

ABSTRACT

Title of Document: GLUTAMINE SUPPLEMENTATION AND
RENAL HEALTH

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Team Juiced designed a multi-faceted research project surrounding the potential risk of college students using protein supplements developing kidney disease. Survey research showed that participants taking protein supplements were ingesting double the recommended allowance based on literature values for average American dietary protein intake. Participants predisposed to kidney disease were no less likely to take protein supplements. Kidney cell modeling showed the molecular response to glutamine, an important protein building block. A significant increase in the gene expression of low-density lipoprotein receptor and two sclerotic markers was found in response to glutamine exposure. Gene expression was time- and dose-dependent. Enzymatic degradation and high performance liquid chromatography showed that three popular protein supplements contained more glutamine than stated by the manufacturers.

GLUTAMINE SUPPLEMENTATION AND RENAL HEALTH

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Chapter 1: Introduction

The University of Maryland, College Park (UMD) sponsors a unique Honors undergraduate research program called Gemstone in which students of all majors work together to design and execute an original investigative study. This thesis describes the work of Gemstone Team Juiced and its multi-faceted project regarding the potential risk for the development of chronic kidney disease among college-aged athletes that use protein and/or glutamine supplements. Glutamine is a building block of protein or an amino acid. It can be taken in the form of a pill or a powder with the intent of increasing normal exercise capabilities. This project is divided into three branches that, when examined together, reveal a great deal about glutamine's effects on specialized cells in the kidney and the implications of the use of glutamine and protein supplements among college students. This thesis explores the protein supplementation patterns of the UMD athletic community, molecular pathways by which glutamine may affect kidney function, and the actual glutamine content of popular supplements as compared to their labels.

Team Juiced was interested in addressing the topic of dietary supplements after observing the rapid rise of the use of these products among the college population. A dietary supplement can be defined as a product intended to supplement diet that contains one or more of the following ingredients: a vitamin, a mineral, an herb or other botanical product, an amino acid, a concentrate, a metabolite, a constituent, an extract, or a combination of these ingredients (United States Food and Drug Administration, 1994). Dietary supplements can reduce the risk of disease, improve recovery from severe illnesses, and enhance exercise capabilities. The Food and Drug Administration (FDA) regulates these supplements as food products (as opposed to drugs). Since the Dietary

Supplement Health and Education Act (DSHEA) of 1994, dietary supplements have not been required to undergo the strict pre-market testing of drugs, thus allowing dietary supplements to enter the market with limited regulation (United States Food and Drug Administration, 1994). Supporters of the legislation argued that the new law would inspire more independent scientific research and consumer education, as well as give buyers greater access to the products. Although some dietary supplements have been studied in detail, the information on safety and efficacy of many of the supplements available to consumers is limited. Considering that related literature has discussed the high percentage of college students and athletes taking dietary supplements, uncertainty concerning the safety and efficacy of these products leaves a growing number of consumers at risk.

The goal of this study is to provide current and accurate information regarding glutamine to both the scientific community and consumers. There are thousands of supplements available on the market but a gap in the knowledge of how these products affect the body, especially in the long term. In addition, there is limited sociological information regarding the demographics of supplementation. It is the responsibility of researchers to make information regarding health concerns involving supplements available to the public so that they can make educated and informed decisions. The dietary supplement industry is a business whose intention is to make profit. In creating a product regulated as a food by the FDA, it is important that the scientific community work to investigate such risks for consumers. Recently, the consequences of the relaxed government regulation have had a significant impact upon American citizens. In an article published by the *New York Times* in February 2009, it was revealed that the

dietary supplement StarCaps, intended to aid in weight loss, was laced with unclaimed chemical additives (Singer, 2009). These additives were drugs that include several serious side effects. StarCaps were imported from China and have been readily available to consumers at major supplement retailers in the United States. The vulnerability of supplement consumers, as shown by such findings and the original research of Team Juiced, brings forward the issue of education for supplement use.

The dietary supplements chosen for this study were protein and glutamine supplements. Glutamine is one of twenty amino acids that, in unique combinations, form all proteins. Glutamine is a naturally occurring amino acid that is produced and used by muscle cells, especially during prolonged exercise. Glutamine-containing supplements are classified as dietary supplements and are therefore regulated by the FDA. Glutamine is a prolific moneymaker, topping the lists of the most popular supplements. Finally, while glutamine has many proven physiological benefits to athletes and patients of certain pathology, there is evidence from *in vitro* studies that suggests the potential for adverse effects in renal (kidney) health (Lagranha, et al., 2005, Lagranha, Doi, Pithon-Curi, Curi, & Sellitti, 2008).

Excess protein has been proven harmful to patients with kidney disease. The kidneys normally function to filter the blood and rid the body of harmful toxins, metabolic waste products, and excess nutrients, however the kidneys can become damaged and scarred in pathological conditions. Chronic kidney disease is irreversible, often leading to renal failure, which requires dialysis and kidney transplantations. Predisposing risk factors for kidney disease include diabetes, hypertension, obesity, or a

family history of any combination of these factors with kidney disease. High protein intake worsens the progression of chronic kidney disease toward loss of function.

While excess protein intake is known to exert a deleterious effect on patients with chronic kidney disease, the effect of high protein consumption on healthy individuals is uncertain. The effects of specific amino acids are better defined, yet few researchers are concerned with their effects in regard to renal health. Some of the most current research involves the study of glutamine in a mouse mesangial cell model. The kidney is composed of several discrete units called nephrons. The glomerulus is the filtration unit of the nephron. Three major cell types are found within the glomerulus: podocytes, endothelial cells, and mesangial cells. Of these cells, the mesangial cells are critical in supporting the capillary beds where filtration occurs. The mesangial cells excrete an extra-cellular matrix (ECM) of proteins that acts as a mesh between the capillaries. In instances where mesangial cells begin to proliferate and excrete too much matrix protein, the capillaries collapse and therefore the glomerulus loses the filtration capabilities. The extracellular matrix proteins harden the tissue and decrease the glomerular function in a process called glomerular sclerosis. Lipids can also accumulate in the sclerotic glomerulus through a mechanism similar to that of an atherosclerotic plaque, especially when the patient exhibits high blood levels of cholesterol. Low-density lipoprotein (LDL) is the main type of particle that circulates in the blood carrying cholesterol, and that infiltrates atherosclerotic plaques and sclerotic kidneys. Evidence suggests that the presence of lipids in glomeruli accelerates the scarring process in chronic kidney disease.

In order to address these research concerns, the current research conducted by Team Juiced was divided into three branches: survey research, kidney cell modeling, and

glutamine analysis. An additional branch of the study, urinalysis, was added after the thesis proposal with the aim of further interlinking the results of survey research and kidney cell modeling branches. The team used survey research to gather information regarding the patterns of protein and glutamine supplementation among the target population, the athletic community at the University of Maryland, College Park. For the purposes of this study, the athletic community consisted of individuals who used the campus exercise facilities, participated in the Reserve Officer Training Corp (ROTC), or were members of an athletic club or team. Anonymous survey research of the target community addressed the following questions:

- 1) What proportion of participants taking protein supplement also take pure glutamine supplements?
- 2) What is the average supplemental protein intake among participants that use protein supplements?
- 3) How long do the participants exercise each week?
- 4) Do participants have individual or family histories of diabetes, hypertension, obesity, or kidney disease?

It was particularly important to know if participants at risk for kidney disease were any less likely to take protein supplements than low risk participants due to the link between protein and kidney disease. In addition to these survey questions, several respondents participated in a follow-up urinalysis study. Both individuals who had and had not reported that they actively used protein supplements had their urine tested for the level of albumin excretion, which can be an early indicator of kidney disease if elevated. By testing participants' urine, it was possible to provide more applicable knowledge to consumers than the kidney cell modeling branch of research could provide.

The goal of the kidney cell modeling branch of the project was to discover how glutamine affects specialized kidney cells that function in glomerular filtration, with specific attention to the expression of low-density lipoprotein receptor (LDL-r). Collaboration with Sonia Q. Doi, M.D., Ph.D., Director of Nephrology Research Laboratory, Department of Medicine, Uniformed Services University (USU), Bethesda, MD, helped address the following sub-questions:

- 1) What is the time dependent (up to 24h) behavior of LDL-r expression in mouse mesangial cells exposed to a supraphysiological level of 2.0 millimolar (mM) glutamine?
- 2) What is the dose dependent behavior of LDL-r expression in cells exposed to increasing concentrations of glutamine up to 20.0 mM at the peak time of expression from the time course study?
- 3) What effect does glutamine have in a chronic setting, after 48 hours of exposure, with respect to the expression of *in vitro* markers of glomerular sclerosis, (collagen I, fibronectin, and collagen IV)? How does LDL-r expression respond to various concentrations of glutamine after 48 hours?

The primary goal of this branch of the project was to define the time- and dose-dependency of LDL-r in response to glutamine. This knowledge would be valuable to the scientific community, specifically molecular biologists in the field of nephrology. Future research building on this knowledge could include the elucidation of specific pathways by which glutamine affects kidney function. In addition to advancing the knowledge base in the scientific community, this information would be critically important for athletes taking protein supplements that have predisposing factors for kidney disease.

The third branch of this project aimed to assess the purity of glutamine supplements and glutamine content of protein supplements. Biochemical analysis of supplements in collaboration with Pierluigi Delmonte, Ph.D., Chemist, Department of

Bioanalytical Chemistry, Center for Food Safety and Applied Nutrition (CFSAN), FDA, College Park, MD, helped address the following questions:

- 1) What is the glutamine content of pure glutamine supplements? Does the content match the amount stated on the product label?
- 2) What is the glutamine content of protein and amino acid supplements that contain glutamine? Does the content match the amount stated on the product label?

Prior to the execution of this research project, a thorough literature review was conducted. Scientific literature puts the present study in context, defines its niche, and provides information to aid in analysis of data. Sources regarding DSHEA were especially important in the early stages of the project. Understanding the legislation helped to clarify the motivations underlying the structure of the research. It also shed light on the government's definition of a dietary supplement and the methods by which they are regulated. Literature about surveys conducted about supplementation among college populations was reviewed to assess what was already known. No literature was found that linked predisposing conditions of kidney disease to protein supplementation.

Literature outlining the physiological benefits of glutamine was also reviewed. Glutamine has been shown to boost the immune system and aid in muscle recovery and tissue re-growth (Lagranha, et al., 2005). Glutamine supplementation was found to be especially beneficial during intense exercise and endurance training. *In vitro* research suggested the potential for adverse effects. One study demonstrated that glutamine might induce mesangial cells to synthesize matrix proteins *in vitro*, the equivalent to the development of chronic kidney disease *in vivo* (Meek, et al., 2003).

Various biochemical analysis methods were also reviewed in the literature. A specific protocol to assess glutamine concentration in protein was not found. High-

performance liquid chromatography (HPLC) was researched with applications to the determination of glutamine concentration. Information regarding enzymatic degradation of proteins was also necessary to develop a method to test glutamine content in popular protein supplements.

The methodology for the current research was defined within the three branches. A survey was used to collect quantitative data from a purposive sample active individuals on the UMD campus. A short questionnaire was administered to gym-goers using the Geary F. Eppley Recreation Center or Ritchie Coliseum, intramural sports players, varsity athletes, and members of the Reserve Officers' Training Corps (ROTC). A total of 947 surveys were collected. A four-digit number was used to maintain the anonymity of participants. Participants were asked how much protein they consumed, and how often they consumed it, as well as how long they used the product. An option was also given to report protein products previously used by the participants. Other questions addressed what other supplements they had been taking, how long they exercised each week, their gender and age, and what their individual and family histories were regarding diabetes, hypertension, clinical obesity, and kidney disease. All data was entered into an SPSS statistics file and analyzed accordingly.

The final page of the survey asked participants if they would be interested in participating in a follow-up study. Several respondents volunteered to give a urine sample to measure urinary albumin excretion, which is used as a screening test for deficient kidney filtration. Participants were asked to provide a sample of urine obtained through the "clean catch" method. The samples were sent to the National Institutes of Health (NIH), Bethesda, MD, for urinalysis. The aim of this sub-study was to compare the

urinary albumin excretion of participants using protein supplements to those that were not. It also served as a free testing for a possible early indication of kidney disease. Participants were told that they would be contacted if their urinary protein was elevated, recommending that they speak with their physicians.

The methodology for the second branch of study used mouse mesangial cells to model the effects of glutamine on the kidneys. Standard cell culture methods, RNA extraction, and one-step reverse transcription and polymerase chain reaction was used to quantify the genes of interest, notably LDL-r. LDL-r was important to study because of its role in bringing what is commonly known as “bad cholesterol” or LDL cholesterol into cells. The first set of experiments was designed to determine the time course of LDL-r expression to a supraphysiological level of 2.0 mM glutamine over a period of 24 hours. The time of exposure when LDL-r expression was found to be optimal was used to elucidate the dose-dependent behavior of LDL-r expression. Establishing such knowledge in the field of molecular nephrology was critical in designing the final set of experiments that used 48 hours of glutamine exposure to simulate conditions of chronic glutamine over-use to assess how glutamine may affect pro-fibrotic matrix proteins, collagen I, fibronectin, and collagen IV.

In the glutamine analysis branch of the project, the glutamine supplements and glutamine-containing protein supplements most frequently used by survey respondents were gathered. After much methodological development, enzymatic degradation, phenyl isothiocyanate (PITC) derivitization, and HPLC were used to determine glutamine concentrations in popular protein supplements.

Each branch of the project contributed to the understanding of various aspects of protein and glutamine supplementation. The results from kidney cell modeling have provided the scientific community a detailed and intricate framework from which the course of chronic kidney disease can be further studied. By determining the time- and dose-dependent behavior of LDL-r expression in response to glutamine, further experiments can be performed to see how the increased LDL-r affects lipid uptake and pro-fibrotic excretion of matrix proteins. It is hypothesized that the up-regulation of LDL-r could play a role in pro-fibrotic excretion of matrix proteins. Our current *in vitro* research provided the groundwork for further studies *in vivo*.

Results from survey research provided a qualitative and quantitative understanding of protein supplementation patterns of the UMD athletic community. Information collected regarding supplemental protein intake gave substantiating evidence for the study of glutamine's effects on the kidneys. It also provided a unique insight into the behavioral patterns of supplement use amongst the college age population.

Results from glutamine analyses at CFSAN will hopefully inspire the development of a more accurate method for determining glutamine concentration in protein samples, as well as draw attention to the regulation and effects of long-term use of glutamine. It is the aim of this thesis to offer a multi-dimensional and in-depth look at the protein supplementation patterns among the UMD athletic community, the potential for adverse side effects from kidney cell exposure to glutamine, and the integrity of commonly used glutamine containing protein supplements.

Chapter 2: Literature Review

2.1 Complete Literature Review Summary

In designing this current research project, much investigation needed to be done regarding glutamine. First, literature regarding the regulation and sale of protein supplements, a major source of glutamine intake, was examined. The DSHEA legislation and its effects proved to be the primary source in understanding the social and political implications of dietary supplement regulation in American society. After reviewing the regulation of glutamine products, it became important to determine an age cohort that was engaged in glutamine supplementation. There was a void in the literature surrounding protein supplementation and renal health that this research team could fill. The literature displayed that college-age individuals commonly participated in protein supplementation but that there was a significant gap in the public body of knowledge regarding glutamine use and risk factors for the development of kidney disease. This void helped shape the survey of the current research. More literature was used in helping to write an effective survey, target a useful and specific subset of a population, and meaningfully analyze the data.

Once the survey had taken shape, it became important to review the link between protein and renal health, as known by literature. Research regarding glutamine's normal biological function, the relationship between high levels of protein and kidney disease, the benefits of protein use in athletes, the expression of certain genes when in the presence of glutamine, and the potential for excess glutamine to harm mesangial cells, was reviewed to help design experiments to study the expression of low-density lipoprotein receptor, or LDL-r, in mouse mesangial cells. An *in vitro* model for studying

the effects of glutamine on kidney cells was used, hoping to lay the groundwork for animal studies and eventually human subject research.

Because literature indicated the importance of glutamine dose when studying mesangial cells in culture, it was assumed that glutamine intake would be a key factor of understanding the effects of glutamine *in vivo*. While the current research could not address *in vivo* studies, it does target an important source of glutamine intake-supplementation. Literature showed the problems with the current system of regulation in regards to consumer safety, directing the current research to focus on the actual glutamine content of various protein and amino acid supplements. Literature regarding high performance liquid chromatography (HPLC) and enzymatic digestion was used to develop a method for glutamine isolation and quantification. The absence of a validated method for analyzing glutamine content in foods or supplements left much room for the current research to advance the field of biochemical analysis.

The possibility of high supplemental glutamine contents and a link between glutamine and glomerular scarring led to the team's study of urinalysis methods- a noninvasive method commonly used to assess early signs of kidney disease. Literature concerning urinary protein excretion was thoroughly reviewed. Overall, the literature used for this research project helped to guide our research aims as well as develop our methodology.

2.2 Survey Research Literature

2.2.1 Executive Summary of Survey Research Literature Review

As research has shown, the regulation of dietary supplements, including protein supplements is a field worth examining. Literature has shown the presence and prevalence of non-vitamin, non-mineral supplements to be a common occurrence amongst university students. These protein supplements are often ingested by a subset of the university's population who exercise on a regular basis, and preliminary research has shown that those who take protein supplements for muscle recovery after rigorous exercise could possibly face detrimental side effects. In particular, L-glutamine is one amino acid that is often contained in protein supplements and is used to supplement recovery and cell growth in humans. The current research has aimed to relate the results of a questionnaire regarding supplement usage with *in vitro* tests to determine the effects of certain dosages, time exposures, and concentrations of L-glutamine on mesangial cells.

2.2.2 Dietary Supplement Health and Education Act (DSHEA)

Dietary supplements are not under strict regulation from the United States Food and Drug Administration (FDA) following the Dietary Supplement Health and Education Act (DSHEA) of 1994. The manufacturers of a dietary supplement must adhere to the following rules in order for their products to be regulated as foods and not drugs by the FDA:

1. "The statement claims a benefit related to a classical nutrient deficiency disease and discloses the prevalence of such disease in the United States, describes the role of a nutrient or dietary ingredient intended to affect the structure or function in humans, characterizes the documented mechanism by which a nutrient or dietary ingredient acts to maintain such structure of function, or describes general well-being from consumption of a nutrient or dietary ingredient,

2. The manufacturer of the dietary supplement has substantiation that such statement is truthful and not misleading, and
3. The statement contains, prominently displayed and in boldface type, the following: ‘This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease’" (United States Food and Drug Administration, 1994).

As long as manufacturers follow these three guidelines, their products will be regulated as foods, even though consumers do not take most dietary supplements in the same way as they do foods. This suggests that dietary supplements may have potentially dangerous side effects since they do not undergo the strict clinical testing of drugs. To eliminate any possible lawsuits, dietary supplements must contain a statement that notes that the FDA has not evaluated the performance claims about the product before it has entered the market.

The law itself was passed after the manufacturers of said products engaged in intensive lobbying of the United States Congress. President Clinton, who signed the act into law, said the act came about as a result of the “efforts [of] manufacturers, experts in nutrition and...consumers at the grassroots level” (Woolley & Peters, 1994). Clinton went on to say that DSHEA “balances [the producers’] interests with the Nation's continued interest in guaranteeing the quality and safety of foods and products available to consumers (Woolley & Peters, 1994). The act was sponsored by Senators Orrin Hatch and Tom Harkin as well as Representative Bill Richardson, and passed in large measure due to the efforts of Senator Edward Kennedy, Congressman John Dingell, and Congressman Henry Waxman.

To illustrate how the process works, one can look back to the recent banning of ephedra. Once a popular dietary supplement, investigations began once there was a

demonstrated and documented list of serious issues surrounding the product, including many deaths directly related to the use and abuse of ephedra. In 2001, two professional football players died as a direct result of ephedra use, prompting Congress to hold hearings regarding ephedra's safety and efficacy. In the end, the FDA banned dietary supplements containing ephedra because it posed an unnecessarily high risk to consumers (Phillips, 2004).

The act provides a very loose and broad definition as to what constitutes a dietary supplement. According to DSHEA, a dietary supplement:

1. "Is a product (other than tobacco) that is intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients,
2. Is intended for ingestion in pill, capsule, tablet, or liquid form,
3. Is not represented for use as a conventional food or as the sole item of a meal or diet,
4. Is labeled as a 'dietary supplement',
5. Includes products such as an approved new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement or food before approval, certification, or license (unless the Secretary of Health and Human Services waives this provision)" (United States Food and Drug Administration, 1994).

In large measure due to the lack of oversight in protein and glutamine supplement use, this current project will test glutamine-containing dietary supplements for their content to be compared against the content claims by manufacturers. Both the dosage and purity of these supplements must be determined in order to see if manufacturers' claims

match the actual content in the supplement bottles determined using high-performance liquid chromatography (HPLC).

In 2007, the FDA issued a Final Rule that established exactly how the dietary supplement needed to be manufactured, labeled, and tested. According to the ruling, manufacturers were required to analyze their products to identify the strength, purity, composition, and identity of dietary supplements. If the products were contaminated or did not contain the supplements that they claim to contain, the FDA has to consider these products adulterated or misbranded (NewsRX, 2007). However, there is no mention as to how the FDA would go about confirming the purity or content of any of these products which are not tested before they enter the market.

A large problem with DSHEA is that unlike highly regulated prescription medications, the burden of proof to demonstrate whether or not the dietary supplement poses an unreasonable risk of illness or injury lies solely on the manufacturer of the dietary supplement. This takes the burden of proof away from the United States Government and allows for most dietary supplements to be put on the market without significant testing. Though the FDA has the authority to conduct random spot checks of products on the market, they have little incentive to do so unless an event such as the aforementioned ephedra incidents shed light on the need for regulation. Therefore, these products could pose a risk to consumers.

According to DSHEA (1994), almost 70% of Americans take a dietary supplement, begging the question of why they go unregulated before their provision to consumers (United States Food and Drug Administration, 1994). The ephedra incident suggests that some products are unsafe to consume at any level. The current team's

research is focused on products, such as glutamine, that have gone relatively unregulated and untested for almost fifteen years.

Though no empirical data was found on the economic impact of DSHEA, one can infer its significance. As previously mentioned, approximately 70% of Americans currently take dietary supplements. Because of the relatively free entry into the marketplace, consumers are flooded with choices ranging from multivitamins to protein powders to obscure herbs. More competition drives down prices, making these products more readily available to people of all socioeconomic classes. This therefore increases the access of consumers to a wide variety of potentially unregulated products.

No empirical data was found for a societal impact either, but some speculations have been made. According to the Coalition to Preserve DSHEA, the ready availability of supplements, mainly multivitamins, should improve the health of the average American. By increasing the general health of Americans, people will save money on health care. For example, if the elderly community increased their doses of calcium with vitamin D, supplements that are readily available in most drug stores, seniors would save over \$13.9 billion over five years (CPDSHEA, 1994). The growing presence of protein supplementation in the general market especially in the context of fad diets and bodybuilding efforts, therefore implies an increase in dietary and protein supplement usage amongst the younger generation.

2.2.3 Presence of Supplementation in the Younger Generation

In a 2006 study, it was shown that about 80% of teenagers had used a form of contemporary or alternative medicine at some point in their lives, and almost 50% of the teenagers surveyed about their use of contemporary and alternative medicine had used it

sometime within the past month (Wilson, et al., 2006). Because contemporary and alternative medicines are defined as including both herbal and dietary supplements, this implies that supplement usage is a growing trend amongst the young population. Several studies have been conducted on the topic of supplementation specifically on college campuses which suggest that it is a prominent activity amongst the student population especially amongst young athletes. The types of supplements used by the college-aged population have ranged from general dietary and protein supplements to specific amino acids for a variety of reasons mostly revolving around physical enhancement.

2.2.4 Non-Vitamin, Non-Mineral Supplement Usage on College Campuses

Generally, the studies have demonstrated the prevalence of students using supplements characterized as non-vitamin, non-mineral (NVNM). NVNM supplements have been defined in a broad sense to include dietary supplements, herbal supplements, protein or amino acid supplements, or other supplements that are used to enhance behavior such as memory, metabolism, energy, exercise, muscle recovery, etc. In a comparison study at Washington State University, researchers suggested that the use of NVNM supplements is more common in college students than in the general population (Newberry, Beerman, Duncan, McGuire, & Hillers, 2001). Other studies have documented the prevalence of NVNM supplement usage on various college campuses, especially in relation to the amount of exercise subjects may be involved in during some increment of time. In a 2004 study at a Turkish university, a stratified random sample of approximately 1,900 students completed a questionnaire regarding NVNM and dietary supplement usage including questions about types of products used, frequency of usage, amount of exercise, and demographic information (Ayranci, Son, & Son, 2005). The data

from this research yielded a positive relationship between exercising and NVNM supplement usage which suggested possible other implications.

In a 2002 study that aimed to determine the usage of NVNM supplements amongst a 1,000 person sample of college students, researchers found that approximately a quarter of the students were using NVNM supplements most of whom were using protein powders or amino acid supplements. This study also indicated a strong correlation between supplement usage and physical activity more than three times per month and discussed further research as necessary for educating the supplement-using population on implications of usage (Perkin, Wilson, Schuster, Rodriguez, & Chabot, 2002).

These findings have suggested that supplementation on college campuses deserves more attention as a research area since it appears to be a common activity amongst the younger generation. Additionally, these studies indicated a positive correlation between increased exercise time and supplement usage which begs further research given the potential health implications of using and possibly over-using and abusing these supplements in conjunction with rigorous exercise training. Data from these experiments also indicated that students may not be fully educated or informed about the consequences of supplement usage and may be under the wrong impressions about the benefits of supplement usage which increase their consumption. These all indicate that further research needs to be conducted in order to address these various findings and concerns.

