitative glucose transporters.

Eight human enzymes are known to utilize ascorbic acid as a cofactor, three participate in collagen hydroxylation (Kivirikko and MyllylÄ, 1985; Peltonen et al., 1985; Peterkofsky 1991; Prockop and Kivirikko, 1995) and two in carnitine biosynthesis (Dunn et al., 1984). Of the three enzymes which participate in collagen hydroxylation, one is necessary for biosynthesis of the catecholamine norepinephrine (noradrenaline) (Kaufman et al., 1991; Levine et al., 1992), one is necessary for amidation of peptide hormones (Eipper et al., 1992, 1993), and one is involved in tyrosine metabolism (Lindblad et al. 1970; Englard and Seifter, 1986). Details about these enzymes and their functional role are described by Padayatty and Levine (2016).

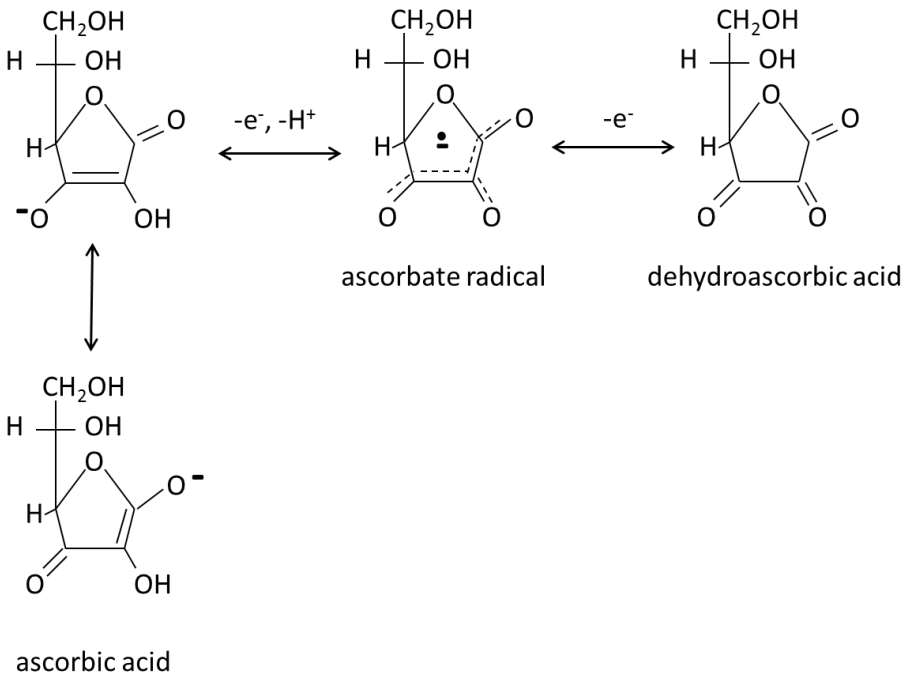

Figure 2.1. The three states of vitamin C. The reduced ascorbic acid exists at the physiological pH of 7.4 as the ascorbic acid anion. The ascorbic acid radical results from the loss of one electron and is a stable radical species, indicated by the dot. Dehydroascorbic acid results from the loss of another electron, and is the oxidized form of Vitamin C, which can be recycled into ascorbic acid through reduction by glutathione or glutathione utilizing enzymes.

As a major water-soluble antioxidant in mammalian physiology, ascorbic acid scavenges potentially harmful oxidizing free radicals and can be irreversibly oxidized in this process, unless it is recycled (Frei et al., 1989, 1990), which leads to increased dietary requirements in situations of oxidative stress. Through redox sensing it also contributes to differential gene expression and mRNA translation, which could also contribute to the prevention of oxidative damage of intracellular proteins and DNA (Hitomi and Tsukagoshi, 1996; Padayatty et al. 2003; Qiao and May, 2011; Sram et al., 2012). Plasma ascorbic acid contributes to the reduction of extracellular oxidants, increased endothelium-dependent vasodilatation, and reduced low density lipoprotein oxidation (Polidori et al., 2004; Traber and Stevens, 2011; Ceriello et al., 2013; Richards et al., 2015).

In the intestinal tract ascorbic acid increases non-transferrin mediated absorption of iron by reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), and also enhances transferrin-mediated uptake

of iron via intracellular reduction of iron (Hallberg et al., 1989; Lane et al., 2013; Lane and Richardson 2014).

Related to the above described biological functions**,** vitamin C has a role in energy-yielding metabolism, collagen synthesis, non-heme iron absorption, and normal functioning of the nervous system. In spite of our knowledge on vitamin C physiology, significant uncertainties remain in the quest to link individual variability in vitamin C metabolism to improved and individualized recommendations. This chapter will update the latest knowledge on the genetic variability influencing Vitamin C utilization and therefore recommendations.

It is well established that a severe dietary undersupply of vitamin C will result in scurvy, and many deficiency symptoms are reflected in the functions of ascorbic acid as a co-factor of known enzymes, such as defects in collagen leading to structural weakening in connective tissue (Padayatty and Levine, 2016). However, ascorbic acids role in the prevention or treatment of common and complex diseases is still uncertain. Even the widely held assumption that ascorbic acid is one of the major biological antioxidants and therefore has a prominent role in disease prevention has not been definitively validated (Padayatty et al. 2003; Padayatty and Levine 2016).

2.4 Current Benchmarks of Vitamin C Status and Dietary Recommendations

Plasma concentrations serve as the most readily available biomarker for vitamin C status. Values below 11 μmol/L specify deficiency coincide with the clinical symptoms of scurvy (Lykkesfeldt and Poulsen, 2010, Robitaille and Hoffer, 2016). The highest concentrations observed in pharmacokinetic studies are between 70-80 μmol/L (Levine et al., 1996, 2001), seldom more than 100 μmol/L has been reported (Padayatty and Levine, 2016), and concentrations plateau in that range even during very high dietary supplementation. However, concentrations as low as 28 μmol/L are considered adequate (Hoffer, 2010), and consequently values between 11 μmol/L and 28 μmol/L indicate marginal deficiency (often referred to as hypovitaminosis C), where scurvy is absent but the risk for chronic diseases is elevated.

The recommended dietary allowance (RDA) for vitamin C has been developed over the years, and the current recommendations in the US and Canada are set to 90 mg/day for adult men and 75 mg/day for adult women. They are based on maintaining near-maximal neutrophil concentrations and to elicit antioxidant protection (Monsen, 2000). Recommendations stratified by age and metabolic status are summarized in **Table 2.1**.

Table 2.1. Recommended Dietary Allowances (RDAs) for Vitamin C in the US and Canada.

Age	Male	Female	Pregnancy	Lactation
0–6 months	40 mg*	40 mg*	n/a	n/a
7–12 months	50 mg*	50 mg*	n/a	n/a
1–3 years	15 mg	15 mg	n/a	n/a
4–8 years	25 mg	25 mg	n/a	n/a
9–13 years	45 mg	45 mg	n/a	n/a
14–18 years	75 mg	65 mg	80 mg	115 mg
19+ years	90 mg	75 mg	85 mg	120 mg
Smokers	Individuals who smoke require 35 mg/day more vitamin C than nonsmokers.			

Recommended dietary allowances (RDAs) are issued if a strong scientific basis exists (Monsen, 2000).

* Adequate Intake (AI) are issued on a less validated scientific basis.

RDAs differ widely across the world, however, which reflects the lack of underlying scientific evidence. For example, for adults in the United Kingdom 40 mg/day are recommended, while in France and Belgium the benchmark lies at 110 mg/day (Birlouez-Aragon et al. 2001), and in Germany at 110 mg/day for males and at 95 mg/day for females (Bechthold et al., 2015).

Due to the large variability in RDAs worldwide, it has been suggested that vitamin C intakes above current RDAs could contribute to the prevention of chronic diseases, in particular cardiovascular diseases (CVD)—principally coronary heart disease (CHD) and stroke—and certain cancers (Frei et al., 2012).

2.5 Vitamin C Status in the General Population

Studies describing the relationship between dietary intake and the plasma concentrations of vitamin C generally show that both low intake and plasma concentrations are common. Available data from the US show mean plasma ascorbic acid concentrations of 48 μmol/L in males, and 54.8 μmol/L in females (Schleicher et al., 2009). However, 8.2% of males and 6% of females had plasma vitamin C concentrations below the deficiency threshold of 11 μmol/L. Among men,

18% of smokers had values below this threshold while only 5.3% of non-smokers had such low values. Among women, 15.3% of smokers and 4.2% of non-smokers had similarly low values (Schleicher et al., 2009).

Around 5.5% of the Canadian general population who did not use supplements had deficient plasma vitamin C concentrations of less than 11 μmol/L (**Figure 2.2**) (Langlois, Cooper et al. 2016), while approximately 11.6% of smokers, 5.8% overweight individuals had deficient vitamin C concentrations. About 13.5% of non-supplement users had marginal deficient plasma vitamin C concentrations between 11-28 μmol/L, while 17.6% of smokers, 9% of non-smokers, 17% of obese individuals, 8.2% of overweight individuals had marginal deficient plasma vitamin C concentrations (Langlois et al., 2016).

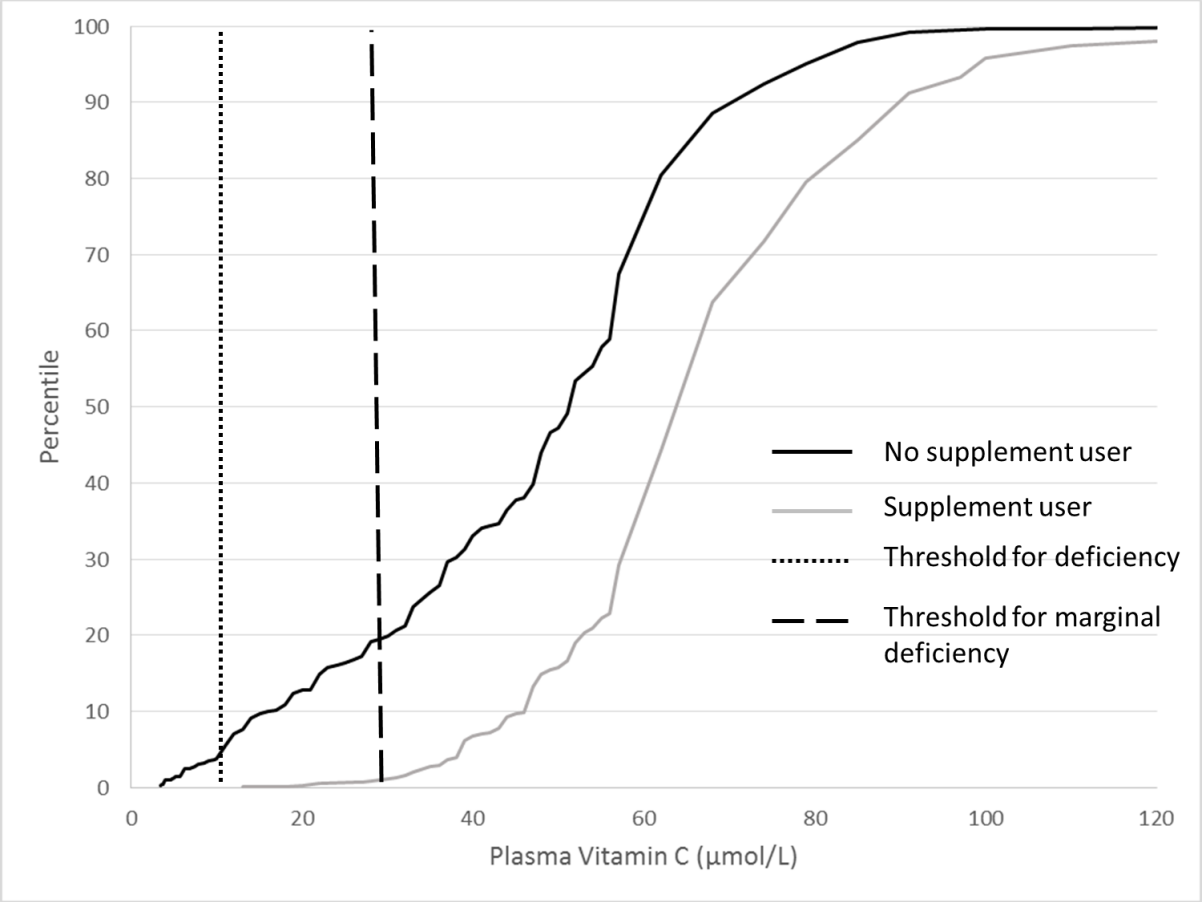

Figure 2.2. Plasma vitamin C concentrations of Canadians. Data sourced from the 2012/2013 Canadian Health Measures Survey (Langlois et al., 2016).

Even in industrialized countries, marginal deficiency or hypovitaminosis C can have a prevalence of about 15% of the general population (Lindblad et al., 2013), 30% of cigarette smokers (Schectman et al., 1991, Pfeiffer et al., 2013), and 60% of hospitalized individuals (Hunt, Chakravorty et al. 1994, Teixeira, Carrie et al. 2001, Fain, Paries et al. 2003, Gariballa and Forster 2006, Gan, Eintracht et al. 2008, Evans-Olders, Eintracht et al. 2009, Zhang, Robitaille et al. 2011, Wang, Liu et al. 2013). The exact health implications of marginal deficient vitamin C status remain unknown, but clinical symptoms may include fatigue or mood disruption (Crandon, Lund et al. 1940, Zhang, Robitaille et al. 2011, Wang, Liu et al. 2013), decreased immunity (Anthony and Schorah, 1982; Hunt et al., 1994; Hemila and Louhiala, 2007), impaired wound healing (Lund and Crandon 1941; Sorensen et al., 2010; Blass et al., 2013), localized pain (Shibuya, Humphers et al. 2013) and cardiovascular disease (Vita et al., 1998; Padayatty and Levine 2000, Frei, Birlouez-Aragon et al. 2012, Juraschek et al., 2012; Rodrigo et al., 2013).

2.5.1 Associations of Vitamin C with Health Outcomes in Observational Studies

Since outcomes in major common and complex diseases are potentially modified by the Vitamin C status, large scale observational studies have examined these relationships. In the following sections the major observations for the most detrimental common and complex diseases, namely cardiovascular disease and cancers, are summarized. It should be noted that men with marginal deficient serum vitamin C concentrations below 28 μmol/L had a 57% higher risk of all-cause mortality after 12–16 years of follow-up than men with the highest vitamin C concentrations above 74 μmol/L, creating the rationale to try to relate this to today's major health problems.

Many observational studies assessed the association between dietary vitamin C intake and the risks of common and complex diseases. However, some of these observational studies did not consider biomarkers of vitamin C status such as plasma concentrations. The following section summarizes studies reporting on the biomarkers of vitamin C status, thereby eliminating the uncertainty of dietary assessments.

2.5.2 The relation of vitamin C to cardiovascular diseases in human observational studies

Serum vitamin C concentrations above 45 μmol/L (45.4 and 79.5 μmol/L) lowered the risk of cardiovascular disease by about 25% compared to individuals with concentrations under 23 μmol/L (Simon et al., 2001). Similarly, a 33% lowered risk for coronary heart diseases was

associated in subjects with mean plasma vitamin C concentrations of 77 μmol/L compared to those with 27 μmol/L (Boekholdt et al., 2006). Moreover, plasma vitamin C concentrations were inversely related to mortality from all causes and CVD (Khaw et al., 2001), where each 20 μmol/L increase in plasma vitamin C was associated with a 20% and 30% reduction in all-cause and CVD mortality, respectively. Concurring evidence was reported for associations of increased vitamin C plasma or serum concentrations (means ranging from 49.5 to 85.2 μmol/L) and decreased CVD risks (Nyyssonen et al., 1997; Simon et al., 1998; Langlois, Duprez et al. 2001). Consistent with these findings, in young Type 1 diabetic patients, poor vitamin C status was found to be associated with an increase in several early cardiovascular risk markers (Odermarsky et al., 2009).

A recent systematic review on the relationship between vitamin C and heart health concluded that in populations with already adequate vitamin C intake, further supplementation with vitamin C did not correlate with the risk of CVD (Moser and Chun, 2016). However, if dietary supply was suboptimal, risks for CVD were elevated (Moser and Chun, 2016).

Older adults with plasma vitamin C concentrations above 28 μmol/L had 30% less deaths from strokes (Gale et al., 1995), and a 42% lower incidence of stroke occurred when plasma concentrations were above 66 μmol/L compared to individuals with vitamin C concentrations below 41 μmol/L (Myint et al., 2008). Similarly, subjects with vitamin C concentrations above 64 μmol/L had a 41% lower risk of stroke than those with vitamin C below 40 μmol/L (Yokoyama et al., 2000). Echoing these data, individuals with plasma concentrations below 28 μmol/L had a two-fold increased risk of stroke compared to individuals with concentrations above 65.0 μmol/L.

Contrary to the positive effects reported, cardiovascular disease mortality increased upon vitamin C supplementation in postmenopausal women with diabetes (Lee et al., 2004), indicating that cases of specific pathologies should be viewed separate from the general population.

In conclusion, observational studies have produced mixed results regarding the relationship of vitamin C and cardiovascular diseases. Generally, no relationship between vitamin C and cardiovascular disease risk was observed at optimal plasma vitamin C concentrations, but suboptimal concentrations seem to be associated with elevated cardiovascular disease risks.

2.5.3 The relation of vitamin C to cancers in human observational studies

More limited evidence exists for a relationship between vitamin C status and the prevention of certain cancers. In a case-control study, individuals with plasma concentrations above 51

μmol/L had a 45% lower risk of gastric cancer compared to individuals with concentrations below 29 μmol/L (Jenab, Riboli et al. 2006). Men with serum vitamin C concentrations below 28 μmol/L had a 62% higher risk of cancer-related deaths after 12–16 years of follow-up than men above 73.8 μmol/L (Loria et al., 2000). Men with mean plasma vitamin C concentrations of 73 μmol/L had a 53% reduced cancer mortality compared to those of 21 μmol/L (Khaw et al., 2001).

Very low vitamin C status was also reported for cases of multiple myeloma (Sharma et al., 2009) and unspecified advanced cancers, where it was associated with increased inflammation and lower survival (Mayland, Bennett et al. 2005). However, in those case-control studies it was undetermined if reduced concentrations are the consequence of rather than the cause of the reported associations.

In conclusion, observational studies indicate that vitamin C plays a role in the prevention of gastric cancer, while the role in the prevention of other cancers is indicated, but requires further validation.

2.5.4 Human Intervention Studies Supplementing Vitamin C

The epidemiologic associations between adequate vitamin C status and decreased risk of CVD and cancers inspired controlled intervention studies to determine if a causal relationship exists. Those intervention studies regularly used vitamin C supplementation in combination with other "antioxidant vitamins" (most often vitamin E and β-carotene), or as part of a multivitamin-mineral mix, which would complicate the interpretation of positive findings. However, if intervention studies report no effect of vitamins supplementations then they would be considered ineffective.

Many trials have used interventions with vitamin C in relation to specific outcomes in patients populations, such as HIV (Guwatudde et al. 2015), type 2 diabetes (Shateri, Keshavarz et al. 2016), depressive disorders (Sahraian et al., 2015), and end stage renal diseases (Biniaz et al., 2014; Shateri et al., 2016). Those small and targeted studies will not be discussed in this chapter, which rather focuses on large-scale long-term studies on health outcomes in the general population. Within these intervention studies, cardiovascular outcomes and cancers had been a targeted most often.

2.5.5 Human intervention studies and health outcomes in common and complex diseases

Major human intervention trials with vitamin C did not show improvements in all-cause mortality (Hercberg et al., 2004), eye health (age-related macular degeneration, age-related cataract, lens opacity, or vision loss) (Age-Related Eye Disease Study Research, 2001; Christen et al. 2012), cognitive performance (Grodstein, O'Brien et al. 2013), infections (Girodon, Galan et al., 1999; Graat et al., 2002; Avenell et al., 2005), and overall quality of life (Avenell et al., 2005).

One clinical trial of moderate size reported the improvement of serum pepsinogen (PG) concentrations, which serve as a biomarker for the progression of gastric mucosal atrophy during *Helicobacter pylori* infection in a group highly supplemented with vitamin C (500 mg/day) versus the low supplemented group (50 mg/day) (Sasazuki et al., 2003).

In conclusion, vitamin C supplementation did not modify all-cause mortality, cognitive performance, overall quality of life, the development of eye diseases, and infections.

2.5.6 Human intervention studies and cardiovascular outcomes

Major coronary events and fatal or non-fatal vascular events (2002) as well as incidences of myocardial infarction, stroke, coronary revascularization, or CVD death were not affected by supplementation with vitamins C + E + β-carotene (Cook et al., 2007). Similar supplementation with vitamins C + E + β-carotene + Zn + Se did not protect from ischemic cardiovascular disease and all-cause mortality (Hercberg et al., 2004). Vitamin C combined with vitamin E did not reduce any cardiovascular events, myocardial infarction, stroke, or death from cardiovascular diseases (Sesso et al., 2008), and did not affect atherosclerotic lesions and carotid artery intima-media thickness (Salonen et al., 2003) or changes in minimum lumen diameter of coronary arteries (Waters et al., 2002). Moreover, cerebrovascular diseases were not reduced upon daily supplementation with 14 vitamins and 12 minerals (Li et al., 1993).

Overall, the attempt to prove causal relationships in controlled clinical feeding trials have negative results for cardiovascular diseases (Moser and Chun, 2016).

2.5.7 Human intervention studies and cancer outcomes

Vitamin C supplementation did not affect incidences or outcomes for major gastrointestinal cancers or overall cancer mortalities in the majority of studies. Specifically, supplementation with vitamin C and molybdenum (Blot et al., 1993), or vitamins C + E + β-carotene + Zinc + Selenium

(Hercberg et al., 2004) did not reduce overall cancer incidence and disease-specific mortality. Similarly, interventions with vitamin C, vitamin E, β -carotene and selenium did not reduce gastric cancer and overall cancer mortality (You et al., 2001). Daily supplementation with 14 vitamins and 12 minerals did reduce esophageal and gastric cancer deaths or total cancer incidences (Li et al., 1993). Occurrences of colorectal adenomas (Greenberg et al., 1994; Gaziano et al., 2012) or the progression of multifocal non-metaplastic atrophy or intestinal metaplasia (Correa et al., 2000) were not correlated with multivitamin-mineral and β-carotene + vitamins C + E supplementation, respectively.

Vitamin C combined with vitamin E did not reduce the risks for prostate and total cancer (Gaziano et al., 2009); similarly, multivitamin supplementation did not reduce the risk for prostate, colorectal and other site specific cancers, however, reduced the risk of total cancer (Gaziano et al., 2012).

One solitary study reported that the positive association between red and processed meat intakes and breast cancer risk was eliminated upon supplementation with vitamins C + E + β-carotene + Zinc + Selenium (Pouchieu et al. 2014). Otherwise, all major intervention studies failed to prove a positive modification of cancer incidences and outcomes.

2.5.8 Human intervention studies and the importance to consider the dose-concentration relationship for vitamin C

Most evidence reported from large epidemiological cohorts, dietary intervention studies, as well as meta-analyses remain ambiguous, with many reporting that vitamin C supplementation had little or no effect on outcome measures (Moser and Chun, 2016; Padayatty and Levine, 2016). Specifically, most intervention studies do not show positive effects, while low systemic vitamin C concentrations in observational studies are associated with negative health outcomes. The explanation for this phenomenon might lay in the saturable relationship between daily intake (dose) and the corresponding systemic concentrations (response). In individuals with depleted plasma vitamin C, concentrations increased only modestly after small doses of up to 30 mg/day but increased more rapidly upon larger doses up to 100 mg/day reaching a plateau at about 200 mg/day (**Figure 2.3**). Consequently, in studies where the control and treatment groups already have an adequate supply at baseline, plasma and cellular concentrations might not differ even upon interventions with high doses of vitamin C, eliminating the chance of any demonstrable effects.

Therefore, the effect of any supplementation would only be observed in groups undersupplied through the basal diet.

This phenomenon is exemplified by a recent meta-analysis on the effects of vitamin C supplementation on glycemic control, where overall no effects were observed on biomarkers of dysglycemia, as measured by glucose, HbA1c, and insulin concentrations (Ashor, Werner et al. 2017). However, in patients with type 2 diabetes, the high blood glucose concentrations were reduced upon vitamin C supplementation (Ashor et al., 2017), which could be attributed to improvements in the low vitamin C status of diabetic individuals (Chen et al., 2006; Tu et al., 2015).

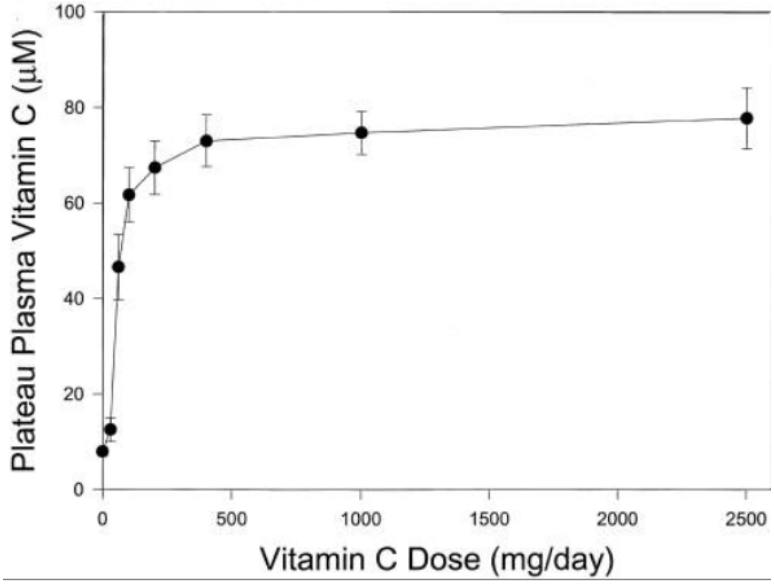

Figure 2.3. Vitamin C concentrations as a function of the daily dose (Levine et al., 2001).

