CHOLESTEROL LOWERING EFFECTS OF BOVINE SERUM IMMUNOGLOBULIN IN HUMAN PARTICIPANTS WITH MILD HYPERCHOLESTEROLEMIA

A Thesis

by

MELINDA LORI BLACK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Nutrition

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Approved by:

Chair of Committee,	William McIntosh
Committee Members,	Mary Bielamowicz
	David McMurray
Chair of Nutrition Faculty,	Nancy Turner

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ABSTRACT

Cholesterol Lowering Effects of Bovine Serum Immunoglobulin in Human Participants with Mild Hypercholesterolemia. (August 2005)

Melinda Lori Black, B.S., Texas Woman's University

Chair of Advisory Committee: Dr. William McIntosh

Hypercholesterolemia is a major risk factor for cardiovascular disease (CVD). Interestingly, the consumption of dairy products, namely milk, has been shown to lower cholesterol. The mechanism of action surrounding this observation has been attributed to the protein fraction of milk. While there have been many studies evaluating the effects of dietary protein sources on cholesterol concentrations, few studies have evaluated specific animal protein components and no human clinical studies regarding the effects of animal plasma protein fractions on cholesterol metabolism have been conducted. This study examined the effect of an oral serum bovine immunoglobulin protein fraction (bIg) derived from US Department of Agriculture approved beef (aged < 30 months) on lipid indices in hypercholesterolemic humans.

Participants included men and women (aged 25 – 70 years) with mild hypercholesterolemia (5.44-6.99 mmol/L) who were not receiving cholesterol-lowering medication. Treatment consisted of the randomized, double blind, parallel group, placebo-controlled administration of 5 grams (g) bIg daily for 6 weeks (W) in 52 participants (n = 26 each in treatment and control groups). Mean ($\pm$ SD) baseline treatment and placebo total cholesterol (TC) was 6.33 $\pm$ 0.1 mmol/L and 6.16 $\pm$ 0.1 mmol/L respectively. A repeated-measures multivariate analysis of covariance

(MANCOVA) covaried for change in total energy and alcohol intake, and a Tukey post-hoc examination of the data showed that the bIg-treated group demonstrated a significant reduction in TC at 3-week (W) (5.98 $\pm$ 0.5 mmol/L; $P < 0.05$) and 6-week (W) (5.97 $\pm$ 0.7 mmol/L; $P > 0.05$) intervals compared to baseline. The 6W concentration was significantly lower than the placebo group ($P < 0.05$). Additionally, study findings displayed no significant changes in the placebo group or in any other lipid indexes or markers associated with hepatorenal or cardiovascular health. Consumption of bIg appears to lower major lipid indexes associated with CVD.

ACKNOWLEDGMENTS

I owe an immense amount of appreciation to Dr. Conrad Earnest, who not only served as primary investigator for this study, but also as my intermediary advisor in completing this paper. Additionally, I greatly appreciate Dr. Tim Church who served as medical director for this project and assisted in the overall study design. I would also like to thank Craig Siefken (Proliant, Inc.) and Melba Morrow for technical assistance during this study, Carrie Finley for data management, Jason Wallace for data management and recruitment services, and Alexandra Jordan for orchestrating the organization, coding and assignment of all treatments. I would also like to thank Dr. Eric Weaver, vice president and manager of scientific research and development for Proliant, Inc., for his direct responsibility in bringing this trial to a clinical population and assisting in study design and data analysis. Dr. Weaver's role in this study was limited to intellectual design functions, and he did not partake in any of the clinical aspects of this trial. Proliant, Inc. provided funding and treatment materials for this study.

Overall, I am extremely grateful to the Cooper Institute Centers for Integrated Health Research, Center for Human Performance and Nutrition Research, located in Dallas, Texas, for providing me with the opportunity to complete my master's degree. Additionally, I would like to thank my committee chair, Dr. William McIntosh, for his unwavering support and patience; my committee members, Dr. Mary Bielomowicz, for her continued encouragement and guidance; and Dr. David McMurray, who not only graciously agreed to serve as my committee member, but also kept me on my toes throughout the course of this research.

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CHAPTER I

INTRODUCTION*

Introduction

Heart disease, one of the principal components of cardiovascular disease (CVD), is the leading cause of death in the United States (1). The most common form of heart disease is coronary artery disease (CAD), the number one greatest killer of both men and women in the United States today (2). Hypercholesterolemia, elevated blood cholesterol, is one of the major risk factors for CVD and for the development of coronary atherosclerosis, which is responsible for nearly three-fourths of all deaths from CVD (2,3). In 2000, the growing numbers of individuals with elevated blood cholesterol levels prompted the National Committee for Quality Assurance, an independent, non-profit organization sponsored by the American Heart Association and American Stroke Association, to choose five quality-of-care performance measures relating to preventing and treating CVD (4). Included in these measures was total cholesterol (TC) screening and control in patients with CAD.

Cholesterol is a "vital sign" for measuring an individual's heart health due to its potential deleterious effects on the inner walls of the arteries that supply blood to the heart (5). An estimated 105 million American adults, (49 million men and 56 million women), ages 20 and older, have borderline high or higher TC levels; a 37.7 million of

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these individuals (16.9 million men and 21.8 million women) have TC levels reaching 6.22 mmol/L (240 mg/dL) or higher (2). As TC levels exceed 4.66 mmol/L (180 mg/dL), the risk for developing coronary heart disease (CHD) increases (2). Current data indicates that during middle age, a 1% increase in TC can increase risk for CHD by 3% (6). Additionally, for individuals diagnosed with high cholesterol, a reduction of TC values by a mere 10 to 15% can roughly drop their incidence risk of CHD by 20 to 30% (7,8).

Considerable evidence has demonstrated that TC reduction can halt or potentially reverse atherosclerosis along with minimizing the threat of myocardial infarction and CHD mortality (9,10). Studies in adults with elevated blood cholesterol have shown that for every 1% reduction in TC levels, an individual can reduce their number of heart attacks by 2%, which equates to cutting the risk of heart attack in half by simply reducing TC by 25% (9). An individual's heart attack risk nearly doubles with a TC that reaches or is greater than 6.22 mmol/L (240 mg/dL) compared to a TC of 5.18 mmol/L (200 mg/dL) (2). Furthermore, a TC value of 5.70 mmol/L (220 mg/dL) correlates to nearly a two-fold elevation in incidence of CHD as compared to a TC of 4.66 mmol/L (180 mg/dL) (2). According to the American Heart Association (AHA), a decrease in an individuals total mortality risk may be achieved by lowering elevated TC levels to a range of 4.15 mmol/L (160 mg/dL) to 5.15 mmol/L (199 mg/dL) for primary heart disease prevention, meaning prior to a cardiac event (11). As noted, modest reductions in TC can significantly influence an individual's CHD risk profile. The latest National Cholesterol Education Program Guidelines for Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (NCEP) recommends that all adults older

than 20 years (y) of age know their cholesterol levels and, in accordance with the AHA guidelines, recommends cholesterol testing at least once every five years for all healthy adults (12,13,14). Elevated serum TC is clearly associated with a high risk of CHD as confirmed by the Lipid Research Clinics-Coronary Primary Prevention Trial (15). However, the prediction of risk for CHD greatly improves when low density lipoprotein cholesterol (LDL-C) levels are measured (16). In order to specifically zero in on heart disease risk, the NCEP guidelines recommend lipid panel testing or a lipoprotein profile, a test that defines all the blood components of TC, as the preferred initial screening protocol for detecting CHD (12).

Elevated TC and LDL-C are significant risk factors in the development of heart or cardiovascular disease (CVD) with elevated blood cholesterol being one of the most modifiable risk factors for CHD. Even mild degrees of hypercholesterolemia, when due to increased levels of LDL-C, are associated with dramatic increases in heart disease, and indirectly, stroke risk (2,12). The lowering of plasma LDL-C has clearly shown a reduction in coronary risk (12,17). Every 1 mg/dL (0.026 mmol/L) reduction in LDL-C can be associated with an approximate 1 to 2% reduction in relative risk of CHD (15,18).

Modifiable dietary risk factors in cholesterol reduction. Controlling the modifiable risk factors most strongly associated with the development of CHD including, but not limited to, TC and LDL-C rely strongly on Therapeutic Lifestyle Change (TLC) recommendations of the Adult Treatment Panel III (ATP III) of the NCEP (3). The NCEP puts strong emphasis on the use of TLC as the first line of intervention in managing CHD risk (3). TLC recommendations are characterized by four lifestyle

change components including, but not limited to, decreased dietary intake of saturated fats (less than 7% of total calories) and cholesterol (less than 200 mg daily), increased intake of viscous fiber (10-25 gm of daily soluble fiber), and the addition of either plant stanols or sterols (2 gm daily) (12). In combination, TLC components can lower LDL cholesterol by ≥ 20% (3). With NCEP's strong emphasis on modifiable risk factors, dietary management and weight reduction, for lowering TC and heart disease risk, studies show the TC lowering effect of the NCEP Step I diet ranging from + 5% to – 40% with an overall average decrease in TC of 20% (12). The NCEP Step I diet, which was intended as the dietary starting point for individuals with high cholesterol, limited total fat and saturated fat to ≤ 30% and 10%, respectively, of total caloric intake and restricted dietary cholesterol intake to < 300 mg/day (d). Average reductions in LDL-C with the NCEP Step I and Step II diet were 12% and 16%, respectively (19). The NCEP Step II diet goals were designed for individual's with TC levels > 240 mg/dL and already following the Step I diet. NCEP's Step II diet goals were lower for saturated fat (< 7 %) and dietary cholesterol (< 200 mg/d) intake. The TLC diet, detailed in **Table 1** (20), is known as the "next generation" of NCEP's Step diets (20).

To enhance the effectiveness of TLC components, the AHA highlights the potential cholesterol-lowering benefits of a daily diet that includes vegetable soy protein and nuts (21). Study observations involving intensive dietary intervention that combine modified NCEP and AHA dietary recommendations into a plant-based meal plan, the Portfolio diet, consisting of soy protein (~ 50 gm/d) replacing dairy protein with the addition of soluble fiber (oats, barley and psyllium), almonds (around 35 gm/d) and plant

sterols have proven to lower LDL-C concentrations by an estimated 30%, which would equate to an average 13-20% reduction in the general population (3,22,23,24).

Table 1
Therapeutic lifestyle change diet in ATP III*

Nutrients	**Percent of Total Calories**
Carbohydrate	50–60%, mainly foods rich in complex carbohydrates
Protein	~ 15%, some animal protein may be replaced with soy protein
Total Fat	25-35%
Saturated Fat	< 7%
Polyunsaturated Fat	≤ 10%
Monounsaturated Fat	≤ 20%
	Recommended Intake Amounts
Fiber **	10-25 gm of viscous (soluble) fiber
Cholesterol	< 200 mg/d
Plant-derived Sterols or Stanols **	2 gm/d
Total Calories	Balance energy intake with moderate physical activity expenditure (contributing ~ 200 calories/day to maintain desirable body weight and prevent weight gain

* American Heart Association 2001. Step I, Step II and TLC Diets.
Internet: http://www.americanheart.org/presenter.jhtml?identifier=4764

** Therapeutic options for lowering LDL-C.

The average cholesterol reduction that can be obtained from a combination of dietary change, weight reduction and regular exercise can vary widely between individuals due to a broad number of parameters, including baseline lipoprotein levels, weight change amounts, genetic factors, exercise consistency and degree of adherence to a modified diet (12,25). Additionally, for many individuals, monumental challenges exist in implementing these changes. Although dietary counseling and modification remain the cornerstones of therapy for virtually all lipid modification trials, an alternative to lowering cholesterol is the use of cholesterol-lowering medications.

Medication management of hypercholesterolemia. Medication management of elevated cholesterol has been shown to be extremely effective in lowering TC and LDL-C values (26,27). When optimal control of LDL-C is unobtainable with nutritional screening and dietary intervention strategies, one or more antilipemic drugs, such as statins, are often prescribed to aid in reducing CHD risk (27). Unfortunately, although study evidence has demonstrated that these medications are effective in reducing cholesterol values and mortality risk, some individuals may experience negative sequealea, primarily musculoskeletal side effects, contraindicating their use (28-33). Franc, et al. concluded that the incidence of adverse muscle-related side effects, including, but not limited to muscle pain, cramps, weakness and tendonitis associated with pain, is likely underreported by published research trials (33). In this study, 815 adult hyperlipidemic individuals taking statins and complaining of muscle-related side effects (noted above) were interviewed about their symptoms and asked to complete a self-administered questionnaire detailing their complaints. Out of the 815 individuals interviewed, 165 reported negative muscle side effects that they attributed to lipid

lowering drug treatment (LLT). The questionnaires completed by 133 of these individuals showed 39% having to resort to analgesics at least once for muscle pain relief. The most frequent symptom reported was muscle cramps, however 40% of the patients in this study also reported associated tendonopathies. Keeping in mind that potential problems can occur with medication management of cholesterol-reduction, the continued investigation of alternative cholesterol-lowering options is warranted.

Bovine immunoglobulin (bIg) supplements may be viewed as one the newest experimental cholesterol-lowering alternatives (34,35). In the event that bIg supplements are found safe and effective in reducing cholesterol, they could become viable weapons in the prevention and treatment of cardiovascular disease.

Objectives of This Research

No known published human studies existed that demonstrated the effect of a bovine serum-derived, highly concentrated, immunoglobulin protein supplement on lipid reduction, specifically TC and LDL-C. The primary objective of this current trial was to assess the cholesterol-lowering effects of a bovine immunoglobulin-rich supplement, Proliant's ImmunoLin™, on blood lipid indices, mainly TC and LDL-C, in hyperchol-esterolemic human subjects. The specific aims of this investigation were:

1. To determine whether Proliant's ImmunoLin™ supplement would result in a statistically significant reduction in TC and LDL-C in the ImmunoLin™ treatment group;
2. To determine if a six-week ingestion of 5g daily of Proliant's ImmunoLin™ supplement would reduce TC and LDL-C concentrations by at least 8 to 10% in the hypercholesterolemic human study participants.

Definitions

The following are operation definitions of key terms in this study:

1. Atherogenic Index (AI): a formula used to compare the atherogenic potential of dietary fats defined as the ratio of non-HDL-C to HDL-C or [(TC – HDL-C) / HDL-C] (36,37). The AI should be as low as possible, below 4.0 for men and 3.5 for woman (37).

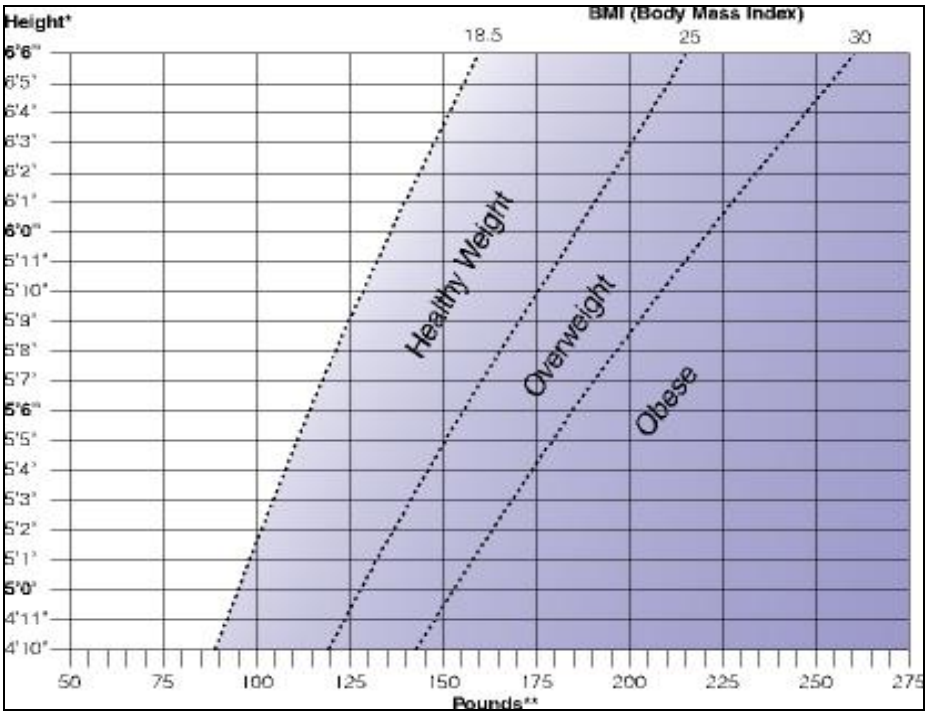
2. Atherosclerosis (coronary artery disease): a slow, progressive disease process that involves a hardening of the arteries, or plaque formation, resulting from excess accumulation and deposition of fatty substances, cholesterol, cellular waste products, calcium and fibrin within the inner lining of the artery. A partial or total block of arterial blood flow can result in hemorrhage into the plaque or formation of a thrombus on the plaque's surface followed by a heart attack or stroke (block of the entire artery). The disease often begins during childhood and progresses with age (38).

3. Body Mass Index (BMI): a measurement, despite the inability to distinguish between excess fat and muscle mass, where weight in kilograms is divided by height in meters squared (kg/m^2) and is strongly correlated with body fat content, disease morbidity and mortality risk in all population groups (**Table 2**) (39). BMI measurements are closely associated with measures of body fat (39). Relative CVD risk factors and incidence rates, average blood pressure (BP), and TC concentrations increase in a graded fashion with rise in BMI accompanied by a decline in average HDL-C (40). Because BMI charts (**Table 3**) (39) can be easily and effectively utilized in predicting the development of health problems related to excess weight, they have recently become the medical standards used to measure overweight and obesity (41).

4. Casein*:* a primary milk protein comprising 75 - 80% of total milk protein (42).

5. Chem-16: a blood chemistry profile measuring the following blood indexes: C-reactive Protein (CRP), total cholesterol (TC), triglycerides (TG), HDL count (HDL-C), LDL count (LDL-C), cholesterol ratios (TC/HDL, LDL/HDL), blood glucose (BG), uric acid (UA), blood urea nitrogen (BUN), creatinine (Cr), BUN/creatinine ratio, sodium (Na), potassium (K), chloride (CL), calcium (Ca), phosphorus (Ph), protein (Pro), albumin (A), globulin (G), A/G ratio, bilirubin (bili), alkaline phosphatase (ALK), lactate dehydrogenase (LDH), liver enzymes (AST/SGOT, ALT/SGPT, GGT), and iron (Fe).

Table 2
Body Mass Index (BMI) chart*



* Weight-control Information Network (WIN): An information service of the National Institute of Diabetes & Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH). Do You Know the Health Risks of Being Overweight? Heart Disease and Stroke. Internet: http://www.niddk.nih.gov.