2.2.5 Protein and Amino Acid Supplementation on College Campuses

The increase of NVNM supplement usage in the general market and on college campuses has led to further research on what specific NVNM supplements are popular

amongst the supplement-using population. Echinacea, ginseng, ginkgo biloba, and protein/amino acid supplements were all amongst the most frequently used NVNM supplements given their perceived benefits on mental and physical enhancement (Ayranci, et al., 2005; Perkin, et al., 2002). Protein and amino acid supplements seem to be used more often in regards to assisting physical attributes such as weight gain and muscle recovery. The following research concerning the use of protein and amino acid supplements with regards to physical enhancement has been conducted which supports the idea that further research on implications of usage needs to be explored.

In a 2004 study at a Division I university that specifically surveyed college athletes, approximately 88% of the subjects reported previous or current use of dietary supplements, 10% of whom specifically stated that they used the amino acid glutamine as a form of their supplement intake. Glutamine was found to be ingested in order to enhance athletic ability or expedite recovery from rigorous exercise (Froiland, Hingst, Kopecky, & Koszewski, 2004). Additionally in a 2007 study, approximately 600 high-performance Canadian athletes responded to a survey that addressed protein supplement usage, reasons for supplementation, sources of supplementation information, and concerns with inherent risks associated with usage (Erdman, Fung, Doyle-Baker, Verhoef, & Reimer, 2007). Data showed that the majority of participants reported taking one or more protein supplements in the previous six months of completing the survey. Additionally, protein drinks were the most frequently used protein supplements across the population sampled.

These studies support the conclusion that protein and amino acid supplementation as a form of NVNM supplements have increased proportionally on college campuses

especially amongst young athletes in recent years. The majority of these studies that have focused on exploring the issue of supplementation on college campuses have been in the form of survey research focusing mainly on self-reporting and anonymous response collecting. With any large-scale survey, it is important to ensure high-quality methodology and survey construction in order to produce reliable results.

2.2.6 Qualitative and Quantitative Research

The current research team constructed their survey using a combination of qualitative and quantitative research methods. When studying human subjects, it is not always advantageous to solely use quantitative methods which may prove impossible for obtaining the desired results. Qualitative research methods focus on phenomena and events that happen in natural, real-world settings which allow various factors all to meet and interplay, thereby creating complexity (Leedy & Ormrod, 2005).

Non-random sampling helps to explain more complex inquiries into human behavior even though the results cannot necessarily generalize to an entire population as is possible with random sampling. First, if a truly random sample were taken, the specific characteristics applicable to the study must be known. Second, random sampling will only produce useful results if beliefs and attitudes of the population are normally distributed. There is little evidence to show that the opinions and values that are crucial to the success of the study will be normally distributed. It is instead more likely that they will be concentrated in non-random subgroups. Lastly, some potential respondents in a population are more knowledgeable about the matter under study and will provide deeper, more useful information that is pertinent to the study (Marshall, 1996). It is more beneficial to seek a targeted population of individuals with characteristics or qualities

related to the studied behavior. This allows information to be garnered about a specific subset of a population concerned with the research area of interest.

2.2.7 Purpose of Using Survey Research

Almost all types of research share the similar goal of providing basic information describing both the topic and the respondents involved (Nardi, 2003). The only way to achieve this with 100% accuracy would be to contact and study each and every member of the population of interest. In most cases, it would be impossible to possess either the time or resources to achieve this goal. If done carefully, it is possible to take a small sample of that population and generalize the findings from that sample to the entire population. This is the basis for survey research methods. For example, in the current study of protein supplement usage on the University of Maryland, College Park campus, it would not be possible to track down and study every single student at the university. Instead with a written questionnaire, a smaller, targeted sample of this population can be studied for habits, trends, behaviors, and attitudes concerning a particular research area. This idea is supported by Peter Nardi in the text *Doing Survey Research* when he says, “Questionnaires are more efficient tools for surveying large samples of respondents in short periods of time than interviews or other research methods, and with less expense than interviews” (Nardi, 2003). For a project with stringent financial and time constraints, a self-administered survey targeting a specific subset of the college population was the best methodology choice for gaining a deeper understanding of the specific research area. Properly conducting survey research requires several aspects to ensure quality results. In all of the literature reviewed, several similar steps must have been taken in order to properly conduct survey research.

2.2.8 Survey Research Methods

The first step for the reviewed studies as well as the current study was usually to determine what specifically will be studied in order to decide on specific research questions. In research efforts, it is critical to come up with a set of well-defined research questions because this governs what will be asked in the survey. The next step was to decide the method for survey administration. Structured interviews, postal/e-mail surveys, and in-person surveys all have their own advantages and disadvantages. However, administering written surveys in person has important advantages over the other types. First of all, unlike personal interviews, one researcher can administer the survey to many respondents at once which saves time. Next, it adds to the reliability of the study to allow respondents to read and answer the items of a survey on their own. It would be less likely that the researchers would affect the outcome of the study either through their presence, phrasing of questions, or asking leading questions (Nardi, 2003). Additionally, even though the researcher is not actually reading each question to the respondents, it is helpful that a researcher be present to clarify the questions if necessary; this is not the case with postal or e-mail surveys.

After deciding which questionnaire method to use, the next and probably most critical step is writing the actual survey questions to address the study's specific research questions. The wording and structure of questions can have a great influence on the results, and often one of the most important aspects of conducting survey research is being able to succeed in "the construction of well-written and manageable questionnaires" (Nardi, 2003). Literature provides guidelines for how to best formulate a questionnaire that would maximize ease of use and minimize biases. First, questions

should be relatively short and simple, usually twenty words or less. It also poses a problem to ask people to recall information that happened more than six months in the past. Since in the case of the current study this was unavoidable, it was important to at least provide reference information to help the respondents recall the information. For example, when asking about a specific brand of a protein supplement product that an individual had used in the past, it was determined helpful to provide a list of popular brands for respondents to consult if needed.

Even after all of the questions have been written and the researchers have decided that the questions cover all the information they wish to learn, the organization of the questions within the survey is very important. Literature suggested that the most effective way to start the survey was to ask general, factual questions which would relax the respondents. Next, the more difficult questions would follow including those that ask the respondent to recall information that occurred in the past. Lastly, identifiable personal questions that were easy to remember would come towards the very end of the survey since this was easily accessible information (Nardi, 2003).

The potential advantages of evaluating and revising the first draft of a questionnaire before distributing it to the targeted population have been discussed in the scientific literature. It is not enough for the researchers to simply read and revise the survey on their own. Researchers who have constructed the survey are most likely already very experienced in the subject matter so there may be questions or directions that seem obvious to them but may cause confusion in respondents. One very effective method of revising survey drafts is to utilize focus groups through cognitive interviewing which are small groups of people from the targeted population who are recruited to fill

out the survey and provide feedback. They can help identify potentially problematic or ineffective questions. The major benefits of using focus groups are that the opinions of a wide range of respondents can be collected at the same time. Additionally, interaction between participants does not happen while people are filling out the survey in a real setting but can help to identify new problems during focus groups.

2.2.9 Purposive (Judgmental) Sampling

A combination of qualitative and quantitative research encompasses several different sampling techniques. For the current study, the research team used purposive, or judgmental, sampling. In purposive sampling, the sample is selected by the researcher for a specific purpose (Leedy & Ormrod, 2005). The subgroup is selected based on attributes it possesses that will help the researcher answer the research question(s) (Marshall, 1996). The researcher should be able to explain exactly why the specific subgroup was chosen (Leedy & Ormrod, 2005). The current study chose to examine a targeted population of students engaged in physical activity as a hobby and/or a requirement. It was presupposed that these students would have more knowledge and experience regarding protein supplementation than students who do not actively engage in physical activity. In addition, the selection of a subgroup should be based on the researcher's previous knowledge of the characteristics of the population and knowledge gained through literature (Marshall, 1996). It was presumed that students who participate in physical activity use protein supplements to enhance their performance. This presumption was later confirmed in several studies that this phenomenon was present on other college campuses especially in a population of students who partake in physical activity (Biemer & Lyberg, 2003).

Purposive sampling is advantageous for several reasons. First, when the study is being conducted under time restraints, purposive sampling allows the researcher to reach the targeted sample quickly (Trochim & October, 2006). The university research program under which this current study was conducted did limit the time for collecting and analyzing data. Second, this type of sampling is inexpensive (Ackoff, 1953). A student research team with minimal university funding does not have the means to conduct research that requires large sums of money to be successful. Purposive sampling is a satisfactory way to achieve results consistent with the academic inquiry by undergraduate students with a university budget. Most importantly, purposive sampling provides directed, valid, and useful information for the researcher because the subgroup has specific interest and/or relevant experience in the topic(s) under scrutiny (Biemer & Lyberg, 2003). This was essential simply because the time and money restraints created pressure to complete efficient yet high-quality research.

Although purposive sampling has its drawbacks, the limitations were not serious enough in nature to pose serious implications for the current study's results. One negative aspect of purposive sampling is that bias is inherent and cannot be eliminated. The sample was specifically chosen for the reason that it is likely that it will provide the information the researcher is seeking (Lunsford & Lunsford, 1995). The bias was actually advantageous because it allowed for a more efficient survey administration to a topic-related targeted population. Additionally, this method must take into consideration the context of the study. It is possible that a respondent may answer one way at one given point in time and a different way at another point in time simply because his or her frame of mind, values, or opinions may have changed or been affected by some unforeseen

variable (Marshall, 1996). However, literature has shown that this does not typically play a significant role on the results garnered from a questionnaire.

There is no established sample size for a purposive study. Literature has discussed that the sample size should simply be large enough to answer the research question adequately (Marshall, 1996). The current study sought to acquire as many respondents within the designated subgroup as possible because it was known that inevitably a portion of the responses would be invalid. In order to evaluate the results of the study, statistical analysis had to be performed on the data.

2.2.10 Purpose of using a Statistical Analysis Program

The program selected to run these descriptive analyses was SPSS (originally, Statistical Package for the Social Sciences) which is a program commonly used in the social sciences. Although other programs exist, SPSS was the most logical choice considering the circumstances. Amongst the many statistical software packages on the market, SAS and SPSS are most frequently used. Each of these programs has its advantages and disadvantages. SPSS has proven to be the most reliable of the aforementioned packages even though it is considered to be more basic program.

The reliability of these packages was determined by how well the programs performed certain tests and features. Specifically, univariate statistics, ANOVA, linear regression, nonlinear regression, and distributions were assessed. Microsoft Excel proved to have errors in each of these five categories. SAS made errors in four out of the five categories. SPSS incorrectly calculated in only three of the five (Altman & McDonald, 2001). In comparison, SPSS was the most reliable of the programs most frequently used.

The fact that undergraduate students with beginner-level experience in statistical analysis were going to be using the program was also a major factor in selecting SPSS over the other packages. On the other hand, SAS is designed for an experienced user who uses the program every day or on a frequent basis and has the skills essential for deciphering its rather confusing design. SAS is a better selection when complex data management is the prime function required. SPSS is a better selection when mainstream, simple data analysis is the prime function required (Acock, 2005). This study will only need basic data analysis functions to produce the desired results.

2.2.11 Statistical Analyses Using SPSS

SPSS can perform both simple and advanced statistical analyses. Some of the most commonly employed statistical applications involve computing frequencies, condescriptive frequencies, independent samples t-tests, Pearson's r , Spearman's Rho, and One-Way ANOVA (Andrews, Davidson, Klem, OMalley, & Rodgers, 1981). Several of the simpler computations fall under a general category called descriptive statistics. Descriptive statistics computed alongside creations of frequency tables, frequency distributions, graphs, and charts (especially pie charts because of their simplicity in data analysis and interpretation) can reveal a great deal of information about the generalities and the specificities in a particular data set (Feeney & Kirkpatrick, 2007).

The purpose of descriptive statistics is to describe and present the data in order to show relationships, comparisons, and correlations amongst all of the variables being tested by the researchers. Typically, these researchers have at least beginner-level experience in statistical analysis (Hagan, 2006). A familiar group of descriptive statistics involves computing the mode, median, and mean of the data set. The mean is the most

commonly used component of this group of descriptive statistics. All three components of this group present certain kinds of averages for the values in a data set.

Another more specific group of descriptive statistics involves computing the range, average deviation, and standard deviation. Standard deviation is the most meaningful constituent in this group for performing more comprehensive statistical analyses for a data set. These values assess the spread of the values in a data set.

In addition to descriptive statistics, there are several more in-depth statistical analyses that a researcher can perform in order to show greater and far more meaningful relationships, comparisons, and correlations amongst the variables in a data set. One of these statistical analyses is called a t-test, also known as a difference of means test. The purpose of a t-test is to compare the sample means of two separate groups. If these two means are different from each other, then the t-test will be statistically significant. The researcher will then be able to conclude that the samples are not from the same population. If these two means are not different from each other, then the t-test will not be statistically significant. The researcher would conclude that the samples either are not different or that the samples are coming from the same population. The latter two conclusions may sound as if they are the same, but in fact are making two separate conclusions which must always be stated together connected by an 'or.'

Another specific statistical analysis that a researcher can perform for clear portrayal of relationships, comparisons, and correlations amongst the variables in any data set is called One-Way ANOVA. This abbreviation stands for analysis of variance. The purpose of ANOVA is to compare three or more sample means in order to assess greater variation between groups and smaller variation within groups.

In addition to t-tests and ANOVA, another meaningful statistical analysis involves the usage of a correlation coefficient called Pearson's r . The range of values for Pearson's r is from 0 to ± 1.00 . 0 represents no statistical relationship. Values of ± 1.00 represent statistically perfect relationships. Values of ± 0.20 represent negligible relationships. Values between ± 0.20 and ± 0.40 represent low relationships. Values between ± 0.40 and ± 0.60 represent moderate relationships. Values between ± 0.60 and ± 0.80 represent highly moderate relationships. Values between ± 0.80 and ± 1.00 represent high relationships. A minus sign in front of a value signifies a negative correlation, while a plus sign in front of a value signifies a positive correlation. Another correlation coefficient for statistical data analysis that reveals specific and meaningful relationships, comparisons, and correlations amongst variables in a data set is Spearman's Rho (r_s), and its range of values is identical to the Pearson's r (Hagan, 2006).

Literature regarding survey construction and analysis had discussed the importance of specifying exactly what a future study would aim to examine. The data collected and analyzed from the University of Maryland, College Park questionnaire was to relate the usage of protein supplements by members of campus. These results were to tie back into the results garnered from data collection from kidney cell modeling and biochemical analysis for a comprehensive analysis of protein supplements and in particular, one specific amino acid: glutamine.

2.3 Kidney Cell Modeling Literature

2.3.1 Executive Summary of Kidney Cell Modeling Literature

Through this review, it has been established that the American diet already provides 150% the daily recommend allowance of protein. The effects of excessive protein are well documented for patients with kidney disease, and a decrease in protein intake has been proven to slow the progression of chronic kidney disease. On the other hand, little is known about the ill effects of excessive protein intake among healthy individuals. This community is likely to include athletes that take protein and amino acid supplements. The issue of hyperfiltration is nullified by its possibility of being a natural response and by the increase in kidney size associated with increased dietary protein. In order to study possible deleterious effects of excessive protein, it is important to focus on a particular amino acid, as different blends of amino acids produce very different physiological effects. For example, human studies have shown the induced change in serum LDL can be opposite for ingestion of different protein sources.

Due to its benefits, glutamine has become a commonly used supplement among athletes. Its benefits are real when glutamine is in high demand by the body, but research data suggest that excess intake of glutamine may be deleterious for the kidney, especially for those who are at risk for chronic kidney disease such as individuals with diabetes, hypertension, obesity, or family histories of kidney disease. Researchers have used the mouse mesangial model in the past to study the effects of glutamine on gene expression for various *in vitro* markers for sclerosis, such as collagen. At the same time, few researchers are concerned with the effects of specific amino acids on renal health. The

most studied amino acid was found to be arginine, which has been reported with conflicting data.

The studies that have been done on glutamine in mesangial cells have indicated the significance of the dose of exposure or concentration of glutamine in experimental groups. The research questions of the current research were designed to address such issues. In addition, it was necessary to know the specific glutamine concentration in protein supplements taken by participants of the survey studies of the current research. A review of biochemical analyses was necessary, with specific attention to chromatography methods.

2.3.2 High Protein Intake and Renal Health

The typical American consumes more protein in their usual diet than the recommended dietary allowance (RDA) suggests. The current RDA intake is 0.8 grams of protein per kilogram of body weight per day. The average American consumes 1.2 g/kg/day, or 15% of total caloric intake (Eisenstein, Roberts, Dallal, & Saltzman, 2002). There are no established definitions for “high protein” diets, but a review of data by Eisenstein et. al. suggests that an intake greater than 1.6 g/kg/day or 25% of caloric intake can be considered a high-protein diet. Intakes of greater than 2.4 g/kg/day or 35% energy intake can be considered extremely high.

Current literature has shown that the use of high protein diets has become popular in the population in general and especially among the athletic community, aiming to increase physical performance and boost the immune system. In addition, the use of protein-rich diets gained the attention of individuals interested in losing weight. Among several measures proposed to control the increasing rate of obesity and development of

diabetes in the population is a low carbohydrate-high protein diet (Eisenstein, et al., 2002). However, the benefits of a high protein intake decreasing the appetite and the levels of glycemia are counterbalanced by deleterious effects including an increased risk to accelerate the progression of chronic kidney disease.

The increased consumption of high protein diets with no medical supervision raised concerns regarding the population at risk for chronic kidney disease. As the primary filtration system for the body, the kidneys are responsible for removing wastes, toxins, and excess nutrients. Nitrogenous waste and acid that accumulate from increasing dietary protein are filtered by the kidneys (Guyton & Hall, 2006). This continuous handling of protein and its byproducts makes renal health critically important. The focus on the effects of dietary protein on the kidneys has been heightened by studies showing that decreased protein intake slows the progression of chronic kidney disease.

Thomas Addis suggested the restriction of protein intake by patients with kidney disease as early as 1948 (Brenner, Meyer, & Hostetter, 1982). The Modification of Diet in Renal Disease (MDRD) study conducted by Dr. Saulo Klahr in 1989 was the springboard for studies recommending that patients suffering from chronic renal insufficiency limit protein intake. The study showed the benefits of decreased protein intake for patients with kidney disease. It measured the decrease of glomerular filtration rate (GFR). Glomerular filtration rate is progressively reduced as kidney disease advances to renal failure. The measurement of GFR is used as an indicator of kidney function. Preservation of filtration is vital to slowing the progression of kidney disease. The experimental group with the lowest protein intake, less than 0.5 g/kg/day, still showed a progressive decrease in GFR, but the decline was significantly less than

patients on a regular protein diet. It was determined that a decrease of 0.2 g/kg/day could slow the decline of GFR by as much as 1.15 mL/min/year. This may reduce the risk of renal failure or death by as much as 50% (Klahr, 1996).

Several researchers have used the data gathered from the MDRD study in various analyses. A analysis by Levey et. al. (1999) supported the claim that lowering protein intake will slow the decline of GFR and the progression of renal disease. The analysis did not suggest dietary protein reductions would always slow GFR decline, but there was evidence to support that lower intake levels with additional intervention would slow the progression of kidney disease (Levey, et al., 1999). High protein diets for those with renal damage has since been discouraged.

While research has supported the notion that high protein diets can be damaging to patients with kidney disease, it has not suggested any significant ill effects to the healthy population. Contrary to the findings relating consumption of high protein with deleterious effects in patients with chronic kidney disease, studies involving renal function in healthy individuals has left no reason to believe that high protein diets poses an unusual risk (Eisenstein, et al., 2002). One of the parameters examined in human renal studies is hyperfiltration. Hyperfiltration is seen early in many forms of kidney disease, and several studies have indicated that increasing protein in the diet, up to a saturation point of 125 g/day, significantly increases hyperfiltration (Eisenstein, et al., 2002).

An increase in hyperfiltration may be a normal response to increased dietary protein. A study involving overweight to obese subjects ($25 < \text{body mass index (BMI)} < 34 \text{ kg/m}^2$) showed changes in renal size and function over six months without any adverse effects. Protein intakes were changed significantly from a 91.4 g/day average to

70.4 g/day in the low protein group and 107.8 g/day in the high protein group (Skov, et al., 1999). Increased protein led to a positive 5.2 ml/min change in GFR, while decreased protein led to a minus 7.1 ml/min change. Kidney volume increased by 9.1 cm³ in the high protein group, and decreased by 6.2 cm³ in the low protein group. While hyperfiltration was noted in the high protein group, net hyperfiltration, which considers the changes in renal size, did not significantly differ between the experimental and control population (Skov, et al., 1999).

2.3.3 Amino Acids and Renal Health

While studies involving high protein diets may not have shown any significant ill effects, studies involving amino acids show more exciting results. Amino acids are the natural building blocks of proteins. When protein is ingested, it is broken down into amino acids so that cells can absorb these vital nutrients from the blood and incorporate them into their cellular metabolism.

In a 120-day feeding study of adult rats, serum lipid levels were examined to determine the influence of two different protein sources- casein and gelatin. Casein is high in glutamic acid, methionine, phenylalanine, and tyrosine, while gelatin is largely composed of arginine, glycine, and hydroxyproline. Both proteins would have been digested by the rats and their composite amino acids would be absorbed into the blood. Each protein source showed varying results. The casein-fed rats showed significant increases in serum levels of triacylglycerols and total cholesterol, while the reverse effect was observed in gelatin-fed rats. The stark contrast between the two protein sources shows how amino acids can produce very different physiological effects (Ratnayake, Sarwar, & Laffey, 1997).

Giving specific attention to amino acids in regards to renal health, administration of high concentrations of amino acids has been shown to increase glomerular flow and filtration rate. These changes were previously attributed to a hemodynamic effect caused by the amino acid load. This idea changed when Meek et. al. (2003) demonstrated that a high concentration of amino acids induced increased expression among proteins considered *in vitro* markers of glomerular sclerosis. A similar response was observed with L-arginine, and the authors suggested that the glomeruli fibrotic response elicited by amino acids was independent of hemodynamic effects (Meek, et al., 2003).

Arginine, a substrate of nitric oxide (NO) is one of the amino acids that have been most studied in the setting of chronic renal disease, mainly because of the NO-related hemodynamic effects. *In vivo* and *in vitro* studies with arginine resulted in controversial conclusions. While some investigators have shown that an increase in arginine may lead to more extensive renal damage (Cook, et al., 1994; Ketteler, Border, & Noble, 1994) others have found a protective role for this amino acid (Ingram, et al., 1995; Lubec, Aufrecht, Amann, Kitzmüller, & Hoyer, 1997; Narita, A., Ketteler, Ruoslahti, & Noble, 1995). Only a few other amino acids have been individually studied as inducing changes in the course of renal diseases (Kaysen & Kropp, 1983). Extensive literature search has supported this notion. Few researchers are concerned with the effects of specific amino acids on renal health.

The effect of different amino acids on human health has found its way from the scientific community into the realm of supplementation by athletes. The physiological benefits of glutamine, in particular, make it an attractive supplement for athletes. Before

the benefits of glutamine to athletes can be discussed, a basic background of glutamine and its physiological role must be established.

2.3.4 Glutamine and its Physiological Role

Glutamine is one of twenty naturally occurring amino acids in the human body and plays a major role in protein synthesis. It is strongly concentrated in cells of the central nervous system where it acts as a precursor for the synthesis of neurotransmitters, nucleotides, and glutathione, a small peptide important for some enzymatic activation (Newsholme, et al., 2003). In addition to its role in the central nervous system and high concentration in the intestinal walls, glutamine is in high demand in skeletal muscle (Connolly, 2004). Glutamine accounts for more than 60% of the total pool of free amino acid in muscle and is the most common amino acid in plasma (Antonio, 2002). In the blood, glutamine plays a strong role in maintaining immune function by acting as a fuel for lymphocytes and macrophages (Castell & Newsholme, 1997). Glutamine is also a substrate for hepatic and renal gluconeogenesis, the synthesis of glucose within the liver and kidneys, respectively, as well as a donor for nitrogen (Newsholme, et al., 2003).

2.3.5 Glutamine in Sport and Exercise

Glutamine has been shown to increase the production and release of plasma interleukin-6 (IL-6), an inflammatory cytokine innately tied with the immune response. In a study by Hiscock et al. (2003), it was shown that among a group of healthy males exercise alone induced an eleven-fold increase in plasma IL-6. Glutamine or glutamine-rich protein potentiated the increase of plasma IL-6. An 18-fold increase was observed in males using a glutamine solution, while a 14-fold increase was observed among glutamine-rich protein users (Hiscock, et al., 2003). IL-6 is a pro-inflammatory and anti-

inflammatory cytokine secreted by T cells and macrophages in trauma. This interleukin regulates immune response and host defense mechanisms. In the kidney, renal tubular epithelial cells express IL-6 during inflammation (Kindt, Osborne, & Goldsby, 2007).

A study by Wilmore and Shabert (1998) demonstrated the close relationship between glutamine availability and cell proliferation. Immunological response and proliferation of bowel mucosal cells increased with glutamine concentration. This effect was associated with repair of intestinal mucosa erosions and maintenance of the bowel barrier function (Wilmore & Shabert, 1998).

Athletes have been shown to be more prone to infection after an exhaustive work out. Exercise reduces the body concentration of glutamine by 20-25% while surgery reduces the body concentration of glutamine by 37% (Lagranha, et al., 2005). A study was conducted in athletes comparing glutamine supplementation to phagocytosis, an important process in the immune system to rid the body of pathogens and cell debris. With glutamine supplementation, there was an increase in phagocytosis as well as oxidative capacity of neutrophils, in comparison to no change without supplementation (Lagranha, et al., 2005). Thus, exercise does not change phagocytosis or reactive oxygen species, but glutamine supplementation increases phagocytosis and has a major effect on the neutrophil function.