2.6 Genetic Influences on Vitamin C Metabolism and Disease Pathology

An individual's vitamin C status depends on the absorption, distribution, and metabolism of ascorbic acid where all of these processes may differ depending on genetic variations. Two genes encoding sodium-dependent ascorbic acid transporters have been identified and polymorphisms in these have been associated with reduced systemic vitamin C concentrations and several diseases. The associations of polymorphisms in the ascorbic acid transporter genes to biomarkers of the vitamin C status and the disease associations will be discussed in separate sections, since it is currently unclear how they correlate. Polymorphisms in two glutathione S-

transferases and haptoglobin had been associated with reduced vitamin C status and those will be summarized in separate sections; however the disease associations for these genes will not be discussed, since their direct involvement in the ascorbic acid metabolism is unclear and correlations to the vitamin C status speculative at best.

2.6.1 Genetic variations in the SLC23A1 gene associated to altered vitamin C status

The *SLC23A1* gene encodes the sodium-dependent ascorbic acid transporter SVCT1 (SLC23A1) which plays a key role in the renal reabsorption of vitamin C (Daruwala et al., 1999; Tsukaguchi et al., 1999; Wang et al., 2000). Genetic variations in *SLC23A1* could lower the renal threshold for ascorbic acid reabsorption and therefore increase urinary losses and decrease steady-state plasma and body vitamin C concentrations (Corpe et al., 2010).

Five alleles of single Nucleotide Polymorphisms (SNPs, variations with frequencies over 1%) in the *SLC23A1* gene have been associated with lowered serum ascorbic acid concentrations; however some genotypes associated differentially, being associated to elevated concentrations in one study and decreased concentrations in others.

The average serum ascorbic acid concentrations were 5.3 μmol/L lower for SNP rs4257763-GG homozygotes in a setting indicating suboptimal values across this cohort (Cahill and El-Sohemy, 2009). Moreover, the SNP rs33972313-G, rs10063949-A, and rs6596473-C alleles were associated with 4.2 μmol/L, 2.9 μmol/L, and 2 μmol/L reductions in circulating concentrations of L-ascorbic acid, respectively (Timpson et al., 2010). These findings could not be replicated for the SNP rs10063949-A allele, but were replicated for the rs33972313-G and rs6596473-C alleles with associated reductions of 8.3 μmol/L and 1 μmol/L, respectively (Timpson et al., 2010). A subsequent pooled analysis across 5 studies associated each additional rs33972313-G allele with a 6 μmol/L reduction in circulating vitamin C. In stark contrast to these results, the rs33972313-G allele was associated with a 3.1 μmol/L elevation in plasma vitamin C concentrations (Kobylecki et al., 2015), where GG homozygotes showed increases of 7.3 μmol/L and AG heterozygotes 4.1 μmol/L plasma vitamin C concentrations, respectively. Similarly, rs33972313-GG homozygotes had plasma ascorbic acid concentrations 6 μmol/L higher than those of GA heterozygotes, while AA homozygotes were found in this study (Duell et al., 2013).

SNP rs11950646-GG homozygotes had 8 μmol/L lower plasma vitamin C concentrations compared to AA heterozygotes, while a 3 μmol/L decrease in plasma vitamin C concentrations was observed in GA heterozygotes (Duell et al., 2013), indicating an allele dosage effect.

To conclude, emerging evidence indicates a role of genetic polymorphisms in the modulation of biomarkers of vitamin C status. However, the inconsistencies in the results need to be addressed in future controlled trials.

2.6.2 Genetic variations in the SLC23A1 gene associated with common and complex diseases

Several variations in the *SLC23A1* gene have been associated with increased risks of certain types of cancers, Crohn's disease, spontaneous preterm delivery, and aggressive periodontitis.

An 80% elevated risk of Non-Hodgkin lymphoma was demonstrated for homozygotes for rs6596473-CC and rs11950646-GG (Skibola et al., 2008). A 150% increase in the risk for Crohn's disease was associated with the rs10063949-AG heterozygotes and a 307% increase with GG homozygotes, clearly showing an allele dosage effect (Amir Shaghaghi, 2014). Certain haplotypes in *SLC23A1* gene were associated with increased risk of spontaneous preterm delivery (Erichsen et al., 2006). The rare allele of rs6596473 was associated with aggressive periodontitis (De Jong et al., 2014).

Overall, evidence of associations in the *SLC23A1* gene with common and complex diseases is currently emerging but lacks enough depth to conclude on the validity.

2.6.3 Genetic variations in the SLC23A2 gene associated to altered vitamin C status

Three SNPs in the *SLC23A2* gene have been associated with lowered serum ascorbic acid concentrations. Plasma vitamin C concentrations were decreased by 5 μmol/L in SNP rs6053005-CC homozygotes and 4.3 μmol/L in CT heterozygotes compared to TT homozygotes (Duell et al., 2013). They were also decreased by 6 μmol/L in carrier of the SNP rs6133175-A allele (Duell et al., 2013). Moreover, rs1279386-GG homozygotes had approximately 1.1 μg/mL lower plasma vitamin C concentrations than the other genotypes (Zanon-Moren et al., 2011).

Current evidence might indicate a role of *SLC23A2* polymorphism in the regulation of steady state plasma vitamin C concentrations, but at present, research is only emerging thus it is premature to make any conclusions.

2.6.4 Genetic variations in the SLC23A2 gene associated with common and complex diseases

Several variations in the *SLC23A2* gene had been associated with the risk of six types of cancer, birth complications, coronary heart disease, and glaucoma.

Reduced risks for gastric cancer were reported for SNP rs12479919-AA homozygotes (Wright et al., 2009), and a haplotype for the common alleles of SNPs rs6139591 + rs2681116 + rs14147458 was inversely associated with gastric cancer (Wright et al., 2009). Similarly, the genotype rs6116569-CT and the two haplotypes, CGTC (rs6052937, rs3787456, rs6116569, rs17339746) and ATC (rs6139587, rs6053005, rs2326576), in the *SLC23A2* gene were associated with the risk for gastric cancer (Duell et al., 2013).

The haplotype GC for SNPs rs4987219 + rs1110277 was associated with a reduction in the risk of colorectal adenoma (Erichsen et al., 2008).

SNP rs12479919-CT heterozygosity interacted with SCARB1 (scavenger receptor class B1) rs4765621 genotypes to elevate the risk for bladder cancer (Andrew, Gui et al. 2009).
The risks for Non-Hodgkin lymphomas were elevated by 80% for genotypes rs6133175-GG, rs1715364-CC, rs1715385-AA, rs1776948-AA, and rs6139587-AA, as well as the two haplotypes rs1776948 + rs6139587 AA and rs1715385 + rs6133175 + rs1715364 AAC (Skibola, Bracci et al. 2008). The alleles rs6133175-G and rs1776948-A elevated the risk for chronic lymphocytic leukemia by about 20% (Casabonne et al., 2017). The haplotype analysis of rs1715364 + rs6133175 supported the genotype results.

The risk of head and neck cancer following HPV16 infections was decreased for rs4987219-CC homozygotes (Chen et al., 2009).

Elevated risks for spontaneous preterm birth were observed in carrier of the minor T allele of SNP rs6139591, showing 1.7-fold and 2.7-fold elevations for hetero- and homozygotes, respectively (Erichsen et al., 2006).

A 5.4-fold elevated risk of acute coronary syndrome was reported in women with the rs6139591-TT genotype with low dietary vitamin C intake (Dalgard et al., 2013). Moreover, women carrying rs1776964-TT at high intake of vitamin C had a 3.4-fold increased risk of acute coronary syndrome, compared with CC-homozygotes with low intake. This might indicate that the genotype effects may not be completely compensated by **a** high dietary intake of vitamin C (Dalgard et al., 2013).

The risk for open-angle glaucoma was elevated by 1.7-fold in rs1279386-GG homozygotes, potentially echoing the lower plasma vitamin C concentration observed for this genotype (Zanon-Moreno et al., 2011).

To conclude, reports on genetic associations in the *SLC23A2* gene mostly consist of observation from single studies, which calls for replications in similar or larger studies in order to validate those results.

2.6.5 Genetic variations in the GSTM1 and GSTT1 genes associated to altered vitamin C status

Glutathione S-transferases (GSTs) are phase 2 enzymes which conjugate glutathione to xenobiotics for the purpose of detoxification. The two major isoforms, GSTT1 and GSTM1, are involved in oxidative stress pathways through the utilization and conjugation of glutathione (Block et al., 2011), and their genes are deleted in ~20% and~50% of the human population. It needs to be noted, that a capacity to recycle dehydroascorbic acid has been demonstrated for GSTO (Linster and Van Schaftingen, 2007), but the participation of the GSTT1 and GSTM1 in the recycling of dehydroascorbic acid have not been proven. However, if they do not participate directly, they could spare the biochemical ascorbic acid consumption indirectly through recycling of other antioxidants.

For individuals carrying *GSTT1* and *GSTM1* null genotypes (**0/*0* gene is non-functional) a rare diet-gene interaction was reported: individuals had an increased risk of vitamin C deficiency when their dietary supply did not meet current recommendations (Cahill et al., 2009). Specifically, the risk of serum ascorbic acid deficiency in marginal vitamin C supply was elevated approximately 12-fold for the *GSTT1*0/*0* genotype, but was only increased approximately 2-fold for carriers of the *GSTT1*1* allele. Moreover, the risk of serum ascorbic acid deficiency with marginal vitamin C intake was approximately 4-fold elevated for the *GSTM1*0/*0* homozygotes, while it was approximately 2-fold for carriers of the *GSTM1*1* allele. Individuals with both nonfunctional genotypes had an approximately 15-fold elevated risk of serum ascorbic acid deficiency in marginal vitamin C supply compared to an approximately 2 fold risk for subjects with both functional genotypes. Serum ascorbic acid concentrations were decreased by approximately 10 μmol/L for homozygotes for both *GSTT1*0/*0* and *GSTM1*0/*0* nonfunctional genotypes in marginal supply (Cahill et al., 2009).

Plasma vitamin C concentrations in *GSTM1*0/*0 homozygotes were decreased by 2.1* μmol/L in the general population and by *5.3* μmol/L in asbestos factory workers (Horska et al., 2011). In workers of a rock wool plant *GSTT1*0/*0 homozygotes showed 9.5 μmol/L lower* concentrations. Homozygotes for *GSTM1*/*GSTT1* deletions had a vitamin C level that was 6.4 μmol/L lower.

In direct contrast to the above reported results, serum vitamin C concentrations were 5 μmol/L higher in *GSTM*0/*0* homozygotes (Block et al., 2011). However, the dual deletions of *GSTM1*0/*0* and *GSTT*0/*0* were not associated with altered serum vitamin C concentrations. The association of vitamin C concentrations with the probability of being *GSTM1*0/*0* was allele dosage dependent (**Figure 2.4**), where subjects in the highest quartile of vitamin C concentrations were 2.6 times as likely to be *GSTM1*0/*0*. SNPs did not modify any associations between dietary vitamin C intake and serum ascorbic acid, as reported in the previous paragraph (Cahill et al., 2009, Block et al., 2011). Furthermore, no associations with plasma vitamin C concentrations have been reported for *GSTM1/T1* polymorphisms (Yuan et al., 2012).

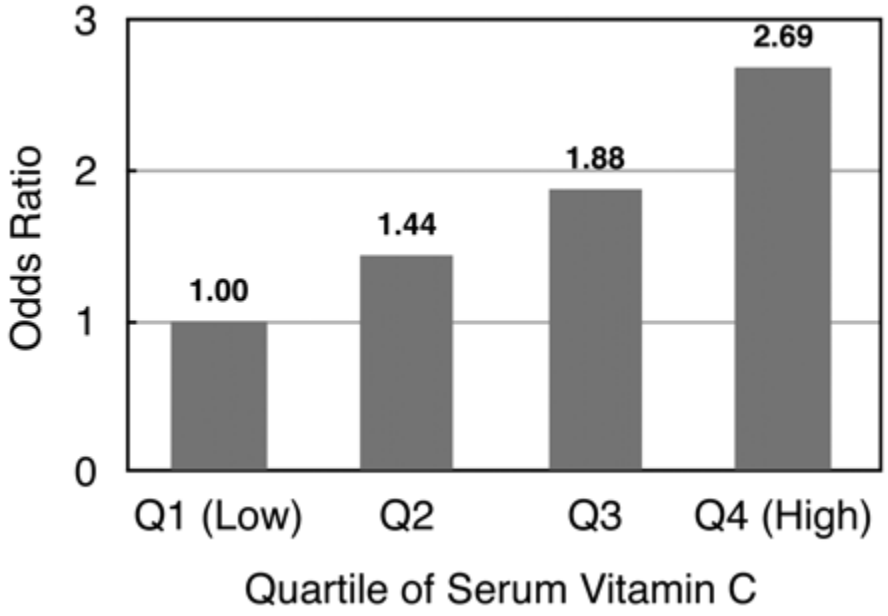

Figure 2.4. Odds ratios for being *GSTM1* null, by quartile (Q) of serum vitamin C, adjusted for age, sex, and BMI. 95% CIs: Q2 (0.80, 2.58), Q3 (1.04, 3.40), Q4 (1.46, 4.93). P-trend = 0.001. Median serum vitamin C: Q1 (41.46 μmol/L), Q2 (59.07 μmol/L), Q3 (67.59 μmol/L), Q4 (82.93 μmol/L). (Block et al., 2011)

Conflicting results of the associations of *GSTT1* and *GSTM1* null genotypes with circulating ascorbic acid concentrations do not allow deducing any causal relationships. In contrast to *GSTO* and glutaredoxin, the participation of the *GSTT1* and *GSTM1* enzymes in the recycling of dehydroascorbic acid have not been proven (Linster and Van Schaftingen, 2007); however, such a proof is necessary to illuminate any mechanistic relevance of the reported genetic variations.

2.6.6 Genetic variations in the Haptoglobin gene associated to altered vitamin C status

The haptoglobin protein binds free hemoglobin in the plasma, inhibits its oxidative activity and therefore acts as an antioxidant. The haptoglobin gene allele *Hp2* comprises a 1.7 kb partial duplication which does not have the same potency as the *Hp1* allele, and its presence could therefore cause excessive metabolic consumption of ascorbic acid (Guthrie et al., 2014).

A 12 μmol/L decrease in serum ascorbate concentrations in the general population has been reported for *Hp2-2* homozygotes regardless of gender (Delanghe et al. 1998), while a reduction of approximately 40 μmol/L was reported for male but not for female *Hp2-2* homozygotes (Na et al., 2006). Similarly, serum ascorbic acid concentrations in HIV patients were approximately 11 μmol/L lower for *Hp2-2* homozygotes (Delanghe et al., 1998). The reduction in serum ascorbate concentrations were limited to *Hp2-2* homozygotes and no differences were observed between subjects with the *Hp2-1* and *Hp1-1* alleles (Delanghe, Langlois et al. 1998, Na, Delanghe et al. 2006). In contrast, haptoglobin genotypes were not associated with vitamin C concentrations in another cohort (Guthrie et al., 2014).

For individuals carrying the *Hp2-2* genotype, a rare diet-gene interaction was reported: they had an increased risk of vitamin C deficiency when their dietary intake did not meet current recommendations (Cahill and El-Sohemy, 2010). Of the subjects who reported not meeting the RDA for vitamin C, those with the *Hp2–2* genotype had 5.7 μmol/L lower average serum ascorbic acid concentrations than individuals with the *Hp1* allele. *Hp2-2* homozygotes had a 4.7-fold elevated risk for deficiency when they did not meet their RDA while this was 1.7-fold for carriers of the *Hp1* allele.

In conclusion, ascorbic acid oxidation might be increased in *Hp2-2* homozygotes. However, the correlations with impaired iron status and lowered vitamin concentrations are unclear, and diet-gene interactions of haptoglobin alleles may extend beyond vitamin C regulation

and should be examined with respect to the role of haptoglobin in iron status as well (Michels et al., 2013).

2.6.7 Conclusions from Large Scale Observational, Intervention, and genetic Association Studies – Implications for Future Research

Around 5% of the general population in industrialized countries has deficient plasma vitamin C concentrations and about 13% have marginal deficient concentrations. Severe deficiency will lead to scurvy, while marginal deficiency is associated to elevated risks of all-cause mortality, cardiovascular disease, and gastric cancer in observational studies.

Supplementation of Vitamin C did not reduce the risks for all-cause mortality, impaired cognitive performance, reduced quality of life, the development of eye diseases, infections, cardiovascular diseases, and cancers. It is suggested that these results related to the fact that additional supplementation did not elevate systemic vitamin C level in individuals already in optimal supply.

Recent genetic association studies indicate that dietary intake is not the sole determinant of systemic vitamin C levels. Polymorphisms in two ascorbic acid transporter genes (*SLC23A1* and *SLC23A2*) are associated with lower system ascorbic acid concentrations. Common variations in three genes participating in the redox (*GSTM1* and *GSTT1*) and antioxidant metabolism (*haptoglobin*) are also associated with lower system ascorbic acid concentrations. The impact sizes are moderate, with reductions ranging between 5-10 μmol/L, which leads to the speculation that these variations would only be relevant in situations of suboptimal dietary intake, as defined by the current RDAs. However, although impact sizes of common polymorphism are expected to be low, epistatic interactions of multiple common variants can dramatically increase effect sizes (Abdullah et al., 2015).

It is anticipated that future research will address these relations, specifically if epistatic effects of common polymorphism will predispose individuals to marginal systemic vitamin C concentrations even if they achieve current intake recommendations.

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Bridge to Chapter III

The following chapter comprises a manuscript of the study protocol for The Manitoba Personalized Lifestyle Research (TMPLR) study. The manuscript details the rationale, methods, and data that will be captured in the areas of diet, physical activity and sleep, genetic and gut microbiome profiles, and healthcare usage data linkage. This chapter is an introduction to the use of observational cohort studies to generate big datasets to health further policymaking and generate hypotheses to investigate. Dylan MacKay and Rebecca Mollard developed the original concept of the study for the original grant application with input from co-investigators. Dylan MacKay prepared the drafts of the study protocol manuscript and compiled feedback and changes from other authors. Rebecca Mollard and **Matthew Granger assisted in the preparation of the study protocol manuscript and was a research assistant involved in the processing and collection of data from participants during TMPLR study**. PF developed the branding and logo for TMPLR study, and the manuscript figures and tables. NCH prepared the data model and was involved in the public engagement. SB (project lead, indigenous health), HB (project lead, nutrition), JC, TAD (project lead, physical activity), PKE (project lead, genetics), EK (project lead, gut microbiome), LML (project lead, biostatistics), DEM (project lead, sleep), SBM, AR, NT, MBA (project lead, developmental origins of chronic disease), and PJJ (Director) are study co-investigators, and were all involved in writing the original grant application. All authors have carefully read, contributed to, and approved the final version of the study protocol manuscript.

Chapter III

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The Manitoba Personalized Lifestyle Research (TMPLR) study protocol: a multicentre bidirectional observational cohort study with administrative health record linkage investigating the interactions between lifestyle and health in Manitoba, Canada

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Keywords: nutrition, physical activity, sleep, chronic disease, genetics, microbiome

3.1 Abstract

Introduction

Lifestyle factors, such as diet, physical activity and sleep, are associated with the development of many chronic diseases. The objective of The Manitoba Personalized Lifestyle Research study is to understand how these lifestyle factors interact with each other and with other factors, such as an individual's genetics and gut microbiome, to influence health.

Methods

An observational study of adults, with extensive phenotyping by objective health and lifestyle assessments, and retrospective assessment of early life experiences, with retrospective and prospective utilization of secondary data from administrative health records.

Study population

A planned non-random convenience sample of 840 Manitobans aged 30–46 recruited from the general population, stratified by sex (equal men and women), body mass index (BMI; 60% of participants with a BMI>25 kg/m^2) and geography (25% from rural areas). These stratifications were selected based on Manitoba demographics.

Measurements

Lifestyle factors assessed will include dietary pattern, physical activity, cardiovascular fitness, and sleep. Factors such as medical history, socioeconomic status, alcohol and tobacco consumption, cognition, stress, anxiety, and early life experiences will also be documented. A maternal survey will be performed. Body composition and bone density will be measured by dual energy X-ray absorptiometry. Blood pressure, pulse wave velocity, and augmentation index will be measured on two consecutive days. Chronic disease risk biomarkers will be measured in blood and urine samples. DNA will be extracted for genetic analysis. A faecal sample will be collected for microbiome analysis. Participants may provide their Manitoba personal health information number to link their study data with administrative health records.

Ethics and dissemination

Ethics approval has been obtained from the University of Manitoba Health Research Ethics Board (protocol # HS18951; 05/01/2016). Data analysis, release of results and publication of manuscripts are scheduled to start in early 2019. Additional information at www.TMPLR.ca.

Trial registration number

NCT03674957; Pre-results.

3.2 Strengths and limitations of this study

- The study is designed to capture extensive phenotyping of participants in the areas of diet, physical activity, sleep, genetic, gut microbiome profiles, and healthcare usage data linkage.
- The use of a mobile research unit to access rural populations makes the study unique as geographic setting can strongly influence health-related behaviours. The study uses non-random convenience sampling for feasibility reasons, which can introduce selection bias and limit generalizability.
- Some of the questionnaires used in The Manitoba Personalized Lifestyle Research (TMPLR) have not previously been validated, or not validated in the specific TMPLR study population.
- The study sample size of 840 individuals was not selected to power a specific primary hypothesis and therefore should be considered exploratory in nature.

3.3 Introduction

Manitoba is a province located in central Canada with a population of just over 1.2 million people. Most Manitobans (~60%) live in Winnipeg, the largest city, with ~27% of the population living in rural areas.[1] Approximately half of Manitobans are living with at least one of the following chronic conditions: obesity, hypertension, type 2 diabetes (T2D), cardiovascular disease (CVD) or chronic kidney disease (CKD).[2] Additionally Manitoba has the highest incidence and prevalence of end stage renal disease in Canada, partly because of the high burden of diabetes.[2] The consequences of these chronic conditions are substantial and the financial burden, both personally and societally, is enormous. In the province of Manitoba, which has a universal healthcare system, over 40% of total provincial revenues are spent on healthcare.[3] The burden of conditions including T2D and CKD is not unique to Manitoba,[4,5] therefore the primary and secondary prevention of these chronic conditions is a major international health research priority.[6]

It is well established that diet, physical activity and sleep influence health and mortality.[7–10] Evidence-based guidelines pertaining to nutrition, physical activity, and sleep exist to educate the public on healthy lifestyle choices. However, most current lifestyle guidelines follow a one-size-fits-all format, even though they are intended for populations comprising individuals with diverse and complex health circumstances and unique factors influencing their ability to follow the guidelines. This format may be a contributing factor to the poor adherence to lifestyle guidelines. For example, although most people are aware that physical activity is important for health, only 15% of the Canadian population achieve the national recommendations.[11] Similarly, it is estimated that 50% of women and 70% of men in Canada have energy intakes that exceed their energy needs, while 50%–90% have deficiencies in calcium and vitamin D.[12]

There is now an increasing interest in the creation of lifestyle strategies or guidelines for specific subpopulations or groups of individuals with specific characteristics.[13–15] It is hoped that such tailored recommendations will be more effective, and that barriers to healthy lifestyle practices can be ameliorated through personalisation. Current one-size-fits-all recommendations and strategies may not be effective due to (1) significant inter-individual variability or (2) shared circumstances, such as geography, sleep/wake patterns or socioeconomic status, of a particular group.

We hypothesise that an individual's lifestyle will be influenced by socioeconomic status and geography, and will interact with their genotype and gut microbiota to affect health.[16,17]

Accordingly, The Manitoba Personalized Lifestyle Research (TMPLR) study will involve the coordinated collection of data related to socioeconomic status, geography, nutrition, physical activity, sleep, early life experiences and health systems usage, in conjunction with the analysis of genetics, gut microbiota and risk factors for chronic conditions such as obesity, hypertension, T2D, CVD and CKD. After establishing the baseline characteristics of this study cohort, administrative health records will be used retrospectively to examine the developmental origins of health and disease,[18] and prospectively to track and investigate the development of chronic disease in the future, starting at 5 years after the initial study is complete. Consent will be obtained to contact study participants for further clinical assessments, contingent on future funding.

Data from this study will provide an ideal opportunity for the exploration and potential discovery of new interactive mechanisms through which lifestyle factors affect health. We will be looking to collaborate with other existing studies[19–21] with overlapping measures to replicate such findings, or increase sample size. Findings from this research may be useful in guiding both clinical and health policy decisions, and will also facilitate the design and testing of personalized health promotion strategies. For example, if we are able to identify interactions between lifestyle factors and disease risk, such as a genetic variant that associates with short sleep to negatively impact health, a follow-up study could be designed looking to improve sleep hygiene specifically in the group with the risk variant.

