COLONIC FERMENTATION, EQUOL STATUS AND THE HYPOCHOLESTEROLEMIC EFFECT OF SOY

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto

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ABSTRACT

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Background: The value of soy, as an effective component of a cholesterol lowering diet, is currently questioned due to smaller lipid reductions than previously reported for the currently approved US FDA health claim for soy. Nevertheless, intrinsic and extrinsic factors may exist that influence the effectiveness of soy, but little research has been done in this area. Such factors include the soy isoflavone content, dietary components that alter colonic fermentation and the colon’s ability to biotransform isoflavones (i.e. equol status).

Objective: To determine if specific factors, such as dose of soy isoflavones and those that alter colonic fermentation, influence the hypocholesterolemic effect of soy. Furthermore, whether cholesterol reductions differ depending on the interindividual variation in isoflavone biotransformation (i.e. equol status), when soy is consumed under different dietary conditions (i.e. with specific factors).

Methods: 85 men and postmenopausal women (42M, 43F) with hyperlipidemia participated in one of three substudies where soy foods, containing 30-52g/d of soy protein, were provided over a one-month period under the following conditions: 1) high-
normal (73mg/d) or low (10mg/d) soy isoflavones (N=41); 2) with or without a prebiotic (10g/d polyfructans) to increase colonic fermentation (N=22); or 3) with a reduced carbohydrate diet (26% of calories) to decrease colonic fermentation (N=22).

**Results:** Unmodified soy foods significantly reduced LDL-C by 5.1%±2.0% (P=0.016). LDL-C reductions were not altered with increased soy isoflavone content nor were the effects dampened with reduced carbohydrate. However, coingestion of soy with a prebiotic improved the cholesterol lowering effect of soy. Equol producers (N=30) showed a relative increase of 5.3±2.5% in HDL-C (P=0.035) after soy compared to nonproducers (N=55), but no significant differences were observed for LDL-C or other lipids. Equol excretion was increased with increased soy isoflavone content, but not with the addition of a prebiotic.

**Conclusion:** The effectiveness of soy, as a cholesterol lowering food, may be improved with the addition of prebiotics, but not with decreased carbohydrate or increased isoflavones. Equol status appears to alter the HDL-C, but not the LDL-C response. These data support the continued use of soy foods as part of the dietary approach to coronary heart disease risk reduction.

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List of Abbreviations

A
AHA – American Heart Association
ApoA1 – Apolipoprotein A1
ApoB – Apolipoprotein B

B
BMI – Body Mass Index
BP – Blood Pressure

C
CH₄ – Methane
CHD – Coronary Heart Disease
CRP – C-Reactive Protein
CV – Coefficient of Variation
CVD – Cardiovascular Disease

D
DASH – Dietary Approach to Stop Hypertension
DHD – Dihydrodaidzein

E
ER – Estrogen Receptor
ESI – Electrospray Ionization

F
FOS – Fructo-oligosaccharides
FFA – Free Fatty Acid

**G**
GC-MS – Gas Chromatography Mass Spectrometry

**H**
H₂ – Hydrogen
HDL-C – High Density Lipoprotein Cholesterol
HN-APCI – Heated Nebulizer Atmospheric Pressure Chemical Ionization
HPLC-MS – High Pressure Liquid Chromatography Mass Spectrometry
HRT – Hormone Replacement Therapy

**L**
LDL – Low Density Lipoprotein
LDL-C – Low Density Lipoprotein Cholesterol
LRC – Lipid Research Clinic

**M**
MUFA – Monounsaturated Fatty Acid

**N**
NCEP – National Cholesterol Education Program
NCEP ATP III – National Cholesterol Education Program Adult Treatment Panel III

**O**
ODMA - O-desmethylandolensin
OMNIHeart - Optimal Macronutrient Intake Trial to Prevent Heart Disease
PUFA – Polyunsaturated Fatty Acid

RCTs – Randomized Controlled Trials

SAS – Statistical Analysis System

SCFA – Short Chain Fatty Acid

SFA – Saturated Fatty Acid

SERMs – Selective Estrogen Receptor Modulators

TC – Total Cholesterol

TG – Triglycerides

US FDA – United States Food and Drug Administration
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Publications and Presentations Arising from Dissertation


Publications


Book Chapters


Oral Presentations


3. **Wong JMW**, Kendall CWC, Esfahani A, Ng VWY, Jenkins DJA. The Effect of a Weight Reducing High Protein, Low Carbohydrate Vegan Diet on Metabolic Risk Factors. 1st Canadian Obesity Student Meeting, Quebec City, Canada, June 2008. *(First place in the Oral Presentation Competition, PhD category)*


7. **Wong JMW**, Esfahani E, Kendall CWC, Jenkins DJA. Effect of a High Protein and Low Carbohydrate Soy Based Vegan Diet on Weight Loss and Serum Lipids. 3rd Natural Health Product Research Conference & Tradeshow, Toronto, ON, March 2006.

8. **Wong JMW** and Jenkins DJA. Colonic Health: Fermentation and Short Chain Fatty Acids. Guidelines for Probiotic Use, Yale University School of Medicine University, New Haven, CT. October 2005.

**Poster Presentations**


5. Wong JM, Esfahani A, Kendall CW, Jenkins DJ. Effect of a vegan based high protein, low carbohydrate diet on weight loss and serum lipids. Fourth Annual Scholarship Reception, Faculty of Medicine, University of Toronto, May 2006. (invited poster presentation)


1. Introduction
Introduction

The value of soy and its potential health benefits have been a topic of great interest since the observation in epidemiological studies that Japanese and Chinese populations with higher levels of soy intake, as part of their traditional diet, tend to have a lower incidence of CVD. This association, coupled with the meta-analysis in 1995 demonstrating that soy foods (47g/d of soy protein) are effective in the treatment of hyperlipidemia where LDL-C was reduced by 13%, drew attention to the potential value in soy. As a result, the US FDA approved a health claim for soy protein (at least 25g/d) and CHD risk reduction. However, recent studies have questioned the effectiveness and value of soy in lowering cholesterol concentrations, suggesting the magnitude of LDL-C reduction is much smaller and is estimated to be closer to 3%. In light of the recent evidence, the US FDA is currently re-assessing the health claim for soy in CHD risk reduction. However, it is worthwhile to note that soy foods, as sources of plant protein, have a number of important roles as components of a plant based diet. Vegetarian diets have been associated with decreased serum LDL-C concentrations and reduced CHD events and progression of CHD. Furthermore, there may be factors associated with these diets that enhance the cholesterol lowering effectiveness of plant proteins, but little attention has been devoted to exploring these possibilities. Factors of particular interest, with respect to soy, include alterations in dietary components that modify colonic fermentation (e.g. administration of prebiotics or reduction in carbohydrate content), isoflavone content of soy foods and the associated interindividual colonic biotransformation (i.e. equol status). Answers to such key questions are of great importance, especially at a time where the availability of equivalent protein alternatives to soy protein foods for the prevention and management
of hyperlipidemia are lacking. Therefore, we believe the search for ways to maximize the effectiveness of soy is particularly relevant at the present time. The aim of the current studies has been to determine if specific factors may influence the effectiveness of soy foods in lowering serum cholesterol concentrations. Such factors include the isoflavone content of soy, altered colonic fermentation via provision of a prebiotic or reduction of dietary carbohydrate content and the influence of interindividual variation in colonic isoflavone biotransformation (i.e. equol status). These specific factors were investigated in a series of sub-studies that provided soy foods commonly found in supermarkets to hyperlipidemic participants for a period of one month during each sub-study.
2. Literature Review
2.1. Coronary Heart Disease

Coronary heart disease (CHD) remains the leading cause of death worldwide. Enormous dietary and pharmacological efforts have been made in the prevention and treatment of hyperlipidemia, specifically LDL-C, an important modifiable risk factor of CHD. It is now recognized that HDL-C is also an important independent risk factor in CHD risk reduction.

The Lipid Research Clinics Coronary Primary Prevention Trial was one of the first large clinical trials to demonstrate that reducing TC and LDL-C can reduce the incidence of CHD morbidity and mortality, providing evidence of a cause-effect association. A number of subsequent studies, with use of lipid lowering agents, have confirmed this observation where every 1% reduction in LDL-C translates into an approximate 1-2% reduction in CHD risk.

Dietary and lifestyle modifications are key modifiable factors for CHD risk reduction. According to the NCEP ATPIII guidelines, elevated serum cholesterol, specifically LDL-C, still remains the primary target of therapy. Therapeutic diets, recommended by the AHA and the NCEP ATPIII, emphasize low intakes of saturated fat and dietary cholesterol, and increased consumption of fruits, vegetables, whole grains and low-fat dairy products.

2.2. Plant Based Diets

Evidence from epidemiological studies of cohorts following vegetarian and vegan diets have been associated with decreased LDL-C and risk of CHD compared to non-vegetarians. Furthermore, decreases in overall total mortality have also been
observed \(^{18}\). In recent studies of the Nurses’ Health Study, it was found that intake of low carbohydrate diets, high in vegetable protein and oil, were associated with reduced CHD events and incidence of diabetes \(^{20,28}\).

Clinical trials with individuals on vegan or vegetarian diets have been shown to reduce the progression of CHD \(^{21}\) and improve diabetes control \(^{29}\). A low-fat vegetarian diet, along with other lifestyle modifications (i.e. smoking cessation, stress management and moderate exercise) for 1 year, was found to significantly reduce serum lipids and reduce the progression of coronary artery lesions, as assessed by the average percentage diameter stenosis using coronary angiography \(^{21}\). In contrast, coronary artery lesions continued to increase in the control group. This study demonstrated that lifestyle changes, with the inclusion of a plant based diet, in those with established CHD, can reduce both serum lipids and disease progression without the use of lipid lowering medications.

Short term studies related to reducing the risk factors for CHD have also supported the findings from epidemiological studies. Diets which emphasize low-fat dairy (i.e. approaching a lacto-ovo-vegetarian diet) or plant protein (i.e. approaching a vegetarian diet) foods with higher intakes of fruits and vegetables, such as the DASH and OMNIHeart diets, have been shown to improve BP and the blood lipids \(^{15,16,30}\). Those who followed a vegan diet (i.e. diets without animal products) based on fruits, leafy vegetables, plant sterols, soy and nuts have resulted in LDL-C reductions of 29%, an effect equivalent to a first generation statin \(^{17}\). A number of components tend to be higher in vegetarian diets, these include vegetable proteins (e.g. soy foods), vegetable oils and viscous fibers, all of which have been shown to individually reduce serum lipids \(^{7,31-34}\). Potential active components responsible for the lipid lowering effect include
MUFA and PUFA, n-3 fatty acids, fibers, plant peptides, sterols, phospholipids, antioxidant, vitamins, minerals and phytochemicals (e.g. isoflavones found in soy) \(^{35}\).

### 2.3. Soy Foods

Higher levels of soy intake among Japanese and Chinese populations have been associated with an improved lipid profile \(^{36,37}\) and reduced rates of CVD \(^1-4\). Extensive research in this area has demonstrated a positive role of soy foods in reducing blood lipids concentrations and CHD risk \(^7,38,39\), which has resulted in a health claim in the United States \(^{40}\). However, the factors which alter the effect of soy foods on blood lipids, and ways to maximize their effectiveness have been largely ignored.

Soy foods have long been consumed as part of the traditional diet in Japan and China. Traditional soy foods can be subdivided into those that are fermented and nonfermented. Fermented soy foods include tempeh, miso, soy sauce, natto and tofu and soymilk products \(^{41,42}\). Whereas, nonfermented soy foods include fresh green soybeans (edamame), whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk and soymilk products, tofu, okara and yuba \(^{41,42}\). In contrast, Western populations consume minimal amounts of these traditional soy foods, other than soy beverage and tofu, and instead, have developed a wide range of soy meat analogues (e.g. soy hot dogs, soy burgers, soy deli slices, etc) containing isolated soy protein, which are second generation products derived from modern processing techniques.

#### 2.3.1. Potential Active Components of Soy

Soy contains many active components that may be responsible for an array of health benefits, including CHD risk reduction \(^{43}\). These components include the active peptides \(^{32,44}\), saponins \(^{45}\), sterols, phospholipids, fiber, fat and isoflavones \(^{46}\). Soy
peptides (i.e. 7S globulin, specifically the α’ subunit \(^{32, 44}\), and active peptides of ~15 amino acid composition \(^{47, 48}\)) and isoflavones (i.e. daidzein and genistein, the 2 main isoflavones in soy) have been the most widely studied, especially for their role in modulating lipid risk factors associated with CHD.

Average intakes of soy protein and isoflavones in older Japanese adults are currently estimated at 6-11g and 25-50mg, respectively and a relatively small (≤10%) proportion of Asian populations consume more than 25g of soy protein or 100mg of isoflavones per day \(^{49}\). Whereas, in Western populations, intakes of soy protein and isoflavones are reported to be very low at <5g/d and <3mg/d, respectively \(^{50-54}\).

### 2.3.2. Soy Foods and Lipid Reduction

Many decades ago, Sirtori and al. demonstrated the cholesterol lowering potential of soy protein for the management of hypercholesterolemia \(^{32}\). In 1995, Anderson et al. \(^7\) completed a meta-analysis on the effect of soy protein on serum lipids. A review of 38 controlled clinical trials indicated that an average intake of 47g/d of soy protein decreased TC by 9.3%, LDL-C by 12.9%, TG by 10.5% and a non-significant increase in HDL-C of 2.4% \(^7\). Based on these findings, the US FDA approved a health claim for soy protein and CHD risk reduction in 1999 \(^8\). This claim was based on clinical trials showing that at least 25g/d of soy protein significantly decreases LDL-C concentrations. However, specific recommendations for other components of soy, including the isoflavones, were not indicated due to the lack of evidence to support such recommendations. Despite this, the specific components of soy responsible for the beneficial effects observed with soy intake have not been fully elucidated.

Since the time of the first meta-analysis over a decade ago, the interest in soy has grown exponentially with recommendations incorporated into national guidelines for
dietary management of hypercholesterolemia. However, the FDA health claim and the benefits of soy in decreasing serum cholesterol concentrations have recently been challenged with a number of subsequent meta-analyses, showing much smaller LDL-C reductions than initially described. The recent meta-analysis by the AHA Nutrition Committee of RCTs suggest that an average intake of 50g of soy protein reduces LDL-C only by 3% with no significant effect of isoflavones whether consumed with or without soy protein. Furthermore, no significant effects were observed in HDL-C (+1.5%) and TG (-5%) and no dose effect was observed on blood lipids. However, a meta-analysis by Reynolds et al. of 41 RCTs supported the initial findings of Anderson et al, but with a much smaller effect size. Significant reductions were observed in TC, LDL-C and TG, as well as a significant increase in HDL-C. Meta-regression analysis indicated dose response relationships where the amount of soy protein and isoflavones were inversely associated with changes in TC, LDL-C and TG, and positively associated with changes in HDL-C.

There may be a number of potential reasons for the observed difference in these meta-analyses and the smaller effect size. These include differences in the studies included in the analyses; study design; food matrix (i.e. soy foods vs. supplements); processing techniques which may alter the presence of active components, such as high temperature treatment in the production of breakfast cereals with denaturation of soy protein, growing conditions, and interindividual variation in colonic soy isoflavone biotransformation.

A number of clinical trials since the most recent meta-analysis have also supported the smaller hypocholesterolemic effect of soy, ranging from 3-5% with 25-38g of soy protein.
2.3.2.1. Mechanisms of Action

The exact mechanism(s) by which soy foods elicit its cholesterol lowering effect still remains a topic of debate. Several potential mechanisms have been put forward including higher plasma thyroxine levels \(^{65,66}\), bile acid balance \(^{66}\), estrogenic effects of soy isoflavones and their metabolites (i.e. equol) \(^{46,62}\), arginine/lysine ratio \(^{65,66}\), displacement of foods higher in saturated fat and cholesterol \(^{9,11}\) and up regulation of LDL receptor activity \(^{65,67,68}\), which is attributed to specific protein components i.e. the 7S globulin and its α-α' subunits \(^{39,44,69}\). Studies continue to be conducted looking at the hypocholesterolemic effect of soy, however, very little research has focused on exploring methods to enhance its effectiveness.

2.4. Soy Isoflavones

Among the foods consumed by humans, isoflavones are most highly concentrated in soybeans \(^{70}\) and have been associated with beneficial effects on certain types of cancer, CVD risk and bone health \(^{71-74}\).

Soy isoflavones are a family of plant-derived polyphenolic compounds with similar structure and properties to mammalian estrogen and possess weak estrogenic activity, hence they are commonly known as phytoestrogens. Soybeans contain three types of isoflavones: daidzein, genistein and glycitein, where concentrations of the latter are much lower than the other two isoflavones \(^{75}\). The isoflavones are found in four chemical forms: aglycones, glucosides, acetylglucosides and malonylglucosides \(^{57}\). The isoflavone content of soy foods varies and is affected by a number of factors including the soybean variety, growing conditions and processing methods \(^{60,61,76}\), but it is estimated to be approximately 1-4mg/g of soy protein \(^{49,76}\).
2.4.1. Isoflavone Content of Soy Foods

The majority of isoflavones naturally found in soy foods (e.g. soy milk, tofu, etc.) are in the β-glucosides form (conjugated), unless the food has been fermented (e.g. tempeh, miso, etc), where the isoflavones are found as the hydrolyzed aglycones (unconjugated). The majority of soy foods consumed in the Western World are nonfermented foods commonly made from whole soybean or highly purified protein extracted from soybeans.

It has been observed that traditional nonfermented soy foods tend to have higher amounts of total isoflavones than fermented soy foods. It has also been demonstrated in analyses of common American and Asian soy foods, that the concentration of soy isoflavones is substantially decreased in soy sauce, alcohol-extracted soy protein concentrate, soy protein isolates and soy fiber. In other words, second generation soy foods tend to have lower total isoflavone content than whole soybeans as a result of certain processing techniques (Table 1). Soy isoflavones have not been found to be present in soybean oil.

The total amount of soy isoflavone has also been shown to remain relatively constant with extraction under room and high temperature and with normal cooking, although changes in the distribution in the mixture of β-glucosides may occur. A decrease in total isoflavone content may occur with heating when accompanied with lowering of the pH (e.g. prepared with tomato sauce) and the when food is charred. It was also observed that lower fat soy products, such as low-fat and nonfat soymilk or low-fat tofu, have a marked depletion in isoflavones, likely as a result of processing. Therefore, the types of soy foods and processing techniques used can have a significant impact on the isoflavone content, as well as the distribution of the isoflavones present. This in
turn, could influence the interpretation of data and outcome of interest in clinical interventional studies with soy, since all soy products are not created equal \(^79\). This may also contribute to variability between studies and their associated results.

As previously mentioned, the processing of soy foods, particularly microbial fermentation used in fermented products, alters the dietary isoflavone content (i.e. glucosides vs. aglycones). Furthermore, a greater urinary recovery of isoflavones has been observed with intakes of fermented (aglycones) versus unfermented (glycosides) isoflavones in soy \(^78\). Once soy isoflavones are ingested, microbial metabolism by the gut microflora can biotransform the β-glucosides to their aglycone form, which in turn

<table>
<thead>
<tr>
<th>Food</th>
<th>Daidzein</th>
<th>Genistein</th>
<th>Glycitein</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miso Paste(^a)</td>
<td>4.4</td>
<td>5.9</td>
<td>0.8</td>
<td>11.1</td>
</tr>
<tr>
<td>Soy Beans(^b)</td>
<td>56.6</td>
<td>44.2</td>
<td>2.8</td>
<td>103.6</td>
</tr>
<tr>
<td>Soy Bean Sprouts(^c)</td>
<td>0.3</td>
<td>0.5</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Soy Milk(^d)</td>
<td>0.9</td>
<td>1.9</td>
<td>0.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Soy Nuts(^e)</td>
<td>28.4</td>
<td>36.3</td>
<td>3.9</td>
<td>68.5</td>
</tr>
<tr>
<td>Soy Protein Powder(^f)</td>
<td>2.5</td>
<td>6.0</td>
<td>0.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Soy Sauce(^g)</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Soy Yogurt(^h)</td>
<td>3.4</td>
<td>6.6</td>
<td>0.3</td>
<td>10.2</td>
</tr>
<tr>
<td>Tempeh(^i)</td>
<td>7.0</td>
<td>10.7</td>
<td>0.6</td>
<td>18.3</td>
</tr>
<tr>
<td>Textured Vegetable Protein(^j)</td>
<td>5.4</td>
<td>10.2</td>
<td>0.5</td>
<td>16.1</td>
</tr>
<tr>
<td>Tofu(^k)</td>
<td>9.3</td>
<td>17.1</td>
<td>0.7</td>
<td>27.1</td>
</tr>
<tr>
<td>Veggie Burger(^l)</td>
<td>0.5</td>
<td>1.1</td>
<td>0.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Table 1. Soy Isoflavone Content of Foods Consumed in Canada.

Adapted from Thompson et al. \(^\text{70}\)

\(^a\) Hanamaruki, white  
\(^b\) dried, no brand, boiled in water, drained  
\(^c\) no brand, raw  
\(^d\) So Good Original Fortified  
\(^e\) no brand, roasted  
\(^f\) NHF Solydelicious  
\(^g\) VH  
\(^h\) So Nice low-fat vanilla  
\(^i\) Noble Bean, organic, frozen, cooked in water  
\(^j\) dried, no brand, cooked in water  
\(^k\) President’s Choice Extra Firm  
\(^l\) Yves, refrigerated, cooked by microwave
could be further biotransformed to various metabolites. It has been demonstrated that the aglycones are more readily absorbed, where a lack of absorption of the glucosides has been observed \(^{80, 81}\). Therefore, the greater availability of aglycones for further biotransformation may affect circulating levels of the parent isoflavone and its associated metabolites. Whether the altered circulating levels of specific isoflavones have an effect on the overall health outcome of interest is still not fully understood.

### 2.4.2. Metabolism of Soy Isoflavones

Upon ingestion of soy isoflavones (daidzin, genistin and glycitin), which are primarily found as \(\beta\)-glucosides, there is a need for them to be deglycosylated to their aglycone form (daidzein, genistein and glycitein) for intestinal absorption \(^{80, 81}\). This process involves \(\beta\)-glucosidase enzymes which are present in the intestinal mucosa and the intestinal microflora \(^{82, 83}\). Once absorbed, the soy isoflavone aglycones are readily conjugated with glucuronic acid and sulfate in the liver and excreted in the bile. As a consequence, they enter the enterohepatic circulation with the potential of further biotransformation and reabsorption in the intestine, where they are ultimately excreted in the urine mainly as glucuronic acid conjugates \(^{84, 85}\). As a result, urinary isoflavone excretions are often used as primary biomarkers when measuring dietary soy intake \(^{86, 87}\). Fecal analyses for soy isoflavones showed that it is a minor route of excretion \(^{83, 88-90}\). Evidence to support the intestinal absorption in the small intestine and colon, and enterohepatic recycling of isoflavones comes from bioavailability studies which observe a biphasic shape (two peaks in blood concentrations) after ingestion \(^{90, 91}\). It has been demonstrated that intake of isoflavones from soy protein isolate as low as 9mg/d are bioavailable as evidenced in isoflavone excretion \(^{92, 93}\).
2.4.2.1. Genistein and Daidzein

The two predominant isoflavones can be further biotransformed by intestinal microflora into various metabolites which have been confirmed in *in vitro* studies where daidzein and genistein were incubated with human fecal flora. Genistein may be further metabolized to 6'-hydroxy-O-desmethyngolensin through the intermediate dihydrogenistein (Figure 1)\(^95,96\). Whereas, daidzein may be metabolized to equol and ODMA through their respective metabolites by intestinal microflora (Figure 1)\(^95,96\).

Equol has been confirmed to be a metabolite of daidzein with isotopically labeled isoflavones\(^89\) and of particular interest, both equol and ODMA production specifically requires the colonic microbial biotransformation of daidzein. Evidence to support this notion comes from studies of germ-free rats and infants fed soy formula, where these

![Metabolic Pathways of Daidzein and Genistein](image)

*Figure 1. Metabolic Pathways of Daidzein and Genistein (from Hwang et al, reprinted with permission).*\(^104\)
metabolites are not produced and studies of adults, in whom the appearance of equol in blood occurs later than the appearance of daidzein. However, not all individuals appear to have the capacity to produce equol and ODMA. It has been observed that approximately 30-40% of adults in Western populations have the ability to produce equol and 80-90% produce ODMA. However, the variability and the significance of ODMA excretion has not been evaluated as extensively relative to equol, since it is believed to be relatively inert. An inverse relationship has been observed between urinary equol and ODMA excretion, suggesting that there are preferred metabolic pathways of daidzein metabolism that differ among individuals. However, others have not observed a significant relationship, with individuals producing both equol and ODMA, suggesting that the metabolism of the metabolites involve different populations of the colonic microflora. It is suggested that the observed interindividual variation in the biotransformation of soy isoflavones and their associated metabolites is dependent on a number of factors, including the composition of the intestinal microflora. Thus, altering the gut microflora, through dietary means as one possibility, may alter the ability to produce the gut bacterial metabolites, especially equol. The daidzein metabolizing phenotype, particularly equol, has been of great scientific interest in recent years, but their clinical relevance is still inconclusive.

2.4.2.2. Pharmacokinetics

Studies using single bolus oral administration of isotopes have found that the $C_{\text{max}}$ for serum $[13C]$daidzein and $[13C]$genistein concentrations were about 7-8h and 6h, respectively, after oral intake. The elimination of the isoflavones followed first order kinetics. The appearance of urinary $[13C]$equol was only in trace amounts within
24h after the single bolus and was predominant on day 2 and 3, suggesting that the delayed appearance of equol is consistent with its colonic origin. Based on the pharmacokinetics of soy isoflavones, daily intake of soy isoflavones throughout the day is required to maintain steady state blood concentrations rather than intake of a single bolus or dose, as would commonly occur with supplements or capsules. Urinary soy isoflavone excretion has been shown to correspond in a dose-dependent manner at low and high doses of dietary soy. However, a decrease in the fractional absorption of isoflavones with increasing intake has been observed in dose response studies. This suggests that there appears to be some sort of upper limit after which there is limited additional advantage to consuming higher amounts of isoflavones. Absorption appears to be rate-limiting and saturable, i.e. increasing serum concentrations of isoflavones with increasing dose was not linear, but flattened at higher intakes (i.e. a curvilinear relationship). This lack of a linear relationship was also reflected in urinary isoflavone excretion.