Glutamine's role in metabolic acidosis may be important for athletes involved in intense anaerobic exercise. During metabolic acidosis glutamine produces ammonium ions after being converted to α -ketoglutarate. The presence of these ammonium ions counterbalances the increased plasma lactate and hydrogen ion concentration generated in the fast glycolysis of anaerobic exercise or in catabolic states, thereby buffering the

effects of metabolic acidosis in the body (Antonio, Sanders, Kalman, Woodgate, & Street, 2002).

Glutamine is particularly beneficial in high-intensity endurance training. An amino acid mixture containing glutamine, leucine, isoleucine, valine, and arginine was administered to 23 Japanese rugby players in a well known *in vivo* study. The amino acid mixture was given at 6.6 g/day for a period of 90 days. A number of positive results including quicker muscle recovery from fatigue, increased blood oxygen-carrying capacity, and decreased muscle damage indices were observed (Ohtani, Masaaki, & Maruyama, 2006). A significant increase in levels of LDL cholesterol was also noted among the experimental group. Normal lipid levels were restored after terminating administration of the amino acid mixture (Ohtani, Maruyama, Titchenal, & Kobayashi, 2001).

Interestingly, some human studies have shown that diets high in animal protein significantly reduce serum triglycerides and LDL cholesterol (Hu, 2005). Hu reviews these studies in which dietary fat intake, cholesterol, and fiber were kept constant while varying protein intake between 11 and 27% of total energy intake. All studies showed significant decreases in serum triglycerides and LDL (Hu, 2005). These opposing findings in serum levels of LDL reinforce the importance of protein source and amino acid compositions. Determining amino acid specific effects becomes important in such situations where protein and amino acid blends were used.

2.3.6 Clinical Applications of Supplemental Glutamine

In addition to its use in endurance training and high intensity exercise situations where glutamine demand may be high, glutamine is used in several clinical applications.

Decreased levels of glutamine may act as an indicator of catabolic states such as infection, surgery, trauma, and acidosis (Rowbottom, Keast, & Morton, 1996). Glutamine also improves nutrient absorption and therefore intestinal health as well as regulates muscle protein turnover (Connolly, 2004).

One study has shown that postoperative patients benefited greatly from parenteral feedings enriched with glutamine. Subjects displayed a more optimal nitrogen balance as well as maintained intestinal permeability (Jiang, et al., 1999). Glutamine, has beneficial effects for burn patients. It has been linked to the proliferation of intra-cellular repletion of glutathione, an important forager of reactive oxygen species. Glutamine's abilities to limit skeletal muscle catabolism and control some inflammatory stimulation may indicate that it can improve a patient's chance of overcoming an acute illness. Improved T cell function *in vitro* and in stressed human patients is also seen in association with glutamine supplementation (Sheridan, et al., 2004).

2.3.7 Adverse Effects of Glutamine on Kidney Cells

Supplementation with amino acids and particularly glutamine has shown beneficial effects in humans when there is a high demand, like in athletics, or a deficiency, like in clinical applications. However, data from studies *in vitro* suggest that amino acids and glutamine in supraphysiological amount cause deleterious rather than beneficial effects on the kidney. Mesangial cells are one of the kidney cells affected by glutamine. They play an important role in glomerular filtration. In the normal kidney these cells maintain a low rate of proliferation, which can be substantially increased in response to an injurious stimulus. An increased mesangial cell growth is often associated

with accumulation of extracellular matrix in kidney disease. Therefore, factors that stimulate mesangial cell proliferation may result in glomerular scarring.

A study of cultured rat mesangial cells in the presence of varying concentrations of an amino acid mixture with and without glucose was performed to determine whether amino acids alone or in association with glucose would pose a risk for kidney disease. This study showed that elevated concentrations of both amino acids and glucose isolated or in combination induced an increase in mesangial cell proliferation and in the expression of markers of glomerular sclerosis (transforming growth factor β , type IV collagen, fibronectin, and thrombospondin-1). Thus, amino acids increase expression of both growth factors and matrix proteins in mesangial cells. The authors conclude that increased amino acids can cause renal damage similarly seen in elevated protein consumption (Meek, et al., 2003).

While it has been shown that an increase in amino acids can cause renal damage, it is important to identify the role of specific amino acids. The demonstration that glutamine induced an increase in mesangial cell proliferation led to the hypothesis that this particular amino acid could induce further damage to these cells. To test that hypothesis, mouse mesangial cells were grown in the presence of varying concentrations of glutamine (0-2 mM) and markers of sclerosis (type IV collagen, tenascin, and fibronectin) and of mesangial cell injury (α -smooth muscle actin, SMA) were studied. Increase in glutamine caused a significant increase in the expression of α -SMA, type IV collagen, and fibronectin. Increased α -SMA expression is seen in many human glomerular diseases and is a marker of mesangial cell increased proliferation induced by

injury. Thus, prolonged high intake of glutamine could cause mesangial cell injury (Lagranha, et al., 2005).

A study with mesangial cells grown for 72 hours in low glucose (5mM) or high glucose (25mM) with glutamine concentrations varying from 0-20 mM glutamine demonstrated that mesangial cell proliferation in low glucose significantly increased and reached a plateau with 1 mM glutamine. In the presence of high glucose, glutamine induced a dose dependent increase in proliferation. In diabetes, high glucose increases the renewal rate of the mesangial cells, mediated by a biosynthetic pathway involving glutamine:fructose-6-phosphate amido-transferase (GFAT). Similarly, glutamine effect on mesangial cell proliferation appears to be mediated by the GFAT pathway. Thus, chronic kidney disease may be induced by elevated levels of glutamine due to supplementation in high risk users, such as diabetes or those with higher blood glucose levels (Lagranha, Doi, Pithon-Curi, Curi, & Sellitti, 2008).

2.4 Biochemical Analysis Literature

2.4.1 Executive Summary of Biochemical Analysis Literature Review

It is important for scientists to study the true content of dietary supplements for consumers' safety, and the passing of DSHEA highlights the importance of this pursuit. This portion of the current study is concerned with quantifying the amount of glutamine in dietary supplements containing glutamine. There are various tools to determine glutamine content, which include HPLC, GC, and CE. While all of these devices are capable of quantifying the amount of glutamine, the literature suggests that HPLC will prove to be the most superior tool in this study. Advantages such as its high resolving power and ability not to alter any component of a substance with heat make HPLC an appropriate tool for this study. HPLC has already been shown to analyze amino acids in mediums, such as supplements and food products, further suggesting that it will serve well in analyzing dietary supplements.

Protein-based dietary supplements cannot be simply dissolved and vaporized into the HPLC column to quantify the amount of glutamine. Instead, these supplements must be hydrolyzed and derivatized before entering the HPLC instrument to be analyzed. Research suggests that enzyme hydrolysis is the best way to quantify glutamine from protein rather than acid hydrolysis. Tsao and Otter (1999) used a three-enzyme system to degrade proteins into its amino acid constituents before HPLC analysis. Enzyme hydrolysis did not underestimate glutamine content like acid hydrolysis did, since acid hydrolysis converts a significant amount of glutamine into glutamic acid. In addition, Baxter et al. (2004) showed that enzyme hydrolysis leads to minimal conversion from

glutamine to glutamic acid, further justifying enzyme hydrolysis as an appropriate means of breaking down protein-based supplements.

As mentioned above, amino acids must be derivatized in order to be detected. PITC has been found to be a good derivatizing agent because it is stable and volatile, and has a fast reaction with amino acids. The combination of enzyme hydrolysis and PITC-derivatization provides us with the best way to detect and quantify the amount of glutamine in protein-based dietary supplements.

2.4.2 Biochemical Analysis Background

Many dietary supplements are not under strict regulation from the United States Food and Drug Administration (FDA) as a result of the Dietary Supplement Health and Education Act of 1994 (DSHEA). As mentioned earlier, there are three basic guidelines that manufacturers of dietary supplements must adhere to in order to be regulated as a dietary supplement and not a drug. Simply put, the statements regarding the benefits must be related to a “classical nutrient deficiency disease” and must specifically say what the ingredient’s main intended action is within the body (United States Food and Drug Administration, 1994). Additionally, the manufacturer must stand by their claim that the statement of benefits is truthful. Finally, the product must contain the warning “This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease” (United States Food and Drug Administration, 1994).

Given the nature of this legislation, manufacturers potentially can add or take out certain amounts of chemicals into their products because the products are not being tested for safety or efficacy before entering the market. Consumers may be unaware of these

and may be ingesting a product without actually understanding what it may contain. Because there is no safety regulation, glutamine-containing dietary supplements were tested to compare their glutamine content against the content claims by manufacturers.

2.4.3 Methods to Detect Amino Acids

Amino acids are commonly detected and quantified using various analytical tools, including high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). The method of HPLC can separate amino acids through the partition of analytes between the mobile and stationary phase. In HPLC, a liquid mobile phase is eluted through a small column that contains a solid, tightly packed stationary phase. Reversed-phase liquid chromatography refers to the use of a nonpolar stationary phase with a relatively polar mobile phase to separate the molecules. The most polar molecules will elute the column first, and the most nonpolar molecules will elute last. Increasing the polarity of the mobile phase (polar solvent) increases the elution time (Skoog, Holler, & Nieman, 2007). One of the most frequent and important applications of HPLC is analyzing amino acids, and this is typically carried out in the reversed-phase (Concha-Herrera, Torres-Lapasio, Vivo-Truyols, & Garcia-Alvarez-Coque, 2006).

The instrument employed for GC is significantly different from HPLC. The major components of a GC system are a column that can be temperature programmed, a sample inlet point, a carrier gas supply and control, a detector, an amplifier, and a data recorder system. Gas chromatography exploits the difference in the partition coefficient between a stationary liquid phase and a mobile gas phase of volatilized analytes as they move through the column. The partition coefficients allow the most volatile analytes to elute first. The temperature of the apparatus is then raised to 50-300°C for volatilization to occur. The

stationary phase is typically a high boiling point liquid material that is either coated onto the column or packed into the column. This tool is particularly useful when coupled to mass spectrometry (Wilson & Walker, 2005).

A third tool that can be used to separate amino acids is CE, and the basis of this device is electrophoresis of samples through narrow tubes. The typical internal diameter of a tube is 50 μm , and this produces a large surface-to-volume ratio. The large surface-to-volume ratio produces enhanced heat dissipation, which is advantageous because CE eliminates both convection currents and zone broadening caused by heat. CE also has the capability to detect concentrations as low as femtomole (10^{-15} moles) (Wilson & Walker, 2005).

All three of the instruments discussed above can be used to separate and detect amino acids. Separation techniques using GC and HPLC have been shown to be well established for analysis (Landers, 1994). While GC is probably the most commonly used form of chromatography, it is used for volatile compounds that do not need derivatization (Wilson & Walker, 2005). The procedure for analyzing amino acids in dietary supplements involves derivatization, which means that the amino acids are generally transformed chemically into another similar substance to be separated in a column. GC analysis includes volatilization; however, the process is time-consuming and the samples must be derivatized before their GC elution and quantification, and the derivatization must take place in water-free conditions (Molnar-Perl, 1994). Furthermore, a considerable number of substances that are tested using chromatography are heat-sensitive. As mentioned above, amino acids that are to be analyzed must evaporate into a gas before entering the GC column. The materials are subjected to temperatures above

their boiling point to achieve this (Hanai, 2005). Therefore, the drawbacks of GC in analyzing amino acids show that this device is not the most appropriate way to detect amino acid concentrations.

HPLC is a better tool for detecting amino acids than GC for many reasons. First, compounds are not subjected to an elevated temperature in HPLC preventing the alteration of any of the components (Hanai, 2005). Also, the process of derivatization is easier to manage in HPLC than GC. HPLC can be paired with an ultraviolet absorption or fluorescence detector in order to detect compounds that have derivatized (Molnar-Perl, 1994). Also, gradient HPLC offers high peak capacity and efficiencies around 50,000 theoretical plates. The resolving power of the chromatographic column increases with the number of theoretical plates per unit length (and the column length). Theoretical plates are directly related to the surface area of the stationary phase. Although small stationary phase particles create more resistance and back-pressure, small particle size stationary phases have been developed to withstand this pressure. This development alone is responsible for the faster and better resolution in HPLC, and explains why HPLC has emerged as the most powerful and versatile form of chromatography (Wilson & Walker, 2005).

Capillary electrophoresis is a newer technique that can also detect amino acids through derivatization. One of the major advantages of using CE is the ability to separate a large number of closely related materials in a very short period of time. Also, a single run in CE uses essentially zero solvent consumption and sample injection volumes are on the level of 5 to 10 nL. CE is a useful tool in protein digestion experiments because of its large peak capacity (Landers, 1994). Overall, this device is a simpler instrument that does

not include a precise high-pressure pump and consumes less reagents than liquid chromatography (Manaenkov, Sidorov, & Sulman, 2003).

Conversely, the main drawback of CE is its poor concentration sensitivity and the fact that the separation buffer and sample diluent must be similar (Landers, 1994). Another drawback of CE is the ultraviolet (UV) laser that is used in the typical capillary electrophoresis-laser induced fluorescence (CE-LIF). The bright and coherent UV laser used is an expensive part. Light-emitting diodes (LED) have been proposed over UV lasers because their small size, low cost, ease of operation, and wider range of excitation wavelengths. However, the light from a LED are divergent and not pure, leading to a relatively high fluorescent background (Chang, Chiu, & Chang, 2006). Also, another disadvantage of CE is that adding substances to the carrier electrolyte through derivatization modifies the surface of the fused-silica capillary and often degrades the precision of analytical results (Manaenkov, et al., 2003). For these reasons, HPLC has been demonstrated to be an appropriate tool to be used in separating and detecting the amino acid glutamine.

HPLC has been used to analyze amino acids in a wide range of mediums, including biological fluids, food, and supplements (Battaglia, et al., 1999; Baxter, Phillips, Dowlati, & Johns, 2004; Korös, Varga, & Molnár-Perl, 2008; Silva, et al., 2007; Tcherkas & Denisenko, 2001). For example, studies have examined the amino acids in human serum or plasma (Tcherkas & Denisenko, 2001; Tcherkas, Kartsova, & Krasnova, 2001). The determination of amino acids in biological fluids, like serum, is important in clinical biochemistry. For example, the change in amino acid concentration in serum and other physiological fluids is believed to be correlated with neurological disorders, such as

Alzheimer's disease. The studies employed isocratic reversed-phase HPLC with either electrochemical detection or fluorescence detection in order to quantify the amount of amino acids in serum, such as leucine and phenylalanine (Tcherkas & Denisenko, 2001; Tcherkas, et al., 2001). These amino acids were pre-column derivatized by o-phthalaldehyde (OPA), a derivatizing agent that will be discussed later in this section.

HPLC can also be used in the analysis of food products, such as red wine. Pre-column derivatization with dabsyl chloride was the method used to determine the amount of all twenty free amino acids in a particular red wine. The researchers then chose to evaluate the effect of the spoilage wine yeast *D. bruzellensis* on the free amino acid composition of the red wine (Silva, et al., 2007). While amino acid analysis has been thoroughly studied in the mediums mentioned above, it is important to note that there is less research completed on the detection of amino acids in supplements. A study that did identify and quantify amino acids in supplements used enzyme digestion, 9-fluorenylmethyl chloroformate (FMOC) derivatization, and HPLC-UV to quantify glutamine in twelve nutritional supplements (Baxter, et al., 2004). Findings from this article will serve as a useful guide in the current study's research, and will be further discussed later in this section.

2.4.4 Use of HPLC and Enzyme Hydrolysis to Detect Glutamine

Glutamine must be released from protein in order for the glutamine content of protein-based supplements to be determined using HPLC. Acid hydrolysis is typically used for amino acid analysis. However, the chemical structure of glutamine is easily modifiable. Glutamine, in its free form alone, is very unstable. In acidic conditions, glutamine is converted into glutamic acid through deamination, which underestimates the

amount of glutamine present. However, there are also chemical modifications under alkaline conditions, and glutamine converts into pyroglutamic acid (PGA). These chemical conversions make it difficult to accurately quantify the amount of glutamine (Tsao & Otter, 1999).

Enzyme hydrolysis provides a more efficient means to quantify the amount of glutamine than acid hydrolysis. Tsao and Otter (1999) developed a procedure to quantify unmodified glutamine using enzyme hydrolysis and a reversed-phase HPLC system. Three milk proteins were used in this study to quantify glutamine in protein, and the milk proteins were α -lactalbumin, β -lactoglobulin, and β -casein. Protein samples containing glutamine were subject to enzyme hydrolysis, and the three enzymes used were pronase E, aminopeptidase M, and prolidase. These enzymes were chosen based on the enzyme hydrolysis described by Tsao and Otter, and originally from the work of Henle et al. (1991). Pronase E is a protease that preferentially cleaves bonds between the hydrophobic amino acids: alanine, valine, leucine, isoleucine, phenylalanine, tryptophan, and tyrosine. Leucine aminopeptidase hydrolyzes bonds between leucine and other amino acids at the amino end of leucine. Prolidase cleaves glycine-proline bonds. The protein samples were also subjected to acid hydrolysis for comparison (Tsao & Otter, 1999).

The average percent recoveries of glutamine following enzyme-hydrolysis for α -lactalbumin, β -lactoglobulin, and β -casein were 77.5 ± 3.9 , 97.5 ± 3.0 , and 100.9 ± 2.7 , respectively. The recovery rates for β -lactoglobulin and β -casein were very high, suggesting the near complete release of glutamine from these proteins. The percent recovery for α -lactalbumin was likely lower than expected because many of the glutamine residues are adjacent to acidic amino acids, i.e. glutamic acid and aspartic acid.

Hydrolysis would be more difficult to achieve in these areas, which accounts for the low percent recovery of glutamine in α -lactalbumin (Tsao & Otter, 1999).

Acidic amino acids are not the only residues that can prevent or slow down hydrolysis of proteins. Proline may be resistant to enzyme hydrolysis as well. Thirty-eight percent of the glutamine residues of β -casein are adjacent to proline residues; however, this did not appear to affect the release of glutamine (average percent recovery = 100.9 ± 2.7). Tsao and Otter (1999) attributed this finding to aminopeptidase M, which specifically releases glutamine and asparagine from peptides. In this study, the rate of hydrolysis of the peptide glutamine-proline (10 minutes) was faster than that of proline-glutamine (2 hours). Although aminopeptidase M releases glutamine from peptides, it does so at a much slower rate when those glutamine residues are adjacent to a proline residue. These results demonstrate how acidic amino acids and proline can prevent complete hydrolysis of protein samples (Tsao & Otter, 1999).

Unlike enzyme hydrolysis, the mean percent recovery for the three proteins using acid hydrolysis could not be determined because glutamine residues were fully converted into glutamic acid residues. The discrepancy in the percent recovery of glutamic acid between enzyme and acid hydrolysis provided evidence for this conversion. The glutamic acid percent recoveries for α -lactalbumin, β -lactoglobulin, and β -casein were 35 ± 2.5 , 46.1 ± 2.9 , and 32.3 ± 2.3 using enzyme hydrolysis, respectively, while the values were 112.7 ± 5.2 , 113, and 103.3 using acid hydrolysis (Tsao & Otter, 1999).

Overall, it appeared that enzyme hydrolysis is a better method for the quantification of glutamine in proteins and peptides than acid hydrolysis. The proteins are not subject to any harsh acidic conditions, allowing the glutamine residues to avoid

chemical modifications. However there are slight drawbacks to this method. In particular, it is difficult to hydrolyze glutamine residues that are adjacent to acidic amino acids and proline. However, the advantages from this procedure outweigh this drawback and provide a more solid technique to quantify glutamine in protein-containing supplements (Tsao & Otter, 1999).

This three-enzyme system has been used in other studies to determine the amount of glutamine (Baxter, et al., 2004). Baxter et al. (2004) has used the enzyme digestion as described by Tsao and Otter (1999) to quantify the amount of glutamine in twelve liquid nutritional products. The study reported that a significant fraction of glutamine deaminated to glutamic acid in the process of enzyme hydrolysis. The glutamine content (mole percentage) for purified proteins was 4.11 ± 0.11 , while the mole percentage for nutritional products was 4.23 ± 0.13 . These values were added to the actual glutamine concentrations measured in this study (Baxter, et al., 2004). This study suggests that enzyme hydrolysis also converts glutamine to glutamic acid, but not to the degree that acid hydrolysis does.

The total glutamine concentrations in 11 of the 12 products were in the range of 4.00 to 6.53 g per 1500 kcal. The extent of glutamine release was also examined in three caseinate-based nutritional products and in three purified proteins (β -casein, β -lactoglobulin, and bovine serum albumin) by calculating the glutamine to GLX ratio. The molar amount of glutamine was determined from enzyme hydrolysis, while the molar amount of GLX was from acid hydrolysis. As mentioned above, each glutamine component includes a 4.1-4.4% component to account for glutamine deamination during enzyme hydrolysis. The average glutamine release from the three nutritional products

was 91%, while the glutamine release from the three purified proteins averaged to be 98%. These data indicated that enzyme hydrolysis was capable of releasing greater than 90% of the total amount of glutamine in casein-dominated proteins (Baxter et al., 2004). Therefore, this study supported the need for enzyme hydrolysis in determining the amount of glutamine in proteins and protein-related products.

2.4.5 Pre-column Derivatization of Glutamine with PITC

Pre-column derivatization has been shown to be necessary in amino acid analysis using HPLC in order to make the amino acids detectable under UV light (Rowley, Martin, & Marsden, 1995). The best derivatizing agents react quickly and quantitatively without significant rapid degradation of the derivatization products. Also, these agents should have low detection limits and react with both primary and secondary amines. Derivatization should yield only one product per amino acid to allow each amino acid to be detected by HPLC. Good derivatizing agents do not interfere with the chromatographic separation (Bank, Jansen, Beekman, & te Koppele, 1996). Finally, derivatizing agents need to be volatile to make these agents more easily removed by evaporation (Heinrikson & Meredith, 1984).

Pre-column derivatization has been proven superior to post-column derivatization due to better resolution and sensitivity (Allison, Mayer, & Shoup, 1984). Pre-column derivatization is also more user-friendly than the classical ion-exchange post-column techniques using ninhydrin (Strydom & Cohen, 1994). Several different agents have been used for the purposes of derivatization including fluorenylmethyl chloroformate (FMOC-Cl), dabsyl chloride, *o*-phthalaldehyde (OPA), and PITC.

2.4.6 Fluorenylmethyl Chloroformate

A study has shown some of the advantages of fluorenylmethyl chloroformate (FMOC-Cl), which include that it reacts quickly and quantitatively with both primary and secondary amines, and that it is relatively stable to other sample matrix components, such as salts. It also has detection limits as low as the picomolar level and is stable at room temperature for several days (Bank, et al., 1996). The major disadvantage of FMOC-Cl as a derivatizing agent is its reactivity with water. FMOC-Cl reacts with water to form FMOC-OH, which elutes in the middle of the chromatogram and often interferes with elution of amino acids. Therefore, the amount of FMOC-OH that elutes must be factored in and subtracted when quantifying some amino acids (Bank, et al., 1996). It is critical to remove any excess FMOC-Cl to ensure as little interference with amino acid quantification as possible (Carratù, Boniglia, & Bellomonte, 1995).

2.4.7 Dabsyl Chloride

Dabsyl chloride derivatives are also stable at room temperature and have detection limits in the low picomolar range. Dabsyl chloride derivatives can also be detected in the visible range at wavelengths from 436-460 nm (Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995). However, similar to FMOC-Cl, the major disadvantage of dabsyl chloride in derivatization is that this agent interferes with its derivatives and forms of multiple derivatives, which complicates quantification (Battaglia, et al., 1999).

2.4.8 O-phthalaldehyde

O-phthalaldehyde (OPA) is the most commonly used derivatizing agent due to its high sensitivity, reactivity, speed, and reliability (Tcherkas, et al., 2001). OPA is used

with a thiol, often mercaptoethanol, to form an isoindole (Concha-Herrera, et al., 2006). One major problem with OPA derivatives is they are unstable, so they require an automated online monitor that gives exact reaction times and analysis must be done immediately (Siebert, Palmer Jr, & Hirsch, 1991). A second problem is that OPA does not derivatize glycine, threonine, or secondary amines, such as proline (Battaglia, et al., 1999).

2.4.9 Phenyl Isothiocyanate

One advantage of Phenyl isothiocyanate (PITC) is that this agent derivatizes both primary and secondary amines (Battaglia, et al., 1999). A second advantage is that PITC-derivatives have detection limits in the low picomolar level and can be quantified over a wide range of concentrations (Molnar-Perl, 1994). PITC is volatile, which means that its removal is possible using high-pressured vacuum (Heinrikson & Meredith, 1984). The removal of excess PITC is the most important step in the derivatization procedure because excess PITC can damage the HPLC column. PITC derivatives are much more stable than OPA derivatives and can be stored in a freezer for a long period of time (Molnar-Perl, 1994). Finally, the PITC reaction is quantitative and fast. Derivatization is more than 99% complete in only five minutes (Heinrikson & Meredith, 1984). However, the reaction is generally allowed 20 minutes for completion to be on the safe side (Molnar-Perl, 1994).

Strydom and Cohen (1994) found the derivatizing agent 6-aminoquinolyl-N-hydroxylsuccinimidyl carbamate (AQC) to be superior to PITC based on greater stability of the AQC derivatives. However, they did not analyze glutamine in their study. Phenyl isothiocyanate has been used to derivatize hydrolyzed milk proteins (Tsao & Otter,

1999). Tsao and Otter (1999) were able to quantify glutamine and obtained 97% recovery of free glutamine with PITC as the derivatizing agent, suggesting that PITC effectively derivatizes glutamine. These authors found that PITC had three peaks on their chromatograms, but none of these peaks interfered with peaks for any of the amino acids. Overall, the literature suggests that PITC can be used as a derivatizing agent in the accurate quantification of glutamine, and PITC has some significant advantages over other derivatization methods.