3.4 Methods

3.4.1 Design

This is an exploratory observational cohort study with retrospective and prospective utilization of secondary data from administrative health records (**Figure 3.1**). The Strengthening the Reporting of Observational Studies in Epidemiology guidelines were followed where applicable in the development of this protocol manuscript.[22]

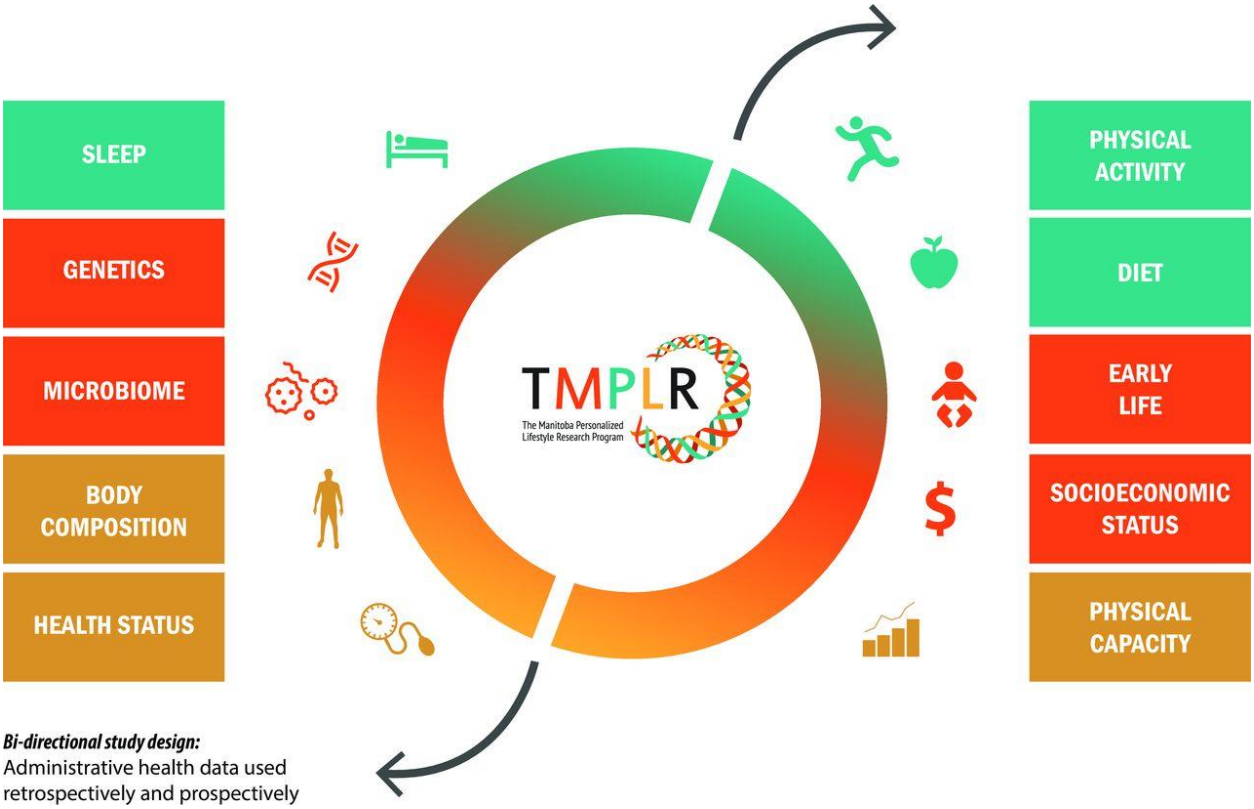

Figure 3.1. The Manitoba Personalized Lifestyle Research study overview.

3.4.2 Setting

Urban (Winnipeg) and rural (Morden, Winkler, Carman and Steinbach) areas with road access in southern Manitoba, Canada.

3.4.3 Objectives of the study

The objective of this study is to explore the complex interactions that exist between lifestyle, genetics and gut microbiota, and how these relate to risk factors for chronic conditions, especially obesity, hypertension T2D, CVD and CKD in Manitoba.

3.4.5 Inclusion and exclusion criteria

A sample of 800 Manitobans aged 30–46, stratified by sex, body mass index (BMI) and geography (**Table 3.1**) are being recruited. Participants must have lived in Manitoba for a minimum of 5 years. Women who are pregnant or lactating are not eligible to participate. Additionally, because it is expected that very few of the 800 Manitobans who join TMPLR study

from the general public will have reduced kidney function (eGFR<30 mL/min), 40 participants from Manitoba (20 women, 20 men, with no set stratification based on BMI or geography) who have severely reduced kidney function are being recruited from the renal health clinic at Seven Oaks General Hospital (SOGH), Winnipeg, Manitoba. Therefore, the study has a recruitment goal of 840 participants.

Table 3.1. The Manitoba Personalized Lifestyle Research study recruitment targets by strata.

Age	30-46 years n = 800							
Sex 50% Men 50% Women	400 Men				400 Women			
Geography 72% Urban 28% Rural	288 Urban men		112 Rural men		288 Urban women		112 Rural women	
BMI 40% Normal (BMI<25) 60% Overweight (BMI≥25)	116 Urban men BMI<25	172 Urban men BMI≥25	45 Rural men BMI<25	67 Rural men BMI≥25	116 Urban women BMI<25	172 Urban women BMI≥25	45 Rural women BMI<25	67 Rural women BMI≥25

- +40 Participants with severely reduced kidney function (eGFR <30 mL/min), 20 women, 20 men, with no set stratification based on BMI or geography.
- BMI, body mass index.

3.4.6 Recruitment

Participants are recruited through the use of printed flyers, online advertisements purchased via Google, Facebook and Twitter ad platforms and social media accounts, appearances in local TV, radio and print media, and direct contact with community groups, such as churches, sports leagues and community clubs. All patients who receive care in the SOGH renal health clinic, who

are aged 30–46, have been living in Manitoba for a minimum of the last 5 years, and are able to provide informed consent are approached to enroll in the study as well.

3.4.7 Sample size

The sample size of TMPLR study was selected based on considerations of feasibility of recruitment, costs and logistics. However, given established values from other sources[23] and our anticipated sample size of 840 participants, we estimate that we will have an 80% power (5% significance, 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). Additional estimated minimum detectable differences are presented in **Table 3.2**. These lower limits should allow for the detection of clinically meaningful changes in these outcomes.

Table 3.2. The Manitoba Personalized Lifestyle Research study estimated minimum detectable differences.

Variable	Mean or median used	SD used	Minimum difference at 10% exposure (percentage of mean)	Minimum difference at 25% exposure (percentage of mean)	References
Body fat (%)	41.3% Women 27.8% Men	7.7% 6.6%	2.5% (6.0)	1.7% (4.0)	**23**
Lumbar bone mineral density (g/cm^2)	1.042 Women 1.058 Men	0.121 0.127	0.041 (3.8)	0.028 (2.6)	**71**
Glomerular filtration rate (mL/min per 1.73 m^2)	107.6	16.8	5.4 (5.0)	3.8 (3.5)	**72**
Systolic blood pressure (mm Hg)	116	12	6.5 (5.6)	4.5 (3.9)	**73**
Fasting glucose (mmol/L)	4.94	0.61	0.20 (4.0)	0.14 (2.8)	**73**

Variable	Mean or median used	SD used	Minimum difference at 10% exposure (percentage of mean)	Minimum difference at 25% exposure (percentage of mean)	References
Fasting insulin (µIU/mL)	7.83	7.50	2.40 (30)	1.67 (21%)	**74**
LDL cholesterol (mmol/L)	2.79	0.67	0.22 (7.8)	0.15 (5.4)	**73**
Waist circumference (cm)	80	10	3.2 (4)	2.2 (2.75)	**73**

3.4.8 Data Collection and Assessments

On two consecutive days, participants come 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 travels to other areas of Winnipeg and southern Manitoba. TMPLR's mobile research unit is a custom built 12 m mobile lab which is equipped with phlebotomy area, a dual-energy X-ray absorptiometry (DXA) and a bicycle ergometer with a metabolic cart. During this visit, participants complete questionnaires, undergo various health assessments, provide urine and faecal samples, and have fasting blood samples taken (**Figure 3.2, Table 3.3**). The same protocols were followed at both sites.

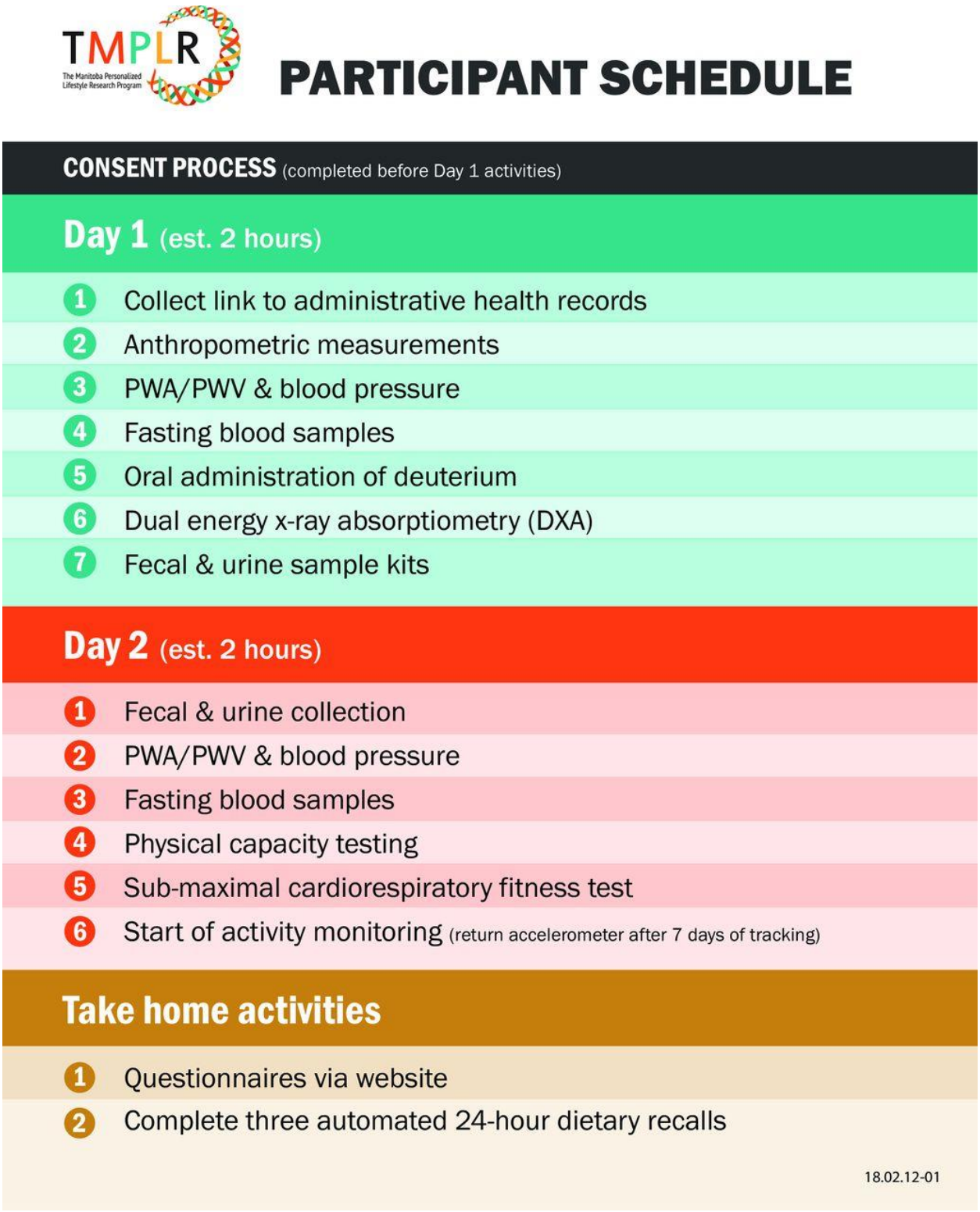

Figure 3.2. The Manitoba Personalized Lifestyle Research study participant schedule.

Table 3.3. The Manitoba Personalized Lifestyle Research (TMPLR) study data, assessment tools and biological samples.

Characteristic	Data	Method, instrument or source
Sociodemographic	Date of birth, sex, ethnicity, marital status	TMPLR Study Questionnaire
Medical	Personal medical history, family medical history, medication(s), pregnancy history	TMPLR Study Questionnaire, administrative health records
	Cognition	Montreal Cognitive Assessment[29]
Lifestyle	Tobacco/smoking/vaping use, alcohol use, unintentional weight loss, exhaustion, depression	TMPLR Study Questionnaire
Physical activity	Frailty	Modified Fried Criteria[27]
	Physical activity	Paffenbarger physical activity index, Actigraphy[41,42]
	Predicted VO_2 max	Modified YMCA bike test with metabolic cart
Nutrition	Dietary patterns and habits	Mindful Eating Questionnaire,[24] Three-factor Eating Questionnaire,[26] automated 24-hour

Characteristic	Data	Method, instrument or source
		dietary recall,[49] Canadian Dietary History Questionnaire[25]
Early life	Childhood health, sociodemographic status and socio-economic status; parental employment history	Childhood Retrospective Questionnaire, adapted from the US Panel Study on Income Dynamics[30]
	Maternal: pregnancy events, obstetrical history, infant feeding	TMPLR Mother's Retrospective Childhood Questionnaire, adapted from the Nurses Health Study[50]
Socioeconomic	Employment, home ownership, educational attainment, income	TMPLR Study Questionnaire
Sleep and stress	Duration of sleep	Actigraphy[46]
	Sleep quality	Pittsburgh Sleep Quality Index[28]
	Perception of stress, daily life stressors	Community-based stress and coping survey
Anthropometric	Height	Wall-mounted stadiometer
	Weight	Digital scale
	Waist circumference, hip circumference	Tape measure

Characteristic	Data	Method, instrument or source
	Body fat, lean mass, bone mineral density	Dual energy X-ray absorptiometry[31]
Blood pressure	Systolic and diastolic	Automated sphygmomanometer
	Pulse wave velocity, augmentation index	Mobil-O-Graph oscillometer[32]
Biomarkers	Blood clinical chemistry and biomarker assays	Fasting blood samples
	Urinary clinical chemistry and biomarker assays	Urine samples
	Microbiome 16S RNA sequencing	Faecal sample[37]

3.4.9 Questionnaires

Questionnaires capture sociodemographic characteristics, personal and family medical history, smoking (including electronic cigarette use), current diet (three Automated Self-Administered 24 hours (ASA24) Dietary Assessment Tool recalls, Mindful Eating Questionnaire,[24] Diet History Questionnaire (DHQ)[25] and The Three-factor Eating Questionnaire[26]), alcohol consumption, physical activities, frailty using the Modified Fried Criteria,[27] stress, sleep (Pittsburgh Sleep Quality Index[28]), cognition (Montreal Cognitive Assessment Questionnaire[29]) and childhood retrospective circumstances (adapted from the US Panel Study on Income Dynamics[30]).

3.4.10 Anthropometric assessment

Weight is measured after participants change into lightweight scrub tops and bottoms, with shoes removed, to the nearest 0.1 kg using a digital calibrated floor scale (7562EF, Taylor Precision Products, Oak Brook, Illinois, USA). Height is measured, without shoes, to the nearest 0.1 cm using a stadiometer (Model 206, SECA North America, Chino, California, USA). BMI is calculated in kg/m^2. Waist circumference is measured in triplicate, to the nearest 0.1 cm at the umbilicus, between the last rib and the iliac crest using a fibreglass tape measure. Hip circumference is measured in triplicate at the widest portion of the buttocks and hips using a fibreglass tape measure. Body composition including fat mass, lean mass, per cent body fat, visceral adipose tissue and bone mineral density are assessed using DXA (Lunar Prodigy Advance, GE Healthcare, Mississauga, Ontario, Canada).[31] Scans are taken of the whole body, femoral neck, L1–L4 of the spine and the non-dominant forearm.

3.4.11 Clinical health assessment

Participants' systolic and diastolic blood pressures are measured in triplicate, on the non-dominant arm in a sitting position using a validated oscillometric blood pressure monitor (BP760CAN, Omron, Burlington, Ontario, Canada). Participants are required to rest for 5–10 min before taking the measurement. Pulse wave velocity and augmentation index are measured on the non-dominant arm in a sitting position using a Mobil-O-Graph PWA Monitor and the HMS Client Server Software (IEM GmbH, Stolberg, Germany) according to the manufacturer's protocol on two consecutive days.[32]

3.4.12 Collection of bio-specimens

Blood, urine and faecal samples are obtained from study participants (online supplementary protocols). Fasting blood samples are collected on two consecutive days via venipuncture by trained phlebotomists. Two blood samples on consecutive days are required to undertake the isotopic assessment of fractional cholesterol and triglyceride synthesis rates. Participants are asked to collect two urine samples at home; one sample is obtained prior to going to bed, and a second of the first morning void on waking up. Participants also collect a faecal sample; they are provided a collection kit and instructed to collect a single sample from three separate places on the stool using a spoon attached to the cap of the collection tube. Participants

are instructed to store the collected faecal samples in their household –20°C freezer with a provided ice pack, and urine samples in the fridge, until transport back to the study centre, using provided ice pack for temperature control, where they are aliquoted and then stored at –80°C for future analysis.[33]

3.4.13 Clinical chemistry in blood and urine

Clinical chemistry, including lipid profile, glucose, insulin and renal and liver profiles will be measured via automated clinical chemistry analyzers (Cobas C111, C311 and e411, Roche Diagnostics Laval, Quebec). Blood and urine biomarkers such as leptin, glucagon and melatonin will be measured via a ligand binding assay or ELISA. Red blood cell and plasma fatty acids will be measured by gas chromatography with flame ionisation detection (GC-FID).[34] Non-cholesterol sterols will be measured in plasma using GC-FID and mass spectrometry.[35] Vitamin C concentrations in the blood will be measured by high pressure liquid chromatography.[36]

3.4.14 Microbiome analyses in fecal samples

Faecal samples will be subjected to genomic DNA extraction (Zymo Research, California, USA) following the manufacturer's protocol. Experimental negative controls will be included in extraction protocols to confirm the reliability and consistency of the extracted nucleic acid. The V4 hypervariable region of 16S rRNA gene will be amplified, the sequencing library will be generated as described previously[37] and sequenced at the Gut Microbiome Laboratory, University of Manitoba. Samples will be multiplexed at the rate of 200 per run aiming for an average sequencing depth of 50 000 sequences per sample. The sequencing data will be deposited into the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) and accession numbers will be provided for future access.

3.4.15 Deuterium oxide administration

After the blood sample collection on day 1, participants are given 0.7 g of deuterium oxide/kg of estimated body water to drink. Body water is estimated as body weight (kg)×0.60. This deuterium administration is used to enrich the body's water pool for the assessment of fractional cholesterol and triglyceride synthesis rates.[38–40]

3.4.16 Physical activity and capacity testing

Physical activity level in TMPLR study participants is assessed using accelerometers (Actigraph GTX3bt, Pensacola, Florida, USA) worn for 1 week.[41,42] Muscle strength is measured using a hand grip dynamometer. Cardiorespiratory fitness is assessed using a submaximal bike protocol which includes heart rate monitoring, and a metabolic cart (VMAX Encore, Carefusion, Unionville, Ontario, Canada) to measure oxygen consumption and CO2 output. Functional walking ability is assessed using a 5 m gait speed test. Additionally, depressive symptoms, obesity history, frailty, low physical activity and cognitive impairment are assessed by validated questionnaires.[43–45]

3.4.17 Sleep assessment

Sleep in TMPLR study participants is measured objectively using accelerometers (Actigraph GTX3bt[46]) worn for a week and subjectively by questionnaire (Pittsburgh Sleep Quality Index[28]). While there is a strong relationship between objective and subjective sleep reports, TMPLR study is collecting both because discrepancies may provide important clinical information reflecting early dysfunction.[47,48]

3.4.18 Dietary assessment

Study participants complete the Canadian version of the DHQ,[25] which estimates the intake of common food items and includes portion size and dietary supplement questions. This questionnaire is on a TELEform for scanning data entry and creation of the data files. Participants also complete the Mindful Eating Questionnaire[24] to assess awareness of the physical and emotional sensations associated with eating, and The Three-Factor Eating Questionnaire[26] to assess dietary restraint, disinhibition and hunger in relation to eating. Participants also complete three dietary recall surveys using the Automated Self-Administered 24 hours Canada (ASA24, NCI, Rockville, Maryland, USA; http://asa24.ca/)[49] dietary assessment tool, a web-based tool that enables multiple, automatically coded, self-administered 24 hours recalls. Participants enrolled from March 2016 to February 2017 used the ASA24-Canada-2014 edition; those enrolled after February 2017 used the ASA24-Canada-2016 edition. Both ASA24-Canada-2014 and ASA24-Canada-2016 use the same nutrient databases.

3.4.19 Early life experiences

Early life exposures spanning the critical time windows of fetal development, birth, infancy and early childhood are documented in three ways: (1) through linkage with administrative health records (see Linkage to administrative health data section), (2) by self-report and (3) by maternal report. Administrative health data will provide method of birth, gestational age, birth weight, diagnosis codes for postdelivery hospitalisation and postdelivery drug prescriptions. Mothers of TMPLR study participants are asked to complete a TMPLR Mother's Questionnaire, adapted from the Nurses' Health Study,[50] capturing key pregnancy, birth and postpartum events such as method of birth; gestational age and birth weight; socioeconomic status at birth; maternal pre-pregnancy BMI and gestational weight gain; maternal smoking and diabetes during pregnancy; maternal prenatal care; breastfeeding initiation, exclusivity and duration; stressful life events during pregnancy and post-partum; and severe illness requiring hospitalization during infancy or early childhood. Early childhood socioeconomic status[51,52] and stressful life events[53,54] are also self-reported by TMPLR participants using the Childhood Retrospective Circumstances Questionnaire, adapted from the US Panel Study of Income Dynamics.[30]

3.4.20 Data quality assurance and control

Methods of data collection (questionnaires, anthropometric assessment and clinical health assessment) were standardized across the urban and mobile TMPLR study sites. Training of TMPLR study staff involved in data collection and data entry is regularly refreshed and all staff handling participant data are trained in compliance with the Manitoba Personal Health Information Act. All data will be entered in the secure digital platform. A TMPLR study data model has been created to help in visualizing the different types of data the digital platform will contain. (**online supplementary Figure 3.3**)

3.4.21 Linkage to administrative health data

At enrollment, TMPLR participants are 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 are accessed through the Manitoba Centre for Health Policy (MCHP) Population Research Data Repository[55] and linkage is achieved using the PHIN, following the standard procedures

established by the MCHP and the Manitoba Health Information Privacy Committee. The data linkage is used to capture retrospective information on early life as well as prospective information on numerous health outcomes, including diagnosis of hypertension, T2D, CVD and CKD.

3.4.22 Statistical analyses

Statistical analyses will be undertaken in consultation with biostatisticians from the George and Fay Yee Centre for Healthcare Innovation at the University of Manitoba. Lifestyle factors will primarily be used as explanatory variables, with chronic disease biomarkers or disease presence/absence as outcomes, in multivariable regression models. Moderating or mediating effects of genetics, gut microbiome, clinical characteristics, socioeconomic status and environmental factors will be explored. The potential confounding effects of health status and healthcare use on variable relationships will be examined using techniques such as propensity score or instrumental variable models.[56–58]

Techniques appropriate for high-dimensional data will be adopted where needed. For example, clustering of lifestyle risk factors will be examined using latent variable modelling techniques (ie, latent class analysis). Dimension reduction techniques for omics data, such as microbiome and genetic markers, will be applied.[59]

The bioinformatics and statistical analyses of microbiome data will be performed as described previously[37] and will be updated based on recommendations and technology advancements between now and the point of processing of samples. Overall microbiota community structures, alpha diversity metrics and relative abundances of operational taxonomic units will be tested for associations with lifestyle and health measures, with appropriate adjustment for multiple comparisons.

Non-response bias or inability to collect certain data may affect the validity of analyses for survey data or biological measures, necessitating the use of multiple imputation methods if the pattern of missing data is deemed to be ignorable.[60] For non-ignorable missing data, selection and pattern mixture models will be examined in sensitivity analyses.[61] Due to the use of non-random sampling there is a risk of selection bias; survey weights and weighting of responses may be used to address this bias. Standardisation or adjustment techniques may be used to address bio-specimen measurement error bias.[62]

Specialised methodological investigations will be conducted for: (1) psychometric analyses of scales, including testing for differential item functioning and measurement invariance,[63–65] (2) development of chronic disease risk prediction models,[66,67] (3) techniques to evaluate the quality of linked databases, including their accuracy, reliability and completeness[68] and (4) robust statistical methods for the analysis of outcome measures with non-normal (eg, skewed) distributions.[69,70]

3.4.23 Patient and Public Involvement

Three focus groups, one for healthcare providers and two for general public, and a public forum were held in the early design stages of this study to obtain input from Manitobans, on the study design and recruitment strategies. A study advisory board was also formed, and meets on a bi-annual basis. This advisory board includes healthcare providers, health researchers and members of the public. The board provides input regarding study recruitment, progress and conduct, and will also provide input and suggestions regarding the dissemination of study results.

3.4.24 Provision of clinical results to participants

Individual results of the anthropomorphic measurements, blood pressure, pulse wave velocity, augmentation index, body composition, bone density, full lipid profile, fasting blood glucose, and renal and liver profile are to be provided to participants. Participants are referred to their primary care providers for further management if their results are beyond clinical reference ranges. Participants will not be provided their genetic and microbiome information.

3.4.25 Ethics and dissemination

Explicit informed consent is obtained from each individual prior to participation in the study. Eligible participants are verbally informed by trained research personnel regarding the nature and purpose of the study, given time to decide whether or not to participate, and have any questions or concerns answered prior to consent and at any point throughout the study. All participants are informed that they may withdraw from the study at any time without penalty and are remunerated for the portion of the study that they have completed up to that point. The full remuneration for study participation is $100 Canadian dollars, provided as cash or as a gift card. Data analysis, release of results and publication of initial manuscripts are scheduled for 2020.

Findings will be shared in peer-reviewed journals, and at regional, national and international scientific conferences. Data and findings will also be presented to healthcare policymakers within Manitoba, to develop preventive strategies that reduce chronic conditions with the intention of reducing healthcare costs. Funding applications for future clinical follow in this study population have been submitted starting in 2017.