Table 3
Risk of associated CVD according to BMI and waist size*

BMI	Classification	Waist less than or equal to 40 in. (men) or 35 in. (women)	Waist greater than 40 in. (men) or 35 in. (women)
18.5 or less	Underweight	--	N/A
18.5 - 24.9	Normal	--	N/A
25.0 - 29.9	Overweight	Increased	High
30.0 - 34.9	Obese	High	Very High
35.0 - 39.9	Very Obese	Very High	Very High
40 or greater	Extremely Obese	Extremely High	Extremely High

* Partnership for Healthy Weight Management: Body Mass Index and Waist Size. Internet: www.consumer.gov/weightloss/bmi.htm

6. Cholesterol: a fat-like substance produced by the liver and transported through the body to perform normal body functions, such as production of bile and Vitamin D (43). Cholesterol is found in the blood, every body cell, and in animal-based foods. TC values can help determine relative risk for developing heart disease because excess blood cholesterol can have deleterious effects on the inner walls of the arteries that supply blood to the heart (atherosclerosis), possibly compromising heart function (12,44). For general cholesterol levels considered acceptable for the average adult with no other know heart disease risk factors, see **Table 4** (12).

Table 4
ATP III (NCEP) classification of LDL-C, TC and HDL-C (mg/dL)[1]

CHOLESTEROL VALUES	
LDL CHOLESTEROL	
Less than 100	Optimal Goal
100 to 129	Near/Above Optimal
130 to 159	Borderline High
160 to 189	High
190 or higher	Very high (definite risk)
HDL CHOLESTEROL	
Less than 40	Low (High Risk)
60 or higher	High (Optimal)
TRIGLYCERIDES	
Less than 150	Optimal Goal
TOTAL CHOLESTEROL	
Less than 200	Desirable Optimal Goal
200 to 239	Borderline High
240 or higher	High

[1]Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). May 2001. Internet: http://www.nhlbi.nih.gov/guidelines/cholesterol.

7. C -Reactive Protein (CRP): an acute phase plasma protein synthesized by liver parenchymal cells and released into the blood stream released into the bloodstream in response to various immune system stressors, such as infection, inflammation or trauma (45). Additionally, CRP levels will rise due to heart attack, stroke or other cardiovascular events (46). Elevated CRP levels may be associated with arterial inflammation and have been shown to predict cardiovascular events

(37,47). According to the *Physician's Health Study,* people with increased CRP levels are at risk for heart attack and stroke (48,49).

8. Coronary artery disease (CAD): disease of the coronary arteries resulting in impaired blood flow and supply to the heart muscle. Commonly, the insufficient or obstructive blood flow can be attributed to atherosclerosis, which can cause a narrowing or blockage of the vessels due to the plaque (cholesterol) buildup in the artery walls (50). Symptoms of CAD may include shortness of breath and/or angina (chest pain) followed by a potential myocardial infarction (heart attack) or disorder of heart rhythm (arrhythmia or dysrhythmia) that could result in sudden death (50).

9. High Density Lipoprotein Cholesterol (HDL-C)*:* the "good" cholesterol that binds and transports cholesterol in the blood away from body cells and tissues and back to the liver for excretion from the body. HDL-C helps keep LDL-C from building up in the walls of the arteries. An inverse correlation exists between HDL-C levels and heart disease risk. HDL-C values < 40 mg/dL result in a substantially higher risk for heart disease and heart attack (estimates are age adjusted) (2), while levels ≥ 60 mg/dL help protect against heart disease (12, 44). The average HDL-C for men is approximately 45 mg/dL and 55 mg/dL for women (12). High levels of HDL-C are clinically more desirable**. Table 4** details HDL-C level classifications.

10. Hypercholesterolemia: borderline to undesirable TC cholesterol levels.

11. Immune milk*:* bovine milk obtained from hyperimmunized cows containing varying amounts of immunoglobulin proteins due to bovine regional variations (51,52).

12. Immunoglobulins (Ig): specialized proteins produced by the body in response to challenge by a foreign molecule. They are found mainly in serum,

colostrum, and milk, and serve to protect the body from microbial, viral, and fungal invasion (53). Immunoglobulin comprises at least 25% of the protein content of serum.

13. ImmunoLin™: a natural, lactose-free, allergy-free, concentrated immuno-globulin protein isolate extracted from bovine serum produced by Proliant, Incorporated (54). ImmunoLin™ (54) is derived from cattle specifically raised for food (54,55). **Table 5** details the contents of the ImmunoLin™ supplement at the treatment dosage level (55).

Table 5
ImmunoLin™ supplemental study product with detailed contents listing and percentage amounts[1]

Components	ImmunoLin™ (10 capsules)
Calories	20
Protein	5 gm
Total Protein	88.6% (by weight)
Immunoglobulin G (IgG)	46% - 53% (of total protein)
- Other Immunoglobulins (Ig)	IgM, IgE, IgD, IgA
(B-lactoglobulin)	0%
Proline-rich polypeptides	5% (of total amino acids)
- Growth factors present[2]	GH, IGF, TGF, etc.
Fat	0.2%
Lactose	0%
Ash	6.5%
Moisture	4%

[1] Wilke Resources: ImmunoLin™ vs. Colostrum. Internet: www.wilkeinternational.com/WILKEresources/ImmunolinColostrum.htm.
[2] GH – Growth Hormone; IGF – Insulin Growth Factor; TGF – Transforming Growth Factor

14. Low Density Lipoprotein Cholesterol (LDL-C): the transporter for most of the cholesterol throughout the body and the primary target for cholesterol lowering

therapy. As the predominant atherogenic protein, excess blood levels of LDL-C, also known as the "bad" cholesterol, can result in cholesterol (plaques) build up on artery walls that can result in damaging accumulation and blockage. Heart disease and stroke risk increases with rising LDL-C levels (12,5). LDL-C levels < 100 mg/dL are desirable (**Table 4**) (12).

15. Lipid panel (profile) test: a blood test used to measure heart health status via assessment of TC, LDL-C, HDL-C, and triglycerides (TG) (12). The latest National Cholesterol Education Program (NCEP) Guidelines for Detection, Evaluation and Treatment of High Blood Cholesterol in Adults lists lipid panel testing as the preferred initial test for detecting coronary heart disease, rather than just screening for TC and HDL-C alone (4,44). **Table 4** (12) displays NCEP guidelines for lipid panel measures (12).

16. Trans fatty acid (TFA): also known as trans fats, TFA are naturally occurring in some meat and dairy products. However, they are also commercially produced during the partial hydrogenation process of vegetable oils, which converts oil into a more stable liquid or semi-solid form (56). Trans fats are present in variable amounts in a wide range of commercially prepared foods containing partially hydrogenated vegetable oils. The AHA reports that partially hydrogenated vegetable oils provide about three-fourths of U.S. dietary TFA intake, which is estimated to range from 2.6 gm/d to 12.8 gm/d (57). Studies suggest that trans fat intake raises LDL-C and lower HDL-C levels, thus, increasing the risk of coronary heart disease (57).

17. Whey protein: a milk protein that accounts for about 20% of the total protein found in milk (58). The purest form of whey protein is whey protein isolate, which is 90% to 95% protein and is virtually fat and lactose free (58).

Assumptions of This Research

The following assumptions may have affected the results of the study:

1. All subjects will answer all questionnaires truthfully and reliably.
2. All subjects will be compliant with required supplement regimen.
3. All subjects will maintain current weight, exercise habits and dietary intake amounts while avoiding the initiation of new vitamin and/or mineral supplements and medications within 6W prior to the study.
4. All subjects currently on standard medical therapy for disease-related conditions will continue current medical regimen during the study.
5. The food frequency questionnaire is a valid and reliable food intake assessment tool.

Limitations of This Research

The following limitations may have confounded the study:

1. Broad age range of subjects included.
2. All subjects must be competent in reading and understanding the food frequency questionnaire.
3. Patient compliance with supplement protocol.
4. Limited sample size.
5. Pill counting utilized to determine treatment compliance.

Significance of This Research

This research is the first to measure the effects of an oral, highly concentrated source of bovine globulin protein on cholesterol indices in mildly hypercholesterolemic human subjects. Overall, this research contribution will add valuable information to the limited research available on animal globulin proteins, immunoglubulins, and their impact on human blood lipids. Furthermore, these results should warrant continued research surrounding medication alternatives and adjunct therapies for cholesterol reduction and management.

CHAPTER II*

REVIEW OF THE LITERATURE

The following chapter will discuss cholesterol in the body and the diet, the components of dairy products responsible for cholesterol reduction, dietary supplement management of elevated cholesterol and ImmunoLin™ product purity standards.

Introduction

High cholesterol levels are a significant contributor to heart disease (HD) and lowering elevated cholesterol levels has been scientifically proven to prevent HD and prolong life**.** Cholesterol, a soft, waxy substance synthesized primarily endogenously via the liver and acquired exogenously via the diet, is transported throughout the plasma as phospholipid, triglyceride and lipoprotein complexes (59). The body produces approximately 80% of its own cholesterol daily, between 800-1000 mg, while daily dietary sources contribute the remaining 20%, about 300 mg, to the total daily input of cholesterol in the body (60,61).

Blood cholesterol and lipid transport. The liver is involved in orchestrating cholesterol balance across the entire body in that it is responsible for cholesterol uptake, breakdown and excretion. The various synthesis and transport pathways of lipids throughout the body can be characterized as exogenous, endogenous or reverse cholesterol transport (62). Focusing on the exogenous lipid pathway, following intake of

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a meal, the epithelial cells of the intestinal mucosa mobilize dietary fat, TG and cholesterol into blood circulation by packaging them inside chylomicrons, complex protein coats (62). Circulating chylomicrons release TG's to the muscle cells and adipose tissue and deliver remaining dietary cholesterol to the liver via chylomicron remnant receptors present on hepatocytes (59). Between meals, the liver manufactures and secretes lipoproteins, TG and cholesterol esters back into plasma (endogenous lipid transport) to form very low density lipoproteins (VLDL), the precursors to LDL-C. Lipoprotein lipase (LPL) metabolizes VLDL cholesterol, releasing fatty acids into the muscle and adipose cells. Remnant VLDL particles are absorbed by liver cell low density lipoprotein (LDL) surface receptors, the remaining LDL-C is cellularly scavenged, and free cholesterol is released into cells whereby excess accumulation can occur within the arteries (62).

Removal of LDL-C is mediated through LDL liver cell surface receptors. These LDL receptors function by binding cholesterol particles from the blood and transporting them into the cells. The number of active LDL receptors on the surface of hepatocytes is directly correlated with the amount of LDL-C that is present in the bloodstream. LDL-C, the main carrier of circulating cholesterol in the body, is responsible for the deleterious formation of arterial plaque and atherosclerosis (62).

The final cholesterol transport process, often referred to as reverse transport, involves the synthesis of HDL-C, a lipoprotein involved in the prevention of atherosclerosis via its participation in the extraction of free cholesterol particles from artery walls and disposal of them through the liver (62). Elevated levels of LDL-C accompanied by depressed levels of HDL-C (high LDL/HDL ratios) are risk factors for

atherosclerosis, while low LDL-C levels accompanied by high HDL-C values (low LDL/ HDL ratios) are desirable.

Exercise and Serum Cholesterol

Serum cholesterol is influenced by many risk factors. Some are non-modifiable, such as heredity, gender, and age, as opposed to those that are more modifiable, such as exercise, diet and weight. The National Cholesterol Education Program (NCEP) promotes exercise, dietary modification and resultant weight loss for the treatment of abnormal lipoprotein. Inactivity is one of the four major risk factors for coronary artery disease (44). Increases in physical activity can benefit the heart and circulation, including improve cholesterol and lipid levels, reduce arterial inflammation, assist in weight loss, and help to keep blood vessels elastic and open (44). Dose response relationships between training volume (15 to 20 miles of brisk walking or jogging) and blood lipid changes exist whereby the amount of physical activity as it relates to large muscle mass usage and caloric expenditure have been deemed as the stimulus for altering blood lipids and lipoprotein levels (63). Based on a cross-sectional analysis report of various exercise-induced lipoprotein change studies, the average individual participating in regular aerobic exercise, which was characterized as jogging and/or brisk walking to produce a weekly energy expenditure $\geq$ 1200 calories, may potentially achieve 2 to 8 mg/dL increases in HDL-C levels and decreases in TC and LDL-C averaging ~ 4 mg/dL (63). Additionally, upon further review of the study literature, baseline study levels of TC and LDL-C along with variations in exercise intensity did not prove to be determinants for exercise-induced alterations in TC and LDL-C values.

However, in studies evaluating exercise-induced HDL-C increases, HDL-C baseline levels were shown to strongly positively correlate with increases in HDL-C levels following exercise, whereby individuals entering the study with normal or slightly elevated HDL-C values attained the greatest HDL-C increases upon trial conclusion (63). Overall, for the majority of individuals, studies indicate that training volumes of 15 to 20 miles of jogging or brisk walking expending between 1200 to 2200 calories per week will produce favorable lipoprotein effects (63).

Body fatness and effects on cholesterol. A second variable that can influence lipid levels dramatically is a loss in body weight, especially body fat loss. Total and LDL cholesterol values are traditionally improved with exercise, especially accompanied by weight-loss (64). Weight loss or body fat loss actually reduces visceral adiposity and fat mass and improves insulin resistance which promotes an increase in circulating HDL-C levels, lowers TG (another risk factor for heart disease) and decreases LDL-C via lipoprotein lipase (64-67). Weight loss accompanied by decreases in body fat can significantly reduce an individual's heart disease risk factors by positively affecting lipid levels (12). Reductions in body fat and weight, coupled by increases in lean body mass (LBM), can be accomplished through adhering to a routine exercise program and a modified caloric, low-fat eating plan. Managing not only the amounts but also the types of foods eaten to control weight will help reduce an individual's cholesterol and cardiac risk (12). For every 10 pounds of weight loss, an individual can lower their TC by 5-8% (12). Weight gain prevention should be emphasized for all individuals (12).

Dietary Cholesterol and Coronary Artery Disease Risk

The body produces all of the cholesterol that it needs to maintain a constant level for the body. However, blood cholesterol may be altered by elevated intake of dietary cholesterol and saturated fat, including foods such as meat, full fat dairy items, and baked goods. The average American man consumes ~ 337 mg of dietary cholesterol daily and the average woman ingests ~ 217 mg/d. (21). On the average, 20% of total blood cholesterol comes from the diet (60). Elevated dietary cholesterol intake can raise blood cholesterol values, which is associated with plaque build-up that can narrow or block blood vessels. The dietary strategy to reduce the risk of CAD focuses primarily on reducing total fat (68,69), saturated fatty acids (68-70), and cholesterol intake (69-71). Furthermore, elevated dietary saturated fat and cholesterol intake down-regulates LDL receptor-mediated clearance function via the liver, increases HMG-CoA reductase activity, and results in an increase in circulating cholesterol (72). Regulation of HMG-CoA reductase activity is the rate-limiting step in cholesterol biosynthesis (72). Additionally, study results have demonstrated a significant positive correlation between plasma TC and LDL-C levels and the susceptibility of LDL to oxidation; consumption of low saturated fatty acid diets decreased both (73). To lower LDL-C concentrations, LDL receptor function must be stimulated, and adhering to a diet that is low in saturated fat and cholesterol can accomplish this goal.

Dietary modifications that may be beneficial for lowering cholesterol and managing weight include (12): replacing animal products containing high saturated fat content with lean meats and skim dairy products to help lower TC values by 8 - 10%, and in turn, reduce CHD risk; limiting total dietary cholesterol intake to < 200 mg daily to

help reduce TC by 5 - 8%; increasing daily soluble dietary fiber intake with the addition of oats and/or psyllium husk to help attain a 3 - 5% reduction in LDL-C; and reducing trans fatty acid intake daily. Controlling the dietary blend of cholesterol lowering foods (noted above) can affect cholesterol reduction up to 20 to 30% (12).

Dairy Products and Cholesterol Reducing Agents

As noted above, lowering cholesterol through dietary management is possible by maintaining an eating plan tailored to include specific cholesterol lowering foods daily (12,26). Milk and dairy products, because of their high saturated fat and cholesterol levels, are often included among the forbidden foodstuffs for people with high serum cholesterol levels. However, despite the fact that whole milk dairy products may negatively affect cholesterol levels, one human study conducted over five weeks compared the effects of daily dietary additions of 2 liters (L) skim milk, yogurt and full cream milk in teenage boys. The results of this study showed no rise in TC concentrations in the full cream milk group. Furthermore, this study also demonstrated that HDL-C and the percentage HDL/TC levels rose highest within the first week with full cream milk consumption (74). A review panel concluded that whole milk may not affect blood lipids due to its fat content and fat composition as previously thought (75).

Cholesterol-lowering effects of milk. Interestingly, some cholesterol-lowering effects have been found to be directly associated with the consumption of whole and skimmed dairy products, namely milk and yogurt (76-80). The potential mildly hypocholesterolemic effect of milk and fermented milk products were initially proposed based on a study conducted by Mann (81) involving the Masai people of Africa. Despite

their low incidence of CVD, the Masai's dietary intake consists of large amounts of meat, milk and blood products, and a diet that could be described as atherogenic. In this report, consumption up to 8.3 liters (L) of milk daily by Masai men aged 16–23 years, resulted in lower TC, despite weight gain in the participants. Of importance to note, this study was terminated after three weeks instead of at the four week study protocol due to the extreme rise in milk consumption from 3-5 L/day (d) before study initiation to 8.3 L/d by the fourth day of the study, lack of encouraged increases in physical activity and resultant weight gain. The study concluded with a positive correlation between increases in milk consumption and cholesterol reduction, noting that the greater the milk intake, the greater the decrease in cholesterol concentration. No rationale was provided to explain the decline in cholesterol values despite subject weight gain, however studies have also focused on inter-individual differences in metabolic responses to dietary saturated fat and cholesterol, along with alternate explanations for low blood cholesterol in African herders, which has been attributed to physiological adaptations that reduce cholesterol synthesis (82,83). Nevertheless, Mann concluded that a component in milk decreased cholesterol concentrations and that milk acts as an inhibitor of cholesterol synthesis. These study findings are difficult to extrapolate to healthy Americans where epidemiological, clinical, and biochemical studies have shown that dietary saturated fat and cholesterol raise total plasma cholesterol and LDL-C levels and induce atherosclerosis (12). Nevertheless, there is considerable evidence in humans that some individuals are more sensitive to a high fat, high cholesterol diet than others (84). Additionally, it is highly likely that there is a major

genetic component of dietary responsiveness, however, the genes involved and their common variants are largely unknown (82).