2.5 Urinalysis Literature

2.5.1 Executive Summary of Urinalysis Literature Review

The detection of proteinuria is important in diagnosing and predicting the outcome of most renal diseases. However, the most common method involves the measurement of protein concentration in timed urine collections over a 24-hour period. Though this method helps to overcome the fluctuations in protein concentration observed throughout the day, it is time consuming and often imprecise. An alternative method that avoids the timed urine collections is the measurement of the ratio of protein to creatinine in single random urine samples. The time at which the spot urine sample is obtained does not impact the proteinuria test when the protein to creatinine ratio is measured. In addition, demographic factors such as the patient's sex, degree of proteinuria, and current renal state also do not impact the accuracy of the results. Since the determination of the urinary protein content is dependent on the creatinine content, a concern is that individuals who are orally consuming creatine (a creatinine precursor) may affect their protein to creatinine ratio; however, creatine supplementation does not alter the urinary protein to creatinine ratio. The effect of glutamine supplementation has also been studied using urinary protein measurements but only in animal models. Little research currently exists in correlating the effect of glutamine supplementation to increases in proteinuria as a precursor to renal disease in humans.

2.5.2 Urinary Protein Excretion

The glomerular filtration rate (GFR) provides the most accurate estimation of renal function (Kang, Han, Kim, & Cha, 2005). Therefore, it is important to have a method to accurately measure the GFR in order to predict the severity and progression of

renal disease and correctly prescribe drug dosages. Since it is difficult to measure the GFR in clinical practice, most physicians measure the serum creatinine concentration as an estimation of the GFR. Creatinine is a useful filtration marker since it is produced endogenously, filtered by the glomerulus, not protein bound, and metabolized in the kidney. It is secreted by renal tubule and affected by factors such as renal disease, muscle mass, meat intake, nutritional status and drugs (Kang, et al., 2005). The method traditionally used to measure creatinine clearance is based on the collection of urine samples over a 24-hour period. However, this method is inconvenient and sometimes inaccurate due to inconsistencies in collection times. Therefore, measurement of protein excretion is often used as a screening test to evaluate glomerular filtration. To eliminate the interference of changes in concentration of urine, the value of protein excretion is usually expressed as a ratio relative to the value of creatinine in the same sample of urine.

Protein excreted by the kidney is a product of the serum proteins that have been filtered through the glomerulus. The kidney may excrete protein normally during congestive heart failure, upon exposure to cold, post-exercise, or during various conditions that cause alterations in renal circulation (King, 1957). Protein excreted by the kidney during chronic renal disease can indicate changes in the pathology of the disease. Increases in protein excretion often indicate progressing renal disease. Proteinuria is defined as an excessive rate of protein excretion relative to creatinine excretion in the urine. Chronic (persistent) proteinuria may be the only clinical evidence of progressive renal disease and should not be overlooked in patients at risk for kidney disease (King, 1957).

2.5.3 Urinary Protein Levels used to Detect Proteinuria

Chronic renal disease affects over 5% of the general population and therefore, there is a need to detect early signs indicative of renal dysfunction when treatments are most effective (Gai, et al., 2006). Normally, small amounts of protein are excreted in the urine (approximately 80 mg/day) and contain both filtered plasma proteins, such as albumin and low molecular weight immunoglobulin, and secreted tubular proteins (CARI, 2004c).

The normal range for creatinine excretion in young and middle-aged males is 1100 to 2000 mg/day (16 to 26 mg/kg body weight per day) and in females is 750 to 1400 mg/day (12 to 24 mg/kg body weight day). Levels of urinary creatinine excretion steadily decrease with age in both sexes from 8 to 15 mg/kg body weight per day once a person reaches the approximate age of 80 (Kampmann, Siersbaek-Neilsen, Kristensen, & Hansen, 1974). Generally, proteinuria is diagnosed in adults when there is greater than 150 mg of protein excreted in a 24-hour period. In newborns (30 days), infants (12 months), and young children (2-10 years), proteinuria is defined as greater than 145 mg/m²/day, 110 mg/m²/day, and 85 mg/m²/day, respectively (Miltenyi, 1979). If proteinuria is detected in an asymptomatic patient, it is usually an indication of either an initial manifestation of a severe renal disease or a temporary nonprogressive renal abnormality of little importance (Miltenyi, 1979).

2.5.4 Proteinuria Classifications

Proteinuria is classified based on different qualities: quantity (non-nephrotic or nephrotic), character (intermittent or constant), posture (orthostatic or nonorthostatic), or the absence of presence of an additional urinary abnormality (isolated proteinuria or

proteinuria with hematuria) (Wingo & Clapp, 2000). In isolated proteinuria, there is no real evidence of renal disease and the proteinuria is likely a result of an urine sediment abnormality. Severe and/or constant proteinuria is often the result of a primary renal disease or it may be a result of a secondary involvement of the kidney from a systemic disease. Nevertheless, patients diagnosed with severe or constant proteinuria often have a decreased life expectancy in relation to the general population (Wingo & Clapp, 2000). As a result, it is vital that the methods used to identify and classify proteinuria are accurate, and that the result is confirmed by multiple measurements.

A positive test for proteinuria has been shown to be a sign of potential renal damage and proteinuria diagnosis is often the first sign of a serious renal disease. If proteinuria is detected in a patient with a nonrenal illness, it is often concluded that the illness may also involve the kidneys. The most common cause of proteinuria is glomerular disease with serious manifestations such as the nephrotic syndrome, hypertension, or progressive renal failure (Abuelo, 1983). Regardless of the cause, diagnosis of proteinuria has several implications for the patient. Due to the severity of renal disease, if the proteinuria is classified as persistent, this may lead to rejection from the armed forces, denial of life insurance, or exclusion from new employment opportunities (Abuelo, 1983). Therefore, accurate diagnosis of proteinuria is important to appropriately evaluate the clinical actions to either treat the condition or, if treatment is unavailable, inform the patient of the prognosis.

2.5.5 Methods for Assessing Proteinuria

There are three main methods that have been used to detect and diagnose proteinuria: urinary protein to creatinine ratio in 24-hour urine collection or in spot urine

collection, and dipstick urinalysis. The standard method for measuring proteinuria is the 24-hour urine collection. In the 24-hour urine collection method, accuracy is decreased due to uncertainties in the time of collection and volume determinations (Steinhauslin & Wauters, 1995). In addition, this method takes an entire day to collect and is, therefore, impractical for routine screening and inconvenient for patients and clinicians who need to make quick, informed decisions. A simple alternative to 24-hour urine collection for assessing urinary protein is to perform qualitative tests for protein concentration on random spot urine samples, either using dipstick urinalysis or measuring the protein to creatinine ratio. As compared to the traditional 24-hour urine collection, spot urine samples are more convenient for patients since only a single morning urine collection is required. The results are available within hours and there is higher sensitivity in the detection techniques (Wheeler, Blackhurst, Dellinger, & Ramsey, 2007). In combination with other signs and symptoms, a rapid screening test can accurately be used to predict 24-hour proteinuria and help guide clinicians in making quick and informed decisions.

The dipstick urinalysis method is often used as an initial rapid screen to measure the concentration of protein in the urine. Unfortunately, this method results in low sensitivity and specificity, potentially due to inter-observer variation (Steinhauslin & Wauters, 1995). The dipstick method is susceptible to fluctuations in the water content of the urine so dilute urine may underestimate the protein concentration that would be measured in a 24-hour urine collection whereas a concentrated urine sample may overestimate it. Previous studies have shown that dipstick urinalysis products vary in degrees of accuracy with sensitivities ranging from 22 to 82% (Dwyer, Gorman, Carroll, & Druzin, 2008). Discrepancies also arise due to differences in the degree of renal

disease in the patients or the time of day that the spot urine sample was obtained. Therefore, the dipstick method is highly limited in its ability to estimate the level of proteinuria and detect small but potentially clinically important changes in the levels of urinary protein excretion. Dipstick protein analysis has also been shown to result in high rates of false positives and false negatives. In studies that involve suitable at-risk populations, the incidence of proteinuria as detected by the dipstick method is less than 10%, which is not consistent with the results of other methods (CARI, 2004b). An alternative method is to use a test strip on a single urine sample to detect proteinuria related to creatinine but this test is not widely available yet (Gai, et al., 2006).

The use of the protein to creatinine ratio in a single urine sample also has advantages over both the 24-urine collection and the dipstick urinalysis. As opposed to the traditional 24-hour urine collection, measuring the protein to creatinine ratio in a single urine sample is time independent because the excretion of protein and creatinine are both expressed per unit time. This provides an estimation of 24-hour protein excretion if stable renal function is assumed. In addition, the ratio is normalized between urinary protein excretion and creatinine clearance (Wheeler, et al., 2007). As opposed to dipstick urinalysis, this technique is not subject to variation due to hydration status because it compares the spot urine protein excretion to the spot urine creatinine excretion, which normalizes the protein excretion to the same rate as glomerular filtration (Dwyer, et al., 2008). It has been shown that the urine protein to creatinine ratio is more sensitive than automated dipstick urinalysis with sensitivities of 96% to 41% ($P < 0.0001$). The protein to creatinine ratio is also a better screening test for early diagnosis in more patients (64% to 19%, $P < 0.0001$) (Dwyer, et al., 2008). It is also more cost-effective than the dipstick

method (Gai, et al., 2006). In addition, using random urine testing based on the ratio of creatinine to protein to assess proteinuria has been shown to have good correlation to results from 24-hour urine total protein measurements in a wide range of patient groups (i.e. normal, known renal disease, diabetes, etc. (CARI, 2004b).

Therefore, the use of the protein to creatinine ratio accurately estimates the level of proteinuria and avoids the potential errors seen in the dipstick and 24-hour sampling methods. In a study of almost 300 outpatients with varying degrees of renal disease, a high correlation was observed between all the tests ($P < 0.0001$) but the highest regression coefficient was observed between the 24-hour urine collection and the protein to creatinine ratio ($R = 0.82$) and the lowest regression coefficient was observed between the protein to creatinine ratio and the dipstick urinalysis ($R = 0.72$). In addition, the dipstick urinalysis method was unable to detect proteinuria in approximately 31% of the patients examined (Gai, et al., 2006).

2.5.6 Correlation Between Protein to Creatinine Ratio and 24-hour Urine Excretion

The protein to creatinine ratio can be used to estimate urinary protein excretion because urinary creatinine clearance at a stable glomerular filtration rate (GFR) is fairly constant. In a study of approximately 40 patients with varying degrees of renal disease, 24-hour urine samples and single-voided urine specimen from four different time points were collected. A strong correlation was observed between the 24-hour urine protein excretion measurements and the protein to creatinine ratio in single voided urine samples at varying degrees of creatinine clearance and ranges of proteinuria (Chu, et al., 1990). A simple ratio of the concentration of protein to the concentration of creatinine in a spot urine sample reflects the cumulative amount of urinary protein excreted in a 24-hour

period because the ratio cancels out time as a variable (Ginsberg, Chang, Matarese, & Garella, 1983). In addition, there is a highly significant ($P = 0.0001$) correlation between the protein to creatinine ratio values in spot morning urine samples and 24-hour protein excretion but the correlation decreases with increases in the ratio value (Ruggenti, Gaspari, Perna, & Rumuzzi, 1998).

2.5.7 Effect of Time and Demographics on Spot Urine Samples for Protein to Creatinine Ratio

The protein to creatinine ratio on both first morning and early morning urine samples and random urine samples correlates well with measurements from 24-hour urine collections. This indicates that the time at which the urine specimen is obtained does not impact the final results (CARI, 2004a). In a study by Ginsberg et al. (1983), the highest correlation between the protein to creatinine ratio and the 24-hour urinary protein excretion was seen when the spot urine samples were obtained after the first voided morning sample and before bedtime. Hence, even though the time at which the spot urine sample is obtained should not significantly impact the protein to creatinine ratio, if abnormally high levels of urinary protein are observed in a random untimed urine specimen, the results should be confirmed with a first morning urine sample to confirm the proteinuria diagnosis (CARI, 2004a).

In a study to investigate the effect of several variables, such as time of samples, patient's sex, degrees of proteinuria, and renal function, the urinary protein to creatinine ratio was measuring in samples obtained at three time points throughout the day and compared to the 24-hour urine excretions. This study by Kristal et al. (1988) involved over 50 patients with varying degrees of renal function and proteinuria. The highest

correlation was observed in urine samples collected at 08.00 and 12.00 hours and the lowest correlation was observed in urine samples obtained at 16.00 hours. This degree of correlation was unaffected by the patient's level of proteinuria or sex, but it was slightly affected by the rate of glomerular filtration (Kristal, Shasha, Labin, & Cohen, 1988). In a study by Chu et al. (1990), no significant differences were found between patients of different age groups or sexes when using the protein to creatinine ratio at different time intervals to estimate urine protein excretion. Therefore, the protein to creatinine ratio is not significantly affected by sex or age (Chu, et al., 1990).

2.5.8 Effect of Oral Creatine Supplementation on Protein to Creatinine Ratio

Creatine is synthesized endogenously by the body and stored in skeletal muscle tissue in a high-energy phosphorylated form. When it is phosphorylated, it plays an important role in the metabolism of energy by supplying phosphate groups to ADP to regenerate ATP (Poortmans, et al., 1997). When muscle contraction occurs, creatine and creatine phosphate are spontaneously converted directly into creatinine. Creatinine is then eliminated from the body in the urine through renal excretion at a relatively consistent rate. Therefore, creatinine is an ideal clinical marker to assess renal function (Ropero-Miller, Paget-Wilkes, Doering, & Goldberger, 2000).

Oral creatine supplementation of 20-30 grams of creatine per day for several days has been shown to increase human skeletal total creatine and phosphorylcreatine (Balsom, Soderlund, & Ekblom, 1994). As a result, oral creatine supplementation has increased among athletes who hope to enhance their performance. However, it has been suggested that ingestion of such nitrogen-rich products might induce chronic renal hyperfiltration and hyperfusion, which would ultimately result in the functional and structural

deterioration of the kidney (Brenner, et al., 1982). If taken in large excess, the two amino and one carboxyl groups of creatine and its high nitrogen content could theoretically add additional strain on the kidney (Poortmans, et al., 1997).

One concern is if creatine supplementation affects blood samples and urine collections analyzed for creatine and creatinine. In a study of five healthy men who ingested either a placebo or 20 grams of creatine monohydrate per day for five consecutive days, the levels of creatine significantly increased in blood and urine excretion levels but the creatinine levels were unaffected (Poortmans, et al., 1997). The non-enzymatic reaction of muscle creatine to creatinine is not affected by a large influx of creatine from supplementation. This indicates that normalization of urinary analyses can still be measured using creatinine values even if patients are using creatine supplements (Poortmans, et al., 1997). Another short-term study did not reveal any apparent effect of oral creatine supplementation administered at recommended daily doses on the integrity of routine urine tests (Ropero-Miller, et al., 2000). Therefore, oral creatine supplementation does not affect creatinine clearance and protein excretion so the protein to creatinine ratio will not be altered if an individual is using creatine supplements.

2.5.9 Testing for Albuminuria as Compared to Proteinuria

Urinary protein excretion can be quantified in terms of total protein or albumin. Albumin, a small protein that is found in large quantities in the blood, is one of the first proteins to pass through the kidneys into the urine upon the development of kidney disease. Detection of albuminuria occurs by the same method as for proteinuria except the albumin to creatinine ratio is measured as opposed to the total protein to creatinine

ratio. The relationship between urinary albumin excretion and total protein excretion and the benefits of one test over the other remains undefined due to a lack of large-scale studies examining albuminuria and proteinuria (Atkins, Briganti, Zimmet, & Chadban, 2003). A study by Shihabi et al. (1991) demonstrated that urinary albumin excretion levels increase seven-fold in patients with kidney disease while urinary total protein levels remain within the normal reference interval. The researchers found that urinary total protein measurements are often subject to analytical difficulties whereas urinary albumin excretion measurements are easier to standardize. Therefore, it was concluded that urinary albumin measurements are a more sensitive indicator of a wide variety of renal disorders than urinary total protein levels (Shihabi, Konen, & OConnor, 1991). A recent study by Collier et al. (2009) examined the relationship between proteinuria and albuminuria as a means to determine which is more sensitive and specific in detecting chronic kidney disease. Urine samples were analyzed using both the albumin to creatinine ratio (ACR) and the protein to creatinine ratio (PCR) and it was determined that either ratio can successfully be used to identify clinical proteinuria (Collier, Greenan, Brady, Murrari, & Cunningham, 2009).

In the first large-scale, population-based study of the relationship between urinary albumin and total protein, a representative cross-section analysis of the Australian adult population showed that albuminuria rather than proteinuria is preferred for the testing of potential renal risk in the general population. Measurements of urine albumin excretion were strongly correlated with measurements for total protein excretion, particularly among the elderly, and those currently diagnosed with diabetes, hypertension, obesity and renal disease ($P < 0.001$) (Atkins, et al., 2003). As a standard screening test, the albumin

to protein ratio was 91.7% sensitive and 95.3% specific, but as a test among patients previously diagnosed with proteinuria, 8% were found to have urinary albumin excretion levels within the normal range. Therefore, it was concluded that while a measurements of the albumin to creatinine ratio may be a suitable test for general population screening for renal disease, overall it should not replace the standard protein to creatinine ratio testing for proteinuria in patients those with known or suspected renal disease (Atkins, et al., 2003).

2.5.10 Urinalysis Study on the Toxicology of Glutamine

L-glutamine (Gln) is a semi-essential amino acid and mammals are unable to synthesize Gln sufficiently during catabolic stress. Therefore, administration of Gln is a potential therapeutic agent in critically ill patients and is contained in several dietary supplements available to the public. Due to the precedence of Gln supplements, a study was performed to examine the toxicological effects of Gln in male and female Sprague-Dawley rats (Tsubuku, Hatayama, Mawatari, Smirga, & Kimura, 2004). Gln was administered as part of a standard diet at doses equal to 1.25%, 2.5%, and 5.0% (w/w). All diets were administered for 13 consecutive weeks following by a 5-week recovery period during which only the standard diet was administered to all animals. Among other tests, a urinalysis study was conducted in all rats during weeks 5 and 13 of Gln administration and also during week 5 of the recovery period post Gln administration. At the end of the administration period, several changes in urine parameters (particularly, total protein) were observed in the 2.5% and 5.0% groups (Tsubuku, et al., 2004). One trend indicated an increase in the number of positive incidences for total urinary protein in females in the 2.5% and 5.0% groups but overall the total protein in the blood did not

increase and no pathological effects in the kidney tissues were observed. Since the changes in the 2.5% and 5.0% groups were infrequent and toxicologically insignificant, the definitive toxic level for Gln was determined to be greater than 5.0% (w/w) (Tsubuku, et al., 2004).

Chapter 3: Methodology

3.1 Survey Research Methodology

3.1.1 Survey Design and Administration

Team Juiced designed a survey in both paper-and-pencil and computerized formats in order to collect data from individuals on the University of Maryland, College Park campus regarding their protein supplement usage (refer to Appendix A.1). The survey was intended to obtain information on the habits and behaviors of protein supplement users that was then used to complement the lab data found in the USU lab and the CFSAN lab at the FDA.

A short, two-page questionnaire was administered to students who frequented the campus gyms, club athletic team members, intramural sports players, varsity athletes, and members of the Reserve Officers' Training Corps (ROTC). The survey was designed to be administered in person. Purposive sampling was used in order to target the student population that would provide the most relevant data efficiently and effectively. Since Team Juiced targeted students who engaged in physical activity, the survey was distributed to students who shared the characteristics of either a) participating in a varsity, intramural, or club sport at the university, b) exercising at one of the university's two recreational facilities on campus: the Eppley Recreation Center or Ritchie Coliseum, or c) being a member of the ROTC program.

The survey immediately separated protein users and protein non-users by asking them to indicate in which category they belonged. Supplement users were asked which product(s) they currently use or have used in the past, how much of the product they used, how often they used the product, how long they used the product, other

supplements or medications that they took concurrently, and their present health status and family medical history regarding diabetes, kidney disease, clinical obesity, and hypertension. Non-users were similarly asked about their personal and family medical history as well as any medications that they were taking. All participants were asked to identify themselves as either gym-goers, club, intramural, or varsity athletes, or ROTC members. All participants were asked to state their sex, their age, and how many hours per week they exercised. In order to maintain participant anonymity, each survey was coded with a four-digit number on each page including the informed consent page. After completion of the survey, the informed consent page with the participant's name on it was separated from the rest of the survey thereby detaching any name association with particular survey responses.

Survey distribution varied depending on which part of the targeted population was being tested. In order to survey students who exercised at one of the gyms on campus, Team Juiced had to schedule twelve different collection dates of five to ten hours per date at the Eppley Recreation Center or Ritchie Coliseum. On each date, Team Juiced set up a table and handed out surveys to students who were interested, also known as purposive sampling. To encourage attendance at the gym during the time of survey collection, members of Team Juiced sent out advertisements to various departmental, academic, and club e-mail listservs. Free water bottles were offered as an incentive for those who took the survey at one of the campus gyms.

Club and intramural team members as well as ROTC cadets were surveyed differently. The presidents of the club and intramural teams were contacted via e-mail by a member of Team Juiced who asked the presidents' permission to distribute the survey

during one of the teams' practices. Upon permission of the president, teams of two or three Team Juiced members went to a practice for each club or intramural team and distributed surveys. Twenty-one of the university's club teams were surveyed. These included the following: tennis, black belt club, women's volleyball, kendo, women's basketball, squash, sailing, women's soccer, weightlifting, water polo, Terp runners, men's crew (novice and regular), men's soccer, cycling, swimming, table tennis, softball, men's rugby, women's rugby, boxing, and men's volleyball. Intramural sports teams proved difficult to reach, but intramural flag football was surveyed and provided many useful data. In addition, members of ROTC were surveyed in the same manner as the club and intramural teams. The Director of the Army ROTC, LTC Ranelle A. Manaois was emailed for permission to distribute surveys. Surveys were given to LTC Manaois, and she distributed them to the cadets. Because of the cooperation from the presidents of each of these teams as well as LTC Manaois, there was no water bottle incentive for any of the club, intramural, or ROTC participants.

To survey varsity athletes anonymously, Team Juiced had to use a new data collection technology method referred to as a computerized self-administered questionnaire (Biemer & Lyberg, 2003). Team Juiced used an online survey website, Survey Monkey to create an identical version of the paper-and-pencil survey available over the internet. Varsity athletes were surveyed over the internet because Team Juiced was not permitted to survey varsity athletes during their team practices. An announcement was sent to the varsity athlete e-mail listserv encouraging them to take the online survey and providing them with a direct web link to the survey. No incentive was offered for participation. Participant responses were labeled with a numeric digit to

ensure anonymity just as the paper surveys were. In total, online surveys accounted for approximately 10% of the overall responses with 96 varsity athletes completing the survey online.

After collecting the responses from 947 total survey respondents, they were entered into an SPSS file for statistical analysis. Several conversions were required for this process in order to organize all of the data into standard measurements. For example, the actual survey listed several options for participants to describe the amount of each protein supplement that they took. They could provide the amount in number of the scoops, pills, or capsules. Due to the wide range of supplements used, each of these forms connoted a varying level of protein per serving size. Reference sheets were therefore generated by Team Juiced detailing the actual amount of protein in grams that the labels of each form (pills or powder) of the products claimed to contain in a serving size. These conversions were then used to generate the amount of protein in grams taken by each respondent. Similar conversions were made for length of usage. The survey allowed the participant to indicate either how many times per day they used a product or how many times per week they used it. For standardization purposes, all responses were entered into the SPSS file in terms of times per week. Therefore, if a participant provided information in times per day, that information was multiplied by seven. Responses in the duration of usage category needed to be standardized as well. Participants could indicate that they had taken a product for a duration of weeks, months, or years. In the SPSS file, Team Juiced converted all of these responses into weeks. If a participant therefore indicated that he or she was taking a product for three months, the response was entered into the SPSS file as twelve weeks, as it was assumed that there are four full weeks per month.

Following entry, the data was “cleaned” by team members. Data cleaning involved double-checking each entered response on the SPSS file for accuracy by comparing it to the original paper-and-pencil or computerized answer sheet. The data cleaning process verified the information originally entered and validated it with the original answer sheet and checked all of the entries for accuracy. Accuracy was especially important in the conversions that were made. The purpose of data cleaning was to eliminate the probability of processing or measurement error to the best of the team’s capability so that those errors would not skew the results.

3.1.2 Data Entry Assumptions and Limitations

There are several significant assumptions and limitations related to the data entry process that are critical to understanding the results of this research. First, when entering data into the “times of use” category, all numbers were converted into weeks with the understanding that there are four weeks in a month and fifty-two weeks in a year. For example, if a survey participant responded that he or she used a supplement two times per day, this was entered into the SPSS computer program as fourteen times per week. Another category was also added for individuals who responded that they used a supplement either rarely or inconsistently. The number “0” in the SPSS file coded for these types of users. A second assumption involved products with long and complicated names. For some surveys with incomplete product information, a specific product was assumed. This was the case for “Muscle Tech Nitro-Tech Hardcore,” “Muscle Tech Cell-Tech Fruit Punch,” and “CytoSport Muscle Milk.” The most commonly incomplete product listed was “100% Whey.” For surveys claiming to be using “100% Whey”, the serving size information for “GNC Pro-Performance Whey Protein” was assumed

because GNC Pro-Performance Whey Protein was the most commonly used whey protein supplement. For surveys that listed “Smoothie Booster” for the type of protein supplement that the respondents were taking, it was assumed that the product was Smoothie Booster C from “The Smoothie Shop.” For the purposes of this study, all pure creatine products were classified as “other supplements” and not as protein.

