Barley protein-enriched flour-Enriched Flour, Coronary Heart Disease and Cancer Risk

By

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

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Abstract

This thesis assessed whether advantages existed for animal (calcium caseinate) versus vegetable protein (barley protein-enriched flour) foods when fed at 30 g protein per day to 23 healthy hyperlipidemic subjects for 4 weeks in a randomized crossover study. Outcomes included serum lipids, serum markers of oxidative stress and the growth response of LNCaP prostate and MCF-7 breast cancer cells when incubated in vitro with individual subject’s study serum as an indication of whether the treatment promoted or inhibited cell growth. There was no treatment effect on blood lipids or biomarkers of oxidative stress nor was in vitro cell growth different between treatments. However, after pooling the two treatments, both MCF-7 and LNCaP cell growth was related positively to the change in oxidized LDL. MCF-7 growth was negatively related to the non-HDL-C: HDL-C ratio suggesting that raising intracellular cholesterol and reducing oxidative stress may have preventive and possibly therapeutic advantages.

Word Count: 148
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8-OHdG – 8-Hydroxydeoxyguanosine
AHA – American Heart Association
apoA1 – apolipoprotein A1
apoB – apolipoprotein B
BMI – Body Mass Index
CD – Conjugated Dienes
CE – Cholesterol Ester
CETP - Cholesterol-Ester Transfer Protein
CHD – Coronary Heart Disease
CV – Coefficient of Variance
DASH – Dietary Approaches to Stop Hypertension
DNA – Deoxyribonucleic Acid
DTNB – 5,5’-Dithio-bis 2-Nitrobenzoic Acid
FDA – Food and Drug Administration
HDL – High-Density Lipoprotein
HDL-C – High-Density Lipoprotein Cholesterol
HL – Hepatic Lipase
hs-CRP – high-sensitivity-C-Reactive Protein
LCAT – Lecithin Cholesterol Acyltransferase
LDL – Low-Density Lipoprotein
LDL-C – Low-Density Lipoprotein Cholesterol
LPL – Lipoprotein Lipase
MDA – Malondialdehyde
MUFA- Monounsaturated Fatty Acids
NCEP – National Cholesterol Education Program
NCEP ATP III – National Cholesterol Education Program Adult Treatment Panel III
Ox-LDL – Oxidized Low-Density Lipoprotein
PUFA – Polyunsaturated Fatty Acid
RCT – Reverse Cholesterol Transport
ROS – Reactive Oxygen Species
S-S – Disulfide Bond
SAS – Statistical Analysis System
SEM – Standard Error Mean
SFA – Saturated Fatty Acid
-SH – Thiol Group
TBARS – Thiobarbituric Acid Reactive Substances
Total-C – Total Cholesterol
TG – Triglycerides
USDA – United States Department of Agriculture
VLDL-C – Very Low-Density Lipoprotein Cholesterol
WCRF – World Cancer Research Fund
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Introduction

1. Introduction

Current dietary advice focuses largely on the quantity and quality of the protein rather than whether the protein is of plant or animal origin. Nevertheless recent publications on cohort studies have suggested a benefit of higher plant protein intakes in terms of cardiovascular disease (4, 5) and studies of legume proteins, especially soy, have demonstrated advantages in terms of LDL-C reduction when compared with animal protein foods (6-8). In addition, plant sources of protein are often sources of antioxidants including the isoflavones of soy and avenanthramides of oats. These components have been suggested to offer further protection from heart disease by reducing the oxidation of LDL (9, 10) and thus its atherogenicity since oxidized LDL is taken up more rapidly by the scavenger system of the arterial wall. In addition, there is interest in oxidation products in general including DNA adducts, such as, 8-hydroxy 2-deoxyguanosine, that the reactive oxygen species (ROS) are responsible for creating (11). They were, therefore, thought to be involved in initiation and have also been implicated in promotion of carcinogenesis (12, 13).

The questions we wished to address were several and included whether barley protein-enriched flour was hypocholesterolemic as was the case for soy. Studies in legumes suggested that the 7s globulin fraction was important for the cholesterol lowering property of a vegetable protein (6-8) but very few proteins have been looked at
in human studies and the emphasis has been largely on soy. However, work by Carroll in rabbits (14) had suggested that vegetable proteins had effects in reducing LDL-C dependent on their essential amino acid content. In view of the current interest in consuming diets higher in protein, we felt it important to explore additional vegetable protein sources in human studies.

Second, we wanted to determine whether the phenolics and other compounds associated with vegetable proteins acted as antioxidants and decreased LDL oxidation as seen with other vegetable protein foods including soy, wheat gluten and also nuts (10, 15-17).

Third, we considered it important to determine whether there were other potential advantages for vegetable proteins, and barley specifically, in terms of effects on cancer cell growth possibly related to the amino acid profile of the vegetable protein through reduced levels of essential amino acids. The model we prepare to use was an in vitro incubation approach using human cancer cells, specifically MCF-7 breast cancer cells and LNCaP prostate cancer cells. The incubation media would contain 10% human serum from the feeding study to determine whether the dietary changes were associated with promotion or inhibition of cancer cell growth.

We recognized that although the potential antioxidant action of increased vegetable protein consumption might benefit both cancer and coronary heart disease (CHD) through less oxidized LDL, the issue of the overall effect in cancer was unclear since the barley protein-enriched flour might lower LDL-C. If cholesterol lowering occurred to the extent that it increased cell growth as has been proposed for humans
(18-20) and demonstrated in rodent studies (21, 22), cancer cell growth, rather than inhibition, may be observed after barley protein-enriched flour consumption.
2. Literature Review
Chapter 2
Literature Review

2.1 Coronary Heart Disease and Cancer

Chronic diseases such as CHD and cancer account for 81 percent of total death in high-income countries such as North America (23). While CHD is the number one cause of death in the whole population, cancer is the second cause of death among Canadians (24). Specifically, breast cancer in women and prostate cancer in men are the second and third causes of total cancer mortality, respectively (25). Genetic predisposition accounts for only 10% of total chronic disease risks. Diets and other lifestyle factors interacting with genetic effects appear to play a more significant role in determining the total disease risk (≈ 90%).

CHD and cancer have strong links to diet and lifestyle. In agriculturally based communities (e.g. the Far East), death from CHD and cancer is far less common. For the last decade, the increasing western influence on the lifestyles of developing economies, particularly diets, has paralleled the rise in CHD and cancer incidence and mortality. This trend has highlighted the possible danger of aspects of contemporary western diets i.e. high in animal products and fats and their negative impact on overall health.

The metabolic syndrome which is a forerunner of CHD and many cancers has been associated with western lifestyles (26). This was seen even among those with the lowest genetic risk (i.e. the Far East). Chinese and Japanese who immigrated to the US had 26 times higher prostate cancer rate than those residing in their birth country (27,
Furthermore, rapid westernization in Korea resulted in 260% increase in prostate cancer mortality over a 10-year period (29).

CHD and cancer have also been shown to be associated with high animal product intake and low fruit and vegetable consumption (30-36). Large epidemiological studies such as the Physicians’ Health Study (n= 21275 men) and the Nurses’ Health Study (n = 80082 women) have shown that a high intake of animal products such as egg proteins and food components such as saturated fats are associated with a higher risk of CHD (30, 33). Furthermore, the recent European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (n=519978 participants) has also shown a positive correlation between animal foods including dairy protein and prostate cancer (36).

2.2 CHD Risk Factors and Assessment

CHD has been the leading cause of death in the United States (US) over the past few decades. One of the major studies to identify CHD as a leading cause of death was the Framingham Heart Study (FHS) which has also played a major role in identifying CHD risk factors.

Lifestyle risk factors identified by the FHS included cigarette smoking. Cigarette smoking may increase CHD risk by 2–4 times that of nonsmokers (37). Smoking may contribute directly to CHD incidence by accelerating coronary plaque development (38, 39), destabilizing and promoting plaque rupture, and coronary thrombosis. Cigarette smoking may also increase CHD risk through worsening other independent risk factors such as raising blood pressure, lowering exercise tolerance and HDL-C.
Aging and hypertension were also identified as independent risk factors for CHD. Most new-onset CHD occurs at the age of 65 and above due to the progressive accumulation of arterial plaque (40, 41). Hypertension refers to blood pressure >140/90 mmHg and is considered an important risk factor for CHD, stroke (42-44) and renal failure (45). The Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure recommended lifestyle modification for the management of hypertension (45).

Serum cholesterol levels have been shown to correlate with CHD risk in the FHS (46-48). However, the National Cholesterol Education Program (NCEP) (49-51) suggested that serum cholesterol levels could be used only in initial detection of high serum cholesterol, while, serum LDL-cholesterol values should be used in risk assessment and evaluation of response to therapy. In addition, NCEP stated that the total cholesterol may overestimate CHD risk in individual patients with high serum HDL-C (45).

Risk factors included in the Framingham predictive 10-y CHD risk equation were therefore age, sex, total cholesterol, HDL-C, systolic blood pressure, type 2 diabetes, smoking and, left ventricular hypertrophy (49).

In addition to these major risk factors, obesity (52, 53), physical inactivity (54, 55), family history of premature CHD (45), small LDL-C particles (56), increased lipoprotein (a) (57), increased serum homocysteine (45, 58), and abnormalities in several coagulation factors (45) have been associated with an increase in CHD risk.

Furthermore, the oxidative damage to serum LDL plays potentially important role in etiology of CHD risk. It has been proposed that oxidized LDL is more likely to be
taken up by macrophages and deposited as atheromatous plaques, leading to increased incidence of CHD (59) (Figure 1).

Figure 1. The Formation of Atherosclerosis (1).

Abbreviations: LDL, Low-density lipoprotein; MM-LDL, minimally modified low-density lipoprotein; ox-LDL, oxidized-low-density lipoprotein; ROS, reactive oxygen species; SR-A, scavenger receptor A.

2.3 Cancer Risk Factors and Serum Biomarkers

In contrast to CHD risk, there is no established equation for cancer risk calculation. However, the World Cancer Research Fund (WCRF) has identified smoking and exposure to radiation as risk factors causally associated with cancer, while obesity,
sedentary lifestyle and alcohol usage are classed as possible risk factors. Currently, diabetes also appears to be a risk factor for the majority of cancers, with the exception of prostate cancer for which it appears to be significantly protective.