Data analysis, release of results, and publication of initial manuscripts are scheduled for 2019. Findings will be shared in peer-reviewed journals, and at regional, national, and international scientific conferences. Data and findings will also be presented to healthcare policymakers within Manitoba, to develop preventive strategies that reduce chronic conditions with the intention of reducing healthcare costs. Funding applications for future clinical follow in this study population have been submitted starting in 2017**.**

3.5 Discussion

TMPLR study has been uniquely designed to provide cross-sectional, retrospective and prospective observations that will improve our understanding of how lifestyle factors interact with each other and factors such as genetics and the gut microbiome to influence health and the risk of obesity, T2D, CVD and CKD. The coordinated collection of lifestyle-gene-environment-microbiota-health data, including objective measurements such as DXA, activity monitoring, stable isotopic tracer methodologies and direct measurement of physiological biomarkers; combined with the ability to retrospectively assess and prospectively follow health outcomes in participants using administrative health records, represents an unprecedented opportunity to collect data which can be used to improve chronic disease prevention and management.

Due to the voluntary non-random recruitment of participants, there may be an under-representation of those with lower health awareness, financial means, access or time to participate. Attempts to counteract this are implicit in the stratified recruitment design. Comparisons between TMPLR study participants and general Manitoban population demographics may allow assessment of potential selection biases. A healthy volunteer effect may impact the ability to detect weak associations between lifestyle and disease risk, but this may attenuate with longer follow-up using administrative health data.

Given a projected sample size of 840 participants may be low for some of the research questions that will be investigated, therefore harmonisation and linking of data across multiple

cohorts may be required. We will be looking to other studies which have undertaken overlapping measurements in order to increase sample sizes. The Canadian Longitudinal Study on Aging,[19] the Toronto Nutrigenomics and Health[20] and The LifeLines DEEP[21] studies among others will be approached regarding the potential of data harmonisation and cross-replication. TMPLR study will also be available to other researchers who are interested in collaboration or using the data for cross-replication.

In summary, TMPLR study will provide a unique platform of extensively phenotyped individuals that will be used to explore the interactions between lifestyle factors that associate with the development of, or protection from, obesity, hypertension, T2D, CVD and CKD. The findings from this research platform will subsequently be used to develop and test preventive and restorative lifestyle and health strategies with the aim of improving the health and reducing healthcare costs at the individual and population levels.

3.6 Study status

Data collection started in March 2016. As of the 15 August 2018, data collection is ongoing and has passed 800 participants. Data collection is expected to end in December 2018.

3.7 Acknowledgements

The authors would like to thank all the Manitobans who have participated in this study, without your valuable contributions we would not be able to undertake this research. The authors would also like to thank the Manitobans who took part in focus groups, and who joined the study advisory board, for their important contributions to this study. Finally, the authors would like to acknowledge the amazing staff involved in making TMPLR study a reality, in particular Stephanie Jew, Sandra Castillo-San Juan, Jeann Buenafe, Meaghan Rempel, Katrina Cachero, Mark Pinder, Eden Vergara and Kamlesh Patel.

3.8 Author contributions

DSM and RCM developed the original concept of the study for the original grant application with input from co-investigators. DSM prepared the drafts of the study protocol manuscript and compiled feedback and changes from other authors. RCM and MG assisted in the preparation of the study protocol manuscript. PF developed the branding and logo for TMPLR study, and the manuscript figures and tables. NCH prepared the data model and was involved in the public engagement. SB (project lead, indigenous health), HB (project lead, nutrition), JC, TAD (project lead, physical activity), PKE (project lead, genetics), EK (project lead, gut microbiome), LML (project lead, biostatistics), DEM (project lead, sleep), SBM, AR, NT, MBA (project lead, developmental origins of chronic disease), and PJJ (Director) are study co-investigators, and were all involved in writing the original grant application. All authors have carefully read, contributed to, and approved the final version of the study protocol manuscript.

3.9 Funding statement

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3.10 Competing interests statement

DSM, RCM, MG, SB, HB, JC, TAD, PKE, PF, NH, EK, LML, DEM, SBM, AR, NT, MBA, and PJJ have no competing interests to declare.

3.11 Ethics Approval

Ethics approval has been obtained from the University of Manitoba Health Research Ethics Board prior to participant recruitment (protocol # HS18951). The study protocol has also been

reviewed and approved by the Manitoba Health Information Privacy Committee in regards to the collection and use of PHIN, The St. Boniface Hospital Research Review Committee in regards to the processing of samples at the hospital, and the Winnipeg Regional Health Authority (WRHA) Research Access and Approval Committee (RAAC), the Southern Health Research Ethics Board, and the Interlake-Eastern Regional Health Authority Regional Ethics Committee, in regards to the study taking place in those health regions.

3.12 Supplementary material

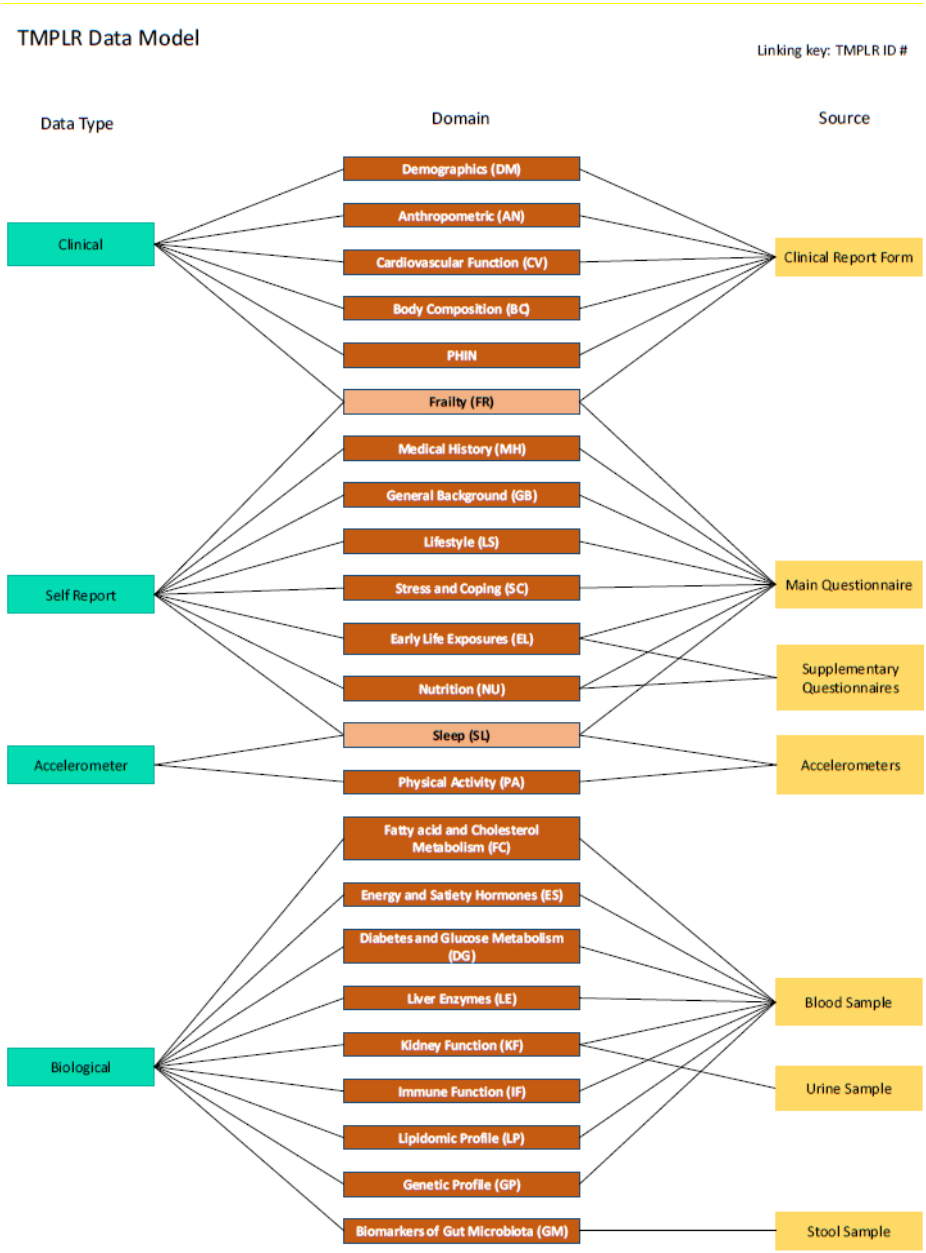
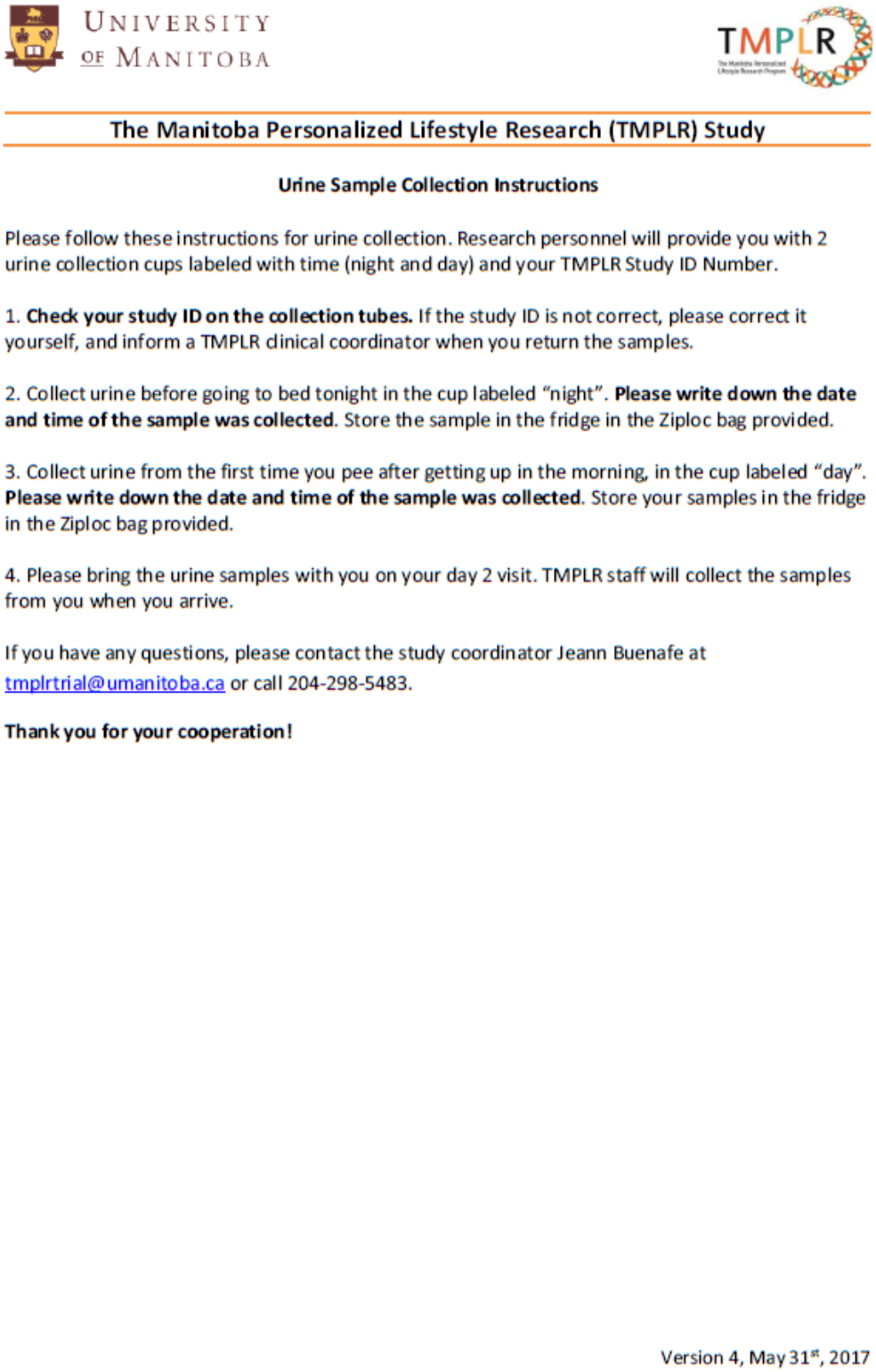


Figure 3.3. TMPLR data model.

3.13 Online Supplementary Protocols



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TMPLR

The Manitoba Personalized Lifestyle Research (TMPLR) Study

Urine Sample Collection Instructions

Please follow these instructions for urine collection. Research personnel will provide you with 2 urine collection cups labeled with time (night and day) and your TMPLR Study ID Number.

1. **Check your study ID on the collection tubes.** If the study ID is not correct, please correct it yourself, and inform a TMPLR clinical coordinator when you return the samples.

2. Collect urine before going to bed tonight in the cup labeled "night". **Please write down the date and time of the sample was collected**. Store the sample in the fridge in the Ziploc bag provided.

3. Collect urine from the first time you pee after getting up in the morning, in the cup labeled "day". **Please write down the date and time of the sample was collected**. Store your samples in the fridge in the Ziploc bag provided.

4. Please bring the urine samples with you on your day 2 visit. TMPLR staff will collect the samples from you when you arrive.

If you have any questions, please contact the study coordinator Jeann Buenafe at tmplrtrial@umanitoba.ca or call 204-298-5483.

Thank you for your cooperation!

Version 4, May 31st, 2017

Figure 3.4. Urine sample collection instructions.

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TMPLR

The Manitoba Personalized Lifestyle Research (TMPLR) Study

Stool Sample Collection Instructions

1. Freeze the ice packs provided by the study once you get home.

2. **Check your study ID** on the collection tubes (the two plastic tubes with blue cap). If the study ID is not correct, please correct it yourself, and inform a TMPLR clinical coordinator when you return the sample.

3. Empty your bladder. Flush toilet. Place the collection unit under the rear part of the toilet seat with the round side pointing towards the back.

4. Have a bowel movement. **Collect 2 samples, one in each plastic tube, from 3 different places of the stool** using the spoon attached to the cap of the collection tubes. **Fill each sample tube about one third of the tube with stool sample**.

5. Close the tube tightly. Place each tube in a Ziploc bag provided. **Write down the date and time of the bowel movement** on the bag. Discard the used collection unit.

6. Wrap the collection tubes with the frozen ice packs, and keep them in the paper bag provided. Keep the collected sample in the freezer.

7. Return the stool samples wrapped with the frozen ice packs on day 2 of the measurements, or as soon as you can. TMPLR staff will collect the sample from you in the paper bag when you arrive.

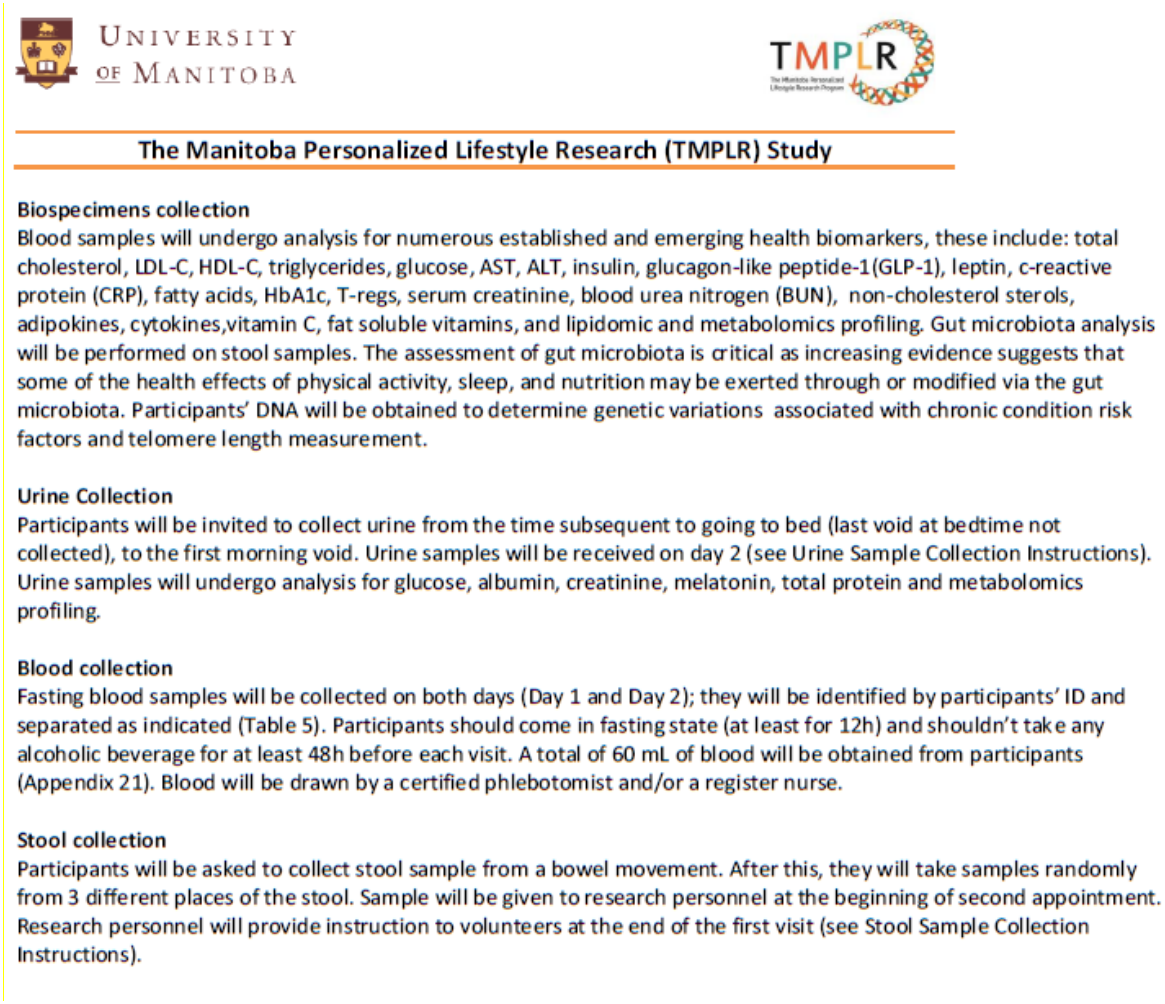
If you have any questions, feel free to contact the study coordinator Jeann Buenafe at tmplrtrial@umanitoba.ca or call 204-298-5483.

Collection Unit

Collection Tube with Spoon

Version 4, May 31st, 2017

Figure 3.5. Stool sample collection instructions.



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The Manitoba Personalized Lifestyle Research (TMPLR) Study

Biospecimens collection

Blood samples will undergo analysis for numerous established and emerging health biomarkers, these include: total cholesterol, LDL-C, HDL-C, triglycerides, glucose, AST, ALT, insulin, glucagon-like peptide-1(GLP-1), leptin, c-reactive protein (CRP), fatty acids, HbA1c, T-regs, serum creatinine, blood urea nitrogen (BUN), non-cholesterol sterols, adipokines, cytokines,vitamin C, fat soluble vitamins, and lipidomic and metabolomics profiling. Gut microbiota analysis will be performed on stool samples. The assessment of gut microbiota is critical as increasing evidence suggests that some of the health effects of physical activity, sleep, and nutrition may be exerted through or modified via the gut microbiota. Participants' DNA will be obtained to determine genetic variations associated with chronic condition risk factors and telomere length measurement.

Urine Collection

Participants will be invited to collect urine from the time subsequent to going to bed (last void at bedtime not collected), to the first morning void. Urine samples will be received on day 2 (see Urine Sample Collection Instructions). Urine samples will undergo analysis for glucose, albumin, creatinine, melatonin, total protein and metabolomics profiling.

Blood collection

Fasting blood samples will be collected on both days (Day 1 and Day 2); they will be identified by participants' ID and separated as indicated (Table 5). Participants should come in fasting state (at least for 12h) and shouldn't take any alcoholic beverage for at least 48h before each visit. A total of 60 mL of blood will be obtained from participants (Appendix 21). Blood will be drawn by a certified phlebotomist and/or a register nurse.

Stool collection

Participants will be asked to collect stool sample from a bowel movement. After this, they will take samples randomly from 3 different places of the stool. Sample will be given to research personnel at the beginning of second appointment. Research personnel will provide instruction to volunteers at the end of the first visit (see Stool Sample Collection Instructions).

Figure 3.6. Biospecimen collection instructions.

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Urine collection processing and collection instructions

Steps	Processing instructions
1	Receive urine sample and store it directly on 4 ˚C
2	Aliquot tubes should be labeled with participant ID
3	Number of labels required: 7 – 2.0 ml urine labels (if a urine sample was received)
4	If a urine sample is received proceed as follows: • Determine the volume of the urine • Pour some urine into a sterile container(to keep) • Aliquot urine into 2 -16 x 100 mm tubes and centrifuge
5	Aliquot as follows: 5 cryovials – 2.0 ml / vial (Seven Oaks) 2 cryovials – 2.0 ml / vial (McMillan)
6	Packaging of samples for transport: These samples must not thaw and must arrive frozen at the research lab Pack a transport box with ice packs and the frozen samples .Place the address label on the box. Ask the courier to return the transport box.

Figure 3.7. Urine collection processing instructions.

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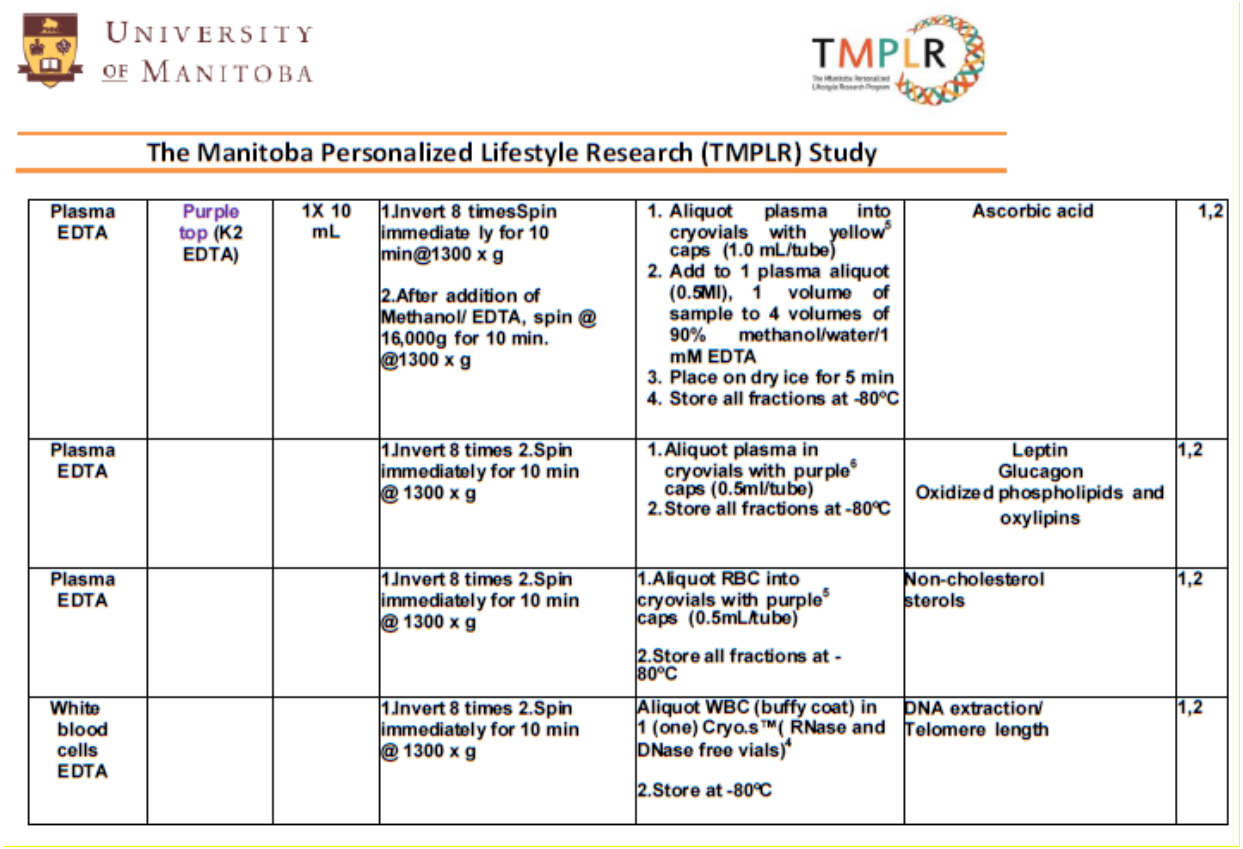
Blood sample processing and collection instructions

Sample	Blood collection tube	Tube volume	Processing instructions	Aliquoting instructions	Analysis	Day
Serum	Red/grey SST tube	1 x 4mL	1. Invert 5 times 2. Room temp for 30 min 3. Spin for 10 min @ 1000 x *g*	1. Aliquot serum into cryovials[1] with brown[2] caps (0.5mL/tube) 2. Store at -80°C	Insulin Lipid profile Glucose CRP GLP-1	1,2
Plasma	CPT tube (sodium heparin)	1 x 8 mL	1. Invert tube 8- 10 times 2. Spin for 30 min @1500- 1800 RCF 3. Resuspend by inverting 4. After addition of PBS spin for 15 min @ 300 RCF 5. Aspirate off as much supernatant without disturbing the pellet 6. Repeat wash in 10mL PBS 7. Resuspend pellet in 3mL freezing medium -10% DSMO (Sigma), 20% FCS (JRH Bioscience) in RPMI1640 (Gibco)	1. Aliquot entire contents above the gel and transfer to 15 mL Falcon tube 2. Add PBS (w/o Ca++ or Mg++) to make 15 mL 3. Store 1mL aliquots in -70°C using a Cyro-1°C/min freezing container.	T-Regulatory cells*	1
Plasma heparin	Green top (lithium heparin)	1x 4 mL	1. Invert 8 times 2. Spin immediately for 10 min @1300 x *g* 3.	1. Aliquot plasma into cryovials with green[3] caps (0.5mL/tube) 2. Store all fractions at -80°C	C-reactive protein	1,2
RBC			1. Invert 8 times 2. Spin immediately for 10 min @ 1300 x g	1. Aliquot RBC into cryovials with red[5] caps (0.5mL/tube) 2. Store all fractions at -80°C	Fatty Acid Analysis	1,2
White blood cells Heparin			1. Invert 8 times 2. Spin immediately for 10 min @ 1300 x g	1. Aliquot WBC (buffy coat) in 1 (one) Cryo.s™(RNase and DNase free	DNA extraction/ Telomere length	1,2

Figure 3.8. Blood processing and collection instructions part 1.