After most of the data had been entered, a second round of “data cleaning” ensued in which each survey in the SPSS system was checked for accuracy and consistency. This process consisted of going through all of the surveys again and making sure that the data had been accurately entered. During this second round of entry, the “amount taken” category was especially revised. This category coded information regarding how much protein an individual consumed at one time. For some surveys, the data recorded for this question was unclear or missing. In these cases, the product used was stated, and an assumption was made that the participant used the standard serving size. For example, if a participant responded that he or she used GNC Pro-Performance Whey Protein product but failed to say how much of the product he or she used at a given time, the serving size provided on this product label, 31 grams was assumed and entered into the SPSS file. In order to keep all of the numbers in this category consistent, each serving size was converted and entered in grams. This required some conversions of pills, scoops, and fluid ounces into grams. If the data showed that a person measured his or her protein intake in cups, it was assumed that one cup equaled one scoop and then the gram conversion was performed.

In addition, for the respondents who were over the age of 25, the team grouped all of these respondents together; in other words the team truncated the age values given for

the respondents over the age of 25 into one category. This is a limitation to the comparison of means for Age of the respondent vs. Amount of protein used for products 1, 2, and 3 since the team is assuming one age for any of the respondents over the age of 25 who in fact comprise a portion of the college-aged population that the team was testing.

Statistical analyses were then performed using SPSS. The data was analyzed using descriptive statistics. Descriptive statistics included sample size numbers for various segments of the tested population, means, standard deviations, and frequency distributions. Tests for associations between variables for comparisons of means included multiple Spearman's Rho bivariate correlational tests. Additionally, depending on the nature of the comparison of means, independent samples t-tests and One-Way ANOVA variances were used to generate results. After completing these varying types of statistical tests and analyses, charts were created to pictorially view the results.

3.2 Kidney Cell Modeling Methodology

3.2.1 Overview

Several studies with humans and animal models have shown the increased development of chronic kidney disease in response to high-protein diets (Brenner, et al., 1982; Klahr, 1996; Levey, et al., 1999). Of the constituent amino acid residues, glutamine is the most prevalent in common proteins such as casein and whey. Extending our efforts to serve the college athletic community, a group that commonly uses protein supplements, the team has collaborated with Dr. Sonia Doi, M.D., Ph.D., Director of Nephrology Research Laboratory, Department of Medicine, Uniformed Services University.

Under the guidance of Dr. Doi, the team aimed to discover the expression patterns of LDL-r in the mouse mesangial cell model in response to varying glutamine conditions. Such knowledge would ultimately contribute to finding a biochemical pathway by which glutamine affects kidney cells. Several researchers have previously used the mouse mesangial cell model (Doi, et al., 2000; Lagranha, et al., 2008; Meek, et al., 2003; Pithon-Curi, et al., 2006).

Gene expression studies were used to detect the increase in the synthesis of several proteins in response to extracellular stimuli, i.e. glutamine concentration. In animal cells, the nucleus shields DNA but allows RNA to pass to the cytoplasm where it is translated into specific proteins. The presence of this messenger RNA is a good representation of the expression of particular protein molecules and their representative synthesis within the cell. Extracting RNA and performing reverse transcription to DNA

and simultaneous polymerase chain reaction to amplify the gene of interest can evaluate the gene expression and related protein synthesis.

Sclerosis of the glomerulus is a major cause of kidney disease. LDL cholesterol plays a role in the development of plaques on the walls of blood vessels, and it is hypothesized that LDL also plays a role in the excretion of matrix proteins by the mesangial cells of the kidneys, causing glomerulosclerosis. This pathway of sclerosis in the kidney must be tested by examining the expression of LDL-receptor. The time- and dose-dependency of LDL-r in response to glutamine. The primary goal of these experiments was to determine the time exposure and dosage related behavior of LDL-r with respect to glutamine. This information would progress research in the field of renal molecular biology with implications to protein supplementation.

3.2.2 Cell Culture

A primary culture of mouse mesangial (passage 16) cells were grown in a T75 flask coated with fibronectin in a 3:1 mixture of DMEM/Ham's F12 media containing 6 mM glucose, 1 mM glutamine, 0.075% NaHCO₃, penicillin (100 U/ml)/streptomycin (100 µg/ml), and 20% fetal bovine serum (FBS, Life Technologies, Rockville, MD, USA). The cells were incubated at 37°C and 5% CO₂ for 96 hours in order to reach confluence. The cells were then removed from the flask with trypsin and counted using a hemocytometer. Using aseptic dilution methods, the cell suspension was reduced to 1.5 x 10⁵ cells per ml. One ml of cells and 2 ml of 20% FBS media were transferred to each well of a 6-well plate coated with fibronectin. The plates were incubated at 37°C and 5% CO₂ for 24 hours. The media was aspirated and changed to DMEM/Ham's solution with

no additional glutamine and incubated for 24 hours at 37°C, 5% CO₂. Glutamine was added as needed according to each experiment.

3.2.3 Time Course Study

A time curve for the expression of LDL-r was found by varying exposure time to supraphysiological (2.0 mM) glutamine in 5% FBS. The starvation media was aspirated from the 6-well plate and media containing either 0 or 2.0 mM glutamine in 5% FBS was added for duplicate wells incubated for 2, 4, 6, 8, 12, and 24 hours at 37°C, 5% CO₂.

3.2.4 RNA Extraction

After the cells were exposed to experimental glutamine conditions, the cells were lysed to extract RNA. After aspirating the treatment media, 400 µl of a freshly prepared RLT buffer with beta-mercaptoethanol was added to each well. The buffer was prepared with a Qiagen RNEASY PLUS kit with DNase. The plate was swirled until the solution reached maximum viscosity. A 20 gauge needle was used to shear the cells in each well by moving the plunger up and down 8 times. Extracted RNA samples were transferred to a 1.5 ml microcentrifuge tube and stored at -80 °C.

3.2.5 OD and Dilutions

To measure the concentration of the extracted RNA, 2.8 µl of extracted RNA was diluted with 67.2 µl of depc water. The optical density (OD) of each sample was measured at 260 nm using a spectrophotometer. Thanks to the ratio of RNA:water used, the OD readings were given in µg/µl. Ten µl of all samples were diluted to 20 ng/µl for use in one-step real time reverse transcriptase polymerase chain reaction (RT-PCR).

3.2.6 Real Time RT-PCR

TAQMAN one-step RT-PCR kit was used with 50 ng of RNA, 300 ng of forward and reverse primers and 200 ng of fluorescent probe per gene (See Appendix for Primers Chart). Beta-actin was used as the housekeeping gene for all experiments. A housekeeping gene allows normalization of genes of interest, such as LDL-r, so that the number of cells per well does not affect the level of expression. Samples were run in the Applied Biosystems 5700 system.

3.2.7 Data Analysis

The software loaded with the Applied Biosystems 5700 gave graphs of fluorescence versus cycle number. Each sample represented one well cultured as specified by the experimental protocol. All samples from an RT-PCR assay were given superimposed on the same graph. The cycle threshold (ct) is a value used to compare the relative expression of each sample. The ct values were found by adjusting a horizontal cursor to intersect the most linear region of the graph. Ct values from genes of interest were normalized using the ct values from beta-actin expression. These normalized values were then compared, as a ratio, to control cells from each experiment that were not exposed to any glutamine. The ratio, or fold change compared to control cells, was used for discussion and included in all figures. All values reported represented mean \pm standard error of the mean (SEM) of replicates. Statistical comparisons were done using one-way ANOVA tests.

3.2.8 Dose Response Study

After discovering the time curve of LDL-r expression from a 2.0 mM supraphysiological level of glutamine, the dose response was found by varying the

glutamine concentrations at 6 hours of exposure. Cells were cultured as in the time course study. Following the starvation step, 5% FBS media with glutamine concentrations of 0, 0.5, 1.0, 2.0, 5.0, 7.0, 10.0, and 20.0 mM were added and incubated in duplicate wells for 6 hours at 37°C, 5% CO₂. RNA extraction, ODs and dilutions, and one-step RT-PCR was performed as in the time course study.

3.2.9 Chronic Study

After discovering the dose-dependent behavior of LDL-r in response to increasing glutamine concentrations at 6 hours, the chronic effects of glutamine were tested by exposing cells to varying glutamine concentrations for 48 hours. Cells were cultured as in the time course study. Following the starvation step, 5% FBS media with glutamine concentrations of 0, 0.5, 2.0, and 10.0 mM were added and incubated in triplicate wells for 48 hours at 37°C, 5% CO₂. RNA extraction, ODs and dilutions, and one-step RT-PCR was performed as in the time course study. In addition to RT-PCR for LDL-r and beta-actin, assays were run for 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG Co-A) reductase, collagen I, fibronectin, collagen IV, and TGF-beta.

3.3 Biochemical Analysis Methodology

3.3.1 Materials

L-glutamine and L-glutamic acid standards were obtained from the Sigma-Aldrich Chemical Company. The enzymes used for the enzymatic hydrolysis were pronase E (5.9 units/mg solid), leucine aminopeptidase (12 units/mg solid), and prolidase (127 units/mg solid), all of which were obtained from the Sigma-Aldrich Chemical Company. The derivatizing solution consisted of a 7:1:1:1 ratio of ethanol, water, PITC, and triethylamine. The redry solution consisted of a 2:2:1 ratio of ethanol, Milli-Q water, and triethylamine. Solid sodium phosphate, acetonitrile, and N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES) buffer were obtained from Sigma-Aldrich as well. The protein and glutamine supplements used are listed below:

- Body Tech L-Glutamine Powder, Recovery Agent
- BSN Cell Mass
- Companion Nutrition Pro-Score 100
- Cytosport Complete Whey Protein
- Cytosport Cytogainer
- Cytosport Evo Pro
- Cytosport Muscle Milk
- EAS L-Glutamine
- EAS Myoplex Original
- GNC L-Glutamine Dietary Supplement
- GNC Pro Performance 100% Whey Protein
- GNC Pro Performance L-Glutamine
- Met-RX Instantized 100% Ultramyosin Whey
- Muscle Tech Mass Tech
- Muscle Tech Nitro-Tech Hardcore
- Optimum Nutrition Pro Complex
- Optimum Nutrition Gold Standard Natural 100% Whey
- Optimum Nutrition Gold Standard 100% Whey
- Optimum Nutrition Glutamine Powder
- Prolab Pure whey
- The Vitamin Shoppe L-Glutamine Powder

Any claims or conclusions made regarding the glutamine content of these products do not reflect the views or findings of the Food and Drug Administration. For the purposes of developing this method, each supplement will be designated a numerical name from Supplement 1 to Supplement 21. All of the supplements were obtained online from bodybuilding.com, with the exception of GNC Pro Performance 100% Whey Protein, which was purchased at a General Nutritional Center store.

3.3.2 HPLC Conditions

The HPLC analysis of the supplements was performed on a Waters2695 Separations Module using a Vydac Denali C18 (250 mm x 4.6 mm, 120 Å, 5 µm, Grace Davison Discovery Science, Deerfield, IL) reversed-phase column. Detection was performed with a Waters2996 Photodiode Array UV detector. The flow rate was 1.0 mL/min with an injection volume of 5 µL. The mobile phase consisted of 25 mM sodium phosphate in water at a pH of 3.30 (solvent A), and acetonitrile (solvent B). The pH of the sodium phosphate solution was adjusted to 3.30 using phosphoric acid. At 0 minutes, the composition of the mobile phase was 95 % A and 5 % B. A gradient was started such that the composition of the mobile phase was 60 % A and 40 % B at 40 minutes. By 42 minutes, the mobile phase was 20 % A and 80 % B. The composition of the mobile phase was maintained at 20 % A and 80 % B from 42 minutes to 50 minutes. The starting condition of 95 % A and 5 % B was reestablished by 51 minutes.

3.3.3 Enzymatic Degradation

The enzymatic degradation method was taken from Baxter et al. (2004). A 0.10 M HEPES buffer solution in water with 0.1 % (w/v) sodium azide was made, and the pH was adjusted to 7.50 with NaOH. A quantity of 50 mg of each sample was dissolved in

50 mL of the 0.1 M HEPES buffer to give a concentration of 1.0 mg/mL. A 12 unit/mL solution of pronase E was made by adding 2 mL of 0.1 M HEPES to 4 mg of pronase E. A 24 unit/mL solution of leucine aminopeptidase was made by adding 1 mL of water to 2 mg of leucine aminopeptidase. The prolidase solution was made by adding 1 mL of water to 1.5 mg of prolidase to give a concentration of 190 units/mL. A volume of 1 mL of each dissolved protein solution was added to a different vial. To each vial was added 50 μ L of pronase E, 20 μ L of leucine aminopeptidase, and 10 μ L of prolidase for a total volume of 1080 μ L. Then, the samples were incubated for 20 hours at a temperature of 37 °C.

3.3.4 PITC Derivatization

The derivatization method was adapted from the method used by Tsao and Otter (1999). After 20 hours of incubation, 50 μ L was taken from each sample. The 50 μ L samples were dried with argon gas in a 2 mL vial. Once the samples were dry, 50 μ L of the redry solution was added, and the samples were mixed in a sonicator for 10 minutes. Then, the samples were dried a second time with argon gas. Next, 50 μ L of the PITC derivatizing solution were added, and the samples were mixed in the sonicator for 20 minutes. It is important to always allow at least 20 minutes for the PITC reaction. Finally, the samples were dried again with the argon gas and were vacuum dried overnight. The next day, 2 mL of ethanol were added to each derivatized sample. The samples were mixed in a sonicator for 10 minutes and then analyzed with reversed-phase HPLC.

3.3.5 Data Analysis

All supplements were tested in duplicates at least once, and some supplements were tested in duplicates a second time. Samples were analyzed with Empower Pro software. Chromatograms were extracted at a wavelength of 254 nm. Peaks were

manually integrated to determine retention time and peak area. Calibration curves were constructed with area on the y-axis and concentration on the x-axis. The slope with a y-intercept was determined using Microsoft Excel and the formula: `LINEST(Y1:Y5, X1:X5, FALSE, FALSE)`. The y-intercept was determined using the formula: `INDEX(LINEST(Y1:Y5, X1:X5), 2)`. The slope with the y-intercept equal to 0 was also determined because the line should have passed through the origin. This slope was used in the final calculations of all samples. The slope was determined with the formula: `LINEST(Y1:Y5, X1:X5, FALSE, FALSE)`. Concentrations of samples were determined using the area determined by integration and the slope of the calibration curve. Finally, standard deviations between trials of the same samples were calculated.

3.4 Urinalysis Methodology

Included at the end of Team Juiced's survey was a page asking participants if they would be willing to take part in a follow-up study that concerned protein excretion in their urine. Survey respondents were asked to indicate if they were willing to participate and to include their contact information if so. To encourage participation in this part of the study, students who agreed to donate urine samples were entered into a raffle for an iPod shuffle with the incentive that the first thirty participants would have their names entered into the raffle five times. The cheapest iPod was used as an incentive to minimize the selective pressure for economically disadvantaged students. Because the participants in this section of the project were voluntary, they represented a convenience sample (Biemer & Lyberg, 2003). Samples were collected twice, once in the spring and fall semesters of 2008. Participants who volunteered were informed to arrive at the University of Maryland, University Health Center at a specified time over the course of three different days. Once there, the members of the surveying team noted the participant's height and weight measurements and provided them with a sterile urine collection cup for the urine sample. Participants were once again identified by the four-digit number from their initial survey response and the urine samples were separated from any identifiable personal information.

After the sample was given, it was stored in a refrigerated container until it was analyzed. The urine samples were taken from the University Health Center to the National Institutes of Technology (NIH) in Bethesda, Maryland. A urinalysis test was performed on the spot urine samples using the protein to creatinine ratio discussed in the literature review. The VITROS Chemistry Products Calibrator Kit 10 (reference number

680 0120) machine was used to detect the amount of protein excreted in the urine based on the protein to creatinine ratio. High levels of urinary protein excretion (proteinuria) are a precursor to renal diseases. The team wanted to draw a link between protein, specifically glutamine, usage and potential risks to kidney damage.

Chapter 4: Results

4.1 Survey Research Results

4.1.1 Calculations Made from Raw Survey Data

Survey data was first separated into frequencies and percentages that were pertinent to the statistical test results. Survey collection generated 947 total respondents, 382 of which were using protein supplements. By examining the data, it was discovered that the most popular protein products taken by participants were GNC Pro-Performance Whey Protein with 96 users (25.13% of protein users), a generic form of whey protein with 65 users (17.02% of protein users), and CytoSport Muscle Milk with 38 users (9.95% of users). Only 13 respondents were taking the pure form of glutamine, called L-glutamine.

The average amount of protein taken by participants per week was also calculated in order to compare it to the Recommended Dietary Allowance's (RDA) suggested protein consumption for humans. The RDA recommends that humans ingest 0.8 grams (g) of protein per kilogram (kg) of body weight per day which translates into 5.6 g of protein per kg of body weight per week. The average American diet consists of 8.4 g of protein per kg of body weight per week (Eisenstein, et al., 2002). Survey data revealed that the average protein supplement intake amongst participants was 183.4 g per week. This 183.4 g demonstrated that survey participants were taking 30% more protein per week than the average American population based on the average American diet. Team Juiced then used 72.1 kg of body weight as a standard measure of body weight for both males and females because a study conducted in 1972 by Van Cott and Kinkade found the average body mass for a college student on the east coast of the United States to be

72.1 kg (Johnson, 1999). Based on 72 kg of body weight and the RDA suggestion for protein intake it was found that survey participants were consuming 50% of the recommended daily allowance from supplements alone.

4.1.2 Statistical Tests Performed on Raw Survey Data

A variety of statistical tests were used to evaluate the survey data. Each test compared an independent variable to the amount of protein taken per week for each product listed by the participant. Each test used 95% confidence. Since the participant could list up to three products, the test evaluated the selected variable in terms of the amount of protein taken per week for the first product, then in terms of the amount of protein taken per week for the second product, and again for the amount of protein taken per week for the third product. The first, second, and third product is not a named product itself, but a grouping of all of the individual products that a participant listed under each category.

4.1.3 Hours of exercise per week vs. amount of protein used for products 1, 2, and 3

The first comparison of means assessed was entitled hours of exercise per week vs. amount of protein used for products 1, 2, and 3. In this comparison of means, hours of exercise per week served as the independent variable, and the level of measurement for this variable was ordinal. It was an ordinal variable since it involved categories that were ordered by rank. The categories include 1-5 hours of exercise per week, 6-10 hours of exercise per week, and greater than ten hours of exercise per week. The amount of protein taken for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Since the independent variable in this comparison is ordinal and the dependent variable is interval-ratio, the appropriate test to use was the Spearman's Rho test. As seen in **Figure 1**, for the individuals exercising anywhere between one hour to greater than ten hours per week and using product 1, the two-tailed significance p-value was 0.136 which was greater than 0.05 and therefore statistically insignificant showing insufficient evidence to support a relationship between the independent and dependent variables and therefore number of hours an individual was exercising and how much protein he or she was consuming. For the individuals exercising anywhere between one hour to greater than ten hours per week and using product 2, the two-tailed significance p-value was 0.330 which was greater than 0.05 and therefore statistically insignificant also depicting insufficient evidence to support a relationship between the independent and dependent variables. For the individuals exercising anywhere between one hour to greater than ten hours per week and using product 3, the two-tailed significance p-value was 0.228 which was greater than 0.05 and therefore statistically insignificant showing insufficient evidence to support a relationship between the independent and dependent variables.

Figure 1: Spearman's Rho Test for Hours of exercise per week vs. Amount of protein used for products 1, 2, and 3

			Correlations			
			Hours of Exercise	How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Spearman's rho	Hours of Exercise	Correlation Coefficient	1.000	.087	.154	-.419
		Sig. (2-tailed)	.	.136	.330	.228
		N	934	293	42	10
	How much Product #1 used (grams)	Correlation Coefficient	.087	1.000	.194	-.060
		Sig. (2-tailed)	.136	.	.243	.887
		N	293	296	38	8
	How much Product #2 used (grams)	Correlation Coefficient	.154	.194	1.000	.847*
		Sig. (2-tailed)	.330	.243	.	.016
		N	42	38	42	7
	How much Product #3 used (grams)	Correlation Coefficient	-.419	-.060	.847*	1.000
		Sig. (2-tailed)	.228	.887	.016	.
		N	10	8	7	10

*. Correlation is significant at the 0.05 level (2-tailed).

A Spearman's Rho test was used to determine whether a statistically significant relationship exists between the comparison of means entitled hours of exercise vs. amount of protein used for products 1, 2, and 3.

4.1.4 Gender of the respondent vs. Amount of protein used for products 1, 2, and 3

The second comparison of means assessed in this study was entitled gender of the respondent vs. amount of protein used for products 1, 2, and 3. In this comparison of means, the gender of the respondent served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories that were not ordered by rank. The amount of protein taken for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Figure 2: Independent Samples T-Test for Gender of the respondent vs. Amount of protein used for products 1, 2, and 3

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
How much Product #1 used (grams)	Equal variances assumed	5.243	.023	2.000	286	.046	19.5202	9.7621	.3056	38.7349
	Equal variances not assumed			4.842	132.879	.000	19.5202	4.0316	11.5457	27.4947
How much Product #2 used (grams)	Equal variances assumed	.968	.331	.751	40	.457	38.60971	51.42631	-65.32675	142.54616
	Equal variances not assumed			2.646	6.081	.038	38.60971	14.59014	3.02383	74.19558

Independent Samples T-Test was used to determine a statistically significant relationship in the comparison of means entitled Gender of the respondent vs. Amount of protein used for products 1, 2, and 3.

A statistically significant relationship exists between the gender of the respondent compared to the amount of protein used for product 1 since more males are using product 1 than females, and more males are using more of product 1 than females. Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. As seen in **Figure 2**, for the males and females using product 1, the two-

tailed significance p-value was 0.046 which was less than 0.05 and therefore statistically significant indicating that there is a statistically significant relationship between the independent and dependent variables. Therefore, the amount of protein that the respondent consumed was dependent on his or her gender. The descriptive statistics above depicted that more males were using more of the protein supplements than females. This test further revealed this statistically-significant finding that deeper reinforced the aforementioned descriptive statistics. In addition, fewer males were using a second or third product. Moreover, no females were using a third product. Therefore, more males were supplementing more of product 1 than females. For the males and females using product 2, the two-tailed significance p-value was 0.457 which was greater than 0.05 and therefore statistically insignificant demonstrating that there is no relationship between the independent and dependent variables.

4.1.5 Assessing Personal and Family Risk Factors as Variables

Additionally, it is important to note that in the comparison of means for each of the risk factors of kidney disease, clinical obesity, hypertension, and diabetes that an individual carries personally or through family history, there can be two pathways for determining any statistically significant relationships. For each risk factor tested individually or through family history, the risk factor served as the independent nominal variable while the amount of protein used served as the dependent interval-ratio variable. The other option for comparison could be switching the two variables so that the independent variable would be the amount of protein used while each risk factor tested individually or through family history would be the dependent variable. No matter which direction chosen, since one variable will always be nominal and the other variable will

always be interval-ratio, the test will always be the same: Independent Samples t-test. The two explanations or interpretations that the reversed comparisons could offer are 1) if the individual has a particular risk factor personally present, will that impact the amount of protein that the individual consumes, and 2) does the amount of protein that the individual consumes impact the risk of whether the individual would have the risk factor in a later stage of his or her life.

4.1 6 Respondent with a family history of diabetes vs. amount of protein used for products 1, 2, and 3

The third comparison of means assessed was entitled respondent with a family history of diabetes vs. amount of protein used. In this comparison of means, respondents with a family history of diabetes served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein used for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. The amount of protein used was compared between participants with and without the stated risk factor. Because it was difficult to combine the amounts reported in Products 1, 2, and 3, the relationships between amount of protein and risk factor was analyzed independently for each “cell.” Products 1, 2, and 3 are not distinct products, rather the first, second, and third cells available for participants to report a product used.

Shown in **Figure 3**, the individuals with a family history of diabetes using product 1, the two-tailed significance p-value was 0.123 and therefore statistically insignificant and demonstrating insufficient evidence to support a relationship between the independent and dependent variables. For the individuals with a family history of diabetes using product 2, the two-tailed significance p-value was 0.519, thereby statistically insignificant and indicating insufficient evidence to support a relationship between the independent and dependent variables. Lastly, for the individuals with a family history of diabetes using product 3, the two-tailed significance p-value was 0.461 and therefore also statistically insignificant portraying insufficient evidence to support a relationship between the independent and dependent variables.

Figure 3: Independent Samples T-Test for respondent with a family history of diabetes vs. amount of protein used for products 1, 2, and 3

		Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means							95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper		
How much Product #1 used (grams)	Equal variances assumed	3.175	.076	1.546	284	.123	9.4292	6.1008	-2.5794	21.4377		
	Equal variances not assumed			1.274	118.934	.205	9.4292	7.4010	-5.2257	24.0841		
How much Product #2 used (grams)	Equal variances assumed	1.220	.276	- .650	39	.519	-14.59747	22.44680	-60.00042	30.80548		
	Equal variances not assumed			- .658	32.889	.515	-14.59747	22.18138	-59.73158	30.53665		
How much Product #3 used (grams)	Equal variances assumed	4.569	.065	.774	8	.461	44.80000	57.88713	-88.68796	178.28796		
	Equal variances not assumed			.774	4.191	.480	44.80000	57.88713	-113.077	202.67680		

Independent Samples t-test was used to determine whether or not a statistically significant relationship exists between an individual with a family history of diabetes vs. amount of protein used for products 1, 2, and 3.