The hypocholesterolemic effect of milk in humans has been demonstrated by several research studies (76,80,81,83-91). One study of particular interest, conducted by Buonopane, et al. (76), concluded that the daily dietary addition of 1.1 L of 2% solids-non-fat fortified skim milk for eight weeks in 64 free-living male and female participants was associated with statistically significant reduction in TC levels. Subjects were equally divided into low (<190mg/dL) and high (≥190 mg/dL) baseline TC groups. Results demonstrated 5.7% and 6% reductions in TC in the low and high baseline TC groups, respectively. With no variations in other hypercholesterolemic factors, such as body weight, the decrease in plasma cholesterol concentration in this study was deemed to have been a direct result of the milk consumption. Study authors believed that subject's compliance to milk supplementation was optimal while the maintenance of a stable body weight throughout this study was attributed to a lack of compliance to baseline dietary intake patterns. Dietary intake alterations, specifically calorie and nutrient intake variations, were not reported, however statistically significant (2-3%) decreases in dietary fat intake were noted in the high cholesterol subject group. Aside from the large quantities of milk consumption in the Mann (81) and Buonopane, et al. (76) studies mentioned above, results remain conclusive that within free-living populations, milk intake lowers TC values. However, the exact mechanisms involved in this reduction along with the factors in milk responsible for the lowering effect continue to remain elusive.

Effects of fermented milk products on cholesterol reduction. In order to

help distinguish the factor in milk responsible for its cholesterol-lowering property, several researchers focused their attention specifically on the cholesterol-reducing ability of fermented milk products, including yogurt (78,85, 86,88,89-94). Human studies detailing the link between fermented milk products and cholesterolemia date back to the 1970s when Hepner, et al. (85) compared the hypocholesterolemic effect of pasteurized and unpasteurized yogurt (three 240-mL portions per week) in a crossover trial involving 17 free-living subjects. Subjects supplemented their daily habitual diet with yogurt or 2% milk (720 mL) for four weeks followed by a 4-week washout period and reverse consumption. After a week of receiving unpasteurized yogurt, TC concentrations decreased by 5%, but continued on a rising trend following the wash out period and milk supplementation. For the milk first group, plasma TC decreased insignificantly, however, following the washout period and one week of yogurt supplementation, TC decreased significantly by 9%. Hepner, et al. (85) conducted a similar secondary experiment and supplemented the daily dietary intake of 36 free-living individuals with 240 mL of unpasteurized and pasteurized yogurt, containing live cultures of *L. bulgaricus* and *S. thermophilus*, for 12 weeks. Study results concluded that both yogurt types, unpasteurized and pasteurized, produced decreases in serum cholesterol of 9% and 5% respectively. The authors of this study concluded that milk may possess a small hypocholesterolemic effect, which may be mirrored by yogurt.

In a later study by Agerbaeck, et al. (91), normocholesterolaemic subjects supplemented their normal daily dietary intake with 200 mL of fermented milk product. Following six weeks of supplementation, the study subjects' TC and LDL-C concentrations were reduced by 6% and 10% respectively. Milk supplementation has

produced a decrease in TC concentrations in several human studies; yet, the components of milk with cholesterol-lowering properties have not been adequately identified. Interestingly, the protein fraction of milk may be a likely candidate for inquiry rather than the fat content because both skim milk and fermented milk (yogurt) have been found to increase HDL-C and lower TC when compared to whole milk (77,85).

Effect of milk proteins on cholesterol. A few studies have been conducted to ascertain the cholesterol-lowering effects of milk proteins. Of the major proteins found in milk, those found in whey present an interesting avenue for investigation as various studies of casein, the primary protein in milk, have resulted in slightly hypercholesterolemic (animal model) and cholesterol neutral (human model) effects, meaning casein produced no effect on cholesterol concentrations when compared to other sources of protein (94, 95). In an animal model, Terpstra, et al. (95) demonstrated that semi-purified diets containing differing proportions of casein protein (10%, 20%, 40%) fed to rabbits over seven weeks compared to a commercial diet feeding regimen resulted in elevated serum cholesterol positively correlated with increases in casein protein within all groups. Rabbits fed the commercial diet containing a lower fat and caloric density (2,800 kcal/kg feed) consumed a much larger amount of food than those in the semi-purified diet groups (3,400 kcal/kg feed). Over the first 4 weeks of the study, the low-casein diets (10%) produced serum cholesterol and growth rates that were significantly lower than high-casein diets (40%). Within the last 3 weeks of the study, rabbits switched from 10% to 40% casein diets exhibited statistically significant increases in levels of serum cholesterol with the highest weight gain being observed in this group. Throughout the study duration, cholesterol remained constant in the groups receiving

the 20% casein and the commercial diets. Additionally, growth rates and weight gain were found to be similar within the 10% and 20% semi-purified diets and none of the rabbits exhibited weight loss. Study findings showed no consistent relationship between the rabbit growth rates and levels of serum cholesterol. Furthermore, study conclusions suggested that the formation of whole serum lipoprotein particles relatively rich in cholesterol were associated with increasing levels of dietary casein intake.

In another study, Damasceno, et al. (96) fed 20 rabbits diets containing 27% casein compared to 27% soy protein isolate for two months and found that the casein feeding contributed to increasing cholesterol concentrations, lipoprotein oxidation and aorta atherosclerotic lesions. In contrast, the soy protein isolate diet, when compared to the casein diet significantly decreased cholesterol concentrations, atherosclerotic lesions and lipid peroxides of LDL fractions.

In agreement with these above mentioned studies, Ho, et al. (97) fed swine high fat, high cholesterol diets with either casein or soy protein for five weeks and documented statistically significant elevated TC levels (334 +/- 46mg/dL) in the casein fed group over the soy protein group (122 +/- mg/dl). In this study, the measured three-hour lymphatic transport of cholesterol in the casein-fed swine was found to be significantly higher than in the soy-fed group and authors concluded that dietary proteins likely affect cholesterol transport into the lymphatics.

In animals, casein ingestion produces a hypercholesterolemic effect, but in humans, more cholesterol neutral effects have been documented (98-100). Sacks, et al. (99) studied the cholesterol modifying effect of 27 grams (g) of casein added to the daily diet of 13 strict vegetarians who consumed no other form of animal protein during

the 40-day study period. In this study, the casein supplementation produced no significant effect on TC or LDL-C concentrations. In support of these findings, Grundy, et al. (100) compared the effects of dietary plant protein (soy) to animal protein (casein) intake on plasma lipoproteins in 14 men on a metabolic ward. Following one month of strictly controlled dietary intake of 15% casein protein, 55% carbohydrates, and 30% fat (as lard), there was no change in plasma TC or LDL-C concentrations. These study findings support the idea that the cholesterol-lowering component of milk is likely not casein.

Ritzel, et al. (101) chose swine in his controlled feeding study in an attempt to examine the effect of large whey protein feedings (57% of the total energy consumed as whey protein powder) on serum lipids in animals. At the conclusion of 112 days, study results demonstrated a significant reduction in TC by 6% with the whey feeding. Kawasa, et al. (102) supported these findings with the examination of the effect of ad libitum feeding of fermented milk supplemented with 80% whey protein concentrate for two weeks on the TC in rats. Study results showed a statistically significant reduction in serum TC levels when compared to the control group. In addition to the rat study, Kawasa, et al. (102) also conducted the only study that could be found demonstrating the effect of whey protein on cholesterol values in human subjects. This 8-week study examined the consumption of 200 mL of fermented milk supplemented with whey protein in 20 healthy adult male subjects. Although a TC reduction was unreported in this study, results showed a statistically significant increase in HDL-C after four weeks compared to no change in the placebo group along with a statistically significant reduction in subjects' mean atherogenic index from 4.24 to 3.52. Overall, the results

from these studies point to a possible effect of the whey protein fraction of milk on serum lipid levels (101,102). However, several other mediating factors have been proposed, including immunoglobulin G (IgG) (52).

In an effort to narrow down the true cholesterol-lowering components in milk, Golay, et al. (52) and Sharp, et al. (51) reported that the daily administration of 90 g of dried skim milk (immune milk from hyper-immunized cows) lowers cholesterol in human subjects with mildly elevated cholesterol concentrations. Golay, et al. conducted their experiment with immune milk produced from dairy cows hyper-immunized with a multivalent bacterial vaccine. Following an eight-week ingestion of 90 g daily immune milk compared to non-immune milk intake (control), a significant reduction in plasma TC and LDL-C was demonstrated in mildly hypercholesterolemic human subjects. Sharp, et al. reproduced this study in 30 subjects for 38-weeks and demonstrated similar cholesterol reduction results due to immune milk consumption. The results of this study concluded that daily supplementation of an individual's normal diet with skim milk from immunized cows when compared to control milk intake can result in a significant reduction of elevated TC and LDL-C concentrations. In both of these studies, authors theorized that the decline in study participant's cholesterol concentrations could be attributed to the immune milk containing higher levels of IgG. Immune milk may be a useful adjunct in the dietary management of hypercholesterolemia due to its higher quantity of immunoglobulins; however, the immunoglobulin content of immune milk requires further study. Whereas milk and whey contain 1% and 3-4% globulin protein concentration, respectively, immunoglobulin comprises at least 25% of the protein content of serum.

Immunoglubulins and Cholesterol Reduction

In an effort to further the research on the cholesterol-lowering effects of immunoglobulins, Proliant, Inc., a biotechnology company, has developed a highly concentrated immunoprotein-rich product, called ImmunoLin™ (34). ImmunoLin™ is an immunoglobulin-rich isolate extracted from the serum of cattle specifically raised for food (34). The result of this process allows consumers to ingest smaller quantities of product with higher levels of immunoglobulin proteins, unlike the impractical intake of 90 grams of immune milk daily as mentioned in the study above.

Based on multiple anecdotal reports of cholesterol reduction in consumers taking the ImmunoLin™ supplement, Proliant conducted an 8-week, in-house pilot study (E Weaver, C Siefken, and R Strohbehn, unpublished observations, 2002). Eleven hypercholesterolemic human subjects (TC > 200 mg/dL, HDL-C < 40 mg/dL) ingested 5g daily supplementation of ImmunoLin™ for one week. TC and LDL-C values were measured at study initiation, midpoint (4W) and conclusion (8W). At 8W, participants with mild hypercholesterolemia showed a statistically significant TC and LDL-C concentration reduction of 15% and 6%, respectively, from baseline. No formal data has been published on the corporation's study findings. Blood lipid level reductions in these individuals may be attributed to the elevated immunoglobulin protein levels in the ImmunoLin™. In addition, this study suggests that ImmunoLin™ may be useful in the management of hypercholesterolemia, specifically TC and LDL-C reduction.

Nutritional Supplements and Cholesterol Reduction

Numerous supplements available on the market today tout heart health benefits along with cholesterol reducing ability (103-106). While supplements are not a substitute for proper medical treatment, many individuals turn to natural alternative supplements for management of medical conditions. As many studies support the value of cholesterol lowering supplements, some natural supplements have been shown to be beneficial adjuncts to dietary modification in controlling cholesterol; therefore, continued investigation is necessary (107-109).

Phytosterols and phytostanols. Only a few of these researched supplements produced statistically significant cholesterol lowering ability in study trials. Phytosterols, naturally occurring plant compounds, are an example of one such supplement that, when ingested in conjunction with a low-fat diet, possess the ability to lower the risk of coronary heart disease (CHD) via profound reductions in TC and LDL-C (107). The primary mechanism responsible for the cholesterol-reducing activity of phytosterols is the inhibition of cholesterol uptake from the intestinal tract in humans (108). However, the actual sites within the intestinal tract where inhibition may occur have not been fully elucidated.

As a food ingredient, phytosterols are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) and they are found naturally in meat and dairy products. Phytosterols are converted to phytostanols through a commercial chemical process called hydrogenation, whereby a liquid vegetable oil is converted to a semi-solid or solid form. Both phytostanols and phytosterols are readily incorporated into food products, such as tub margarine spreads (107). The most common dietary

phytosterol is sitostanol (mixtures of soy sterols), a saturated derivative of sitosterol. In a comparison review of three randomized control trials, Law (107) reported a study average of 14% LDL-C reduction with a $\geq$ 2 g dose of plant sterol or stanol per day, which equals the amount currently added to an average daily portion of fortified margarine. Additionally, following review of six randomized phytosterol trials, Law concluded that cholesterol reduction and decreased incidence of heart disease by one quarter with the use of sitostanol-containing spreads was common in all six interventions. To further support these findings, Jones, et al. (108) demonstrated a statistically significant reduction in LDL-C of 24%, when combined with dietary change, in subject's whose diets were supplemented with 1.7 g of a plant-sterol product containing sitostanol as compared to a 9% decrease in the diet-only part of the trial. Additional controlled and double-blind trials have confirmed these results (110-113).

Guggulipid. Another cholesterol-reducing alternative medicinal therapy, guggulipid, derived from an extract of guggul gum, is actually an approved treatment for elevated cholesterol concentrations in India (106). Often referred to by a number of names, such as guggul gum, guggul and guggulsterone, guggul is a natural herb whose resin is extracted from the Commiphora tree, has been an Ayurvedic medicine approach to preventing atherosclerosis for centuries, and has been studied in Eastern clinics and hospitals for over 20 years (y). Two compounds, Z-guggulsterone and E-guggulsterone, appear to account for guggul's TC and LDL-C lowering affects and the boosting of HDL-C levels. Urizar, et al. (114) found that guggulsterone, the active agent in guggul extract, targets and blocks the activity of the Farnesoid X receptor (FXR), a

nuclear hormone receptor present in the liver cells involved in the regulation of cholesterol via its function in monitoring levels of bile acids, which are produced from cholesterol and released by the liver. In this study, wild-type and FXR-null mutant mice were fed a guggulsterone supplemented high-cholesterol diet for one week and results demonstrated a statistically significant decrease in hepatic cholesterol levels in the wild-type mice that was absent in the FXR-null mice. However, the mechanism for guggulsterone's effect on the FXR remains unidentified (114). In another study by Singh, et al. (109) researchers reported that the combined effect of a fruit- and vegetable-enriched prudent diet with 50 mg of guggulipid capsules taken twice daily for 24 weeks by 61 hypercholesterolemic individuals produced an 11.7% and 12.5% decrease in TC and LDL-C levels, respectively, compared to the pre-diet levels. The TC and LDL-C levels in the placebo group were unchanged. In this study, the subjects were reported to be > 96% compliant to ingestion of the guggulipid capsules. Final study comments noted that, at 36 weeks, the effect of diet and guggulipid was equal to that of traditional lipid-lowering drug treatments, however, the subjects' final (36-week) cholesterol percentage reductions were not reported.

Red yeast rice. One of the more promising nutritional supplements, an ingredient in red yeast rice, called monocolin K, has been shown to lower cholesterol (104,105). For numerous centuries, red yeast rice has been a component of Chinese medicine and a dietary staple in many Asian countries with usual consumption ranging from 14 – 55 g per day (0.5 – 2 ounces) (104,115). Red yeast rice was documented by Heber, et al. (115) in a double-blind 12-week clinical trial to lower LDL-C concentrations

by 22% as compared to 1% in the placebo group within 83 men and women with above average LDL-C concentrations. Within this study, the supplemental red yeast rice product inhibited the production of cholesterol by halting the action of the primary liver enzyme, HMG-CoA reductase, which is responsible for cholesterol synthesis. These authors noted the low cost of red yeast rice supplements in comparison to prescription medications, and concluded that this supplement could provide a more cost effective and innovative approach to the maintenance of healthy cholesterol concentrations. However, since red yeast rice extracts are not pure, meaning they contain additional substances, their effects may be less predictable than drug treatments.

Monacolins, compounds of the statin class, were found to behave similarly to the drug, lovastatin, which is currently used for cholesterol-reduction. Yet, significantly lower amounts of the monacolins contained in the red yeast rice were necessary to elicit a reduction response comparable to the cholesterol-lowering drug (104,105,115,116). However, due to the similarities between statin drugs and the contents in red yeast rice supplements, an impending infringement on patent rights was filed resulting in the sale of red yeast rice being banned in the United States.

ImmunoLin™ Supplement Product Purity

Unfortunately, following the conclusion of this study, the first case of a bovine spongiform encephalopathy (BSE)-infected cow within the United States food supply was documented. Treatment group subjects became slightly concerned about having taken the bovine-derived ImmunoLin™ product. However, subjects' questions were

thoroughly addressed and concerns were quelled as they were assured that the ImmunoLin™ product was produced under stringent quality control procedures. In addition, subjects were informed that the raw material for the ImmunoLin™ product were collected in USDA-inspected facilities and approved for use in food products (**Appendix D**).

The purification process of Proliant's ImmunoLin™ product was conducted in a closed system, which rigidly insures against product contamination. The Proliant manufacturing facility itself is compliant with FDA Good Manufacturing Practices (GMP) for food products, uses only United States beef cattle and cattle less < 30 months of age in the production of their product. Furthermore, Proliant's ImmunoLin™ product was derived from FDA-approved blood products, excluding any brain or spinal tissue. These above mentioned measures aid in eliminating any threat of contracting BSE (Mad Cow disease) from ingestion of Proliant's ImmunoLin™ supplement.

CHAPTER III*

MATERIALS AND METHODS

This chapter will describe experimental subject selection criteria, method of selection, instruments used in measuring experimental variables, experimental procedures, and data analysis.

Subjects

In this study, 52 men and women aged 25-70 years (y) volunteered for this investigation conducted at The Cooper Institute (CI) Center for Human Performance and Nutrition Research (Dallas, TX USA).

Recruitment. Individuals were recruited through eligibility screening for Cooper Clinic (CC)** employees and the surrounding Dallas/Ft. Worth community in person, by word-of-mouth, radio, television and newspaper advertisements (**Appendix A**).

Initially, 500 potential participants were screened by phone. Two hundred and fifty eligible participants were qualified based on answers to key questions on the medical history questionnaire. Qualified participants completed medical history, food

*Part of the data reported in this chapter is reproduced with permission by the *American Journal of Clinical Nutrition.* © Am J Clin Nutr. American Society for Clinical Nutrition from "Cholesterol-lowering effects of bovine serum immunoglobulin in participants with mild hypercholesterolemia" by Earnest CP, Jordan AN, Safir M, Weaver E, Church TS. *Am J Clin Nutr* 2005;81:792-8.

** While the CI and CC are both located on the same grounds of the Cooper Aerobics Center campus, they function as fully separate entities. The CI is a non-profit, tax exempt, and public corporation by virtue of its scientific research and professional training/certification endeavors, that operates with its own Board of Trustees and Scientific Advisory Council. The Institute also maintains financial independence from any other Cooper Aerobic enterprises.

frequency and qualities of life questionnaires via an online Internet data collections system (Vitalink, Bellevue, WA, USA) or were mailed questionnaires (without Internet access) (**Appendix B**). Upon completion of the questionnaires, participants provided written informed consent approved by The Cooper Institute Institutional Review Board and Texas A&M Internal Review Board prior to entering the investigative, blood cholesterol-screening portion of the study protocol (**Appendix C**). The elapsed time between the initial phone screening and the first blood sample was less than two weeks.

Exclusion criteria. Participants were excluded from the trial if their plotted body mass index (BMI; in kg/m^2) was < 18.5 or > 30; they had recently donated blood (< 3 months) or failed to agree to donate blood during the trial period; they were pregnant, lactating or considering pregnancy during the study period. Also excluded were participants with elevated blood pressure, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) or fasting plasma glucose requiring immediate drug therapy according to national guidelines (Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, Adult Treatment Panel II, and American Diabetes Association). Lastly, those individuals who were already taking lipid-altering medications, planned to move from the area during the trial, smoked, consumed alcoholic beverages exceeding an average of 3 drinks/day (d) or consumed coffee > 710 mL/d (in excess of 3 cups/d) were excluded from the study. Coffee drinkers were excluded from this study due to potential impact of coffee intake on TC and LDL-C levels (117,118).