The Manitoba Personalized Lifestyle Research (TMPLR) Study

Plasma EDTA	Purple top (K2 EDTA)	1X 10 mL	1.Invert 8 timesSpin immediate ly for 10 min@1300 x g 2.After addition of Methanol/ EDTA, spin @ 16,000g for 10 min. @1300 x g	1. Aliquot plasma into cryovials with yellow[5] caps (1.0 mL/tube) 2. Add to 1 plasma aliquot (0.5Ml), 1 volume of sample to 4 volumes of 90% methanol/water/1 mM EDTA 3. Place on dry ice for 5 min 4. Store all fractions at -80°C	Ascorbic acid	1,2
Plasma EDTA			1.Invert 8 times 2.Spin immediately for 10 min @ 1300 x g	1.Aliquot plasma in cryovials with purple[5] caps (0.5ml/tube) 2.Store all fractions at -80°C	Leptin Glucagon Oxidized phospholipids and oxylipins	1,2
Plasma EDTA			1.Invert 8 times 2.Spin immediately for 10 min @ 1300 x g	1.Aliquot RBC into cryovials with purple[5] caps (0.5mL/tube) 2.Store all fractions at -80°C	Non-cholesterol sterols	1,2
White blood cells EDTA			1.Invert 8 times 2.Spin immediately for 10 min @ 1300 x g	Aliquot WBC (buffy coat) in 1 (one) Cryo.s™(RNase and DNase free vials)[4] 2.Store at -80°C	DNA extraction/ Telomere length	1,2

Figure 3.9. Blood processing and collection instructions part 2.

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Bridge to Chapter IV

The following chapter comprises a manuscript of a protocol for a targeted nutrigenetic study. Special attention is given to study design, blood lipid and sterol measurement, and selecting genetic variants for study. This chapter is an introduction to the use of a randomized controlled trial to investigate nutrigenetic effects of plant sterols. Maryam Shamloo sought ethical approval and prepared the drafts of the manuscript and compiled feedback and changes from other authors. **Matthew Granger assisted with the preparation and feedback of the draft manuscript, and was a research assistant involved in the telephone and in-person screening and data collection of participants during the trial.** Dylan MacKay designed the study protocol and sought funding and ethical approval. James House contributed to the development of the study protocol, ethical approval, and manuscript. Dylan MacKay designed the selection criteria of the participants. All authors contributed to and made critical revisions to the final manuscript before submission. All authors read and approved the final manuscript.

Chapter IV

Manuscript III

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Genetic basis for prediction of non-responders to dietary plant sterol intervention (GenePredict-PS): a study protocol for a double-blind, placebo controlled, randomized two-period crossover study

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4.1 Abstract

Background

Functional food ingredients and natural health products have been demonstrated to reduce disease risk and thereby help to lower health care costs across populations at risk for chronic or degenerative diseases. However, typically a wide range of interindividual variability exists in response across individuals to nutritional and natural health product bioactives, such as plant sterols (PS). This study aims to determine and utilize information on the associations between genosets and the degree of responsiveness to dietary PS intervention, with a long-term objective of developing genetic tests to predict responses to PS.

Methods

This clinical trial is designed as a double-blind, placebo controlled, randomized two-period crossover study. Sixty-four eligible participants with the specific a priori-determined single nucleotide polymorphisms (SNPs) associated with a responsiveness to PS will consume PS or a placebo treatment for two 4-week periods. The PS treatment consists of two daily single portions of margarine, each providing 1 g PS during the PS period (2.0 g/day of PS in total). The placebo will be an identical margarine containing no added PS. Low-density lipoprotein cholesterol (LDL-C) responsiveness to the controlled administration of PS will be investigated as the primary outcome, and the associations between interindividual genoset variabilities and response to PS consumption will be determined.

Discussion

This research will provide further insight into whether the associations between previously identified SNPs and the response of LDL-C to PS consumption can be used in a predictive manner. It will also provide insight into the complexities of undertaking a nutrigenetic trial with prospective recruitment based on genotype.

Trial registration

ClinicalTrials.gov: Identifier: NCT02765516. Registered on 6 May 2016.

4.2 Background

Elevated blood concentrations of low density lipoprotein cholesterol (LDL-C) is an important risk factor for cardiovascular disease (CVD) [1]. Evidence suggests that the incidence of coronary heart disease (CHD) is proportionally reduced by lowering the LDL-C [2] and that CHD is the primary target when initiating lipid-lowering interventions in the current guidelines [3]. LDL-C reduction strategies include diet and lifestyle changes, pharmaceutical therapies, intestinal bypass surgery, and lipid apheresis [3].

Changes in dietary habits can play a critical role in the reduction of LDL-C concentrations; for example, supplementation with functional food ingredients such as dietary fibers and plant sterols (PS) has been demonstrated to reduce LDL-C [4] and thereby help to lower healthcare costs across populations with chronic or degenerative diseases [5]. However, a wide range of interindividual variability in responsiveness to natural health product bioactives, such as soluble fiber and PS, has been reported [6]. Understanding this existing interindividual variability in responsiveness is important for public health and for functional food manufacturers because it may help them predict which individuals might or might not receive benefits from consuming a particular functional food or natural health product.

A better understanding of the cause of such interindividual variability, especially the impact of genetics, can help to inform individuals about optimal dosing strategies and can contribute to the development of a method that can determine, before use of a product begins, whether an individual will benefit from a particular bioactive. For instance, if an individual knows that they are a non-responder to a bioactive, they then may be able to choose other therapeutic products or approaches.

A series of predictive response tests for nutritional bioactives based on genotype would represent a genomics-derived solution and would promote a better understanding of the wide range of interindividual variability in responses to nutritional bioactives.

PS are a nutritional bioactive for which such a predictive test would be helpful. The clinical efficacy of consuming added PS for lowering LDL-C is demonstrated in the vast number of clinical studies, as summarized in several meta-analyses [7,8,9]. The concept of a predictive responsiveness test for PS supplementation is based on our research findings of previously completed human nutrition intervention trials [10,11,12]. In a recent intervention trial, the response of LDL-C to PS consumption was associated with SNPs in cholesterol 7 alpha-hydroxylase

(CYP7A1, rs3808607) and apolipoprotein E (ApoE, rs7412 and rs429358) (**Table 4.1**) [13]. A key discovery from these trials was that combinations of these SNPs (known as genosets) were found to interact with each other to form stronger associations with the magnitude of LDL-C lowering in response to PS consumption than for each SNP alone. However, these associations were established post hoc in a trial that selected for individuals with high or low cholesterol synthesis. An a priori approach replicating these findings is required to provide evidence that these genosets could indeed be used as a predictive responsiveness test.

Table 4.1. Single nucleotide polymorphisms (SNPs) for plant sterol responsiveness testing.

Gene *SNP*	**Function of gene**	**Association with plant sterol response**
CYP7A1 *rs3808607*	The rate-limiting enzyme in the synthesis of bile acid in the classic pathway.	T/T = non-responsive G/T = responsive G/G = responsive
APOE Variant	Apolipoprotein E is a glycoprotein present in human plasma; ApoE is associated with triglyceride-rich lipoproteins (chylomicrons and VLDLs) and HDL.	ε2/- = Unknown ε3/ε3 = neutral ε4/- = responsive

To the best of our knowledge, no clinical trial so far has investigated the associations between certain SNPs and/or genosets and the degree of responsiveness to a dietary PS intervention in an a priori fashion.

Therefore, the objective of this study is to determine and utilize information on the associations between SNPs and the degree of responsiveness to dietary PS intervention, with the long-term goal of developing a predictive responsiveness test.

Therefore, the primary specific hypothesis of this study after these protocol amendments are 1) the genoset formed from CYP7A1 rs3808607T/T and APOE E3/3 predict nonresponse, and 2) APOE isoform ε4/− and CYP7A1 rs3808607 G/− will independently predict response to PS consumption in a pattern that reflects the current gene-biomarker associations outlined in **Table**

4.1. For these hypotheses, response is being defined as a reduction in the LDL-C concentrations due to plant sterol consumption.

4.3 Methods/Design

Study design

To formally validate whether APOE isoform, which is formed by rs7412 and rs429358, and CYP7A1 rs3808607 can predict responsiveness to PS consumption across the general population, the present proposal is to carry out a double-blind, placebo-controlled, randomized two-period crossover study to investigate the LDL-C responsiveness to the controlled administration of PS. The PS treatment will consist of two daily single portions of margarine, providing 1 g each of PS during the PS period (2.0 g/day of PS in total). The placebo treatment will be an identical margarine, except it will not contain any added PS. Both the PS and placebo margarine treatments will be coded by the industrial partner organization, Unilever, and provided to the research group to maintain blinding of both the researchers and participants throughout the clinical trial.

We have two original specific hypotheses. 1) APOE isoform and CYP7A1 rs3808607 will independently predict the response to PS consumption in a pattern that reflects the current gene-biomarker associations as outlined in **Table 4.1**. APOE ε4/− will be more responsive to PS than ε3/ε3. The CYP7A1 rs3808607 G allele will predict responsiveness to PS consumption in a dose-responsive fashion, with T/T predicting nonresponse. 2) The genosets formed by combinations of APOE isoform and CYP7A1 rs3808607 will follow the pattern as predicted in **Table 4.2**.

Table 4.2. Original plant sterol trial genotype recruitment targets and predicted response.

APOE	CYP7A1	Predicted response	Planned Recruitment
ε2/−	T/T	Nonresponder	*n* = 8
ε2/−	G/−	Responder	*n* = 8
ε3/ε3	T/T	Non-responder	*n* = 8
ε3/ε3	T/G	Responder	*n* = 8
ε3/ε3	G/G	Responder	*n* = 8
ε4/−	T/T	Responder	*n* = 8
ε4/−	T/G	Responder	*n* = 8
ε4/−	G/G	Responder	*n* = 8

Because of the amount of time spent on recruitment and the difficulty in finding participants who were eligible with rare combinations of genosets, the APOE 2 groups were removed, and other groups were combined. To maintain the study power, we increased the n in the other groups as described in **Table 4.3**.

Table 4.3. Amended plant sterol trial genotype recruitment targets and predicted response.

ApoE	CYP7A1	Predicted response	Planned Recruitment
ε3/ε3	T/T	Nonresponder	*n* = **20**
ε3/ε3	G/−	Responder	*n* = 22
ε4/−	−/−	Responder	*n* = 22

The trial will use a priori recruitment of 64 individuals (**Table 4.3**) with the specific SNPs associated with responsiveness to PS at a) the University of Manitoba's Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) and b) Seven Oaks General Hospital (SOGH) in Winnipeg, Manitoba, Canada. The present trial will therefore select individuals from the general population with specific SNPs and then test their responsiveness to PS consumption. These responsiveness characterizations will generate the required data to validate the genoset-based classifications of responders and nonresponders.

Each treatment period will consist of 28 days, with a minimum washout of 21 days between periods. **Figure 4.1** shows the schematic flow diagram of the trial protocol.

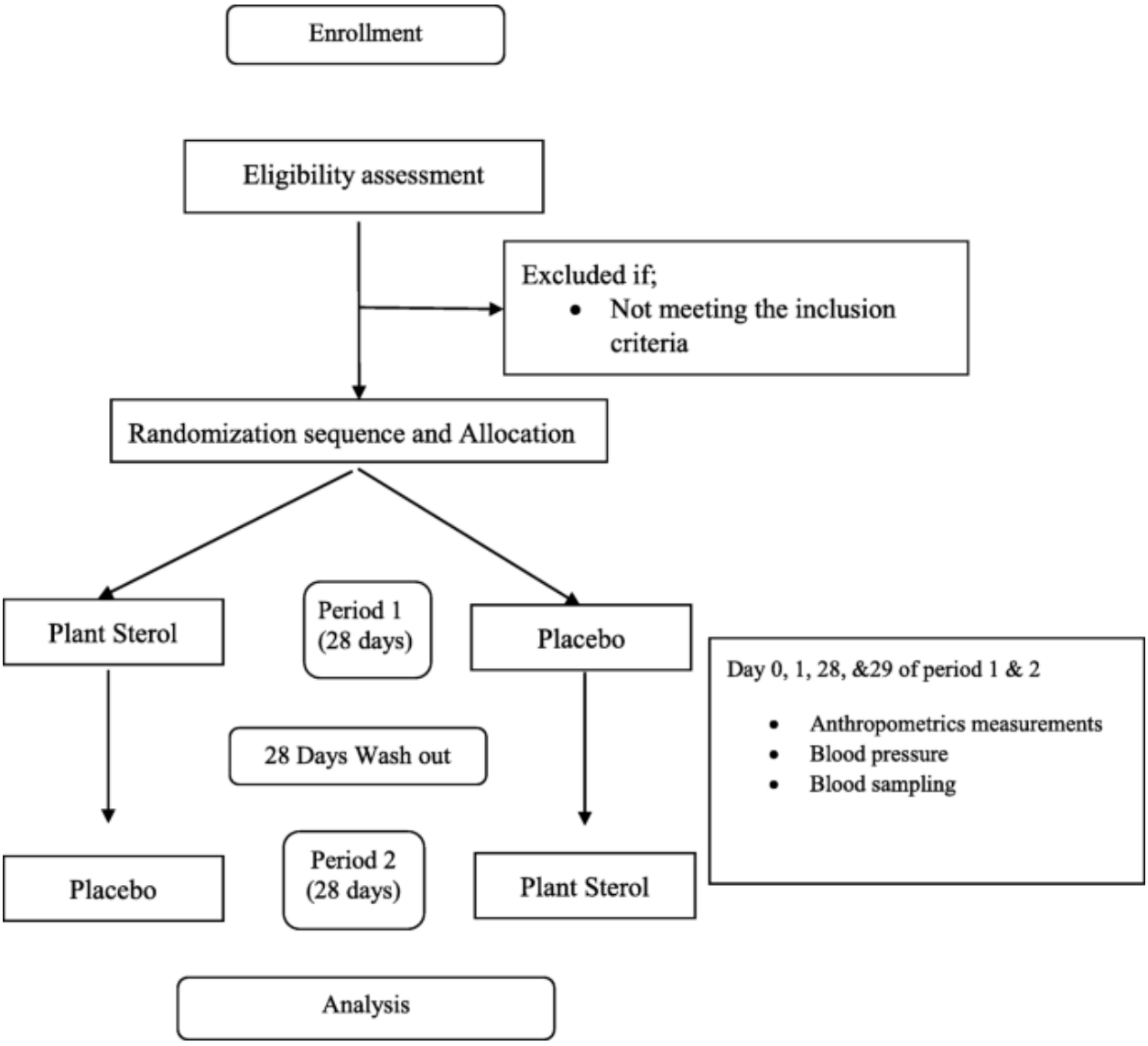

Figure 4.1. Schematic flow diagram of the trial protocol.

Participants will be required to attend breakfast at the RCFFN or Seven Oaks General Hospital (SOGH) and consume a meal containing one daily portion of margarine under supervision from Monday to Friday; the additional daily portion will be consumed with their evening meal. Participants will be given their evening and weekend margarine portions to take home for consumption. Participants will be provided diaries in which they are instructed to record when they

ate the margarine in the evenings and on the weekends. During the week, participants will be required to return the empty margarine tubs on the following day to help monitor compliance, with those margarine tubs used on Saturday and Sunday being returned on Monday. The return of the empty tubs and the record of the consumption in the study diaries will be verified by clinical coordinators using a compliance checklist. Additionally, serum noncholesterol sterols, including sitosterol and campesterol, the two main PS in the margarine, will be measured to monitor compliance. Partial supervision of treatment consumption in person and the monitoring of unsupervised treatments by return of the empty container represent a compromise between participant compliance and participant burden.

Missed treatment consumption and return of margarine tubs will be recorded for each participant. Noncompliance of a participant will be defined as 1) missing supervision, 2) failing to return at least 80% of the total empty margarine tubs per treatment period, and 3) missing two consecutive measurements or blood sampling days. Noncompliant participants will be asked to leave the trial; however, they will be compensated on a prorated basis according to the duration of their involvement in the trial. Participants were asked to maintain their typical diet and physical activity levels throughout the study.

Additionally, on a weekly basis, clinical coordinators will ask participants to report any changes in diet, lifestyle (sleep), or physical activity, which may interfere with the results of the trial and any other health outcomes or symptoms they may experience during the trial. Fasting blood samples are collected from participants on 2 consecutive days at the beginning (Days 0 and 1) and at the end (Days 28 and 29) of each trial period as described in the **Table 4.4**.

Table 4.4. Schedule of enrollment, interventions, and assessments.

	Study Period (weeks)					
Visit #	**Screening**	**1, 2**	**3, 4**	**4 week wash out**	**5, 6**	**7, 8**
Study week		**0 (±0 d)**	**4 (+5/-2 d)**		**9 (+5/-2 d)**	**12 (+5/-2 d)**
Informed consent, demographic information, inclusion/exclusion criteria, and medical history	+					
Vital signs and anthropometric measures (body weight, hip and waist circumference, BMI, blood pressure, arterial stiffness)	+	+	+		+	+
Concomitant medications	+	+	+		+	+
Genotyping of DNA samples	+					
Blood lipid profile (TG, TC, LDL-C, HDL-C) and glucose	+	+	+		+	+
Blood sterol and sterol precursor profile		+	+		+	+

Gastrointestinal (GI) tolerability questionnaires		+	+		+	+
Treatment Dispensation		+			+	
Treatment Accountability (Participant Consumption Checklist)			+			+
Treatment Checklists			+			+
Adverse Events			+			+
Study Termination						+

Study participants

The participants (64 in total) will be recruited using various established methods, including the use of flyers around the University of Manitoba and Seven Oaks General Hospital (SOGH), newspaper advertisements, direct mail advertisements within the City of Winnipeg, and digital advertisements at the Active Living Center of University of Manitoba, as well as SOGH social media, websites and newsletter advertising among 6500 members of SOGH. An internal list of previous volunteers who have expressed interest in participating in other clinical studies will also receive an advertisement. Participants will be initially screened for eligibility over the telephone by the study coordinator if they respond to an advertisement. If eligible, potential participants will be invited to the clinical research unit at the RCFFN or SOGH for an information session to introduce the research staff and provide further information about the study. Those expressing further interest will be invited to consent to and have a blood sample taken to ensure they meet all other trial criteria as listed below. Blood samples will be taken by RCFFN or SOGH phlebotomists or registered nurses. Screening blood samples are analyzed for the following: fasting lipid profiles including LDL-C, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations, as well as, glucose, serum creatinine, blood urea nitrogen (BUN),

aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), serum total protein, and serum albumin; all will be measured using the automated enzymatic methods on the Cobas 311 Analyzer (Roche Diagnostics GmbH, Mannheim, Germany) or measured by Diagnostic Services Manitoba (DSM) according to their standard protocols. DNA will be extracted from the blood sample buffy coat using commercially available column-based DNA extraction kits (DNeasy Blood and Tissue Kit, QIAGEN Sciences) according to the manufacturer's instructions. The concentration and integrity of the genomic DNA will be assessed by micro-volume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). DNA samples will then be genotyped by TaqMan SNP genotyping assays (CYP7A1-rs3808607, assay identification (ID) C2749212120; APOE rs7412, assay ID C2749212120; APOE-rs429358, assay ID C308479320; Life Technologies) on a StepOnePlus Real-Time PCR System (Applied Biosystems; Life Technologies). Data from the screening blood sample will be used to screen participants based on the predefined inclusion and exclusion criteria.

Inclusion criteria

Men and women aged 18–70 years and with LDL-C concentrations of 3.0–4.9 mmol/L will be recruited into the trial. Participants must have a fasting glucose concentration of < 6.1 mmol/L. A prospective recruitment scheme based on genotype will recruit 20–22 individuals in each of the three most common combinations, also called genosets, (outlined in **Table 4.3**). This approach would leave a minimum sample size of 20 participants for each individual genotype. The prospective recruitment based on genosets of interest will require screening of 200–400 potential participants. Such lengthy screening is required to find sufficient individuals who have the rarer genosets and meet all the inclusion and exclusion criteria. Additionally, participants must be willing to fast for 10–12 h before blood sampling, abstain from alcohol for 2 days prior to blood sampling, and abstain from coffee and physical exercise for at least 12 h before measurements and blood sampling. All participants must be able and willing to give informed consent to participate in the trial prior to their inclusion.

Exclusion criteria

Participants will be excluded if they are consuming or have consumed in the last 3 months medications or nutritional supplements known to affect lipid metabolism (such as cholestyramine,

colestipol, niacin, clofibrate, gemfibrozil, probucol, HMG-CoA reductase inhibitors (statins), methotrexate, high-dose dietary fiber supplements, or plant sterols or stanols), or have any dietary restrictions which would prevent them from consuming the trial treatments. Participants who have a BMI > 40 kg/m2 will be excluded. Participants must not have self-reported weight gain or loss greater than 3 kg in the past 3 months. Participants must be free of active cardiovascular disease including stroke; congestive heart failure; myocardial infarction; unstable angina pectoris; coronary artery bypass graft; percutaneous transluminal coronary angioplasty; temporal ischemic attacks; anemia; abnormal electrolytes; proteinuria; and abnormal liver, kidney, or thyroid function. Participants will be excluded if they have clinically significant biochemistry defined as: LDL-C < 3.0 mmol/L or > 4.9 mmol/L, TC > 6.2 mmol/L, fasting glucose > 6.1 mmol/ L, fasting TG > 4.52 mmol/L, AST > 100 U/L, ALT > 100 U/L, or at the investigator's discretion, for any other clinically significant abnormalities in hematology and/or biochemistry.

Participants will be excluded if they have phytosterolemia, type 1 or type 2 diabetes, a history of cancer or malignancy in the last 5 years, or any metabolic disease, gastrointestinal disorder, or other clinically significant disease/disorder that could interfere with the results of the study or the safety of the participant. Participants will be excluded if they are smokers, tobacco/snuff/nicotine users, recreational drug users, or if they consume more than 14 alcoholic beverages a week. Participants who are pregnant or plan to become pregnant during the trial period will be excluded. Lactating women will also be excluded. Patients with unstable or serious illness, for example, dementia, terminal illness, recent bereavement, or recent significant medical diagnosis, will also be excluded. Employees of Unilever, Nutritional Fundamentals of Health (NFH) and the research institutes conducting the research will not be allowed to participate in the study.

Randomization

Eligible participants will be randomly allocated to two groups: the PS treatment group or the placebo group for the first period, and then, participants will switch treatments for the second period after the washout between periods. Randomization will be done by an assistant outside of the research team using a block randomization method through sealed envelopes with stratification by sex and genoset. Randomization in blocks of eight and four, each with equal numbers of treatment orders will be used. This blocking is being done to minimize imbalances in treatment

orders within each genoset group or by sex. Administration of the intervention will be conducted in a double-blind manner. Single portion tubs of PS treatment and placebo margarine are being created for this study by Unilever and are being delivered to the research team in identical packages labeled either A or B.

Remuneration

Study participants will receive up to a total of CAD $400 (i.e., $200/period × 2 periods) for study completion. This amount will be divided into two portions. Participants will receive $200 after the completion of period 1 and another $200 after the completion of period 2. If a participant withdraws early from the study, the participant will receive an appropriate prorated fraction of this amount.

4.4 Outcome measures

Primary outcome

Serum LDL-C concentration and its change in response to PS consumption is considered the primary outcome of this trial. This outcome was chosen to measure plant sterol response in terms of lowering of the LDL-C concentration between the placebo and plant sterol consumption period. Blood samples (20 mL) will be collected on days 0, 1, 28, and 29 of the intervention period. The serum lipid profile (TC, LDL-C, HDL-C, and TG) will be measured using the Cobas 311 Analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The average value for days 0 and 1 will be used as the baseline, and the average of days 28 and 29 will be the endpoint values.

Secondary outcomes

At baseline and at the end of the two intervention periods, anthropometric measurements, including body weight, BMI, hip and waist circumference, and blood pressure, will be taken. Blood pressure will be measured in an office setting on. Days 0, 1, 28, and 29 of each treatment period. Participants will be asked to rest 10 min prior to having their blood pressure taken, in case they had rushed into the setting. This measurement will take place in a quiet room while the participant is in a seated position, with the arm rested on an armrest at heart level. Participants will be advised to rest quietly throughout the measurements. Blood pressure measurement will be performed four times at 2-min intervals. Gastrointestinal tolerability questionnaires will be completed by the

participants at the beginning and the end of each intervention period. The 10-year CVD risk score will be calculated for each participant during each intervention period utilizing the ACC/AHA Cholesterol Guideline risk calculator. Upon completing the trial, participants will be asked to complete a questionnaire that asks them whether they think they know which treatment they received during each treatment period. This information will be used to verify participant blinding.