However, the prognosis, surveillance, and monitoring of cancer rely upon serum tumor markers (60). Multiple serum markers have been identified for site-specific cancers including serum prostate specific antigen (PSA) for prostate cancer (61) and CA 15-3 and carcinoembryonic antigen (CEA) for breast cancer (62) (Table 1). However, the use of these serum markers is limited due to a lack of sensitivity for early disease detection and lack of specificity of CEA and CA 15-3 for breast cancer (60). There is also the issue of false positive values for PSA in diagnosing prostate cancer since the size of non-tumorous prostate also affect serum PSA levels i.e. a large prostate gland may result in high PSA secretion (63).

### Table 1. Commonly Used Serum Tumor Markers in Different Cancers.

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<th>Malignancy</th>
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<td>(62)</td>
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<tr>
<td>Trophoblastic disease</td>
<td>HCG</td>
<td>(64)</td>
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<tr>
<td>Ovarian cancer</td>
<td>CA 125</td>
<td>(65)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>CEA</td>
<td>(66)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>PSA</td>
<td>(61)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>CEA, CA 15-3</td>
<td>(62)</td>
</tr>
</tbody>
</table>

### 2.4 Diets and CHD

Large Cohorts such as the Nurses’ Health Study and the Health Professionals’ Follow-up Study have shown a strong inverse correlation between fruit and vegetable
intake and CHD risk (34). Diets rich in low-fat dairy or plant protein with higher intakes of fruit and vegetables, such as the DASH and OMNI Heart diets, have been shown to improve blood pressure and blood lipids, and hence CHD outcomes (67, 68). The NCEP ATP III Guidelines recommended low saturated fats and dietary cholesterol reduction (49). These recommendations were based on previous work, much of it relating to the early studies of Ancel Keys and Seven Countries Study from the 1960s and 1970s (69, 70). These studies established that saturated fats were associated with CHD and supported findings that saturated fat was potent in raising LDL-C while polyunsaturated fat tended to reduce serum cholesterol.

As a result, equations were proposed by Keys (71) and also by Hegsted (72) and later by Mensink and Katan (73) which have been used to predict the change in cholesterol associated with a change in diet. However, most recently, a meta analysis by Hu and Krauss failed to support the view that saturated fat consumption was associated with CHD (74). Our interpretation of this is that possibly with the increasing incidence of obesity, the importance of saturated fats may be over shadowed by the association of refined carbohydrates (high glycemic index foods) which may have a more deleterious effect in glucose intolerant obese individuals found in the present era (75, 76). In short, in the past, lean people were at obvious risk of CHD when they ate saturated fat, most recently, obese people who are glucose intolerant suffer from the effects of too rapidly absorbed carbohydrates (74).

Little work has been done on protein, although studies by Halton and Willet have indicated that vegetable proteins and oils are protective for CHD and diabetes incidence in comparison with their animal product equivalents (4, 77). These results do not appear
to be confounded by fiber which has also been shown to be negatively associated with CHD and diabetes (78).

Epidemiological studies of the 7-d Adventists have also detailed the effects of food components on CHD and cancer risk (79-81). In general, studies suggest that increased plant food intakes are associated with a reduced risk of CHD. Thus, 7-d Adventist vegetarians have lower levels of CHD than 7-d Adventist non-vegetarians (82). The effect of stricter vegetarian or vegan diets appears to increase the protectiveness of the diet in reducing the risk of CHD, although, overall mortality may not be improved above that seen with a vegetarian diet (83). Similar reports have been seen with studies of British Vegetarians (84), although the most recent assessment of those individuals showed no advantage for a vegetarian possibly related to the changing dietary pattern with less red meat consumption by the general population (85-87).

Finally, randomized controlled trials using plant-based diets are few. Ornish and colleagues have conducted a series of studies of 1 or 4 year duration which demonstrated that high soy containing vegan diets reduced the size of atheroma assessed by coronary angiography (88). However, these studies have often involved multiple interventions including exercise, meditation and stress reduction.

2.5 Diets and Cancer

There has been a long recognition of a potential association of diet and cancer and an early review of the topic by Doll and Peto suggested that diets contributed 10-70% to all cancer incidence (89). Later estimates have been more modest in their claim
of this association (90). The chief cancers for which diet is a causative factor include breast, colon and prostate cancers (90). In general, distinctions have been drawn between factors which cause initiation and those involved in promotion of tumor growth. For the most part, the emphasis has been placed on the role of diet in promotion of cancer. In general, those nutrients which nourish healthy cells are also those which may increase the growth of transformed cells. Thus, increased calorie intake per se has been associated with increased cancer risk (91) as has supplementation with micronutrients such as folate and B12 (92). The current lists of nutrients implicated in promotion or prevention of tumor growth for breast and prostate are given in Table 2 and 3.

**Table 2. Observational Studies Investigating the Link between Foods and Breast Cancer (93)**

<table>
<thead>
<tr>
<th>Dietary Component</th>
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<th>Effect</th>
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</tr>
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<td>Quality of Carbohydrate</td>
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<td>Fiber</td>
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<td>Red Meat</td>
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<td>Soy</td>
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<td>Caffeine</td>
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<tr>
<td>Alcohol</td>
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</tr>
<tr>
<td>Vitamin D</td>
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<td>Decrease Risk</td>
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<tr>
<td>Other Antioxidants</td>
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<td>No Effect</td>
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<tr>
<td>Folate</td>
<td>2</td>
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<tr>
<td>Carotenoids</td>
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<td>Decrease Risk</td>
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</table>

Strength of Evidence: 1 = weak, 2 = moderate, and 3 = strong evidence
Table 3. Evidence on the impact of dietary components in preventing prostate cancer (94).

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<tr>
<td>Selenium</td>
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<td>Decrease Risk</td>
</tr>
<tr>
<td>Vitamin E</td>
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<td>Decrease Risk</td>
</tr>
<tr>
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<td>Decrease Risk</td>
</tr>
<tr>
<td>Soy/Isoflavones</td>
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</tbody>
</table>

Strength of Evidence: 1 = systematic review, 2 = cohort study, and 3 = case-control study

It is worth noting that dietary restriction renders laboratory animals resistant to the effects of carcinogens both in initiation and promotion of tumorigenesis. Obesity and diabetes are, therefore, risk factors for many cancers (95, 96) as are the associated increases in growth factors including insulin, IGFs (Insulin-like Growth Factors), and the reduction in their binding proteins (97, 98).

Despite current controversies, the advice most generally agreed on for the prevention of cancer appears to include a reduction in total calorie intake (99) and an increase in energy expenditure (exercise) (100).

The specific effects on cancer cell growth of low serum cholesterol and reduced oxidative stress are detailed below.
2.6 Serum Lipids and CHD

Risk factors for CHD include serum lipids (e.g. LDL-C, HDL-C and TG etc), markers of inflammation (e.g. CRP) and measures of oxidative stress (e.g. oxidation of serum proteins and LDL). Evidence suggests that the consumption of fruit and vegetables may reduce CHD risk through improving these multiple risk factors. Various plant food components, such as soy proteins, isoflavones and 7S globulins have been shown to decrease serum cholesterol (6, 7).

In the first meta analysis of soy protein and serum LDL-C reduction, consumption of 47 g/d soy protein reduced LDL-C by 13%, total cholesterol by 9% and TG by 10% while significantly raising serum HDL-C (101). Subsequent meta analyses have indicated more modest effects. The soy peptide containing 7s globulin is a promising soy component that may be responsible for serum cholesterol reduction. In rats, the consumption of 200 mg/kg body weight/d of 7s globulins together with a high cholesterol diet was shown to decrease serum cholesterol by 36% through upregulating liver clearance by 96% (102).

In addition, vegetable proteins may reduce LDL-C by providing a lesser stimulus to cholesterol biosynthesis than animal protein (102). This in turn may also relate to lower essential amino acid levels (14), lower ApoB synthesis rates (14) and reduced HMG CoA reductase activity (8). Other factors in plant foods which may reduce LDL-C include viscous fiber, which results in increased bile acid losses in the feces (103, 104). Additionally, plant sterols found in oils, nuts, seeds and leafy vegetable may block cholesterol absorption resulting in increased cholesterol losses in the feces (105). In this
way, plant foods together with their polyunsaturated fatty acid content exert a favorable
effect in lowering LDL-C (106) and reducing CHD risk (107).

Plant food diets, however, may be associated with increased carbohydrate intake.
Higher carbohydrate intakes themselves may increase serum triglyceride levels. In this
situation, a serum enzyme, cholesterol ester transfer protein (CETP), exchanges the
cholesterol in the HDL for the triglyceride in the VLDL (108-110). As a result, there may
be a corresponding reduction in HDL-C levels brought about by increased catabolism of
triglyceride rich HDL (111). The resulting lower HDL levels result in reduced reverse
cholesterol transport, and hence, cholesterol taken up by the LDL receptor in liver or
muscle may accumulate within the cell (108-110). Low HDL-C levels may therefore be
associated with increased risk of CHD and are found in states where serum triglyceride
levels are raised including individuals with carbohydrate intolerance, obesity, diabetes,
and in what is increasingly referred to as the metabolic syndrome.

2.7 Antioxidants and CHD

The role of antioxidants and CHD risk reduction is highly controversial. Very few
randomized controlled trials investigating the use of high doses of vitamin E
supplementation have demonstrated a significant reduction in oxidized-LDL (112, 113).
Most major trials investigating antioxidant supplementation have shown no benefit for
cardiovascular disease (114-117). The MRC/BHF (Medical Research Council and
British Heart Foundation) Heart Protection Study concluded that long term
supplementation of vitamin E, C and beta-carotene daily did not result in significant a
reduction in the 5-year mortality from, or incidence of CHD or cancer (118). Furthermore,
many studies have documented harmful effects of supplement use. Supplementation of β-carotene in smokers increased the risk of lung cancer and cardiovascular disease (117). Similarly, the HOPE (Heart Outcomes Prevention Evaluation) Study showed a significant increase in heart failure in high CHD risk subjects given 400 IU of vitamin E daily for a mean of 7 years (119).