4.1.7 Respondent with a family history of kidney disease vs. amount of protein used for products 1, 2, and 3

The fourth comparison of means assessed was entitled respondent with a family history of kidney disease vs. amount of protein used for products 1, 2, and 3. In this

comparison of means, respondent with a family history of kidney disease served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein used for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. As seen in **Figure 4**, for the individuals with a family history of kidney disease using product 1, the two-tailed significance p-value was 0.639 and therefore statistically insignificant and demonstrating insufficient evidence to support a relationship between the independent and dependent variables. For the individuals with a family history of kidney disease using a second product, the two-tailed significance p-value was 0.561, thereby statistically insignificant and indicating insufficient evidence to support a relationship between the independent and dependent variables.

Figure 4: Independent Samples T-Test for respondent with a family history of kidney disease vs. amount of protein used for products 1, 2, and 3

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
How much Product #1 used (grams)	Equal variances assumed	.125	.724	-.469	284	.639	-13.2124	28.1581	-68.6375	42.2127
	Equal variances not assumed			-.954	2.186	.433	-13.2124	13.8494	-68.1873	41.7625
How much Product #2 used (grams)	Equal variances assumed	.	.	-.586	39	.561	-42.67246	72.80933	-189.943	104.59831
	Equal variances not assumed			.	.	.	-42.67246	.	.	.

An Independent Samples t-test was used to determine whether a statistically significant relationship exists between the comparison of means for the respondents with a family history of kidney disease vs. the amount of protein used for products 1, 2, and 3.

Since there were no individuals with a family history of kidney disease using a third product, the independent variable in this comparison of means regarding a third product could not be tested against the dependent variable and therefore, a statistical test could not be executed to determine a two-tailed significance p-value.

4.1.8 Respondent with a family history of clinical obesity vs. amount of protein used for products 1, 2, and 3

The fifth comparison of means assessed was entitled respondent with a family history of clinical obesity vs. amount of protein used for products 1, 2, and 3. In this comparison of means, respondent with a family history of clinical obesity served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein used for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. For the individuals with a family history of clinical obesity using product 1, the two-tailed significance p was 0.433 and therefore statistically insignificant and indicating insufficient evidence to support a relationship between the independent and dependent variables.

As seen in **Figure 5**, for the individuals with a family history of clinical obesity using product 2, the two-tailed significance p-value was 0.537 and thereby statistically insignificant and representing insufficient evidence to support a relationship between the independent and dependent variables. Lastly, for the individuals with a family history of clinical obesity using product 3, the two-tailed significance p-value was 0.549 and statistically insignificant portraying insufficient evidence to support a relationship between the independent and dependent variables.

Figure 5: Independent Samples T-Test for respondent with a family history of clinical obesity vs. amount of protein used for products 1, 2, and 3

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
How much Product #1 used (grams)	Equal variances assumed	.656	.419	.785	284	.433	11.2307	14.2984	-16.9136	39.3751
	Equal variances not assumed			.595	11.532	.563	11.2307	18.8693	-30.0678	52.5293
How much Product #2 used (grams)	Equal variances assumed	.176	.677	.623	39	.537	32.45133	52.11051	-72.95212	137.85478
	Equal variances not assumed			.515	1.069	.693	32.45133	63.04571	-655.972	720.87469
How much Product #3 used (grams)	Equal variances assumed			-.626	8	.549	-61.11111	97.66360	-286.324	164.10156
	Equal variances not assumed						-61.11111			

An Independent Samples t-test was used to determine whether there was a statistically significant relationship between an individual with a family history of clinical obesity using greater quantities of products 1, 2, and 3.

4.1.9 Respondent with a family history of hypertension vs. amount of protein used for products 1, 2, and 3

The sixth and final comparison of means assessed by the team was entitled respondent with a family history of hypertension vs. amount of protein used for products 1, 2, and 3. In this comparison of means, respondent with a family history of hypertension served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and

“no” that were not ordered by rank. The amount of protein used for products 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. For the individuals with a family history of hypertension using product 1, the two-tailed significance p-value was 0.458 and therefore statistically insignificant indicating insufficient evidence to support a relationship between the independent and dependent variables. As seen in **Figure 6**, for the individuals with a family history of hypertension using product 2, the two-tailed significance p-value was 0.707 and thereby also statistically insignificant demonstrating insufficient evidence to support a relationship between the independent and dependent variables.

Figure 6: Independent Samples T-Test for respondent with a family history of hypertension vs. amount of protein used for products 1 and 2

		Independent Samples Test									
		Levene's Test for Equality of Variances		t-test for Equality of Means						95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper	
How much Product #1 used (grams)	Equal variances assumed	.818	.367	-.744	283	.458	-5.8172	7.8198	-21.2095	9.5751	
	Equal variances not assumed			-.916	81.191	.362	-5.8172	6.3481	-18.4474	6.8130	
How much Product #2 used (grams)	Equal variances assumed	.010	.923	-.378	38	.707	-16.57833	43.83795	-105.324	72.16696	
	Equal variances not assumed			-.456	2.529	.685	-16.57833	36.34809	-145.440	112.28380	

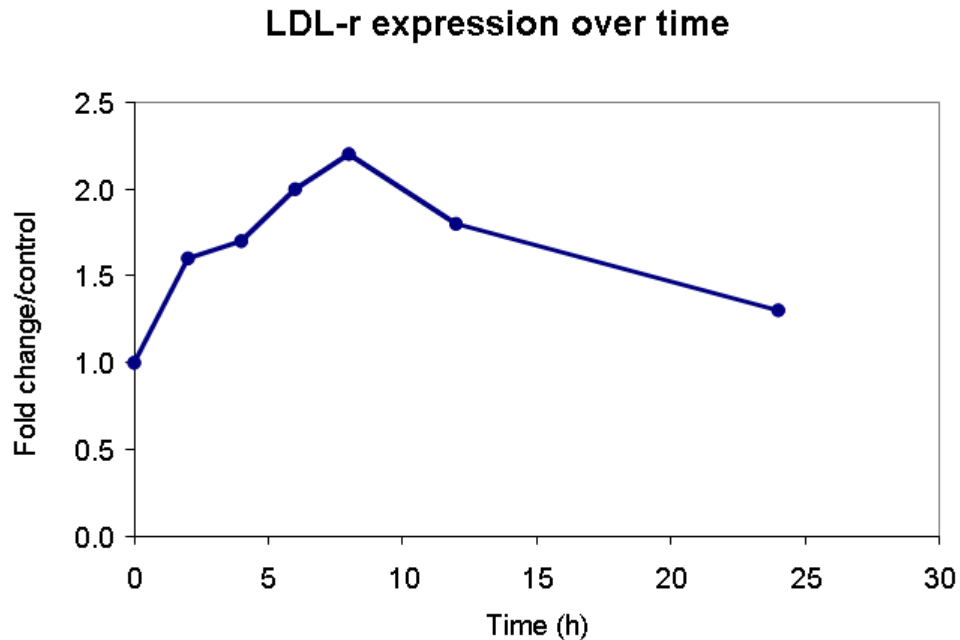
An Independent Samples t-test was used to assess the relationship between whether an individual with a family history of hypertension uses greater quantities of products 1 and 2.

4.2 Kidney Cell Modeling Results

4.2.1 Time Course Study

In the time course study a supraphysiological level of 2.0 mM of glutamine was used to determine the time dependent behavior of LDL-r expression. **Figure 7** shows that as early as two hours, an increase of greater than 50% or 1.5-fold was observed when comparing the beta-actin normalized LDL-r expression in cells grown in 2.0 mM glutamine versus control cells grown in the absence of glutamine. Experimental cells in 2.0 mM of glutamine showed increasingly elevated expression for LDL-r up to greater than twofold the baseline of control cells between 6 and 8 hours. The fold change versus control cells dropped at 12 and 24 hours; expression remained elevated in experimental cells, still showing an increase of 1.5-fold after 24 hours.

Figure 7: LDL-r time course in 2.0 mM glutamine

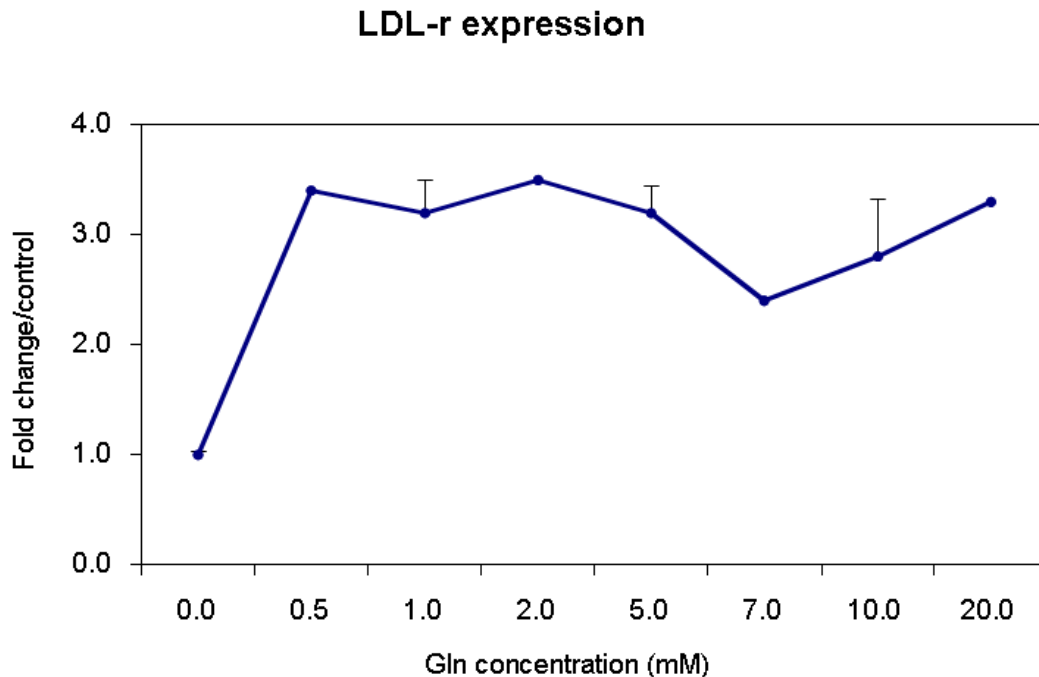


Fold change/control = ratio of normalized LDL-r expression in experimental versus control cells (no glutamine). Data points are given for 2, 4, 6, 8, 12, and 24 hours.

4.2.2 Dose Response Study

It was apparent from the time course study that LDL-r expression was responding optimally between 6-8 hours, when it showed the highest increase in expression as compared to control cells. Six hours was chosen for the dose response study so that culture and extraction could be done comfortably within one work day. The findings of the dose response study are summarized in **Figure 8**. As little as 0.5 mM of glutamine showed an increase in LDL-r expression of approximately 3.5-fold as compared to control cells not exposed to any glutamine after 6 hours of exposure. This elevated level of expression was maintained through 20.0 mM of glutamine. LDL-r expression was no higher than 3.5-fold at any concentration of glutamine higher than 0.5 mM.

Figure 8: LDL-r dose curve at 6 hours

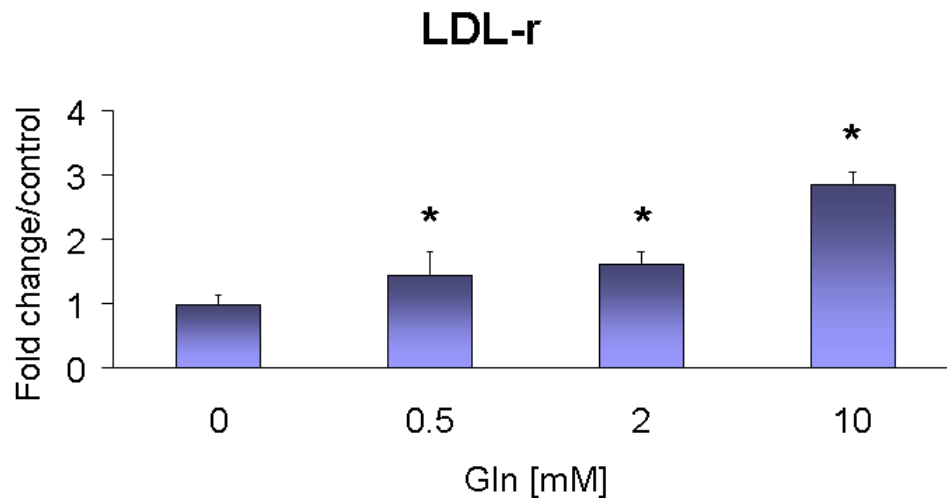


Fold change/control = ratio of normalized LDL-r expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Data points are given for 0.0, 0.5, 1.0, 2.0, 5.0, 7.0, 10.0, and 20.0 mM. Note the horizontal axis of concentration is not drawn to scale. Error bars are given in the figure based on variation between duplicate wells. Only positive error bars are shown.

4.2.3 Chronic Study

The dose dependent studies conducted at 48 hours of exposure to glutamine were conducted to gain insight on conditions of chronic glutamine over-use. LDL-r, as well as HMG CoA reductase, which is the key enzyme in the cholesterol synthesis pathway, matrix proteins (collagen I, fibronectin, and collagen IV), and growth factor TGF- β were tested for expression at 0.5, 2.0, and 10.0 mM of glutamine at 48 hours of exposure. LDL-r expression was found to be dose dependent, unlike at 6 hours of exposure. As shown in **Figure 9**, a statistically significant increase was observed for LDL-r expression compared to control cells for 0.5 and 2.0 mM, both showed a 1.5-fold increase in expression. Dose dependency was apparent when analyzing cells exposed to 10.0 mM of glutamine where the fold increase was nearly threefold, approximately double the expression seen at 0.5 and 2.0 mM glutamine.

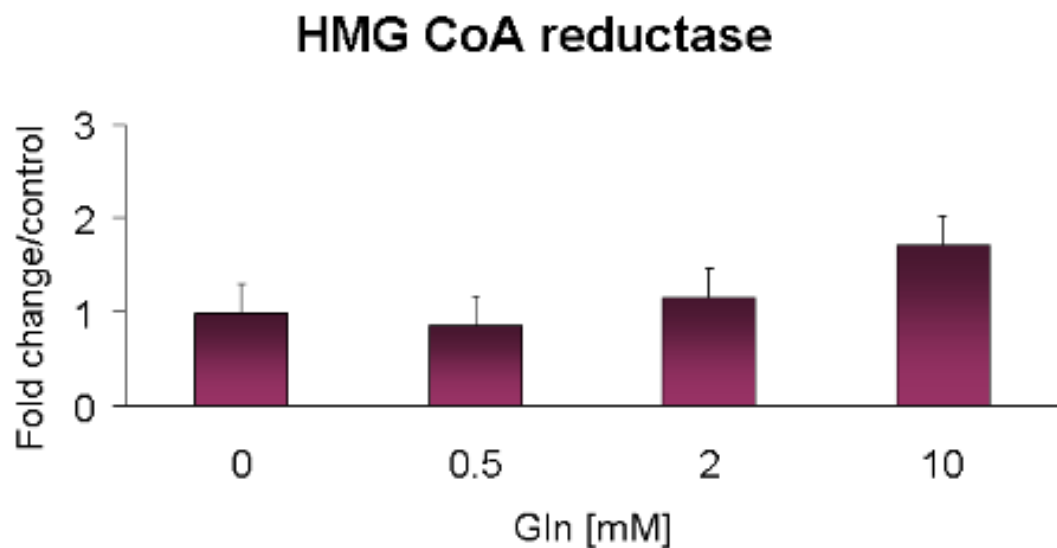
Figure 9: LDL-r dose response at 48 hour



Fold change/control = ratio of normalized LDL-r expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells. Asterisks = statistical significance of $P < 0.05$ compared to control cells at 0.0 mM glutamine.

While LDL-r shows an extrinsic path for LDL to enter the cells, HMG CoA reductase represents an internal mechanism by which the cellular metabolism generates lipids. Unlike LDL-r expression, cells did not show a statistically significant increase in the expression of HMG CoA reductase in response to increasing concentrations of glutamine for 48 hours of exposure. **Figure 10** shows a variation between triplicate wells in error bars that cover nearly a 0.5-fold range. Cells exposed to 10.0 mM of glutamine showed a higher level of HMG CoA reductase expression, up to nearly twofold higher than baseline, but the variation between wells was still too large to prove statistical significance.

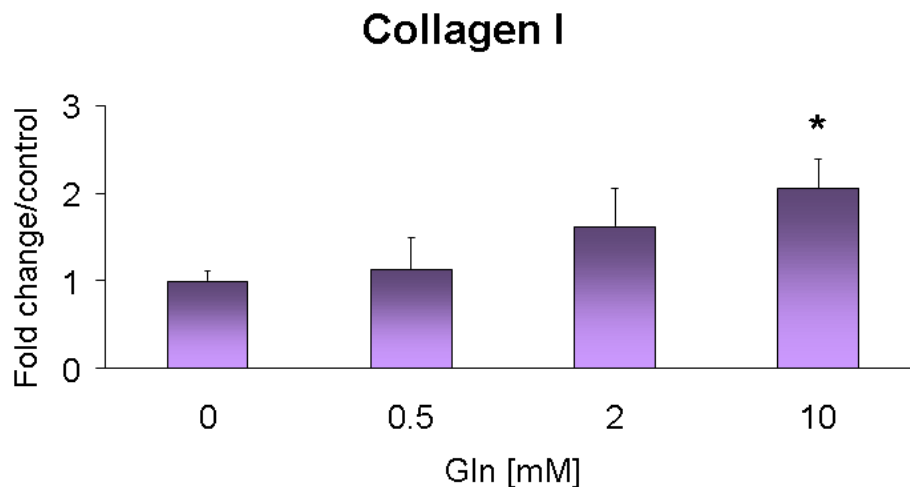
Figure 10: HMG CoA reductase dose response at 48 hours



Fold change/control = ratio of normalized HMG CoA reductase expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells.

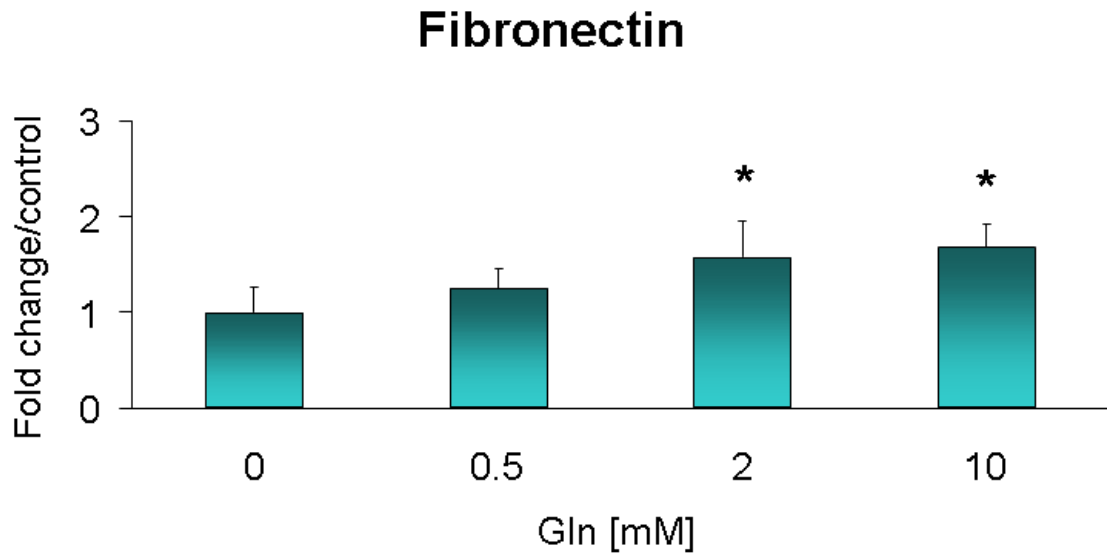
All three matrix proteins were found to have a trend towards increased expression as compared to control cells over the increasing doses of glutamine for 48 hours of exposure. Collagen I and fibronectin both showed statistically significant increases as compared to control cells. As seen in **Figure 11**, cells exposed to 10.0 mM of glutamine were significantly higher in collagen I expression, showing a level approximately twofold greater than control cells. For fibronectin expression, **Figure 12** illustrates a significant increase of approximately 1.5-fold as compared to control cells for cells exposed to both 2.0 and 10.0 mM of glutamine. This increase in expression for fibronectin was slightly less than the increase noted for collagen I. As shown by **Figure 13**, expression of collagen IV largely mirrored that of collagen I expression, but with larger variation between wells. Without this variation, the approximately 1.5- to twofold increase in collagen IV expression for cells exposed to 10.0 mM of glutamine may also have been significant.

Figure 11: Collagen I dose response at 48 hours



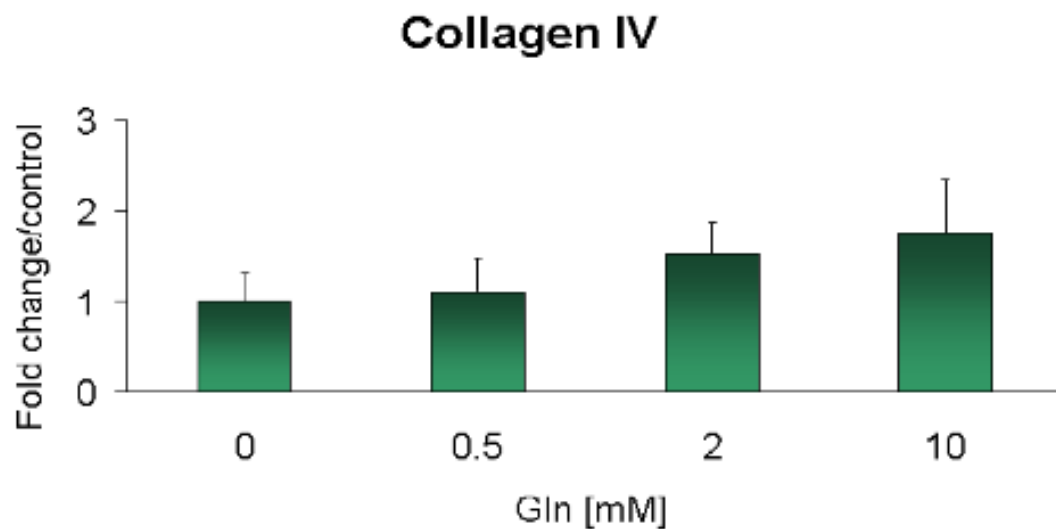
Fold change/control = ratio of normalized collagen I expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells. Asterisks = statistical significance of $P < 0.05$ compared to control cells at 0.0 mM glutamine.

Figure 12: Fibronectin dose response at 48 hours



Fold change/control = ratio of normalized fibronectin expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells. Asterisks = statistical significance of $P < 0.05$ compared to control cells at 0.0 mM glutamine.

Figure 13: Collagen IV dose response at 48 hours

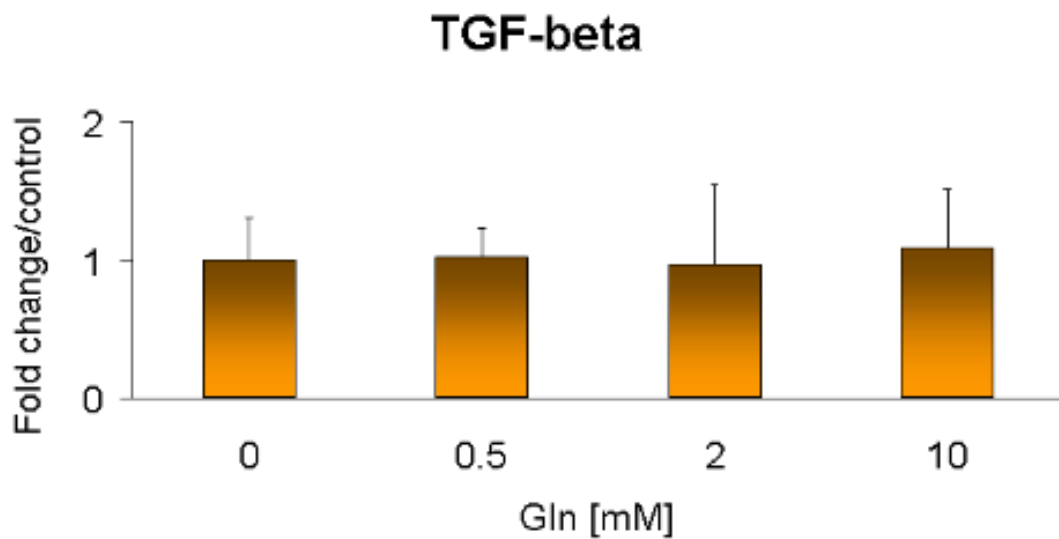


Fold change/control = ratio of normalized collagen IV expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells.

The growth factor, TGF- β was found to be constant throughout increasing concentrations of glutamine exposure for 48 hours. Unusually large amounts of variation were observed between triplicate wells, but the averages of TGF-beta expression were held constant at the baseline established by control cells not exposed to any glutamine.

Figure 14 shows the variation as error bars.