Fasting serum glucose will be measured with the Cobas 311 Analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Plasma samples will be used to quantify concentrations of blood sterols and sterol precursors (noncholesterol sterols, NCS) according to a previously established method [11]. Authenticated internal standards will be added to plasma samples, which will then be saponified with methanolic KOH solution. Sterols will then be extracted twice with petroleum ether. Extracted sterols will be derivatized using a trimethylsilylation (TMS) procedure. The TMS-derivatized samples and sterol analysis will be carried out by gas chromatography with flame ionization detection. Campesterol, sitosterol, campestanol, sitostanol, and cholestanol, as well as lanosterol, desmosterol, and lathosterol will be measured.

Fractional cholesterol synthesis will be measured by deuterium incorporation according to previously established procedures [10, 13, 14]. Twenty-four hours before the end of each treatment period participants will be asked to consume deuterium water (D2O) given at a dose of 0.7 g/kg body water (estimated at 60% of total body weight). D2O is a stable isotopic tracer and poses no radiation hazard and can be safely administered to human participants. D2O water will be administered orally. A fasted blood sample will be taken at baseline and on day 28 prior to isotope administration, in addition to the fasting samples taken on day 29. The change in deuterium enrichment within red blood cell free cholesterol will be determined as an index of cholesterol synthesis over days 28 and 29.

4.5 Sample size calculation and statistical analysis

The sample size, with a minimum of 20 participants for each individual genotype and n = 20–22 for each individual genoset, is based on previous work performed by this research group [13]. A power calculation was performed using PROC POWER SAS Institute (version 9.4) using the paired means statement to model the AB/BA crossover design (corr = 0.75, a = 0.05 and b = 0.80); based on an average reduction in LDL-C of 0.34 mmol/L resulting from PS consumption according to the meta-analysis findings of Demonty et al. [8], the standard deviations in LDL-C

for placebo (0.67 mmol/L) and PS (0.7 mmol/L) from the MacKay et al. [11] and a correlation in LDL-C values of 0.75, which was an estimate based on variability in LDL-C concentrations over time from MacKay et al. [11] and on a within-persons correlation in cholesterol response to plant sterols [15]. From this power calculation, we determined that 18 participants would be needed for each genoset to detect a response, in the form of a significant reduction in LDL-C from placebo to plant sterol consumption period, in the group. Our objective was to test if each genoset would respond, with the hypothesis that the CYP7A1 rs3808607T/T and APOE E3/3 genoset would not respond to plant sterol consumption with LDL-C lowering.

Given the crossover design, the study outcomes measures will be analyzed in a per-protocol population where only participants who received both treatment and placebo are included. The effects of treatment, comparing the endpoint values of the treatment and placebo periods, will be analyzed by the SAS MIXED procedure. Sequence and sex will be included in the model as fixed factors, while participants will be included as a random and repeated factor. Genoset and treatment by genoset will be included as fixed factors to assess the impact of the genoset on the treatment. The impact of the individual genotypes will also be investigated individually. Significant treatment-by-genoset or treatment-by-genotype effects will be examined by the SAS SLICE function, with Bonferroni correction for the number of slices. Treatment effect sizes by genoset or genotype, from significant interactions, will be compared by t test or ANOVA using the difference in mixed-model least squares means summary statistics for the treatment effect slices, with Tukey-Kramer adjustment for multiple comparisons [13].

4.6 Discussion

In a recent clinical trial by our group, the response of LDL-C to PS consumption was associated with SNPs in cholesterol 7 alpha-hydroxylase (CYP7A1, rs3808607) and apolipoprotein E (ApoE, rs7412 and rs429358) [13]. This ongoing GenePredict-PS clinical trial is investigating if this previous association identified between the SNPs and the LDL-C response to PS consumption can be used in a predictive manner. Individuals with genosets that fail to reach significant reductions in plasma LDL-C levels in response to PS consumption will be classified as nonresponders, whereas those who do exhibit LDL-C lowering will be classified as responders (see predicted response in **Table 4.3**). Individuals who are classified by the genosets as responders could be advised to consider PS-added products for lowering their elevated blood total and

especially LDL-cholesterol, while non-responders could be either recommended to modify the dose of PS or use other pharmaceutical or natural health products that may lower cholesterol through other pathways. Very few studies in nutrigenetics and nutrition have yet to explore recruitment of participants a priori based on genotype, let alone based on combinations of genotypes (genosets). Previously, the impact of rs1801133, a variant in the methylenetetrahydrofolate reductase (MTHFR) gene, on riboflavin supplementation and blood pressure has been explored [16]. In that trial, Wilson et al. were able to use an available population of 1427 patients with hypertension from which they were able to recruit individuals based on genotype. The strategy of recruiting directly from a previous genotyped population can be highly recommend given the difficulty that the current trial has faced with de novo recruitment from the general public. Recruitment of previously genotyped individuals may be the most suitable way a priori nutrigenetic studies can be carried out in a suitable fashion, especially if the studies will be recruiting based on genosets or polygenic risk scores [17].

4.7 Statements

Trial status

This trial is ongoing and has been recruiting since July 2016. The trial is expected to continue until approximately June 2020. The current protocol number and date is version 5 and 20 July 2018, respectively.

Availability of data and materials

The de-identified datasets, that will be used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

Acknowledgements

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4.8 Ethics declarations

Ethics approval and consent to participate

The Bannatyne Campus Biomedical Research Ethics Board (BREB) in Winnipeg, Manitoba, Canada has approved this study protocol (HS19441 (B2016:011). All amendments to this studies protocol are reviewed and approved by the U of M BREB, and changes to the protocol have been updated on clinicaltrials.gov. This trial is registered at clinicaltrials.gov (Identifier: NCT02765516). All participants must provide a written informed consent before they are enrolled in this trial, and these consent forms will be dated and signed by participants and an appropriate trial staff prior to participant entry into the trial. Participants sign a general consent to participate in the trial as well as a consent for genetic analysis. Participants receive a copy of the consent forms they have signed.

Consent for publication

All participants provide consent to publish or present their information gathered during the trial in a fashion where their personal information such as their name, address, telephone number and/or any other identifying information are not revealed.

Competing interests

ET is employed by Upfield™ marketed food products with added plant sterols. All other authors declare that they have no competing interests.

4.9 References

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Bridge to Chapter V

The following chapter comprises the results manuscript of the protocol from chapter IV investigating the nutrigenetic effect of plant sterols on LDL cholesterol in a randomized clinical trial. The authors' responsibilities were as follow: Dylan MacKay, Peter Eck, and Peter Jones designed the study protocol, sought funding, and submitted ethics for approval; Itzel Vasquez, **Matthew Granger, and Maryam Shamloo conducted the clinical trial and collected the data**; Maryam Shamloo and Matthew Granger analyzed all the samples; Itzel Vasquez and **Matthew Granger conducted the statistical analysis and wrote the manuscript drafts**; Elke Trautwein provided critical revisions and input to the manuscript and assisted in seeking funds for the trial; James House was the trial principal investigator. James House and Dylan MacKay shared responsibility for the oversight of the trial and the final content of this manuscript. All the authors read and approved the final paper.

Chapter V

This manuscript will be submitted for publication in 2021

Genosets for APOE and CYP7A1-rs3808607 variants do not predict low-density lipoprotein cholesterol lowering upon intervention with plant sterols – results of the Genetic Basis for Prediction of Non-responders to Dietary Plant Sterol Intervention (GenePredict-PS) a double-blind, placebo-controlled, randomized two-period crossover trial

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Chapter V

5.1 Abstract

Background

The consumption of 2 g/day plant sterols (PS) reduces circulating low-density lipoprotein cholesterol (LDL-C) up to 10%. The degree of LDL-C lowering was associated with specific APOE and CYP7A1 genosets in previous post hoc analyses of randomized controlled trials. However, since post-hoc analyses do not conform to the randomization model, there is a greater potential that the findings may be due to confounding. warranting validation through an *a priori* designed intervention trial.

Objective

The GenePredict Plant Sterol study (GPS) was designed to validate associations of LDL-C lowering with specific APOE and CYP7A1 genosets through a priori recruitment of individuals carrying pre-specified genosets.

Methods

A two center, double-blind, placebo-controlled, randomized two-period crossover dietary intervention with 2g/day of plant sterols was undertaken. A priori recruitment of individuals with slightly elevated LDL-C was based on genosets of APOE isoforms and CYP7A1 rs3808607. Randomization was performed with stratification by sex and genoset.

Results

The recruitment target of 64 participants with pre-specified genosets could not be reached, despite the screening of 477 individuals; 42 participants completed the feeding trial. Reductions of total cholesterol, LDL-C, high-density lipoprotein cholesterol, were similar across all genosets. Suggesting the shortfall in recruitment may not have stopped the trial from meeting the objective.

Conclusions

APOE and CYP7A1 genotypes did not influence the efficacy of LDL-C reductions upon dietary intervention with PS. Findings of previous post-hoc analyses could not be validated in a trial using a priori genotype based recruitment. Obtaining adequate numbers of participants is challenging in trials using genoset based recruitment, even for common variants.

Trial Registration: Clinical Trials #NCT02765516

Funding: GenePredict-PS has received research funding from the University of Manitoba, Mitacs, Unilever R&D, and Nutritional Fundamentals for Health.

5.2 Introduction

Decreasing hypercholesterolemia is the main target for primary and secondary prevention of cardiovascular diseases. In dietary intervention trials, supplementation with esterified plant sterols has repeatedly resulted in clinically relevant reductions in circulating low-density lipoprotein cholesterol (LDL-C), which is recognized as a major risk biomarker for atherosclerotic cardiovascular disease.[1] Specifically, ingestion of 1.5-2g/d of esterified plant sterols reduces circulating LDL-C up to 10%, by reducing the intestinal cholesterol absorption, altered circulating cholesterol distribution or enhanced bile acid synthesis. [2,3,4,5] Therefore, functional claims have been approved by governments' institutions in Europe (EFSA), the US (FDA) and Health Canada). Moreover, plant sterol supplementation, as a component in a broader cholesterol-lowering strategy, is integrated into the guidelines of stakeholder societies such as the Canadian Cardiovascular Society, Heart and Stroke Association, American Heart Association, and British Heart Foundation. [6,7,8,9,10]

It has been established that the efficacy of LDC-C reduction by plant sterol interventions' shows large and repeatable inter-individual variability, pointing towards an intrinsic determinant of response. [11] In *post-hoc* analyses of two intervention trials variations in the *CYP7A1* and *APOE* genes, which are determinants of cholesterol absorption and distribution, consistently associated with the efficacy of LDL-C reduction. [12,13] Specifically, the genoset constituted of single nucleotide polymorphism (SNP) rs3808607-T/T homozygosity in combination with the *APOE* ε3 showed slightly increased circulating LDL-C, while all other combinations showed reductions. Herein, the *APOE* ε4 isoform may attenuate the non-response phenotype of the SNP rs3808607-T allele.

Therefore, it was hypothesized that these genosets would predict the response to plant sterol intervention. However, since recruitment in these trials was not based on genotypes, the number of individuals in each genosets were unequal, and the *post-hoc* comparisons were at risk of confounding. To appropriately test the predictive associations from *post-hoc* findings, the Gene Predict Study (GPS) used an *a priori* genotype based recruitment to tests the hypothesis that the genoset of SNP rs3808607-T/T-*APOE* ε3 predicts non-response to plant sterol consumption (**Table 5.1**). [12] We here report on the primary outcome, reduction in circulating LDL-C, as well as the challenges in recruiting individuals carrying specific genosets.

Table 5.1. Genotype distribution among selected genosets *a priori* the clinical trial.

Gene	Genosets		
	GPS1	GPS2	GPS3
APOE (*rs7412, rs429358*)	ε3/ε3	ε3/ε3	ε4/ε4
CYP7A1 (*rs3808607*)	T/T	T/G G/G	T/G G/G T/T
Predicted response	Non-responder	Responder	Responder
GPS Group	1	2	3
Female	6	10	11
Male	2	6	7
Initial recruitment target	20	22	22
Final number recruited	8	16	18

Abbreviations: APOE = apolipoprotein E; CYP7A1 = cholesterol 7-α-hydroxylase; GPS = GenePredict study group.

5.3 Material and Methods

Study Design

A double-blind, placebo-controlled, randomized two-period crossover dietary intervention trial with *a priori* recruitment based on genoset was designed (**Table 5.1**). Methodological details of the GPS study have been published. [14] Randomization was completed by an assistant external to the research team using a block randomization method through sealed envelopes with stratification by sex and genoset. Randomization occurred in blocks of eight and four with equal numbers of treatment for each block to minimize any potential imbalances in the order of the treatment within any group. The primary outcome measure was LDL-C reduction associated with specifics genosets. The GPS study was registered on ClinicalTrial.gov #NCT02765516. The clinical trial ran from July 5, 2017 to December 31, 2019. The original protocol outlined the recruitment and grouping of eight different genosets with *APOE*-(ε3/ε3)/*CYP7A1*-(T/T) and *APOE*-(ε2/−)/*CYP7A1*-(T/T) genotypes predicted to be non-responders, and the other genotypes as responders. Changes were made to the study design in response to the amount of time spent on recruitment and the difficulty in recruiting participants who were eligible with rare combinations

of genosets, genosets including *APOE*-ε2 were removed, and multiple smaller genosets were collapsed into three genosets which still allowed the trials hypothesis to be tested. [14] The Bannatyne Campus Biomedical Research Ethics Board (BREB) in Winnipeg, Manitoba, Canada has approved this study protocol (HS19441 (B2016:011).

Study Population

Residents of Winnipeg, Manitoba, Canada and the surrounding area between the ages of 18 to 70 years old were screened and recruited to participate at Richardson Centre for Functional Foods and Nutraceuticals or the Seven Oaks General Hospital. Individuals with circulating LDL-C between 3.0 to 4.9 mmol/L and glucose concentration <6.1 mmol/L and the genosets described in Table 5.1 were participating. Individuals with phytosterolemia, type 1 or type 2 diabetes, a history of cancer, gastrointestinal diseases, or any other chronic disease were ineligible.

Intervention

The full protocol is published.[14] Briefly, the GPS study consisted of two treatment periods 28 days in length with a minimum of a 28-day washout period. Participants consumed their regular diet and two daily portions of margarine, a serving with breakfast and dinner, containing 2 grams/day of plant sterols (PS) or placebo. The interventions for the trial were manufactured by Unilever (Vlaardingen, Netherlands) and provided to the researchers in blinded single-serve portions. The PS intervention contained 70% sitosterol, 14% campesterol, 8% sitostanol, 3% brassicasterol and other minor plant sterols.

Blood sampling and analyses

Fasting blood samples were taken by a phlebotomist or registered nurse at the beginning (Days 0 and 1) and end (Days 28 and 29) of each period. Genomic DNA was extracted from buffy coat white bloods using a column-based DNA extraction kit (DNeasy Blood and Tissue Kit; QIAGEN Sciences) as per the manufacturer's instructions. Concentration and DNA integrity was measured via spectrophotometer. (NanoDrop 2000; Thermo Fisher Scientific). Genotyping of DNA samples was done with TaqMan SNP genotyping assays (CYP7A1-rs3808607, assay identification (ID) C2749212120; APOE rs7412, assay ID C2749212120; APOE-rs429358, assay ID C308479320; Life Technologies) on a StepOnePlus Real-Time PCR System (Applied

Biosystems; Life Technologies). Blood cholesterol and sterols were analyzed via gas chromatography with flame ionization detection. [15] Fasting serum glucose, total cholesterol, LDL-C, HDL-C, and triglycerides were measured with the Cobas 311 Analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analysis

The effects of PS treatment comparing the endpoint values of treatment and placebo periods were analyzed using the SAS MIXED procedure (Statistical Analysis Software, version 9.4). Sequence and sex were included in the model as fixed factors, while participant ID was included as a repeated factor. Genotype/genoset and treatment-by-genotype/treatment-by-genoset were individually included as fixed factors to assess the impact of genotype or genosets. Significant treatment-by-genotype or treatment-by-genoset effects were examined by the SAS SLICE function, with Tukey correction for the number of slices. Treatment effect sizes by genotype or genoset, from significant treatment-by-genotype or -genoset interactions, were compared using t-test or ANOVA with the difference in mixed-model least squares means summary statistics for the treatment effect slices, with Tukey's *post hoc* adjustment for multiple comparisons. Statistical significance was set at $p < 0.05$ in all analyses.

5.4 Results

Recruitment and biomarkers at baseline

The study was terminated due to futility in recruitment (**Table 5.1**). 477 individuals were screened and 42 participants between the ages of 23 to 68 years completed the trial (**supplementary Figure 5.4**). Baseline characteristics are shown by sex (**supplementary Table 5.4)** and by genosets **(Table 5.2)**. No participants reported any adverse effects of the treatments. The planned numbers of individual recruited per genosets were not met. 8 participant in GPS1, 16 in GPS2 and 18 in GPS3 were enrolled, this was below the target for each group (**Table 5.1**).

Table 5.2. Baseline participant characteristics by genoset.

	GPS1	**GPS2**	**GPS3**	**P-value**[1]
Sex balance F/M	6/2	10/6	11/7	NS
Age, years	58.00 ±7.71	57.81 ±10.26	54.72 ±13.64	NS
Body weight, kg	77.94 ±20.88	79.54 ±15.58	82.16 ±21.69	NS
Body mass index, kg/m2	28.19 ±5.53	28.26 ±4.91	29.04 ±5.04	NS
Total cholesterol, mmol/L	5.97 ±0.69	6.14 ±0.69	6.09 ±0.67	NS
LDL-C, mmol/L	4.09 ±0.62	4.10 ±0.60	4.17 ±0.58	NS
HDL-C, mmol/L	1.51 ±0.28	1.45 ±0.39	1.44 ±0.31	NS
Triglycerides, mmol/L	1.24 ±0.40	1.73 ±1.01	1.52 ±0.48	NS
Glucose, mmol/L	5.13 ±0.67	5.17 ±0.45	5.20 ±0.53	NS
Cholestanol ratio†	1.58 ±0.34	1.73 ±0.91	1.59 ±0.36	NS
Campesterol ratio†	1.73 ±0.63	2.24 ±2.77	1.44 ±0.37	NS
Desmosterol ratio†	0.65 ±0.23	0.61 ±0.21	0.62 ±0.39	NS
Lathosterol ratio†	1.68 ±0.93	1.64 ±0.73	1.97 ±0.99	NS
Sitosterol ratio†	1.78 ±0.43	1.56 ±0.82	1.05 ±0.30	**0.0011**

1 All values are differences in estimated least-squares means ± SEMs. P values were derived by using SAS MIXED model.

†µmol sterol or stanol per mmol cholesterol

Abbreviations: NS, not significant; GPS1, *APOE*-*ε*3/*ε*3 and *CYP7A1*-T/T; GPS2, *APOE*-*ε*3/*ε*3 and *CYP7A1*-G/G and G/T; GPS3, *APOE*-*ε*4/*ε*4 and CYP7A1-T/T, G/G and G/T.

Circulating LDL-C and related lipids

As anticipated, when not stratified by genoset, the plant sterol intervention reduced circulating LDL-C (-0.3012 mmol/L, p=0.0002), total cholesterol (TC, -0.3788 mmol/L, p=0.0001), and HDL-C (-0.0587 mmol/L, p=0.0198) compared to the placebo (**supplementary Table 5.5**). Lathosterol-to-cholesterol ratio (Δ0.6077 µmol/mmol, p=0.0468) and sitosterol-to-

cholesterol ratio (Δ0.8823 μmol/mmol, p=0.0003) increased consistent with plant sterol consumption.

LDL-C reductions following plant sterol consumption compared to placebo were similar across all genosets (**Table 5.3**) with reductions of -0.2979 ±0.16, -0.3572 ±0.1153, -0.2934 ±0.1087 mmol/L (p=0.0002) were measured for GPS1, GPS2, and GPS3, respectively. Consistent reductions in TC were measured with reductions of -0.3552 ±0.1734, -0.3912 ±0.1223, and -0.4105 ±0.1152 mmol/L (p<0.0001) for GPS1, GPS2, and GPS3, respectively. Additionally, HDL-C and triglyceride concentrations and parameters of cholesterol synthesis did not differ between genosets (**Table 5.3**).

Moreover, upon plant sterol consumption, reductions of LDL-C and TC did not differ when stratified by *CYP7A1* rs3808607 genotypes (**supplementary Table 5.6**) or APOE isoforms (**supplementary Table 5.7**). The rs3808607-T/T had reductions of -0.3572 ±0.1396 and -0.4070 ±0.1485 mmol/L, in LDL-C and TC, respectively. The rs3808607-G/T had reductions of -0.2869 ±0.0882 and -0.3779 ±0.0938 mmol/L in LDL-C and TC respectively. The rs3808607-G/G had reductions of -0.4505 ±0.2410 and -0.4663 ±0.2564 mmol/L, in LDL-C and TC respectively. The *APOE*-ε3/ε3 genotype had reductions of -0.3376±0.0935 and -0.3793 ±0.09907 mmol/L, for LDL-C and TC, respectively, while *APOE*-ε4/- genotype had reductions of -0.2935 ±0.1074 and -0.4106 ±0.1138mmol/L, for LDL-C and TC, respectively.

Table 5.3. Changes in blood lipids and non-cholesterol sterols after PS consumption by genoset.

	Least squares means (Treatment – Placebo)			**P value[1]**	
	GPS1	**GPS2**	**GPS3**	**Treatment**	**Treatment x Genotype**
LDL-C, mmol/L	-0.2979 ±0.1636	-0.3572 ±0.1153	-0.2934 ±0.1087	**0.0002**	0.9126
TC, mmol/L	-0.3552 ±0.1734	-0.3912 ±0.1223	-0.4105 ±0.1152	**<0.0001**	0.9650
HDL-C, mmol/L	-0.02284 ±0.05148	-0.01611 ±0.03629	-0.1056 ±0.03420	0.0525	0.1677
TG, mmol/L	-0.05903 ±0.1467	-0.1612 ±0.1034	-0.09716 ±0.09749	0.1312	0.8272
Cholestanol ratio†	0.03915 ±0.1394	-0.00245 ±0.09350	-0.06043 ±0.08813	0.9016	0.8085
Campesterol ratio†	-0.00823 ±0.05674	-0.02775 ±0.03896	-0.02769 ±0.03586	0.4193	0.9525
Desmosterol ratio†	-0.2432 ±0.6644	0.8782 ±0.4511	0.6611 ±0.4252	0.1644	0.3741
Lathosterol ratio†	0.01103 ±0.3059	-0.2401 ±0.2093	-0.06883 ±0.1919	0.4815	0.7480
Sitosterol ratio†	1.8445 ±0.4704	0.8334 ±0.3235	0.3483 ±0.2982	**<0.0001**	**0.0365**

1 All values are differences in estimated least-squares means ± SEMs. *P* values were derived by using SAS MIXED model.

†µmol sterol or stanol per mmol cholesterol

Abbreviations: GPS1, *APOE*-ε3/ε3 and *CYP7A1*-T/T; GPS2, *APOE*-ε3/ε3 and *CYP7A1*-G/G and G/T; GPS3, *APOE*-ε4/ε4 and CYP7A1-T/T, G/G and G/T.

5.5 Discussion

The purpose of this study was to determine if the genoset *APOE*-(ε3/ε3)/*CYP7A1*-rs3808607-T/T was predictive of non-responsiveness of LDL-C lowering to PS. Despite the trial not reaching the recruitment goals for each genoset, a reduction in LDL-c was observed across all genosets, indicating that the genoset of GPS1 (*APOE*-(ε3/ε3)/*CYP7A1*-rs3808607-T/T) is likely not predictive of non-response of cholesterol to PS. Therefore, this study does not support the genotyping of rs7412, rs429358, or rs3808607 in advance of PS consumption to predict responsiveness. [16] In agreement with many previous trials investigating PS and cholesterol, this study found an overall average decrease of -0.3788 and -0.3012 mmol/L in TC and LDL-C, respectively, as well as increases in sitosterol and lathosterol to cholesterol ratios from the consumption of 2 g/day of PS over a four-week period. [17]

A recent analysis of a previous genome-wide association study (GWAS) added more novel loci to the more than 380 existing lipid and cholesterol genetic associations. [18] While network mapping and analysis like this provide invaluable insight into the big picture of genetics, there seems to be an imbalance with respect to the necessary mechanistic studies that can further explicate these associations. These genes have widespread physiological effects, which may not be influenced by nutrient intake, so while GWAS can be used to generate variant candidates for nutrigenetics studies, there may be variants that do have gene by nutrient interactions that are not captured in GWAS because there is no nutritional intervention or deficit to amplify the signal of that interaction.

Many of the nutrigenetic associations reported in the literature are from epidemiological studies which often measure nutrient intake via dietary recall tools and are not replicated, and where there is replication in separate cohorts, very few are tested in clinical trials.[19] Several of these gene by nutrient associations are assumed to be real and used to form the basis of genetic based tests that are sold as genetic based diets. A 2008 paper found a positive association of rs4148217 in *ABCG8* with LDL-C reduction to consumption of PS, but a follow-up study found no association.[12,20] That same 2008 study also found an association between LDL-C response and PS in rs2072183 of *NPC1L1*, which was not observed in RCTs conducted in 2008 and 2016.[20,21,22] Similarly, a positive association was found between LDL-C response and PS consumption in rs5882 of cholesteryl ester transfer protein (*CETP*) and then no such association was observed in a follow-up study.[12,23] The present study attempted to validate previous

findings from clinical trials with the hypothesis that perhaps a combination of SNPs was the predictive of LDL-C response to PS.[12] However our hypothesis was not supported and that previous association of rs3808607 in *CYP7A1* and LDL-C response to plant sterols appears to have been spurious given that the LDL-C response across all genosets was proportionally of the same magnitude.