However, it is important to consider the antioxidant-pro-oxidant equilibrium hypothesis. This hypothesis proposes that antioxidants may become pro-oxidants in an oxidative environment. Therefore, supplementing high CHD risk individuals with a high dosage of a single vitamin supplement may promote pro-oxidant activity, and hence increased CHD risk (120). It may be argued that consumption of whole foods rich in vitamins, which are natural antioxidants, may be more beneficial. The interaction of all food components may provide more appropriate environment for antioxidants to quench free radicals. The DASH (Dietary Approaches to Stop Hypertension) trial has supported this hypothesis. The consumption of DASH diets rich in vegetables, fruit, whole grains and low-fat dairy has resulted in significant reductions in blood pressure and serum markers of oxidative damage (67, 121, 122). Additionally, a number of epidemiological studies have also found a negative relationship between CHD risk and consumption of plant foods rich in antioxidant vitamins (vitamin E) such as nuts, seeds, and vegetable oils (15, 123).

Despite the failure of large antioxidant trials, smaller randomized controlled studies (10, 16, 124, 125) and in vitro studies have shown antioxidant benefits of plant phenolics such as soy isoflavones (10, 126) and oat avanenthramides on markers of oxidative stress (9). Soy isoflavones at a consumption dose of 73 mg/d have been
shown to reduce conjugated dienes in the LDL fraction (oxidized LDL) by approximately 10% (8). An *in vitro* study has also documented a synergy between isoflavones and ascorbic acid (127) which resulted in a 5-fold increase in lag time of resistance to LDL oxidation. Oat phenolics including avenanthramides have been shown to work synergistically with vitamin C and enhance resistance to Cu²⁺-induced human LDL oxidation in a dose-dependent manner (127). Barley grains have been shown to have a comparable total antioxidant capacity to oats (Table 4) (3).

**Table 4. The Level of Total Phenolic Compounds in Barley and Oat Grains (3)**

<table>
<thead>
<tr>
<th>Raw materials</th>
<th>Dry weight of extract (mg)</th>
<th>Total Phenolics (mg of GAE/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat grain</td>
<td>(Avena sativa)</td>
<td>18</td>
</tr>
<tr>
<td>Barley grain</td>
<td>(Hordeum sativum)</td>
<td>17</td>
</tr>
</tbody>
</table>

The content of total phenolics in the extracts was determined spectrometrically according to the Folin–Ciocalteu procedure and calculated as gallic acid equivalents (GAE).

Similar to soy and oats, barley kernel extract has also been shown to decrease Cu²⁺-induced human LDL oxidation *in vitro* (128). However, no study has been undertaken to investigate the clinical effect of barley feeding on serum oxidative markers in human.

### 2.8 Serum Lipids and Cancer

It has been known for sometime that cholesterol biosynthesis is linked to cell division (2, 21, 22, 129). Serum LDL delivers cholesterol to the cell via the LDL receptor mechanism, and in healthy cells the level of intracellular cholesterol determines the activity of HMG CoA reductase i.e. the lower the intracellular cholesterol concentration, the greater the activity of HMG CoA reductase, the rate limiting enzyme in cholesterol
biosynthesis (2). When cholesterol biosynthesis is stimulated by low intracellular cholesterol, the resulting mevalonate production provides the substrate for both nucleotide and ubiquinone synthesis which may stabilize DNA and also provides squalene for membrane cholesterol synthesis (2) (Figure 2 and 3). Therefore, the level of LDL receptor activity and the concentration of cholesterol in the LDL particle may be determinants of cell division.

**Figure 2. Pathway of Isoprenoid Synthesis (2)**

**Figure 3. Role of Cholesterolgenesis in the Cell Cycle and DNA Synthesis (2)**
Furthermore, by removing cholesterol from cells through reverse cholesterol transport, HDL may have the opposite effect by reducing intracellular cholesterol levels and therefore contributing to the stimulation of both endogenous cholesterol synthesis and hence an increase in cell division.

Therefore, it is of interest that low serum cholesterol levels have been suggested to be associated with increased risk of cancer (130). However, this suggestion has been challenged by the observation that preexisting cancer may itself result in lower serum cholesterol levels, either by depression of appetite or, in the case of leukemia, through the use of serum cholesterol by the tumor cells for cell membranes during the course of rapid cell division (the leukemoid crisis). On the other hand, the concept has been revived again in relation to possible cancer promoting effects of hydrophilic statins, such as pravastatin. These hydrophilic statins enter the cell through a sodium-independent bile acid transporter which is only present in liver and ileum (131-133). Therefore, pravastatin may inhibit hepatic cholesterol biosynthesis while leaving HMG CoA reductase active in peripheral tissues. To compensate for a reduction of serum cholesterol following pravastatin ingestion, HMG CoA reductase levels are increased in extrahepatic tissues. As a result, mevalonate levels increase and since mevalonate is the essential starting point for both cholesterol biosynthesis and DNA synthesis, cancer cell proliferation is stimulated (131-133).

Furthermore, the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial demonstrated an increased risk of cancer related to ezetimibe since again peripheral tissues would be deprived of cholesterol. In this situation, serum LDL-C is reduced through the loss of cholesterol from the bowel by inhibition of cholesterol absorption
Similarly, in rodents, studies with ezetamide have demonstrated increased cancer growth, while in contrast, studies with cholesterol supplementation have demonstrated reduced breast cancer cell growth (MCF-7) (135). Rodent and cell culture studies have also indicated that mevalonic acid may stimulate endogenous cholesterol synthesis and provide the substrate for cell division (129).

2.9 Antioxidants and cancer

Fruit and vegetables have been shown to be protective against prostate cancer in a number of epidemiological and clinical studies (136-145). Various plant foods and their components exert protective effects through different mechanisms. Plant components such as phyto-SERMs (e.g. isoflavones and lignans which also have antioxidant activity), specific peptides (e.g. soy and barley lunasin which inhibits DNA acetylation and cell replication) and antioxidative phenolics have been suggested to reduce the prevalence of breast and prostate cancer (137-140, 142, 143, 145).

We suggest that part of the debate surrounding antioxidants and cancer may be explained by the dual role which kinases may play under different degrees of oxidative stress. In an environment which could be considered as high oxidative stress, damage to DNA by reactive oxygen species (ROS) resulting from radiation or chemotherapeutic agents, as used therapeutically, may promote apoptosis by stimulating apoptotic signaling kinase 1 (ASK1), JNK and p38 (146-148). In contrast, in the absence of significant oxidative stress, ROS stimulation of the JNK pathway may lead to cell proliferation (149-152). Thus, feeding rodents with 0.3% oxidized cholesterol diets was
shown to non-significantly increased breast tumor size and the number of preneoplastic colonic foci in rodents compared to a cholesterol supplementation alone. This effect was seen despite the fact that the oxidized cholesterol comprised as little as 0.5% of the total cholesterol in the diet (21, 22, 135). Had larger amount been fed, the result may well have been significant.

The effects of antioxidants on the endogenous antioxidant systems, superoxide dismutase (SOD) and glutathione peroxidase, are not clear. It is possible that antioxidant supplementation depresses overall activity of the endogenous antioxidant system and is the reason why exogenous (dietary) antioxidants have been unsuccessful in reducing cancer. On the other hand, increased oxidative stress (as with exercise) may be associated with upregulation of the endogenous antioxidant system and has been shown to be associated with reduced tumor incidence, especially of breast and colon (100, 153). An alternative explanation may be that increased exercise reduces insulin resistance that is associated with increased cancer risk (154). Therefore, we conclude that there is sufficient complexity in relation to the current data on antioxidants and cancer to warrant further studies in this area.
3. Hypotheses and Aims
Chapter 3
Hypotheses and Aims

3. 1 Hypotheses

3.1.1 General
The substitution of a vegetable for an animal protein source in the diet will improve risk factors for heart disease and cancer.

3.1.2 Specific

1) Barley protein-enriched flour versus casein consumed against the background of an otherwise healthy diet by hyperlipidemic subjects will result in improvements in the blood lipid profile.

2) Barley protein-enriched flour will reduce oxidative damage to LDL and serum proteins.

3) Serum from subjects consuming barley protein-enriched flour will stimulate growth of breast and prostate cancer cells less than serum from subjects consuming casein.

3.2 Aims

3.2.1 General
To assess the possible health benefit of unmodified barley protein-enriched flour and its associated components prepared from air classification of barley flour compared to calcium caseinate in subjects with hypercholesterolemia.

3.2.2 Specific

1) To feed breads containing either 30 g/d of barley protein-enriched flour or 30g/d of calcium caseinate to hyperlipidemic subjects and determine the blood lipid response.
2) To determine the possible oxidative damage to serum LDL.

3) To assess the effect of serum derived from the feeding study on cell growth of prostate cancer (LNCaP) and breast cancer (MCF-7) cells in culture.

(Please see Appendix 2 for Candidate’s role in the study)
Chapter 4


4.1 Abstract

High protein diets have been advocated for weight loss and the treatment of diabetes. Yet animal protein sources are often high in saturated fat and cholesterol. Vegetable protein sources, by contrast, are low in saturated fat and without associated cholesterol. We have, therefore, assessed the effect on the serum lipids of raising the protein intake by 5% using a cereal protein, barley protein-enriched flour, as part of a standard therapeutic diet. Twenty-three hypercholesterolemic men and postmenopausal women completed a randomized cross-over study comparing a bread enriched with either barley protein-enriched flour or calcium caseinate (30 g protein/8,374 kJ (2,000 kcal)) taken as two 1-mo treatment phases separately with a minimum 2 wk washout. Body weight, and diet history were collected weekly during each treatment. Fasting blood samples were obtained at wk 0, 2 and 4. Palatability, satiety and compliance were similar for both the barley protein-enriched flour and casein enriched breads with no differences between treatments in LDL cholesterol, measures of oxidative damage, serum C-reactive protein and blood pressure. Nevertheless, since no adverse effects were observed on cardiovascular risk factors, barley protein-enriched flour remains an additional option for raising the protein content of the diet.

Word Count: 191
4.2 Introduction

With the exception of soy protein (7, 10, 101, 155-161) and some trials of wheat gluten (17), few concentrated vegetable protein sources have been studied. Nevertheless, high protein diets have been recommended for treatment of obesity as an alternative to more conventional high carbohydrate diets. Contemporary diets low in fiber and high in refined carbohydrate with a high glycemic index may increase the risk of diabetes, impair glycemic control in diabetes, reduce HDL cholesterol (HDL-C) and raise serum triglycerides (75, 76, 162-164). On the other hand, low carbohydrate diets are often rich in animal protein sources and relatively high in saturated fat and cholesterol may increase low density lipoprotein (LDL-C) levels, despite active weight loss (165, 166).