2.4.3. Soy Isoflavones and Lipid Reductions

2.4.3.1. Soy Protein with Varying Levels of Isoflavones

The specific active components responsible for the hypocholesterolemic effect of soy are still not fully elucidated. Numerous studies have investigated the role of soy isoflavones, whether consumed alone or with soy protein, yet their contribution to the cholesterol lowering effect of soy still remains controversial. Studies have been inconclusive, as intake of soy protein with isoflavones has shown hypocholesterolemic effects, whereas other studies have shown them to be ineffective. In a study by Crouse et al, 156 healthy men and women (post-menopausal) were
randomized to one of five diets for nine weeks. All the diets followed a NCEP Step 1 diet with 25g of casein protein (isoflavones free) or 25g of isolated soy protein containing 3, 27, 37 or 62 mg of isoflavones. Hence, in this study, the additive effect of isoflavones on the hypocholesterolemic effect of soy could be determined since the level of soy protein in each diet was kept constant. The soy containing diet with the highest amount of isoflavones (62mg) significantly lowered TC and LDL-C by 4% and 6%, respectively, compared with the casein control. In addition, those with higher baseline LDL-C (>4.24mmol/L) had greater improvements in their lipid profile with intakes of 37mg and 62mg of isoflavones, where the latter isoflavone intake (62mg) decreased TC by 9% and LDL-C by 10%.

Similar findings were observed in other studies where a greater lipid lowering effect was seen in those with higher baseline values. Furthermore, those who had higher baseline LDL-C values also responded in a dose dependent fashion, where increasing amounts of soy isoflavones resulted in greater reductions in TC and LDL-C. The lipid lowering effect of soy isoflavones was further supported by the lack of significant lipid changes with ethanol-extracted soy protein which contained minimal amounts of soy isoflavones (3mg treatment).

A number of meta-analyses have been conducted to determine if consumption of soy isoflavones, when taken with soy protein, has an independent effect on serum cholesterol. Taku et al. compared equivalent amounts of soy protein with or without soy isoflavones in RCTs. The mean intake of soy isoflavones was 102mg/d. After controlling for the amount of soy protein, significant reductions in TC (1.77%) and LDL-C (3.58%) were observed, with no significant effect on HDL-C or TG. The authors suggest that soy isoflavones play an important role in lowering cholesterol and has
additive effects when consumed as soy protein containing isoflavones. Similar findings were observed in an earlier meta-analysis which found that intake of 90mg/d of soy isoflavones, regardless of soy protein content present, significantly decreased LDL-C \(^{120}\). Zhan et al. found that soy protein with isoflavones intact was associated with significant reductions in TC (3.77%), LDL-C (5.25%) and TG (7.27%) and a significant increase in HDL-C (3.03%). It was noted that in those studies where isolated soy protein contained >80mg/d of soy isoflavones showed greater improvements in the blood lipid profile, including greater increases in HDL-C. Further subgroup analysis indicated that LDL-C reductions still remained significant between isolated soy protein with isoflavones compared to isolated soy protein alone, suggesting that the soy isoflavones may have an independent cholesterol lowering effect \(^{121}\). Despite the observed hypocholesterolemic effect of soy isoflavones, no linear relation between the amount of soy isoflavones and reductions in blood lipids were observed \(^{119, 120, 122}\). However, differences in the dose and distribution of the isoflavones (i.e. daidzein, genistein and glycine) used in the studies, in conjunction with interindividual differences in soy isoflavone biotransformation, may have added some degree of variability resulting in a lack of association between soy isoflavones and blood lipids. One meta-analysis has shown that soy isoflavones (mean 52 mg/d), when consumed with soy protein (mean 36g/d), did not have an independent effect on LDL-C reduction \(^{122}\). Furthermore, no dose response relation was observed with soy isoflavones and changes LDL-C and HDL-C cholesterol \(^{122}\). However, in this study intake of soy protein in combination with soy isoflavones did result in a significant 4% LDL-C reduction and a 3% increase in HDL-C \(^{122}\).
2.4.3.2. Isolated Soy Isoflavones

When soy isoflavones are given alone (i.e. supplements or capsules), studies have consistently shown a lack of significant reduction in blood lipids\textsuperscript{123-125}. This has been further supported by a meta-analysis of 11 trials with use of isolated soy isoflavones in over 400 subjects, with doses ranging from 28.5 to 150mg/d over a span of 2 to 26 weeks. No significant effects were seen in TC, LDL-C, HDL-C or TG\textsuperscript{126}. The authors concluded that intake of isoflavone tablets of up to 150mg/d has insignificant effects on serum lipids. Possible explanations for the lack of lipid lowering effect may be related to the inactivation of isoflavones during the process of purification and/or soy isoflavones may require other components found in soy to exert their effects, which are also removed during processing. However, there has been one recent study that has shown that soy isoflavones without the presence of soy protein have beneficial effects, which is contrary to previous studies. A novel soy germ pasta, naturally enriched with soy isoflavone aglycones, containing 33mg of soy isoflavones per serving per day with negligible soy protein resulted in significant improvements in serum lipids, CRP, brachial artery flow-mediated vasodilatation and urinary 8-isoprostanes\textsuperscript{127}. A number of differences in this current study may contribute to the observed effect which has not been seen in previous studies. These include the form of isoflavones (glycosides versus aglycones), the food matrix (pasta versus capsules), processing techniques (naturally occurring versus purification) and interindividual variation in colonic soy isoflavone biotransformation (equol producer versus equol nonproducer). In this soy germ pasta study, a greater benefit was also observed in those who were equol producers than equol nonproducers\textsuperscript{127}. Furthermore, the processing of the pasta resulted in a much higher proportion of isoflavone glucosides being hydrolyzed to their
absorbable aglycone form, due to the presence of β-glucosidase in the semolina wheat and a prebiotic effect may have also been present. Although there appears to be a lack of cholesterol lowering ability of isolated isoflavones, they have been shown to be effective in reducing BP. Intake of isoflavones from either soy or red clover supplements have been shown to improve systemic arterial compliance.

2.4.3.3. Summary

When considering the totality of evidence there appears to be a cholesterol lowering effect of soy isoflavones when they are consumed with soy protein, but lack any benefit when consumed as purified isoflavones. This suggests that a synergistic relationship may exist between soy protein and its associated isoflavones for cholesterol reduction or that the removal of isoflavones is also associated with damage to the soy protein structure, including the 7S globulin fraction. It is possible that dietary manipulations, such as the addition of prebiotics, altering the form of isoflavones (i.e. aglycones) or enhancing colonic isoflavone biotransformation (i.e. equol production), may enhance the effectiveness of soy foods. However, evidence to support this effect is limited as few studies have been conducted to address these issues.

2.5. Equol

Equol is not naturally present in plants, but is formed by microbial biotransformation of its precursor, daidzein. 7-hydroxyl-3-(4′hydroxyphenyl)-chroman [(-)(S-equol)] was first isolated in the urine of pregnant mares in 1932 and was named for its equine origins. The source of dietary isoflavones comes from plant products, such as red clover. Equol has been subsequently identified in other species including goat, cow, hen and sheep.
Equol was first identified in the urine of adults consuming soy foods over 2 decades ago. Equol is a chiral molecule that can exist as two enantiomeric forms, S-equol and R-equol and it has been shown that humans exclusively produce the S-equol diastereoisomer. Much interest has been generated with the emergence of the “equol hypothesis”, which suggests that those individuals who have a gut microflora capable of biotransforming daidzein to equol, have greater beneficial effects with consumption of soy foods than those who produce equol in low concentrations or have a lack of production.

There appear to be interspecies differences in the metabolism of isoflavones, especially in relation to equol. This may explain some of the discrepancies in the physiological effects of soy in different animal models compared with humans, especially if the outcome of interest is safety and biological potency related to disease risk factors. Sprague-Dawley rats and cynomolgus monkeys fed diets with soy protein isolate had a similar isoflavone metabolic phenotype, which was different from pigs and women. Both monkeys and rats have a gut bacteria profile that favors equol biotransformation, although this capacity is limited in pigs and women. A large proportion of the total isoflavones excreted in rats and monkeys is in the form of equol, with a lower proportion as daidzein, indicating that the majority of ingested daidzein is biotransformed to equol. In addition, rats and monkeys excrete a high percentage of isoflavone as aglycones, whereas pigs and women mainly excrete isoflavones as glucuronides, with a small fraction as sulfates. Therefore, it is crucial when designing studies to account for interspecies differences, especially in relation to disease outcomes.
Evidence for the colonic biotransformation of daidzein to equol is supported by studies where lack of equol excretion is observed in germ-free animals \(^97, 108\) and infant fed soy formula \(^98-100\), as a result of an underdeveloped gut microflora. Furthermore, pharmacokinetic studies in humans demonstrated a delayed appearance of equol in serum and urine \(^90, 138\). Other evidence comes from \textit{in vitro} studies, where incubation of human fecal flora from equol producers, biotransforms daidzein to equol \(^94, 139\). Compared to daidzein and genistein, equol remains in the circulation longer and is elevated in urine longer after a soy challenge, therefore, it could be one of the reasons why greater health benefits are suggested based on equol status with soy consumption \(^62\).

\textbf{2.5.1. Prevalence of Equol Production in Humans}

Humans appear to differ in their capacity to produce equol compared to other species. It is estimated that only 30-40% of adults in Western populations are equol producers while consuming soy \(^10, 63, 89, 123, 139-145\). However, adults living in the Orient, where soy is consumed as part of their traditional diet, have a prevalence of equol producers that is higher at 50-60% \(^101, 146, 147\). This was further supported by a study by Song et al. which showed that after a standardized soy challenge, the prevalence of equol producers was higher in Korean women and girls than Caucasian women and girls \(^148\). Interestingly, the high prevalence of equol producers seen in the Orient has been recently documented in two clinical trials, one trial in a Western population of vegetarians (59%) \(^149\) and the other trial in an Italian population (69%) \(^127\). It is noteworthy that the increased prevalence of equol producers in these two trials may be related to alterations in the diet, where in the former study there was increased intake
of carbohydrate and soluble fiber (i.e. fermentable substrate) from a vegetarian diet and in the latter study, the consumption of soy germ pasta which was high in aglycone isoflavones. It has been suggested that those who are equol producers have more favorable responses to soy containing diets than equol nonproducers \(^{62}\). Therefore, based on the difference observed in the prevalence among different populations, it has been of great scientific interest to determine the factors that influence equol production in the hopes of possibility converting an equol nonproducer into a producer. Despite great efforts, equol production has been observed to be quite stable in an individual. In other words, an equol producer will always be an equol producer \(^{89, 107, 142}\). However, there have been a few occasions where a change in equol status, from either an equol producer to a nonproducer or vice versa, have been observed \(^{145, 150}\). However, whether this is a true phenomenon is still to be confirmed.

Variations in the ratio of equol producers and nonproducers may be influenced by a number of factors. Many of these studies are epidemiological or case control studies where a standardized soy challenge was not conducted to determine equol status. Therefore, prevalence of equol producers will be dependent on the soy intake, i.e. substrates are a prerequisite for metabolites. Whereas, in the clinical trials, although a soy challenge may not have been provided prior to the start of the study, subjects usually receive a predetermined amount of soy and equol status could be determined post intervention. However, this could be influenced by subject adherence to the soy treatment. Furthermore, differences in the methodology in determining equol status and the analytical techniques used could also add to the variability between studies. Beyond just reasons related to study design and variability, there may be specific factors inherent in these populations that may determine or influence equol production,
these include differences in the macronutrient composition of the diet, gut microflora, types of soy foods consumed, genetics and other unknown factors.151

2.5.2. Definition of Equol Status

The circulating levels and excretion of equol vary considerably among individuals with the consumption of soy foods. However, there is a large demarcation between equol producers and equol nonproducers of several orders of magnitude in the difference in urinary equol concentrations.149, 151. Excretion of specific metabolites, such as equol, after low and high doses of soy intake is dose-dependent.92 As a result, studies have used a threshold concentration of equol measured in serum or urine, which was established based upon the distinct clustering of individuals into low or negligible levels of equol and those with significantly higher levels.139, 141, 143, 149 To date, there has been a lack of a standardized protocol in defining equol status (i.e. different cutoff values) which has added a degree of heterogeneity to the current literature. As this area of research continues, accurate equol status classification is needed to allow for comparison between studies. The most widely used approach to define equol status is based on urinary equol concentrations. Setchell et al. defined an equol producer as excreting >1000 nmol/L in urine.62, 141, 143, this definition has added considerably to the field of equol research by attempting to establish a standardized method to define equol status. However, in some respects, it is an approximation based on references quoted.141, 143, since Rowland et al. suggested that an equol producer is defined as >1000nmol/d urinary equol excretion (intake of 56mg/d of soy isoflavones for 17 days)143 and Lampe et al. suggested >2000 nmol/d (intake of 30mg/d for 4 days)141. Unfortunately, either reference provided 24-hr urine volumes. Recently, this method has been further refined by Setchell and Cole, who have proposed a new approach to
classify an individual’s equol status \(^{149}\). In this method, the precursor-metabolite relationship is utilized in the classification of equol producers and nonproducers. An equol producer is defined as an individual with a urinary \(\log_{10} S\text{-equol}:\text{daidzein} \) ratio of \(>1.75\) \(^{149}\). The advantage of this method compared to previous approaches of defining equol producers is that it eliminates the issue of different exposure levels of soy isoflavones, the interindividual variations in isoflavone pharmacokinetics and systemic errors in analytical techniques used for isoflavone measurement. However, equol nonproducers may produce low levels of equol where their ratio falls above 1.75, which would classify them as an equol producer. Therefore, a combination of the two approaches (i.e. total equol excretion and urinary \(\log_{10} S\text{-equol}:\text{daidzein} \) ratio) would increase the accuracy in equol status classification. In the current thesis, we have used a combination of these two approaches (see Chapter 6 – Isoflavone and Equol Method).

### 2.5.3. Equol Analysis

As previously mentioned, the identification of equol in human urine was first isolated over 2 decades ago \(^{136}\). Urinary equol was extracted and isolated using a gas chromatography-mass spectrometry (GC-MS) analysis and furthermore, the determination of equol status (i.e. cutoff values) has been predominantly established based on this analytical method \(^{62,141,143}\). Earlier work documenting the metabolic fate of soy isoflavones, specifically daidzein and genistein, has also used a similar method of GC-MS to measure soy isoflavones in blood and urine samples \(^{95,96,139,141}\). Modifications of the earlier GC-MS method have improved this analytical technique. Currently, GC-MS is a validated assay for the analysis of soy isoflavones and it is considered a reference method that is both sensitive and specific \(^{152,153}\). GC-MS
involves a multi-stage process including purification, extraction, hydrolysis and derivatization of samples. GC-MS does not allow for the analysis of conjugated isoflavones as they appear in blood, urine and food (i.e. they are first enzymatically or chemically hydrolyzed prior to analysis). Therefore, measurements obtained from GC-MS reflect the total hydrolyzed soy isoflavone content. Over the years, due to the cost and time-consuming nature of GC-MS, newer methods have been developed. Recently, another commonly used method for soy isoflavone analysis has involved another chromatographic technique, high pressure liquid chromatography (HPLC) coupled with MS. HPLC-MS has been combined with electrospray ionization (ESI) or heated nebulizer atmospheric pressure chemical ionization (HN-APCI) interfaces. If the amount and distribution of both aglycones to conjugates are of interest, these methods allow for the detection of these various forms of isoflavones. Furthermore, HPLC-MS does not need hydrolysis and derivatization of samples, i.e. fewer steps and less time consuming. A disadvantage of HPLC-MS is that measurements are dependent on the availability of standards for both the aglycone and their equivalent conjugate and some of these materials may not be available. As such, some have continued to use the enzymatic hydrolysis step, as used with GC-MS, with HPLC-MS. Recently, HPLC with tandem MS/MS has increased the specificity and allows for the direct injection of biological fluids. Specifically for the measurement of equol, it has been reported that the use of HPLC with ultraviolet (UV) detection lacks reliability and sensitivity for the isolation and quantification of equol because of its poor UV absorption characteristics.

Another emerging technique is the use of immunoassay, which is less costly and time consuming relative to GC-MS or HPLC-MS. Hence, immunoassays allow for rapid
analysis of a large number of samples. However, cross-reactivity with other similar compounds may occur in biological samples.

To date, various methods have been used to determine urinary isoflavone excretion, however, GC-MS and HPLC-MS are the two most common. In specific reference to the determination of equol status, as measured from urinary equol excretion, the recently refined method by Setchell and Cole utilizes the precursor-metabolite relationship as outlined in the previous section on the definition of equol status. An equol producer is defined as an individual with a urinary log$_{10}$S-equol:daidzein ratio of $>\text{1.75}$ 149. Hence, determination of this ratio eliminates the issue of variation and systemic errors in different analytical techniques used for isoflavone measurement.

The technique used in this thesis was based on the original technique of Setchell et al., but is not the technique currently used in his laboratory, which is HPLC-ESI-MS. Setchell et al. has reported that the correlation coefficient for isoflavones measured by GC-MS and ESI-MS was 0.95 89.

### 2.5.4. Possible Determinants of Equol Production

There is a suggestion that many factors may exist that could potentially influence the capacity to produce equol. These include habitual diets, addition of other dietary components (i.e. prebiotic and probiotics), the food matrix and colonic microflora profile. Other less studied factors include gender, age and genetics.

#### 2.5.4.1. Habitual Diet and Macronutrient Composition

Diet has the potential to influence the composition of the intestinal microflora, as a consequence, equol production may also be altered based on the habitual diet of an individual. An *in vitro* study of the metabolism of daidzein in a colonic model of
fermentation using human fecal flora showed that the rate of conversion of daidzein to the intestinal bacterially derived metabolite equol, is influenced by the presence or absence of carbohydrates (Figure 2) \textsuperscript{46, 156}. When isoflavones were incubated with a low carbohydrate containing media, there was little evidence for the biotransformation of daizein to equol. Levels of daidzein remained relatively stable over time, whereas levels of equol were low. However, when a mixture of carbohydrate was added to the media, a different pattern emerged. The levels of equol increased over time and levels of daidzein became very low, suggesting almost all of the daidzein was biotransformed to equol (Figure 2). Therefore, it appears that dietary components may have the capacity to increase or decrease the gut microflora’s ability to biotransform daidzein to equol and that carbohydrate fermentation may play a role in this process. This was furthered supported by a study of 22 individuals, which found that in those following a Western-type diet, equol producers tended to consume a greater percentage of energy from carbohydrates (55\% vs. 47\%) and a lower percentage of energy from fat (26\% vs. 28\%).
35%) compared to those who were equol nonproducers (Figure 3) \(^{143}\). It was also noted that the relationship for equol production was the strongest for fat and the authors suggested that dietary fat intake decreases the capacity of the gut to synthesize equol. The benefits of higher carbohydrate or lower fat in equol production have also been observed in another study \(^{140}\). Interestingly, Rowland et al. found no relationship with equol status and nonstarch polysaccharides, suggesting that carbohydrates may be more important than dietary fiber, which was also seen in a study where coingestion of wheat bran and soy protein did not alter equol production \(^{142}\). However, this was not supported in other studies where equol production was associated with other components of the diet. Equol production was related to higher intakes of plant protein and dietary fiber, both as soluble and insoluble fiber \(^{141}\), fat \(^{109, 157}\), meat \(^{109, 158}\) and alcohol consumption \(^{157}\). Furthermore, a greater prevalence of equol producers has
been observed among vegetarians than non vegetarians. Based on the current evidence, the role diet plays in equol production is unclear and conflicting. Part of the reason for this inconsistency may be a result of studies that were not designed primarily to determine the effect of diet on equol production, i.e. to manipulate the diet with a specific intervention and measure equol production. Post-hoc analyses and cross-sectional studies only provide associations of potential relationships used to generate hypotheses. Therefore, these data suggest the urgent need for more clinical trials to specifically test and confirm these hypotheses.

### 2.5.4.2. Long-term intake of Soy Foods

As discussed previously, there is a greater prevalence of equol producers in the Orient that consume soy as part of their traditional diet. Despite the suggestion that equol status is quite stable it has been observed that subjects with a long-term intake of soy (≥30mg soy isoflavones/d for at least 2 years) showed a higher probability of being equol producers than those who consumed ≤5mg/d. It is possible that long-term dietary habits may allow for adaptation and selection for a certain colonic microflora profile that favors biotransformation of daidzein to equol. However, further studies are needed to confirm this observation. To date, most intervention studies have been short term and may not be adequate in time to allow for adaptation or changes to gut microflora needed to alter isoflavone biotransformation.

### 2.5.4.3. Prebiotics and Probiotics

The *in vitro* study by Cassidy demonstrated that colonic fermentation may be important in equol production since daidzein was biotransformed to equol only in the presence of
carbohydrates (Figure 2) \(^{156}\). This has sparked interest in using dietary manipulations with pre- and probiotics to alter colonic fermentation, which in turn may influence colonic isoflavone metabolism and outcomes of interest, such as the blood lipid profile. Intake of a soy protein isolate with 44mg of isoflavones and a probiotic (\(10^9\) colony-forming units of \(lactobacillus\) acidophilus DDS-1 and \(Bifidobacterium\) longum) for a period of 6 weeks did not significantly affect the plasma and urine isoflavone concentrations \(^{145}\). Fecal counts of bifidobacteria were significantly increased, however, the data were not presented. Although it was noted that 67% of the equol producers had increased equol excretion with the soy and probiotic combination, the change was not reflected in excretion of daidzein and ODMA. Furthermore, no significant correlations were observed between individual plasma isoflavones and any lipid endpoints \(^{118}\). A lack of significant changes to circulating soy isoflavones and urinary excretion was also observed in another study in which a probiotic or a prebiotic (resistant starch) was added to a soy containing diet \(^{159}\). Interestingly, the intake of resistant starch (i.e. prebiotic) with soy resulted in significant lipid reductions which were not observed when a probiotic was taken with soy \(^{160}\). However, it cannot be concluded if the addition of a prebiotic enhanced the cholesterol lowering effect of soy because this treatment was not compared to the intake of soy alone without a prebiotic. Furthermore, the use of resistant starches increase overall colonic fermentation, however, not all resistant starches are bifidogenic, unlike fructo-oligosaccharides, which selectively stimulate bifidobacteria counts. The coingestion of inulin and soy was also studied in postmenopausal women who consumed 40mg of isolated isoflavones with 3.66g inulin twice daily for 21 days \(^{161}\). It was found that plasma concentrations increased for daidzein (+38%) and genistein (+91%) with the combined intake of soy.
isoflavones and inulin in comparison to isoflavones without inulin. It is possible that the addition of inulin may have increased the hydrolyzation of the β-glucosides to the aglycones, facilitating increased absorption. Unfortunately, other metabolites, including equol were not measured in this study\textsuperscript{161}, therefore, it is unclear whether the total isoflavone concentrations were altered or just the distribution of isoflavones.

### 2.5.4.4. Other Factors

It has been suggested that older individuals are less likely to be equol producers compared to younger individuals, where equol status was not affected by gender or anthropometric measures\textsuperscript{140}. However, this observation is confounded by the possibly that gut absorption of soy isoflavone decreases with age\textsuperscript{151}, as observed with certain micronutrients\textsuperscript{162}. At this time, there are few studies that have assessed both younger and older individuals with an adequate sample size to establish the link between age and equol status.

Equol status was also positively associated with greater education and a suggestion of a positive correlation with current or past oral contraceptive use\textsuperscript{140}. Ethnicity/race may also be related to equol status as demonstrated in the difference in prevalence in Western populations compared to countries in the Orient with higher intakes of soy. However, studies to date are lacking and have not included ethnically diverse populations and measured the prevalence of equol producers to nonproducers. These associations, whether they truly exist or not, need to be interpreted with caution.

In a family study, it was found that equol production may be under some degree of genetic control, however, other environmental factors may also be contributing to this phenotype\textsuperscript{140}. 
Differences in the frequency of equol producers between the sexes are inconclusive due to the limitation of available data. Men have been observed more likely to be equol producers than women\textsuperscript{149}, whereas in a large clinical trial of 90 subjects, the number of equol producers did not differ between the sexes\textsuperscript{10}. However, many of the studies looking at equol status include small sample sizes, which were most likely underpowered to detect any difference between the sexes, if they exist. Future studies of larger sample sizes are needed to determine if the prevalence of equol production differs between males and females, and if such a difference exists, what factors influence equol production in these two groups.

2.5.5. Colonic Microflora and Equol Production

The “equol hypothesis” has not only generated much interest in looking at differences between producers and nonproducers, but also the quest to identify the specific microflora responsible for the biotransformation of daidzein to equol. However, a large microflora population is present in the human colon at $10^{10}$ to $10^{11}$ cfu/g wet wt\textsuperscript{163}, with more than 50 genera and over 400 species of bacteria already identified in human feces\textsuperscript{164,165}. Therefore, the goal of isolating the specific microflora responsible for the biotransformation is a daunting task. The ultimate goal is to develop strategies to target these microbes with the hope to increase equol production, but even more relevant, converting an equol nonproducers to a producer if the benefits of equol production can be clearly defined.

Initially, it was believed that one bacterium was responsible for biotransforming daidzein to equol, but recent evidence suggests that different bacteria have the capacity to convert daidzein to its intermediates, and another set of bacteria can
convert the intermediates to equol\textsuperscript{106, 107}. Furthermore, the bacteria responsible for converting daidzein to equol may not be the same as those that convert daidzein to ODMA, i.e. different bacteria are involved in the different steps of the conversion of daidzein to equol, which has been supported by both \textit{in vivo} and \textit{in vitro} studies\textsuperscript{106, 107}. As research continues in this area, the picture is becoming more complicated than initially believed and the exact bacteria responsible are still unclear, since what is observed \textit{ex-vivo} may not be the same as seen \textit{in vivo}.

It has been shown that equol is produced under anaerobic conditions as demonstrated by inhibition of equol production by antibiotics that target anaerobic bacteria\textsuperscript{107}. The microflora involved in the process of biotransforming daidzein to equol may be different between individuals, since use of antibiotics did not inhibit equol production equally in individuals\textsuperscript{107}. This variation suggests that the conversion of daidzein to DHD, and DHD to equol, might be carried out by different bacteria that are able to co-exist and that the specific bacteria involved may differ among individuals\textsuperscript{107}. \textit{In vitro} studies of equol producers noted that no DHD, which is an intermediate of equol and ODMA, was found in samples, suggesting that a bacterial population exists that very efficiently converts DHD through the two pathways to the final products, equol and ODMA, leaving very little time for the intermediate to be measured\textsuperscript{106}. It has also been observed in studies of urinary isoflavone excretion that the production of equol does not exclude the formation of ODMA\textsuperscript{106, 107}, confirming the notion that different bacteria are involved in the biotransformation of daidzein. Competition between microbes for substrate may exist, since some have observed an inverse relationship between the amount of equol and ODMA\textsuperscript{96, 102, 166}, whereas others have not\textsuperscript{92}. In this latter study, the authors suggested the lack of an inverse relationship may be related to short
duration (9 day) and the low doses of isoflavones used (9-36mg/d) ⁹². But it is unlikely that equol producers absorb more isoflavones because no significant differences in total isoflavone excretion have been observed between equol producers and nonproducers ⁹², ¹⁴⁵, ¹⁵⁹.

_In vitro_ studies using human fecal flora have identified a number of bacteria involved in the conversion of daidzein to equol. In one study, bacteria with high β-glucosidase activity were isolated from an equol producer and a gram-positive bacterium, strain HGH6 (ATCC number BAA-96), was found to have β-glucosidase activity, which could deconjugate daidzin to daidzein, and then metabolize daidzein to DHD ¹⁶⁷. However, this strain did not metabolize DHD to equol, which suggests that more than one bacterial species is involved in the biotransformation of daidzein, since the total colonic flora from the individual was able to convert daidzein to equol ¹⁶⁷.