Inclusion criteria. Inclusion criteria for this study necessitated that all participants, men and women, possess borderline to undesirable elevated TC ranging between 5.44 to 6.99 mmol/L (210 mg/dL to 270 mg/dL), with HDL-C less than 1.81 mmol/L (70 mg/dL). In addition, subjects were to be free of cholesterol-lowering medications. If a potential participant was unsure of their TC concentration prior to baseline screening, but thought it may be high, these participants were informed of their baseline TC concentration so that they could make an informed decision whether to enter the trial or seek medical counsel. In addition, advice was provided to those participants with LDL-C $>$ 4.14 mmol/L (160 mg/dL) regarding the risks associated with LDL-C concentrations of this nature and these individuals were encouraged to see their physician. If the subject chose to continue participation in the study, a written medical clearance from their physician was requested before study initiation.

Habituated vitamin and/or nutritional supplement users for at least 3 months prior to the study inclusion qualified for the study if they were willing to maintain their current supplement intake throughout the duration of the study. All individuals who agreed to maintain their current dietary intake and exercise habits were also eligible for study participation. Postmenopausal women, both on and off hormone replacement therapy (HRT), were accepted into the study. Finally, participants on standard medical therapy at study initiation (for conditions such as hypertension, arthritis or other chronic diseases) were included in the study if they agreed to remain on their current therapy throughout trial duration. All of these variables were examined during the trial via online and mail return questionnaires. A quality of life questionnaire was administered pre-

and post-study in order to assess not only the subject's mood and feelings, but also to identify any beneficial supplemental affects on study outcome.

Confidentiality. All subject names remained confidential throughout the duration of the study. An assigned number coding system ensured subject confidentiality throughout the study duration. In addition, group selection or subject randomization was computer-generated.

Estimated benefits. Subjects taking ImmunoLin™ may have benefited from a reduction in serum TC and LDL-C, thereby improving their cardiovascular risk factors. In addition, subjects participated in the study without cost and received a complimentary copy of their blood lipids and laboratory test results at study conclusion. Following trial completion, each subject was awarded a $100 compensation fee for his or her willingness to comply with the daily ingestion of ten capsules and the multiple blood collections. If for any reason a subject was unable to complete the entire eight-week study period, full compensation was still awarded for their participation. After completing the trial, any individual assigned to the placebo group received 6 weeks worth of the active ImmunoLin™ product to try on their own.

Possible risks. The risks associated with participation in this study were nominal (119). Upon each of the 5 blood draws, subjects may have experienced a temporary stinging pain along with the chance of possible bruising at the puncture site (119). To prevent any discomfort and risk, only trained and licensed CI staff phlebotomists were responsible for collection of blood samples. In addition, the study was conducted at a medical facility, the CI, with licensed staff nurses and board-certified physicians on site. An informational hotline, 972-341-3284, was also

maintained by the CI's Principal Investigator and Project Research Director, Dr. Conrad Earnest, to answer study participant questions or allow them to report any side effects that may have occurred throughout the duration of the study.

Subjects reported no side effects to the ImmunoLin™ supplement except a small amount of weight gain and intolerance to the product delivery system of 10 capsules to ingest daily resulting in poor compliance (~75%) to our study treatment.

Survey Instrument

The food frequency questionnaire (**Appendix B**) utilized in this study consisted of questions concerning the types of foods the study participants consumed throughout the duration of the study. The questionnaire contained a list of food items and required participants to indicate whether or not they consumed a particular food by marking the average number of times they had eaten that food within the past month, week or day. Additionally, to ensure that there were no questions left unanswered on the questionnaire, participants were to record a no response if they did not consume a particular food within the month.

The CI's food frequency questionnaire was developed using 3-day dietary records of individuals from a similar study population (120). The questionnaire was specifically designed to focus on five key dietary components, including energy and fat intake. Non-quantitative portion sizes, based on the USDA standard portion size amounts for common foods, were used to estimate the amounts of particular foods recorded as consumed (121). Because these portion sizes are only estimates of the exact amounts actually eaten, this type of food frequency questionnaire is most

effective for estimating intake amounts for key dietary components across a group of individuals versus for measuring individual dietary intake (122).

Food frequency questionnaires were administered at baseline and at 6 weeks (W) to examine total energy intake and macronutrient partitioning. Following completion of the questionnaires, each was computer-scanned. Based on subject question responses, summary dietary information was generated for total energy, fat (including the different types), cholesterol, and alcohol using the Food Intake Analysis System (FIAS; version 3.9, Human Nutrition Center, University of Texas Health Science Center School of Public Health, San Antonio). The FIAS system was selected for this study because it is linked with the Pyramid Serving Database (PSDB) (123). The USDA food codes generated after analysis of the dietary recalls in FIAS are correlated with PSDB to determine the number of servings of each of the major food groups consumed by study participants. The database was developed to analyze the number of servings in each of the Food Guide Pyramid's major food groups, including the amounts of discretionary fat and sugars consumed (124). Results from the study food frequency questionnaires are reproducible, valid and reliable (122).

Pre-Trial Screening and Baseline Testing

A four-phase approach was utilized in performing this trial. These phases included a telephone screening procedure to determine potential eligibility; three baseline visits, inclusive of two run-in screening visits; 6 weeks (W) of treatment, inclusive of a 3W mid-study visit; and a post-test assessment. A general schematic of the trial is presented in **Figure 1.**

During the first phase of the trial, 500 potential participants were screened by telephone resulting in 250 individuals who were qualified to begin the baseline-testing portion of the trial. Once participants were determined eligible to continue the trial, during this second phase, each participant was requested to participate in three baseline visits prior to formal randomization into the trial. During the first two visits of this testing phase, each subject participated in two run-in visits within 7 days of each other. Successful continuation to the third baseline visit was predicated on obtaining two TC concentrations differing by $\leq$ 10%. If the TC difference between these two visits was > 10%, a requested third baseline measurement was performed. Averages of the two closest baseline measures were accepted for the entry criterion lipid variable. Following this run-in procedure, 90 participants remained eligible to continue to the third baseline visit of the trial where blood collection was performed to examine other blood markers using a basic blood chemistry panel (Chem-16) measuring 16 variables associated with alterations in hepatorenal or cardiovascular function.

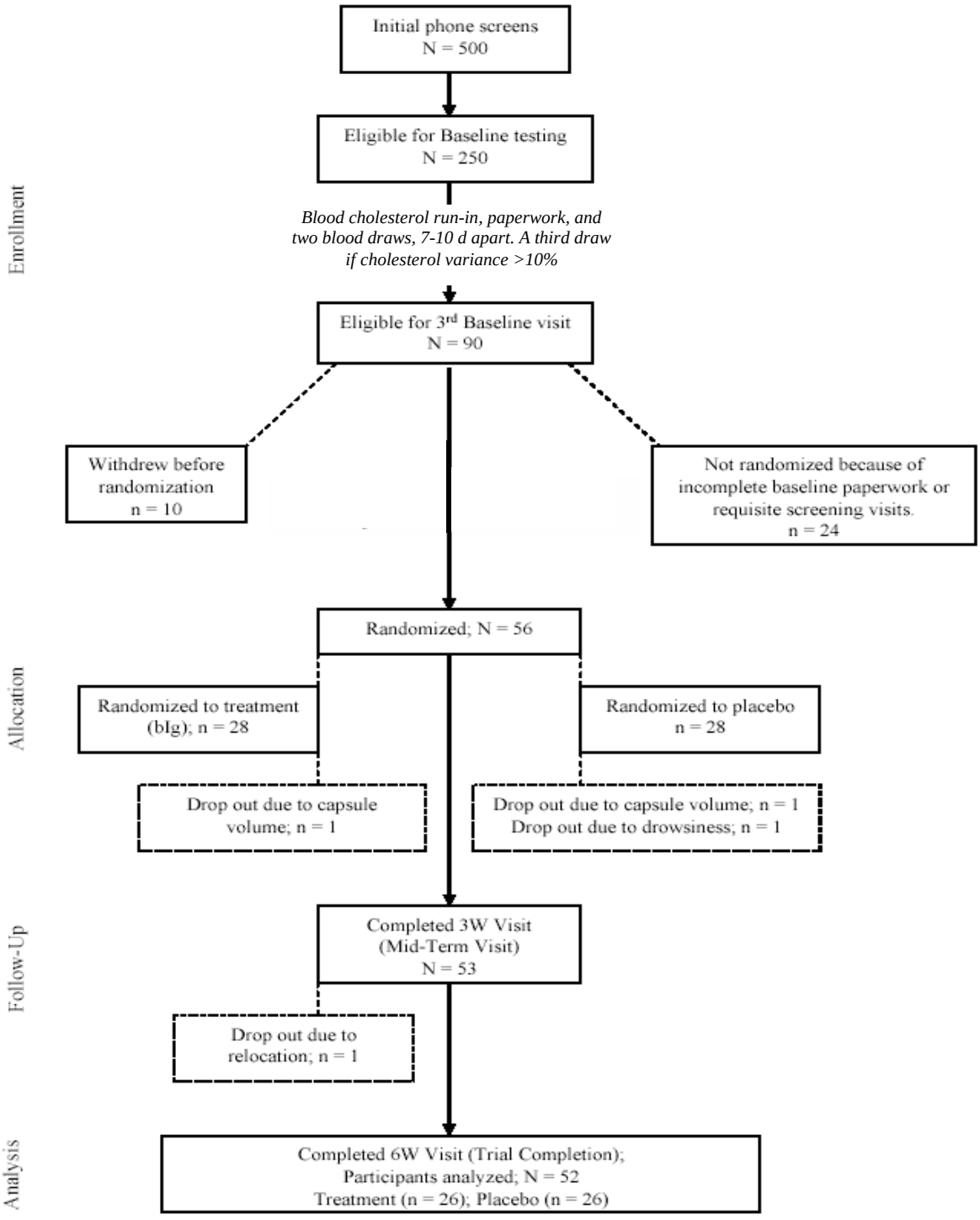

Figure 1. Schematic representation of study timeline**.** bIg, bovine serum immunoglobulin.

In conjunction with the third baseline visit, each participant was required to complete several questionnaires detailing their medical history and dietary habits. These questionnaires were made available via an online Internet data collection system (Vital Link, Bellevue, WA, USA). Questionnaires were mailed to those participants without Internet access. Once participants completed all forms and the three baseline visits, they became eligible for formal random assignment into the treatment portion of the trial. If these conditions were not met, the participant was not randomized into the treatment portion of the study. The time elapsed between the first run-in visit and the third baseline collection period ranged from 10 to 12 days.

The rationale for using this type of enrollment procedure was to establish stable baseline entry criteria for TC, as well as attempt to minimize dropouts and maximize protocol compliance by dissuading participants who were less likely to complete the trial given their low willingness to adhere to the study procedure. Of the 90 participants eligible for random assignment, 10 withdrew prior to randomization due to “lack of continued interest” in pursuing the study and 24 failed to complete all baseline visits and/or necessary paperwork. Thus 56 participants received formal random assignment randomized to undergo treatment and enter the third phase of the study, i.e. the treatment phase.

Treatment and Assessment Indices

The treatment source of bIg utilized in this study is an immunoglobulin-enriched serum protein fraction obtained through the partial removal of fibrinogen, lipids and albumin. The resulting total protein fraction was 88.6%, with the remainder of the chemical constituents being moisture (< 8%) and ash (< 3%). The total IgG was 48.4%

as determined by radial immunodiffusion assay. Proliant's ImmunoLin™ treatment was obtained from the plasma of USDA inspected and approved beef cattle raised for food that were < 30 months of age, purified by membrane filtration and spray dried. Hydrolyzed gelatin, a cholesterol-free, GRAS protein source was utilized as the source of protein for the control group as it was a cost-effective and easily manufactured treatment option (125). All treatment bottles and capsules were distributed using identical bottles, size and coloring via a randomized number sequence for treatment distribution so that in the case of side effects, the treatment code of that particular individual could be broken rather than sacrifice the integrity of the entire treatment group. One of the investigators was in charge of this aspect of the study (ANJ) and had no contact with any of the study participants at anytime nor was involved in the data analysis process.

During this third trial phase, each participant was randomly assigned, in a double-blind manner, to consume ten treatment (bIg) or placebo capsules, containing 5 g of the appropriate treatment, daily (5 capsules; BID) for 6 W and verbally instructed to take them with a meal (5 capsules in the morning, 5 capsules at lunch or dinner with the latter being their choice). The 10 capsule treatment regimen provided 5 g of Proliant's ImmunoLin™ per day (34). The ImmunoLin™, treatment dosage amount for this study was determined based on Proliant's in-house pilot data showing this amount to be efficacious (unpublished study results, Proliant Inc. 2002). Additionally, due to the use of capsules, the possibility of using a larger dosage was prohibitive. Subsequently, the sample size for this trial was chosen on the basis of power calculations obtained from this pilot effort.

Given that this was a study using a “free living” population, and recognizing the fact that alterations in body mass and energy intake can affect cholesterol concentrations, participants were weighed and food frequency questionnaires were collected before (baseline) and following (6W) study intervention (fourth phase of the trial) to examine total energy intake and macronutrient partitioning. At trial initiation, 3W, and trial close (6W) all participants were questioned about noted side effects and encouraged to report any noticed side effects throughout the 6-week study duration. Additionally, all participants agreed to maintain their current dietary intake and exercise habits and to avoid beginning the consumption of any new supplement or medication.

At study initiation, participants received enough capsules for 3W worth of treatment (bIg) or placebo. All participants were requested to return at the 3W study interval for interim blood measurement and determination of capsule compliance by pill count. Following the 3W visit, new bottles were provided to study participants with enough treatment or placebo capsules to complete the trial (6W). Upon return of all study participants at 6W, a final pill count was performed.

The validity of pill counting may be affected by loss of pills or fabrication of pill ingestion, both of which can result in overestimation of treatment intake (126,127). However, the pill count method is still one of the most common methods used in assessing medication compliance instead of self-reported pill ingestion via patient interview, which has been regarded as unreliable for accurately assessing adherence to a medical regime (126,127). Unfortunately, there appear to be no viable alternatives to this method of obtaining data on medication compliance.

Blood was obtained in a fasting condition (> 12 hours) at pretrial, 3W and 6W for several analyses, including a blood lipid profile, blood glucose concentration, and

muscle, kidney, and hepatorenal indexes (Chem-16). At blood collection, ≈ 50 mL of blood were divided into one serum separator evacuated tube (10 mL) and four K3 EDTA-coated tubes (≈ 40 mL). Licensed CI staff phlebotomists drew the blood for this study. Subsequently, all blood samples were spun within 3 minutes of venous collection in a cold centrifuge at 2383 x *g* for 15 minutes at 4°C (Celsius). Separated plasma and red blood cells were divided into four cryovials and placed in –80 °C freezers.

Blood lipid profiles, fasting blood glucose concentration, and muscle and hepatorenal indices were analyzed at a commercial laboratory, (Lab Corp, Dallas, TX USA). The Friedewald formula (128) was utilized to calculate LDL-C, where LDL-C = [TC – (HDL-C + TG/5)]. In order to validate the use of an external laboratory, where minor differences in cholesterol may be related to the time of day of the analysis, changing technicians and/or different reagents used over the course of the clinical trial, 40 frozen blood samples were randomly chosen for a reliability analysis and sent to an alternate laboratory. These samples were tested in batch on the same day. The results of this secondary sample analysis showed no difference in TC or LDL-C with the data obtained from LabCorp (CV = 0.08; r^2 = 0.96) subsequently utilized in this study report.

Statistical Analysis

The primary outcome variables examined in this trial were TC and LDL-C. All other lipid sub-fractions inclusive of very low density lipoprotein cholesterol (VLDL-C), HDL-C and TG were treated as secondary outcome measurements. As tertiary measures, blood glucose and the Chem-16 panel were examined. All baseline, primary and secondary outcome measurements were examined for normal distribution

characteristics. As such, each variable was found to be normally distributed; thus, data were not adjusted.

In order to examine potential treatment differences in this study, primary, secondary, and tertiary variables were examined separately using a repeated measures multivariate analysis of covariance (MANCOVA), covaried for a change in total energy expenditure and alcohol intake for both groups (see below) with the use of a 3-step approach. First, an initial MANCOVA was performed to determine the overall time effects and treatment time-by-treatment interaction for statistical significance. Secondly, if a significant statistical or interaction effect was observed for the overall MANCOVA, changes in individual lipid variables were examined by treatment effect at each time point (i.e. baseline, 3W and 6W). Thirdly, a Tukey-Kramer post-hoc test was used to examine appropriate within or between group changes at 3W and 6W. All food frequency data was assessed using a 2 x 2 analysis of variance (ANOVA) to denote before and after and between- and within-group food values. All statistical analyses were performed using JMP statistical software (version 5.0.1.2; SAS Inc, Cary, NC) Statistical significance referred to a P value of < 0.05. All data were reported as mean $\pm$ SD.

In an attempt to clarify the affect of food intake on changes in the lipid characteristics, several regression analyses of food intake *a posteriori* were performed (**APPENDIX E**). However, these interpretations must be considered with caution as the use of repeated regression analyses on co-related variables increases the likelihood of inflating the experiment-wise error rate of the study and decreasing the likelihood of detecting a true statistical relationship when one does not exist (i.e., Type I error).

CHAPTER IV*

RESULTS

Results

Study population demographics and compliance analyses. After formal randomization to the treatment phase of this study protocol (N = 56), four participants dropped out of the study. These individuals were equally distributed between the placebo and treatment groups. Three of these individuals (2 and 1 in the placebo and bIg groups, respectively) dropped out prior to the 3W visit. Two of these dropouts cited daily capsule number intake as the reason for stopping the study. One participant asked to drop out because, "the treatment made her feel drowsy." However, after breaking this subject's treatment code, it was determined that she was in the placebo group. The one treatment participant continuing past the 3W visit dropped out due to an unexpected job move to a different state. As three of the randomized participants did not attend the 3W visit, the authors of this study elected not to perform an intent-to-treat analysis. This decision was based on the observation that inclusion of these three subjects and/or the one participant that surpassed the 3W visit would not alter study outcome or any data reported below.

No differences for age, body mass index (BMI) or any lipid variable were evident in this study population. The mean (±SD) age of study participants was 51 ± 8.2 years (y). Baseline body mass was 79.96 ± 10.2 kg. The treatment (bIg) group was comprised of 16 men and 10 women and the placebo group consisted of 15 men and

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11 women. No gender interactions for any of the subsequent treatment analyses were observed in this study. Throughout the study, each group remained ~75% compliant to the study protocol, citing daily pill ingestion number as the primary reason for lack of compliance. No difference in compliance between the treatment (78%) and placebo group (73%) was observed ($P = 0.33$). However, a significant main effect of time ($P<0.01$) on total energy consumption was observed for both study groups, demonstrating a decrease in total energy consumption during the study period.