By contrast, low carbohydrate diets, high in vegetable protein and oils, have been shown to reduce LDL-C and in cohort studies have been associated with reduced risk for chronic disease (4, 77).

To expand the options for high vegetable protein therapeutic diets, we therefore assessed the effect of barley protein-enriched flour on serum lipids and oxidized-LDL.

4.3 Participants and Methods

4.3.1 Participants. A total of 687 hypercholesterolemic participants responded to newspaper advertisements (Supplemental Figure 1) for men over the age of 21 y or post-menopausal women with LDL-C > 3.5 mmol/L (Table 1). Participants taking statin medications were allowed to participate if their family physicians permitted them to discontinue medication use at least 2 wk prior the study. None of the participants had a
history of diabetes, renal or liver disease, xanthoma or premature cardiovascular
disease or untreated hypertension. Of thirty-three participants, 16 were randomized first
to the casein group and 17 to the barley protein-enriched flour group (Supplemental
Figure 1). 23 participants completed the study.

4.3.2 Study Protocol. Participants were randomized to receive either a barley protein-
enriched flour or casein containing bread (30 g protein per 8,374 kJ (2,000 kcal) of
diets) for 4 wk separated by a 2 wk washout period in a cross over design. Fasting
serum lipids were obtained at wk 0, 2 and 4. At each weekly visit, body weights and
blood pressure were measured, diet and exercise histories from the previous 7-d were
collected and discussed with the dietitian to optimize diets and ensure exercise was
maintained constant. Satiety and palatability were assessed weekly using two bipolar
semantic scales (-4 represented extremely hungry, 0 was neutral and +4 was satiated,
and -3 was unpalatable, 0 was neutral and 3 was extremely palatable, respectively).

The Ethics Committee of St. Michael’s Hospital, the University of Toronto, and
the Therapeutic Products Directorate of Health Canada approved the study. The
participants provided written informed consent. The clinical trials registration number is
NCT00334308.

4.3.3 Diets. Prior to the start and during the study, participants were instructed to follow
a low-fat therapeutic diet with a mean macronutrient profile which was close to the
current National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP
III) guidelines (<7% energy from saturated fat and <200 mg/d dietary cholesterol)
although the total fat was lower at 21% than ATP III guidelines (25 to 35% energy from total fat) (Table 2) (49). Participants were instructed to consume either 30 g of barley protein-enriched flour or casein (see Appendix 1) per 8,374 kJ (2,000 kcal) of their diet in the form of study breads which were exchanged for their regular breads and provided at weekly intervals (Table 2). To the extent acceptable to individual participants, advice was given to follow a low fat lacto vegetarian diet using low fat dairy products. This diet encouraged reduction of meat intake and the maintenance of a low saturated fat and dietary cholesterol intake during both treatments (Table 2).

The barley protein-enriched flour was prepared by air classification, in which flour is separated on an air bed to isolate the more protein rich and dense particles and obtain a 25% protein flour (Parrheim Foods, Saskatchewan, Canada). The process avoids chemical separation or additional heat other than that generated in the milling process. Casein was prepared from defatted milk by precipitation at pH 4.6, separated from whey by a fine filter, washed 4-5 times to remove residual lactose and acid, and spray dried with an inlet temperature of 93 °C (200 °F) and outlet temperature of 204 °C (400 °F) (American Casein Company, New Jersey).

4.3.4 Laboratory Analyses. Serum cholesterol, HDL-C and triglyceride concentrations were determined on serum stored at 70°C at the Nutraceuticals & Functional Foods Institute, Laval University (167, 168). Serum HDL-C concentration was measured as previously described (168). LDL-C was calculated by the method of Friedewald et al. (LDL-C = total cholesterol – (triglycerides/2.2 + HDL-C)) in mmol/L (169).

CRP was analyzed by end-point nephelometry (coefficient of variation, 3.5%)
(Dade-Behring BN Prospec, N high-sensitivity CRP reagent, Dade-Behring, Mississauga, Ontario).

LDL oxidation was estimated in 22 participants in triplicate by measuring LDL conjugated dienes (170) in the LDL fraction (CV, 5.0%) (171), and in 21 participants by the malondialdehyde (MDA)-thiobarbituric acid (TBA) assay (CV, 9.1%) (172).

Protein oxidation was measured in 22 participants to assess the loss of reduced thiol groups (173) as a measure of oxidation (CV, 4.7%) (174).

Diets were analyzed using a computer program based on USDA data (175).

4.3.5 Statistical Analysis. All results are expressed as mean ± SE. The primary outcome was the treatment difference in LDL-C. The analyses were based on those participants who completed the crossover study using PROC MIXED in SAS 9.1 (176) with the wk 4 value as the response variable, and treatment, sex, sex by treatment and a participant ID nested in sex by sequence as main effects, and baseline as a co-variate. Student’s t-test (two-tailed) was used to assess the change from baseline within treatment. Results were considered significant when p<0.05.

4.4 Results

4.4.1 Dietary intake, Compliance, Satiety and Palatability. The macronutrient profiles were very similar for both treatments (Table 2) with a 5% to 8% increase in protein intake during the barley protein-enriched flour and casein treatments, respectively. There was also a small but significantly higher fiber intake during the casein diet reflecting the higher wheat fiber content of the casein bread supplement (Table 3).
There were no differences in compliance and satiety or palatability ratings (scale -3 to +3) reported between the treatment groups. The compliance to barley protein-enriched flour bread was 97.0±1.5% versus 97.9 ±1.0% during casein treatment.

4.4.2 Serum Lipids. There were no treatment differences at wk 4 in serum lipids (Table 4). However, at the end of the casein treatment, there was a reduction in HDL-C (-4.8±1.9%, \( p =0.02 \)), while HDL-C levels were constant throughout the barley protein-enriched flour treatment.

4.4.3 Body Weight. There was no significant treatment difference at wk 4 in body weight, although, there was a reduction during the casein treatment (-0.5 ± 0.2 kg, \( p =0.003 \)) (Table 4). In contrast, the change in body weight during the barley protein-enriched flour treatment was not significant (-0.3±0.2 kg).

4.4.4 Oxidation Products. There were no significant differences in measurements of oxidative stress. However, there was a significant increase of 10.1±4.4% (\( p=0.034 \)) in TBARS in the LDL fraction during the casein phase after adjustment for LDL-C concentration.

4.4.5 Blood Pressure and CRP. There were no significant treatment differences in changes in blood pressure or CRP within or between treatments (Table 4).
4.5 Discussion

Both barley protein-enriched flour and casein were similar in terms of their effect on serum lipids, oxidative damage and CRP. Nevertheless, dairy foods high in casein in the context of diets with a higher proportion of plant based foods, and higher intakes of calcium and low sodium have been associated with reduced blood pressure in the DASH diet (Dietary Approaches to Stop Hypertension) and a more favorable serum lipid profile (177). Therefore, since barley protein-enriched flour was similar to casein, its use in diets where higher protein intakes are desired may still be justified.

In the present study, the weight loss during the casein diets was associated with an apparent increase in caloric intake. We believe this increase was an artifact which we have observed in previous studies where dietary change has been advocated or supplements provided. It may be due to more accurate dietary recording where higher caloric intakes are documented despite modest weight loss (75, 178).

Soy and wheat proteins have been shown to reduce oxidized LDL. However, no difference was seen here between barley protein-enriched flour and casein despite a considerable phenolic content in barley (179) nor was any overall antioxidant difference seen in markers of oxidative damage to serum proteins and lipids, respectively. The lack of antioxidant effect may be due to the fact that most of the barley phenolics are linked to fiber and not the protein fraction used in the present study (179).

Nevertheless, foods made from barley protein-enriched flour would fit well with current low saturated fat and cholesterol dietary recommendations (49) and with the beneficial metabolic effects of low carbohydrate diets reported in weight loss programs (165, 166, 180).
The major limitation of this study is that neither barley protein-enriched flour nor casein, as the positive control, lowered serum LDL-C. However, this lack of effect may be the result of the participants relatively low baseline LDL-C concentration of 3.9 mmol/L and saturated fat intake at 5.7% of energy with cholesterol intakes of less than 60 mg/4187 kJ (1,000 kcal). In this situation small differences in serum lipids may be more difficult to detect. Furthermore, the fiber intake was higher during the casein period than during the barley protein-enriched flour diet. This discrepancy reflected the higher fiber content of the casein bread. However, the fiber was wheat fiber, an insoluble fiber, found to be lipid neutral in previous studies (181). There was also a non significantly higher protein intake during the casein treatment but the physiological effect of this difference in terms of serum lipids is not clear especially since the fatty acid profiles of both diets were so similar. Additionally, the results obtained could be dependent on the method of processing the protein sources. Aqueous methods of barley protein-enriched flour extraction may give different results from the air classification used here. Similarly, casein in whole milk may behave differently from spray dried casein, although casein is resistant to denaturation because of its lack of tertiary structure and the encapsulation structure of pure micellar casein (182).