In a subsequent _in vitro_ study by the same research group, a gram positive anaerobic bacterium capable of metabolizing daidzein to ODMA was identified as a _Clostridium sp._ ¹⁶⁸. Another study found that equol production correlated negatively with _Clostridium coccoides-Eubacterium rectale_ counts and positively with sulfate reducing bacteria in postmenopausal Caucasian women ¹⁵⁷. A mixed microbial culture (EPC4) was also found to produce equol ¹⁶⁶. The addition of EPC4 to fecal cultures of an equol nonproducer, resulted in equol production ¹⁶⁶. It was also demonstrated that the bacteria involved in equol production used hydrogen gas, since the removal of this gas resulted in the lack of equol production. The authors suggested that hydrogen most likely acts as an electron donor in the biotransformation of equol ¹⁶⁶. Interestingly, the presence of increasing concentrations of propionate and butyrate did not inhibit equol production, whereas acetate did ¹⁶⁶.
Adding to the complexity in addressing the question of which bacteria are involved in equol production is the observation that over a period of 9 months, fecal samples collected from the same individuals at different times showed differences in daidzein metabolism (i.e. equol status) \(^{106}\). However, this study included a small number of subjects (N=6) and therefore, conclusions cannot be drawn, since most studies to date have suggested that equol status remains relatively stable over time \(^{89,107}\). However, as previously mentioned, due to the short duration of most studies, long-term effects are unclear. One long-term study where samples were collected over a period of years showed the relative stability of equol status \(^{150}\). Based on the \textit{in vitro} studies conducted to date, it appears that a number of bacteria are involved in different stages of the biotransformation of daidzein to equol. Whether these specific bacteria play a significant role \textit{in vivo} is unclear, since the gut is a dynamic organ where conditions cannot be entirely replicated \textit{in vitro}.

\textbf{2.5.6. Equol Status and Lipid Reduction}

Those who have the capacity to produce equol have been suggested to exhibit greater health benefits than seen in equol nonproducers \(^{62}\). One particular outcome of interest is serum cholesterol, a risk factor for CHD, where results have been mixed. Meyer et al. (n=23) found that although there were no differences in plasma lipids between the soy and dairy diets, a post-hoc analysis where subjects were divided based on equol status, showed that equol producers had significant reductions in TC (8.5%), LDL-C (10%), LDL-C:HDL-C (13.5%) and TG (21%), compared to equol nonproducers while consuming soy \(^{144}\). These data suggest that equol production may be an effect modifier of the cholesterol lowering action of soy, where those who are equol producers have
greater lipid improvements relative to equol nonproducers. This hypothesis was further supported by Clerici et al. (n=29 on soy germ pasta arm) showing that intake of a soy germ pasta significantly reduced serum lipids (TC and LDL-C), CRP and flow mediated vasodilatation in equol producers compared to equol nonproducers. However, a number of studies have shown equol status does not influence lipid reductions. A large clinical trial of over 90 participants on soy diets demonstrated that compared to the dairy control, TC significantly decreased by 3% during the soy treatment. Furthermore, no significant differences were observed with HDL-C, LDL-C and the TC:HDL-C ratio. Importantly, no significant differences were seen when the participants were divided based on equol status. In another large study of 117 participants, consumption of isolated soy isoflavones (50mg/d) enriched in cereal bars for 8 weeks did not result in significant reductions in lipids nor were there differences between equol producers and nonproducers. However, as previously discussed, this study used isolated soy isoflavones which have been consistently shown to be ineffective at reducing cholesterol concentrations. Intake of soy milk over a period of 4 weeks in 28 individuals showed a reduction of 5% in LDL-C relative to the dairy control, but in a post-hoc analysis, the LDL-C reduction was comparable between equol producers and nonproducers. Again, as previously mentioned, studies that included a pre- or probiotic to enhance the cholesterol lowering ability of soy found no significant differences based on equol status. Therefore, whether equol status plays a role in lipid reduction is unclear. Since the primary objective of many of these studies was not to determine differences between equol producers and nonproducers, they may not have been adequately powered to detect significant differences.
2.6. Estrogens, Lipids and CHD

The risk of CVD has been suggested to be related to hormone levels, specifically endogenous estrogen (i.e. 17β-estradiol), since it has been observed that the incidence of CVD is lower in premenopausal women and higher in postmenopausal women. The protective effects of estrogens may be related to improvements in serum lipid concentrations, coagulation and fibrinolytic systems, antioxidant systems, and the production of other vasoactive molecules, such as nitric oxide and prostaglandins, all of which can influence the development of vascular disease.

As a result, the use of HRT had great hopes as a therapeutic agent in reducing the risk of CHD. However, the once widely recommended use of HRT for CHD prevention based on positive findings from clinical trials, is now not recommended as a result of evidence showing the lack of benefit and possibly increased risk with use. Studies such as the Heart and Estrogen/Progestin Replacement Study and the Women’s Health Initiative have concluded that HRT is ineffective in the prevention of CHD and is accompanied by side effects including breast and uterine cancers.

Despite the negative findings, estrogens tend to improve the blood lipid profile (Table 2) and although their exact mechanism of action is unknown, they may provide clues into how phytoestrogens may also improve serum lipids. Estrogen has been associated with increasing HDL-C and lowering LDL-C. Studies using oral estrogen therapy have been associated with increased HDL-C and ApoA1 and decreased in LDL-C and Lp(a). However, it is also associated with increased TG and CRP. It has been shown in kinetic studies to selectively raise ApoA-1 in postmenopausal women, by increasing the ApoA-1 production rate without altering the fractional catabolic rate. This
indicates that estrogen can influence HDL metabolism which has been suggested to play an important role in reverse cholesterol transport, thus reducing CVD risk \(^{179, 180}\).

### 2.7. Selective Estrogen Receptor Modulators (SERMs)

In light of the negative findings with HRT use, alternatives that mimic the physiological benefits of estrogens are being sought intensively. SERMs have the ability to bind to the ERs and elicit an effect, which could be either estrogenic or antiestrogenic. This is dependent on a number of factors including site of action (i.e. tissue), preferential binding to specific ERs and concentration of both endogenous estrogen and the SERM in circulation. The differences in tissue specific actions of SERMs is probably related in part to their ability to induce unique conformational changes in the ER complex which

### Table 2. Comparative Effects of Oral Hormone-Replacement Therapy and SERMs on Serum Lipids, Apolipoproteins and C-reactive Protein.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hormone Replacement Therapy</th>
<th>Tamoxifene</th>
<th>Toremifene</th>
<th>Raloxifene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-density lipoprotein cholesterol (LDL-C)</td>
<td>-12†</td>
<td>-19†</td>
<td>-21†</td>
<td>-12†</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol (HDL-C)</td>
<td>7†</td>
<td>-2</td>
<td>14†</td>
<td>0</td>
</tr>
<tr>
<td>Triglycerides (TG)</td>
<td>18†</td>
<td>31†</td>
<td>-4</td>
<td>-4</td>
</tr>
<tr>
<td>Apolipoprotein A-I (ApoA1)</td>
<td>13†</td>
<td>5</td>
<td>13†</td>
<td>3</td>
</tr>
<tr>
<td>Apolipoprotein B (ApoB)</td>
<td>-4</td>
<td>-9†</td>
<td>-10†</td>
<td>-9†</td>
</tr>
<tr>
<td>C-reactive protein (CRP)</td>
<td>84†</td>
<td>-</td>
<td>-</td>
<td>-7</td>
</tr>
</tbody>
</table>

† \( P<0.05 \) for the comparison with placebo. Adapted from Riggs et al. \(^{238}\)
include the recruitment of other proteins, such as coactivators and corepressors, resulting in different responses depending on the ligand binding to the ER and the series of molecular interactions \(^{181}\).

There are two known ERs, ER\(\alpha\) and ER\(\beta\) \(^{182}\), both of which are members of the superfamily of steroid hormone receptors, which are transcription factors that alter gene expression when they are activated. The two ERs are different in many ways including their distribution in tissues, structure and functionality.

There are currently a number of SERMs that are being used in the treatment of a number of diseases. Perhaps the best known SERM is tamoxifen, a commonly used drug for the treatment of breast cancer, which has antiestrogenic effects in the mammary gland, but has estrogenic effects in the bone and in the uterus \(^{183}\). Another SERM is raloxifene, which is used in the treatment of osteoporosis and has similar actions to tamoxifen, but no uterine effects, thereby decreasing the risk of developing endometrium cancer \(^{184}\). Both of these SERMs have also been shown to have effects on blood lipids, where both decrease LDL-C, but have no effect on HDL-C (Table 2). However, another SERM, toremifene, has been found to decrease LDL-C and increases HDL-C (Table 2). Therefore, SERMs themselves may have different lipid effects. Phytoestrogens, including soy isoflavones, are of particular interest because these are considered to be non-pharmacological SERMs and have the ability to bind to ERs, where they have been proposed to elicit the benefits associated with estrogen and pharmacological SERMs, especially in improving the blood lipid profile.
2.7.1. Equol and Other Isoflavones as Potential SERMs

Equol has been suggested to be the most estrogenic isoflavone and may behave similarly to SERMs, such as tamoxifen and raloxifene, due to its structural similarity to 17β-estradiol (Figure 4). However, the clinical relevance of equol production still remains to be fully established.

The ability of soy isoflavones, which are phytoestrogens, to bind to ERs and elicit estrogenic or antiestrogenic activity has been demonstrated in in vitro studies, thereby acting as SERMs, similar to the drug tamoxifen. It has been shown that the soy isoflavone glucosides have poor binding affinity to both ERα and ERβ and are poor inducers of transcription, whereas the aglycones generally bind to and induce transcription 185.

Soy isoflavones and their metabolites bind to ERs and may elicit either an agonist (estrogenic) or antagonist (antiestrogenic) effect. However, the binding affinity of soy isoflavones has been found to be weaker (100-1000 fold weaker) than that of 17β-estradiol, but it is more strongly competitive with 17β-estradiol for binding to ERβ than ERα 104, 185. Binding of commonly known SERMs, such as tamoxifen, to ER have also been demonstrated in these in vitro studies 186. Genistein has the highest binding affinity for both ERs where the intermediate metabolite has a much weaker binding affinity 104, 185-187. Similarly, the intermediate metabolites of daidzein were observed to
have the lowest binding affinity to both ERs. Among the metabolites of daidzein, equol has the highest binding affinity, especially to ERβ. Binding to ERs does not imply either an agonist or antagonist effect, *in vitro* studies have used induction of transcription as a marker of activity of the ligand once binding occurs to the ERs. For example, both daidzein and equol (in the absence of estradiol) did not affect growth of MCF-7 cells at concentrations from $10^{-11}$ to $10^{-9}$, but at concentrations from $10^{-9}$ to $10^{-5}$, cellular proliferation was stimulated in a dose-dependent manner, suggesting an estrogenic potential. The estrogenic potency of the soy isoflavones found in food compared to estrogen has been observed to be $17\beta$-estradiol $>$ genistein $>$ daidzein. Equol was shown to cause a higher induction of transcription activity to ERβ than its precursor, intermediates and ODMA.

Therefore, equol appears to have the highest binding affinity and estrogenic activity among the metabolites of daidzein. Although a greater estrogenic activity is exhibited by equol relative to the other isoflavones and metabolites, in the presence of $17\beta$-estradiol, there is a greater reduction in overall estrogenic activity compared to its precursor, suggesting it has a greater antagonistic effect and does not result in an additive effect.

An interesting aspect that has been observed with soy isoflavones is their dual role (i.e. biphasic response) which is dependent on estrogen concentrations as demonstrated *in vitro*. Soy isoflavones act as estrogen agonist in the presence of estrogen concentrations observed in postmenopausal women ($\sim 10^{-11}$ M), whereas, transactivation by a premenopausal dose of estrogen ($\sim 10^{-9}$ M) was inhibited by isoflavones, i.e. acting as an estrogen antagonist. This inhibitory effect on estrogen activity was more prominent with ERβ than ERα. It is likely that the antagonistic
(antiestrogenic) effect shown at the physiological premenopausal doses of estrogen is a result of competitive inhibition of endogenous estrogens for the ERs, and the weaker transcription activity of phytoestrogens relative to estrogen. Whereas, in the presence of a low estrogen, i.e. levels found in postmenopausal women, isoflavones may have some additive effect \(^{104}\).

2.8. Carbohydrate Fermentation

Relevant sections adapted from Wong et al, 2006 and Wong and Jenkins, 2007 \(^{189,190}\)

Carbohydrates resistant to digestion and those that escape absorption in the small intestine are available for colonic bacterial fermentation resulting in the production of short chain fatty acids (SCFAs - acetic, butyric, and propionic acids) together with gases (CO\(_2\), CH\(_4\), and H\(_2\)) and heat \(^{191,192}\). The role of SCFAs has expanded to include their role as nutrients for the colonic epithelium, as modulators of colonic and intracellular pH, cell volume, and other functions associated with ion transport, and as regulators of proliferation, differentiation, and gene expression \(^{193}\). Increases in SCFAs result in decreased pH, which indirectly influences the composition of the colonic microflora (e.g., reduces potentially pathogenic clostridia when pH is more acidic), decreases solubility of bile acids, increases absorption of minerals (indirectly), and reduces the ammonia absorption by the protonic dissociation of ammonia and other amines (i.e., the formation of the less diffusible NH\(_4^+\) compared with the diffusible NH\(_3\)) \(^{194-197}\).

The major source of fermentable carbohydrates are the resistant starches. It is estimated that 5–20% of dietary starch is not absorbed in the small intestine \(^{198-202}\). Soluble and insoluble fibers are fermented to varying degrees. However, insoluble
fibers (e.g., lignans, cellulose, and some hemicelluloses) that are resistant to colonic fermentation may carry with them fermentable carbohydrate substrate, including starches and sugars, although their major role is in fecal bulking. Soluble fibers (e.g., pectins, β-glucans, gums, mucilages, some hemicelluloses, as well as inulin-type fructans) are generally more completely fermented with little effect in increasing fecal bulk. Most fiber containing foods contain about one-third soluble and two-thirds insoluble fiber.

The production of SCFA is determined by a number of factors, including the number and types of microflora present in the colon, type of substrate, and gut transit time. In general, fecal SCFA production is in the order acetate > propionate > butyrate in a molar ratio of ~60:20:20, respectively. Absorption of SCFA in the cecum and the colon is a very efficient process with only 5–10% being excreted in the feces. Once absorbed, SCFA are metabolized at 3 major sites in the body: 1) cells of cecocolonic epithelium that use butyrate as the major substrate for maintenance-energy-producing pathways; 2) liver cells that metabolize residual butyrate with propionate used for gluconeogenesis and 50–70% of acetate is also taken up by the liver; 3) muscle cells generate energy from the oxidation of residual acetate. The primary interest in SCFA has been in relation to colonic function as a result of their uptake and metabolism by colonocytes, specifically butyrate, although SCFA are also metabolic substrates for other tissues of the host.

2.8.1. Short Chain Fatty Acids and Cardiovascular Disease

Acetate and propionate have been proposed to have opposing effects in hyperlipidemia, a risk factor for coronary heart disease. Subjects given rectal infusions of acetate and
propionate in equivalent ratios showed a dose-dependent increase in serum total cholesterol and TG levels, providing indirect evidence that SCFA are utilized for lipid synthesis \(^{209}\). In a subsequent study by the same research group, rectal infusions of a mixture of acetate and propionate attenuated the serum cholesterol increase observed when acetate infusion was given alone. However, rectal infusions of propionate alone did not affect lipids or TG in healthy young men and women \(^{210}\). These results support the idea that propionate inhibits the utilization of acetate for cholesterol synthesis. However, dietary trials have been inconsistent. One-week intakes of 2.7 g of sodium propionate given in bread \(^{211}\) and 7.5 g sodium propionate taken as a capsule \(^{212}\) did not affect serum lipids, although one study showed that 5.4 g of propionate given daily for 2 wk lowered LDL-C and total cholesterol in subjects with total cholesterol >5.5 mmol/L \(^{213}\). Animal studies suggest that propionate inhibits cholesterol synthesis by inhibiting both 3-hydroxyl-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-coA reductase \(^{214,215}\).

Inulin-type fructans are bifidogenic and have been associated with hypolipidemic effects. Although a number of mechanisms have been proposed, increased propionate production, resulting in a decreased acetate:propionate ratio, has been one of the suggested modes of action. Increased production of propionate, through fermentation, may inhibit cholesterol synthesis \(^{214,216-221}\). This has been supported in studies with hyperlipidemic experimental animals \(^{216,222}\), but not supported in other animal studies \(^{223-225}\). The effect of inulin-type fructans on blood lipids in humans have yielded inconsistent results \(^{226}\) compared with the animal data; this may be related to species differences. Furthermore, few studies have quantified the synthesis of SCFA, specifically acetate and propionate, with use of prebiotics.
The lack of agreement on the relation between increased colonic fermentation and lipid metabolism may be a result of differences in the chemical composition of the substrate source. Studies with resistant starch have been consistent in showing raised fecal butyrate\textsuperscript{227-230}. Starch fermentation primarily yields acetate and butyrate, whereas fermentation of pectin and xylan yields acetate alone as the main product\textsuperscript{231}. Recent human studies found that acute ingestion of a nondigestible monosaccharide, L-rhamnose (25 g), increased serum propionate without increasing acetate\textsuperscript{232}, but longer-term studies have not shown reduced serum lipids\textsuperscript{233}. Lactulose, a rapidly fermented dietary fiber, has been shown to result in higher serum cholesterol levels, possibly as a consequence of increased production and absorption of colonic acetate resulting in increased hepatic lipogenesis\textsuperscript{234}. Oligofructose and inulin, in common with other fermentable carbohydrates, may improve glucose tolerance through suppression of serum FFA concentrations\textsuperscript{235}. It has been shown that short-chain organic acids, including b-hydroxybutyrate, acetate, and propionate, may suppress FFA release and possibly stimulate increased insulin secretion\textsuperscript{209, 210, 236}.

\subsection*{2.8.2. Serum Lipids and the Equol:Propionate Link}

The picture emerging from the relationship between serum lipids and colonic fermentation is not clear. There is some evidence that increased acetate production may raise serum cholesterol and that possibly this increase in serum cholesterol may be modulated by propionate\textsuperscript{209, 210, 216, 221}. This phenomenon may be of relevance to the equol story, since increased propionate synthesis is likely to be seen amongst equol producers and whether directly or indirectly linked to equol synthesis may be one
of the reasons for the different lipid response to diet change sometimes reported in equol producers.

2.9. Summary

The cholesterol lowering potential of soy has been demonstrated in early studies \textsuperscript{32} and later confirmed in the first meta-analysis by Anderson et al. in 1995 \textsuperscript{7}. As a result, the FDA approved a health claim for soy protein in coronary heart disease risk reduction in 1999 \textsuperscript{8}. Since that time, soy has been recommended as part of a cholesterol lowering diet in the management of hypercholesterolemia. However, the effectiveness of soy as cholesterol lowering foods has been recently challenged with subsequent meta-analyses suggesting the magnitude of effect is much smaller than initially observed in the first meta-analysis \textsuperscript{9, 11-13}. However, there are a number of factors that may influence the effectiveness of soy as a hypocholesterolemic food, but very little research has focused on exploring these factors. The specific factors of interest in the current thesis were the isoflavone content of soy, altered colonic fermentation via provision of a prebiotic or reduction of dietary carbohydrate content and the influence of interindivdual variation in colonic isoflavone biotransformation (i.e. equol status). These specific factors were investigated in a series of sub-studies that provided soy foods commonly found in supermarkets to hyperlipidemic participants for a period of one month during each sub-study. Results of the current studies will provide further evidence of the value of soy foods as part of a dietary strategy for the management of hypercholesterolemia and may also provide possible methods by which the magnitude of effect may be amplified.
3. Aims and Hypotheses
3.1. Overall Aim

The overall aim was to determine if specific factors may influence the effectiveness of soy foods in lowering serum cholesterol concentrations. Such specific factors include the isoflavone content of soy, altered colonic fermentation via prebiotic administration, carbohydrate reduction or individual differences in isoflavone biotransformation (i.e. equol status) (Figure 1).

![Figure 1. Summary of current gaps in the literature and questions addressed in the current thesis related to the hypocholesterolemic effect of soy, these include the effectiveness of soy, altered colonic fermentation and individual differences in isoflavone biotransformation (i.e. equol status).]

3.1.1. Specific Aims

1) **Prebiotic Study:** To determine if coingestion of soy foods with a non-absorbable carbohydrate (i.e. prebiotic to increase colonic fermentation) will alter the cholesterol lowering effect of soy.
2) **Eco-Atkins Study:** To determine if intake of soy with reduced carbohydrate (i.e. decreased colonic fermentation) will alter the cholesterol lowering effect of soy.

3) **Equol Study:** To determine if the cholesterol lowering effect of soy is dependent on equol status. Furthermore, if factors such as soy isoflavone content, increased or decreased colonic fermentation will result in differences between equol producers and nonproducers.

### 3.2. Overall Hypothesis

Soy foods are effective in reducing LDL-C when consumed as part of a cholesterol lowering diet. The isoflavone content of the soy foods, altered colonic fermentation via a prebiotic or carbohydrate reduction, and isoflavone biotransformation (i.e. equol status) are all factors that will influence the hypocholesterolemic potential of soy.

### Specific Hypotheses

1) **Prebiotic Study:** Coingestion of soy foods with a non-absorbable carbohydrate (i.e. prebiotic) will significantly increase the cholesterol lowering effect of soy.

2) **Eco-Atkins Study:** Intake of soy with reduced carbohydrate will lessen the cholesterol lowering effect of soy.

3) **Equol Study:** The cholesterol lowering effect of soy is dependent on equol status. Overall, equol producers will have greater reductions in LDL-C compared to equol nonproducers after consumption of soy foods. Furthermore, equol producers will have greater LDL-C reductions with greater isoflavone intake and increased fermentable substrate, whereas, low intakes of soy isoflavones and decreased
colonic fermentation will not result in differences between equol producers and nonproducers.
4. Prebiotic Study

“Can the Potential Cardioprotective Effects of Soy be Further Improved by Co-ingestion of a Prebiotic? A Randomized Controlled Trial”
Can the Potential Cardioprotective Effects of Soy be Improved by Co-ingestion of a Prebiotic? A Randomized Controlled Trial

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Running Title: Cardioprotective Effects of Soy and Prebiotics
4.1. Abstract

Introduction: The value of soy protein, as part of the cholesterol lowering diet, has been questioned by recent studies. The apparent lack of effect may relate to the absence of dietary factors which increase colonic fermentation and potentiate the cholesterol lowering effect of soy. Therefore, unabsorbable carbohydrates (prebiotics) were added to the diet to increase colonic fermentation and so increase the hypocholesterolemic effect of soy.

Methods: Twenty-three hyperlipidemic adults (11M, 12F; 58±7y; LDL-C, 4.18±0.58mmol/L) completed three 4-week diet intervention phases: 10g/d of oligofructose-enriched inulin (fermentable carbohydrate – prebiotic) and low-fat dairy diet, and soy foods (30g/d soy protein, 61mg/d isoflavones from soy foods) with and without 10g/d prebiotic (prebiotic plus soy and soy alone, respectively), in a randomized controlled crossover study.

Results: Intake of prebiotic plus soy resulted in a greater reductions in LDL-C (-0.32±0.14mmol/L, P=0.019) and TC:HDL-C (-0.61±0.18, P<0.001) compared to prebiotic alone. Both soy alone and prebiotic plus soy reduced apoB and ApoB:ApoA1 similarly compared to the prebiotic alone. An increase in mean hydrogen production was observed on the prebiotic alone (4.2±1.9 ppm, P=0.032) and prebiotic plus soy (3.56±1.93 ppm, P=0.070), compared to soy alone. No significant differences were observed in fecal microbiology and blood pressure among the three treatments.

Interpretation: Soy foods in conjunction with a prebiotic resulted in greater improvements in the lipid profile than intake of either prebiotic or soy alone. Coingestion of prebiotics may increase the effectiveness of soy foods as part of the dietary strategy to lower serum cholesterol.
4.2. Introduction

The value of soy as an integral component of the cholesterol lowering diet has been questioned by the results of recent studies. Studies in the late 1970’s by Sirtori et al. indicated the potential clinical significance of soy \(^{32}\). The first meta-analysis of the effect of soy in 1995 suggested a 13% LDL-C reduction following soy supplementation \(^{7}\) and resulted in the acceptance of a full CHD risk reduction health claim for soy protein by the US FDA \(^{8}\). Subsequently, soy foods have been widely recommended as cholesterol lowering and cardioprotective foods \(^{55,56}\). However, a recent AHA Nutrition Committee meta-analysis suggested only a 3% reduction in serum cholesterol \(^{9}\) and so challenged the existing health claim. This challenge has come at a time when much emphasis has been placed on lowering serum cholesterol to reduce CHD \(^{24}\) and relatively few cholesterol lowering dietary strategies exist. Therefore, methods for enhancing the effectiveness of soy and other cholesterol lowering food components are of continuing importance. It has been suggested that one means of increasing the effectiveness of soy is through enhancing the effectiveness of the isoflavone component of soy. Part of the reason for the disparity between studies in LDL-C reduction may relate to the differences in isoflavone content or bioavailability from different soy products. Soy isoflavones may act as SERMs \(^{237}\) and, as seen with tamoxifen \(^{238}\), may enhance the cholesterol lowering effect of soy proteins. However, their activation through the colonic biotransformation is driven by the microbial metabolism of fermentable carbohydrates \(^{46,156}\). Therefore, to determine whether the clinical utility of soy in lowering serum cholesterol can be enhanced, we have added a non-absorbable fermentable carbohydrate (prebiotic), in the form of a polyfructan, to a diet containing soy to determine its effect in maximizing cholesterol reduction.
4.3. Methods

4.3.1. Participants

Participants were recruited from patients attending the Clinical Nutrition and Risk Factor Modification Centre at St. Michael’s Hospital and from newspaper advertisements. Subject recruitment for this study occurred concurrently with two other clinical trials (Figure 1). Those subjects who met the eligibility criteria for more than one study were placed in their preferred study. Thirty-seven men and postmenopausal women with hyperlipidemia were randomized with a mean age of 59.6±7.7 years (range, 44-83 years), body mass index of 26.3±3.3kg/m², and LDL-C of 4.19±0.53 mmol/L (Table 1). No participant had a history of cardiovascular disease, untreated hypertension (blood pressure >140/90 mmHg), diabetes, renal or liver disease. No participants were taking medications known to influence serum lipids apart from four women who were on stable doses of a cholesterol lowering medication and were admitted to the trial in error and one man who was taking levothyroxine before and during the study. Data from the statin users did not differ from the group as a whole and are included in the final analysis. All participants were non-smokers and none had taken antibiotics in the last 3 months. Three participants were taking antihypertensive medications at a constant dose prior to and during the study with one participant starting the medication during the second phase. Participants were asked to keep constant across treatments the intakes of prescription and non-prescription medications and supplements, and level of physical activity. Of the 37 participants randomized, 23 completed all three phases and their data have been used in the final analysis (Figure 1). For those who completed the study, the mean duration of the study was 146±33 days (range: 111-219 days) with a mean washout period of 31±19 days between phases.
4.3.2. Study Protocol

The study consisted of three 4-week diet phases in a randomized controlled crossover design, with each phase separated by a minimum two-week washout period. During all study phases and washout periods, the diets were self-selected and approached the NCEP ATPIII recommendations for patients treated without medications with this degree of hyperlipidemia (<7% of total energy as saturated fat and < 200 mg/day dietary cholesterol) 23. Participants were randomized to complete the following treatments in random order: oligofructose-enriched inulin (prebiotic) with a diet containing very low-saturated fat (<7% of energy) dairy and egg protein food supplements (prebiotic alone), or soy food supplements with (prebiotic plus soy) or without prebiotic (soy alone).