Figure 14: TGF-beta dose response at 48 hours



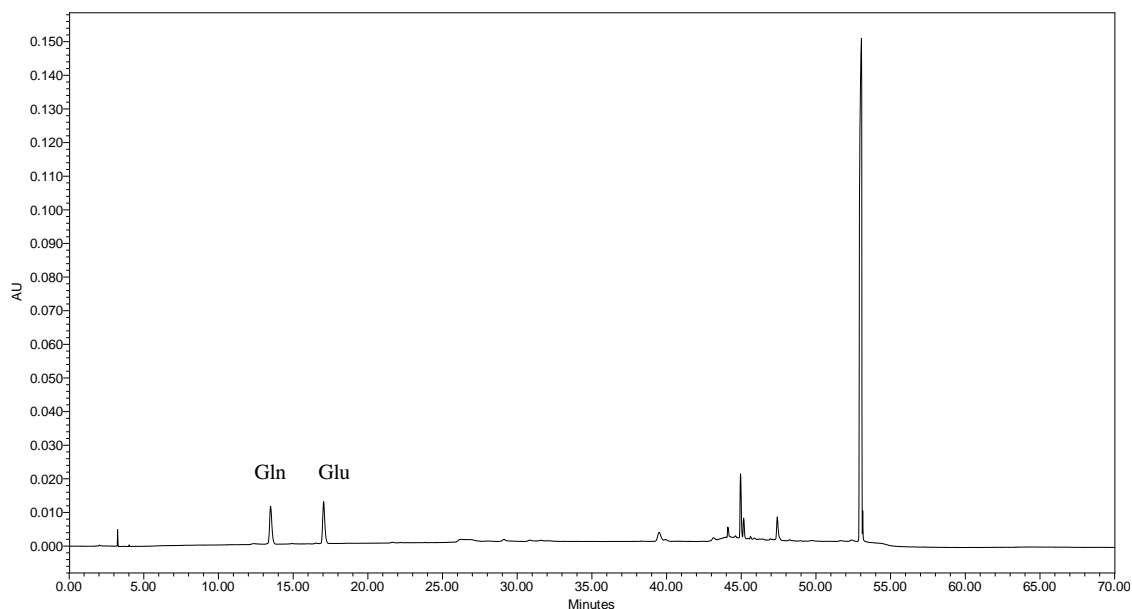
Fold change/control = ratio of normalized TGF-beta expression in experimental versus control cells exposed to no glutamine. Gln = glutamine. Error bars are given based on the variance between triplicate wells. Note the large error bars.

4.3 Biochemical Analysis of Glutamine Results

4.3.1 Determination of Retention Time and Calibration Curve for Glutamine

The retention time (RT) of glutamine was determined in order to quantify the concentration of glutamine in each supplement. Also, it was observed whether the enzyme hydrolysis and PITC derivatization processes caused any glutamine to convert into glutamic acid. Glutamine and glutamic acid standards were first tested separately using HPLC to determine the RT for both of these amino acids. The glutamine standard produced a peak with a RT of approximately 14 minutes, while the glutamic acid standard peak had a RT of approximately 17 minutes. The chromatogram shown in **Figure 15** displays these retention times for glutamine and glutamic acid in the same HPLC separation.

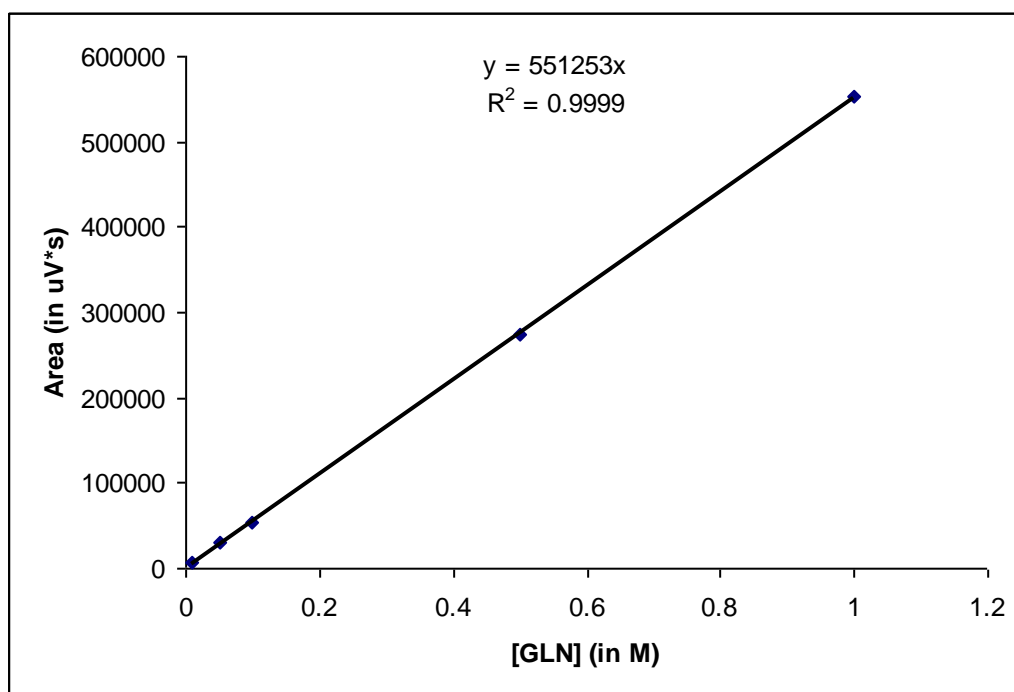
Figure 15: Glutamine/Glutamic Acid Chromatogram



An equal mixture of glutamine (gln) and glutamic acid (glu) was tested. High performance liquid chromatography was used to detect glutamine and glutamic acid after phenyl isothiocyanate derivatization. The retention time for glutamine was approximately 14 minutes, while the retention time for glutamic acid was approximately 17 minutes.

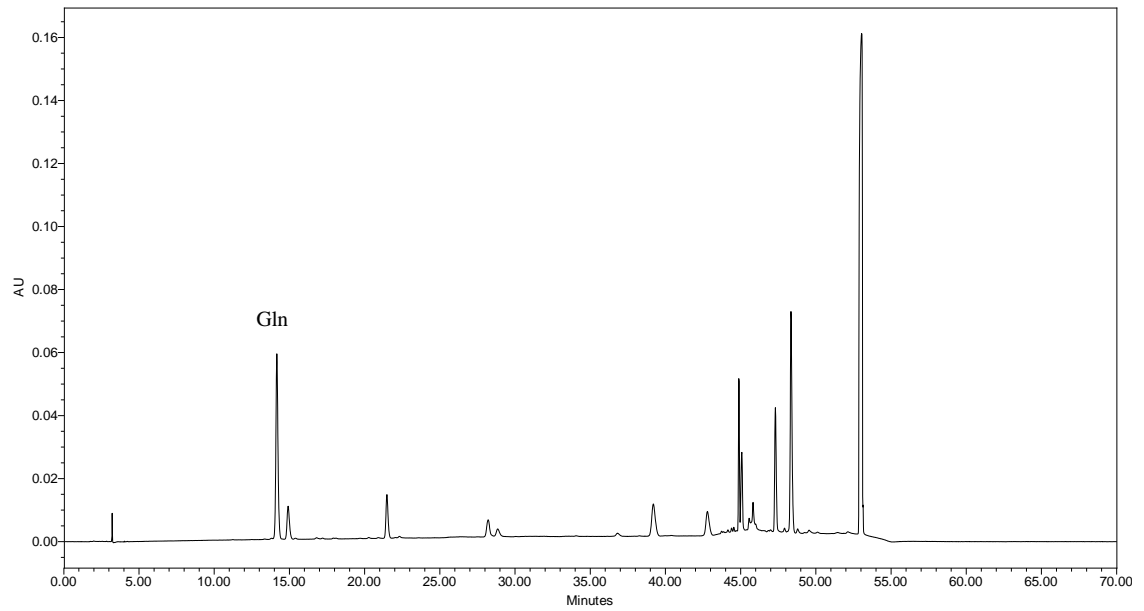
Different concentrations of a glutamine standard were then tested for calibration. A calibration curve was completed to establish the relationship between the HPLC machine measuring glutamine concentration and the actual glutamine concentration. The calibration curve, which is shown in **Figure 16**, had a slope of 551253.1 with an R^2 value of 0.9999. This slope was used to measure the concentrations in the supplements. The average RT for glutamine was 14.05 ± 0.0055 s. Figure 17 shows there were no peaks around 17 minutes (RT for glutamic acid) in any of the glutamine standard runs for the calibration curve.

Figure 16: Glutamine Calibration Curve



High performance liquid chromatography was used to measure the concentrations of five different glutamine (gln) solutions: 0.01, 0.05, 0.10, 0.50, 1.00 mg/mL after phenyl isothiocyanate derivatization. Glutamine was eluted from the high performance liquid chromatography column using a sodium phosphate/acetonitrile mobile phase after 14.0514 ± 0.0055 s. The areas of the peak for each concentration were measured and graphed, as seen above, to calculate the slope.

Figure 17: Glutamine Chromatogram



High performance liquid chromatography was used to measure five concentrations from a glutamine standard after phenyl isothiocyanate derivatization. This chromatogram is taken from the high performance liquid chromatography run with a 0.1 mg/mL concentration of glutamine. This chromatogram clearly shows a peak at ~ 14 minutes, which indicates the presence of glutamine (gln).

4.3.2 Pure Glutamine Supplements

The supplements tested were split into two groups: glutamine and protein. The five glutamine supplements tested were Supplement 1, 8, 12, 19, and 21. All pure glutamine supplements were expected to have 100% recovery of glutamine. The five glutamine supplements listed in **Table 1** yielded similar concentrations of glutamine as compared to the sample concentration. The average percent recovery for these three supplements was between 98% and approximately 102%. The chromatograms for these five supplements are included in **Figure 18** on the following page. There was a clear peak with a RT of approximately 14 minutes for each pure glutamine supplement. There were no peaks at approximately 17 minutes, which would indicate the presence of glutamic acid.

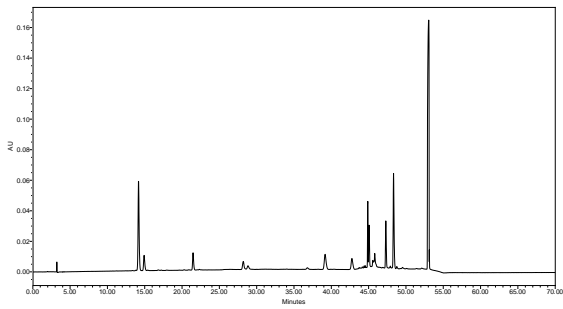
Table 1: Measured Glutamine Concentrations from Pure Glutamine Supplements

Supplement #	[Supplement] (mg/mL)	Measured [Gln] (mg/mL)	Percent Recovery
1	1.040	1.035 ± 0.0053	101.41
8	1.003	1.024 ± 0.0370	102.01
12	1.022	1.011 ± 0.0255	98.89
19	1.022	1.017 ± 0.0053	99.46
21	1.035	1.006 ± 0.0241	98.29

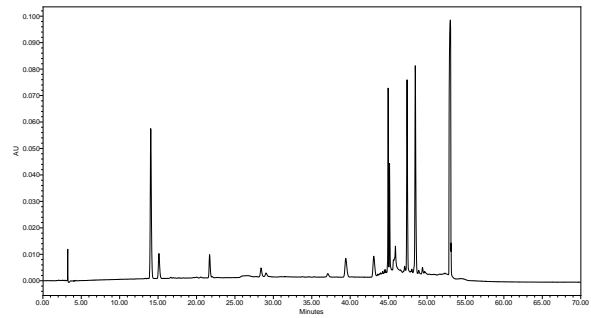
Five pure glutamine (gln) supplements with a concentration of approximately 1 mg/mL were derivatized, and the glutamine content was measured using high performance liquid chromatography. This was completed a total of two times for each pure glutamine supplement.

Figure 18: Pure Glutamine Chromatograms

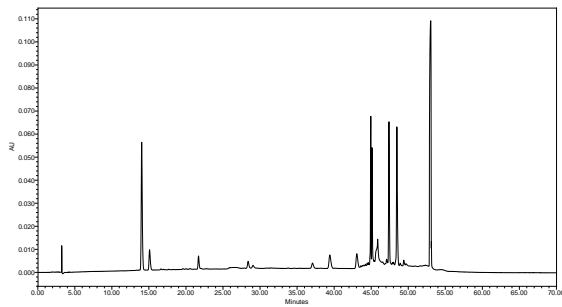
Supplement 1



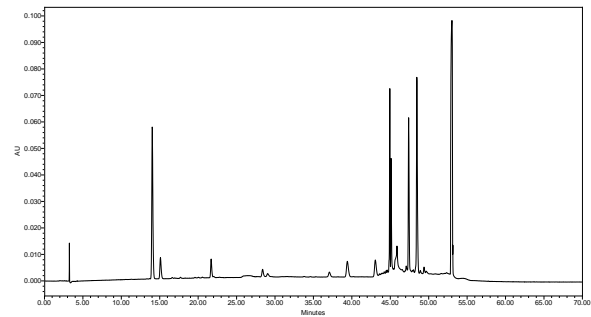
Supplement 8



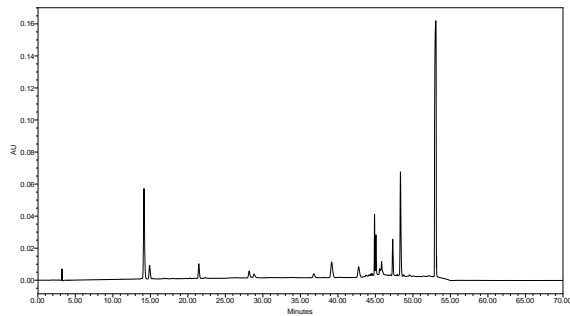
Supplement 12



Supplement 19



Supplement 21

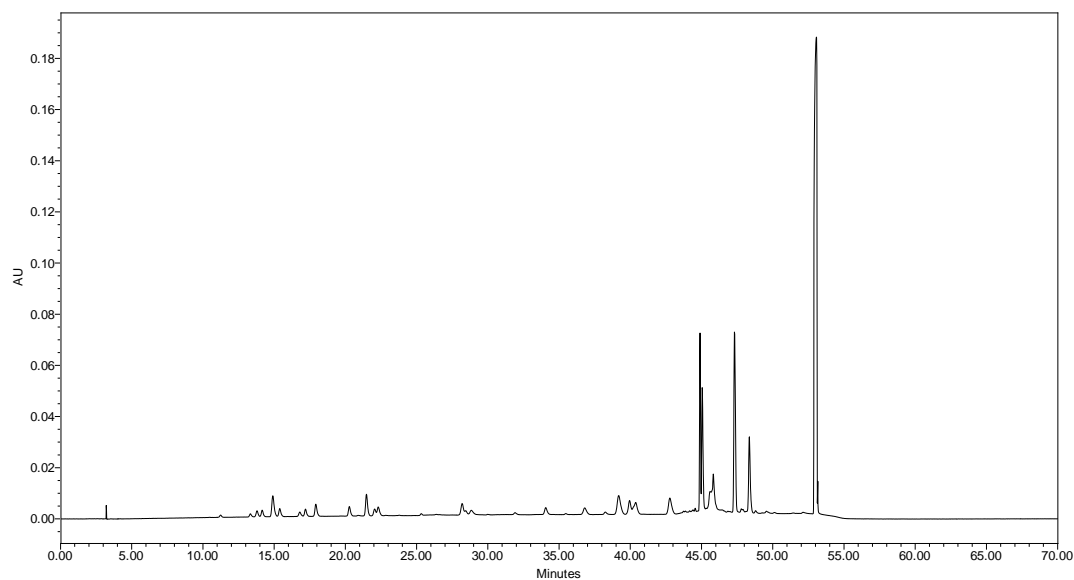


High performance liquid chromatography was used to test the glutamine concentration in five pure glutamine supplements after phenyl isothiocyanate derivatization. Each chromatogram has a peak at around 14 minutes, which is the peak for glutamine.

4.3.3 Protein Supplements with Glutamine Content on Label

The concentrations of glutamine were then measured in protein supplements with and without glutamine content on the supplement bottle's label by manufacturers. The nine protein supplements with glutamine content on the label will be discussed first, and these supplements were Supplement 2, 4, 5, 6, 11, 13, 16, 17, and 18. These supplements were hydrolyzed and derivatized with PITC according to the procedure. An example of a chromatogram from one of these protein supplements is shown in **Figure 19**.

Figure 19: Chromatogram of Supplement 11



Supplement 11 was hydrolyzed with enzymes and derivatized with phenyl isothiocyanate to produce this chromatogram after high performance liquid chromatography.

The concentration of glutamine was measured, and the amount of glutamine detected per serving size for these nine supplements are listed in **Table 2**.

Table 2: Measured Amount of Glutamine from Protein Supplements, with Glutamine Content on Label

Supplement #	[Supplement] (mg/mL)	Measured [Gln] (mg/mL)	Amount of Gln Detected, per serving size (g)
2	0.99	0.0377 ± 0.0002	0.61
4	1.11	0.0510 ± 0.0003	1.01
5	1.05	0.0371 ± 0.0010	5.33
6	1.33	0.1866 ± 0.0014	4.51
11	1.02	0.0419 ± 0.0001	1.15
13	1.00	0.0400 ± 0.0004	1.24
16	1.02	0.0550 ± 0.0002	3.99
17	1.01	0.0472 ± 0.0000	1.47
18	1.03	0.0304 ± 0.0001	0.95

Protein supplements with a concentration of about 1 mg/mL were derivatized with phenyl isothiocyanate, and then run through high performance liquid chromatography. The chromatograms were used to determine the glutamine concentration in each protein supplement. Each supplement had two trials. The amount of glutamine per serving size is listed in the last column.

The comparison of the amount of glutamine detected versus the amount of glutamine expected to be in the supplements, as determined by the manufacturer’s labels, is displayed in **Table 3**. Supplement 2, 4, 11, 13, 16, 17, and 18 measured less glutamine than listed on their labels. Supplement 5 and 6 measured more glutamine than is stated on the labels by manufacturers.

Table 3: Comparison of Measured Glutamine Recovery and Percent Glutamine on Label of Protein Supplements

Supplement #	Amount of Gln Detected, per serving size (g)	Amount of Gln, from Label (g)
2	0.61	2.00
4	1.01	12.50
5	5.33	2.00
6	4.51	3.00
11	1.15	3.56
13	1.24	4.00
16	3.99	10.50
17	1.47	4.08
18	0.95	4.00

4.3.4 Protein Supplements without Glutamine Content on Label

Glutamine concentrations in protein supplements without the glutamine content listed on the label by manufacturers were also measured. These six supplements were Supplement 3, 7, 9, 14, 15, and 20. The glutamine concentrations measured in these six protein supplements are listed in **Table 4**. The amount of glutamine detected in each sample was approximately 3 grams or less. Since the manufacturers do not list any information about the glutamine content on their labels, it was not possible to compare the amount of glutamine detected with what was expected.

Table 4: Measured Amount of Glutamine in Protein Supplements Without an Amount of Glutamine on Label

Supplement #	[Supplement] (mg/mL)	Measured [Gln] (mg/mL)	Amount of Gln Detected, per serving size (g)
3	1.02	0.0437 ± 0.0115	1.73
7	1.03	0.0450 ± 0.0014	3.06
9	0.99	0.0169 ± 0.0029	1.33
14	2.05	0.0191 ± 0.0003	2.12
15	1.06	0.0179 ± 0.0032	0.48
20	1.05	0.0472 ± 0.0006	1.44

Protein supplements (without a glutamine amount on the label) with a concentration of about 1 mg/mL, except Supplement 14, were derivatized with phenyl isothiocyanate. High performance liquid chromatography was used to measure glutamine (gln) concentration. Two trials were run for each supplement.

4.4 Urinalysis Results

In addition to the data collected from the survey, urine samples of survey respondents were gathered and tested for the presence of abnormal amounts of protein. The samples were then sent to the National Institute of Health (NIH) for processing and analysis. At the time this thesis was written, the results from the urinalysis have yet to be completed. The researchers are optimistic that the samples will be received from the NIH in May 2009. See addendum for additional information.

Chapter 5: Discussion

5.1 Survey Research Discussion

5.1.1 Assumptions

Given the nature of this survey study, a few basic assumptions were made in order to account for links between any data found and patterns noticed to ensure reliability and validity. First, it was assumed that some of the protein supplements used by the surveyed population of college students at the University of Maryland, College Park contained the amino acid glutamine. A significant component of this study involved lab research at USU that was concerned with the effects of varying dosages and concentrations of glutamine on rat mesangial cells. Because the purpose of the survey data was to connect results pertaining to human supplement consumption to the lab research focusing on effects of consumption, it was important that the same amino acid be studied. This assumption was verified by an extensive search of the content labels of protein supplements taken by the survey participants. Nearly every label either listed glutamine or whey protein as a key ingredient. Whey protein was assumed to contain glutamine for the purposes of this study. This assumption could be made because all of the protein supplements tested for glutamine content at the FDA that listed whey protein as an ingredient were found to contain glutamine. Second, it was assumed that in answering the survey questionnaire, respondents were honest about their answers regarding supplement usage as well as family and personal medical history. The current study was designed to discern possible links between protein supplement usage and long-term health risks so it was imperative that the data be as accurate as possible. Dishonesty in survey responses is likely to occur in a situation where the questions asked are of a

personal nature or in regard to socially stigmatized behavior (Biemer & Lyberg, 2003). Because the questions asked did not involve discussing a sensitive topic, respondents had no incentive or motivation to lie which reduced the possibility of intentional misstatement. Given the lack of means to verify subjects' responses, respondent honesty was a required assumption. In addition, because the survey was anonymous and the respondents therefore had little to fear about others knowing what information they revealed, the assumption that they were being honest was strengthened.

Third, in designing and administering the survey, it was assumed that any bias on the part of the respondent, the experimenters, or the survey design itself would not significantly affect the results in a manner that would skew the data in favor of a particular trend. Due to the limited interaction between the subjects and the experimenters, as this was either a paper-and-pencil questionnaire or computerized questionnaire as opposed to an interview, experimenter influence on subject response was minimal. In terms of respondent selection bias, it was assumed that people who were interested in the topic of protein supplements were more likely to fill out the survey than those who were not. However, because the project does not address a question of the prevalence of protein supplementation on campus, it was actually beneficial to target subjects likely to be more knowledgeable in order to obtain the most focused data. Respondent questionnaire bias could have played a role if the subjects did not understand the intent of the questions asked (Leedy & Ormrod, 2005). Another potential type of error was specification error which often occurs when questions appear confusing to the respondent. Specification error means that the concept measured is different from the concept that the survey intended to measure (Biemer & Lyberg, 2003). In addition,

sampling bias on the part of the experimental design is usually present when attempting to generalize from a small population sample to a larger more comprehensive population. Sampling bias often occurs because the experimenters do not verify absolute randomness of the sample or do not take into consideration characteristics about those who did not respond (Leedy & Ormrod, 2005). Sampling bias is assumed not to have a large effect on this study because the study intentionally targeted members of what was considered an active population at a university including varsity, intramural, and club athletes as well as non-athlete students that exercise at either of the university's recreational facilities (Geary F. Eppley Recreation Center or Ritchie Coliseum). The study did not aim to collect information about a sample in order to generalize about a larger and more comprehensive population.

Lastly, it was assumed that any non-sampling error was unlikely to have a significant effect on the results of the survey analysis. Due to the team's extensive data cleaning procedures, the assumption was made that any error in measurement or processing was double-checked and eliminated through this process as to not significantly affect the results or discussion of the data (Biemer & Lyberg, 2003).

5.1.2 Discussion of Results

It was the goal of the survey portion of this research project to better understand the supplementation habits of protein users at the University of Maryland, College Park. This included gathering information regarding the types of products commonly used, the average protein intake, how much exercise was completed each week, and the relationship between individual protein use and health and family risk factors for kidney disease.

Before administering the survey, the team had assumed that college students were in fact using protein supplements. Past studies have shown that college students are more likely to be using supplements than the general population (Newberry, et al., 2001). The team did discover that approximately 40% of the targeted population was using protein supplements therefore providing a sample that would produce pertinent information to the study. In addition, Team Juiced believed that protein supplements would be the most commonly taken form of NVNM supplements based on an earlier study that stated the high popularity of protein supplements amongst college students (Ayranci, et al., 2005; Perkin, et al., 2002). The large percentage of participants using protein supplements also verified the team's belief.

Arguably the most important finding of the survey research was that individuals with more risk factors for kidney disease took no less protein than individuals with fewer or no risk factors at all. There was no statistically significant relationship between risk factors and supplemental protein intake. Participants suffering from diabetes, obesity, or hypertension or those with a higher likelihood of developing these illnesses due to their family health history were at higher risk of developing kidney disease. The survey data showed that these individuals were taking no less supplemental protein than low risk participants. There was also no significant difference in the percentage of people with and without risk factors who used protein supplements. These findings were extremely important because of the known link between protein and kidney disease and because of the possible link between glutamine and glomerular scarring. The comparisons performed on participant's risk factors and their protein intake have not, to the team's knowledge,

been done in any other studies in the past decade, making these findings an integral part of emerging research.

The findings concerning protein intake and risk factors for kidney disease suggested that participants either did not understand the risks associated with protein and renal health or that they did not know how to properly regulate their protein intake. The indifference of this at risk population was concerning. Since protein supplements are regulated as foods and not drugs, it is not a far reach to assume that most users did not consult their doctors before engaging in a supplementation regime. Protein users need to be better educated and informed on the potential health risks of high protein intake. An advocacy, awareness, or educational program may be necessary for individuals interesting in supplementation regimens. It cannot be expected that the regulatory industry will jump to act upon the findings of this study because of the market ramifications of any changes.

Another surprising and very important finding of the present study was the overwhelming amount of protein taken by the survey participants that used these supplements. Among those using protein supplements, the average participant was taking 50% of the recommended daily allowance (RDA) of protein from supplements alone. Literature states that the average dietary intake of protein among Americans is already 150% of the RDA (Eisenstein, et al., 2002). It was assumed that the survey participants consumed similar diets to those stated in literature. If anything, the survey participants may be increasing their dietary protein intake for the same reasons that they take supplemental protein. When combining the 150% dietary intake of protein with the 50% supplementary intake of protein, it is clear that the survey participants were taking as

much as double the RDA of protein. A value this high was not previously found in the literature. Participants that used protein supplements may not understand the significance of how much dietary protein they were consuming. They also may not be aware of the possible detrimental effects of excess protein, especially for those with family risk factors for kidney disease. The fact that participants were who taking protein supplements could have been consuming double the RDA of protein from a combination of dietary and supplementary sources reiterates the importance of consumer education. Consumers need to know how much protein they are consuming, what the recommended amount of protein is, and what the possible consequences of high protein intake are.