Energy partitioning and macronutrient analyses. A description of this study population's macronutrient energy partitioning is presented in **Table 6**. Analysis of individual macronutrients showed no other significant difference between the treatment and the control group findings other than the main effect for time ($P < 0.01$) and related decrease in total energy consumption noted above. These included carbohydrates, protein, total fat, and associated subfractions of fat (saturated, polyunsaturated, or monounsaturated fat), dietary cholesterol and dietary fiber. A significant main effect of time with regard to alcohol intake ($P < 0.01$) was also observed. A decline in alcohol intake may be attributed to limitations of self-reported food intake data, or possibly a concern by study participants that alcohol intake might interfere with treatment efficacy (121).

Changes in dietary behaviors, as measured by food frequency intake records, were noted in each study group with profound variations occurring in the placebo versus the treatment group from baseline to study conclusion. However, despite performing a regression analysis in an attempt to clarify the affect of food intake on changes in lipid characteristics, only weak, yet statistically significant findings, summarized as LDL-C decreases for increases in protein, fat, carbohydrates and dietary cholesterol intake, for

the treatment group only were observed. And, it is contradictory for increases in these variables, specifically dietary cholesterol and fat intake, which largely contribute to raising cholesterol levels, to be related to declines in LDL-C values.

Table 6
Nutrient characteristics for the study cohort during the experimental period[1]

	Treatment (n = 26)		Placebo (n = 26)	
Nutrient Intake	**Baseline**	**Week 6**	**Baseline**	**Week 6**
Total Energy (kJ • d^{-1})[2]	7862 ± 3472.4	6814 ± 1968.2	7644 ± 2784.1	6459 ± 3548.9
Protein (g • d^{-1})	77.70 ± 41.3	70.50 ± 47.4	84.40 ± 62.2	62.20 ± 40.8
Carbohydrates (g • d^{-1})	223.80 ± 110.6	199.7 ± 96.9	235.60 ± 146.3	195.30 ± 133.6
Fat Intake (g • d^{-1})				
Total Fat	70.70 ± 38.8	67.2 ± 50.5	74.80 ± 55.1	56.90 ± 38.8
Saturated Fat	23.2 ± 12.7	21.60 ± 16.8	25.00 ± 20.4	18.60 ± 12.7
MUFA	26.8 ± 14.8	25.90 ± 19.4	28.40 ± 21.4	21.50 ± 14.8
PUFA	15.10 ± 8.7	14.60 ± 10.2	15.00 ± 10.2	12.10 ± 8.7
Cholesterol (mg • d^{-1})	264.30 ± 189.2	221.70 ± 158.1	273.40 ± 262.1	193.20 ± 139.7
Alcohol (g • d^{-1})[2]	10.50 ± 27.5	4.91 ± 7.1	8.10 ± 12.2	5.91 ± 7.6
Fiber (g • d^{-1})	18.10 ± 8.7	16.40 ± 7.6	20.10 ± 13.8	16.10 ± 12.7

[1] All values are $\bar{x}$ ± SD. MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

[2] Significant main effect for time $P < 0.001$ (ANOVA). There were no other significant main effects as interactions.

Furthermore, emerging research has shown a relationship between body weight and milk intake (129,130), as well as potentially influencing lipid outcomes; the subjects' dietary milk intake was examined. No between or within group differences for total milk intake, low fat intake (< 2% fat) and high fat milk intake (≥ 2%) were found. Total milk

intake ranged between 0.20L and 0.43L servings (227 mL each) per day for both the placebo and treatment groups.

Primary lipid variables. With repeated measure MANCOVA analysis, a statistically significant overall main effect of time ($P < 0.001$), treatment ($P < 0.03$) and time-by-treatment interaction ($P < 0.0001$) was observed. Subsequently, each lipid variable was examined individually, and both TC and LDL-C were found to be statistically significant for time and treatment at 3W and 6W (both: $P < 0.0001$). At 6W, statistically significant between group differences were observed for TC ($P < 0.005$) and LDL-C ($P < 0.007$). Based on these results, a Tukey post-hoc assessment of TC and LDL-C for the within (i.e., time) group effects observed at 3W and 6W was performed. However, only the between group effects at 6W were examined as no statistically significant treatment effect was noted at 3W. The results of this analysis are fully detailed in **Table 7**.

Table 7
Serum lipid characteristics of groups during the experimental period[1]

Lipid Characteristics	Treatment (n = 26)	Placebo (n = 26)
Total cholesterol (mmol/L)[2-4]		
Baseline	6.33 ± 0.1	6.16 ± 0.1
3-week change[3]	5.98 ± 0.5[5]	6.13 ± 0.6
6-week change[3,4]	5.97 ± 0.7[5,6]	6.06 ± 0.5
LDL cholesterol (mmol/L)[2]		
Baseline	4.12 ± 0.6	3.95 ± 0.5
3-week change[3]	3.92 ± 0.7[5]	4.00 ± 0.6
6-week change[3,7]	3.84 ± 0.6[5,6]	3.92 ± 0.6
HDL cholesterol (mmol/L)		
Baseline	1.35 ± 0.05	1.49 ± 0.04
3-week change	1.28 ± 0.3	1.46 ± 0.3
6-week change	1.29 ± 0.3	1.52 ± 0.3
VLDL cholesterol (mmol/L)		
Baseline	0.87 ± 0.5	0.71 ± 0.3
3-week change	0.78 ± 0.4	0.73 ± 0.3
6-week change	0.84 ± 0.5	0.62 ± 0.2
Triglycerides (mmol/L)		
Baseline	1.84 ± 0.9	1.51 ± 0.6
3-week change	1.65 ± 0.8	1.43 ± 0.7
6-week change	1.77 ± 1.0	1.35 ± 0.8

[1] All values are $\bar{x}$ ± SD.

[2] Significant overall main effects of time ($P < 0.001$) and treatment ($P < 0.03$) and significant time × treatment interaction ($P < 0.0001$) (repeated-measures multivariate analysis of covariance).

[3] Statistically significant for time × treatment interaction (both: $P < 0.0001$)

[4,7] Statistically significant for time × treatment (i.e., between-group interaction): [4]$P < 0.005$, [7]$P < 0.007$

[5] Significant within-group (i.e. time) difference from baseline group, $P < 0.05$ (Tukey-Kramer post hoc analysis).

[6] Significantly different from placebo $P < 0.05$ (Tukey-Kramer post hoc analysis).

Overall, post-hoc assessment demonstrated a statistically significant within treatment group reduction in TC from baseline to 3W (-0.35 ± 0.5 mmol/L, $P < 0.05$) and 6W (-0.36 ± 0.6 mmol/L, $P < 0.05$). By 6W, the reduction in TC from baseline was statistically significantly different from that in the placebo group ($P < 0.05$). No statistically significant within group difference from baseline for the placebo group at 3W (-0.03 ± 0.5 mmol/L) or 6W (-0.10 ± 0.4 mmol/L) was observed. Mean change data in TC and LDL-C during the 3W and 6W intervals for the treatment and the placebo groups are detailed in **Figures 2a and 2b** with error bars representing 95% confidence intervals.

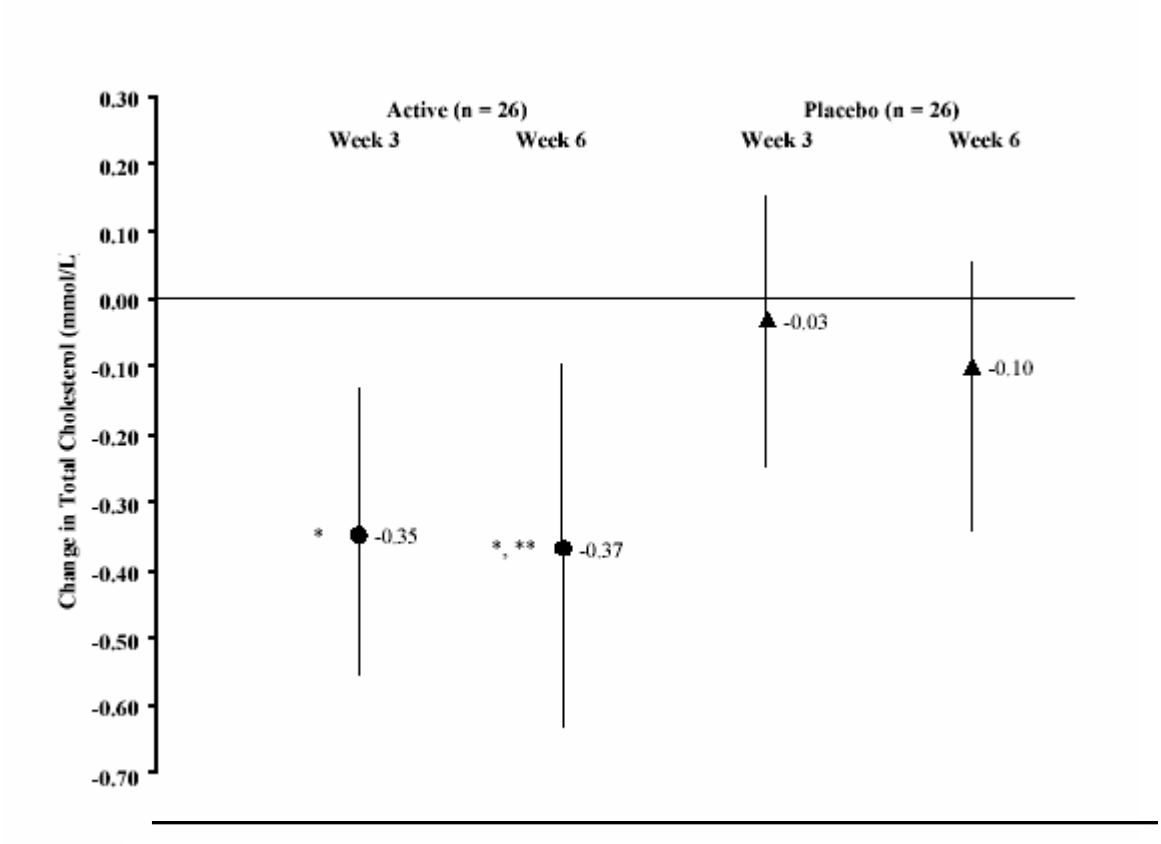

Figure 2a. Mean change data for TC (mmol/L) in study cohorts. Data analysis via repeated measures MANCOVA is co-varied for change in total energy and alcohol intake with Tukey post-hoc assessment following significant parameter estimates. Level of significant at $P < 0.05$ for within group analysis vs. baseline is denoted by * symbol. Significant difference between treatment and placebo groups ($P < 0.05$) is represented by ** symbol.

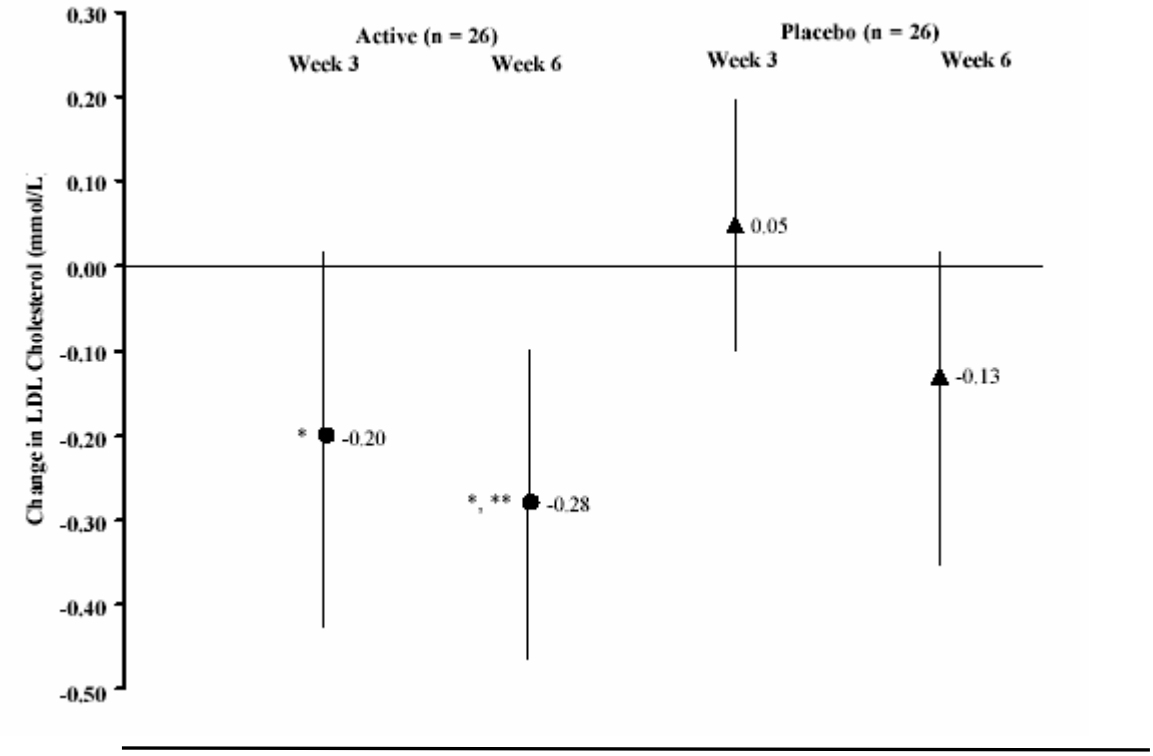

Figure 2b. Mean change data for LDL-C (mmol/L) in study cohorts. Data analysis via repeated measures MANCOVA is co-varied for change in total energy and alcohol intake with Tukey post-hoc assessment following significant parameter estimates. Level of significant at $P < 0.05$ for within group analysis vs. baseline is denoted by * symbol. Significant difference between treatment and placebo groups ($P < 0.05$) is represented by ** symbol.

The observed reduction in TC could be attributed to the statistically significant reduction in LDL-C subfraction, where a significant within treatment group reduction from baseline in LDL-C at 3W (-0.20 $\pm$ 0.3 mmol/L; $P < 0.05$) and 6W (-0.28 $\pm$ 0.4 mmol/L; $P < 0.05$) was found. By 6W, a statistically significant difference between groups was observed, whereby the Tukey post-hoc examinations showed LDL-C to be significantly lower in the treatment group than in the placebo group ($P < 0.05$). No within-group

treatment differences from baseline were observed in the placebo group at 3W (0.05 $\pm$ 0.4 mmol/L) or 6W (-0.03 $\pm$ 0.4).

Secondary lipid parameters and tertiary outcomes analysis. An examination of these data (i.e. HDL-C, VLDL-C and TG) revealed statistically significant main effects or time ($P < 0.001$), treatment ($P < 0.0001$) and the time-by-treatment interaction ($P < 0.001$). However, despite this overall MANCOVA result, no other statistically significant results for the marker estimates associated for HDL-C, VLDL-C, TG or glucose were found. Furthermore, no significant changes in other blood chemistry markers associated with hepatorenal or cardiovascular function were seen.

CHAPTER V*

GENERAL DISCUSSION AND CONCLUSION

Bovine Immunoglobulin Lowers Cholesterol

The primary purpose of this experiment was to assess the cholesterol-lowering properties of a bovine immunoglobulin (bIg)-rich supplement on blood lipids, mainly total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), in a cohort of hyper-cholesterolemic human subjects. This chapter will discuss the outcomes of our study in relation to our hypotheses.

Hypothesis one. The first hypothesis of this study was there would be a statistically significant reduction in TC and LDL-C in the treatment group compared to the placebo group. By 6W, the reduction in TC from baseline in the treatment group was statistically different from that in the placebo group. No significant within-group differences in the placebo group from baseline to 3W or 6W were observed. However, because a reduction in TC and LDL-C occurred within the control group, it was specu-lated that this finding could be due to variations in single cholesterol measurements or the placebo effect within the control group. As normal physiologic variations can occur in cholesterol measurements simply due to such variables as stress or posture, these variables could account for the change in the placebo cholesterol concentrations (131).

The second plausible explanation for the cholesterol variations in the placebo group may be attributed to what is called the “placebo effect”, whereby a medical improvement is seen in relation to no treatment (132-134). The placebo effect has been

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documented in a variety of study conditions, including mortality in coronary artery disease (CAD) (134). One study of a lipid-lowering drug in coronary heart disease (CHD) patients demonstrated that subjects taking the placebo, who were only 80% compliant with pill intake, had a reduction in mortality rate of 28.3% after 5 years when compared with a 15.1% mortality rate reduction for subjects who were compliant with placebo intake (135). In another study, Horowitz, et al. (136) found that patients with CAD who were noncompliant with placebo intake experienced two-fold higher death rates than those subjects who were compliant with their placebo pill intake. These studies demonstrate that the placebo effect can have clinical consequences.

The third plausible explanation for changes in cholesterol within the placebo group could be attributed to the dietary changes, specifically the reduction in dietary TC, total fat, and saturated fat intake. Though a thorough analysis of data was performed, the reason for noted changes in reported dietary behaviors was not found. The placebo hydrolyzed gelatin capsules might have triggered a decrease in appetite or an increase in fluid consumption, resulting in a decline in total food intake, however no studies could be found to document this hypothesis.

Statistically significant reductions were observed in blood lipids, mainly TC and LDL-C, within treatment group subjects. The fraction of cholesterol most associated with the TC change was LDL-C, though, minor variations in HDL-C may account for part of the observation despite lack of statistical significance at 6W.

Hypothesis two. The second hypothesis was that following an 8-week ingestion of 5 g daily of ImmunoLin™, TC and LDL-C concentrations would be reduced by at least 8 to 10% in hypercholesterolemic human study participants. Results demonstrated a 5.8% and 6.8% reduction in TC and LDL-C respectively within the

experimental group at trial completion. Despite the statistically significant reductions observed, they did not meet the required percentage to prove the hypothesis of an 8 to 10% reduction in TC and LDL-C nor match Proliant's pilot study results of a 15% reduction in TC. Some explanations accounting for differences in percent reductions between this research study and the Proliant pilot study could be the following: sample size, treatment compliance, HDL-C baseline values, and a "threshold effect" theory.

The unpublished Proliant study contained only 11 treatment subjects with TC within the 5.44 to 6.99 mmol/L (210 mg/dL to 270 mg/dL) versus the 52 subjects in this study. The smaller sample size in the Proliant study may account for inflated cholesterol percentage reductions and may not be representative of the general population as larger sample sizes generally result in increased study reliability. Aside from sample size, treatment compliance may also have produced variations in results.