Designing nutrigenetic experiments in a way that results in only finding associations has led to an all-too-common common pattern in nutrigenetic research of finding statistical associations and then not observing those findings in repeated experiments, or vice versa. Studies like this do not help to clarify the unknowns of human genetics. While our present study did not find any association with LDL-C response and PS consumption, De Castro-Orós et al. did include some mechanistic binding and expression experiments to demonstrate an increased expression of CYP7A1 which at least indicates some possible mechanism to explain the association despite the inconsistent findings of association previous and subsequent studies. [13]

An emphasis on greater collaboration between mechanistic and association researchers is of paramount importance if this pattern of finding associations that fail to replicate is to end. Many proposed nutrient by gene associations have been reported, it would be advisable for future studies to begin investigating these associations mechanistically as well as trying to replicate them in clinical trials, rather than continuing to publish data suggesting new nutrient by gene associations that may just be spurious statistical associations.

5.6 Strengths and Limitations

The primary limitation of this study was the failure to reach the recruitment for each genoset in the study. These recruitment issues resulted in a lower power than originally planned, so any genetic associations found within should be interpreted with this shortcoming in mind. Despite this issue with recruitment, the study was able to show a reduction in LDL-C in each group in response to PS consumption, the lack of power would have biased towards not seeing a reduction in LDL-C relative to the control. Recruitment from an existing database of willing participants whose relevant genetic profiles are known would significantly expedite future nutrigenomic studies with a similar design. Dietary intake of fat or fibre was not controlled for, nor measured at baseline or throughout the study and could have influenced the changes in lipids.[24] However, one of the benefits of a crossover design is participants act as their own control and often have

consistent lifestyles. [25] The basal cholesterol metabolism and synthesis of participants was not evaluated in participants *a priori* as part of the inclusion criteria. [15] Cholesterol synthesis levels may be a predictor of cholesterol lowering in response to PS consumption. Study strengths include the *a priori* genetic recruitment and grouping of participants as well as the thorough design of the study, specifically the cross-over design, block randomization, and adherence monitoring during consumption of the PS treatment.

5.7 Conclusions

In consensus with previous studies, consumption of 2 g/day of PS was observed to significantly reduce blood cholesterol concentrations, regardless of genotypes investigated. The *APOE*-(ε3/ε3)-CYP7A1-(T/T) genoset was not predictive of non-response of cholesterol to PS consumption, reductions in cholesterol were still observed, regardless of genoset. This suggests that most people may achieve a reduction in cholesterol from the consumption of 2 grams of PS per day, and that genotyping in advance of PS consumption may be unnecessary. Future nutrigenomic studies should seek to adopt the *a priori* recruitment strategy used in this study as it is far more robust to pre-specify a genetic association to be tested. Unrestricted exploration of potential genetic associations after a study has been completed has the potential to lead to p-hacking and/or selective reporting of results, which increases of the risk of spurious findings.

5.8 Financial Support

GenePredict-PS has received research funding from the University of Manitoba, Mitacs, Unilever R&D, and Nutritional Fundamentals for Health. Funding to MITACS was provided by Unilever and Nutritional Fundamentals for Health. Unilever also provided product support in the form of margarine for this GPS study.

5.9 Supplemental tables and figures

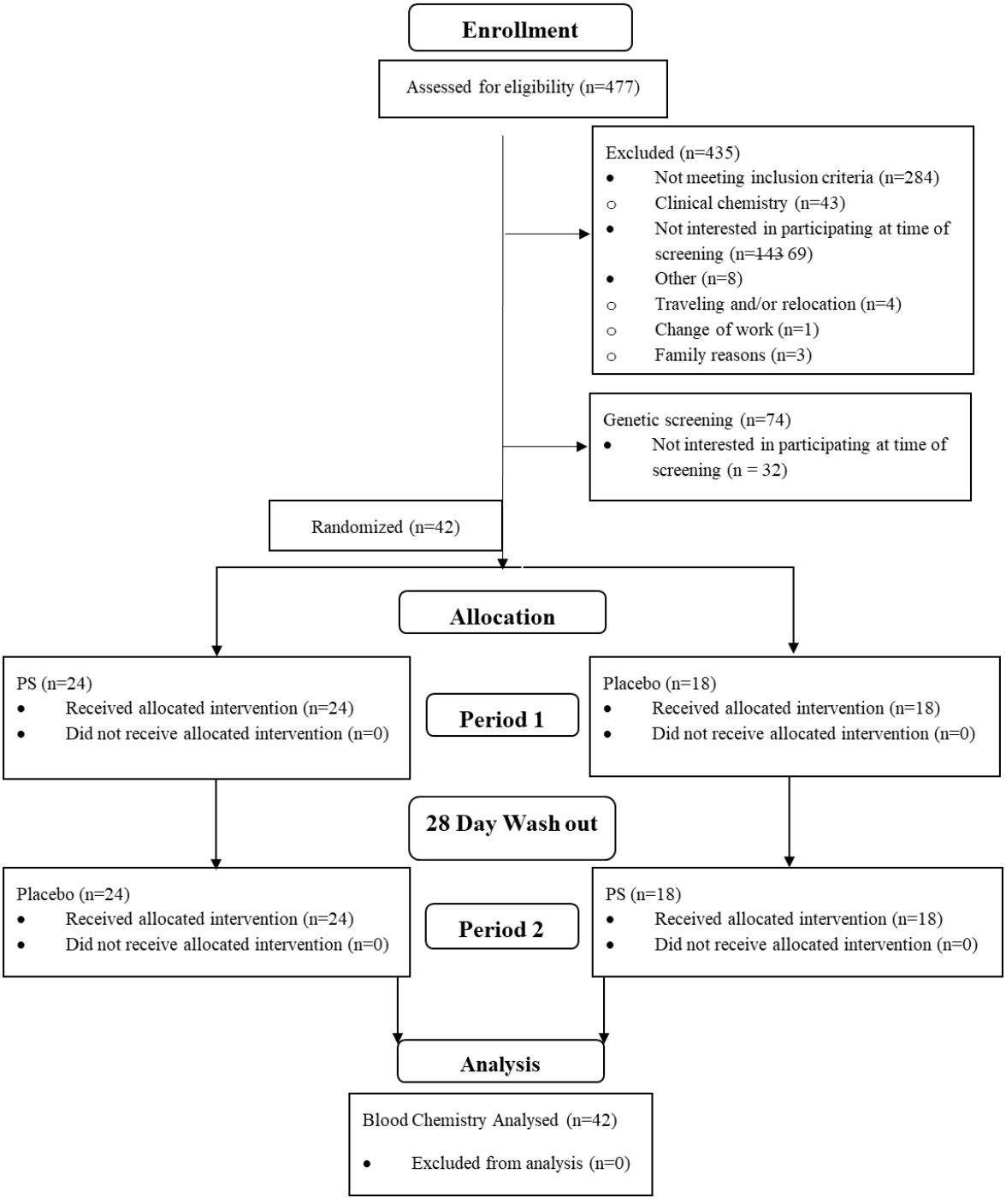

Figure 5.1. GenePredict-PS Trial Flow chart.

Table 5.4. Baseline participant characteristics by sex.

	Total (n=42)	**Males (n=15)**	**Females (n=27)**	**P-value**[1]
Age, years	56.52 ±11.33	54.47 ±14.23	57.67 ±9.46	NS
Body weight, kg	80.35 ±19.0	92.24 ±13.34	73.75 ±18.62	**0.001**
Body mass index, kg/m^2	28.58 ±4.98	29.49 ±3.26	28.08 ±5.71	NS
Total cholesterol, mmol/L	6.09 ±0.67	5.96 ±0.82	6.16 ±0.57	NS
LDL-C, mmol/L	4.13 ±0.58	4.12 ±0.66	4.13 ±0.55	NS
HDL-C, mmol/L	1.46 ±0.33	1.31 ±0.27	1.55 ±0.34	**0.033**
Triglycerides, mmol/L	1.55 ±0.73	1.59 ±0.93	1.52 ±0.60	NS
Glucose, mmol/L	5.17 ±0.52	5.22 ±0.48	5.15 ±0.55	NS
ALT, mmol/L	26.71 ±11.9	30.03 ±15.99	24.86 ±8.70	NS
AST, mmol/L	26.41 ±6.35	28.37 ±7.90	25.33 ±5.15	NS
Cholestanol ratio†	1.64 ±0.62	1.78 ±0.95	1.56 ±0.31	NS
Campesterol ratio†	1.80 ±1.75	2.31 ±2.83	1.52 ±0.54	NS
Desmosterol ratio†	0.62 ±0.29	0.80 ±0.38	0.52 ±0.18	**0.0021**
Lathosterol ratio†	1.79 ±0.88	2.09 ±1.09	1.64 ±0.73	NS
Sitosterol ratio†	1.38 ±0.64	1.30 ±0.42	1.31 ±0.51	NS

1 *P* values were calculated by T-test between males vs. females. Values are mean ± SD.

†µmol sterol or stanol per mmol cholesterol

Table 5.5. Changes in blood lipids and non-cholesterol sterols after PS consumption for all participants (n=42).

	Least squares means[1]		**Change** (mmol/L)	**P value[2]**
	Treatment	**Placebo**		**Treatment**
LDL-C, mmol/L	3.9754 ±0.09531	4.2766 ±0.09531	-0.3012	**0.0002**
TC, mmol/L	5.8353 ±0.1168	6.2141 ±0.1168	-0.3788	**<0.0001**
HDL-C, mmol/L	1.4573 ±0.05259	1.5160 ±0.05259	-0.0587	**0.0198**
TG, mmol/L	1.3879 ±0.09563	1.5060 ±0.09563	-0.1181	0.082
Cholestanol ratio†	1.5287 ±0.06414	1.5252 ±0.06325	0.0035	0.9532
Campesterol ratio†	0.6103 ±0.02729	0.6380 ±0.02648	-0.0277	0.2733
Desmosterol ratio†	2.3711 ±0.2265	1.7634 ±0.2219	0.6077	**0.0468**
Lathosterol ratio†	2.2728 ±0.2313	2.4226 ±0.2281	-0.1498	0.2746
Sitosterol ratio†	2.4735 ±0.2074	1.5912 ±0.1997	0.8823	**0.0003**

1 All values are differences in estimated least-squares means ± SEMs.

2 *P* values were derived by using SAS MIXED model

†µmol sterol or stanol per mmol cholesterol.

Table 5.6. Changes in blood lipids and non-cholesterol sterols after PS consumption by rs3808607 variant.

	Least squares means[1] (Treatment – Placebo)			**P value[2]**	
	rs3808607				
	T/T (n=27)	**G/T** (n=11)	**G/G** (n=4)	**Treatment**	**Treatment x Genotype**
LDL-C, mmol/L	-0.3572 ±0.1396	-0.2869 ±0.0882	-0.4505 ±0.2410	**0.0007**	0.7850
TC, mmol/L	-0.4070 ±0.1485	-0.3779 ±0.0938	-0.4663 ±0.2564	**0.0003**	0.9451
HDL-C, mmol/L	-0.07144 ±0.0459	-0.05635 ±0.02899	-0.00426 ±0.07924	0.1835	0.7570
TG, mmol/L	-0.05026 ±0.1255	-0.1491 ±0.07925	-0.03847 ±0.2166	0.3777	0.7590
Cholestanol ratio†	0.000155 ±0.1086	-0.08405 ±0.06634	0.4245 ±0.1814	0.1370	0.0423
Campesterol ratio†	-0.02256 ±0.04759	-0.02317 ±0.02906	-0.06487 ±0.08851	0.3023	0.9009
Desmosterol ratio†	0.1366 ±0.5615	0.6193 ±0.3458	1.6352 ±0.9456	**0.0476**	0.3862
Lathosterol ratio†	-0.06485 ±0.2566	-0.1763 ±0.1559	0.2297 ±0.4818	0.9842	0.7108
Sitosterol ratio†	1.1192 ±0.4285	0.6692 ±0.2628	0.8719 ±0.7898	**<0.0080**	0.6722

1 All values are differences in estimated least-squares means ± SEMs.

2 *P* values were derived by using SAS MIXED model.

†µmol sterol or stanol per mmol cholesterol

Abbreviations: GPS1, *APOE*-ε3/ε3 and *CYP7A1*-T/T; GPS2, *APOE*-ε3/ε3 and *CYP7A1*-G/G and G/T; GPS3, *APOE*-ε4/ε4 and CYP7A1-T/T, G/G and G/T.

Table 5.7. Changes in blood lipids and non-cholesterol sterols after PS consumption by *APOE* variant.

	Least squares means[1] (Treatment – Placebo)		**P value[2]**	
	APOE			
	ε3/ε3 (n=24)	ε4/- (n=18)	**Treatment**	**Treatment x Genotype**
LDL-C, mmol/L	-0.3376±0.0935	-0.2935 ±0.1074	<**0.0001**	0.7572
TC, mmol/L	-0.3793 ±0.09907	-0.4106 ±0.1138	<**0.0001**	0.8361
HDL-C, mmol/L	-0.01833 ±0.0294	-0.1056 ±0.03377	**0.0089**	0.0575
TG, mmol/L	-0.1274 ±0.08415	-0.09735 ±0.09665	0.0887	0.8147
Cholestanol ratio†	0.01163 ±0.07687	-0.06053 ±0.08704	0.6776	0.5362
Campesterol ratio†	-0.02141 ±0.03176	-0.02772 ±0.03540	0.3097	0.8950
Desmosterol ratio†	0.5299 ±0.3766	0.6618 ±0.4281	**0.0441**	0.8176
Lathosterol ratio†	-0.1622 ±0.1716	-0.06898 ±0.1904	0.3743	0.7176
Sitosterol ratio†	1.1516 ±0.2740	0.3475 ±0.3061	**0.0008**	0.0572

1 All values are differences in estimated least-squares means ± SEMs.

2 *P* values were derived by using SAS MIXED model.

†μmol sterol or stanol per mmol cholesterol

Abbreviations: GPS1, *APOE*-ε3/ε3 and *CYP7A1*-T/T; GPS2, *APOE*-ε3/ε3 and *CYP7A1*-G/G and G/T; GPS3, *APOE*-ε4/ε4 and CYP7A1-T/T, G/G and G/T.

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Chapter VI

Overall conclusions

6.1 Summary and implications

The results presented within this thesis have implications for the precision nutrition industry and nutrigenetic researchers. The chapter on the nutrigenetics of AA in human health and disease detailed the numerous putative genetic associations between AA status and various diseases as well as many SNPs in AA transporters that have been demonstrated to affect circulating AA concentrations.

The findings from the PS clinical trial detailed above should help inform potential precision nutrition consumers who are interested in managing their LDL-C and are considering having their genotype(s) determined in advance of utilizing functional foods enriched with plant sterols (PS) therapeutically. Purveyors of precision nutrition products should find the information within useful when considering which genetic variants they ought to be utilizing in the development of their algorithms and subsequent products. Finally, nutrigenetic researchers should find the contents of this thesis of use when it comes to the design and implementation of nutrigenetic studies, considerations to make, and pitfalls to avoid. There needs to be caution when relying on data from observational studies when it comes to translating that data into research hypotheses and dietary recommendations as the nutrigenetic effect of a nutrient may not be present without intervention.

When it comes to the consumption of PS as a way to manage LDL-C, the data from the GPS study does not indicate that consumption of PS needs to be genetically targeted for an individual.

6.2 Limitations and future directions

A limitation of the GPS study was the failure to meet our estimated recruitment targets. However, a consistent and significant effect was measured with respect to PS consumption on C LDL-C reduction across all genosets, genotypes, and for both sexes. This indicates that the effect of this failure was likely negligible, but this still deserves to be acknowledged. One solution to remedy the recruitment challenges of the GPS study would be to establish a database of participants who have either been genotyped or sequenced and are interested in participating in trials, this was the goal of the planned TMPLR cohort. Establishing such a database would streamline the recruitment process and result in fewer resources being expended on the screening

stages of a study. While selection bias in participants is a legitimate concern, the more efficient utilization of study resources would doubly allow for larger study sizes, more thorough dietary observation, and numerous other possibilities.

A potential limitation was that GPS study was not a controlled full feeding trial, so there remains the possibility that other nutrients such as fibre could have affected the outcome. While participants were required to consume their first dose of PS in front of a research assistant, there were no other dietary controls or restrictions in place. Dietary fiber is known to reduce LDL-C, and while the effect is small, with an average reduction of 0.13 mmol/L of LDL-C per 3 grams of soluble fiber, it could still be of consequence proportionally given the average reduction of 0.3012 mmol/L in LDL-C observed in the GPS study.[1] The consistent response in the reduction of LDL cholesterol across all genoset groups suggests that this was of little consequence to the final outcomes. However, a larger sample size might help smooth out any differences that may have been attributable to the consumption of fiber or any other nutrients.

A fixed shortcoming of highly specific genetic research projects like GPS study is that researchers are limited to selecting a few SNPs to investigate. Expanding the number of genotypes and genoset combinations requires significantly larger sample sizes. There remains numerous other SNPs and combinations thereof that may have an effect on LDL-C response to PS consumption. Along with the SNPs mentioned above, a recent study in children (n=26) found a positive response in LDL-C to PS consumption in participants with genetic variants in the hepatic triglyceride lipase (LIPC) rs1800588 (C514T) and the transcription factor peroxisome proliferator-activated receptor alpha (PPAR-α) rs1800206 (L162V) genes, so there are still newly discovered genetic associations that require further testing.[2] The simplest solution, relatively speaking, for this issue is to utilize more comprehensive genomic sequencing technologies or microarrays which in terms of dollars are becoming more affordable for research with costs still in the high hundreds or low thousands of dollars and if ethical, to build a database of participants with whose genetics are known.[3] While the SNPs investigated in GPS study did not predict the response of LDL cholesterol to PS, future research projects should consider incorporating these technologies into their methodologies in order to more comprehensively test putative predictive associations between nutrients and biomarkers or other health outcomes.

6.3 Final conclusions

The intricacies of nutrigenetic research are still being fine-tuned, however, there are numerous promising technological advancements that are available and in development that should assist in the execution and improvement of nutrigenetic studies. Through a robust, comprehensive, and critical assessment of nutrigenetic associations and methodologies, researchers can design stringent studies that lead to reliable and hopefully consistent conclusions. Relying on potentially spurious associations will only further obfuscate the literature as the reasoning for selecting those variants are often not well-founded when critically analyzed. Despite the GPS trial not observing a predictive response of LDL cholesterol to PS consumption, clinical trials with *a priori* genetic recruitment should become the standard for future nutrigenetic studies.

6.4 References

1. Brown L, Rosner B, Willett WW, Sacks FM. Cholesterol-lowering effects of dietary fiber: a meta-analysis. Am J Clin Nutr. 1999 Jan;69(1):30-42. doi: 10.1093/ajcn/69.1.30. PMID: 9925120.

2. San Mauro Martín I, Garicano Vilar E, Sanz Rojo S, et al. Gene Influence in the Effectiveness of Plant Sterols Treatment in Children: Pilot Interventional Study. Nutrients. 2019;11(10):2538. Published 2019 Oct 21. doi:10.3390/nu11102538

3. Gordon LG, White NM, Elliott TM, Nones K, Beckhouse AG, Rodriguez-Acevedo AJ, Webb PM, Lee XJ, Graves N, Schofield DJ. Estimating the costs of genomic sequencing in cancer control. BMC Health Serv Res. 2020 Jun 3;20(1):492. doi: 10.1186/s12913-020-05318-y. PMID: 32493298; PMCID: PMC7268398.

Appendices

Appendix 1: Copyright licenses for previously published materials

Chapter II: Manuscript 1

Vitamin C in Human Health

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Licensed Content Publication	Elsevier Books
Licensed Content Title	Advances in Food and Nutrition Research
Licensed Content Author	Matthew Granger,Peter Eck
Licensed Content Date	Jan 1, 2018
Licensed Content Volume	83
Licensed Content Issue	n/a
Licensed Content Pages	30
Start Page	281
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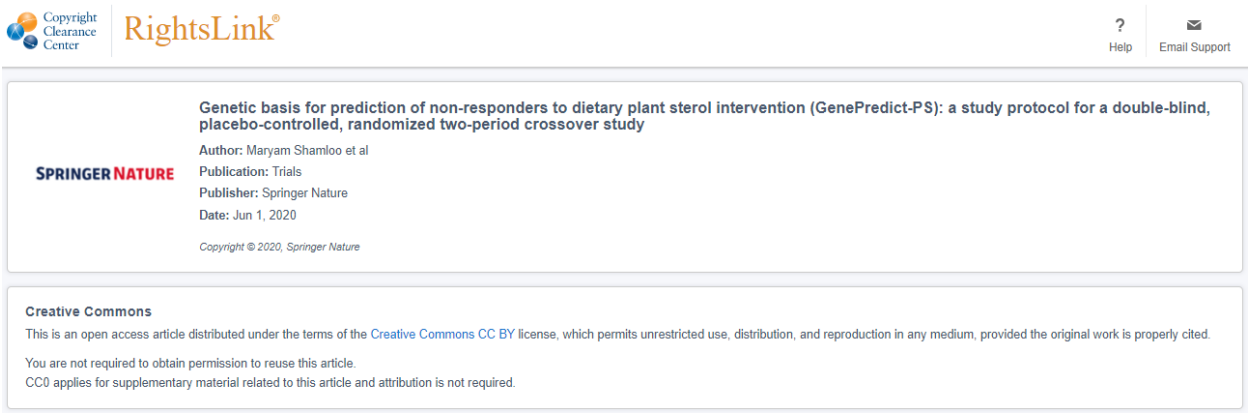
Chapter III: Manuscript 2

Genetic basis for prediction of non-responders to dietary plant sterol intervention (GenePredict-PS): a study protocol for a double-blind, placebo-controlled, randomized two-period crossover study



Chapter IV: Manuscript 3

Genetic basis for prediction of non-responders to dietary plant sterol intervention (GenePredict-PS): a study protocol for a double-blind, placebo-controlled, randomized two-period crossover study

Appendix 2: : Bannatyne Research Ethics Board approval letters

Appendix 2.1: Bannatyne Research Ethics Board approval letter for study in chapter III

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

UNIVERSITY OF MANITOBA

Research Ethics - Bannatyne
Office of the Vice-President (Research and International)

HEALTH RESEARCH ETHICS BOARD (HREB)
CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES
Full Board Review

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. Peter Jones	U of M and RCFFN/Food Sciences	HS18951 (H2015:367)
HREB MEETING DATE:	**APPROVAL DATE:**	**EXPIRY DATE:**
December 14, 2015	January 5, 2016	**December 14, 2016**
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (if applicable):		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
NA	The Manitoba Personalized Lifestyle Research (TMPLR) Program
SPONSORING AGENCIES AND/OR COORDINATING GROUPS:	
Research Manitoba	

Submission Date(s) of Investigator Documents:	REB Receipt Date(s) of Documents:
September 8, November 16 and December 18, 2015	September 8, November 16 and December 21, 2015

THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol:		
TMPLR Program Protocol	V. 2	October 20, 2015
Consent and Assent Form(s):		
Research Participant Information and Consent Form	V. 3	December 18, 2015
Research Participant Information and Consent Form – Additional Research Participant Information and Consent Form for Genetic Analysis and Long Term Storage of Samples for Future Analysis (Both Optional)	V. 2	December 16, 2015
Research Participant Information and Consent Form (Parents or Mother)	V. 2	November 16, 2015
Other:		
Pre-Screening Form (Telephone Screening)	V. 1	August 18, 2015
Participant Results Package	V. 2	November 10, 2015 submitted November 16, 2015
Diet History Questionnaire		
ASA24	Updated	January 24, 2014
Questionnaire Appendices	V. 1	August 11, 2015
Advertisement	V. 1	August 7, 2015
Data Collection Forms	V. 1	September 3, 2015

CERTIFICATION
The University of Manitoba (UM) Health Research Board (HREB) has reviewed the research study/project named on this ***Certificate of Final Approval*** at the ***full board meeting*** date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM HREB.

HREB ATTESTATION
The University of Manitoba (UM) Health Research Board (HREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba.

In respect to clinical trials, the HREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

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

CONDITIONS OF APPROVAL:

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

[redacted]
John Arnett, PhD., C. Psych.
Chair, Health Research Ethics Board
Bannatyne Campus

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

Appendix 2.2: Bannatyne Research Ethics Board approval letter study in chapter IV and V

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

UNIVERSITY OF MANITOBA | Research Ethics - Bannatyne
Office of the Vice-President (Research and International)

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

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. James House	U of M/Agricultural and Food Sciences/Human Nutritional Sciences	HS19441 (B2016:011)
BREB MEETING DATE: February 22, 2016	**APPROVAL DATE:** April 12, 2016	**EXPIRY DATE:** February 22, 2017
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
MC00009	MC00009 Genetic Basis for Prediction of Non-Responders to Dietary Plant Sterol Intervention (GenePredict-PS)
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Mitacs Inc.	

Submission Date(s) of Investigator Documents:	REB Receipt Date(s) of Documents:
January 29 and April 11, 2016	February 1 and April 11, 2016

THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol:		
Protocol Clarifications as per Letter received April 11, 2016	V. 1	April 11, 2016
Consent and Assent Form(s):		
Research Participant Information and Consent Form	V. 2	April 1, 2016
Additional Research Participant Information and Consent Form for Genetics Analysis and Request for Long Term Storage of Samples for Future Research	V. 2	April 11, 2016
Other:		
Poster 1	V. 2	April 11, 2016
Poster 2	V. 2	April 11, 2016
Telephone Screening Form	V. 2	April 11, 2016
Participant Consumption List	V. 2	April 11, 2016
Participant Diary	V. 1	April 11, 2016
Screening Log	V. 1	April 11, 2016
Case Report Form	V. 2	April 11, 2016

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on this ***Certificate of Final Approval*** at the ***full board meeting*** date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.