We conclude that the effect of barley protein-enriched flour as used here was similar to casein in terms of serum lipids, blood pressure, and antioxidant responses. Despite the lack of advantage over casein, the use of barley protein-enriched flour is a possible option to displace saturated fat and cholesterol rich protein sources from the diet especially when higher protein intakes are considered as an advantage in therapeutic diets.
### 4.6 Tables

**Table 1.** Baseline characteristics of randomized and completer participants

<table>
<thead>
<tr>
<th></th>
<th>Randomized</th>
<th>Completers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>33</td>
<td>23</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td>56 ± 2 (41 - 69)</td>
<td>57 ± 2 (41 - 69)</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>68.2 ± 2.6 (49.7 - 108.8)</td>
<td>68.8 ± 3.1 (49.7 - 108.8)</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>26 ± 1 (18 - 35)</td>
<td>26 ± 1 (18 - 35)</td>
</tr>
<tr>
<td><strong>Sex (Men/Women)</strong></td>
<td>9/24</td>
<td>7/16</td>
</tr>
<tr>
<td><strong>Serum lipids²</strong>, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.99 ± 0.18 (4.54 - 8.03)</td>
<td>5.84 ± 0.19 (4.54 - 8.03)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>4.04 ± 0.14 (2.70 - 5.84)</td>
<td>3.91 ± 0.15 (2.70 - 5.84)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.34 ± 0.06 (0.96 - 2.03)</td>
<td>1.32 ± 0.06 (0.96 - 2.01)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.33 ± 0.10 (0.58 - 2.79)</td>
<td>1.33 ± 0.10 (0.67 - 2.40)</td>
</tr>
<tr>
<td><strong>Blood Pressure, mmHg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>119 ± 2 (97-141)</td>
<td>119 ± 2 (97-141)</td>
</tr>
<tr>
<td>Diastolic</td>
<td>71 ± 2 (50-95)</td>
<td>70 ± 2 (50-82)</td>
</tr>
<tr>
<td><strong>Medications, n=12 randomized participants⁵, n=10 completers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid-Lowering³</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Antihypertensive</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Thyroid Hormone</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Ethnicity⁴ n = 29 randomized participants⁶, n=23 completers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Indian</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Oriental</td>
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</tr>
<tr>
<td>Black</td>
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<td>1</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

¹Values are the mean ± SEM (range), n=23. ²Serum was used for lipids.³Lipid Lowering Medication discontinued 2 weeks before study baseline. ⁴Ethnicity is as determined by participant.⁵Data based on 29 participants who started the study. Four participants did not start the study, therefore, their baseline values were not available. ⁶4 participants did not complete the ethnicity survey.
Table 2. Nutrient profiles of hypercholesterolemic participants during barley protein-enriched flour and casein periods

<table>
<thead>
<tr>
<th></th>
<th>Barley protein-enriched flour</th>
<th>Barley protein-enriched flour</th>
<th>Casein</th>
<th>Casein</th>
<th>P-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 4</td>
<td>wk 0</td>
<td>wk 4</td>
<td></td>
</tr>
<tr>
<td>Energy intake, kJ/d</td>
<td>5925±298</td>
<td>7257±368$^a$</td>
<td>6171±333</td>
<td>6890±403$^a$</td>
<td>0.128</td>
</tr>
<tr>
<td>Protein, % energy</td>
<td>18 ± 1</td>
<td>23 ± 1$^a$</td>
<td>17 ± 1</td>
<td>25 ± 1$^a$</td>
<td>0.085</td>
</tr>
<tr>
<td>Plant protein, % energy</td>
<td>9 ± 0</td>
<td>17 ± 1$^a$</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
<td>0.000</td>
</tr>
<tr>
<td>Available carbohydrate, % energy</td>
<td>61 ± 2</td>
<td>57 ± 1$^a$</td>
<td>62 ± 1</td>
<td>56 ± 1$^a$</td>
<td>0.349</td>
</tr>
<tr>
<td>Fat, % energy</td>
<td>21 ± 1</td>
<td>20 ± 1</td>
<td>21 ± 1</td>
<td>20 ± 1</td>
<td>0.695</td>
</tr>
<tr>
<td>Saturated</td>
<td>6 ± 0</td>
<td>5 ± 0</td>
<td>6 ± 0</td>
<td>5 ± 0</td>
<td>0.71</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>7 ± 0</td>
<td>0.752</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>6 ± 0</td>
<td>6 ± 0</td>
<td>5 ± 1</td>
<td>6 ± 0</td>
<td>0.325</td>
</tr>
<tr>
<td>Cholesterol, mg</td>
<td>83 ± 9</td>
<td>59 ± 7$^a$</td>
<td>72 ± 10</td>
<td>64 ± 6</td>
<td>0.477</td>
</tr>
<tr>
<td>Fiber, g</td>
<td>28 ± 2</td>
<td>29 ± 2</td>
<td>30 ± 2</td>
<td>36 ± 2$^a$</td>
<td>0.002</td>
</tr>
<tr>
<td>Alcohol, % energy</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.0 ± 0.0$^a$</td>
<td>0.024</td>
</tr>
</tbody>
</table>

$^1$Values are the mean ± SEM, $n$ =23 participants during both barley protein-enriched flour and casein treatments. Means with superscripts are significant different from the wk 0 value within the treatment period, $P <0.05$.

$^2$P-values represent test for difference at wk 4 between barley protein-enriched flour and casein treatments, $P <0.05$. 
Table 3. Nutritional Composition of barley protein-enriched flour- and casein-containing Breads

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Barley protein-enriched flour bread</th>
<th>Casein bread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kJ</td>
<td>979</td>
<td>867</td>
</tr>
<tr>
<td>Protein, g (% energy)</td>
<td>19 (33)</td>
<td>18 (34)</td>
</tr>
<tr>
<td>Available Carbohydrate, g (% energy)</td>
<td>36 (61)</td>
<td>30 (58)</td>
</tr>
<tr>
<td>Dietary Fiber, g (% energy)</td>
<td>3.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Fat, g (% energy)</td>
<td>1.7 (7)</td>
<td>1.8 (8)</td>
</tr>
<tr>
<td>Saturated</td>
<td>0.4 (2)</td>
<td>0.5 (2)</td>
</tr>
<tr>
<td>Monounsaturated,</td>
<td>0.3 (1)</td>
<td>0.3 (1)</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>0.9 (4)</td>
<td>0.9 (4)</td>
</tr>
<tr>
<td>Cholesterol, mg</td>
<td>&lt;1</td>
<td>13</td>
</tr>
</tbody>
</table>

1Values are the nutrient contents of one sample of the study supplements analyzed
Table 4. Body weight, serum lipids, serum CRP and blood pressure of hypercholesterolemic participants during both the barley protein-enriched flour and casein periods

<table>
<thead>
<tr>
<th></th>
<th>Barley protein-enriched flour</th>
<th>Casein</th>
<th>P-value&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 4</td>
<td>wk 0</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.3 ± 3.1</td>
<td>68.0 ± 3.1</td>
<td>68.6 ± 3.1</td>
</tr>
<tr>
<td>Serum lipids&lt;sup&gt;2&lt;/sup&gt;, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.95 ± 0.21</td>
<td>5.90 ± 0.19</td>
<td>5.91 ± 0.19</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.33 ± 0.10</td>
<td>1.42 ± 0.11</td>
<td>1.39 ± 0.12</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>4.00 ± 0.17</td>
<td>3.95 ± 0.16</td>
<td>3.95 ± 0.16</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.35 ± 0.06</td>
<td>1.30 ± 0.06</td>
<td>1.33 ± 0.06</td>
</tr>
<tr>
<td>Serum CRP&lt;sup&gt;2&lt;/sup&gt;, mg/L</td>
<td>1.65 ± 0.49</td>
<td>1.40 ± 0.42</td>
<td>1.54 ± 0.38</td>
</tr>
<tr>
<td>Blood Pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>118 ± 2</td>
<td>118 ± 3</td>
<td>117 ± 3</td>
</tr>
<tr>
<td>Diastolic</td>
<td>67 ± 2</td>
<td>69 ± 2</td>
<td>68 ± 2</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are the mean ± SEM, n =23 participants during both barley protein-enriched flour and casein treatments. Means with superscripts are significant different from the wk 0 value within the treatment period, P <0.05.

<sup>2</sup>Serum was used for lipids, and CRP.

<sup>3</sup>P-values represent test for difference at wk 4 between barley protein-enriched flour and casein treatments, P <0.05.
Supplemental Figure 1. Study flow diagram showing progress of participants through the trial.
5. Oxidized Serum LDL and Low Serum Lipids from Healthy Subjects on High Protein Diets Stimulate Breast and Prostate Cancer (LNCaP and MCF-7) Cell Growth *in vitro*
Chapter 5

Oxidized Serum LDL and Low Serum Lipids from Healthy Subjects on High Protein Diets Stimulate Breast and Prostate

5.1 Abstract

Aim: Vegetable protein sources rich in antioxidants may lower serum cholesterol compared to animal protein foods and may be associated with reduced cancer risk, especially prostate cancer.

Methods: 23 hyperlipidemic subjects were randomized into a crossover study to consume either barley protein-enriched flour or casein enriched bread supplements. Serum collected at baseline and end of the study was incubated with MCF-7 human breast and LNCaP human prostate cancer cells. Differences in cell growth between treatments were compared and the effects of serum oxidative products and markers of lipid metabolism on cell growth were determined.

Results: No difference in cancer cell growth was observed after incubation with serum from either barley protein-enriched flour or casein treatments. However, breast and prostate cancer cell growth was positively related to oxidized LDL. Prostate cancer cell growth was additionally related to serum protein thiol loss. Furthermore, non HDL-C and the non HDL-C: HDL-C ratio both related negatively to cell growth of MCF-7 cells in vitro. No effect of serum lipids was seen with LNCaP cells.

Conclusion: No difference was seen in cell growth of cancer cells incubated with serum from animal or vegetable protein sources. However, serum markers of oxidative
damage related positively to cell growth and serum markers of increased intracellular cholesterol related negatively to cell proliferation, but only for MCF-7 cells. These data have possible implications for cancer therapy and tumor growth suppression.

**Word Count:** 228
5.2 Introduction

A number of studies have drawn attention to the metabolic differences between the dietary effects of animal and vegetable proteins on cholesterol metabolism (4, 101, 160), antioxidant activity (10, 183) and cancer risk (184, 185). Vegetable proteins, such as soy, lower serum cholesterol (6, 7, 10, 101, 160, 186) and reduce oxidative damage to LDL and serum proteins (10, 16). On the other hand, animal protein consumption has not been associated with these benefits but has been associated with increased cancer risk (184, 187, 188).

Both reduced serum lipids and increased oxidative damage have been implicated in increasing cancer risk. Where differences in cancer have been observed between consumption of animal and vegetable proteins, part of the explanation could be their influence on serum lipids and oxidative damage. However, the data are not clear in terms of providing an advantage for vegetable protein despite a suggestion of benefit from certain epidemiological studies (80). On the one hand, plant proteins have been associated with reduced oxidative stress reducing a stimulus to cancer cell growth. On the other hand, plant proteins may lower serum cholesterol resulting in increased cholesterol biosynthesis and increased risk of cancer.

We have therefore assessed the effect of feeding a plant protein, barley protein-enriched flour, compared to an animal protein, casein, on cancer cell growth when prostate (LNCaP) and breast cancer (MCF-7) cells were incubated in vitro with serum samples derived from a previously published feeding study (189). The study also allowed us to determine whether individual subject differences in blood lipids and oxidative products predicted cell growth.
5.3 Methods

5.3.1 Participants. Thirty-three hypercholesterolemic participants took part in the study of which 16 were randomized first to the casein group and 17 to the barley protein-enriched flour group. 23 participants (7 men, 16 women, age 57 ± 2 y, BMI 25.9 ± 0.8 kg/m²) completed the study (189).