Throughout this research, each group remained ~75 % compliant to the study protocol as measured by pill counting. Threats to validity of pill counting include loss of pills or fabrication of pill ingestion, which can result in overestimation of treatment compliance. An alternate method used in assessing medication (pill) compliance is self-reported pill ingestion. However, as previously noted, self-reported compliance information is also characterized by potential threats in validity (126). Despite the validity threat associated with pill counting, it remains as one of the most common methods utilized in medication compliance studies (126). However, subjects did cite pill ingestion number as the primary reason for lack of compliance.

The subject's mean HDL-C levels in this study were 13 points lower than subject's values in the Proliant study, which resulted in higher LDL-C baseline values in the Proliant study subjects. Speculation is that the bIg supplement affects TC values

mostly through reducing LDL-C as demonstrated by the lack of statistically significant variations in HDL-C or VLDL-C observed in this study. If the LDL-C values in the Proliant study were indeed higher due to the lower HDL-C levels, one would expect greater reductions in TC, as noted in the Proliant study.

Another possible explanation for greater effects seen in the Proliant study may be due in part to a "threshold effect," meaning LDL-C must be above or at a certain "threshold" to see a supplement effect on cholesterol reduction (137). With subjects in the Proliant study having higher LDL-C values, potentially above or equal to this study's proposed threshold value, reductions observed in LDL-C in the Proliant study may have potentially been greater than in this study, however further research would be necessary to potentially elucidate this supposition. This research found that the greatest change within the treatment group occurred at the 3W period for TC and LDL-C, with results demonstrating a 5.5% and 4.9% reduction, respectively. At the 6W interval, there was only an additional 0.3% and 1.9% reduction in TC and LDL-C, respectively, despite the additional 3W of treatment. Additionally, a statistically significant reduction in VLDL-C of 10% at the 3W interval was observed within the treatment group, however, the percent change for VLDL-C at the 6W interval was not statistically significant. These results support a "leveling off" supposition, whereby when LDL-C levels fall below the "threshold" as mentioned earlier, reductions in cholesterol gradually begin to plateau as noted at the 6W conclusion of this study.

Immunoglobulins. Milk constituents, such as immunoglobulin G (52), magnesium (76), riboflavin (76), orotic acid (76), and an unknown inhibitory factor (81) have all been proposed as potential mediators of cholesterol reduction. Additionally, declines in cholesterol have been attributed to increased bacterial gut activity (138). The major

constituents of milk that can be eliminated as potential cholesterol-lowering mediators (given their hypercholesterolemic nature) include saturated fat, lactose, calcium, and casein (139).

In support of the immunoglobulins being responsible for the decline in cholesterol concentrations, Golay et al. (52) compared the daily intake of 2 L standard skim milk with skim milk from immunized cows for in 11 male subjects with primary hypercholesterolemia for 8 weeks. Subject's dietary intake composition remained unchanged throughout the duration of the study. Though small sample size may limit outcome reliability, the researchers found a TC and LDL-C reduction of 8% and 4%, respectively. Because immune milk differs from standard milk in immunoglobulin content, study researchers speculated that the increased immunoglobulin amounts found in immune milk were responsible for the cholesterol-reduction. Additionally, the authors directly attributed reductions in TC concentrations to the increased specific activity of the milk immunoglobulin G fraction against human gut microorganisms, which they proposed potentially, may alter cholesterol metabolism within the intestinal micro-flora. However, a factor that complicates comparing this research to previous studies involving immune milk is the observation that the bIg protein concentrations used in these previous studies involving immune milk products are unknown.

The amount of immunoglobulin necessary to produce a lipid-lowering effect, specifically 5.8% for TC, in this study was 2.4 g/day. These results are similar to the results observed in a human study using an immune milk product that achieved a 6.6% reduction in TC, while subjects consumed only 0.5 g of bIg/day (51,52). In light of these similarities, one can infer that greater intake amounts of bIg may not necessarily equate to higher reductions in TC. Additional studies will be necessary to determine the opti-

mum level of supplementation of bIg to produce a desirable cholesterol-lowering effect. The effects of bIg supplementation reported in this study may point to a means of modulating circulating cholesterol such as with previous dietary approaches focusing on phytosterols, soluble fiber, or soy protein, which affect intestinal cholesterol absorption. In conclusion, Proliant's ImmunoLin™ product positively impacted cholesterol profiles in hypercholesterolemic human subjects and further investigation into its use is warranted.

Bovine immunoglobulins and cholesterol reduction mechanisms. Currently, the exact mechanism of action for the bIg lipid lowering effect is unknown. However, several previous research findings provide insight into potential mechanisms, including the lipogenic effects of endotoxin and cytokines (140) and factors in plasma, such as plasma apolipoproteins, which may affect cholesterol absorption (141,142). The bIg utilized in this study was reported by Proliant, Inc. to contain minimal amounts of endotoxin and cytokines based on pre-tested Standard Plate Count (SPC) results of < 1000/ mL (personal conversation with Dr. Eric Weaver, vice president and manager of scientific research and development for Proliant, Inc.). SPC results should be < 10,000/ mL. The exact amounts of endotoxin and cytokine content within the ImmunoLin™ product were not measured in this study.

Data on the effects of feeding plasma protein components on cholesterol metabolism is scarce. The only previously reported animal study to evaluate the effects of plasma protein components on cholesterol absorption or metabolism found no differences in serum or liver cholesterol concentrations between animals fed soy protein and plasma protein, whereas significant increases in liver cholesterol concentration was reported for casein (94). However, bIg is not absorbed in the gut of healthy adults so its effects are mostly luminal. A luminal effect is possible since intestinal cholesterol

absorption is an important origin of circulating LDL-C (143,144). Cholesterol enters the lumen of the small bowel via the diet and directly from biliary cholesterol excretion. Intestinal cholesterol undergoes micellar adaptation by bile salts and is absorbed into intestinal cells whereby it is esterified via acyl coenzyme A cholesterol acyl transferase (ACAT), packaged into chylomicrons within the intestinal epithelial cell, and transported through the lymphatic system to the liver (142,143).

Endotoxin, a pro-inflammatory molecule produced by immune cells in the gut, has been shown to be lipogenic and is proposed to be a contributor to atherosclerosis and cardiovascular disease because of its pro-inflammatory effects (145). Oral administration of bIg may possess gut endotoxin-binding capacity because it contains endotoxin-binding proteins. Endotoxin-mediated lipogenesis was found in animal models to be mediated via increased activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and reduced cholesterol 7-alpha-hydroxykase activity (146-149). Interestingly, immunoglobulin and other plasma proteins possess an endotoxin-binding capacity which when administered orally in sufficient quantity have the potential to bind endotoxin in the lumen and subsequently affect inflammatory cytokine production in the intestine (150). Helping to substantiate this premise is the finding that the oral administration of an IgA-bIg fraction of human plasma affects inflammatory cytokine production (151). Antibodies and other plasma proteins may also be involved in reducing cholesterol absorption. Antibodies to cholesterol were present in normal human and animal plasma (143,152). It is unknown whether antibodies to cholesterol were present in the study treatment (bIg) protocol and this was not evaluated. However, if present, these antibodies may directly bind cholesterol in the lumen and hinder absorption. At this time, no known studies exist that directly link oral

bIg intake and endotoxin effects. Future studies are necessary to elucidate the exact mechanism of action for bIg on endotoxin binding capacity.

Concluding Remarks

An immunoglobulin effect, as evidence by a statistically significant reduction in TC and LDL-C reductions within the treatment and placebo group was observed in this study. Furthermore, despite the possible threshold and leveling effect of LDL-C reduction within the treatment group, it is speculated that the ImmunoLin™ product positively impacted the subject's TC values and CHD risk (153). In addition, if a follow-up study were conducted with better control for HDL-C values, a greater LDL-C reduction effect may have been observed. In retrospect, this study may also have shown a greater immuno-globulin related cholesterol lowering effect via modifying the delivery system of the treatment product as treatment compliance was minimized by the need for the large quantity of daily pill ingestion.

This research demonstrated that the ImmunoLin™ had a cholesterol lowering effect that may be a useful adjunct combined with or in lieu of medications for individuals who are intolerant to drug side effects. In addition, more dramatic results may be obtained utilizing ImmunoLin™ in conjunction with lifestyle modifications, specifically diet and exercise, so individuals may be able to avoid or prolong the need for cholesterol medication; assessing this combination is worthy of future study. In view of the size of the hypercholesterolemia problem in industrialized countries, the results observed in his research should encourage further studies of the effects of globulin protein supplementation on cholesterol metabolism in humans.

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APPENDICES

APPENDIX A*

SUBJECT RECRUITMENT FLYER

*Reprinted with permission from Dr. Conrad P. Earnest, Director of the Center for Human Performance & Nutrition Research at The Cooper Institute Centers for Integrated Health Research, Dallas, TX.

CHOLESTEROL RESEARCH AT

THE COOPER INSTITUTE

The Cooper Institute Center for Human Performance and Nutrition Research is investigating a new nutrition supplement to lower blood cholesterol. The supplement is serum-derived, allergy-free, lactose-free protein.

Pilot study data suggests that this product may be as effective as contemporary cholesterol lowering medications and appears to target LDL cholesterol**.**

ELIGIBLE PARTICIPANTS WILL NEED TO:

1. Be between 25 and 70 years of age,

2. Have a total cholesterol between 210 and 270 mg/dL,

3. Not be taking any cholesterol-lowering medications,

4. Not be taking new vitamins for less than 3 months. If you have been taking vitamins for more than 3 months and are willing to continue their usage, this is okay,

5. Not be pregnant or breast-feeding,

6. Not be a diabetic individual.

**** The length of the study is 8-weeks**.

Eligible participants for the study will be expected to make an eight-week commitment to the project, which will include five visits to the Cooper Institute to have blood drawn. All participants will be financially reimbursed for their time.

If you are interested in participating or would like to learn more, please contact Dr. Conrad Earnest at: 972-341-3239.

APPENDIX B*

SUBJECT RECRUITMENT FLYER

*Reprinted with permission from Dr. Conrad P. Earnest, Director of the Center for Human Performance & Nutrition Research at The Cooper Institute Centers for Integrated Health Research, Dallas, TX.

Draft

Proliant

Cholesterol Trial

MEDICAL HISTORY QUESTIONNAIRE

Name: _____ Nickname or name used: ☐☐☐☐☐☐☐☐☐☐

Date of Examination: ☐☐ / ☐☐ / ☐☐☐☐
Month Day Year

This is your medical history form for your Cooper Complete visit. All information will be kept confidential. The physician will use this information in evaluating your health. Obviously, you will want to make it as accurate and complete as possible.

Please print your responses.

INSTRUCTIONS

1. Please read each question carefully.
2. To avoid errors, please keep this form free from wrinkles, stray marks and extra folds.
3. Please complete this form with a black ink pen.
4. Mark a heavy, dark mark that fills the circle (bubble) completely when answering questions that require a bubbled in answer.
5. If you make a mistake please clearly identify the correct answer by either crossing out the incorrect answer or circling the correct answer.
6. Do not divide form into single sheets.
7. For optimum accuracy, it is recommended that characters be written block style without touching sides.

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I. GENERAL INFORMATION

2

Title
○ Mr.
○ Mrs.
○ Ms.
○ Miss.
○ Dr.
○ Rev.
○ Other _____

Last Name:

First Name:

Middle Name:

Maiden Name, if applicable:

Number & Street Address:

City:

State:

Zip Code:

Country:

(Area Code) Home Phone Number:

Last Four Digits of Social Security Number:

(Area Code) Day Time Phone (Work)

Age:

Date of Birth: / / (Month / Day / Year)

(Area Code) Fax Number:

CURRENT OCCUPATION: Are you currently employed? ○ Yes ○ No (Skip to Present Work Situation)

Type of Business:

Your Position, Title, or Type of work:

What is your present work situation? (Mark all that apply.)
○ Employed Full-time ○ Semi-Retired ○ Self-Employed ○ Housewife
○ Employed Part-time ○ Fully-Retired ○ Unemployed ○ Student

If retired, give retirement date. ____/____/____

Name, Address, and Phone Number of person to be notified **in case of emergency:**

Name: _____ Relationship to Patient: _____

Number and Street Address: _____

City: _____ State: _____ Zip code: _____

Home Phone Number: (_____)_____ Work Phone Number: (_____)_____

PERSONAL PHYSICIAN

Dr.

Last Name:

First Name:

M. I.

(Area Code) Phone Number:

(Area Code) Fax Number:

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II. PERSONAL PROFILE

Sex: ○ Male ○ Female **Race:** ○ White ○ Black ○ Hispanic ○ Asian ○ Other _____
Place of Birth: _____

A. Marital History

1. Are you now or have you ever been married? ○ Yes ○ No
2. Current marital status: ○ Single ○ Married ○ Divorced ○ Widowed If currently married, how many years? _____
3. Number of children? _____

B. Education: Indicate highest level attained.

			Degree	Field	College/University
Grade: ○ 7 ○ 8 ○ 9 ○ 10 ○ 11 ○ 12	BACHELOR	○ Yes ○ No	_____	_____
College: ○ 13 ○ 14 ○ 15 ○ 16	MASTERS	○ Yes ○ No	_____	_____
Post Graduate: ○ 17 ○ 18 ○ 19 ○ 20	DOCTORATE	○ Yes ○ No	_____	_____

III. CURRENT MEDICAL STATUS

A. PRESENT MEDICAL CONDITIONS: Please list any known significant medical problems that you have at present. If female, are you currently pregnant, breast feeding or anticipating becoming pregnant within the time frame of this study.

PROBLEM	DATE OF ONSET	COMMENTS

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IV. REVIEW OF SYSTEMS

4

Please indicate whether you have ever had a significant problem with any of the symptoms or conditions listed below.

GENERAL	Yes	No	Don't Know	If yes, year of onset? Example: 1996	Is this still a problem? (Circle Yes or No)	Comments
1. Unexplained weight loss	○	○	○		Yes No	
2. Unexplained weight gain	○	○	○		Yes No	
3. Chronic fatigue	○	○	○		Yes No	
4. Change in appetite	○	○	○		Yes No	
5. Night sweats	○	○	○		Yes No	
6. Fever or chills	○	○	○		Yes No	
7. Any type of cancer	○	○	○		Yes No	
8. HIV Positive/AIDS	○	○	○		Yes No	

HEART/VASCULAR						
9. Chest pain or pressure	○	○	○		Yes No	
10. Chest pain with exertion	○	○	○		Yes No	
11. High blood pressure	○	○	○		Yes No	
12. High blood cholesterol	○	○	○		Yes No	
13. High blood triglycerides	○	○	○		Yes No	

ENDOCRINE						
14. Thyroid disease	○	○	○		Yes No	
15. High blood sugar	○	○	○		Yes No	
16. Diabetes	○	○	○		Yes No	

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IV. REVIEW OF SYSTEMS (CONT.)

5

ALLERGIES

103. Do you have any allergy problems?	○ Yes	○ No	○ Don't Know
104. Do you have hay fever symptoms?	○ Yes	○ No	○ Don't Know
105. Do you have food allergies?	○ Yes	○ No	○ Don't Know

CURRENT MEDICATIONS AND SUPPLEMENTS

Do you take insulin? ○ Yes ○ No

Do you take other medications or vitamins? ○ Yes ○ No

Do you take any medications to lower cholesterol? ○ Yes ○ No If yes, please list: _____

Do you take Niacin? ○ Yes ○ No If yes, please list: _____

Have you taken any over-the-counter supplements for less than three months? ○ Yes ○ No

If yes, please list _____

Please list all medication and supplements here:

MEDICATION	DOSAGE	DOSES PER DAY	FOR WHAT?	WHEN

DRUG ALLERGIES: Are you aware of any allergic reactions to any medications? ○ **Yes** ○ **No**

If so, list medication and reaction to it.

MEDICATION	TYPE OF ALLERGIC REACTION	YEAR

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IV. REVIEW OF SYSTEMS (CONT.)

GYNECOLOGICAL HISTORY: (WOMEN ONLY)

1. When was your last menstrual period? ☐☐ / ☐☐ / ☐☐☐☐ (Month / Day / Year)
2. When was your last pelvic examination? ☐☐ / ☐☐ / ☐☐☐☐ (Month / Day / Year)

   Was the pelvic examination abnormal? ○ Yes ○ No Was the Pap Smear abnormal? ○ Yes ○ No
3. Are (or were) your menstrual periods abnormal? ○ Yes ○ No
4. Do you have urine loss when you cough, sneeze, or laugh? ○ Yes ○ No
5. Have you had a hysterectomy? ○ Yes ○ No
6. Are you currently using a form of birth control? ○ **Yes** ○ **No (Skip to question 7)**

   If yes, what kind? ○ Oral (Specify): _____

   ○ Other (Specify): _____
7. Number of pregnancies? ☐☐
8. Number of live births? ☐☐
9. Year of last pregnancy? (Example: 1996) ☐☐☐☐

V. PAST MEDICAL HISTORY

A. SIGNIFICANT PAST ILLNESSES: Please list any other significant illnesses you had as a child or adult.

ILLNESS	YEAR(S)	COMMENTS

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VI. PERSONAL HABITS

7

A. TOBACCO

1. Do you live with people who smoke? ○ Yes ○ No
2. Have you ever used tobacco? ○ **Yes** ○ **No (If no, skip to Alcohol section)**
3. Do you currently use tobacco? ○ **Yes** ○ **No (If no, skip to question 4)**

(Example: 1996)

a. If you smoke cigarettes now, how many per day?		What year did you start?	
b. If you smoke cigars now, how many per day?		What year did you start?	
c. If you smoke a pipe now, how many pipefuls per day?		What year did you start?	
d. If you use "smokeless tobacco" now, how many times per day?		What year did you start?	

4. Have you used any of the following in the past, but do not use them now? ○ **Yes** ○ **No** (skip to Alcohol section)

	How many per day?	What year did you start?	What year did you stop?	Comments
a. Cigarettes				
b. Cigars				
c. Pipe				
d. "Smokeless" Tobacco				

B. ALCOHOL

1. Do you drink alcoholic beverages? ○ Yes ○ No

If yes, how many drinks per week? Beer (12 oz.) Wine (5 oz. glass) Hard Liquor (1.5 oz.)

2. Have you used alcohol in the past but subsequently quit? ○ Yes ○ No

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FOOD FREQUENCY QUESTIONNAIRE

date completed ☐☐ / ☐☐ / ☐☐☐☐

Directions: Please use a black pen to complete this form. For each food listed, indicate the average number of times per month, week, or day you ate the food during the last month in the boxes provided. If you did not eat the food during this time, please mark NO by the food.