Clinic visits were at weekly intervals during the one month treatment phases. At each visit, body weight and blood pressure were measured after an overnight fast (10-12h). Body weights were determined with shoes removed, in indoor clothing using a stationary beam balance scale (Healthometer, Continental Scale Corp, Bridgeview, IL). Blood pressure was measured after sitting for 15-20 minutes three times in the non-dominant arm by an automated digital blood pressure machine (OMRON Healthcare Inc., Vernon Hills, IL). Blood lipids were measured at weeks 0, 2 and 4 of each diet phase.

Seven-day weighed diet records and supplement checklists were completed and returned weekly during each 4-week diet phase. These were checked by one of the study dietitians who also recorded weekly exercise and ensured that it was constant over the study period. Symptom diaries (i.e. flatus, bloating and abdominal discomfort) were also completed daily during each 1-month phase using a 7-point semantic scale,
where 0 was none, +3 was moderate and +6 was severe. Participants rated their overall feeling of satiety on the diet at weekly intervals using a 9-point bipolar semantic scale, where –4 was very hungry, 0 was neutral and +4 was uncomfortably full.

Breath, fecal and urine collections were obtained at baseline and at the end of each treatment. Three-day fecal collections were obtained, as well as microbiological samples collected by core suction biopsy of 1 to 2 mL of fresh fecal material and added to peptone water containing 0.03% cysteine hydrochloride\textsuperscript{239,240}. Samples were stored on frozen CO\textsubscript{2} until transported to the laboratory where they were stored at -70°C until plating and enumeration. Breath gases were obtained at hourly intervals for 12 hours over the course of the day for H\textsubscript{2} and CH\textsubscript{4} using a modified Haldane-Priestley tube\textsuperscript{241}. One participant who completed the study chose not to complete the breath, fecal and urine collections.

Participants were randomized based on sex and baseline LDL-C using a random number generator and SAS software (SAS Institute Inc, Cary, NC)\textsuperscript{242} by the statistician in a location removed from the clinic. Study dietitians were not blinded to the diet since they were responsible for packaging and providing the study foods to the participants and for reviewing their diet records. The laboratory staff responsible for analyses were blinded to the treatment and received samples labelled with participant codes and dates.

The study was approved by the Ethics Committees of the University of Toronto and St. Michael’s Hospital. Written informed consent was obtained from the participants. The study clinical trial registration number is NCT00516594, which contains two independent clinical trials (only Study 2 is presented here).
4.3.3. Diets

Subjects were expected to be following a therapeutic low saturated fat and cholesterol diet prior to starting the study (Table 2). Two weeks prior to each dietary treatment and throughout the study period, subjects were instructed to avoid foods containing prebiotics, fructo-oligosaccharides (FOS) such as Jerusalem artichokes, chicory, onions, garlic and bananas \cite{196}; and foods with FDA approved health claims for coronary heart disease reduction including soy containing foods, soluble fiber (oats, barley and psyllium), plant sterols and nuts \cite{8, 243-246}. Participants were also asked to avoid any herbal supplements that contained soy isoflavones and/or fructo-oligosaccharides (FOS). These guidelines were intended to ensure that the run-in and baseline diets, and treatment periods were not influenced by specific cholesterol lowering foods other than those being tested. Participants were counseled initially and at follow-up visits on strategies to ensure weight maintenance.

The three phases consisted of: 1) oligofructose-enriched inulin (10 g/d) and low-fat dairy and egg protein foods (prebiotic alone); 2) soy protein foods (30 g/d soy protein, total isoflavone 61 mg/d) with prebiotic (10g/d) (prebiotic plus soy); or 3) soy protein foods without prebiotic (soy alone), based on a 2000 kcal diet (Table 3). The dairy and egg protein foods included skim milk, fat-free cheese and yogurt, and egg substitute and liquid egg white. The soy protein foods included soy beverage, low fat tofu, and a variety of soy meat analogues such as deli slices, hot dogs, burgers and breakfast patties. The soy and control supplements were matched for macronutrient composition except that soy protein replaced other proteins in the soy treatment arms. Fatty acid composition was balanced between the two diets with the use of butter and a mixture of sunflower and safflower oil. Dietary fiber was slightly higher and dietary cholesterol was
slightly lower on the soy foods (based on a 2000kcal diet: +3.9g and -9.5mg, respectively). The amount of soy protein and dairy foods were estimated based on the Lipid Research Clinic (LRC) Tables for energy requirements \(^{247}\) for each participant in order to provide 30g of soy or dairy protein per 2000 kcal diet. This level of soy protein was chosen to be in excess of the minimal level of soy protein (25g/d) in the current FDA health claim for CHD risk reduction (Table 3) \(^8\). The amount of prebiotic consumed (10g/day) remained constant for all participants and the prebiotic used was Synergy1\(^\circledR\) (Orafti Group, Belgium), a 50/50 mixture of inulin and oligofructose. Maltodextrin (10g/d) was provided on the soy alone phase as a placebo (Table 2). Participants were blinded to the prebiotic and maltodextrin during the study treatments.

All participants were instructed to weigh all foods consumed prior to and during the study period with self-taring electronic foods scale provided (Salter Housewares, Kent, England). During the study period all food supplements to be consumed by the participants were provided weekly at clinic visits. Participants were given a 7-day rotating menu of control or soy supplements (Table 4) on which they checked off each item as eaten and confirmed the weight of the foods. The same menu was used for all participants, but when necessary, the menu was modified to suit individual preferences providing the goals for soy protein, or dairy and egg protein were met. Compliance was assessed from the completed weekly checklists and from the return of uneaten food items.

### 4.3.4. Analyses

Serum was analyzed according to the LRC protocol \(^{218}\) for total cholesterol, triglyceride, and high-density lipoprotein cholesterol, after dextran sulphate-magnesium chloride
precipitation in the J. Alick Little Lipid Research Laboratory. LDL-cholesterol was calculated by the method of Friedewald et al. in mmol/L (LDL-cholesterol = total cholesterol – (TG/2.2 + HDL-cholesterol)).

Dietary isoflavone concentrations were measured as the 3 aglycones (genistein, daidzein, and glycistein) in study supplements. The samples were analyzed in duplicate using a modification of a previously described TMS derivatization procedure for isoflavones.

Freeze-dried soy and control foods were analyzed using methods of the Association of Official Analytical Chemists for fat, protein and fiber with available carbohydrate by difference. Bacterial populations were expressed as the Log$_{10}$ of colony forming units per gram of fresh sample (Log$_{10}$ CFU/g). Total aerobes, total anaerobes, bacteroides, bifidobacteria and fusobacteria were determined on serially diluted fecal samples after plating and colonies were identified on the basis of their morphology and confirmed through microscopic observation and fermentation characteristics.

Forced end-expiratory samples of alveolar air were collected with a modified Haldane-Priestley tube for the analysis of breath H$_2$ and CH$_4$ using a Quintron gas chromatograph (Quintron Microanalyzer Model DP, Quintron Co, Milwaukee, WI).

Diet histories were assessed for macronutrients, fatty acids, cholesterol and fiber using a computer program based on USDA data.

4.3.5. Statistical Analysis

Data analysis was conducted using SAS software Version 9.1 (SAS Institute Inc, Cary, NC). Results are expressed as mean±SE. Twenty-three subjects who completed all three treatments were included in the statistical analyses. For variables which were
measured at baseline and at weekly or two-weekly intervals during each treatment, differences from baseline were analyzed using a mixed linear model with random intercept to assess the effect of interaction between treatment and time, controlling for the factors including treatment, time and sequence. Results of the primary outcomes (serum lipids) were confirmed using least square means (PROC MIXED) with baseline as a covariate with phase and subject ID in the model with a Tukey-Krammer adjustment for multiplicity of comparisons. Where only baseline and week 4 were measured, differences from baseline were assessed using a mixed linear model with random intercept to assess the treatment effect controlling for the confounders including sequence and baseline values for the respective analysis. Pair-wise comparisons were conducted when the overall F-test showed a significant interaction effect between treatment and time or a significant overall treatment effect (significance level set at P<0.10) for outcomes with only week 0 and 4 values. Values in Tables and Figures are unadjusted values, and values in the text are values adjusted for confounders unless otherwise stated. P-values are adjusted for confounders unless indicated otherwise. The level of statistical significance was set at p<0.05, unless indicated otherwise.

4.4. Results

Compliance was good for the majority of the participants as assessed from completed supplement sheet checklists and return of uneaten food items. When expressed as a percentage of prescribed soy or dairy protein recorded as eaten during all 4 weeks of each treatment, mean compliance for the protein was 99% on the prebiotic alone phase, 95% on soy alone and 94% on the prebiotic plus soy phase. Mean compliance for the
prebiotic or maltodextrin over the 4 weeks was 98% on the prebiotic alone phase, 99% on the prebiotic plus soy phase and 99% on the maltodextrin plus soy (soy alone) phases (Table 2).

4.4.1. Lipids, Apolipoproteins and C-reactive Protein

No between-group differences were seen in serum lipids at baseline (see Appendix I). Although no significant differences were seen between soy alone and prebiotic plus soy, only the lipid reductions on the prebiotic plus soy were significantly different from prebiotic alone (Table 5). Greater mean reductions in LDL-C, TC:LDL-C and LDL-C:HDL-C ratios were observed on the prebiotic plus soy versus the prebiotic alone (unadjusted: -0.30±0.10 vs. 0.02±0.09 mmol/L, -0.36±0.11 vs. 0.25±0.10 and -0.31±0.11 vs. 0.22±0.08, respectively). The corresponding adjusted treatment differences were LDL-C: -0.32±0.14 mmol/L (P=0.019), TC:LDL-C: -0.61±0.18 (P<0.001) and LDL-C:HDL-C : -0.54±0.16 (P<0.001). The significant differences between prebiotic alone and prebiotic plus soy for LDL-C, TC:HDL-C and LDL-C:HDL-C remained after inclusion of baseline values as a covariate.

On the other hand, both soy alone and prebiotic plus soy significantly improved apoB and the apolipoprotein ratio compared to prebiotic alone. There was a greater reduction on the prebiotic plus soy versus the prebiotic alone for both Apo B (unadjusted: -0.06±0.02 vs. 0.02±0.02 g/L) and ApoB:ApoA1 ratio (unadjusted: -0.03± 0.02 versus 0.05±0.01). The corresponding adjusted treatment differences were ApoB: -0.08±0.03 (P=0.018) and ApoB:ApoA1 ratio : -0.08±0.02 (P<0.001). The same pattern of change was observed for soy alone compared to prebiotic alone in both ApoB (-0.07±0.03 g/L, P=0.036 and ApoB:ApoA1 ratio (-0.08±0.02, p<0.001) (Figure 2). No significant
differences were observed in TC, HDL-C, TG and apoA1 between the three treatments (Figure 2). C-reactive protein was not altered among the three dietary treatments (Table 5).

4.4.2. Body Weight, Satiety and Gastrointestinal Symptoms

No treatment differences were seen in body weight at baseline, nor in changes from baseline to end of treatment (Table 5), nor in feelings of satiety. Significant differences in mean ratings of bloating and abdominal discomfort, measured using a 7-point semantic scale (0=none and +6=severe), were not significantly different between the three treatments. Whereas, the mean rating of flatus was significantly increased on the prebiotic alone compared to soy alone (unadjusted mean values: 1.7±0.3 vs. 1.3±0.2), where the adjusted treatment difference was 0.60±0.25 (P = 0.014). No other treatment differences were observed in the mean rating of flatus.

4.4.3. Blood Pressure

No significance changes were observed in either systolic or diastolic blood pressure among the three treatments (Table 5).

4.4.4. Breath Data and Fecal Microbiology

Mean change in hydrogen production was increased during the prebiotic alone compared to soy alone (4.2±1.9ppm, P=0.032). A similar relationship was observed between prebiotic plus soy and soy alone (3.56±1.93ppm, P=0.070). No differences from baseline between treatments in mean 12-hr methane production between the three treatments and from baseline (Figure 3).
No treatment differences were seen in fecal microbiology.

4.5. Discussion

Intake of soy foods in conjunction with a prebiotic, compared to prebiotic alone, resulted in significant improvements in LDL-C (~7%) and the lipoprotein ratios, as predictors of CHD events. The observed LDL-C reduction on the soy alone phase (~4%), relative to prebiotic alone, was similar to the predicted effect as outlined in the latest American Heart Association Scientific Advisory on soy. The improvements in ApoB and ApoB:ApoA1 observed in the current study have also been supported by others studies that have shown similar effects of soy on apolipoproteins.

The significant reductions in LDL-C and the lipid and lipoprotein ratios during the prebiotic plus soy treatment compared to the prebiotic control treatment may have been due to presence of active components found in soy including the soy peptides, saponins, and isoflavones. To date, a possible link between altered colonic metabolism and the soy peptides or saponins has not been suggested, however, increased colonic metabolism has been associated with increased colonic soy isoflavone biotransformation, resulting specifically in equol production, a metabolite of daidzein, has been suggested to be the most estrogenic isoflavone with the ability to bind to both ER-α and ER-β (as SERMs). SERMs, such as tamoxifen, have been shown to lower LDL-C.

Evidence for an effect of soy isoflavones proposed in the present study comes from a recent study in which participants consumed a soy germ pasta containing soy isoflavones and which resulted in a decrease in both TC and LDL-C (7.3% and 8.6%, respectively). The pasta study contained minimal amounts of soy protein and was
therefore a test of the isoflavone effect on serum lipids. Although the total isoflavone
dose (33mg/d) in the study was less than the current study, over 80% of the isoflavones
were in the aglycone form, which are more readily absorbed than the glycosides\textsuperscript{81, 259}. However, the cholesterol lowering effect of soy isoflavones and their metabolites (i.e.
equol) is still debated. A meta-analysis of isolated isoflavones given alone has failed to
demonstrate a lipid lowering effect\textsuperscript{126}. The soy isoflavone lipid lowering effect has
therefore only been seen in the context of food. Whether the soy pasta has to be part of
the food matrix, at present remains an open question.

Colonic metabolism or the numbers of specific bacteria may be altered by providing
either substrate for bacteria (prebiotic) or direct supplementation with live bacteria
(probiotic). Prebiotics, as used here in the form of fructo-oligosaccharides (FOS), may
make the soy isoflavones more bioavailable for absorption by providing substrate for
bacteria to enhance colonic fermentation. In this way, the aglycone form is produced,
which is more readily absorbed\textsuperscript{81, 259}. In one study, the effect of resistant starch, as a
prebiotic which would provide substrate for colonic fermentation, was tested in
combination with soy and resulted in a significant reduction in LDL-C despite no effect
on isoflavone concentration\textsuperscript{159, 160}. These data suggest that other products of increased
colonic fermentation, including the short chain fatty acids, may be important in
potentiating the effects of soy. For example, bacterially produced propionate from
carbohydrate fermentation has been suggested to inhibit hepatic cholesterol
biosynthesis\textsuperscript{210} and so increase the effectiveness of the soy and prebiotic combination.
Two studies have assessed the effects of altering the colonic microflora to enhance the
cholesterol lowering effect of soy by providing live bacteria or probiotics\textsuperscript{118, 160}. Both
studies used strains of Lactobacillus and Bifidobacteria provided in yogurt or in capsule
form and observed modest or no additional effect on LDL-C\textsuperscript{118, 160} and no effect on isoflavone concentrations in blood and urinary output when taken with soy\textsuperscript{145, 159}. Furthermore, there was no evidence that microbial metabolism was altered\textsuperscript{118, 160} as would be desired with the so called probiotic intervention\textsuperscript{260}. FOS, including the prebiotic used in the current study, have been demonstrated to be effective in increasing bifidobacterial numbers\textsuperscript{165, 261} at the dose provided\textsuperscript{262, 263} in short term studies of 7-21 days\textsuperscript{262} and these bacteria may be useful in enhancing isoflavone biotransformation through cleaving of the sugar from the isoflavone. However, significant changes in colonic flora were not seen. Nevertheless, increased carbohydrate fermentation evidenced by increased breath hydrogen production after prebiotic, may have increased colonic production of isoflavone aglycones and equol synthesis\textsuperscript{46, 62, 156} or influenced lipid metabolism by other means (e.g. short chain fatty acids)\textsuperscript{166, 189}. Interestingly, after adjustment for baseline, HDL-C was also significantly higher after the prebiotic plus soy compared to soy alone (\(P=0.023\)). This finding may explain why inclusion of HDL-C or ApoA1 in the ratios of TC:HDL-C or ApoB:ApoA1 greatly increased the significance of the TC and Apo B effects. Although HDL-C and ApoA1 increases on prebiotic plus soy did not reach significance, there is a plausible explanation why they might with larger subject numbers through the SERM effects of the isoflavones and as shown with the SERM toremifene\textsuperscript{238}, which has been shown to lower LDL-C and raise HDL-C.

A potential weakness of the study is the difference between treatment LDL-C concentrations at baseline, even though non-significant (see Appendix I). Such a finding would have to be confirmed by further analysis both chemical and statistical of
the data prior to acceptance as valid, although its elimination does not alter the significance of the data. Changes in LDL-C, TC:HDL-C and LDL-C:HDL-C on the prebiotic plus soy remained significantly greater than prebiotic alone when baseline values were included as a covariate, while soy alone remained not significantly different from the prebiotic alone.

The study strengths are the crossover design, the use of a variety of soy foods rather than a single soy isolate supplement, as used in some studies; partial blinding through use of a maltodextrin control for the fructo-oligosaccharides (both white powders) and assessment of both microbial change by fecal bacterial enumeration together with assessment of microbial metabolic activity by measurement of breath hydrogen production. This combined approach to assessing the effect of pre- or probiotics on colonic microflora and their activity has not been reported in other studies which have attempted to enhance the effect of soy by increasing colonic fermentation.

The treatment implications have relevance for the design of cholesterol lowering diets in general. It also has implications for heart health claims for soy which are now being reconsidered by the US FDA due to the relatively poor performance of soy in recent studies. There are relatively few specific foods or food components which actively lower serum cholesterol and for the most part, all of these have been singled out for heart health claim status by the FDA. The foods and food components include soy, nuts, viscous fibers (psyllium, β-glucan in oats and barley), and plant sterols (in vegetable oils and leafy green vegetables and enriched in certain commercial margarines). Although individually these dietary components may only lower serum cholesterol by 5-10%, when incorporated into the same diet as a dietary portfolio and provided under
metabolically controlled conditions, they have been shown to result in LDL-C reductions of up to 30%, similar to early statins\textsuperscript{17}. We believe the present study therefore confirms the value of soy as a cholesterol lowering food, in the 5% reduction range, especially when given with fermentable substrates such as would be present when in diets that also contained viscous fibers to lower serum cholesterol.

In conclusion, both soy treatments were effective in reducing the apolipoprotein B and the apolipoprotein ratio compared to prebiotic alone, however, a significant reduction in LDL-C and the lipid ratios was only observed with soy after co-ingestion of a fermentable substrate or prebiotic. The provision of fermentable substrates may be one means, possibly through colonic microbial biotransformation of lipid lowering components including isoflavones or production of specific short chain fatty acids, to increase the effectiveness of soy foods as part of a dietary strategy for cardiovascular disease risk reduction.
4.6. Figures

Figure 1. Patient Flow Diagram.

* Chose not to participate (106): not interested (36), busy lifestyle (26), study too demanding (19), no compensation (3), decided to start statin (1), currently on another diet (1)

** Other reasons (212): unable to contact (70), joining Portfolio study (48), joining FOS-study (36), unable to come to clinic (21), away during study period (11), medical issue unrelated to study (11), GP not consent (5), wants to lose weight (3), does not want to discontinue statin (2), personal issue (2), B12 deficiency (1), Hepatitis B (1), cancer history and personal issues (1)
Figure 2. Changes in lipids and apolipoproteins (A) and the associated ratios (B) (N=23).

Mean changes unadjusted for confounders.

*Significantly different from prebiotic alone, p-value <0.05 for diet x time interaction, adjusted for treatment, time and sequence.

†Units of mmol/L for TC, HDL-C, LDL-C. Units of g/L for Apo A1 and Apo B.
Figure 3. Mean Breath Hydrogen and Methane Production (N=22).

Values unadjusted for confounders.

*Significantly different from soy alone based using a mixed linear model with random intercept to assess the treatment effect controlling for period and baseline values, P<0.05.
4.7. Tables

Table 1. Subjects’ Baseline Characteristics at Randomization.

<table>
<thead>
<tr>
<th></th>
<th>Completers (n=23)</th>
<th>Non completers (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>58.1 ± 6.8</td>
<td>62.1 ± 8.8</td>
</tr>
<tr>
<td>Males/Females</td>
<td>11/12</td>
<td>3/11</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>74.2 ± 14.8</td>
<td>72.8 ± 7.6</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>25.9 ± 2.6</td>
<td>26.8 ± 4.3</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.96 ± 0.66</td>
<td>6.11 ± 0.75</td>
</tr>
<tr>
<td>LDL-C</td>
<td>4.18 ± 0.58</td>
<td>4.20 ± 0.45</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.12 ± 0.31*</td>
<td>1.39 ± 0.48</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.44 ± 0.78</td>
<td>1.16 ± 0.32</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tchol:HDL-C</td>
<td>5.62 ± 1.21*</td>
<td>4.74 ± 1.15</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>3.96 ± 0.94*</td>
<td>3.30 ± 0.94</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>122.8 ± 10.9</td>
<td>118.8 ± 10.3</td>
</tr>
<tr>
<td>Diastolic</td>
<td>76.7 ± 7.6</td>
<td>75.0 ± 9.4</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid lowering prior to and during the study</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Data expressed as mean±SD unless otherwise noted.

To convert total cholesterol, LDL-C, and HDL-C to mg/dL, divide by 0.0259; to convert triglycerides to mg/dL, divide by 0.0113.

*Significantly different from non-completers using two sample t-test.
Table 2. Mean Nutrient Profiles During the Study Treatments (n=23)*.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Mean Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prebiotic Alone</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Calories (kcal)</td>
<td>1581.9 ± 99.9</td>
<td>1590.8 ± 105.0</td>
</tr>
<tr>
<td>% of Total Calories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>17.9 ± 1.0</td>
<td>18.9 ± 1.1</td>
</tr>
<tr>
<td>Vegetable Protein</td>
<td>8.0 ± 0.3</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Available Carbohydrate</td>
<td>57.3 ± 1.8</td>
<td>56.9 ± 2.2</td>
</tr>
<tr>
<td>Dietary Fibre (g/1000 kcal)</td>
<td>17.8 ± 1.0</td>
<td>19.6 ± 1.5</td>
</tr>
<tr>
<td>Fat</td>
<td>22.9 ± 1.1</td>
<td>22.1 ± 1.4</td>
</tr>
<tr>
<td>Saturated</td>
<td>6.1 ± 0.5</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>8.9 ± 0.4</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>5.4 ± 0.4</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000 kcal)</td>
<td>82.8 ± 10.8</td>
<td>86.2 ± 11.0</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.9 ± 0.7</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>Soy Protein (g/d)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Prebiotic/Maltodextrin (g/d)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Unadjusted mean values.

Superscripts with a different letter in a row denote significant differences (P<0.05 for diet x time interaction) based on mean change from baseline using a mixed linear model with random intercept to assess interaction between diet and time controlling for treatment, time and sequence.
Table 3. Nutritional Profiles of Prescribed NCEP (Control) and Soy Food (Test) Supplements (based on 2000 kcal diet).

<table>
<thead>
<tr>
<th></th>
<th>NCEP (Control) Supplements</th>
<th>Soy Food (Test) Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>416.1</td>
<td>415.7</td>
</tr>
<tr>
<td>Total Protein (g/day)</td>
<td>38.3</td>
<td>38.1</td>
</tr>
<tr>
<td>Soy Protein (g/day)</td>
<td>0.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Soy Isoflavones (mg/d)</td>
<td>0.0</td>
<td>60.5</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.0</td>
<td>28.4</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.0</td>
<td>29.7</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Available Carbohydrate (g/day)</td>
<td>39.1</td>
<td>39.0</td>
</tr>
<tr>
<td>Total Dietary Fiber (g/1000kcal)</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Total Fat (g/day)</td>
<td>11.8</td>
<td>12.1</td>
</tr>
<tr>
<td>SFA (g/day)</td>
<td>2.4</td>
<td>2.6</td>
</tr>
<tr>
<td>MUFA (g/day)</td>
<td>4.4</td>
<td>4.6</td>
</tr>
<tr>
<td>PUFA (g/day)</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000kcal)</td>
<td>5.4</td>
<td>0.6*</td>
</tr>
</tbody>
</table>

SFA - saturated fatty acids.
MUFA - monounsaturated fatty acids.
PUFA - polyunsaturated fatty acids.

*The source of cholesterol was from butter, which was used in conjunction with sunflower and safflower oil to balance the fatty acids between treatments.
Table 4. Example Diets Based on 2000kcal.