5.3.2 Study Protocol. Participants were provided with either a barley protein-enriched flour or casein containing bread (30 g protein per 8,374 kJ (2,000 kcal) of diets) for 4 wk separated by a 2 wk washout period in a cross over design. Fasting serum lipids were obtained at wk 0, 2 and 4. At each weekly visit, body weights and blood pressure were measured (189).

The Ethics Committee of St. Michael’s Hospital, the University of Toronto, and the Therapeutic Products Directorate of Health Canada approved the study. The participants provided written informed consent. The clinical trials registration number is NCT00334308.

5.3.3 Diets. Prior to the start and during the study, participants were instructed to follow a low-fat therapeutic diet National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) guidelines (<7% energy from saturated fat and <200 mg/d dietary cholesterol). Participants were provided with either 30 g of barley protein-enriched flour or casein in breads to be eaten daily and advice was given to follow a low fat lacto vegetarian diet using low fat dairy products to reduce meat intake and lower saturated fat and dietary cholesterol intake during both treatments.
5.3.4 Laboratory Analyses. Serum cholesterol, HDL-C and triglyceride concentrations were determined on serum stored at -70°C (167, 168). LDL-C was calculated by the method of Friedewald et al. (LDL-C = total cholesterol – (triglycerides/2.2 + HDL-C)) in mmol/L (169).

LDL oxidation was estimated in 22 participants in triplicate by measuring LDL conjugated dienes (170) in the LDL fraction (CV,5.0%) (171), and in 21 participants by the malondialdehyde (MDA)-thiobarbituric acid (TBA) assay (CV,9.1%) (172).

Protein oxidation was measured in 22 participants to assess the loss of reduced thiol groups (173) as a measure of oxidation (CV, 4.7%) (174).

Diets were analyzed using a computer program based on USDA data (175).

5.3.5 Cells and in vitro Proliferation Assay. LNCaP human prostate cancer epithelial cells and MCF-7 human breast cancer epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in T-75 culture flasks (base area of 75 cm²) at 37°C in 5% CO₂.

Authentication testing was performed by Genetica® DNA Laboratories, Inc. (Cincinnati, OH, USA) which included DNA typing with analytical procedures for DNA extraction using QIAamp® DNA Blood Mini kit (Qiagen), polymerase chain reaction (PCR), and capillary electrophoresis on a 3130xl genetic analyzer (Applied Biosystems). The thirteen core CODIS short tandem repeat (STR) loci plus D2S1338, D19S433, and the sex-determining locus, amelogenin, were analyzed using the commercially available AmpF/STR® Identifiler® kit and GeneMapper ID v3.2 software (Applied Biosystems). Appropriate positive and negative controls were used concurrently throughout the analysis. A STR DNA profile of 15 loci plus the sex determining marker Amelogenin was
obtained and compared to the reference profile reported by ATCC (HTB-22 for MCF-7 cells and CRL-1740 for LNCaP cells). The DNA profiles of MCF-7 and LNCaP cells used in this study are 100% and 95% identical to the profiles of the reference human cell lines designated by ATCC (HTB-22 and CRL-1740, respectively). Experiments were conducted using MCF-7 and LNCaP cells with the passage number below 14 from the same cell pool as those authenticated. Experiments were conducted within 2 months after initial cell culture.

LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). MCF-7 cells were cultured in Dulbecco’s Modified Eagle’s Medium-Ham’s F12 (DMEM-F12 1:1) supplemented with 10% blood type AB pooled human serum (PHS, Valley Biomedical Products & Services, Inc., Winchester, VA, USA). The cells were passaged routinely (75% confluent culture) by trypsinization and the medium was renewed every 2 days. For the \textit{in vitro} proliferation assay (the CellTiter 96AQ, MTS assay, Promega, Madison, WI, USA), 5x10³ cells/ well were plated in 96-well plates in 100 µl volume of 10% PHS supplemented RPMI-1640 medium for LNCaP cells and in DMEM-F12 1:1 medium for MCF-7 cells and incubated for 24-h to allow cell attachment. The control medium was removed (100 µl for MCF-7 and 70 µl for LNCaP due to the less stable adhesion of LNCaP) and replaced with 100 µl of 10% PHS supplemented medium for the negative controls, or 100 µl of 10% subject serum supplemented medium from 22 subjects for MCF-7 or 15% subject serum supplemented medium for LNCaP to adjust for an additional 30 µl of control medium (total volume of 130 µl). Analysis of each subject’s serum sample was replicated 8 times together with 16 PHS control samples and placed in the incubator (37 °C, 5% CO₂) for 48 h prior for the assessment of cell proliferation. In using the 96 well-plate, only the
middle 6 of the 12 columns were used since this technique avoided the evaporation of medium which had been seen to take place in the peripheral columns. Instead, two of the empty lateral columns on each side were now filled with serum-free medium to further reduce evaporation in the central 6 columns. The central columns 1 and 4 were filled with 10% PHS, the mean of these values was used to standardize the other columns which were filled with 0 and 4 week sera from both barley protein and casein treatments. The data for the test sera were expressed as a percentage of the mean of the 10% PHS. The final data were expressed as percentage change in cell growth from wk 0 to wk 4.

The CellTiter 96AQ, MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay protocol was as follows. 100 µl of serum supplemented medium in each well was replaced with the mixture of 20 µl MTS dye and 100 µl serum free medium, following the manufacturer’s instructions. Samples were incubated for 2-3 hrs prior to reading at absorbance 485 nm.

5.3.6 RT-PCR for HMG CoA Reductase mRNA Expression. MCF-7 and LNCaP cells were plated in DMEM with 10% FBS 48 h prior to harvest, and were ~90% confluent when total RNA was isolated using TRIzol Reagent (Invitrogen, Burlington, ON, Canada). For reverse transcription, cDNA was synthesized from 3.5 µg of total RNA by oligo(dT) priming using Supercript II reverse transcriptase (Invitrogen). HMG-CoA reductase mRNA level was determined by amplification of 1 µl of cDNA with forward primer: 5’-tcgtggccagtgtgctc-3’ and reverse primer: 5’-ccagccatgccagccacc-3’ to yield a 464 bp fragment under the following condition: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min,
and extension at 72°C for 1 min, with the final extension at 72°C for 10 min. The level of 36B4 was determined by amplification of 1 μl of cDNA with forward primer: 5’-tgccagctgctgctcgggct-3’ and reverse primer: 5’-agttgggtacccgatctgca-3’ to yield a 380 bp fragment under the following condition: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min, with the final extension at 72°C for 10 min. The optimal cycle number for each primer set was determined so that the product was obtained during the exponential phase of amplification (data not shown). PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and images were captured under UV light using a Canon powershot SD 780IS camera. Band densities were determined in arbitrary units using Adobe Photoshop software and were normalized to the corresponding 36B4 band, and expressed relative to values for MCF-7 cells.

5.3.7 Statistical Analysis. All results are expressed as mean ± SE. The primary outcomes were the percentage change from baseline in cell growth for both LNCaP and MCF-7 cells incubated with serum from the participants who completed both barley protein-enriched flour and casein phases. All analyses were undertaken using paired sample t-test (2-tailed). Student’s t-test (two-tailed) was used to assess the change from baseline within treatment. Results were considered significant when p<0.05. Pearson correlations were used to define the association of changes in cell growth with other variables of interest including blood lipids and oxidative products.
5.4 Results

5.4.1 Serum Lipids and Measures of Oxidative Damage

No treatment differences were seen in serum lipids and measures of oxidative damage (Table 1).

5.4.2 Breast and Prostate Cancer Cell Proliferation

There was no significant percentage change from baseline in MCF-7 cell proliferation after incubation with serum from subjects who consumed barley protein-enriched flour (p=0.971) or casein (p=0.469). Nor was a difference observed between the barley protein-enriched flour and casein treatments (p=0.656).

There was, however, a significant percentage reduction from baseline in LNCaP cell proliferation after incubation with serum from subjects both after consumption of barley protein-enriched flour (-8.2% ± 1.6%, p<0.001) and casein (-8.0%±1.6%, p<0.001) (Figure 1), however the difference between reductions in cell proliferation after barley protein-enriched flour versus casein was not significant (p=0.934).

LNCaP prostate cancer cell proliferation was not significantly related to MCF-7 breast cancer cell proliferation (p=0.103).

5.4.3 Relationship between Cancer Cell Proliferation and Serum Oxidative Products

Percentage change from baseline in MCF-7 breast cancer cell proliferation related positively to percentage change from baseline in oxidized serum LDL (as conjugated dienes (CD) in the LDL fraction: r=0.37, n=44, p=0.013 and as TBARS in the LDL fraction: r=0.45, n=42, p=0.003) (Figure 3). However, there was no significant
relationship between the serum protein thiol content and MCF-7 cancer cell proliferation 
\((r=-0.20, n=44, p=0.193)\) (Figure 2).

Oxidized LDL content in serum measured as CD in the LDL fraction also related positively to LNCaP cell proliferation \((r=0.42, n=44, p=0.005)\). However, no significant relation was seen with oxidized LDL measured as TBARS \((r=0.05, n=42, p=0.753)\). Nevertheless, LNCaP prostate cancer cell proliferation related negatively to serum protein thiol concentrations \((r=-0.36, n=44, p=0.018)\), where lower serum thiol concentrations are markers of oxidized serum proteins.

The data were confirmed by Spearman correlation. For the relationship between TBARS and MCF-7 cell growth, the relationship remained significant \((r=-0.46, p=0.006)\). No other relationship was significant using spearman correlation.

5.4.4 Relationship between Cancer Cell Proliferation and Serum Lipids

Increases in serum non-HDL-C, calculated VLDL-C, non-HDL-C:HDL-C, Total-C: HDL-C, LDL-C:HDL-C, and calculated VLDL-C:HDL-C as markers of increased intracellular cholesterol availability related to reductions in MCF-7 breast cancer cell proliferation \((\text{non-HDL-C: } r=-0.30, n=44, p=0.049, \text{ calculated VLDL-C: } r=-0.32, p=0.034, \text{ non-HDL-C:HDL-C: } r=-0.39, p=0.009, \text{ total-C: HDL-C: } r=-0.40, p=0.008, \text{ LDL-C:HDL-C: } r=-0.37, p=0.015, \text{ and calculated VLDL-C:HDL-C: } r=-0.33, p=0.027)\) (Figure 4).