FOOD ITEM	*Did you eat the food item last month?* NO	If YES, enter number of times. per month *OR* per week *OR* per day
Total, Corn Flake or Raisin Bran Total, Basic 4 or All Bran Cereal	○	M ☐☐ W ☐☐ D ☐☐
Other ready-to-eat cereal	○	M ☐☐ W ☐☐ D ☐☐
Cooked cereal such as grits, oatmeal, or cream of wheat	○	M ☐☐ W ☐☐ D ☐☐
Pancakes, waffles, or French toast	○	M ☐☐ W ☐☐ D ☐☐
Doughnut, muffin, pastry, sweet roll, or croissant	○	M ☐☐ W ☐☐ D ☐☐
Biscuit or scone	○	M ☐☐ W ☐☐ D ☐☐
Cornbread, hush puppies, or corn fritter	○	M ☐☐ W ☐☐ D ☐☐
Bread, toast, English muffin, roll, or bagel (not in a sandwich)	○	M ☐☐ W ☐☐ D ☐☐
Stuffing, all types	○	M ☐☐ W ☐☐ D ☐☐
Tortilla, including flour or corn	○	M ☐☐ W ☐☐ D ☐☐
Spanish rice	○	M ☐☐ W ☐☐ D ☐☐
Macaroni or other pasta and cheese	○	M ☐☐ W ☐☐ D ☐☐
Stuffed pasta such as lasagna, manicotti, ravioli, or tortellini	○	M ☐☐ W ☐☐ D ☐☐
Other pasta such as spaghetti with tomato or meat sauce	○	M ☐☐ W ☐☐ D ☐☐
Rice or noodles as a side dish	○	M ☐☐ W ☐☐ D ☐☐
Pizza or calzone	○	M ☐☐ W ☐☐ D ☐☐
Bean soup with or without meat	○	M ☐☐ W ☐☐ D ☐☐
Vegetable, tomato, noodle, or broth soup with or without meat	○	M ☐☐ W ☐☐ D ☐☐
Cream-based soup or chowder	○	M ☐☐ W ☐☐ D ☐☐
Regular peanut butter alone or in a sandwich, with or without jelly	○	M ☐☐ W ☐☐ D ☐☐
Cheese sandwich	○	M ☐☐ W ☐☐ D ☐☐
Bologna, corned beef, salami, or pastrami, with cheese, alone or in a sandwich	○	M ☐☐ W ☐☐ D ☐☐
Bologna, corned beef, salami, or pastrami, without cheese, alone or in a sandwich	○	M ☐☐ W ☐☐ D ☐☐
Ham, chicken, or turkey cold cuts, with cheese, alone or in a sandwich	○	M ☐☐ W ☐☐ D ☐☐

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FOOD ITEM	NO	Did you eat the food item last month? If YES, enter number of times. per month OR per week OR per day
Ham, chicken, or turkey cold cuts, without cheese, alone or in a sandwich	○	M ☐☐ W ☐☐ D ☐☐
Beef patty, hamburger, hot beef, pork, or chicken sandwich with cheese	○	M ☐☐ W ☐☐ D ☐☐
Beef patty, hamburger, hot beef, pork, or chicken sandwich without cheese	○	M ☐☐ W ☐☐ D ☐☐
Hot dog with or without bun, or corn dog	○	M ☐☐ W ☐☐ D ☐☐
Taco, tostado, or chalupa	○	M ☐☐ W ☐☐ D ☐☐
Taco salad	○	M ☐☐ W ☐☐ D ☐☐
Cheese nachos with any topping	○	M ☐☐ W ☐☐ D ☐☐
Enchiladas, burritos, or quesadillas with cheese	○	M ☐☐ W ☐☐ D ☐☐
Enchiladas or burritos without cheese	○	M ☐☐ W ☐☐ D ☐☐
Beef or chicken fajitas	○	M ☐☐ W ☐☐ D ☐☐
Tamales or flautas	○	M ☐☐ W ☐☐ D ☐☐
Chile rellenos	○	M ☐☐ W ☐☐ D ☐☐
Chili con carne	○	M ☐☐ W ☐☐ D ☐☐
Beef stew or pot pie with sauce and vegetables	○	M ☐☐ W ☐☐ D ☐☐
Beef mixed dishes such as meatloaf, chili, or oriental dishes	○	M ☐☐ W ☐☐ D ☐☐
Beef or lamb as a main dish such as steaks, roast, or ribs	○	M ☐☐ W ☐☐ D ☐☐
Pork or ham mixed dishes such as oriental dishes	○	M ☐☐ W ☐☐ D ☐☐
Pork or ham as a main dish such as steak, roast, or chops	○	M ☐☐ W ☐☐ D ☐☐
Meatless bacon or sausage	○	M ☐☐ W ☐☐ D ☐☐
Bacon	○	M ☐☐ W ☐☐ D ☐☐
Sausage or chorizos	○	M ☐☐ W ☐☐ D ☐☐
Beef liver	○	M ☐☐ W ☐☐ D ☐☐
Poultry or pork liver	○	M ☐☐ W ☐☐ D ☐☐
Soy burger, vegetable patty, or meatless meats	○	M ☐☐ W ☐☐ D ☐☐
Tofu or soy mixed dishes such as casseroles or oriental dishes	○	M ☐☐ W ☐☐ D ☐☐
Chicken or turkey mixed dishes such as stews or oriental dishes	○	M ☐☐ W ☐☐ D ☐☐

Draft

date completed ☐☐ / ☐☐ / ☐☐☐☐

FOOD ITEM	Did you eat the food item last month? NO	If YES, enter number of times. per month OR per week OR per day
Fried chicken or turkey and chicken nuggets	○	M ☐☐ W ☐☐ D ☐☐
Baked, broiled, or grilled chicken or turkey	○	M ☐☐ W ☐☐ D ☐☐
Fish and shellfish mixed dishes such as tuna casserole, crab or tuna salad, or tuna fish sandwich	○	M ☐☐ W ☐☐ D ☐☐
Fried fish, fish sticks, or fish sandwich	○	M ☐☐ W ☐☐ D ☐☐
Raw or cooked oysters	○	M ☐☐ W ☐☐ D ☐☐
Canned salmon or sardines	○	M ☐☐ W ☐☐ D ☐☐
Fresh mackerel, salmon, bluefish, or swordfish	○	M ☐☐ W ☐☐ D ☐☐
White fish such as trout, perch, tuna, halibut, or flounder	○	M ☐☐ W ☐☐ D ☐☐
Shrimp, lobster, scallops, clams, or crab	○	M ☐☐ W ☐☐ D ☐☐
Eggs prepared any way with cheese	○	M ☐☐ W ☐☐ D ☐☐
Eggs prepared any way without cheese	○	M ☐☐ W ☐☐ D ☐☐
Egg roll	○	M ☐☐ W ☐☐ D ☐☐
Vegetable combinations such as stir-fry, stew, or mixed vegetables	○	M ☐☐ W ☐☐ D ☐☐
Refried beans	○	M ☐☐ W ☐☐ D ☐☐
Dried beans and peas	○	M ☐☐ W ☐☐ D ☐☐
Potato salad	○	M ☐☐ W ☐☐ D ☐☐
Boiled or mashed potatoes, or baked potato without cheese topping	○	M ☐☐ W ☐☐ D ☐☐
Stuffed baked potato with cheese or au gratin potatoes	○	M ☐☐ W ☐☐ D ☐☐
Fried potatoes - hash browns, home fries, tater tots, or fries	○	M ☐☐ W ☐☐ D ☐☐
Fried onion rings	○	M ☐☐ W ☐☐ D ☐☐
Sweet potatoes or yams	○	M ☐☐ W ☐☐ D ☐☐
Broccoli with cheese	○	M ☐☐ W ☐☐ D ☐☐
Broccoli without cheese	○	M ☐☐ W ☐☐ D ☐☐
Cauliflower	○	M ☐☐ W ☐☐ D ☐☐
Green beans	○	M ☐☐ W ☐☐ D ☐☐
Corn	○	M ☐☐ W ☐☐ D ☐☐

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FOOD ITEM	Did you eat the food item last month? NO	If YES, enter number of times. per month OR per week OR per day
Summer squash, yellow or zucchini	○	M W D
Raw spinach, including salads made with spinach	○	M W D
Cooked greens such as spinach, mustard, turnip, or collards	○	M W D
Avocado or guacamole	○	M W D
Green salad or chef salad with cheese	○	M W D
Green salad or chef salad without cheese	○	M W D
Reduced calorie or light salad dressing	○	M W D
Regular salad dressing (not low fat)	○	M W D
Raw or cooked carrots other than in green salads	○	M W D
Raw tomatoes other than in salads or sandwiches	○	M W D
Green hot chili peppers (jalapenos and green chilis)	○	M W D
Sweet green or yellow peppers	○	M W D
Sweet red peppers	○	M W D
Cabbage, coleslaw, or sauerkraut	○	M W D
V-8, tomato, or vegetable juice	○	M W D
Calcium-fortified orange juice	○	M W D
Regular orange juice	○	M W D
Fruit juice other than orange	○	M W D
Fruit flavored drinks such as Kool-Aid, lemonade, or fruit punch	○	M W D
Apples	○	M W D
Bananas	○	M W D
Grapes	○	M W D
Oranges or tangerines	○	M W D
Grapefruit	○	M W D
Peaches or nectarines	○	M W D
Watermelon	○	M W D

Draft

date completed ☐☐ / ☐☐ / ☐☐☐☐

FOOD ITEM	NO	Did you eat the food item last month? If YES, enter number of times. per month OR per week OR per day
Cantaloupe or honeydew melon	○	M ☐☐ W ☐☐ D ☐☐
Pears	○	M ☐☐ W ☐☐ D ☐☐
Pineapple	○	M ☐☐ W ☐☐ D ☐☐
Fresh or dried apricots	○	M ☐☐ W ☐☐ D ☐☐
Plums	○	M ☐☐ W ☐☐ D ☐☐
Strawberries	○	M ☐☐ W ☐☐ D ☐☐
Berries other than strawberries	○	M ☐☐ W ☐☐ D ☐☐
Fruit salad or fruit cocktail	○	M ☐☐ W ☐☐ D ☐☐
Dried fruits (not apricots)	○	M ☐☐ W ☐☐ D ☐☐
Soy milk	○	M ☐☐ W ☐☐ D ☐☐
Skim or 1/2% milk	○	M ☐☐ W ☐☐ D ☐☐
1% milk	○	M ☐☐ W ☐☐ D ☐☐
2% milk or buttermilk	○	M ☐☐ W ☐☐ D ☐☐
Whole milk, chocolate milk, or milkshakes	○	M ☐☐ W ☐☐ D ☐☐
Liquid meals such as instant breakfast, Slimfast, Sego, or Dynatrim	○	M ☐☐ W ☐☐ D ☐☐
Tofutti, tofu frozen desserts	○	M ☐☐ W ☐☐ D ☐☐
Low fat or nonfat yogurt	○	M ☐☐ W ☐☐ D ☐☐
Whole milk yogurt	○	M ☐☐ W ☐☐ D ☐☐
Low fat or nonfat ice cream, frozen yogurt, sherbet, mellorine, or ice milk	○	M ☐☐ W ☐☐ D ☐☐
Regular ice cream or ice cream bar	○	M ☐☐ W ☐☐ D ☐☐
Pudding or custard	○	M ☐☐ W ☐☐ D ☐☐
Low fat or nonfat sour cream or sour cream dip	○	M ☐☐ W ☐☐ D ☐☐
Regular sour cream or sour cream dip	○	M ☐☐ W ☐☐ D ☐☐
Low fat or nonfat cream cheese	○	M ☐☐ W ☐☐ D ☐☐
Regular cream cheese	○	M ☐☐ W ☐☐ D ☐☐
Low fat cottage cheese	○	M ☐☐ W ☐☐ D ☐☐

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FOOD ITEM	Did you eat the food item last month? NO	If YES, enter number of times. per month OR per week OR per day
Regular cottage cheese	○	M ☐☐ W ☐☐ D ☐☐
Soy cheese	○	M ☐☐ W ☐☐ D ☐☐
Low fat or nonfat cheese	○	M ☐☐ W ☐☐ D ☐☐
Low sodium cheese	○	M ☐☐ W ☐☐ D ☐☐
Mozzarella or string cheese	○	M ☐☐ W ☐☐ D ☐☐
Regular cheese	○	M ☐☐ W ☐☐ D ☐☐
Low fat cakes such as angel or sponge cake	○	M ☐☐ W ☐☐ D ☐☐
Other cake	○	M ☐☐ W ☐☐ D ☐☐
Pie	○	M ☐☐ W ☐☐ D ☐☐
Low fat cookies, brownies, or granola bars	○	M ☐☐ W ☐☐ D ☐☐
Regular cookies, brownies, or granola bars	○	M ☐☐ W ☐☐ D ☐☐
Chocolate candy	○	M ☐☐ W ☐☐ D ☐☐
Candy other than chocolate	○	M ☐☐ W ☐☐ D ☐☐
Rice cake, matzo, or low sodium crackers	○	M ☐☐ W ☐☐ D ☐☐
Pretzels or low fat crackers	○	M ☐☐ W ☐☐ D ☐☐
Regular crackers	○	M ☐☐ W ☐☐ D ☐☐
Popcorn, plain or air-popped	○	M ☐☐ W ☐☐ D ☐☐
Popcorn, cooked or eaten with oil or butter	○	M ☐☐ W ☐☐ D ☐☐
Low fat corn chips, tortilla chips, or potato chips	○	M ☐☐ W ☐☐ D ☐☐
Regular corn chips, tortilla chips, or potato chips	○	M ☐☐ W ☐☐ D ☐☐
Chile con queso or cheese sauce	○	M ☐☐ W ☐☐ D ☐☐
Taco sauce, picante sauce, or salsa	○	M ☐☐ W ☐☐ D ☐☐
Soy nuts	○	M ☐☐ W ☐☐ D ☐☐
Unsalted nuts or seeds such as sunflower seeds, peanuts, or almonds	○	M ☐☐ W ☐☐ D ☐☐
Regular nuts or seeds such as sunflower seeds, peanuts, or almonds	○	M ☐☐ W ☐☐ D ☐☐
Diet soft drinks, all flavors	○	M ☐☐ W ☐☐ D ☐☐

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date completed ☐☐ / ☐☐ / ☐☐☐☐

FOOD ITEM	*Did you eat the food item last month?* NO	If YES, enter number of times. per month *OR* per week *OR* per day
Regular soft drinks, all flavors	○	M ☐☐ W ☐☐ D ☐☐
Coffee with or without milk, cream, sugar, or sweetener	○	M ☐☐ W ☐☐ D ☐☐
Hot tea with or without sugar or sweetener	○	M ☐☐ W ☐☐ D ☐☐
Iced tea with or without sugar or sweetener	○	M ☐☐ W ☐☐ D ☐☐
Regular or light beer	○	M ☐☐ W ☐☐ D ☐☐
Red wine	○	M ☐☐ W ☐☐ D ☐☐
Other wine or wine coolers	○	M ☐☐ W ☐☐ D ☐☐
Liquor or mixed drinks	○	M ☐☐ W ☐☐ D ☐☐

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Quality of Life Questionnaire

SECTION1: HEALTH AND DAILY ACTIVITIES

The first part of the Health Questionnaire is about your health and your daily activities. Please try to answer every question by marking the appropriate circle and as accurately as you can.

1. In general, would you say your health is:

   ○ Excellent
   ○ Very good
   ○ Good
   ○ Fair
   ○ Poor

---

2. How much bodily pain have you generally had during the past 4 weeks?

   ○ None
   ○ Very mild
   ○ Mild
   ○ Moderate
   ○ Severe
   ○ Very severe

---

3. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

   ○ Not at all
   ○ Slightly
   ○ Moderately
   ○ Quite a bit
   ○ Extremely

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SECTION 2: PHYSICAL HEALTH
These questions are about your physical activities and symptoms.

4. The following items are activities you might do during a typical day. Does your health limit you in these actvities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a. Vigorous actvities, such as running, lifting heavy objects, participating in strenuous sports	O	O	O
b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	O	O	O
c. Lifting or carrying groceries	O	O	O
d. Climbing several flights of stairs	O	O	O
e. Climbing one flight of stairs	O	O	O
f. Bending, kneeling, or stooping	O	O	O
g. Walking more than one mile	O	O	O
h. Walking several blocks	O	O	O
i. Walking one block	O	O	O
j. Bathing or dressing	O	O	O

5. How satisfied are you with your physical ability to do what you want to do?

- O Completely satisfied
- O Very satisfied
- O Somewhat satisfied
- O Somewhat dissatisfied
- O Very dissatisfied
- O Completely dissatisfied

6. When you travel around your community, does someone have to assist you because of your health?

- O Yes, all of the time
- O Yes, most of the time
- O Yes, some of the time
- O Yes, a little of the time
- O No, none of the time

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7. Are you in bed or in a chair most or all of the day because of your health?

- O Yes, every day
- O Yes, most days
- O Yes, some days
- O Yes, occasionally
- O No, never

8. How often during the past 4 weeks...

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. Did you feel worn out?	O	O	O	O	O	O
b. Were you discouraged by your health problems?	O	O	O	O	O	O
c. Did you have a lot of energy?	O	O	O	O	O	O
d. Did you feel weighed down by your health problems?	O	O	O	O	O	O
e. Did you feel full of pep?	O	O	O	O	O	O
f. Were you afraid because of your health?	O	O	O	O	O	O
g. Did you have enough energy to do the things you wanted to do?	O	O	O	O	O	O
h. Was your health a worry in your life?	O	O	O	O	O	O
i. Did you feel tired?	O	O	O	O	O	O
j. Were you frustrated about your health?	O	O	O	O	O	O
k. Did you feel despair over your health problems?	O	O	O	O	O	O

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9. How often have you had any of the following symptoms during the past 4 weeks?

	Never	Once or twice	A few times	Fairly often	Very often
a. Stiffness, pain, swelling or soreness of muscles or joints	○	○	○	○	○
b. Coughing that produced sputum	○	○	○	○	○
c. Backaches or lower back pains	○	○	○	○	○
d. Nausea (upset stomach)	○	○	○	○	○
e. Acid indigestion, heartburn, or feeling bloated after meals	○	○	○	○	○
f. Heavy feelings in arms and legs	○	○	○	○	○
g. Headaches or head pains	○	○	○	○	○
h. Lump in throat	○	○	○	○	○
i. Sore throat	○	○	○	○	○
j. Nasal congestion, runny or stuffy	○	○	○	○	○
k. Loose stool	○	○	○	○	○
l. Constipation	○	○	○	○	○
m. Abdominal pain	○	○	○	○	○
n. Gas	○	○	○	○	○
o. Abdominal cramping	○	○	○	○	○
p. Fever	○	○	○	○	○
q. Chills	○	○	○	○	○
r. Loss of energy	○	○	○	○	○
s. Loss of appetite	○	○	○	○	○
t. Weight loss	○	○	○	○	○
u. Depression	○	○	○	○	○

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SECTION 3: PAIN

10. Did you experience any bodily pain in the past 4 weeks?

- ○ Yes ----> Continue with Question 11, below
- ○ No ----> Skip to SECTION 4

The following questions are about the pain or pains you experienced in the past 4 weeks. If you had more than one pain, answer the questions by describing you feelings of pain in general.