<table>
<thead>
<tr>
<th></th>
<th>Soy + Prebiotic or Maltodextrin</th>
<th>Dairy + Prebiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td>Bran Flakes cereal 1c</td>
<td>Bran Flakes cereal 1c</td>
</tr>
<tr>
<td></td>
<td>Soy Beverage 1c</td>
<td>Milk – skim 3/4c</td>
</tr>
<tr>
<td></td>
<td>Whole Wheat toast with 2 slices Light Margarine and Double Fruit Jam</td>
<td>Whole Wheat toast 2 slices</td>
</tr>
<tr>
<td></td>
<td>1T</td>
<td>1T</td>
</tr>
<tr>
<td></td>
<td>Strawberries 1c</td>
<td>Strawberries 1 c</td>
</tr>
<tr>
<td></td>
<td>Tea/coffee/water</td>
<td>Tea/coffee/water</td>
</tr>
<tr>
<td></td>
<td>Prebiotic(^a) or Maltodextrin 5g</td>
<td>Prebiotic(^a) 5g</td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td>Grapes 1/2c</td>
<td>Grapes 1/2c</td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td>Sandwich:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soy Deli Slices 3 slices</td>
<td>Fat Free Cheese Slices 2 slices</td>
</tr>
<tr>
<td></td>
<td>Whole Wheat Bread 2 slices</td>
<td>Whole Wheat Bread 2 slices</td>
</tr>
<tr>
<td></td>
<td>Tossed Salad (mixed greens and lettuce, tomato, cucumber) 1.5c</td>
<td>Tossed Salad (mixed greens and lettuce, tomato, cucumber) 1.5c</td>
</tr>
<tr>
<td></td>
<td>With Vinaigrette</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Olive Oil 1.25T</td>
<td>Olive Oil 1.25T</td>
</tr>
<tr>
<td></td>
<td>Balsamic Vinegar 1T</td>
<td>Balsamic Vinegar 1T</td>
</tr>
<tr>
<td></td>
<td>Pear 1</td>
<td>Pear 1</td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td>Stir Fry:</td>
<td>Egg Omelette:</td>
</tr>
<tr>
<td></td>
<td>Extra Firm Low Fat Tofu 82 g (1/4 block)</td>
<td>Egg White and Egg Substitute with</td>
</tr>
<tr>
<td></td>
<td>Broccoli, red peppers and onions 1c</td>
<td>Broccoli, red peppers and onions 1c</td>
</tr>
<tr>
<td></td>
<td>Butter or Sunflower and Safflower Oil(^bc) 1/2t</td>
<td>Sunflower and Safflower Oil(^b) 1 t</td>
</tr>
<tr>
<td></td>
<td>Brown Rice 1.25c</td>
<td>Brown Rice 1.25c</td>
</tr>
<tr>
<td></td>
<td>Whole Wheat Toast 1 slice</td>
<td>Whole Wheat Toast 1 slice</td>
</tr>
<tr>
<td></td>
<td>Cantaloupe 1c</td>
<td>Cantaloupe 1c</td>
</tr>
<tr>
<td></td>
<td>Prebiotic(^a) or Maltodextrin 5g</td>
<td>Prebiotic(^a) 5g</td>
</tr>
<tr>
<td><strong>Snack</strong></td>
<td>Whole wheat crackers 8 each</td>
<td>Whole wheat crackers 8 each</td>
</tr>
<tr>
<td></td>
<td>Light Margarine 1T</td>
<td>Light Margarine 1 T</td>
</tr>
<tr>
<td></td>
<td>Soy Beverage 1c</td>
<td>Milk - skim 3/4 c</td>
</tr>
</tbody>
</table>

Abbreviations: T, Tablespoon; t, teaspoon; c, cup.
\(^a\)Prebiotic used was Synergy1® (Orafti Group, Belgium), a 50/50 mixture of inulin and oligofructose.
\(^b\)On the soy plus prebiotic or maltodextrin treatments, butter was used two days/week and safflower and sunflower oil were used the remaining days of the week.
\(^c\)Butter and vegetable oils were used to balance the fatty acid profile of the soy and dairy foods prescribed.
Table 5. Effect of study treatments on body weight, blood lipids, apolipoproteins, blood pressure and C-reactive protein (N = 23)*

<table>
<thead>
<tr>
<th>Study Treatment</th>
<th>Baseline Mean</th>
<th>Treatment</th>
<th>Baseline Mean</th>
<th>Treatment</th>
<th>Baseline Mean</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight, kg</td>
<td>72.9 ± 3.1</td>
<td>Prebiotic Alone</td>
<td>73.4 ± 3.1</td>
<td>Prebiotic Plus Soy</td>
<td>72.9 ± 3.1</td>
<td>Prebiotic Plus Soy</td>
</tr>
<tr>
<td>Body Mass Index (BMI), kg/m²</td>
<td>25.5 ± 0.5</td>
<td>Soy Alone</td>
<td>25.7 ± 0.5</td>
<td>Soy Alone</td>
<td>25.5 ± 0.5</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Waist Circumference</td>
<td>89.9 ± 2.3</td>
<td>Prebiotic Alone</td>
<td>90.6 ± 2.3</td>
<td>Prebiotic Plus Soy</td>
<td>89.4 ± 2.2</td>
<td>Prebiotic Plus Soy</td>
</tr>
<tr>
<td>Satiety (-4 to 4)</td>
<td>0.4 ± 0.2</td>
<td>Soy Alone</td>
<td>0.7 ± 0.2</td>
<td>Soy Alone</td>
<td>0.8 ± 0.2</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.18 ± 0.19</td>
<td>Soy Alone</td>
<td>6.13 ± 0.20</td>
<td>Soy Alone</td>
<td>6.13 ± 0.18</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>LDL-C</td>
<td>4.20 ± 0.16</td>
<td>Soy Alone</td>
<td>4.34 ± 0.16</td>
<td>Soy Alone</td>
<td>4.21 ± 0.15a</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.23 ± 0.08</td>
<td>Soy Alone</td>
<td>1.19 ± 0.08</td>
<td>Soy Alone</td>
<td>1.16 ± 0.07</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.66 ± 0.15</td>
<td>Soy Alone</td>
<td>1.70 ± 0.15</td>
<td>Soy Alone</td>
<td>1.65 ± 0.10</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC:HDL-C</td>
<td>5.27 ± 0.22</td>
<td>Soy Alone</td>
<td>5.69 ± 0.34</td>
<td>Soy Alone</td>
<td>5.52 ± 0.27a</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>3.61 ± 0.19</td>
<td>Soy Alone</td>
<td>3.96 ± 0.27</td>
<td>Soy Alone</td>
<td>3.84 ± 0.24a</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Apolipoproteins, g/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo A1</td>
<td>1.57 ± 0.06</td>
<td>Soy Alone</td>
<td>1.55 ± 0.06</td>
<td>Soy Alone</td>
<td>1.50 ± 0.05</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.31 ± 0.04</td>
<td>Soy Alone</td>
<td>1.34 ± 0.05</td>
<td>Soy Alone</td>
<td>1.33 ± 0.05a</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Apo B: Apo A1</td>
<td>0.86 ± 0.04</td>
<td>Soy Alone</td>
<td>0.89 ± 0.05</td>
<td>Soy Alone</td>
<td>0.91 ± 0.05a</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>C-reactive Protein</td>
<td>1.59 ± 0.35</td>
<td>Soy Alone</td>
<td>1.29 ± 0.20</td>
<td>Soy Alone</td>
<td>1.98 ± 0.43</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Blood Pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>122.8 ± 2.9</td>
<td>Soy Alone</td>
<td>125.6 ± 3.3</td>
<td>Soy Alone</td>
<td>120.9 ± 2.5</td>
<td>Soy Alone</td>
</tr>
<tr>
<td>Diastolic</td>
<td>75.8 ± 1.8</td>
<td>Soy Alone</td>
<td>77.7 ± 1.9</td>
<td>Soy Alone</td>
<td>74.8 ± 1.4</td>
<td>Soy Alone</td>
</tr>
</tbody>
</table>

*Unadjusted mean values.
Superscripts with a different letter in a row denote significant differences (P<0.05 for diet x time interaction) based on mean change from baseline using a mixed linear model with random intercept to assess interaction between diet and time controlling for treatment, time and sequence.
5. Eco-Atkins Study

“The Effect of a Plant Based Low-Carbohydrate ("Eco-Atkins") Diet on Body Weight and Blood Lipids in Hyperlipidemic Subjects”

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The Effect of a Plant Based Low-Carbohydrate ("Eco-Atkins") Diet on Body Weight and Blood Lipids in Hyperlipidemic Subjects

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Number of Tables: 4

Number of Figures: 4
5.1. Abstract

Background: Low-carbohydrate, high animal protein diets, advocated for weight loss, may not promote the desired reduction in low-density lipoprotein cholesterol (LDL-C). The effect of exchanging the animal proteins and fats for those of vegetable origin has not been tested.

Aim: To determine the effect on weight loss and LDL-C of a low-carbohydrate diet, high in vegetable proteins, from gluten, soy, nuts, fruits, vegetables and cereals, and vegetable oils compared to a high-carbohydrate diet based on low fat dairy and whole grain products.

Methods: Forty-seven overweight hyperlipidemic men and women consumed either a low-carbohydrate (26% of total calories), high vegetable protein (31%, from gluten, soy, nuts, fruit, vegetables and cereals), and vegetable oil (43%) plant based diet, or a high-carbohydrate lacto-ovo vegetarian diet (58% carbohydrate, 16% protein and 25% fat) for four weeks each in a parallel study design. Study food was provided at 60% of calorie requirements.

Results: 94% of subjects (92% test, 96% control) completed the study. Weight loss was similar for both diets (~4.0kg). However, reductions in LDL-C, and TC:HDL-C and apolipoprotein B:A1 ratios were greater on the low-carbohydrate than high-carbohydrate treatment (-8.1%, P=0.002; -8.7%, P=0.004; and -9.6%, P=0.001, respectively). Reductions in systolic and diastolic blood pressure were also seen: -1.9% (P=0.052) and -2.4%, (P=0.017).

Conclusion: A low-carbohydrate plant based diet had lipid lowering advantages over a high-carbohydrate, low fat weight loss diet, in improving heart disease risk factors not seen with conventional low fat diets of animal origin.
5.2. Introduction

There is a dilemma relating to the proportion and source of fat, protein and carbohydrate that constitutes the optimal weight loss and cholesterol lowering diet. Newer dietary approaches for the prevention and treatment of chronic disease increase consumption of fruit and vegetables, but reduce meat consumption either directly as part of the dietary strategy \(^{264}\) or displace meat by advocating increased intakes of fish, poultry and low-fat dairy foods \(^{15,\,30}\). Running counter to this advice has been the promotion of low-carbohydrate diets with increased meat consumption for body weight reduction and also in the longer term for the prevention and treatment of diabetes and coronary heart disease (CHD). These diets not only challenge the concept that red meat intakes should be reduced, but also reverse the dietary macronutrient profile such that fat and protein are the major macronutrients, while carbohydrate is now the minor macronutrient. Such low-carbohydrate diets have been shown to be effective in inducing weight loss \(^{264-268}\), reducing insulin resistance \(^{268}\), lowering serum triglycerides and raising HDL cholesterol \(^{264,\,266,\,267}\). However, the higher meat diets have not resulted in lower LDL-cholesterol (LDL-C) concentrations, but have tended to increase LDL-cholesterol except where vegetarian sources of fat and protein were included \(^{264}\). This lack of a benefit for LDL-C control is a major disadvantage in using this dietary strategy in those already at increased risk of CHD.

In view of the apparent success of low-carbohydrate diets for weight loss and the demonstration that relatively high-carbohydrate diets low in animal products, lower CHD risk factors \(^{15,\,17,\,269,\,270}\), we have determined the effect on serum lipids a low-carbohydrate weight loss diet without the use of animal products compared to a higher carbohydrate diet.
5.3. Methods

5.3.1. Participants

Fifty overweight participants, recruited by newspaper advertisement and hospital clinic notices, were randomized. Forty-seven subjects were available to start the study. Of these, 44 (18 men and 26 postmenopausal women) completed the one-month metabolic study (Figure 1). Over half the participants reported being of European origin (n=32: Northern, n=21; Eastern, n=8; Southern, n=3). The remainder had their origins in Sub-Saharan Africa (n=4), the Indian Subcontinent (n=4), Latin America (n=2), the Orient (n=2), and the Middle East (n=2). Four subjects did not provide their ethnic origins. Race was assessed by the participants using a classification provided by the investigators. Participants’ baseline characteristics are shown in Table 1. Study inclusion criteria included healthy men and postmenopausal women, between the ages of 21-70y, with a high normal or raised LDL-C (>3.4mmol/L at diagnosis), triglycerides > 0.5mmol/L, but <5 mmol/L, a body mass index (BMI) >27 kg/m² and who were not currently involved in a weight loss program. Exclusion criteria included lipid lowering medications, hormone replacement therapy, alcohol consumption >2 drinks/day, tobacco use, major cardiovascular event or surgery in the preceding 6 months, diabetes, untreated hypothyroidism, blood pressure >140/90 mmHg, renal or liver disease, cancer (excluding non-melanoma skin cancer) or any food allergies. At recruitment, thirteen subjects were taking lipid lowering medications, however, these were discontinued at least 2 weeks prior to the start of the study after obtaining approval from their family physician. Thirteen participants were taking anti-hypertensive medications at a constant dose prior to and during the study. One participant altered
the anti-hypertensive medication dosage during the study. Three participants took thyroxine at a constant dose prior to and during the study.

5.3.2. Study Protocol

The intervention was a randomized parallel study stratified by sex in which participants were assigned to either low or high-carbohydrate, calorie-reduced diets. The one month study was metabolically controlled with all food provided and participants were seen at weekly intervals. At each visit, fasting body weights and blood pressure were measured. Blood samples were obtained after 12h overnight fasts pre-treatment and at the end of weeks 2 and 4. Body weights were determined, with shoes removed, in indoor clothing using a stationary beam balance scale (Healthometer, Continental Scale Corp, Bridgeview, IL). Blood pressure was measured three times in the non-dominant arm after sitting for 15-20 minutes using an automated digital blood pressure machine (OMRON Healthcare Inc., Vernon Hills, IL). Food to be eaten by subjects for the entire metabolic month was pre-packed and delivered to subjects by courier. A “no starch” high protein nut bread was obtained from the clinic at weekly intervals. Participants were asked to hold exercise constant over the metabolic period. Exercise dairies were also completed weekly with type of exercise, duration and intensity recorded as light, moderate or vigorous in accordance with the Guidelines of the Centers of Disease Control and Prevention and the American College of Sports Medicine, and calculated as metabolic equivalents (METs)\textsuperscript{271, 272}. Body fat percentage was measured by bioelectrical impedance (Quantum II, RJL Systems, Clinton Twp., MI) and waist and hip measurements were measured biweekly. Waist measurements were made at the
umbilicus and hip measurements at the maximum lateral protrusion of the greater trochanter of the femur.

Subjects rated their overall feeling of satiety for the previous week at each study visit using a 9-point bipolar semantic scale where –4 was extremely hungry, 0 was neutral and +4 was uncomfortably full. Palatability was rated at the end of the study using a 7-point bipolar semantic scale where -3 was very unpalatable, 0 was neutral and +3 was very palatable.

The Ethics Committees of St. Michael’s Hospital and the University of Toronto, and the Therapeutic Products Directorate of Health Canada approved the study. Written informed consent was obtained from the participants. The study’s clinical trial registration number is #NCT00256516.

5.3.3. Diets

Metabolically controlled diets were consumed by the participants in which all food was provided. The low-carbohydrate diet provided the minimum level of carbohydrates currently recommended (130g/d) and eliminated common starch containing foods, such as bread, baked goods, potatoes and rice. The protein content was provided by gluten (54.8% of total protein), soy (23.0%), fruit and vegetables (8.7%), nuts (7.5%) and cereals (6.0%). Gluten was provided in the nut bread and seitan products and, together with soy, in burgers, veggie bacon, deli slices and breakfast links. In addition, soy was provided as tofu and soy beverages. Nuts included almonds, cashews, hazelnuts, macadamia, pecans and pistachios. The fat was provided by nuts (43.6% of total fat), vegetable oils (24.4%), soy products (18.5%), avocado (7.1%), cereals (2.7%), fruit and vegetables (2.3%) and seitan products (1.4%). The diet was designed to
provide 26% of calories as carbohydrates, 31% as protein and 43% as fat. The high-carbohydrate diet was a low-fat lacto-ovo vegetarian diet (58% carbohydrate, 16% protein and 25% fat) using low fat or skim milk dairy products and liquid egg whites or egg substitute to ensure a low saturated fat and low cholesterol intake. All diets were provided at 60% of estimated calorie requirements using a modified Harris Benedict equation with allowance for exercise.

The low-carbohydrate diet featured viscous fiber containing foods, including oats and barley, for the relatively limited amount of carbohydrate allowed, and the production of a “no starch” high protein bread made entirely from ground almonds, hazel nuts and wheat gluten. The carbohydrate foods and low starch vegetables, emphasizing okra and eggplant, provided 6-7 g of viscous fiber per 2000 kcal diet. The bread was provided as part of the diet.

Food preparation by participants was made as straightforward as possible by provision of commercial dishes or food items which were ready for microwave or oven cooking or could be reconstituted with boiling water, as with instant soups. Diet foods were packed in a central location and shipped by courier in separate boxes for dry, refrigerated and frozen goods. Egg substitutes and soy dairy foods were shipped in their commercial packages to be refrigerated on receipt by the participants.

On the low-carbohydrate diet, plant or microbially derived vitamin and mineral supplements were also provided, including vitamin B12 1000 mcg per week (microbially synthesized B12, Genestra Brands, Toronto, Canada), and vitamin D 200 IU per day (VegLife, Park City, UT). Women were also provided with calcium 500 mg per day and magnesium 250 mg per day (VegLife, Park City, UT).
Self-taring electronic scales (My Weigh Scales, Vancouver, BC or Tanita Corporation, Arlington Heights, IL) were provided to all participants to weigh all food items consumed during the study and record weights on the menu plan.

Adherence was assessed from the completed menu plans and subjects were also requested to weigh any leftover food items. Subjects were asked to record intake of prescribed vitamin and mineral supplements as a further measure of compliance throughout the study. Supplement bottles were returned at the end of the study. Participants were offered no financial compensation for participation in the study.

5.3.4. Analyses

Serum was analyzed according to the Lipid Research Clinics protocol for total cholesterol, triglycerides, and high-density lipoprotein cholesterol, after dextran sulphate-magnesium chloride precipitation (Bayer Technicon RA1000, Bayer Healthcare, Toronto, ON, Canada) or by detergent solublization and measurement of HDL-C (Roche Hitachi 917, Roche Diagnostics, Laval, QC, Canada), in the J. Alick Little Lipid Research Laboratory. LDL-cholesterol was calculated by the method of Friedewald et al. in mmol/L (LDL-cholesterol = total cholesterol – (TG/2.2 + HDL-cholesterol)). Apolipoproteins A1 and B were measured by a nephelometric method (Dade Behring BN ProSpec, Dade Behring Canada Inc.). High sensitivity C-reactive protein (hs-CRP) was measured by end-point nephelometry (Dade Behring BN ProSpec, Dade Behring Canada Inc.). CRP values > 10mg/L were eliminated providing they spiked >5mg/L above the mean for the individual’s series.

Blood glucose was measured in the hospital routine analytical laboratory by a glucose oxidase method (SYNCHRON LX Systems, Beckman Coulter Canada Inc.,
Insulin was measured by Access Ultrasensitive Insulin Assay which is a simultaneous one-step immunoenzymatic (“sandwich”) assay (Beckman Coulter Canada Inc., Mississauga, ON, Canada). A measure of insulin resistance was derived for fasting glucose and insulin using the HOMA-S model: fasting glucose(mmol/L) × insulin(mU/L) ÷ 22.5. HbA1c was measured by a designated HPLC method (Tosoh G7 Automated HPLC Analyzer, Grove City, OH, USA).

Diets were assessed for macronutrients, fatty acids, cholesterol and fiber using a computer program based on the USDA database and developed in our laboratory to allow addition of the macronutrient content of study foods obtained from food labels or directly from food manufacturers.

5.3.5. Statistical Analyses

The results are expressed as mean ± standard error (SE). Blood lipids are expressed as absolute values in Tables and percentage changes from baseline in text and Figures, unless otherwise stated. Differences between groups in baseline variables were assessed by two sample t-test (two-tailed). Intention-to-treat (ITT) analysis was undertaken with baseline observation carried forward for subjects who dropped out. ITT data are presented throughout unless otherwise stated. Time zero was utilized as baseline. Within treatment groups, blood lipids and other measurements were not found to be significantly different between weeks 2 and 4 during the metabolic phase. For these reasons, the respective treatment differences were assessed by the CONTRAST statement in SAS using all available data and reported as changes from baseline to weeks 2 and 4 in Table 4. The model specified change from baseline as the response variable with week as the main effect and baseline as covariate, except where
percentage changes from baseline were assessed. A significant difference was found between weeks 2 and 4 for body weight and BMI, therefore, the end of treatment values were assessed with baseline observation carried forward using the General Linear Model in SAS. Dietary data were analyzed using two sample t-tests for mean differences between the two treatment diets and at baseline.

5.4. Results

Baseline characteristics, obtained at randomization, for the control and low-carbohydrate diets are provided in Table 1. No significant differences were found in any of the variables between the two groups. There were no significant differences in the mean macronutrient profiles between individuals assigned to the test and control diets at baseline (Table 2). Both diets were well complied with, with no significant difference between treatments (Table 3). Over ninety percent of calories provided were consumed on test (95%) and control (94%) diets. Of those who started, all but three subjects completed, two withdrew from the control diet and one withdrew from the low-carbohydrate plant based diet for reasons unrelated to the study protocol (Figure 1). Mean weight loss after 4 weeks of the metabolic phase was similar on both treatments at 4.7±0.4% (3.9±0.4kg) on test and 4.9±0.3% (4.2±0.3kg) on control (P = 0.938). There were no absolute differences in calculated change in energy expenditure between the test and control treatments (-3.6±2.7 versus 1.4±1.7 METs, P=0.120). Subjective satiety ratings were significantly higher on the low-carbohydrate diet (low-carbohydrate 1.5±0.3 vs. high-carbohydrate 0.8±0.3, P=0.003) (Table 4). Mean satiety
scores were positive for both treatments indicating that the diets tended to satisfy participants (scale -4 to +4).

5.4.1. Lipids
Both treatments groups had similar lipid values at baseline (Table 1). During the metabolic phase, reductions in LDL-C and TC:HDL-C were greater on the low-carbohydrate diet versus the high-carbohydrate diet (LDL-C: \(-20.4\pm2.8\%\) vs. \(-12.3\pm2.6\%, P=0.002\) and TC:HDL-C: \(-15.6\pm3.4\%\) vs. \(-6.8\pm2.4\%, P=0.004\)). In addition, total cholesterol and triglycerides were reduced on the low versus the high-carbohydrate diet (TC: \(-19.8\pm2.4\%\) versus \(-12.7\pm2.2\%, P=0.001\) and triglycerides: \(-29.2\pm5.5\%\) versus \(-17.8\pm4.5\%, P=0.024\), respectively). Similar patterns of significance were seen with the absolute lipid and lipoprotein concentrations (Table 4) and with the completers (Figure 3). No treatment differences were seen in HDL-C.

5.4.2. Apolipoproteins
Apolipoprotein A1 (apoA1) fell on both treatments and there was no difference between treatments (P=0.355). However, both the apolipoprotein B (ApoB) and the ApoB:A1 ratio fell significantly more on the low-carbohydrate versus the high-carbohydrate diet by \(-21.1\pm2.8\%\) vs. \(-13.2\pm2.2\%\) (P=0.001) and \(-13.8\pm3.4\%\) vs. \(-4.2\pm2.1\%\) (P=0.001), respectively. A similar pattern was seen in completers (Figures 3).

5.4.3. C-Reactive Protein
No significant treatment differences were seen in hs-CRP (P=0.735) (Table 4).
5.4.4. HbA1c, Blood Glucose, Serum Insulin and Insulin Resistance

HbA1c, fasting blood glucose, insulin and insulin resistance (calculated using the HOMA model) fell similarly on both treatments during the course of the study (Table 4).

5.4.5. Blood Pressure

Small, but significantly greater reductions in systolic blood pressure of -2.2mmHg (-1.9%, P=0.052) and diastolic blood pressure of -1.7mmHg (-2.4%, P=0.017) were seen on the low-carbohydrate versus high-carbohydrate diet.

5.5. Discussion

The present study demonstrated that consumption of a low-carbohydrate plant based diet resulted in body weight reductions of 4kg that were similar to those reported for low-carbohydrate “Atkins-like” diets $^{264-268}$. In addition to weight loss, the consumption of a low-carbohydrate diet containing vegetable proteins and oils was associated with significantly reduced concentrations of LDL-C, not reported in the majority of low-carbohydrate diet studies in which the protein and fat are largely of animal origin. These diets result in increases in LDL-C compared to routinely used higher carbohydrate therapeutic diets $^{265-268, 280}$. The reduction in LDL-C is a potentially important attribute of the diet in reducing CHD risk $^{24, 281}$.

Our data support earlier conclusions that differences in weight loss between treatments are likely to result from a reduction in caloric intake rather than metabolic changes associated with an altered macronutrient profile $^{265, 266, 268, 280}$, despite the possibility that high protein low-carbohydrate diets might enhance postprandial thermogenesis $^{282}$. Additionally, the satiating effect of protein on self-selected diets, together with mild
ketonemia following low-carbohydrate intake, might also favour weight reduction. In the present study, carbohydrate intake on the low-carbohydrate diet met the minimum recommended level, and thus, significant ketonuria is unlikely, although it was not assessed.

Low glycemic load diets have been associated with greater weight loss in adolescents. Increased subjective ratings of satiety were also found during the present study, which by virtue of being low-carbohydrate, was also a low glycemic load diet. A similar finding of increased satiety was found in a metabolically controlled study using a low glycemic load weight loss diet in younger adults.

High-carbohydrate vegetarian diets such as the original Ornish diet, which emphasized soy and legume protein, have been associated with reduced progression of coronary artery disease, as well as weight loss and reduced LDL-C concentrations. However, such diets also lower HDL-C due to the impressively low intake of fats. Concern has been expressed over the use of high-carbohydrate diets which may depress already low HDL-C concentrations further. The present diet, while lowering LDL-C, did not depress HDL-C significantly and resulted in a 16% reduction in the TC:HDL-C ratio. These changes would be expected to reduce CHD risk.

Both soy and nuts, as key components of the present study, have been shown to increase HDL-C when included in low-fat diets. Triglyceride levels were lower with consumption of the low-carbohydrate diet by comparison with the control diet, possibly reflecting the lower glycemic load and the presence of gluten, soy protein and nuts, all of which have been associated with lower fasting serum triglyceride concentrations.
Most low-carbohydrate diets have not reported the effects on apolipoproteins. The reduction in ApoB and the ApoB:A1 ratio observed in the present study is a further confirmation of the potential CHD benefit that might be expected from this dietary approach to body weight reduction. In some studies, the apolipoprotein concentrations have been claimed to have greater predictive value for CHD events than more conventional lipid variables.

Both diets tended to reduce systolic and diastolic blood pressure as expected relative to the degree to which body weight was reduced, but with a greater blood pressure reduction on the low-carbohydrate diet. High protein diets have been associated with lower blood pressure. No treatment difference in hs-CRP was seen in the present study, possibly related to the great variability in this measurement. In other studies, hs-CRP tended to be lowest on the diets containing the highest proportion of carbohydrate, although low glycemic index and low glycemic load diets have also been associated with a lower hs-CRP concentration.

No randomized controlled trials have been undertaken to assess the effect of low-carbohydrate diets on CHD events. Nevertheless, a recent cohort study reported that a low-carbohydrate diet high in protein and oil from vegetable rather than animal sources, was associated with reduced CHD risk and incidence of diabetes. The median level of vegetable protein and oil in the cohort study was considerably less than used in the present study, being only 4.2-5.6 g/d for vegetable protein and 9.6-18.9 g/d for vegetable oil. Had the intakes been similar to the levels in the present study the effects might have been greater.