The data were confirmed using Spearman correlation. The relation of non-HDL-C: HDL-C ratio to MCF-7 cell growth remained significant \((r = -0.33, p=0.032)\).

Unlike MCF-7 cells, no relationship between serum lipids and LNCaP prostate cancer cell proliferation was observed.
5.4.5 HMG CoA Reductase mRNA Expression in Cells

The level of HMG-CoA reductase mRNA was assessed by RT-PCR in MCF-7 human breast cancer cells and LNCaP human prostate cancer cells. Prior to harvesting and analysis, cells were grown for 48 h in DMEM containing 10% FBS that is a rich source of LDL-C. Under these conditions it was found that HMG-CoA reductase mRNA level was ~3.5-fold higher in LNCaP cells than in MCF-7 cells (Figure 5).

5.5 Discussion

Lower serum concentrations of oxidative products were associated with reduced MCF-7 and LNCaP cell growth. By contrast, higher serum lipid concentrations reduced only MCF-7 cell growth. No difference in cell growth was seen in response to barley protein-enriched flour versus casein for either cell line.

The markers of the overall level of oxidative stress in the present study were measured as loss to oxidation of thiol groups in serum proteins and oxidized LDL measured as conjugated dienes and TBARS in the LDL fraction (178). Both the MCF-7 and LNCaP cells showed reduced cell growth associated with at least 2 of the three measures of oxidative damage. The conjugated dienes represent intermediate markers of lipid oxidative damage whereas malondialdehydes or TBARS are end products of the process. Protein thiols may be regenerated relatively rapidly and act as the first line defense for metabolizing Reactive Oxygen Species (ROS) (190). HDL-C concentrations may be depressed in situations of increased inflammation and inflammation may, in turn, be related to increased oxidative damage. The question, therefore, arises as to whether correlations between cell growth and both serum lipid ratios and measures of oxidative
stress may have been due to the association of oxidative stress with the lipoprotein fraction HDL. This does not appear to be the case since no relation was seen between HDL and TBARS (r=-0.09, p= 0.558).

The generation of ROS induced by x-radiation or chemotherapeutic agents may promote apoptosis by stimulating apoptotic signaling kinase 1 (ASK1), JNK and p38 (146-148), under conditions of chemical or metabolic stress. Thus under conditions of extreme stress brought on by cancer therapy, the associated oxidative stress and the production of ROS can be expected to promote cell death. Antioxidants would therefore be expected to increase cancer cell survival.

On the other hand, in the absence of stress, stimulation of the JNK pathway leads to cell proliferation (149-152). Furthermore, when superoxide dismutase (SOD) that is part of the endogenous antioxidant system was over expressed in a multistage skin carcinogenesis model, the reduced oxidative stress closed down the JNK pathway and prevented tumor formation (191). It may be that generation of a certain amount of ROS is needed for optimal cell growth, while excessive ROS production, as in cancer therapeutic situations, leads to apoptosis. However, low levels of oxidative damage as seen in some individuals in the present study also results in apoptosis, suggesting a possible optimal range of oxidative activity to promote cell growth and multiplication.

In terms of cancer prevention, the use of antioxidants has had minimal success. Selenium and lycopene have shown initial promise in prevention of prostate cancer (136, 183). However, recent prospective studies of lycopene showed no associations with prostate cancer risk (114), although a further meta analysis suggests a benefits for selenium (115). β-carotene supplementation in smokers appeared to increase the incidence of lung cancer (116, 117). Perhaps in this situation, the apoptosis resulting
from ROS generation under the chronic stress of smoking was reduced to levels where JNK began to stimulate cell growth rather than cancer cell apoptosis. Other antioxidant studies have reported no beneficial effects in cancer prevention. No studies have reported the use of antioxidants in cancer therapy.

Although the oxidative stress story has been a topic of much interest stimulated by potential preventive and therapeutic implications, more recently considerable interest has been generated by the concept that cholesterol metabolism may be manipulated to achieve therapeutic objectives in cancer treatment (192). It is known that the processes that drive cellular cholesterol biosynthesis are linked to cell proliferation. The therapeutic potential of this linkage has been highlighted by the recent debate over the possible cancer prevention or promoting properties of the cholesterol lowering drugs (133). It has been suggested that the conflicting data in this respect (131, 132) may be due to lack of separation of the lipophilic (most statins) from the hydrophilic statin drugs (e.g. Pravastatin). Unlike the lipophilic statins that can easily diffuse into all types of cells, pravastatin requires the sodium-independent bile acid transporter for uptake (193). This transporter is present only in the liver and ileum, leaving most extrahepatic tissues with unopposed HMG CoA reductase activity during pravastatin-mediated lipid reduction therapy. Therefore, when serum cholesterol levels are decreased following pravastatin ingestion, a compensatory increase in endogenous cholesterol production can freely occur at extra-hepatic sites. This process may promote tumor growth in peripheral tissues by providing increased mevalonate and cholesterol in these tissues that is linked to cell replication. Indeed, a recent meta-analysis of trials of pravastatin found an increased risk of cancer specifically in elderly individuals, as well as a significant trend towards increasing cancer risk in users of pravastatin with advancing age (194). It is
expected that this group would harbor an increased number of occult malignant cells, and that their growth may be stimulated by an increase in cellular mevalonate and cholesterol levels to form detectable tumors. The Simvastatin and Ezetamide in Aortic Stenosis (SEAS) trial (134) also suggested that the lower the concentration of serum cholesterol the greater the risk of cancer. The effect did not show significance for individual sites and, of relevance to the present study, no difference was seen between breast and prostate which both showed non significant increases in cancer incidence of 35% (134). Subsequent meta analyses have failed to demonstrate a significant effect of ezetamide (195) or hydrophilic statins (196). However, meta analyses have failed to correct for the non HDL-C: HDL-C ratio and the effect may therefore have been missed.

Our own study suggests that not only are circulating total cholesterol, LDL-C and VLDL-C linked to cell proliferation but so also is HDL-C. HDL is involved in reverse cholesterol transport which delivers cholesterol from extrahepatic tissues to the liver. It is therefore the balance between LDL and HDL activities which determine the intracellular cholesterol availability i.e. the more cholesterol that is delivered to the cell as LDL-C and the less cholesterol that is taken away by HDL, the greater the intracellular cholesterol content. Through negative feedback regulation, the higher intracellular cholesterol content will inhibit HMG-CoA reductase activity (2). HMG CoA reductase is a rate-limiting enzyme in cholesterogenesis that converts HMG-CoA to mevalonate, an intermediate product required in cholesterol biosynthesis for cell membranes and for DNA synthesis, both of which are required for cell division (2).

MCF-7 cells expressed low levels of HMG-CoA reductase mRNA when grown in medium containing 10% FBS, suggesting that the feedback down regulation of HMG-CoA reductase is activated in the presence of exogenous serum cholesterol levels (197).
Conversely, LNCaP cells maintained a much higher level of HMG-CoA reductase expression, despite the presence of abundant LDL-C in serum, suggesting less stringent feedback regulation. Since inhibition of HMG-CoA reductase inhibits cancer cell growth (198), this difference in feedback regulation may explain, at least in part, why MCF-7 cells but not LNCaP cells are growth inhibited by increasing concentrations of cholesterol in human serum.

5.6 Conclusion

The present study is small but the effects achieved reasonable significance levels. The findings fit well with much of the basic biology although the clinical data are mixed. The therapeutic implications of combining antioxidant strategy with inhibition of cholesterol synthesis in reducing the rate of cancer progression require further exploration.
### 5.7 Table

**Table 1:** Serum Lipids, Markers of Oxidative Damage and Cancer Cell Proliferation of Participants during Barley protein-enriched flour and Casein Diets (n=22)

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Barley protein-enriched flour</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 0</td>
<td>Wk4</td>
</tr>
<tr>
<td>Serum lipids, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.32 ± 0.10</td>
<td>1.37 ± 0.11</td>
</tr>
<tr>
<td>Total-C</td>
<td>5.88 ± 0.20</td>
<td>5.84 ± 0.19</td>
</tr>
<tr>
<td>Non-HDL-C</td>
<td>4.53 ± 0.18</td>
<td>4.54 ± 0.18</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>0.60 ± 0.05</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td>LDL-C</td>
<td>3.92 ± 0.17</td>
<td>3.91 ± 0.16</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.35 ± 0.07</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td>TG: HDL-C</td>
<td>1.04 ± 0.10</td>
<td>1.15 ± 0.13</td>
</tr>
<tr>
<td>Total-C: HDL-C</td>
<td>4.48 ± 0.16</td>
<td>4.69 ± 0.22</td>
</tr>
<tr>
<td>Non-HDL-C: HDL-C</td>
<td>3.48 ± 0.16</td>
<td>3.69 ± 0.22</td>
</tr>
<tr>
<td>VLDL-C: HDL-C</td>
<td>6.59 ± 0.51</td>
<td>6.87 ± 0.55</td>
</tr>
<tr>
<td>LDL-C: HDL-C</td>
<td>3.00 ± 0.13</td>
<td>3.16 ± 0.18</td>
</tr>
<tr>
<td>Markers of Oxidative Damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein thiols (mM)</td>
<td>323 ± 14</td>
<td>321 ± 14</td>
</tr>
<tr>
<td>CDs adjusted for LDL-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol/mmol)</td>
<td>17.4 ± 0.6</td>
<td>17.5 ± 0.7</td>
</tr>
<tr>
<td>TBARs adjusted for LDL-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol/mmol)†</td>
<td>0.23 ± 0.02</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Cell Proliferation (% of PHS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7 Human Breast Cancer</td>
<td>91.3 ± 0.9</td>
<td>91.4 ± 1.0</td>
</tr>
<tr>
<td>LNCaP Human Prostate Cancer</td>
<td>135.0 ± 4.7</td>
<td>123.7* ± 4.6</td>
</tr>
</tbody>
</table>

Abbreviation: Total Chol, total cholesterol, CDs, conjugated dienes, TBARs, thiobarbituric acids.