11. During the past 4 weeks, how often have you had pain or discomfort?

- ○ Once or twice
- ○ A few times
- ○ Fairly often
- ○ Very often
- ○ Every day or almost every day

12. When you had pain during the past 4 weeks, how long did it usually last?

- ○ A few minutes
- ○ Several minutes to an hour
- ○ Several hours
- ○ A day or two
- ○ More than two days

13. During the past 4 weeks, how much did pain interfere with the following things?

	Not at all	A little bit	Moderately	Quite a bit	Extremely
a. Your mood	○	○	○	○	○
b. Your ability to walk or move about	○	○	○	○	○
c. Your sleep	○	○	○	○	○
d. Your normal work (including both work outside the home and housework	○	○	○	○	○
e. Your recreational activities	○	○	○	○	○
f. Your enjoyment of life	○	○	○	○	○

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14. Please circle the one number that best describes your pain on the average over the past 4 weeks?

No Pain — Pain as bad as you can imagine

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

15. Please circle the one number that best describes your pain on the at its worst over the past 4 weeks?

No Pain — Pain as bad as you can imagine

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

SECTION 4: YOUR FEELINGS

These questions are about how you feel and how things have been with you during the past month. For each question, please bubble the one answer that comes closest to the way you have been feeling.

16. How happy, satisfied, or pleased have you been with your personal life during the past month?

- O Extremely happy, could not have been more satisfied or pleased
- O Very happy most of the time
- O Generally satisfied, pleased
- O Sometimes fairly satisfied, sometimes fairly unhappy
- O Generally dissatisfied, unhappy
- O Very dissatisfied, unhappy most of the time

17. During the past month, how often has feeling depressed interfered with what you usually do?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

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18. How much of the time, during the past month, did you have difficulty reasoning and solving problems; for example, making plans, making decisions, learning new things?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

19. During the past month, how much of the time have you generally enjoyed the things you do?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

20. During the past month, how much of the time did you have difficulty doing activities involving concentration and thinking?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

21. During the past month, how much of the time did you feel depressed?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

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22. How much of the time, during the past month, have you felt calm and peaceful?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

---

23. How much of the time, during the past month, did you have trouble keeping your attention on any activity for long?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

---

24. During the past month, how much of time have you ben anxious or worried?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

---

25. During the past month, how much of the time have you been a person?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

26. During the past month, how depressed (at its worst) have you felt?

- O Extremely depressed
- O Very depressed
- O Quite depressed
- O Somewhat depressed
- O A little depressed
- O Not depressed at all

SECTION 6: SOCIAL ACTIVITIES
The next questions ask about your social activities.

27. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

---

28. Compared to your usual level of social activity, has your social activity during the past 6 months decreased, stayed the same, or increased because of a change in your physical or emotional condition?

- O Much less socially active than before
- O Somewhat less socially active than before
- O About as socially active as before
- O Somewhat more socially active than before
- O Much more socially active than before

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29. Compared to others your age, are your social activities more or less limited because of your physical health or emotional problems?

- O Much more limited than others
- O Somewhat more limited than others
- O About the same as others
- O Somewhat less limited than others
- O Much less limited than others

SECTION 7: YOUR HEALTH

Next are some general questions about your health and health-related matters.

30. How TRUE or FALSE is each of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a. I am somewhat ill	O	O	O	O	O
b. I feel about as good now as I ever have	O	O	O	O	O
c. I have been feeling bad lately	O	O	O	O	O
d. I am in poor health	O	O	O	O	O
e. I am as healthy as anybody I know	O	O	O	O	O
f. My health is excellent	O	O	O	O	O
g. I seem to get sick a little easier than other people	O	O	O	O	O
h. I expect my health to get worse	O	O	O	O	O

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SECTION 8: YOUR SLEEP

31. How often during the past 4 weeks did you...

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. feel that your sleep was not quiet (moving restlessly, feeling tense, speaking, etc., while sleeping)?	○	○	○	○	○	○
b. get enough sleep to feel rested upon waking in the morning?	○	○	○	○	○	○
c. awaken short of breath or with a headache?	○	○	○	○	○	○
d. feel drowsy or sleepy during the day?	○	○	○	○	○	○
e. have trouble falling asleep?	○	○	○	○	○	○
f. awaken during your sleep time and have trouble falling asleep again?	○	○	○	○	○	○
g. have trouble staying awake during the day?	○	○	○	○	○	○
h. take naps (5 minutes or longer) during the day?	○	○	○	○	○	○
i. get the amount of sleep you needed?	○	○	○	○	○	○

APPENDIX C*

PARTICIPANT INFORMED CONSENT DOCUMENT

*Reprinted with permission from Dr. Conrad P. Earnest, Director of the Center for Human Performance & Nutrition Research at The Cooper Institute Centers for Integrated Health Research, Dallas, TX.

Informed Consent Document:

Consent Form

The Cooper Institute

12330 Preston Road

Dallas, TX 75230

Principal Investigator: Dr. Conrad Earnest

You have volunteered as a candidate for this study because you have a total cholesterol concentration ranging between 210 and 280 mg/dL, are between the ages of 25 and 70 years old, and have no known major health problems. The following information describes the study and your role as a participant. The investigators will answer any questions you may have about the study, this consent form, and your participation in the project. Please read this form carefully, and do not hesitate to ask questions about any aspect of the study.

Purpose

The purpose of the study is to evaluate the effectiveness of a serum-derived nutritional supplement, consumed in capsular form, on selected blood cholesterol concentrations, namely total and LDL blood cholesterol concentrations.

Procedures

Individuals who agree to participate and who meet all eligibility criteria will be randomly assigned to one of two groups. **Random assignment** means your group assignment is determined by chance, and **you will not be able to choose one group over the others**. However, if you are

assigned to the placebo group, you will receive 8 weeks worth of the active product following your successful completion of the trial to try on your own.

All groups will participate in an 8-week trial.

Individuals will be assigned to 1 of 2 groups.

1) Receive a serum-derived treatment in capsule form for an 8 week period.

2) Receive a placebo capsule for a 8 week period.

ALL STUDY PARTICIPANTS WILL BE ASKED TO COMPLETE QUESTIONNAIRES CONCERNING YOUR MEDICAL HISTORY, YOUR FOOD INTAKE, AT BASELINE AND 8 WEEKS. BLOOD DRAWS WILL TAKE PLACE FIVE (5) TIMES THROUGHOUT THE STUDY PERIOD.

Discomforts and Risks

The risks of participating in the Proliant Health Industries Cholesterol study are small. You may experience temporary pain during the blood drawing, with later bruising at the puncture site. Only specially trained staff will be responsible for collection of blood samples. Also, any samples collected during the course of the trial will be tested for biological hazards if they are involved in an incident that results in exposure of any individual to potentially hazardous biologic material. Emergency equipment and trained personnel are available to deal with unusual situations that may arise.

Exclusions

IF YOU DONATE BLOOD DURING THE TRIAL, IF YOU SMOKE, CONSUME ALCOHOLIC BEVERAGES EXCEEDING AN AVERAGE OF 3 DRINKS PER DAY, OR CONSUME COFFEE IN EXCESS OF 3 CUPS PER DAY, YOU WILL BE EXCLUDED FROM THE STUDY. IN

ADDITION, IF YOU HAVE PLANS TO BECOME PREGNANT; OR ARE BREASTFEEDING, YOU WILL BE EXCLUDED FROM THE STUDY.

Voluntary Participation

Participation in the Proliant Health Industries Cholesterol study is voluntary. Refusal to participate will involve no loss of benefits to which you are entitled. Further, you may withdraw from the study at any time without penalty. If you decide to withdraw from the study, we request that you notify the Project Director, Dr. Conrad Earnest, of your decision in writing. At that time, we also request that you complete an exit interview by mail or telephone to enable us to determine the reason for withdrawing from the study.

Significant Findings

You will be told of any significant findings that may occur during the course of this study that could relate to your willingness to continue to participate. The investigator and the sponsor reserve the right to terminate the study and discontinue your participation at any time for any reason, in order to ensure your safety.

Benefits to Participants

There is no cost for participation in this study. At the conclusion of the trial, you will receive an individualized report on your free lipids and other lab test results.

Project Funding

This project is funded by private donations.

Compensation for Medical Treatment

If you suffer any adverse experience during the testing, study staff will render first aid and emergency care. The project physician will not provide medical care outside test supervision.

Confidentiality

Only you and the investigators will have access to your study records and other data obtained from the study as required by the Privacy Act, 5, U.S.C. 522a. Details from your medical records will be stored on a private computer, but your name will not be used as an identifier. Information stored on the computer may be seen by Proliant Health Industries Cholesterol study staff. Your name will not appear in any publications; only group data will be used. If for any reason you desire for us to share any and/or all your data with someone else, a signed letter stating your desire to release this information and to whom it should be released will be required.

Questions

Your signature indicates the investigators or other members of study staff have answered all your questions about the study and your participation in the study. If you should have additional questions during the course of the study, or if any problems arise, you should contact the Principal Investigator and Project Director, Dr. Conrad Earnest at 972-341-3284. If you have questions about your rights as a research study participant, contact Dr Steve Farrell the Institutional Review Board Chairman, at The Cooper Institute at 9720-341-3275.

Authorization

You are making a decision whether or not to participate in this study. You should not sign until you understand all the information presented in the previous pages and until all your questions about the research have been answered to your satisfaction. Your signature indicates that you have decided to participate after having read (or been read) the information provided above.

I understand that I am not waiving any legal rights or releasing the local institution sponsoring this study or its agents from liability for negligence. I understand that in the event of physical injury resulting from the research procedures, the local institution sponsoring this study does not have funds budgeted for compensation either for lost wages or for medical treatment. Therefore,

aside from the emergency care previously described, the local institution does not provide for treatment or reimbursements for such injuries.

Name (Please print.)_____

Signature _____

Date _____

Witness _____Date _____

APPENDIX D

PROLIANT IMMUNOLIN™ PRODUCT CERTIFICATE OF ORIGIN AND PRODUCT PURITY FROM BSE CONTAMINATION

Proliant
HEALTH INGREDIENTS
The Art of Service.
The Science of Protein.

February 20, 2004

To Whom It May Concern:

Certificate of Origin

This is to certify that the raw material used to manufacture Proliant™ ImmunoLin WP was derived exclusively from USDA-regulated abattoirs located in the United States. The animals received ante- and post-mortem inspection under a veterinarian's supervision and were passed as free of infectious or contagious diseases and injurious parasites.

The USDA announced the finding of a cow in Washington State testing positive for BSE on Tuesday, December 23, 2003. Now that laboratories in the United Kingdom have confirmed the testing, this is the first case of BSE in the United States. Trace-back and trace-forward investigations have confirmed that this animal originated from Canada.

We first want to assure our customers that there is no chance that any tissues from this animal would be in the products we manufacture. The slaughtering facility where the animal was found does not supply Proliant Inc. with any raw materials used in the manufacture of our products.

Our products are manufactured exclusively from tissues which are known to be those that do not harbor the agent which causes BSE. The infective tissues are those of the central nervous system such as the brain, spinal cord and distal ileum. OIE (World Organisation for Animal Health) considers blood to be a low-risk tissue and not considered a Specified Risk Material (SRM).

Bonnie J. Humm
Director of Quality Assurance

BJH:pjm
40000244 Proliant Health

Proliant Health Ingredients • 7425 SE Oak Tree Court • Ankeny, Iowa 50021 • USA
phone (866) 440-1797 • fax (515) 289-4360 • www.proliantinc.com

Proliant

The Art of Service.
The Science of Protein.

December 30, 2003

This communication addresses customer inquiries and concerns regarding Proliant Inc.'s beef and milk products with respect to the recent USDA announcement that a Holstein cow from Washington State tested presumptive positive for BSE (Bovine Spongiform Encephalopathy).

The USDA announced the finding of a cow in Washington State testing positive for BSE on Tuesday, December 23, 2003. Now that laboratories in the United Kingdom have confirmed the testing, this is the first case of BSE in the United States. Trace-back and trace-forward investigations are continuing to determine the birth herd of the cow.

We first want to assure our customers that there is no chance that any tissues from this animal would be in the products we manufacture. The slaughtering facility where the animal was found does not supply Proliant Inc. with any raw materials used in the manufacture of our products.

Our products are manufactured exclusively from tissues which are known to be those that do not harbor the agent which causes BSE. The infective tissues are those of the central nervous system such as the brain, spinal cord and distal ileum.

The USDA is aggressively investigating the circumstances that may have resulted in this animal contracting BSE. At this time, the information is too sparse for USDA to provide any definitive information on this matter.

Further actions of the USDA will be based on their findings during the investigation regarding the history of this animal and likelihood of finding other animals associated with the circumstances which caused this animal to become infected. The USDA, like Canada, has taken strong precautions to prevent BSE from occurring in the United States. Hopefully, as we saw in the Canadian BSE incident, this will prove to be an isolated case with no other animals involved.

Proliant Inc. is committed to providing safe products and will be in continuous contact with all government agencies and associations to respond in any manner to assist and assure that our products are safe.

Proliant Inc.'s beef stocks, flavors and fats are processed in excess of 133°C for 20 minutes at 3 bars pressure which is considered an effective means by European regulatory agencies to minimize any chance for the transmission of BSE if it is present in the tissue.

After extensive research, blood products have been demonstrated to be one of the most unlikely tissues in an animal to contain the infectious agent of BSE, as is the case for milk and milk products. The global scientific community does not consider meat, blood and milk as risk tissues in regards to BSE, and therefore their consumption as food is not restricted.

We encourage our customers, colleagues, or employees to feel free to contact Proliant Inc. directly with any questions. Please address your questions to Quality Assurance by calling 515/296-7100.

For the latest information regarding this situation, or for more information about BSE or Creutzfeldt-Jacob (vCJD), please refer to the following websites:

www.usda.gov (United States Department of Agriculture)
www.bseinfo.org (BSE, CJD, vCJD Information Resource)
www.cdc.gov (Centers of Disease Control)
www.fda.gov (Food and Drug Administration)
www.agr.gc.ca (Agriculture and Agri-Food Canada)
www.beef.org (National Cattlemen's Beef Association)

Proliant BIOLOGICALS
The Art of Service. The Science of Protein.

January 5, 2004

The following statements are in response to the recent announcement from the USDA on a presumptive case of BSE in the United States:

- There is no possibility that any tissue from the affected animal is in Proliant's BSA products. The herd and the slaughter facility where the animal was located do not supply Proliant with any raw material. Our source abattoir is located in Kansas.
- The infective tissues for BSE are the central nervous system, such as brain, spinal cord, and distal ileum. BSA is manufactured from Bovine Plasma, a blood derivative. Blood plasma is considered an extremely low risk material by international regulatory authorities.
- Proliant bovine albumin is produced from bovine plasma that has been collected, handled and processed in such a manner as to minimize any potential contamination from other bovine or animal tissues, particularly specified risk materials.
- Proliant Biologicals Bovine Albumin (BSA) is manufactured in a plant that is completely dedicated to the handling and processing of bovine plasma.
- All source animals are from US feed lots; no animals are shipped directly from Canadian feed lots.
- All animals are steers and heifers under 36 months of age and there is no dairy or cull cattle kill at our plants. BSE has never been identified in young animals.
- The USDA has implemented a ban on the slaughter of "downer" cattle. Previously, "downer" animals were tested negative for BSE under the USDA surveillance program.
- Proliant's BSA received a Certificate of Suitability from the European Pharmacopoeia in 2001 confirming traceability, compliance to cGMP's and minimizing TSE risk and contamination potential.
- Published BSA prion clearance studies suggest that certain of our BSA manufacturing conditions (heating in the presence of caprylate followed by solids removal) are effective in clearing or inactivating up to 4 logs of a TSE infectious agent. (Blum, Budnick *et al.* BioPharm, April, 1998).

The USDA is aggressively investigating the circumstances around the finding of a BSE-case, apparently in a Canadian import cow. The USDA and Proliant are also working closely with our trading partners to reassure them that our products are safe and that import barriers are not necessary.

Proliant Biologicals is committed to providing the highest level of safety in our products and will be in continuous contact with all government agencies and associations for the latest developments. Updates may be viewed on our web site:

http://www.proliantinc.com/biologicals/main/Biologicals.asp

Contacts:
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APPENDIX E

REGRESSION ANALYSES

Table A-1
Linear regression analysis of change in TC within treatment group regressed on change in dietary protein intake

Independent variable	Regression coefficient
Change in dietary protein	- 0.19 (0.05)[1,2]
Constant	- 11.75 (2.18)
Adjusted R^2	0.13
n	83

[1] SE in parentheses.
[2] Significantly different from zero, *P* <.0005

Table A-2
Linear regression analysis of change in TC within treatment group regressed on change in dietary carbohydrate intake

Independent variable	Regression coefficient
Change in dietary carbohydrates	- 0.07 (0.02)[1,2]
Constant	- 11.46 (2.22)
Adjusted R^2	0.11
n	83

[1] SE in parentheses.
[2] Significantly different from zero, *P* <.0014

Table A-3
Linear regression analysis of change in TC within treatment group regressed on change in dietary fat intake

Independent variable	Regression coefficient
Change in dietary fat	- 0.17 (0.05)[1,2]
Constant	- 12.50 (2.19)
Adjusted R^2	0.11
n	83

[1] SE in parentheses.
[2] Significantly different from zero, *P* <.0011

Table A-4
Linear regression analysis of change in TC within treatment group regressed on change in dietary cholesterol intake

Independent variable	Regression coefficient
Change in dietary cholesterol	- 0.04 (0.01)[1,2]
Constant	- 11.23 (2.22)
Adjusted R^2	0.11
n	83

[1] SE in parentheses.
[2] Significantly different from zero, *P* <.0010

Table A-5
Linear regression analysis of change in TC within placebo group regressed on change in dietary protein intake

Independent variable	Regression coefficient
Change in dietary protein	- 0.01 (0.04)[1,2]
Constant	- 1.42 (2.05)
Adjusted R^2	- 0.01
N	86

[1] SE in parentheses.
[2] Significantly different from zero, *P* <0.8202

Table A-6
Linear regression analysis of change in TC within placebo group regressed on change in dietary carbohydrate intake

Independent variable	Regression coefficient
Change in dietary carbohydrates	0.01 (0.01)[1,2]
Constant	- 2.07 (1.97)
Adjusted R^2	- 0.01
N	86

[1] SE in parentheses.
[2] Significantly different from zero, *P* <0.4664

Table A-7
Linear regression analysis of change in TC within placebo group regressed on change in dietary fat intake

Independent variable	Regression coefficient
Change in dietary fat	0.02 (0.04)[1,2]
Constant	- 2.00 (2.03)
Adjusted R^2	- 0.01
N	86

[1] SE in parentheses.
[2] Significantly different from zero, *P* <0.6182

Table A-8
Linear regression analysis of change in TC within placebo group regressed on change in dietary cholesterol intake

Independent variable	Regression coefficient
Change in dietary cholesterol	- 0.01 (0.01)[1,2]
Constant	- 1.12 (2.01)
Adjusted R^2	- 0.01
N	86

[1] SE in parentheses.
[2] Significantly different from zero, *P* <0.4986

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