In the present study, the high-carbohydrate control diet can be seen as providing a positive control since lacto-ovo vegetarians appear to be at generally lower risk of CHD...
than non-vegetarians with notable studies demonstrating reduced CHD in cohorts of California Seventh Day Adventists and the earlier assessment of British vegetarians 18, 19. Furthermore, low fat dairy diets emphasizing higher intakes of fruit and vegetables, such as the DASH and OMNI diets, have been associated with lower blood pressure and improved blood lipid profiles 15. As such, the benefits for CHD risk reduction seen with the present study might have been much more marked had the low-carbohydrate diet been compared to those from a more typical low fat diet.

According to the Mensink and Katan equation, the LDL-cholesterol reductions seen on the low-carbohydrate diet (-0.96±0.15mmol/L) were greater than predicted (-0.42±0.03mmol/L) even when adjusted for weight loss (P<0.001) 298, 299. We believe the greater than expected cholesterol reductions are likely to be due in part to the cholesterol lowering properties of soy protein and nuts which have been demonstrated in previous studies 7, 32, 34, 286, 289. Furthermore, the small amount of carbohydrate included in the low-carbohydrate treatment was associated with viscous fiber in low starch vegetables and β-glucan in oats and barley. Viscous fiber is also expected to contribute to the overall cholesterol lowering effect of the diet 27, 31, 281. Vegetable protein has also been shown to be inversely related to blood pressure in the cross-sectional epidemiological INTERMAP study of 4680 individuals aged 40-59 from 4 countries 300. Soy protein consumption has been associated with lower blood pressure in a number of feeding trials (i.e. meta-analysis) 301 providing a further reason why vegetable proteins would be expected to reduce the risk of CHD.

Lower saturated fat intake may have other advantages, in addition to LDL-cholesterol reduction including reduced insulin resistance, chronic inflammation and improved endothelial function 302, 303, all of which would contribute to the lower risk of coronary
heart disease associated with reduced saturated fat intake. On the other hand, polyunsaturated fats and vegetable oils in general in epidemiological studies, have been shown to be associated with a reduced risk of CHD as opposed to saturated and trans fats which are associated with increased risks. Key characteristics of a plant based diet include fiber, vegetable oils and vegetable proteins, and foods such as nuts and seeds. These foods and food components benefit coronary heart disease risk factors and it is therefore not surprising that plant based diets have been associated with reduced CHD events in epidemiological studies.

We conclude that low-carbohydrate diets emphasizing vegetable sources of protein, such as gluten, soy and nuts, together with vegetable oils can be used in weight reduction diets to improve serum lipids. There are, however, currently no trials of diets high in vegetable protein and oils with disease end-points. The impact of low-carbohydrate diets in primary and secondary CHD prevention, therefore, remain to be determined. Nevertheless, recent studies indicate beneficial effects of vegetable oils and proteins on both CHD risk factors and CHD risk. Consumption of foods rich in these components including nuts and soy have been shown to reduce serum lipids and nut consumption has been associated with lower CHD risk. Important questions remain. Can the advantages be maintained if some of the vegetable protein is replaced by vegetable oil and in this context, can carbohydrate be further reduced or is there an optimal carbohydrate load, perhaps determined by an individual’s BMI and insulin resistance. There may also be certain advantages for higher carbohydrate intakes providing the carbohydrate comes from high fiber, low glycemic index foods. Pending answers to these questions, a plant based low-carbohydrate diet, high in vegetable
proteins and oils, may be an effective option in treating those with dyslipidemia for whom both weight loss and lower LDL-C concentrations are treatment goals.
5.6. Figures

Figure 1. Patient Flow Diagram.

*Choose not to participate (29): busy lifestyle (13), not interested (6), study too demanding (3), currently on another diet (2), no compensation (2), work-related (2), dislike prepackaged foods (1)

**Other reasons (44): unable to contact (19), unable to come to clinic (13), away (5), throat surgery (1), bowel resection (1), high potassium and BP (1), high potassium (1), raised liver function tests (1), not interested (1), medical insurance issue (1)
Figure 2. Weight loss on both treatments during the 4 weeks of the study ($P = 0.975$) for completers.
Figure 3. Mean percent change from baseline in completers on both treatments for (A) LDL-C (P = 0.001), (B) TC:HDL-C (P = 0.003), (C) apolipoprotein B (apoB) (P = 0.001) and (D) ApoB:ApoA1 ratio (P = 0.001) (significance of difference between treatments).
Figure 4. Mean percent change from baseline in completers on both treatments for triglycerides (P= 0.017) (significance of difference between treatments).
### 5.7. Tables

**Table 1. Baseline Characteristics at randomization (n=50).**

<table>
<thead>
<tr>
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<th>Step 2 Control (n=25)</th>
<th>Low Carbohydrate Test (n=25)</th>
<th>p-value*</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>56.1 ± 7.5</td>
<td>57.8 ± 7.1</td>
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<td>Males/Females</td>
<td>12/13</td>
<td>10/15</td>
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<td>Body Weight (kg)</td>
<td>87.4 ± 11.7</td>
<td>82.7 ± 11.1</td>
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<tr>
<td>Body Mass Index (kg/m²)</td>
<td>31.0 ± 2.4</td>
<td>30.6 ± 2.9</td>
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<td>Blood pressure, mmHg</td>
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<tr>
<td>Systolic</td>
<td>126.1 ± 10.2</td>
<td>127.7 ± 13.7</td>
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<tr>
<td>Diastolic</td>
<td>79.4 ± 8.1</td>
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<td>Cholesterol, mmol/L</td>
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<tr>
<td>Total</td>
<td>6.32 ± 1.12</td>
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<td>LDL-C</td>
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<td>HDL-C</td>
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<td>Triglycerides</td>
<td>1.61 ± 0.82</td>
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<td>Ratios</td>
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<td>Tchol:HDL-C</td>
<td>5.18 ± 1.42</td>
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<td>LDL-C:HDL-C</td>
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<td>Glucose (mmol/L)</td>
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<td>Exercise (METs)*</td>
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Data expressed as mean±SD unless otherwise noted.

*Calculated by two-sample t-test or chi-square test.

Body mass index was calculated as weight in kilograms divided by the square of height in meters.

To convert total cholesterol, LDL-C, and HDL-C to mg/dL, divide by 0.0259; to convert triglycerides to mg/dL, divide by 0.0113.

*Baseline data for control (n=23) and test (n=22).
Table 2. Nutritional Profiles at Baseline.

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<tr>
<th></th>
<th>High Carbohydrate</th>
<th>Low Carbohydrate</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>1726.2 ± 113.8</td>
<td>1779.0 ± 129.3</td>
<td>0.761</td>
</tr>
<tr>
<td>% of Total Calories</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Available Carbohydrate</td>
<td>46.4 ± 1.7</td>
<td>45.0 ± 1.9</td>
<td>0.596</td>
</tr>
<tr>
<td>Protein</td>
<td>20.1 ± 0.8</td>
<td>19.6 ± 0.9</td>
<td>0.680</td>
</tr>
<tr>
<td>Vegetable Protein</td>
<td>5.9 ± 0.3</td>
<td>5.7 ± 0.3</td>
<td>0.690</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>---</td>
</tr>
<tr>
<td>Fat</td>
<td>31.5 ± 1.5</td>
<td>34.0 ± 1.6</td>
<td>0.254</td>
</tr>
<tr>
<td>Saturated</td>
<td>10.8 ± 0.7</td>
<td>11.8 ± 0.8</td>
<td>0.361</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>12.5 ± 0.7</td>
<td>12.9 ± 0.6</td>
<td>0.659</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>5.5 ± 0.4</td>
<td>6.4 ± 0.5</td>
<td>0.142</td>
</tr>
<tr>
<td>Alcohol</td>
<td>2.0 ± 0.8</td>
<td>1.4 ± 0.7</td>
<td>0.531</td>
</tr>
<tr>
<td>Dietary Fibre (g/1000 kcal)</td>
<td>12.0 ± 0.9</td>
<td>12.4 ± 1.1</td>
<td>0.758</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000 kcal)</td>
<td>144.2 ± 9.7</td>
<td>152.4 ± 10.9</td>
<td>0.582</td>
</tr>
</tbody>
</table>

*Calculated using two sample t-test.
Table 3. Nutritional Profiles During the Study.

<table>
<thead>
<tr>
<th></th>
<th>High Carbohydrate</th>
<th>Low Carbohydrate</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>1488.2 ± 48.1</td>
<td>1451.4 ± 47.3</td>
<td>0.588</td>
</tr>
<tr>
<td>% Calorie Compliance</td>
<td>94.2 ± 1.3</td>
<td>94.8 ± 1.4</td>
<td>0.758</td>
</tr>
<tr>
<td>% of Total Calories</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Available Carbohydrate</td>
<td>58.2 ± 0.4</td>
<td>26.8 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein</td>
<td>16.8 ± 0.1</td>
<td>30.0 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Vegetable Protein</td>
<td>7.0 ± 0.1</td>
<td>29.9 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>0.2 ± 0.0</td>
<td>6.8 ± 0.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fat</td>
<td>24.5 ± 0.4</td>
<td>43.1 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Saturated</td>
<td>4.6 ± 0.0</td>
<td>6.3 ± 0.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>7.9 ± 0.2</td>
<td>25.0 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>9.1 ± 0.2</td>
<td>9.6 ± 0.1</td>
<td>0.015</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0.3 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.150</td>
</tr>
<tr>
<td>Dietary Fibre (g/1000 kcal)</td>
<td>21.3 ± 0.2</td>
<td>28.3 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000 kcal)</td>
<td>30.1 ± 1.4</td>
<td>0.4 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Calculated using two sample t-test between high vs. low carbohydrate diets.
Table 4. Effect of high and low carbohydrate diets on body weight, blood lipids, apolipoproteins and C-reactive protein (intention-to-treat analysis).

<table>
<thead>
<tr>
<th></th>
<th>High Carbohydrate</th>
<th>Low Carbohydrate</th>
<th>P-Value$^\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0</td>
<td>Week 2</td>
<td>Week 4</td>
</tr>
<tr>
<td>Body Weight, kg</td>
<td>86.6</td>
<td>83.9</td>
<td>82.3</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>31.0</td>
<td>30.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Body Fat, %</td>
<td>36.2</td>
<td>-</td>
<td>34.6</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>103.7</td>
<td>101.8</td>
<td>100.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.7</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Satiety (-4 to 4)</td>
<td>1.1</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Cholesterol, mmol/L$^\dagger$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.59</td>
<td>5.73</td>
<td>5.75</td>
</tr>
<tr>
<td>LDL-C</td>
<td>4.34</td>
<td>3.82</td>
<td>3.79</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.29</td>
<td>1.20</td>
<td>1.21</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.11</td>
<td>1.56</td>
<td>1.66</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tchol:HDL-C</td>
<td>5.37</td>
<td>4.92</td>
<td>4.94</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>3.52</td>
<td>3.27</td>
<td>3.24</td>
</tr>
<tr>
<td>Apolipoproteins, g/L$^\ddagger$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo A1</td>
<td>1.62</td>
<td>1.46</td>
<td>1.47</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.37</td>
<td>1.18</td>
<td>1.18</td>
</tr>
<tr>
<td>Apo B: Apo A1</td>
<td>0.86</td>
<td>0.83</td>
<td>0.81</td>
</tr>
<tr>
<td>C-reactive Protein, mg/L</td>
<td>2.13</td>
<td>1.22</td>
<td>1.44</td>
</tr>
</tbody>
</table>

$^\dagger$To convert total cholesterol, LDL-C, and HDL-C to mg/dL, divide by 0.0259; to convert triglycerides to mg/dL, divide by 0.0113.

$^\ddagger$To convert apolipoprotein A1 and B to mg/dL, multiply by 100.

$^\gamma$P-values based on intention-to-treat analysis, with baseline observation carried forward for subjects who did not complete the study, assessed by the CONTRAST statement in SAS (using all available data) except for body weight and body mass index which were carried forward for all subjects.
6. Isoflavone and Equol Method
6.1. Introduction

The isolation and quantification of soy isoflavones and their metabolites in biological fluids are of particular importance when determining their physiological role, specifically in relation to the hypocholesterolemic effect of soy. Therefore, both accurate and precise analytical methodology is needed for the identification and quantification of soy isoflavones. In clinical trials with dietary interventions containing soy foods, measurement of soy isoflavones in blood or urine is often used as a biomarker for adherence to the study protocol. In epidemiological studies, measurement of soy isoflavones is often used as an indicator of usual level of soy intake. To date, the most commonly used techniques for measurement of soy isoflavones include: HPLC-UV, GC-MS and LC-MS. A new and emerging technique is the use of immunoassay.

6.2. Description of GC-MS

The following was the method used for the analysis of urinary soy isoflavones in this thesis.

For the isoflavone analysis, the urine sample went through solid-phase extraction, β-glucuronidase hydrolysis, and another solid-phase extraction. An aliquot of the urine sample was passed through a C18 solid phase extraction column (SPE column, Octadecyl C18/14%, 200 mg/3 mL; Applied Separations, Allentown, PA) that was preconditioned with 5 mL methanol and 5 mL water. The column was then washed with 5 mL water and eluted with 4 mL methanol. The eluent was evaporated to dryness in a rotary evaporator at 60°C under vacuum. To the residue was added 5 mL 0.1 mol/L sodium acetate buffer and 50 µL β-glucuronidase and the dispersion was then incubated overnight at 37°C. The β-glucuronidase hydrolysate was again subjected to solid-phase extraction as described previously. The eluent was evaporated to dryness
at 60°C under vacuum and the residue was reconstituted with 1 mL methanol and stored at –20°C until analysis by gas chromatography–mass spectrometry (GC-MS). When ready for analysis, 100 µL of internal standard solution (5α-androstane-3β, 17β-diol, 50 µg/mL methanol) was added to the above sample in methanol solution. The sample was dried under nitrogen flow, and after adding 300 µL of pyridine and 100 µL of BSA, was incubated for 1 h on room temperature. The silylating agent was then removed under nitrogen gas flow, and the trimethyl-silylated sample was dissolved in 100 µL of hexane. Then 1µL aliquot was injected to GC-MS. An Agilent 6890 series GC system (Agilent Technologies, Wilmington, DE) interfaced with an Agilent 5973 network mass selective detector and with an HP-5ms (5%-phenyl)-methylpolysiloxane capillary column (25 m × 0.12 mmi.d. × 0.25 µmfilm thickness; Agilent Technologies) was used. Data acquisition and processing were carried out using enhanced Chemstation software (G1701CA version C.00.00; Agilent Technologies). Operating conditions were as follows: injection port temperature 250°C; splitless mode; carrier gas He at a constant flow rate of 1mL/min; oven program temperature 100°C held initially for 1 min, increased at 15°C/min to 280°C and held for 17 min at 280°C (total run time 30 min); GC-MS interface temperature 280°C; MS source temperature 230°C; MS quad temperature 150°C; ionization by electron ionization (70 eV). Analysis was performed in the scan ion monitoring mode and two or three characteristic ions were chosen for each analyte or standard. The selected ions for each analyte were as follows: m/z 421.3 and 436.4 for 5α-androstane-3β, 17β-diol; m/z 281.2 and 459.2 for ODMA; m/z 386.2 and 371.2 for equol; m/z 398.2 and 383.1 for daidzein; m/z 471.2 and 399.1 for genistein, m/z 414.2 and 399.1 for genistein (2nd pair); and m/z 428.2 and 413.1 for glycitein.
6.3. Method Refinement

A series of analyses was conducted to refine the current GC-MS method of isolation and quantification of equol in urine samples.

Urine samples from two known equol producers (identified as Subject 1 and 2) were used in the method refinement of equol analysis. The two equol producers were identified out of a group of 7 individuals after consuming 2-3 servings of soy foods for 3-4 days. On Day 4, a 24-hr urine collection was completed. Samples from this 24-hr urine collection were used for the analyses below. In addition, one equol nonproducer (Subject #3) was also identified using the same soy challenge with a 24-hr urine collection.

6.3.1. Equol Recovery

Description: Urine samples of two equol producers (Subject 1 and 2) were spiked with known quantities of an equol standard. The following quantities were added to 0.5mL of urine: 0, 1, 5, and 10ug/mL (Table 1). Duplicates were also completed (Table 2).

Table 1. Percent Recovery of Spiked Quantities of Equol in Urine Samples from Subject 1 and 2 (Run #1). *

<table>
<thead>
<tr>
<th>Subject</th>
<th>Equol Conc. Added (ug/mL)</th>
<th>Equol Measured (ug/mL)</th>
<th>Recovery of Equol (ug/mL)</th>
<th>Recovery of Equol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2.6</td>
<td>1.1</td>
<td>106.0</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6.5</td>
<td>5.0</td>
<td>100.2</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>11.6</td>
<td>10.1</td>
<td>100.8</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>3.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4.4</td>
<td>1.3</td>
<td>107.0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>8.3</td>
<td>5.2</td>
<td>102.6</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>13.3</td>
<td>10.2</td>
<td>101.6</td>
</tr>
</tbody>
</table>

*Columns represent the following: equol conc. added = amount of spiked equol; equol measured = total urinary equol measured; recovery of equol = amount of spiked equol recovered in urine sample in both ug/mL and %.
Table 2. Percent Recovery of Spiked Quantities of Equol in Urine Samples from Subject 1 and 2 (Run #2).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Equol Conc. Added (ug/mL)</th>
<th>Equol Measured (ug/mL)</th>
<th>Recovery of Equol (ug/mL)</th>
<th>Recovery of Equol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2.5</td>
<td>1.3</td>
<td>112.1</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6.1</td>
<td>4.9</td>
<td>98.5</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>10.6</td>
<td>9.4</td>
<td>94.6</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4.0</td>
<td>1.0</td>
<td>101.2</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>7.9</td>
<td>5.0</td>
<td>99.7</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>12.4</td>
<td>9.4</td>
<td>95.4</td>
</tr>
</tbody>
</table>

*Columns represent the following: equol conc. added = amount of spiked equol; equol measured = total urinary equol measured; recovery of equol = amount of spiked equol recovered in urine sample in both ug/mL and %.

**Results:** The percent recovery of the spiked equol ranged from 94.6-112.1. The coefficient of variation (CV) was 10% for Run #1 and 12% for Run #2. The correlation coefficient between the duplicates was 0.999 (P<0.001) (Figure 1).

![Figure 1. Correlation Between Duplicates of Equol Recovery.](image-url)
Conclusion: Known quantities of spiked equol can be isolated, measured and adequately recovered from urine samples from equol producers.

6.3.2. Standard Curves in Methanol or Urine

Description: Standard curves, used to calculate equol concentrations from study samples, are created using soy isoflavone standards in methanol. However, study samples are in the form of urine. Therefore, standard curves were created: 1) in methanol and 2) in urine from an equol nonproducer (Subject #3). The presence of other compounds contained in urine may or may not interfere with the measurement of equol.

Urine samples (0.5mL) from the same individual were spiked with either 0.1, 1, 5 or 10 ug of equol to create standard curves in methanol and in urine.

Table 3. Equol Recovery from Standard Curves Constructed in Methanol and in Urine.

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Equol Added (ug)</th>
<th>Equol Recovered (ug)</th>
<th>Equol Recovery (%)</th>
<th>Equol Recovered (ug)</th>
<th>Equol Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-A</td>
<td>0.1</td>
<td>0.07</td>
<td>70</td>
<td>0.09</td>
<td>91</td>
</tr>
<tr>
<td>1-B</td>
<td>0.1</td>
<td>0.08</td>
<td>83</td>
<td>0.10</td>
<td>98</td>
</tr>
<tr>
<td>2-A</td>
<td>1</td>
<td>0.90</td>
<td>90</td>
<td>1.06</td>
<td>106</td>
</tr>
<tr>
<td>2-B</td>
<td>1</td>
<td>1.03</td>
<td>103</td>
<td>0.98</td>
<td>98</td>
</tr>
<tr>
<td>3-A</td>
<td>5</td>
<td>5.59</td>
<td>112</td>
<td>5.34</td>
<td>107</td>
</tr>
<tr>
<td>3-B</td>
<td>5</td>
<td>5.62</td>
<td>112</td>
<td>5.27</td>
<td>105</td>
</tr>
<tr>
<td>4-A</td>
<td>10</td>
<td>9.51</td>
<td>95</td>
<td>9.33</td>
<td>93</td>
</tr>
<tr>
<td>4-B</td>
<td>10</td>
<td>9.79</td>
<td>98</td>
<td>10.07</td>
<td>101</td>
</tr>
<tr>
<td>5-A**</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

*Sample A and B represent duplicates.
**Sample only completed for standard curve in urine.
Results: The CV for the standard curve in methanol was 15.3% and in urine 5.9%. Furthermore, the correlation coefficient was 0.996 (P<0.001) and 0.998 (P<0.001), respectively (Figure 2).

Conclusion: Standard curves constructed in methanol or in urine were similar. Therefore, the presence of other compounds contained in urine does not appear to interfere with the isolation and quantification of equol.

6.3.3. Standard Curves – Graded Ratios of Urine:Water

Description: Soy isoflavones, including equol, present in urine samples need to be enzyme hydrolyzed to their aglycone form prior to analysis by GC-MS. The process of enzyme hydrolysis, with a β-glucuronidase preparation, may be affected by compounds contained in urine.
Samples from two equol producers (Subject 1 and 2) were diluted with graded ratios of urine:water and were analyzed for equol content using the same quantity of the enzyme preparation for each sample (Table 4).

**Results**

Table 4. Equol Concentration in Urine Samples with Graded Ratios of Urine:Water.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Urine (mL)</th>
<th>Water Added (mL)</th>
<th>Final Volume (mL)</th>
<th>Enzyme (uL)</th>
<th>Buffer (mL)</th>
<th>Equol Concentration (ug/mL)</th>
<th>Equol Concentration (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-A</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>2.13</td>
<td>2.44</td>
</tr>
<tr>
<td>1-B</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>2.12</td>
<td>2.15</td>
</tr>
<tr>
<td>2-A</td>
<td>0.4</td>
<td>0.1</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>1.94</td>
<td>2.22</td>
</tr>
<tr>
<td>2-B</td>
<td>0.4</td>
<td>0.1</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>2.02</td>
<td>2.17</td>
</tr>
<tr>
<td>3-A</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>1.85</td>
<td>1.61</td>
</tr>
<tr>
<td>3-B</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>1.17</td>
<td>1.58</td>
</tr>
<tr>
<td>4-A</td>
<td>0.1</td>
<td>0.4</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>1.64</td>
<td>1.07</td>
</tr>
<tr>
<td>4-B</td>
<td>0.1</td>
<td>0.4</td>
<td>0.5</td>
<td>50</td>
<td>5</td>
<td>1.34</td>
<td>1.61</td>
</tr>
</tbody>
</table>

*A and B represent duplicates.

The overall CV was 19.0% for Subject #1 and 23.2% for Subject #2. When only urine volumes of >0.4mL were included, the CV improved to 3.8% and 5.2%, respectively. The correlation coefficient for dilutions compared to measured equol concentration were 0.984 (P<0.001) for Subject #1 and 0.994 (P<0.001) for Subject #2 (Figure 3).
**Conclusion:** The enzyme hydrolysis process and the measurement of urinary equol concentration does not appear to be affected by compounds contained in urine. Urine samples of 0.5mL will continue to be used in the analysis of urinary soy isoflavones.

### 6.3.4. Within and Between Day Coefficient of Variation of Urine Samples and Standard Curves

**Description:** Construction of standard curves for equol and analysis of 6 urine samples from an equol producer (Subject #2) were completed on Day 1. On Day 3, 2-4 additional urine samples from Subject #2 were analyzed along with another standard curve. The constructive of standard curves and analysis of additional urine samples were repeated 4 times (Table 5).
**Results:** The within day CV was <11%, whereas the between day CV was ~26%. A similar variation between days was also observed in the standard curves (~26%).

Table 5. Within Day and Between Day Coefficient of Variation.

<table>
<thead>
<tr>
<th>Run</th>
<th>Sample</th>
<th>1 (ug/ml)</th>
<th>2 (ug/ml)</th>
<th>3 (ug/ml)</th>
<th>4 (ug/ml)</th>
<th>5 (ug/ml)</th>
<th>6 (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>2.13</td>
<td>1.49</td>
<td>1.46</td>
<td>1.17</td>
<td>1.18</td>
<td>1.69</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2.15</td>
<td>1.71</td>
<td>1.35</td>
<td>1.02</td>
<td>1.04</td>
<td>1.69</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.13</td>
<td>1.71</td>
<td>1.35</td>
<td>1.02</td>
<td>1.06</td>
<td>1.68</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.08</td>
<td></td>
<td></td>
<td></td>
<td>1.24</td>
<td>1.64</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>2.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td></td>
<td>2.12</td>
<td>1.60</td>
<td>1.40</td>
<td>1.10</td>
<td>1.13</td>
<td>1.67</td>
</tr>
</tbody>
</table>

| Within Day CV | 3.3% | 9.6% | 5.7% | 10.3% | 8.6% | 1.3% |
| Between Day CV| 25.7%|      |      |       |      |      |

**Conclusion:** Samples from the same individual should be analyzed on the same day including all duplicates. Construction of additional standard curves should increase the accuracy of equol quantification.

6.4. Summary

6.4.1. Justification of GC-MS Method for the Determination of Equol Status

Equol status was determined in subjects who participated in the three sub-studies presented in Chapter 7. Based on the method refinement analyses presented here, our current method of GC-MS can adequately isolate and quantify urinary equol after soy intake. The CV was <11% for samples analyzed on the same day, however, it was increased to ~26% for samples analyzed on different days. However, based on the
urinary equol excretion of the subjects in the current sub-studies (Chapter 7), the lowest urinary equol concentration among the equol producers (n=30) was at least 2-fold higher than the highest urinary equol concentration among the equol nonproducers (N=66) (Table 6). This was true for all soy treatments in the three sub-studies, except for the low isoflavone phase in the high and low isoflavone study (Table 6). The low isoflavone treatment was the only intervention where the soy foods were alcohol washed to remove the majority of the soy isoflavones (10mg/d in low isoflavone soy foods versus 73mg/d in high normal isoflavone soy foods). In the other soy treatments in the sub-studies (Table 6), the magnitude of equol excretion between equol producers and nonproducers was much larger than the observed CV between days. Therefore, this provided justification for the use of the current GC-MS technique to determine equol status.