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

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

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

Sincerely,

[redacted]

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

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

Appendix 3: Study forms

Appendix 3.1: Study forms for Chapters III

Advertisements

Participant consent forms

General consent form

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

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

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

"The Manitoba Personalized Lifestyle Research (TMPLR) 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 Khafipour	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 Carlberg	University of Manitoba
Dr. Diana McMillan	University of Manitoba
Dr. Heather Blewett	St. Boniface Hospital/ CCARM
Dr. Todd Duhamel	University of Manitoba

Sponsor: Research Manitoba, 205-445 Ellice Ave., Winnipeg, Manitoba, R3B 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 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 (t-regs), 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.

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 first2 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

tablespoons). 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.

Station	Day 1	Estimated time (min)
1	Consent process	20
	Anthropometric Measurements	5
	PWA/PWV and blood pressure	10
2	Fasting blood samples	10
	Oral administration of deuterium	5
Participants will have the option of coming later on day 1 to complete tests		
Snack will be provided		
3	DXA	20
4	Questionnaires including General Health Questionnaire	30
5	Fecal and urine sample kits (bring the next day)	0
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 cardiorespiratory 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 assed 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

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.

Station	Day 2	Estimated time (min)
Participant will give urine and fecal sample to coordinator		
1	PWA/PWV and blood pressure (Mobil-O-Graph)	15
2	Fasting blood samples	10
3	Physical capacity (Assessing Frailty using the Modified Fried Criteria)	10
	Sub-maximal cardiorespiratory fitness test (YMCA submaximal cycle ergometer)	30
Snack can be provided after physical test		
4	Questionnaires (frail scale, obesity history, mindful eating quest, three factor eating questionnaire, Pittsburgh sleep quality index)	30
5	Instructions for activity monitor	5
Total time		100

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.

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.

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,

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.

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.

Investigator:	Dr. Peter Jones	Tel No.	204 474 9787
Coordinator:	Dr. Dylan Mackay	Tel No.	204 782 8124

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

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 take part in the retrospective study section of this study ☐ Yes ☐ No

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

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

Genetic analysis consent form

Richardson Centre for Functional Foods and Nutraceuticals

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM
FOR GENETIC ANALYSIS AND LONG TERM STORAGE OF SAMPLES FOR
FUTURE ANALYSIS
(BOTH OPTIONAL)

"The Manitoba Personalized Lifestyle Research (TMPLR) 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 Khafipour	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
Dr. Sharon Bruce	University of Manitoba
Dr. Jared Carlberg	University of Manitoba
Dr. Heather Blewett	St. Boniface Hospital/ CCARM
Dr. Todd Duhamel	University of Manitoba
Dr. Diana McMillan	University of Manitoba

Sponsor: Research Manitoba, 205-445 Ellice Ave., Winnipeg, Manitoba, R3B 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 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, in Canadians over the age of 20, three out of five 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. This study will include men and women aged 30-46, stratified by BMI, and geography.

Nature and Duration of Procedure

From the blood drawn during the clinical study entitled "The Manitoba Personalized Lifestyle Research (TMPLR) program", we would like to extract DNA and perform genetic analyses using a laboratory technique that augments and recognizes specific genes. This will allow us to determine the gene-environment interactions that modulate the susceptibility to chronic diseases in Manitobans. This genetic analysis may include looking at individual points or portions of you DNA, up to and including your entire genome, which is your full set of DNA including all your genes.

Confidentiality and Safekeeping of DNA Samples

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your samples a specific code. This code will link you to your samples containing genetic information and can only be decoded by the principal researcher or an individual authorized by the latter. Samples containing your genetic information will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of

Dr. Peter Jones for 10-year period following the end of the research project (January 2027). After this time, all samples will be destroyed, unless you have given consent for long term storage of your samples for future analyses. Your samples containing genetic information will only be used for the purpose of this research project.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Due to the research purposes of the planned analyses, we will not inform you, or your own doctors, of the results of the genetic tests on your samples. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee or a representative from the Richardson Centre for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. Project updates will be mailed at the end of the project. However, we will not communicate any individual results to you.

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 and kidney diseases.

Risk and Discomfort

As the DNA will be extracted from blood samples that have already been taken, there are no additional invasive procedures to undergo and no physical risks to you. Receiving information regarding susceptibility to genetic disease or identification of blood relationships may cause distress. There are potential psychological and social risks of genetic analysis related to how the results could change a person's life. You could have emotional reactions to learning that you do or do not carry a gene change for a certain condition. Additionally, some genetic analyses could reveal unexpected

relationships, such as non-paternity (a different biological father). **However, we will not be providing you with your personal results of the genetic testing that will be undertaken in the TMPLR program because the planned genetic tests are for research purposes, not the type used to determine disease risks or paternity**. While there may be no direct benefits to you for taking part in these additional analyses, we hope that the results will provide novel information on the influence of genetic characteristics of Manitobans.

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 (January 2027), 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, 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.

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 treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204 474 9787
Coordinator:	Dr. Dylan Mackay	Tel No.	204 782 8124

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.

Long term storage of samples for future analyses (optional)

In a study such as TMPLR, the samples that are collected have the potential to be an invaluable resource for future research.

We would like you to consider allowing us to store your blood and biological samples and questionnaire data for a maximum of 25 years for the purposes of future analyses related to lifestyle and chronic disease risk, other than those currently planned for the TMPLR program, after this time, all samples will be destroyed. 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 could provide new information on chronic disease in Manitoba. These future analyses could include sending your samples to other research centers to have additional analyses performed. Your samples and data would continue to be coded and your name and personal information such as your name, address or telephone number would not leave the Richardson Centre for Functional Foods and Nutraceuticals. All future analyses on samples would only occur after having received approval from the University of Manitoba Research Ethics Board.

This part of the program is optional, and if you decide not to participate you can still take part in the main preplanned pat of the TMPLR program.

Your blood and biological samples will still be stored for the purposes of future analyses that currently planned for the TMPLR program. There is no additional physical risk involved in allowing long-term storage samples. It does not require any additional visits, or any additional samples. You will not receive any financial compensation for allowing

your samples to be stored, or for any discoveries made using specimens held in storage.

By accepting this request to allow your samples and data to be for a maximum of 25 years, you are making an enormously valuable contribution to health research.

I agree that my biological samples (blood, stool and urine) may be stored for a maximum of 25 years for future analyses including DNA testing and take part in the continuing study ☐ Yes ☐ No

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 Research Ethics Board at the 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 am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.
4. 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.
5. By signing and dating this document, I am aware that none of my legal rights are being waived.

Participant signature: _____ Date: _____

Printed name of above: _____

I confirm that I have explained the purpose, duration etc. 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.

Research staff signature: _____ Date: _____

Printed name of above: _____ Study role: _____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Appendix 3.2: Study forms for Chapters IV and V

Advertisements

UNIVERSITY OF MANITOBA

Richardson Centre for Functional Foods and Nutraceuticals

Poster 2
April 11, 2016, Version 2

Do you have high cholesterol?

The University of Manitoba is conducting a study to investigate the effect of natural plant compounds on blood cholesterol concentrations

The study is open to men and women who meet the following criteria:

- **Aged 18 - 70 years**
- **Have elevated cholesterol**
- **Not taking medication to lower cholesterol**
- **Non-smokers**

Genetic testing will be done in this study

Participants will be compensated for their contribution to this study

For more information:
Phone: (204) 474-7091
Email: GenePredict-PS@umanitoba.ca, Website: www.rcffn.ca
James House, PhD, Principal Investigator

Participant consent forms

General consent form

UNIVERSITY OF MANITOBA

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study:	Genetic basis for Prediction of non-responders to dietary Plant Sterol intervention (GenePredict-PS)
Investigator:	James House, PhD Human Nutritional Sciences W569 Duff Roblin Building University of Manitoba 190 Dysart Road Winnipeg, Manitoba, Canada R3T 2N2 Phone: 204-474-6837
Sponsors:	Mitacs A250, Agricultural Engineering Building 96 Dafoe Rd, University of Manitoba Winnipeg, Manitoba R3T 5V6 Unilever R&D PO Box 290 3130 AG Vlaardingen Nutritional Fundamentals for Health 3405 FX-Tessier Vaudreuil-Dorion, Quebec J7V 5V5

July 20, 2018, Version 3

Participant initials _____

You are being asked to participate in a clinical trial (a human research study). Please take your time to review this Participant Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand. Should you decide to participate in this clinical study, please be advised that you are not allowed to participate in any other study for the duration of this clinical study. The institution is receiving professional fees and financial support to conduct this study.

Purpose of study

This clinical trial is being conducted to study the effect of plant sterols (PS) on cholesterol concentrations. You are being asked to take part in this study because you have specific genetic single nucleotide polymorphisms (SNPs) which have been associated with the degree to which people respond to PS and you are between the ages of 18-70 years, have LDL cholesterol (LDL-C) between 3.0 and 4.9 mmol/L, fasting glucose concentration of <6.1 mmol/L, and fasting triglycerides (TG) < 4.52 mmol/L. A total of 64 participants will participate in this study.

This research is being done because it has been shown that the consumption of plant sterols favorably alter blood cholesterol concentrations, however not all individuals respond to plant sterol consumption with the same amount of cholesterol lowering. The purpose of this study is to determine and utilize information on associations between genetic variations (SNPs) and the degree of responsiveness of cholesterol-lowering to plant sterols. The long term goal of this study is to develop ways to predetermine which individuals will respond best to plant sterols.

Study procedures

Pre-screening procedures

If you agree to take part in this study, you will be asked to give a fasting (nothing to eat or drink 12 hours before the test) blood sample (approximately two teaspoons) to measure your blood lipid levels and additional biochemistry parameters. In addition, we will measure your blood pressure and waist circumference.

Prior to beginning the study staff will review your medical history and ask questions to determine whether you are eligible to participate. Any change in your health status at any point during the study needs to be reported to the study investigators.

Study procedures

In this study, you will be "randomized" into one of 2 treatment orders described below. "Randomized" means that you are put into a treatment order by chance, like flipping a coin. You will have an equal chance of being placed in any order. The study will consist of 2 periods of 28 days each during which you will consume your assigned treatment

foods. Consumption of treatment foods will be from days 1 to 28. There will also be a washout period of a minimum of 21 days between the 2 treatment periods where you can consume your habitual diets. The entire study is designed to take 12 weeks from start to completion. During each study period, you will be provided with packaged, single portions of margarine which are shelf-stable food products. You will be required to consume 2 portions of margarine each day; one portion of margarine will be consumed at the RCFFN **or Seven Oaks General Hospital (SOGH)** under the supervision from Monday to Friday; an additional portion will be given to you to be consumed with an evening meal at home. You will be given weekend margarine portions to take home for consumption. You will be provided diaries in which you will record when you consumed the margarine in the evenings and on the weekends. You will need to return empty margarine containers daily during the weekdays and Saturday and Sunday containers will need to be returned on Monday.

The 2 periods of treatment will include:

1) Treatment period: Two daily single portions of margarine, providing 1 g each of plant sterol during the plant sterol period, for a total of 2.0 g/day of plant sterols

2) Control period: The control product will be an identical margarine, except it will not contain the additional plant sterols.

This study has a double-blind design, which means that neither you nor the clinical study staff will know which treatment you will be receiving in each period. You will receive both treatments, which will be given to you in one of 2 possible orders, to which you will be randomly assigned (randomized). In the unlikely event of an emergency, the information on which treatment period you are receiving will be made available.

If you take part in this study, you will have the following tests and procedures:

During days 0, 1, 28 and 29 of each of the treatment period of the trial, fasting blood samples (approximately 4 teaspoons will be taken on each blood draw day) will be obtained for assessment of blood fatty acid composition blood lipid profile, blood glucose, blood sterols and sterol precursors. Each blood test will take approximately 5 minutes.

On days 0, 1, 28 and 29 of each phase, we will also measure your body weight, hip and waist circumference, blood pressure and arterial stiffness. Any change in your health status at any point during the study needs to be reported to the study investigators.

On day 28, you will be required to consume a small amount of deuterated water (about 2-3 tablespoons). The movement of these tagged materials will permit assessment of the change in cholesterol metabolism of your body in response to your diet. All of the above tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you.

No alcoholic beverages are to be consumed within 48 hours prior to blood draws during the study periods. No caffeinated beverages consumption within 12 hours prior to blood draws during the study periods

Your personal cholesterol values measured at the beginning and end of each study period will be provided to you following the completion of the trials and you will be informed of the order in which you received the treatments. Digital copies of the published works produced from this research will be sent to you as they become available, provided you have given consent to future contact and provided an email address which the study staff can use to contact you.

Risks and discomforts

While on the study, you are at risk for certain side effects. As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

In case you feel any discomfort during the experimental trial our study staff and the principal investigator will be available to contact at any time. Our study staff can be reached at 204-474-7091, and the principal investigator James House, PhD can be reached at 204-474-6837.

Benefits

By participating in this study, you will be providing information to the study investigators that will show the effects of plant sterol for the treatment of high cholesterol. There may or may not be a direct benefit to you from participating in this study; however, the study should contribute to a better understanding of the inter-individual variability in responsiveness of cholesterol to plant sterol consumption. Improved understanding as to what contributes to the variability in response to plant sterol consumption may lead to improvements in the use of plant sterols to control elevated cholesterol concentrations. You will also receive access to your test results when they become available (as outlined above).

Costs

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

Remuneration for participation

You will receive up to a maximum of $400.00 at completion of this study for your time and inconvenience of the study schedule. This amount will be divided into 2 portions.

You will receive $200 after completion of phase 1 and another $200 after completion of phase 2. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives

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

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN or Seven Oaks General Hospital (SOGH) staff involved with your care may review/copy medical information that may reveal your identity. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned participant code. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the RCFFN or Seven Oaks General Hospital (SOGH), except for a single transfer of records from Seven Oaks General Hospital to the RCFFN for storage after the study is complete.

The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes. Organizations that may also inspect/copy your research records for quality assurance and data analysis include groups such as: the study sponsors (Mitacs, Unilever R&D, Nutritional Fundamentals for Health) and their representatives, and other researcher groups who are performing meta-analysis or knowledge synthesis work who request access to the raw data.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the University of Manitoba.

Study samples will be stored in at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of James House, PhD. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 5 years following the end of the research project, nor shared with any other group, other than is indicated in the protocol, without your specific consent. After this

time, all samples will be destroyed, unless you have given consent for long term storage of your samples for future analyses.

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. However, if you decide to stop participation in the study, we encourage you to talk to the study staff first.

We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study. You will be advised to follow-up with a physician if your test results suggest medical follow-up may be required.

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

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

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study. Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

Medical care for injury related to study

In the event of an injury that occurs to you as a direct result of participating in this study you should immediately notify the study investigator, James House, PhD at 204-474-6837 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, James House, PhD will be contacted, who will inform you of the results.

Questions

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

Investigator:	James House, PhD	Tel No.	204-474-6837
Coordinator:	Dylan MacKay, PhD	Tel No.	204-474-7091

For questions about your rights as a research participant, you may contact: The Biomedical Research Ethics Board, University of Manitoba at 204-789-3389

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

This study is registered on a publicly available Registry Databank at Clinicaltrials.gov. ClinicalTrials.gov is a website that provides information about federally and privately supported clinical trials. A description of this clinical trial will be available on http://ClinicalTrials.gov. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

Statement of Consent

Future contact

In order to be informed of, and receive copies of, research publications that arise from this study there may be a need to contact you after you have completed your participation in the study.

I agree to being contacted in relation to this study.

Yes ☐ No ☐

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

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my research records by Mitacs, Unilever R&D, Nutritional Fundamentals for Health, and The University of Manitoba Biomedical Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature:_____ Date: _____
(day/month/year)

Participant printed name:_____

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

Signature:_____Date: _____
(day/month/year)

Printed name of above:_____

Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Genetic analysis consent form

UNIVERSITY OF MANITOBA

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS AND REQUEST FOR LONG TERM STORAGE OF SAMPLES FOR FUTURE RESEARCH

Title of Study:	Genetic basis for Prediction of non-responders to dietary Plant Sterol intervention (GenePredict-PS)
Investigator:	James House, PhD Human Nutritional Sciences W569 Duff Roblin Building University of Manitoba 190 Dysart Road Winnipeg, Manitoba, Canada R3T 2N2 Phone: 204-474-6837
Sponsors:	Mitacs A250, Agricultural Engineering Building 96 Dafoe Rd, University of Manitoba Winnipeg, Manitoba R3T 5V6 Unilever R&D PO Box 290 3130 AG Vlaardingen Nutritional Fundamentals for Health 3405 FX-Tessier Vaudreuil-Dorion, Quebec J7V 5V5

July 20, 2018, Version 3
Participant initials _____

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

Nature and duration of procedure

From the blood drawn during the clinical study entitled "Genetic Basis for Prediction of Non-Responders to Dietary Plant Sterol Intervention (GenePredict-PS)" as outlined in the Research Participant Information and Consent Form, we would like to extract genetic information from your blood cells and perform analyses using laboratory techniques that augment and recognize specific genes. Genetic information, in this case DNA, is found in the cells of your body and are organized into genes that contain all of the information needed to make the proteins that perform specific biological functions in your body.

Benefits

There may or may not be a direct benefit from this genetic analysis, however the genetic analysis is required to determine your eligibility to participate in this study. This study should contribute to a better understanding of the inter-individual variability in responsiveness of cholesterol to plant sterol consumption. Improved understanding as to what contributes to the variability in response to plant sterol consumption may lead to improvements in the use of plant sterols to control elevated cholesterol concentrations.

Risks and discomfort

As the DNA will be extracted from blood samples that have already been taken, there are no additional invasive procedures to undergo and no physical risks to you. Receiving information regarding susceptibility to genetic disease or identification of blood relationships may cause distress. There are potential psychological and social risks of genetic analysis related to how the results could change a person's life. You could have emotional reactions to learning that you do or do not carry a gene change for a certain condition. Additionally, some genetic analyses could reveal unexpected relationships, such as non-paternity (a different biological father). However, we will not be providing you with your personal results of the genetic testing that will be undertaken in this study because the planned genetic tests are for research purposes, not the type used to determine disease risks or paternity.

Confidentiality and safekeeping of biological samples containing genetic information

All of the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your samples a specific code. This code will link you to your samples containing genetic information and can only be decoded

by the principal researcher or an individual authorized by the latter. Samples containing your genetic information will be kept at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, under the supervision of James House, PhD for a 5-year period following the end of the research project. After this time, all samples will be destroyed, unless you have given consent for long term storage of your samples for future analyses. Your samples containing genetic information will only be used for the purpose of this research project.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee or a representative from the RCFFN **or Seven Oaks General Hospital (SOGH)** may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project.

Study records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN **or Seven Oaks General Hospital (SOGH)** staff involved with your care may review information that may reveal your identity. The Bannatyne Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave RCFFN **or Seven Oaks General Hospital (SOGH), except for a single transfer of records from Seven Oaks General Hospital to the RCFFN for storage after the study is complete.**

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. You will be advised to follow-up with a physician if your test results suggest medical follow-up may be required.

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 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 treatment and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the study staff.

Investigator:	James House, PhD	Tel No.	204-474-6837
Coordinator:	Dylan MacKay, PhD	Tel No.	204-474-7091

For questions about your rights as a research participant, you may contact:

The Bannatyne 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.

Long term storage of samples for future analyses (optional)

In a study such as GenePredict-PS, the samples that are collected have the potential to be an invaluable resource for future research. We would like you to consider allowing us to store your genetic material (DNA) and data for 25 years (until 2041) for the purposes of future analyses other than those currently planned for the GenePredict-PS study, after this time, all samples will be destroyed. These analyses could include additional single nucleotide polymorphism (SNP) testing, sequencing of sections of genomic DNA, and or whole genome/exome sequencing to look at genetic associations with lipid metabolism and/or the magnitude of lipid response to plant sterol consumption. These future analyses could include sending your samples to other research centres to have additional analyses performed. Your samples and data would continue to be coded and your name and personal information such as your name, address or telephone number would not leave the Richardson Centre for Functional Foods and Nutraceuticals. All future analyses on samples would only occur after having presented for review and received approval from the University of Manitoba Research Ethics Board and or from the Research Ethics Boards of other research centres where the samples would be sent.

This part of the program is optional, and if you decide not to participate you can still take part in the main pre-planned clinical trial of the GenePredict-PS study.

Your blood samples will still be stored for the purposes of future analyses that currently planned for the GenePredict-PS study. There is no additional physical risk involved in allowing long-term storage samples. It does not require any additional visits, or any additional samples. You will not receive any financial compensation for allowing your samples to be stored, or for any discoveries made using specimens held in storage.

By accepting this request to allow your samples and data to be held for 25 years, you are making an enormously valuable contribution to a resource health research.

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS AND REQUEST FOR LONG TERM STORAGE OF SAMPLES FOR FUTURE RESEARCH: Genetic basis for Prediction of non-responders to dietary Plant Sterol intervention

Voluntary participation/withdrawal from long term storage of samples for future analyses

Your decision to allow your samples to be stored for 25 years is voluntary. You may refuse to participate or you may withdraw your samples from future research use at any time. If the study staff feels that it is in your best interest to withdraw your samples from future research, they will remove you without your consent.

To withdraw your samples from future research, please contact:

Investigator:	James House, PhD	Tel No.	204-474-6837
Coordinator:	Dylan MacKay, PhD	Tel No.	204-474-7091

I agree that my genetic material (DNA) may be stored for a 25 year period (ending in 2041) for future analyses ☐ Yes ☐ No

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS AND REQUEST FOR LONG TERM STORAGE OF SAMPLES FOR FUTURE RESEARCH: Genetic basis for Prediction of non-responders to dietary Plant Sterol intervention

Signature of participant

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

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

_____ _____

Signature of Participant Date

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

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Participant baseline information form

Telephone Screening Form

Participant code:	**Date of call:**
Eligible for study: Y or N	**Reason if not eligible:**
Name:	
Telephone number: (home):	(other):
Email:	
Age:	Birth Date (Y/M/D):
Weight: kg (2.2 lb/kg)	Height: m (2.54 cm/in)
BMI: kg/m^2 (exclude of > 40)	
Smoker: Y or N	

Medical History:

Do you have any chronic disease such as: diabetes, heart disease, liver or kidney disease, inflammatory bowel disease, pancreatitis, gallbladder or biliary disease, hypertension?	Y or N
Have you ever been diagnosed with a specific type of hypercholesterolemia/hyperliproteinemia?	Y or N
Have you ever been diagnosed with phytosterolemia?	Y or N
Are you of Hutterite ancestry?	Y or N
Have you been diagnosed with cancer (i.e., non-skin cancer, melanoma, etc.)? If yes, occurrence or any therapy within past 5 year?	Y or N
Are you taking any cholesterol-lowering, triglyceride-lowering or blood pressure medication? If yes, what medication, frequency and length of time:	Y or N
Have had weight gain or loss greater than 3 kg in the past three months?	Y or N
Are you taking any other prescription medication? If yes, what medication, frequency and length of time:	Y or N

Do you have any other underlying health issues?	Y	or	N
(Only if female) Pregnant, breastfeeding or planning to become pregnant during the course of this trial?	Y	or	N
Postmenopausal	Y	or	N

Diet:

Have you taken lipid-lowering supplements (e.g., omega- 3 supplements, plant sterols/stanols foods or supplements, fiber, etc.) within last 3 months?	Y	or	N
For fiber or stimulant laxatives, exclude if > 2 doses/days Are you taking vitamin supplements or any natural health/herbal/food supplements? (units/day) (Multi, Vit E, Vit A, beta-carotene, etc.)	Y	or	N
Do you consume alcohol? (exclude if >2 alcoholic drink/day)	Y	or	N
Allergies (food or medication):_____	Y	or	N
Food intolerances:_____			
Do you follow a special diet:_____			
Vegetarian:_____			
Do you a history of eating disorders?	Y	or	N
How much do you exercise? (exclude if > 15miles/wk or 4,000kcal/wk)			

Mitacs Gene Predict-PS Study Calendar Group 3, March 2018

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
March 12	March 13	March 14	March 15 Day 0 Fasting Blood Breakfast 6:30 am – 10:30 am	March 16 Day 1 Fasting Blood Treatment 1 Breakfast 6:30 am – 10:30 am	March 17 Day 2 Breakfast consumed at home.	March 18 Day 3 Breakfast consumed at home.
March 19 Day 4 Breakfast 6:30 am – 10:30 am	March 20 Day 5 Breakfast 6:30 am – 10:30 am	March 21 Day 6 Breakfast 6:30 am – 10:30 am	March 22 Day 7 Breakfast 6:30 am – 10:30 am	March 23 Day 8 Breakfast 6:30 am – 10:30 am	March 24 Day 9 Breakfast consumed at home.	March 25 Day 10 Breakfast consumed at home.
March 26 Day 11 Breakfast 6:30 am – 10:30 am	March 27 Day 12 Breakfast 6:30 am – 10:30 am	March 28 Day 13 Breakfast 6:30 am – 10:30 am	March 29 Day 14 Breakfast 6:30 am – 10:30 am	March 30 HOLIDAY Day 15 Breakfast consumed at home.	March 31 Day 16 Breakfast consumed at home.	April 1 Day 17 Breakfast consumed at home.
April 2 Day 18 Breakfast 6:30 am – 10:30 am	April 3 Day 19 Breakfast 6:30 am – 10:30 am	April 4 Day 20 Breakfast 6:30 am – 10:30 am	April 5 Day 21 Breakfast 6:30 am – 10:30 am	April 6 Day 22 Breakfast 6:30 am – 10:30 am	April 7 Day 23 Breakfast consumed at home.	April 8 Day 24 Breakfast consumed at home.