†TBARs were measured in 21 subjects. *p<0.05 from baseline. No significant differences in measurements between 2 dietary treatments at wk 4.
5.8 Figures

Figure 1: Percentage change from baseline in a) breast cancer and b) prostate cancer cell proliferation after barley protein-enriched flour and casein treatments. *P< 0.05 from baseline. No significant between treatments at P<0.05.
Figure 2: Relationship between percentage change from baseline in serum protein thiols and percentage change in a) MCF-7 breast cancer cell proliferation ($r=-0.20$, $p=0.193$) and b) LNCaP prostate cancer cell proliferation ($r=-0.36$, $p=0.018$).
Figure 3: Relationship between percentage change from baseline in MCF-7 breast cancer cell proliferation and percentage change in a) CD adjusted for LDL-C (µmol/mmol) and b) TBARS adjusted for LDL-C and LNCaP prostate cancer cell proliferation c) CD adjusted for LDL-C (µmol/mmol) and d) TBARS adjusted for LDL-C (µmol/mmol)
Figure 4: Relationship between percentage change from baseline in MCF-7 breast cancer cell proliferation and percentage change in a) serum calculated VLDL-C, b) Calculated VLDL:HDL-C ratio, c) Total-C: HDL-C ratio and d) LDL-C:HDL-C ratio. Proliferation c) CD adjusted for LDL-C (µmol/mmol) and d) TBARS adjusted for LDL-C (µmol/mmol)
Figure 5: HMG-CoA reductase mRNA expression. The level of HMG-CoA reductase mRNA was determined by RT-PCR in MCF-7 human breast cancer cells and LNCaP human prostate cancer cells grown in medium containing 10% FBS, as described in the Methods. The mRNA level of the 36B4 gene is also shown as a control.
6. Overall Discussion: Conclusion and Limitations
Chapter 6

Overall Discussion: Conclusion and Limitations

6.1 Conclusion

Our study of barley protein-enriched flour failed to demonstrate a difference from casein in changing CHD lipid risk factors and measures of oxidative damage (189). We also failed to demonstrate a difference in \textit{in vitro} cancer cell proliferation when subjects’ sera were incubated with breast and prostate cancer cells.

However, markers of oxidative stress related to increased cancer cell proliferation as did a low ratio of non HDL-C: HDL-C.

6.1.1 Barley protein-enriched flour and Serum Lipids

A major reason for assessing a cereal protein such as barley protein-enriched flour was to provide an alternative to legumes since a proportion of the population have an allergy to legume proteins. Serum lipid reduction has been ascribed to both barley and oats related to their content of \(\beta\)-glucan fiber (104). The possible effect of the protein had not been explored.

A further reason for assessing barley protein-enriched flour was that animal proteins have been postulated to increase serum lipids related to their higher content of essential amino acids (160, 199). Barley protein-enriched flour is lower in the essential amino acid, lysine, but has a higher arginine to lysine ratio than casein which may confer an additional cardiovascular benefit through enhanced nitric oxide synthesis (200, 201).
The lack of treatment effect in terms of serum lipids may be explained by the absence of the 7s-globulin fraction in barley protein-enriched flour. This fraction is found mostly in legumes and has been considered responsible for the hypolipidemic effect of vegetable proteins (8).

6.1.2 Barley protein-enriched flour and Cancer Cell Proliferation

Epidemiologically, cancer risk has usually been associated with red meat and processed meat consumption (187, 188, 202-207), possibly through the prooxidant activity of heme iron in red meat and nitroso compounds in processed meat (187, 188, 202-207). Our use of casein as a low-fat dairy protein may have minimized the potential difference between animal protein and vegetable protein since this protein source contains neither heme nor nitroso compounds. Casein may, however, stimulate cancer cell growth through its stimulation of IGF-1 production, its abundance of essential amino acids by comparison with many vegetable proteins, and its content of calcium, which in epidemiological studies, has been linked to prostate cancer (208).

Components which may have anti-cancer properties in plant foods such as soy and barley include lunasin peptide that in cell culture studies has been shown to reduce MCF-7 cancer cell proliferation, possibly through inhibition of histone acetylation (137-139, 142, 209). However, no feeding studies have been undertaken to assess whether barley lunasin has an effect in vivo. Our study represents the first in this area and the dose of barley protein-enriched flour at 30 g/d is high, ensuring that any effect of barley protein-enriched flour was unlikely to be missed. Nevertheless, the inhibition in cell growth with barley protein-enriched flour was no greater than was seen with casein.
Despite the lack of treatment difference, after pooling the treatments, associations were seen between cancer cell growth and oxidative stress. Previous studies have shown inhibition of cancer cell growth after addition of antioxidants to cancer cell culture (210-213). Nevertheless, our study is the first we know of to have assessed the direct effect of differences in oxidative status of unmodified human serum on changes in cancer cell growth. These data are also in line with studies on ROS and stimulation of JNK/p38/ASK1 and the effect of p38 in increasing cancer cell growth.

In addition, our study supported the concept that intracellular cholesterol bioavailability is a determinant of cell growth. Previous studies have indicated that low LDL-C and high HDL-C independently may relate to cancer risk (21, 22, 214) and HDL has been shown to promote MCF-7 cell growth \textit{in vitro} (215, 216). Our study demonstrated that a low ratio of non HDL-C: HDL-C in unmodified human serum stimulated MCF-7 breast cancer cell growth. We believe the importance of this study is the demonstration that cell growth may relate to intracellular cholesterol resulting from both the delivery of cholesterol to the cell through Apo B containing particles (LDL and VLDL) and the removal of cholesterol by Apo A1 containing particles (HDL).

\section*{6.2 Limitations}

The weaknesses include the relatively small number of subjects, the nature of the diet which was self-selected rather than metabolically controlled, and the duration which may not account for changes that require more than one month. Having said this, the study represented a real life approach, although the amount of barley protein-enriched flour was high. Nevertheless, we wished to run studies which were comparable to the soy studies where 25 g/d of soy protein was the level of intake for which the FDA
have permitted a CHD health claim to be made (217). Furthermore, a one month period has proved sufficient duration to see significant changes in serum lipids for other plant derived lipid lowering components including soy protein, viscous fibers, plant sterols and nuts.
7. Future Studies
Chapter 7

Future Studies

7. Future Studies

We do not believe that there is a need for further studies of barley protein-enriched flour of the sort that we have undertaken since we are convinced that there is no advantage in terms of serum lipid reduction or antioxidant activity by comparison with casein even when the barley protein-enriched flour was provided at a high dose (30 g protein/d) since the lipid changes on both treatments were similar. Nevertheless, despite the lack of benefits in comparison with dairy protein, barley protein-enriched flour still provides the option for use of an additional low fat low cholesterol protein source.

By contrast, we believe that considerably more work is required to explore the effects of serum lipids and antioxidants on cancer cell growth. We believe that dose response studies to assess the effect of graded levels of lipids and antioxidants are required on cancer cells in culture, and that not only cancer cells but also non-cancer cells e.g. MCF-10 could usefully be assessed. It is known that the inhibitory effect of cholesterol may be lost in certain tumor cell lines and during tumor cell dedifferentiation. It may be that from the preventive stand point certain tumors and certain stages of tumorigenesis may be most susceptible for therapeutic intervention. These studies can be combined with mRNA studies to determine effects on HMG CoA reductase and LDL receptor activity in relation to lipid and antioxidant interventions. Furthermore, attention should be paid to a range of HDL-C concentrations since understanding the effect of reverse cholesterol transport in depleting intracellular free cholesterol through ApoA1.
linked cholesterol efflux through the ABCA1 transporter may be a further way of maximizing therapeutic interventions. Studies of different dietary interventions including weight loss studies may also prove more useful in determining the effects of dietary change than the modest intervention which we employed. Finally, other factors in serum other than cholesterol and ROS may be important determinants of cancer cell growth and required testing. These factors may include not only a range of oxidative products and phenolics found in the serum but also intermediate products in the biosynthetic pathway of cholesterol and their interaction with sex hormones and growth factors including insulin and IGF-1.
8. Summary
Chapter 8

Summary

8. Summary

8.1 General

The substitution of a vegetable for an animal protein source in the diet did not provide additional benefit to risk factors for CHD or cancer.

8.2 Specific

1) Barley protein-enriched flour versus casein consumed against the background of an otherwise healthy diet by hyperlipidemic subjects did not result in improvements in the blood lipid profile.

2) Barley protein-enriched flour did not reduce oxidative damage to LDL as CD and TBARs or to serum proteins as protein thiols.

3) Serum from subjects consuming barley protein-enriched flour did not stimulate growth of breast and prostate cancer cells differently from serum from subjects consuming casein. However, increased levels of oxidative damage increased both LNCaP and MCF-7 cell growth while increased serum non-HDL-C and its ratio with HDL-C decreased MCF-7 cancer cell growth. The latter data supported the hypothesis that high intracellular cholesterol levels by suppressing HMG CoA Reductase reduce cell proliferation.
9. References
9. Reference

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10. Appendices
### Appendix 1. Composition of Barley Flour and Casein Powder

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<tr>
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<th>Barley Protein Flour</th>
<th>Calcium Caseinate</th>
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<tr>
<td>Total Weight (g)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Kcal</td>
<td>361.1</td>
<td>363.4</td>
</tr>
<tr>
<td>Moisture (% total weight)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>25.5</td>
<td>88.6</td>
</tr>
<tr>
<td>Animal</td>
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<tr>
<td>Vegetable</td>
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</tr>
<tr>
<td>Wheat</td>
<td>0</td>
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</tr>
<tr>
<td>Fat (g)</td>
<td>4.5</td>
<td>1</td>
</tr>
<tr>
<td>Chol (mg)</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>60.5</td>
<td>0</td>
</tr>
<tr>
<td>Fiber (viscous fiber) (g)</td>
<td>5.7 (2.9)</td>
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</tr>
<tr>
<td>Available Carbohydrate (g)</td>
<td>54.8</td>
<td>0</td>
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Appendix 2. Candidate’s involvement in Study 1.

I was responsible for checking and calculating participants’ dietary record, organizing study data, assisting the post-doctoral fellow in the measurement of biomarker of oxidative stress, initial statistical analyses, manuscript preparation together with the principle investigator, manuscript submission, and extensive revisions required by the journal together with resubmission of the revised manuscript.