Table 6. Urinary Equol Excretion at the End of Treatments (in nmol/L and nmol/d) During the Sub-studies Presented in Chapter 7.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study Phase</th>
<th>Equol Producers</th>
<th></th>
<th></th>
<th>Equol Nonproducers</th>
<th></th>
<th></th>
<th>Equol Producers</th>
<th></th>
<th></th>
<th>Equol Nonproducers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmoL</td>
<td></td>
<td></td>
<td>nmoL</td>
<td></td>
<td></td>
<td>nmoL/d</td>
<td></td>
<td></td>
<td>nmoL/d</td>
</tr>
<tr>
<td>Prebiotic and Soy</td>
<td>Soy Alone</td>
<td>mean±SE (range)</td>
<td></td>
<td></td>
<td>3221 ± 1130 (1298-8609)</td>
<td>60 ± 21 (0-360)</td>
<td>7137 ± 3088 (1909-22210)</td>
<td>139 ± 47 (0-786)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy Alone</td>
<td></td>
<td>mean±SE (range)</td>
<td></td>
<td></td>
<td>3459 ± 1640 (936-11309)</td>
<td>65 ± 16 (0-214)</td>
<td>5819 ± 3060 (1756-21034)</td>
<td>162 ± 42 (0-520)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High and Low</td>
<td>Low Isoflavone</td>
<td>mean±SE (range)</td>
<td></td>
<td></td>
<td>8945 ± 2689 (731-36606)</td>
<td>82 ± 19 (0-265)</td>
<td>14637 ± 3988 (1340-50902)</td>
<td>129 ± 38 (0-659)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoflavone Study</td>
<td></td>
<td>mean±SE (range)</td>
<td></td>
<td></td>
<td>983 ± 388 (0-5458)</td>
<td>17 ± 10 (0-206)</td>
<td>1733 ± 639 (0-8460)</td>
<td>22 ± 15 (0-347)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eco-Atkins Study</td>
<td>Eco-Atkins</td>
<td>mean±SE (range)</td>
<td></td>
<td></td>
<td>9918 ± 2983 (775-21609)</td>
<td>43 ± 12 (0-140)</td>
<td>21387 ± 7062 (2670-56618)</td>
<td>63 ± 17 (0-181)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.4.2. Justification of Equol Status Classification

The clear demarcation between equol producers and nonproducers as shown in Table 6 for all the soy treatments in these sub-studies (except for the low isoflavone treatment in the high and low isoflavone study as explained in the above section) also confirms the use of urinary equol excretion in the determination of equol status. The definition of an equol producer, as suggested by Setchell et al., was >1000nmol/L urinary equol excretion \(^{62}\). This classification has added great value to the field of equol research in establishing a standardized cutoff value. However, in some respects, it is an approximation based on the references quoted. Rowland et al. suggested that >1000nmol/d urinary equol excretion \(^{143}\) would classify an individual as an equol producer and Lampe et al. suggested >2000nmol/d urinary equol excretion \(^{141}\). Unfortunately, neither reference provided 24-hr urine volumes. We have therefore selected >1000nmol/d as per Rowland et al. \(^{143}\), since this value clarify differentiates those who excrete significant amounts of equol compared to those who excrete little equol in the current sub-studies (except for the low soy isoflavone treatment) (Table 6). Furthermore, there was no overlap in urinary equol measurements between equol producers and nonproducers (Table 6). Setchell and Cole recently refined their method of equol status classification and proposed that the urinary log\(_{10}\)S-equol:daidzein ratio of >-1.75 would be defined as an equol producer \(^{149}\). We have also selected this ratio as the \textit{sine qua non} for equol excretion, i.e. all equol producers excrete >1000nmol/d and all have a urinary log\(_{10}\)S-equol:daidzein ratio of >-1.75. On the other hand, equol nonproducers may also have this ratio >-1.75, but did not produce significant amounts of equol (>1000nmol/d).
7. Equol Study

Equol Status and the Blood Lipid Profile in Hyperlipidemia
Following Diets Containing Soy Foods
Equol Status and the Blood Lipid Profile in Hyperlipidemia
Following Diets Containing Soy Foods

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Number of Tables: 4
Number of Figures: 8
Running Title: Equol Status and Blood Lipids
7.1. Abstract

**Background:** Limited LDL-C reductions in recent studies have challenged the effectiveness of soy as part of a cholesterol lowering diet.

**Aim:** To determine whether equol status determines the effectiveness of soy foods to lower serum cholesterol and whether dietary changes to increase equol production improve the effect of soy on serum lipids.

**Methods:** 85 hypercholesterolemic men and postmenopausal women (42M, 43F) who took part in one of three sub-studies where soy foods were provided for one month periods at dose of 30-52g/d: 1) with high normal (73mg/d) or low (10mg/d) isoflavones; 2) with or without a prebiotic to enhance colonic fermentation (10g/d polyfructans); or 3) with a low carbohydrate diet (26% carbohydrate).

**Results:** Soy in unmodified form (n=63 subjects), without prebiotic or other dietary change, reduced LDL-C by 5.1±2.0% (P=0.016). Separation of the larger group (n=85 subjects) into equol (n=30) and equol nonproducers (n=55) demonstrated no differences in the LDL-C response when all soy treatments were combined, but an increase HDL-C in equol producers compared to nonproducers (+1.0±2.7% vs. -4.3±1.1%, P=0.035). However, equol production did not related to blood lipid changes on soy consumption nor was it increased by fermentable carbohydrate administration.

**Conclusion:** Soy showed a modest overall effect in reducing serum LDL-C. However, in equol producers, approximately 35% of our study population, it may have the added benefit of maintaining HDL-C concentrations as a further justification for soy use in individuals at increased risk of cardiovascular disease.
7.2. Introduction

Several recent studies including a meta-analysis which formed the basis for the recent AHA Science Advisory for Professionals from the Nutrition Committee on soy protein and CVD have found much smaller reductions in serum lipids than were reported at the time of the US FDA approved CVD health claim for soy. At the same time, a study of soy germ pasta naturally enriched with isoflavones, with little soy protein has been shown to result in relatively large reductions in LDL-C of 8.6% in a study population with large numbers of equol producers (69% as opposed to the more usual 30%) Furthermore, the improvement in LDL-C was more pronounce in equol producers than nonproducers. This study rekindled interest in the concept that isoflavones, especially equol production from daidzein in equol producers, might be one of the factors responsible for the LDL-C reduction in subjects consuming soy.

Earlier studies had suggested that equol producers tended to eat higher carbohydrate diets and that addition of carbohydrate to fecal cultures in the presence of daidzein, the equol precursor, resulted in greatly increased production of equol. Data such as these opened the possibility that equol production may not be a fixed characteristic of an individual, but may be subject to dietary manipulations which increase colonic microbial fermentation, and hence, biotransformation of daidzein to equol. In turn, equol, the most estrogenic of the isoflavones, would act as a SERM, such as tamoxifen, which results in lower serum cholesterol levels.

Our study therefore was designed to test whether equol status influenced the cholesterol reduction seen with soy under three conditions including high and low isoflavone intake; the use of oligofructose-enriched inulin (prebiotic) to increase colonic fermentation; or the reduction of dietary carbohydrate to reduce colonic carbohydrate
fermentation. Other studies have assessed the effect of altering the colonic microbial flora by ingestion of live bacteria (probiotics)\textsuperscript{118,159} or resistant starch (prebiotic)\textsuperscript{159} to increase colonic fermentation. The results have been mixed in terms of cholesterol reduction. The present study combines data from three soy food feeding studies\textsuperscript{257, 309, 310} in which equol status has been determined and urinary isoflavones measured.

7.3. Methods

7.3.1. Participants

Men and post-menopausal women were recruited from hyperlipidemic patients attending the Risk Factor Modification Centre, St Michael's Hospital (Toronto, ON) and from newspaper advertisements. A total of 85 subjects (42M, 43F; BMI 26.9±0.4kg/m\textsuperscript{2}; age 59.9±1.0yrs) completed one of three one-month soy studies in which 15-26g/1000kcal of soy was consumed. Forty-one subjects (23M, 18F) in study 1 (high and low soy isoflavone study), twenty-two subjects (10M, 12F) in study 2 (prebiotic study) and twenty-two subjects (9M, 13F) in study 3 (Eco-Atkins study). All participants had mild to moderately elevated LDL-cholesterol concentrations (LDL>3.4mmol/L) at recruitment. None of the participants had a history of cardiovascular disease, untreated hypertension (blood pressure > 140/90 mm Hg), diabetes, or renal or liver disease and none were taking medications known to influence serum lipid concentrations apart from three who were on stable doses of statins throughout the study (Table 1)\textsuperscript{257, 309, 310}. A detailed description of other medications at baseline and changes during the study has been previously described in greater detail for 2 of the three studies\textsuperscript{257, 309, 310}. The Ethics Committees of the University of Toronto and St. Michael's Hospital, and the Therapeutic Products Directorate of Health Canada (only for study 3) approved the
studies. The study clinical trial registration numbers are #NCT00877825 (sub-study 1), #NCT00516594 (sub-study 2) and #NCT00256516 (sub-study 3). Written informed consent was obtained from all participants.

7.3.2. Study Protocol

All participants completed one of three soy studies. Dietary treatments within each study where 4-weeks in duration in which 30-52g/d soy protein containing 10-73mg/d of isoflavones was provided as soy foods (milk, tofu, deli slices, burgers, hot dogs, etc). Treatments were separated by a minimum two-week washout period. Both study 1 and 2 where randomized controlled crossover designs and study 3 was a parallel study. Details of study design and measurements are reported elsewhere. At each visit, fasting body weights, waist and hip measurements and blood pressure were measured. Blood samples were obtained after 12h overnight fasts at 2-wk intervals. Breath and urine collections were obtained at baseline and at the end of each treatment in 2 of the three studies (prebiotic and Eco-Atkins studies). Breath gases were obtained at hourly intervals for 12h over the course of the day for H₂ and CH₄ using a modified Haldane-Priestley tube. Urine was collected over a 24h period.

7.3.3. Diets

The 4-wk study periods were weight-maintaining diets in study 1 and 2 and a weight reducing diet in study 3 with foods provided at 60% of estimated caloric requirements. In study 1 and 2, subjects consumed a self-selected background diet that conformed to a NCEP ATPIII diet (< 7% of energy from saturated fat and <200 mg dietary cholesterol/d). The soy foods (milk, tofu, deli slices, burgers, hot dogs, etc.) or low-fat
dairy foods (low fat milk, yogurt, cheese and egg substitute and liquid egg white) with a similar macronutrient profile were provided on the test and control phases respectively. The mean soy protein intakes for the three diets ranged from 30-52g/d (study 1, 30g/d; study 2, 45g/d; study 3, 50 or 52g/d). Study 1 and 2 had two soy phases. In study 1, the soy was high normal isoflavone soy foods (73mg/d total isoflavones) or low isoflavone soy foods (10mg/d total isoflavones). In study 2, either a prebiotic polyfructan (Synergy1®, Orafti Group, Belgium) or a control sugar (maltodextrin) was given with the soy as a dose of 10g/d. In study 3, the weight reducing control diet included similar low-fat dairy foods to studies 1 and 2, but the test diet was a low carbohydrate diet, high protein and fat (26% carbohydrate, 31% protein, 43% fat), which in addition to the soy foods, was also high in gluten, nuts and monounsaturated fat. Only the subjects who completed the soy containing treatment are included in the current analysis.

7.3.4. Analyses

Serum was analyzed according to the Lipid Research Clinics protocol \textsuperscript{218} for total cholesterol, triglycerides, and high-density lipoprotein cholesterol, after dextran sulphate-magnesium chloride precipitation (Bayer Technicon RA1000, Bayer Healthcare, Toronto, ON, Canada) \textsuperscript{248} or by detergent solubilization and measurement of HDL-C (Roche Hitachi 917, Roche Diagnostics, Laval, QC, Canada), in the J. Alick Little Lipid Research Laboratory. LDL-cholesterol was calculated by the method of Friedewald et al. in \textit{mmol/L} (LDL-cholesterol = total cholesterol – (TG/2.2 + HDL-cholesterol)) \textsuperscript{249}.

Dietary isoflavone concentrations were measured as the 3 aglycones (genistein, daidzein, and glycinein) in study supplements. The samples were analyzed in duplicate
using a previous described method with modification in TMS derivatization procedure of isoflavones 70. In brief, the extracted isoflavone samples were added 100 μL of internal standard solution (5α-androstane-3β, 17β-diol, 50 μg/mL methanol) and dried under nitrogen flow. And then dried samples were added 300 μL of pyridine and 100 μL of BSA, and incubated in room temperature for 1 hr. The silylating agent was then removed under nitrogen gas flow, and the trimethylsilylated samples were dissolved in 100 μL of hexane. One μL of aliquot was injected to GC-MS. Urinary metabolites of isoflavones (genistein, daidzein, glycinein, equol, and ODMA) were measured by means of a previous established method with modification in TMS derivatization procedure of isoflavones as above 311. Equol producers and nonproducers were determined based on the creatinine-corrected urinary equol concentrations. The following criteria were used: 1) having a minimal concentration of 1000nmol/d equol 62, 143 and 2) A log10-transformed urinary S-equol:daidzein ratio of greater than -1.75 149. Diets were assessed for macronutrients, fatty acids, cholesterol and fiber using a computer program based on the USDA database 254.

7.3.5. Statistical Analysis

The results are expressed as mean ± standard error (SE). Differences between groups in baseline measurements were assessed by two sample t-test (two-tailed). Changes in lipids between unmodified soy and control treatments and differences in outcomes between equol producers and nonproducers were assessed using two-sample t-test. All analyses were conducted using the SAS (Version 9.1.3, SAS Institute, Cary NC).
Correlations were determined \textit{a priori} and assessed using Spearman’s correlation. Equol and ODMA excretion on the soy treatments for both equol producers and nonproducers were related to changes in serum lipids (LDL-C, HDL-C, TC:HDL-C), apolipoprotein (ApoA1, ApoB, ApoB:ApoA1 ratio). Excretion of daidzein (precursor of both metabolites), as a ratio relative to the total isoflavone excretion, was also related to the excretion of equol and ODMA. No adjustment for multiple comparisons was conducted on these exploratory analyses.

7.4. Results

Baseline characteristics for both equol producers and nonproducers are presented in Table 1. No significant differences were observed in any of the variables among the two groups. Furthermore, there were no significant differences in mean macronutrient profile among equol producers and nonproducers at baseline and at the end of treatments (Table 2 and 3).

7.4.1. Lipids

No differences were seen at baseline in the mean lipid values of the test and control treatments for sub-studies 1, 2 and 3. For the high normal isoflavone and soy without prebiotic treatments which represented the unmodified effect of soy (n=63 subjects), the soy differences from control were total cholesterol \( -0.21\pm0.10\text{mmol/L} \) (-3.2\pm1.5\%,
\( P=0.046 \)); LDL-C \(-0.22\pm0.09\text{mmol/L} \) (-5.1\pm2.0\%,
\( P=0.016 \)); HDL-C \( 0.38\pm0.02\text{mmol/L} \) (3.4\pm1.6\%,
\( P=0.042 \)); TC:HDL-C \(-0.41\pm0.10 \) (-7.1\pm1.8\%,
\( P<0.001 \)); ApoA1 \( 0.05\pm0.02\text{g/L} \) (3.3\pm 1.2\%,
\( P=0.009 \)); ApoB \(-0.07\pm0.02\text{g/L} \) (-4.7\pm1.6\%,
\( P=0.005 \); and -0.08\pm0.01 (-8.3\pm1.6\% (P<0.001) (Figure 1). Assessment of data from 85 subjects from
the 3 sub-studies indicated significant differences dependent on equol excretion status. For HDL-C and ApoA1, significant differences were observed between equol producers compared to nonproducers (0.07±0.03mmol/L (5.3%), P=0.040 and 0.06±0.03g/L (3.7%), P=0.032, respectively) (Figure 2), whereas no significant differences were observed when only unmodified soy treatments were included (Figure 3). No difference in response was seen for LDL-C or other lipids and lipoproteins.

A difference between treatment LDL-C concentrations at baseline was noted in sub-study 2, even though non-significant (see Appendix I). Such a finding would have to be confirmed by further analysis both chemical and statistical of the data prior to acceptance as valid, although its elimination does not alter the significance of the data.

7.4.2. Body Weight, Blood Pressure and CRP

No significant differences were observed in changes in body weight, waist circumference, blood pressure and CRP among equol producers and nonproducers (Table 4).

7.4.3. Effect of Equol Status in Sub-studies

In sub-studies 1-3, no differences were seen between equol producers and nonproducers indicating a lack of equol related difference in blood lipids in response to changing the isoflavone content of soy, or changing the carbohydrate substrate for colonic fermentation either by provision of fructo-oligosaccharides or reducing the proportion of carbohydrate in the diet (Figure 4, 5 and 6). Part of the reason for the lack of effect seen may have been the limited power of individual studies.
7.4.4. Breath Hydrogen: Evidence of Increased Colonic Fermentation

No differences were seen in baseline measurements of breath hydrogen related to equol status nor were differences seen after prebiotic administration or carbohydrate restriction. Furthermore, despite the interventions, no significant differences were seen in breath hydrogen between treatments.

7.4.5. Urinary Isoflavones

The mean 24h urinary isoflavone output on equol producers and nonproducers were similar (Figure 7), except that in equol producers, the equol output was 1333.7±254.9nmol/mmolCr compared to 16.4±3.1nmol/mmolCr in equol nonproducers (P<0.001) and the ODMA, as the alternate metabolite of daidzein, showed the inverse effect with a lower value in equol producers was 914.6±179.6nmol/mmolCr versus 1574.6±180.9nmol/mmolCr in nonproducers (P=0.020).

In sub-study 1, the higher isoflavone output was seen following the higher isoflavone intake from soy 12320.3±1126.6nmol/mmolCr (high isoflavone) versus 1403.6±300.5nmol/mmolCr (low isoflavone), (P<0.001) (Figure 8). No differences was seen in the ratio of equol to total isoflavone output on either high or low isoflavone treatments in equol producers (P=0.151) and nonproducers (P=0.314).

In sub-studies 2 and 3, neither the provision of a prebiotic to increase colonic fermentation or the reduction in carbohydrate to reduce colonic fermentation resulted in differences in isoflavone output, including equol, after subjects were divided into equol producers and nonproducers.
7.4.6. Correlations

As a ratio of the total urinary isoflavone excretion, excretion of daidzein was negatively associated with excretion of equol \((r = -0.777, P <0.001)\) and non-significantly with excretion of ODMA \((r = -0.326, P = 0.078)\) in equol producers. Whereas excretion of ODMA was negatively associated with excretion of daidzein in \((r = -0.421, P = 0.001)\) equol nonproducers.

In equol producers, there was no association between urinary isoflavone excretion \((\text{nmol/mmol Cr})\) and changes in serum lipids \((\text{LDL-C, HDL-C, TC:HDL-C})\), apolipoproteins and ApoB:ApoA1 ratio. However, in equol nonproducers excretion of ODMA was negatively associated with changes in LDL-C \((-0.350, P = 0.009)\), HDL-C \((r = -0.415, P = 0.002)\) and apoA1 \((r = -0.270, P = 0.046)\), although the reason for this association is not clear.

Neither equol or ODMA excretion related to breath hydrogen production, nor was there a change in breath hydrogen production in equol producers versus non producers either when the prebiotic was given in sub-study 2 or when all breath hydrogen were combined from sub-study 2 and 3.

7.5. Discussion

We believe this study is the first to demonstrate that soy consumption results in a significant increase in HDL-C and ApoA1 in equol producers as opposed to nonproducers. No difference was observed between producers and nonproducers in LDL-C reduction resulting from soy consumption which was approximately 5\% in both treatments for equol producers and nonproducers and of similar magnitude to that reported in recent meta-analyses\(^9,\,12,\,121\). However, HDL-C showed a relative rise of 5\%
in equol producers compared to nonproducers and this prevented the fall in HDL-C often seen when LDL-C is lowered. As a result of the 5% reduction in LDL-C and the HDL-C stabilization, a reduction in CHD risk of 10% might be expected in equol producers which would be considerably more if soy foods were also used to displace from the diet foods high in cholesterol and saturated fat. These data therefore confirm the potential value of soy for cardiovascular health especially in equol producers.

A number of recent studies have assessed the effect of equol status on the cholesterol lowering potential of soy with mixed results. Two studies tested the hypothesis that increased colonic microbial activity might increase equol synthesis and so potentiate the action of soy. One study supplemented the diet with Lactobacillus and Bifidobacteria in capsules (probiotic) in an attempt to change the patterns of the fecal microflora. No increase was seen in urinary equol output nor was there a difference in the cholesterol lowering effect of those who were equol producers and those who were not.

A further study using a similar probiotic approach, but given as yogurt, saw an increase in total cholesterol reduction in response to soy in those receiving the probiotic supplement. However, they saw more consistent effect with both greater total cholesterol and LDL-C reductions with soy when a resistant starch supplement was provided to increase colonic microbial fermentation (prebiotic). In our own study, the subgroup who received prebiotic (oligofructose-enriched inulin) demonstrated a potentiation of the soy cholesterol lowering effect supporting the possible use of co-administration of a prebiotic with soy. However, we also failed to show an increase
in urinary equol excretion nor was there a difference between equol producers and nonproducers.

There are several plausible mechanisms for the observed HDL-C lowering effect. Equol has been shown to be more estrogenic than its precursor daidzein and has the ability to bind to both ER\(\alpha\) and ER\(\beta\), compared to ODMA, another metabolite of daidzein\(^{103, 104}\). Mammalian estrogen and estrogen therapy has been shown to alter cholesterol and apolipoprotein metabolism and is known to increase HDL-C and reduce LDL-C\(^{176, 314}\). A similar HDL-C raising effect has been observed in transsexual men on estrogen therapy\(^ {315}\). Selective estrogen receptor modulators (SERMs), such as toremifene, have similar actions as 17B-estrodiol in raising HDL-C\(^ {238}\). Whereas, other SERMs such as tamoxifen, only demonstrate an LDL-C lowering effect\(^ {238}\). Therefore, not all SERMs behave in a similar manner on serum lipids, and this may be reason why equol appears to have a benefit on HDL-C and not LDL-C in the current study.

There are few dietary strategies available to raise HDL-C other than weight reduction, monounsaturated fat and moderate alcohol intake\(^ {316}\). Apart from nicotinic acid and fibric acid derivatives, pharmacological strategies used to raise HDL-C, such as CETP inhibitors and HRT, have failed to reduce cardiovascular events\(^ {173, 174, 317}\). Furthermore, intake of soy foods containing isoflavones does not appear to have the negative side effects associated with HRT such as raised CRP and triglycerides\(^ {177, 314}\) a further advantage in cardiovascular disease risk reduction. Estrogens have also been shown to stimulate the immune system\(^ {318}\), which has also been observed with soy isoflavones with increases in IL-6 concentrations\(^ {319}\). This is likely to have a beneficial effect in priming the immune system as a natural defense mechanism against potential pathogens or infections rather than a negative effect of increasing autoimmune
disorders since increases in other proinflammatory markers such as CRP and TNF-α were not changed. However, further research is needed in this aspect.

In the current studies, 35% of the subjects were defined as equol producers, which is consistent with 30-40% estimation from the literature based on Western populations. No differences in baseline macronutrient profile were observed between equol producers and nonproducers in the current analysis, a similar finding in other studies. Other studies have suggested that diet may play a role in an individual’s equol status, as it has been observed that equol producers have higher intakes of carbohydrate and lower intakes of fat in Western populations, whereas higher intakes of fat, meat and the fat-to-fiber ratio was observed in equol producers in Japan. It is worthwhile to note that in our study, where subjects were given the same soy foods with or without a prebiotic, the same individuals were classified as equol producers in both treatments. This is consistent with the suggestion that that equol producer status does not change.

Intake of soy foods in the current study appears to have a significant effect in increasing HDL-C in equol producers in one month studies. Whether this effect is maintained in the long-term, it is unclear. However, a significant rise in HDL-C was reported in a one year study of soy consumption. In the meta-analysis by Anderson et al., soy intake resulted in a non-significant rise in HDL-C by 2.4%, however, subjects were not analyzed based on their equol status, nor has any subsequent meta-analyses assessed the effect of soy based on equol status.

We conclude that equol status improves the effect of soy consumption on cardiovascular risk by maintaining HDL-C. Whether soy is also more effective in reducing LDL-C in equol producers is less easy to determine and was not observed in
the present study where attempts to increase equol synthesis failed and urinary isoflavone output did not appear to relate to the LDL-C lowering effect of soy. On the other hand, equol status was associated with an increased HDL-C concentration in producers compared to nonproducers on soy consumption and together with the LDL-C reduction seen in both equol producers and nonproducers supports the use of soy as part of the cardiovascular risk reduction diet.
Figure 1. Percent change from baseline of serum lipids and apolipoproteins for unmodified soy and dairy control treatments (N=63).

*Significantly different from dairy control using a paired t-test, P<0.05.
Figure 2. Percent change from baseline of serum lipids and apolipoproteins in equol producers and equol nonproducers for all soy treatments (N=85). *Significantly difference from equol nonproducers using 2 sample t-test, P<0.05.
Figure 3. Percent change from baseline of serum lipids and apolipoproteins in equol producers and equol nonproducers for unmodified soy treatments (N=63).

No significant differences between equol nonproducers and nonproducers using 2 sample t-test, P>0.05.
Figure 4. Percent change from baseline of serum lipids and apolipoproteins in equol producers (N=17) and equol nonproducers (N=24) for the isoflavone study: (A) low isoflavone and (B) high normal isoflavone treatments. No significant differences between equol producers and nonproducers using 2 sample t-test, P>0.05.
Figure 5. Percent change from baseline of serum lipids and apolipoproteins in equol producers (N=6) and equol nonproducers (N=16) for the prebiotic study: (A) soy alone and (B) prebiotic plus soy. No significant differences between equol producers and nonproducers using 2 sample t-test, P>0.05.
Figure 6. Percent change from baseline of serum lipids and apolipoproteins in equol producers (N=7) and equol nonproducers (N=15) for the soy containing treatment on the Eco-Atkins study.

*Significantly different from equol nonproducers using 2 sample t-test, P<0.05.
Figure 7. End of treatment urinary soy isoflavone excretion in equol producers and nonproducers for all soy treatments (N=85).

*Significantly different from equol nonproducers using 2 sample t-test, P<0.05.
Figure 8. End of treatment urinary soy isoflavone excretion in equol producers and nonproducers for the isoflavone study while consuming soy containing low and high-normal isoflavone (N=41).

*Significantly different from equol nonproducers for the same soy treatment using 2 sample t-test, P<0.05.
### 7.7. Tables

Table 1. Baseline Characteristics (n=85).

<table>
<thead>
<tr>
<th></th>
<th>Equol Producers (n=30)</th>
<th>Equol Nonproducers (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males/Females</td>
<td>16/14</td>
<td>26/29</td>
</tr>
<tr>
<td>Body Weight, kg</td>
<td>73.6 ± 12.8</td>
<td>76.0 ± 13.8</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>26.1 ± 3.6</td>
<td>27.4 ± 3.7</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>123.5 ± 17.6</td>
<td>127.3 ± 16.1</td>
</tr>
<tr>
<td>Diastolic</td>
<td>76.0 ± 9.1</td>
<td>77.3 ± 6.9</td>
</tr>
<tr>
<td>Cholesterol, mmol/L†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.50 ± 0.81</td>
<td>6.52 ± 1.05</td>
</tr>
<tr>
<td>LDL-C</td>
<td>4.34 ± 0.70</td>
<td>4.50 ± 0.83</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.28 ± 0.33</td>
<td>1.21 ± 0.31</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.93 ± 1.20</td>
<td>1.79 ± 0.90</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC:HDL-C</td>
<td>5.34 ± 1.30</td>
<td>5.63 ± 1.23</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>3.56 ± 0.89</td>
<td>3.90 ± 0.99</td>
</tr>
<tr>
<td>Apolipoproteins (apo), g/L‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo A1</td>
<td>1.63 ± 0.21</td>
<td>1.59 ± 0.28</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.38 ± 0.20</td>
<td>1.41 ± 0.25</td>
</tr>
<tr>
<td>Apo B: Apo A1</td>
<td>0.86 ± 0.18</td>
<td>0.91 ± 0.19</td>
</tr>
<tr>
<td>C-reactive Protein, mg/L</td>
<td>1.45 ± 1.53</td>
<td>2.35 ± 2.38</td>
</tr>
<tr>
<td>Medications, No. of Subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid lowering</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

No significant differences between equol producers and nonproducers at baseline.

Data expressed as mean±SD unless otherwise noted.

Body mass index was calculated as weight in kilograms divided by the square of height in meters.

†To convert total cholesterol, LDL-C, and HDL-C to mg/dL, divide by 0.0259; to convert triglycerides to mg/dL, divide by 0.0113.

‡To convert apolipoprotein A1 and B to mg/dL, multiply by 100.
Table 2. Nutrient Profiles at Baseline (n=85).

<table>
<thead>
<tr>
<th></th>
<th>Equol Producers (n=30)</th>
<th>Equol Nonproducers (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>1756.5 ± 105.0</td>
<td>1727.5 ± 64.4</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>79.9 ± 4.7</td>
<td>79.9 ± 3.1</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(18.4)</td>
<td>(18.9)</td>
</tr>
<tr>
<td>Vegetable Protein</td>
<td>31.2 ± 2.0</td>
<td>29.3 ± 1.5</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(7.3)</td>
<td>(6.8)</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>1.1 ± 0.5</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(0.3)</td>
<td>(0.1)</td>
</tr>
<tr>
<td>Available Carbohydrate (g)</td>
<td>233.9 ± 13.0</td>
<td>220.0 ± 9.4</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(54.2)</td>
<td>(51.2)</td>
</tr>
<tr>
<td>Dietary Fibre (g/1000 kcal)</td>
<td>15.8 ± 0.9</td>
<td>15.7 ± 0.9</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>52.2 ± 5.2</td>
<td>53.0 ± 3.0</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(26.0)</td>
<td>(27.2)</td>
</tr>
<tr>
<td>Saturated (g)</td>
<td>15.3 ± 2.0</td>
<td>16.6 ± 1.2</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(7.5)</td>
<td>(8.4)</td>
</tr>
<tr>
<td>Monounsaturated (g)</td>
<td>20.9 ± 2.4</td>
<td>20.1 ± 1.2</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(10.3)</td>
<td>(10.3)</td>
</tr>
<tr>
<td>Polyunsaturated (g)</td>
<td>10.6 ± 0.8</td>
<td>11.1 ± 0.8</td>
</tr>
<tr>
<td>(% of Energy)</td>
<td>(5.4)</td>
<td>(5.7)</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000 kcal)</td>
<td>100.6 ± 9.3</td>
<td>113.3 ± 7.8</td>
</tr>
<tr>
<td>Alcohol</td>
<td>4.4 ± 1.8</td>
<td>7.2 ± 1.9</td>
</tr>
<tr>
<td>(% of energy)</td>
<td>(1.5)</td>
<td>(2.7)</td>
</tr>
</tbody>
</table>

Values represent mean ± SE.

No significant differences between equol producers and nonproducers.
Table 3. Nutrient Profiles During Soy Treatments (n=85).

<table>
<thead>
<tr>
<th></th>
<th>Equol Producers (n=30)</th>
<th>Equol Nonproducers (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>1760.6 ± 86.4</td>
<td>1648.2 ± 59.5</td>
</tr>
<tr>
<td>Protein (g) (% of Energy)</td>
<td>97.3 ± 3.8</td>
<td>94.9 ± 3.1</td>
</tr>
<tr>
<td>Vegetable Protein (% of Energy)</td>
<td>88.9 ± 4.5</td>
<td>87.5 ± 3.2</td>
</tr>
<tr>
<td>Soy Protein (% of Energy)</td>
<td>40.2 ± 2.9</td>
<td>37.8 ± 1.9</td>
</tr>
<tr>
<td>Available Carbohydrate (g) (% of Energy)</td>
<td>236.1 ± 19.8</td>
<td>202.8 ± 11.7</td>
</tr>
<tr>
<td>Dietary Fibre (g/1000 kcal)</td>
<td>18.7 ± 1.2</td>
<td>19.0 ± 0.9</td>
</tr>
<tr>
<td>Fat (g) (% of Energy)</td>
<td>45.5 ± 3.5</td>
<td>46.7 ± 2.8</td>
</tr>
<tr>
<td>Saturated (g) (% of Energy)</td>
<td>9.5 ± 0.7</td>
<td>9.6 ± 0.5</td>
</tr>
<tr>
<td>Monounsaturated (g) (% of Energy)</td>
<td>20.3 ± 2.3</td>
<td>21.2 ± 1.8</td>
</tr>
<tr>
<td>Polyunsaturated (g) (% of Energy)</td>
<td>12.3 ± 0.7</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>Dietary Cholesterol (mg/1000 kcal)</td>
<td>34.2 ± 4.7</td>
<td>28.7 ± 2.9</td>
</tr>
<tr>
<td>Alcohol (% of energy)</td>
<td>2.5 ± 0.7</td>
<td>5.3 ± 1.4</td>
</tr>
</tbody>
</table>

Values represent mean ± SE.

No significant differences between equol producers and nonproducers.
Table 4. Effect of body weight, blood lipids, apolipoproteins and C-reactive protein for equol producers and nonproducers on all soy treatments (N=85).

<table>
<thead>
<tr>
<th></th>
<th>Equol Producers (N = 30)</th>
<th>Equol Nonproducers (N=55)</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0</td>
<td>Mean Treatment</td>
<td>Week 0</td>
</tr>
<tr>
<td>Body Weight, kg</td>
<td>73.6 ± 2.4</td>
<td>72.6 ± 2.2</td>
<td>75.8 ± 1.9</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>26.1 ± 0.7</td>
<td>25.7 ± 0.6</td>
<td>27.3 ± 0.5</td>
</tr>
<tr>
<td>Waist Circumference, cm</td>
<td>89.7 ± 2.0</td>
<td>89.1 ± 2.0</td>
<td>92.5 ± 1.5</td>
</tr>
<tr>
<td>Cholesterol, mmol/L†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.60 ± 0.14</td>
<td>6.07 ± 0.15</td>
<td>6.60 ± 0.13</td>
</tr>
<tr>
<td>LDL-C</td>
<td>4.39 ± 0.11</td>
<td>3.93 ± 0.13</td>
<td>4.49 ± 0.10</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.30 ± 0.07</td>
<td>1.30 ± 0.07</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.00 ± 0.19</td>
<td>1.85 ± 0.26</td>
<td>1.91 ± 0.14</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcholesterol:HDL-C</td>
<td>5.42 ± 0.25</td>
<td>4.99 ± 0.26</td>
<td>5.58 ± 0.17</td>
</tr>
<tr>
<td>LDL-C:HDL-C</td>
<td>3.62 ± 0.18</td>
<td>3.22 ± 0.16</td>
<td>3.82 ± 0.13</td>
</tr>
<tr>
<td>Apolipoproteins, g/L‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo A1</td>
<td>1.63 ± 0.04</td>
<td>1.61 ± 0.04</td>
<td>1.61 ± 0.04</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.39 ± 0.04</td>
<td>1.28 ± 0.04</td>
<td>1.42 ± 0.03</td>
</tr>
<tr>
<td>Apo B: Apo A1</td>
<td>0.88 ± 0.03</td>
<td>0.82 ± 0.04</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>C-reactive Protein, mg/L</td>
<td>2.35 ± 0.51</td>
<td>1.82 ± 0.28</td>
<td>2.60 ± 0.36</td>
</tr>
<tr>
<td>Blood Pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>120.2 ± 2.5</td>
<td>118.0 ± 2.5</td>
<td>127.2 ± 2.0</td>
</tr>
<tr>
<td>Diastolic</td>
<td>75.7 ± 1.4</td>
<td>74.4 ± 1.3</td>
<td>78.2 ± 1.0</td>
</tr>
</tbody>
</table>

*Significant differences in change from baseline between equol producers and nonproducers using 2 sample t-test, P<0.05.

†To convert total cholesterol, LDL-C, and HDL-C to mg/dL, divide by 0.0259; to convert triglycerides to mg/dL, divide by 0.0113.
‡To convert apolipoprotein A1 and B to mg/dL, multiply by 100.
8. General Discussion
8.1. General Discussion

Soy is an effective cholesterol lowering food when consumed as part of a diet for the management of hyperlipidemia. Consumption of soy foods with a mean of ~43g/d of soy protein and ~62mg/d of soy isoflavones (high-normal soy isoflavones and soy without prebiotic treatments) resulted in an overall LDL-C reduction of 5% compared to a diet based on low-fat or fat-free dairy products and whole grains. This reduction was achieved without the combination of prebiotics, low isoflavone or reduction in carbohydrate intake. It is likely that the LDL-C reduction observed is beyond simple displacement of foods high in saturated fat and dietary cholesterol as some have suggested\(^9,11\) because a similar displacement was achieved on the control treatments where dairy protein (from low-fat or fat-free dairy foods) and egg protein (from egg substitute and egg whites) were used. The current 5% LDL-C reduction achieved is less than the original estimation\(^7\) and closer to estimates of recent studies on the cholesterol lowering effect of soy\(^9,13,63\), but a 5% reduction is still clinically relevant. Based on the current estimate that every 1% LDL decrease translate to a 1-2% reduction in CHD risk, it can be estimated that soy foods in the current study equate to a 5-10% CHD risk reduction\(^{23,25,26}\). Furthermore, participants were counseled to consume a NCEP ATPIII diet\(^{23}\) during the run-in period and diets that conform to the current ATPIII criteria (<7% saturated fat/d and <200mg cholesterol/d) have been shown to reduce LDL-C by ~15%\(^{321}\). Therefore, the combination of a low saturated fat and cholesterol diet with soy foods could presumably further reduce LDL-C concentrations. Soy foods are only one component of a cholesterol lowering diet. The combination with other foods known to lower cholesterol (i.e. viscous fibers, plant...
sterols and nuts) have been shown to result in LDL-C reductions of ~29%, similar to a first generation statin\textsuperscript{17}.

The effectiveness of soy appears to be improved with the additional of a prebiotic, likely through increased colonic fermentation. Although there are a number of active components found in soy, to date, a link between altered colonic fermentation and soy isoflavone biotransformation has been suggested\textsuperscript{46, 156}. The suggested link is through an increase in deconjugation of the soy isoflavone $\beta$-glucosides to the aglycones and thus, potentially more substrate available for further gut metabolism, specifically equol, a gut metabolite of daidzein. However, although colonic fermentation was increased, no difference in total isoflavone excretion was observed when soy was consumed alone, or with a prebiotic, nor were there differences among the equol producers in equol excretion. It is possible that the sample size in the prebiotic study was too small to detect any differences. It was also interestingly to note that there were no significant changes in the fecal microbiology, which was an unexpected observation.

FOS, including the prebiotics used in the prebiotic study, have been demonstrated to be effective bifidogenic prebiotics\textsuperscript{322, 323} at the dose provided\textsuperscript{262, 263}. The inability to detect differences in bifidobacteria may be related to the collection method and storage prior to plating, however, this same process did not prevent the confirmation of increased bifidobacteria with antibiotic use in a previous 10-day study\textsuperscript{324}. It is possible that changes in the colonic microflora may have occurred earlier in the supplementation period and adaptation may have resulted by the end of one month. It has also been suggested that the indigenous microflora remain rather constant over time\textsuperscript{325}, despite changes in dietary intake. In the current prebiotic study, fecal samples were only collected at the end of the supplementation period, therefore we cannot determine if
there were any acute changes and whether the apparent consistency of the colonic microflora was the result of a rapid return to status quo ante. Nevertheless, an increase in mean breath hydrogen production (i.e. increased colonic fermentation) was seen with prebiotic administration which was significant when the prebiotic was taken alone, indicating increased colonic microbial metabolism of carbohydrate with prebiotic consumption despite no change in bacterial type or numbers.

It is also possible the by-products of fermentation (e.g. propionate) may have contributed to the greater cholesterol lowering effect of soy if taken with a prebiotic. It has been demonstrated that rectal infusions of a mixture of acetate and propionate attenuated the serum cholesterol increase observed when acetate infusion was given alone. Whereas, rectal infusions of propionate alone did not affect lipids. These results support the idea that propionate inhibits the utilization of acetate for cholesterol synthesis and the ratio of acetate:propionate may be of greater importance. This notion has been supported in studies where increased propionate inhibits cholesterol synthesis, but not in other studies. In animal studies, it has been observed that propionate inhibits cholesterol synthesis by inhibiting both 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-coA reductase. However, SCFAs were not measured in any of our substudies. Therefore, it is possible that altered soy isoflavone biotransformation or SCFAs in the colon could act either separately or together to lower LDL-C when prebiotics are consumed in conjunction with soy. It also may be that equol production is simply a marker of the nature of the SCFA produced in the colon. In this respect, it has been shown in an in vitro study that increased hydrogen, propionate and butyrate did not inhibit the biotransformation of
daidzein to equol, whereas acetate did. Thus, equol production could indicate an environment in the colon in which propionate rather than acetate is produced.

In our current Eco-Atkins study, the observed LDL-C reduction was 20.4% (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Changes in LDL-C Reduction for the Eco-Atkins Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL Reduction</td>
</tr>
<tr>
<td>m mol/L</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Fatty Acid Profile</td>
</tr>
<tr>
<td>Weight Reduction</td>
</tr>
<tr>
<td>Soluble Fiber</td>
</tr>
<tr>
<td>Nuts</td>
</tr>
<tr>
<td>Soy</td>
</tr>
<tr>
<td>Total LDL-Reduction</td>
</tr>
</tbody>
</table>

It is predicted that the following components of the Eco-Atkins diet contributed to the overall LDL-C reduction, these include the fatty acid profile, weight reduction, viscous fiber, nuts and soy. According to the Mensink and Katan equation, the fatty acid profile of the diet is predicted to result in a LDL decrease of 7.2% and the weight loss achieved would add a further 1.7% reduction (Table 1). The mean intake of 4.7g of viscous fiber on the Eco-Atkins diet is predicted to result in a 3.1% LDL-C reduction (Table 1). The mean nut consumption on the Eco-Atkins diet was ~32g/d and it is predicted to result in a 2.4% LDL-C reduction (Table 1). Based on the predicted LDL-C contribution of each component of the Eco-Atkins diet, the remaining cholesterol lowering contribution by soy is calculated to be ~6% (Table 1), which is similar to the
overall reduction of 5% achieved (high-normal isoflavones and soy without prebiotic treatments) in the current substudies. Thus, it suggests that the hypocholesterolemic effect of soy was not lessened by a low fermentation diet, which questions the role of colonic fermentation in the biotransformation of soy isoflavones in relation to the cholesterol lowering effect of soy.

It is also possible the vegetable protein and fat in the Eco-Atkins diet could contribute to the maintenance of soy isoflavone biotransformation as suggested in studies were correlations seen with higher plant protein and fat with equol production [109, 141, 149, 157]. Although we did not see any difference in the prevalence of equol producers in this study, we cannot determine if equol status had changed, since the same individuals did not consume a higher fermentation diet with soy for direct comparison (i.e. study was parallel in design).

The prevalence of equol producers in the current substudies are similar to those observed in Western populations at ~30-40% [10, 63, 89, 123, 139, 141-143, 145]. Previous studies have shown a difference in LDL-C response to soy intake based on equol status, where greater reductions were observed in equol producers [127, 144]. However, this has not been a consistent finding [10, 63, 118, 123]. In the current substudies, LDL-C reductions were independent of equol status. However, to our surprise, a difference in the HDL-C and apoA1 response was seen between equol producers and nonproducers. In equol producers, HDL-C remained unchanged with soy intake, whereas in equol nonproducers, HDL-C decreased. The resulting relative difference between the two groups was 5% for HDL-C and 4% for ApoA1. To our knowledge, this is the first study to show such differences based on equol status. Soy foods, in general, have been shown to increase HDL-C significantly in some studies [67, 116, 118, 327], but not in others [7, 9].
However, in those studies where increases in HDL-C were observed, the effect was smaller than the LDL-C reductions and many of these studies did not determine equol status. The observation that the HDL-C response to soy is based on equol status as demonstrated in the current substudies, could be a possible explanation for the lack of consistency in the HDL-C effect seen in previous studies, where equol status is not assessed. For the studies that did determine equol status, the HDL-C effect could be small and the studies may not be adequately powered to detect significant differences. If the HDL-C effect observed in our current substudies is indeed a real phenomenon and confirmed with further studies, soy foods have an added advantage of modulating HDL-C responses, beyond LDL-C. Again, this would have clinical relevance since based on epidemiological data, a 1 percent decrease in HDL-C is associated with a 2–3 percent increase in CHD risk.

Equol, which has the strongest binding affinity and is the most estrogenic of the soy isoflavone metabolites, may be a potential mechanism for the observed HDL-C effect. Primarily, equol may be acting as a SERM. Decreased LDL-C and/or increased HDL-C has been observed with other SERMs such as tamoxifen, raloxifene and toremifene. These three SERMs do not have the same action on the blood lipid profile. In general, although these SERMs have been shown to significantly reduce LDL-C, only toremifene significantly increases HDL-C, whereas the other two SERMs do not. Therefore, it is possible that soy isoflavones and specifically equol also have similarities and differences in their actions on lipid responses, specifically HDL-C, where increases have also been observed with use of HRT.

Other known factors that raise HDL-C may contribute to the differences between equol producers and nonproducers. Factors that raise HDL-C include increased MUFA,
physical activity and moderate alcohol consumption with sustained weight loss. No differences in alcohol and MUFA intake were observed between equol producers and nonproducers. It is possible that physical activity may have changed, but participants were asked to maintain their level of physical activity in all three substudies. Furthermore, two of the substudies were weight maintaining and only one substudy was weight reducing. However, the calorie reduced diet was provided to both equol producers and nonproducers, with no difference in weight loss between the two groups. The total isoflavone excretion did not differ between equol producers and nonproducers, with the exception equol excretion. Daidzein can be biotransformed through two possible pathways, either to equol or ODMA which was reflected in the distribution of soy isoflavones excreted. Addition of prebiotic did not result in any differences between equol producers and nonproducers in terms of isoflavone output. However, further studies are needed to confirm the effect of altered colonic fermentation on soy isoflavone biotransformation as our current substudies were individually small in sample size.

8.2. Weaknesses

There are a number of limitations to the current studies. Two of the three substudies were crossover studies, whereas one substudy was parallel in design. Either design has its pros and cons. The use of a crossover design with three treatments arms in the high and low soy isoflavone study and the prebiotic study may not be adequately powered to detect differences beyond the primary outcomes due to adjustments for multiple comparisons, i.e. detecting differences based on equol status. Crossover studies also tend to be long in duration, where dropouts and adherence to the protocol
become a concern, possibly due to diet or study fatigue. Carryover effect (treatment*period interaction) between treatments may also occur in crossover studies if appropriate washout periods are not considered in the study design. The use of a parallel design may address the concern of study duration and adherence, but adequate sample sizes to detect differences are also an issue in this design due to interindividual variation.

Another potential weakness of the current studies is the difference between treatment LDL-C concentrations at baseline, even though non-significant (see Appendix I). Such a finding would have to be confirmed by further analysis both chemical and statistical of the data prior to acceptance as valid, although its elimination does not alter the significance of the data.

The exploration of equol status as a modulator of the cholesterol lowering effect of soy was underpowered with each substudy. Therefore, definitive conclusions could not be drawn as to whether dose of soy isoflavones and alterations in colonic fermentation are factors that influence the effectiveness of soy as it relates to equol status. Nonetheless, the results of the substudies provide support for potential hypotheses for future exploration and study design. In the three substudies, equol status was not determined at the time of recruitment with a soy challenge in order to randomize equal numbers of equol producers and nonproducers.

The current substudies were all one month in duration, thus short-term studies. It is unclear whether similar effects would be observed with longer term consumption of soy foods.
8.3. Strengths

There are a number of advantages to the current studies. Soy foods, typically found in supermarkets shelves were used in all three substudies, where results could be easily translated into clinical practice. The participants in the substudies had a similar demographic and clinical profile, since the inclusion/exclusion criteria were consistent between the substudies. Furthermore, the duration of each treatment in the substudies was the same at one month in length. These similarities allowed for the successful combination of the three substudies (i.e. low heterogeneity).

This is one of first studies to test the cholesterol lowering effect of soy under a number of dietary conditions that reflect “real” clinical practice. In all three substudies, soy foods were consistently demonstrated to be effective at reducing LDL-C concentrations. Furthermore, this is one of the first comprehensive analyses that explored these dietary conditions and their relation to equol status. In all three substudies, equol status was defined using the same criteria, was analyzed by the same technician using the same methodology and lab equipment within the same period of time. This significantly decreased the potential sources of error associated with the analyses. In all three substudies, the observation that HDL-C tends to be improved in equol producers relative to nonproducers when soy is consumed under different dietary conditions, suggests that the effect is quite robust and less likely due to chance.
9. Future Research
In any future soy study where one of the outcome is HDL-C, it is important to determine the equol status of the study participants and this should be reflected in the study design (i.e. power calculation), since the lipid response differs between equol producers and nonproducers, i.e. it is an effect modifier. This should be determined using a soy challenge at the time of recruitment. The advantages will be two-fold; it will allow prospective determination of changes in equol status as a result of the intervention and the number of equol producers and nonproducers will be adequately represented in the study population.

Based on the results of the current studies, increased colonic fermentation appears to enhance the effectiveness of soy in lowering cholesterol concentrations. One of the proposed mechanisms of action is the increased deconjugation of soy isoflavones from the β-glucosides to the aglycones for absorption. Although there were no significant differences in total isoflavone excretion in our study, this could be a result of inadequate power to detect such differences. If this mechanism of action is indeed likely, similar effects should be observed when individuals consume soy foods that are fermented (aglycone form) compared to those that are unfermented (conjugated form), i.e. the deconjugation occurs ex-vivo versus in vivo.

Another proposed mechanism of action associated with increased colonic fermentation potentiating the hypocholesterolemic effect of soy is the generating of by-products of fermentation such as the SCFAs. Diets of low fermentation and increased fermentation would be provided to an equal number of equol producers and nonproducers in a crossover design with measurements of SCFA production, serum cholesterol and breath gases. These measurements will determine if the response to altered colonic
fermentation is influenced based on equol status and whether this has an influence on cholesterol levels.

The current study is the first we know of to show that equol producers and nonproducers have different HDL-C, but not LDL-C, responses to soy intake. Therefore, with any new findings, additional studies will need to be conducted to confirm such results. Such studies would be designed with HDL-C as the primary outcome. For example, the inclusion criteria will specifically target individuals with low HDL-C concentrations, so that any changes from the study intervention will likely be detected. Furthermore, it would be interesting to determine if the HDL-C advantage in equol producers has an additive effect when soy intake is combined with other known strategies to raise HDL-C such as increased MUFA, small amounts of alcohol and physical activity with sustained weight loss. Such a study would provide a comprehensive approach to target HDL-C, an independent risk factor for CHD.

Finally, longer term studies are needed to confirm the current findings to determine if the effects are sustained, diminished or enhanced with long-term consumption of soy foods, as part of a cholesterol lowering diet.
10. Summary
In carrying out this thesis, we were able to demonstrate the following (Figure 1):

1) **Prebiotic Study:** Coingestion of soy foods with a non-absorbable carbohydrate (i.e. prebiotic) significantly increased the LDL-C lowering effect of soy compared to prebiotic alone, but was not different from soy consumed alone.

2) **Eco-Atkins Study:** Intake of soy with reduced carbohydrate did not lessen the LDL-C lowering effect of soy.

3) **Equol Study:** Soy foods resulted in an overall LDL-C reduction of 5%. The HDL-C response, and not LDL-C, was dependent on equol status. Overall, there was a relative increase in HDL-C concentrations of 5% in equol producers relative to equol nonproducers. However, equol producers did not have greater LDL-C reductions compared to equol nonproducers overall or under any of the following dietary conditions: high or low isoflavone intake and increased or decreased colonic fermentation. The actual amount of equol excretion was not associated with changes in serum cholesterol, and total isoflavone excretion was not altered with the addition of a prebiotic.
Figure 1. Summary of current gaps in the literature addressed in the current thesis related to the hypocholesterolemic effect of soy, these include the effectiveness of soy, altered colonic fermentation and individual differences in isoflavone biotransformation (i.e. equol status).
11. References


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12. Appendices
Appendix I
Figure 1. Effect on LDL-C of sequence of treatments taken in the prebiotic study; P = prebiotic alone, S = soy alone, and SP = prebiotic plus soy.
Figure 2. Effect on HDL-C of sequence of treatments taken in the prebiotic study; P = prebiotic alone, S = soy alone, and SP = prebiotic plus soy.
Figure 3. Effect on TC:HDL-C of sequence of treatments taken in the prebiotic study; P = prebiotic alone, S = soy alone, and SP = prebiotic plus soy.
Figure 4. Effect on LDL-C:HDL-C of sequence of treatments taken in the prebiotic study; P = prebiotic alone, S = soy alone, and SP = prebiotic plus soy.
Appendix II
Power Calculations

1. High and Low Isoflavone Study (3-phase crossover study)

LDL-C effect size = 4% (for low isoflavone and equal increment for high isoflavone, both relative to control; estimated based on literature); SD of effect = 10%; α = 0.017 (Bonferroni correction); 1- β = 0.8

**Sample Size Required Without Dropouts**: 58 subjects

The attrition rate was assumed to be 20%, therefore,

**Required Sample Size Including Dropouts**: 73 subjects

**Number of Subjects Recruited**: 73 subjects

**Number of Subjects Completed**: 41 subjects

2. Prebiotic Study* (3-phase crossover study)

LDL-C effect size = 6% (for soy alone and an equal increment for prebiotic plus soy, both relative to prebiotic alone; estimation based on previous high and low isoflavone study); SD of effect = 10%; α = 0.017 (Bonferroni correction); 1- β = 0.8

**Sample Size Required Without Dropouts**: 27 subjects

The attrition rate was assumed to be 25% (based on the observed rate from high and low isoflavone study), therefore,

**Required Sample Size Including Dropouts**: 36 subjects

**Number of Subjects Recruited**: 37 subjects

**Number of Subjects Completed**: 23 subjects
*Posthoc analysis using the observed 3% LDL-C effect size between the soy alone and prebiotic and soy treatments would require 101 subjects based on the other previous assumptions.

3. Eco-Atkins Study (2-phase parallel study)

LDL-C effect size = 10% (Eco-Atkins diet compared to NCEP control diet, estimation based on current literature); SD of effect = 10%; \( \alpha = 0.05 \), \( 1 - \beta = 0.8 \)

Sample Size Required Without Dropouts: 34 subjects (17 per group)

The attrition rate was assumed to be 25% (based on the observed rate from our previous studies), therefore,

Aim for Sample Size Including Dropouts: 45 subjects

Number of Subjects Recruited: 50 subjects

Number of Subjects Completed: 44 subjects