After careful analysis of the data, one statistically significant comparison was established. The important trend revealed through the survey data in this study is that the average number of grams of protein taken per week is overwhelmingly higher for men than for women. While it may seem obvious that men take more protein than their female counterparts, it is important to understand the rationale that motivates this conventional wisdom conclusion as well as the reasons why the data supports it. The first logical conclusion is that the serving size for men is larger based on height, weight, and body composition. However, on product labels only one serving size is given, and it is the personal responsibility of the individual to determine if this recommendation is appropriate. Product labels almost never encourage users to tailor the serving size to their personal dimensions. Therefore, another explanation for why men take more protein than women is that the perceived benefits of protein supplementation as well as the bodybuilding culture appeal predominantly to men. The media and the male fitness industry have reinforced the belief that strength, big muscles, and a “cut” figure are

physical features that equate to career success, social dominance, and sexual power. Consequently, protein supplements are marketed as a fast and effective way to achieve these types of physical goals. For women, the ideals of physical perfection as dictated by the media and norms of societal behavior are very different. The benefits of protein supplementation are less attractive to women who generally aim for lean, fit bodies rather than ones overwrought with muscles and bulk. This finding that men use protein supplements more than women highlights important social issues such as body image and the gender gap and should signal to the Food and Drug Administration that perhaps more attention should be paid to such an influential societal force.

It is noteworthy to mention that the current research team uncovered a surprising finding that there was not a statistically significant p-value for the comparison between the number of hours that an individual was exercising per week and the amount of protein that he or she consumed. Based on previous research that stated that there was a strong correlation between supplement usage and exercise, Team Juiced believed that data would display a relationship between hours of exercise and protein supplement consumption (Ayranci, et al., 2005). Therefore the team had made an earlier assumption prior to distributing and administering the surveys to the various segments of its targeted population that the large sample size being tested would reveal a statistically significant p-value especially since varsity athletes comprised a segment of the team's sampled population. Varsity athletes were a major part of the study based on the previously established fact that supplement usage correlates with exercising more than three times per month (Perkin, et al., 2002). Another study also had shown that 88% of varsity athletes were using protein supplements (Froiland, et al., 2004). Team Juiced's research

did not produce such a high number; in fact only 33% of varsity athletes surveyed were found to be using supplements. This low percentage may have played a role in the lack of statistical significance. When comparing hours of exercise and amount of protein used, the p-value was greater than 0.05. Although the p-value is not statistically significant, it is important to take note that it still indicates the existence of a relationship between the two variables. Despite the fact that the team's earlier assumptions were unconfirmed in this particular study, it is important to recognize the potential relationship between protein use and hours spent exercising. If more research is aimed at discovering the link between these variables, the scientific community will have an easier time at isolating and studying individuals participating in these behaviors. The isolation of these groups becomes especially important when relating behavioral trends to physiological effects. By understanding this potential statistical relationship, scientists will be better able to target protein users should laboratory evidence reveal harmful or useful biological effects of supplementation. This data may also be important to advertisers because they will now have more information to adjust and improve their marketing strategies to their desired consumer population.

5.1.3 Possible Limitations-Survey

Given the scope and breadth of this survey as well as the sheer size of the potential target population and the rules and regulations for soliciting survey responses at the university, the experiment was limited by several factors. First, the survey was limited by the amount of available times for the group to distribute surveys both in regard to obtaining club sport and intramural team responses and obtaining responses at the campus gyms. Ideally, a team member would be at the gym at all hours of the day for

several weeks to target as many people as possible. However, the Campus Recreation Services at the university and the schedules of team members limited the survey distribution to twelve scheduled dates for a certain allotted period of time per date. This may have played a role in affecting the types of individuals who responded to the survey. For example, because surveying at the facilities was usually conducted in the afternoon, there was a chance that early morning users of the gym facilities were not able to take the survey. However, because the survey was designed to gain an understanding of frequency and amounts of protein supplementation by protein supplement users as opposed to prevalence of supplementation on campus, this is unlikely to have significantly affected the results.

Second, this study was also limited by the fact that the team could not actively solicit individuals to take the survey. In accordance with the policy of Campus Recreation Services in regard to maintaining a level of comfort for their facility users, only respondents who approached the team out of their own curiosity or interest were allowed to take the survey. In order to reduce the possibility that people would not take the survey, an incentive of a free water bottle for participants at the gym was offered. Third, the most active and athletic group of students, the varsity athletes, were not able to be surveyed to the extent that was desirable. Due to hesitation and general inapproachability by sports coaches and managers, survey responses from varsity athletes only consisted of approximately 10% of the total responses collected. Fourth, the length of the survey may have deterred certain individuals from taking the survey due to time constraints. The actual survey itself was two pages, but it appeared longer to the viewer due to the addition of the informed consent pages, the protein supplement product reference list, and

the follow-up study information sheet. Respondent preference for a shorter survey may have limited the number of responses. Fifth, the reference list of commonly used protein supplements included with the survey may not have been comprehensive. Some participants may have been unable to recall the name of the supplement that they used and could not find it on the list making it impossible for their responses to be entirely complete or accurate. Finally, as alluded to above, this study was limited by the time constraints of the participants. The people surveyed at the gym tended to be in a rush or had other obligations which again may have limited the number of responses collected.

In addition to the above limitations, there are certain characteristics about the interviewer or experimenter that may have affected the nature of the responses collected. The age range for the surveyors on this team was between 19-21 years old which may have deterred respondents from participating due to their perception of the experimenters as peers as opposed to an authoritative figure. Additionally, this was the first time conducting a large-scale survey for many of the surveyors. This may have affected the results in that research has shown that interviewers with a greater amount of experience tend to correlate with higher response rates in a survey questionnaire (Biemer & Lyberg, 2003). Lastly, a lack of interviewer success in obtaining approval to conduct the study especially with regard to gaining varsity athlete responses may have affected the response rate (Biemer & Lyberg, 2003). These characteristics of the surveyors are likely to have played a role in limiting the response rate from the varied members of the university's athletic population.

5.1.4 Possible Limitations-Data Analysis

The values for standard deviations in many of the comparisons of means performed could have limited the team's statistical findings. Standard deviation represents variation around the mean. The reason why the team may not have been able to uncover more statistical findings in the comparisons of means tested could be a direct consequence of the massive amount of variation in the responses provided by the respondents for these particular questions asked on the team's survey. Moreover, there may have been a lot of random error as a result of the large standard deviation values present for each comparison of means tested as well as the observations that the relationships were too small to detect with the available data. It is possible that had there been less variation around the mean, the team would have uncovered more statistically significant results.

5.2 Kidney Cell Modeling Discussion

5.2.1 Assumptions

There were two critical assumptions in the studies conducted using the mouse mesangial cell model. The first assumption was made when deciding what concentration of glutamine the control cells should be exposed to. Two possibilities were considered: either control cells would be exposed to physiological levels of glutamine to simulate normal *in vivo* conditions, or control cells would be exposed to no glutamine at all. The decision was made to not introduce any glutamine after the starvation step of cell culture for the control cells. This decision was largely made due to the knowledge of glutamine's importance to the normal growth of cells. Any amount of glutamine immediately after a period of 24 starvation in 5% FBS and no glutamine would stimulate some response. It was important that control cells did not exhibit any growth response or division. To draw comparisons to physiological levels of glutamine, the concentrations of 0.5 and 1.0 mM glutamine were added to the dose response study. This allowed a comparison to control cells with no glutamine as well as comparisons to physiological levels.

The second assumption was made in designing the chronic study. The aim of the study was to simulate conditions of chronic glutamine over-use. It would have been very impractical based on our time and laboratory constraints to expose the cells to high concentrations of glutamine over an extended period in the magnitude of years or even months. Drawing an analogy to many other fields of testing, such as tire wear, it was assumed that exposing the cells to the glutamine concentrations of interest for an unusually long time would provide reasonably accurate data for extrapolating to chronic conditions- over-use in the magnitude of years. If the analogy was extended, this

approach would equate to Bridgestone continuously running its tires for days until failure. It is practically unreasonable to think that drivers would drive continuously for such an extended period, but this method gives the manufacturer an idea of the life of their tire. Similarly it would be wrong to assume that serum levels of glutamine would stay elevated at 2.0 or 10.0 mM of glutamine for 48 hours consecutively, but this still gives an idea as to what the long-term effects of such transient changes over years may be.

5.2.2 Discussion of Results

The three experiments conducted, the time course study, the dose response study, and the chronic study, were designed to answer the following research questions, respectively:

- 1) What is the time dependent behavior of LDL-r expression in mesangial cells exposed to a supraphysiological level glutamine up to 24 hours of exposure?
- 2) What is the dose dependent behavior of LDL-r expression in mesangial cells exposed to increasing concentrations of glutamine up to 20.0 mM at the peak time of expression from the time course study?
- 3) What effect does glutamine have on a chronic scale with respect to the expression of various proteins that may contribute to glomerular sclerosis, hardening of the kidney tissue that ultimately results in renal failure?

Unpublished results from Dr. Sonia Doi's lab showed the intracellular accumulation of neutral lipids in response to glutamine conditions. Knowing of this accumulation, it was important to elucidate both the time- and dose-dependency of LDL-r expression in response to glutamine.

The time course was first of the three experiments conducted. Mesangial cells were exposed to a constant concentration of glutamine for the following time periods: 2,

4, 6, 8, 12, and 24 hours. Because each control and experimental set of cells required at least duplicate wells per time of exposure, it was necessary to divide the experiment into two sets containing three-time points each. The experimental groups that were exposed to 2.0 mM glutamine, more than double the physiological serum level, showed increased expression of LDL-r at all time points. At as early as 2 hours of exposure to 2.0 mM glutamine, the mesangial cells showed a significant increase in the expression of LDL-r indicating a quick rate of genetic up-regulation. The phenomenon may be accounted for by the fact that cells are normally exposed to a baseline amount of glutamine, between 0.5 and 0.9 mM of glutamine in physiological conditions. The control cells in this experiment were grown in the absence of glutamine. The fact that LDL-r expression was increased in 2 hours shows the importance of glutamine to the functioning of the cell.

The up-regulation of the receptor continued to increase through 8 hours of glutamine exposure. This suggested that intracellular demand for LDL remained high within this period of time. Considering that the cells were starved for 24 hours in conditions of low glucose (6 mM) and 5% FBS serum, it was not unreasonable to see that the cells required more lipids than they held at the end of starvation. While LDL-r expression remained elevated compared to the control cells throughout the entire time course of the experiment, its fold increase as compared to the control cells was less after 8 hours. The 12 and 24 hour time points each showed decreasing levels of expression. This suggested that after 8 hours of LDL-r mRNA being translated to receptor proteins, the cell might be signaled to decrease its manufacturing of LDL-r. Most likely, LDL itself would be responsible for this negative feedback. Because of the variance between duplicates, it was unclear whether this decrease after 8 hours was significant.

After determining that the highest response of LDL-r expression as compared to the control cells occurred between 6 and 8 hours, the dose experiment was conducted to determine the dose-dependent behavior for up-regulation of LDL-r expression in mesangial cells. Testing increasing concentrations of glutamine at 6 hours of exposure showed that glutamine levels as low as 0.5 mM produced a significant increase in LDL-r expression- over threefold greater than the baseline of no glutamine ($P < 0.05$). This threefold increase was maintained through 20.0 mM glutamine conditions. Normal serum levels of glutamine range between 0.5 and 0.9 mM. It was important to note that there was no significant increase in expression relative to this baseline at higher concentrations. Mesangial cells showed relatively the same level of LDL-r expression at 1.0, 2.0, 5.0, 7.0, 10.0, and 20.0 mM glutamine. This suggested that supraphysiological levels of glutamine did not affect the expression of LDL-r for up to 6 hours of exposure. The results of the dose response study did not provide insight to if this constant level of LDL-r expression would remain true for longer periods of exposure. At 6 hours of exposure, the limiting factor in LDL-r expression may be the saturation of cellular machinery rather than the lack of stimulus by environmental glutamine. The observed plateau effect may change at later time periods of exposure. For this reason, the chronic study followed to determine the prolonged effect of glutamine.

The chronic study tested the expression of LDL-r, HMG CoA reductase, three matrix proteins (collagen I and IV and fibronectin), and TGF-beta in increasing glutamine conditions for 48 hours of exposure. The purpose of this extensive exposure time was not to suggest that serum levels of glutamine could remain elevated for as long as two days, but rather to extrapolate the results to any chronic changes that may occur

after years of excessive glutamine use. An evaluation of the LDL-r expression at this time was intended to bring insight to the significance of the constant LDL-r expression with respect to physiological level after 6 hours of exposure.

Consistent with the 6-hour findings of the dose experiment, a glutamine level of 0.5 mM significantly increased the level of LDL-r expression ($P < 0.05$). Instead of seeing an increase of greater than threefold more than the control cells, an increase of 1.5-fold was observed for 0.5 mM of glutamine. The same 1.5-fold increase was seen for 2.0 mM of glutamine. At the highest concentration tested for 48 hours, 10.0 mM glutamine, the increase in LDL-r was nearly threefold. It is unclear whether this increase was significantly higher than what is observed at 0.5 and 2.0 mM glutamine without the use of statistical analysis. The nearly two-fold difference in expression between the 10.0 mM glutamine samples and both the 0.5 and 2.0 mM glutamine samples would indicate that this increase at 10.0 mM glutamine was significantly higher. The fact that 10.0 mM glutamine induced a higher up-regulation of LDL-r expression as compared to the 0.5 mM physiological level and 2.0 mM supraphysiological level was critically important. This finding suggested that if serum spikes in glutamine after dietary overload exceed a certain level, possibly 2.0 mM, more receptor proteins may be present after periods of chronic glutamine over-use. If more receptor is present for extended periods of time, LDL may begin to accumulate within the cell and cause changes in matrix production.

Finding that glutamine significantly increased the expression of collagen I after 48 hours of exposure to mouse mesangial cells supported the findings in literature concerning glutamine and collagen expression. Glutamine had previously been shown to increase collagen expression in cultured human fibroblasts as well as in mouse mesangial

cells (Bellon, Chaqour, Wegrowski, Monboisse, & Borel, 1995; Pithon-Curi, et al., 2006). The current research specifically identified the significant increase in expression of collagen I, while Pithon-Curi et. al. (2006) discussed the glutamine dependence of collagen IV expression.

Finding a significant increase in the expression of matrix proteins collagen I and fibronectin was more important because it could contribute to the sclerosis of renal tissue. Sclerosis of the glomerulus occurs when extracellular matrix proteins accumulate and occupy the space of the capillary beds, affecting filtration.

It would be valuable to investigate the height to which serum levels of glutamine can reach after dietary overloads. If the serum increases are above a threshold level, 10.0 mM glutamine for example, the chronic changes seen in the 48 hour experiment would be very important. Assuming the 48 hours of exposure time reasonably model a period of chronic glutamine over-use, serum spikes of greater than 10.0 mM glutamine would contribute to greater expression of collagen I and fibronectin. This could mean hardening of kidney tissue and decreased renal function. Fibronectin showed a significant increase in expression even at concentrations of glutamine as low as 2.0 mM ($P < 0.05$).

While statistical significance was not attained for the increase in expression of collagen IV, higher concentrations of glutamine show a trend towards increased collagen IV expression. Literature has shown that glutamine is required for the expression of collagen IV in mesangial cells (Pithon-Curi, et al., 2006). Naturally, it was thought that there may be a dose-dependent behavior for this matrix protein. Compared to the control cells containing no glutamine, cells exposed to 0.5 mM glutamine did not show any notable increase in collagen IV expression. While 2.0 mM glutamine reached just above a

1.5 fold increase in collagen IV expression as compared to the control cells, the variance in the duplicates did not provide statistical significance. The highest concentration of 10.0 mM glutamine also had issues of variability between duplicate wells, but showed an increase of approximately twofold compared to the control cells. A repeat of this experiment using triplicate wells would be beneficial to indicate the significance of collagen IV up-regulation in the 10 mM glutamine range.

The increased expression of matrix proteins, collagen IV and fibronectin, was particularly important because TGF-beta expression remained constant over the increasing glutamine range. The TGF-beta pathway is known to increase matrix protein excretion; thus, it could be argued that the effects of glutamine were non-specific to these matrix proteins if TGF-beta were also up-regulated. Because the expression of TGF-beta remained constant over increasing glutamine concentrations, it may mean that glutamine increased the expression of collagen I and fibronectin by some other, unknown pathway. It is premature to jump to this conclusion without further study because, like the dose response study, the chronic study showed only a snap-shot in time. The expression of TGF-beta may have been up-regulated at an earlier point, causing the non-specific increase in matrix proteins. Determining the exact pathway by which collagen I and fibronectin were up-regulated would be the focus of further research. Small interfering RNA (siRNA) used to knockout gene expression or protein inhibitors that specifically block signaling molecules could be used effectively to test various related biochemical pathways.

The expression of HMG CoA reductase was investigated to determine if intracellular lipid metabolism was occurring in conjunction with increased lipid uptake.

HMG CoA reductase is an enzyme that is critical in the synthesis of lipids and cholesterol, including LDL. While there was some trend towards increased expression of HMG CoA reductase with increasing glutamine concentrations, variance between duplicate wells did not allow statistical significance. If the increase in HMG CoA reductase expression was found to be significant, it would only contribute to the conclusion that overloads of glutamine could be deleterious via chronic changes.

5.2.3 Implications of Research

The culmination of the three experiments to determine the time- and dose-dependent expression of LDL-r in response to glutamine and the effect of prolonged glutamine exposure to matrix protein expression showed that LDL-r expression increased in a both a time- and dose-dependent manner. This was an important and novel finding-gaining recognition by the American Society of Nephrology for abstract publication and poster presentation at their 2008 annual meeting (Sharma, et al., 2008). Showing that glutamine significantly increases the expression of LDL-r in mouse mesangial cells elucidates a pathway by which LDL may accumulate within the cell. This is supported by unpublished findings of neutral lipid accumulation within mesangial cells in response to glutamine exposure from Dr. Doi's laboratory. Using oil-red-O staining methods and microscopy imaging, it would be important to clearly show an increase in intracellular LDL in the presence of high levels of glutamine. Using fluorescent tags to illuminate the actual LDL-r molecules would also be a valuable addition to the research.

Doi et al. (2000) had previously shown that glutamine increases the glucose-induced proliferation of mesangial cells. This proliferation, via glutamine:fructose 6-phosphate aminotransferase and cyclic-adenosine monophosphate dependent pathways,

suggested that glutamine could pose a risk for kidney disease in individuals with diabetes (Doi, et al., 2000). The increased expression of LDL-r in response to glutamine found by the current research was significant in the absence of additional glucose, extending its importance to non-diabetics. In addition, the results of the chronic study gave insight to the possible long-term implications of higher levels of LDL-r.

With the increasing incidence of chronic kidney disease in the United States, the rates of atherosclerotic cardiovascular disease among these individuals is notably higher than in the general population. This can be largely attributed to the abnormalities in lipid metabolism. The oxidation of LDL is especially important, a process that is usually prevented to a certain extent by high density lipoprotein, or HDL cholesterol. The oxidized LDL loses its affinity for LDL-receptor and instead of being taken in by cells it is “ingested” by macrophages, stimulating them to become foam cells. Foam cells play a role in cardiovascular disease, and similar phenomenon with foam cells is seen in the glomeruli. The activity of foam cells play a role in the progression of chronic kidney disease and glomerular sclerosis (Vaziri, 2008). The complexity of LDL metabolism and its possible avenues to contribute to kidney disease show that in order to fully understand the up-regulation of matrix proteins, more than LDL-receptor may need to be studied. Specifically, an *in vivo* model may be necessary.

The expression of matrix proteins collagen I and fibronectin was significantly increased in a dose-dependent manner. These findings add to the already known glutamine dependency of matrix protein collagen IV (Pithon-Curi, et al., 2006). The current research could not show statistical significance for the observed increase in collagen IV dependency, but that could well be contributed to an unusual variation

between triplicate samples. A repeat of the chronic study experiment could show significance for collagen IV, as well. The observation that TGF-beta did not increase over increasing glutamine concentrations adds to the value of the matrix protein findings. Because the TGF-beta pathway can contribute to non-specific increases in the expression of matrix proteins, it was valuable to negate the increased expression of TGF-beta for the 48 hour time point. It would be unreasonable to jump to the conclusion that because TGF-beta was unchanged for this snap-shot in time that it was unrelated to the increase in expression of matrix proteins. Still it is suggested from the data that a glutamine-dependent pathway involving the accumulation of LDL within the cell may be involved in the increased expression of collagen I and fibronectin. Knowing the exact pathway would be critically important to preventing cells from excreting these pro-fibrotic proteins, contributing to glomerular sclerosis and renal pathology, especially with the contributions of mesangial cell proliferation. Glutamine was shown to increase the proliferation of mesangial cells in culture, thereby increasing the number of cells that can excrete extra-cellular matrix protein in response to glutamine (Lagranha, Doi, Pithon-Curi, Curi, & Sellitti, 2008). Further studies with siRNA or inhibiting proteins could elucidate a more specific pathway for the observed up-regulation of collagen I and fibronectin.

The risk from excessive glutamine of induced mesangial cell proliferation and lipid accumulation, possibly leading to excretion of pro-fibrotic matrix proteins, becomes especially important for the college-aged community taking protein supplements. The risk is only amplified by considering results from the survey branch of research showing that participants taking protein were ingesting double the recommended daily allowance,

and that participants with predisposing risk factors for kidney disease were just as likely to report protein supplementation as those without risk factors. The novelty of this social finding among college-aged athletes combined with the previously unknown increase in LDL-r expression in response to glutamine levels prove to be of great relevance to both the athletic community and the scientific community. There is both a need for awareness for indiscriminate supplementation glutamine, as well as a need for researchers to renew investigation in the possible deleterious effects of glutamine in regards to renal health.

5.2.4 Possible Limitations

It is important to note that significant findings from cell culture methods are usually followed up by in vivo animal studies. To make the conclusion that glutamine poses a definite risk for glomerulosclerosis or at least deleterious effects to lipid balance in mesangial cells would not be appropriate without validating studies in which animals, such as mice or rats, are given glutamine and their mesangial cells are found to react in the same way as seen by the current research. The distinction lies in the path by which glutamine would present itself to the mesangial cells in the kidney. In practical applications, ingested glutamine must be absorbed by the gut and maintained in the blood stream. Among the complexities of the physiological responses, the uptake of glutamine by muscle cells may be the most significant. Future research testing for LDL-r expression in mesangial cells as well as the expression of matrix proteins in response to various conditions of glutamine ingestion would be critically important to further substantiating the current findings. Human subject testing, depending on the magnitude of the findings and ethics of the issue could follow animal models.

5.3 Biochemical Analysis Discussion

5.3.1 Assumptions

Given the small scale of the reactions used in the method developed in this study, a couple of assumptions were made. One assumption was that all protein supplements were water-soluble and would dissolve in aqueous buffer, even though the solutions often appeared cloudy when protein supplements were mixed with HEPES, because the labels for all protein supplements directed the user to mix the supplements in water. In addition, the supplements were mixed very well in the sonicator in the lab, so it is reasonable to assume these supplements were sufficiently dissolved. The second assumption was that vacuum drying the derivatized samples overnight sufficiently dried the samples before analysis. This assumption was necessary because the quantity of sample in each vial was so small that it was difficult to see whether or not a sample was completely dry. Finally, lipids can interfere with solubility in ethanol, but it was assumed in this study that the high lipid content of some of the samples would not interfere with solubility.

5.3.2 Conversion of Glutamine to Glutamic Acid

Research by Tsao and Otter (1999) suggested that glutamine converts to glutamic acid during the enzymatic degradation and PITC derivatization procedure, so both glutamine and glutamic acid must be quantified in order to correctly quantify the glutamine content. However, there was no peak correlating to glutamic acid (retention time of approximately 17 minutes) on any chromatograms for pure glutamine samples. This demonstrates that glutamine was never converted to glutamic acid in these samples. Therefore, a correction for the conversion of glutamine to glutamic acid was not necessary in quantifying the glutamine content in the protein samples tested.

5.3.3 Glutamine Quantification in Pure Glutamine Samples

The method designed in this study was proven to be reproducible and effective for quantifying glutamine in pure glutamine samples. The method was refined using an L-glutamine standard until 100% recovery was consistently determined. Then, some of the pure glutamine products were preliminarily tested and found to have recoveries near 100%. The glutamine recovery was 98% or higher for all glutamine supplements tested: Supplement 1, Supplement 8, Supplement 12, Supplement 19, and Supplement 21. This shows that these supplements, which claim to be pure glutamine, have glutamine contents consistent with those claimed on their labels. This means a person taking these glutamine products is actually ingesting the amount of glutamine they believe to be ingesting. The supplements were not tested for specific additives, so it is possible that they also contain trace amounts of other chemicals.

5.3.4 Glutamine Quantification in Protein Samples

The results gathered from the present study cannot be used to confidently determine whether the amount of glutamine in commonly used protein supplements is in accordance with that claimed on the label because the method was not consistently reproducible when quantifying glutamine in protein samples. Previous studies using methods similar to the method designed accurately quantified glutamine in milk proteins (Tsao & Otter, 1999) and commercial liquid nutritional products (Baxter, et al., 2004). This suggests that these methodologies may need further revision to accurately quantify glutamine in protein supplements. Very few other studies have been conducted that specifically quantify glutamine, so it is difficult to compare the results obtained in this study to results from other studies.

In the current study, chromatograms for six protein supplements had standard deviations of the glutamine peak area greater than 6% of the total glutamine peak area: Supplement 3, Supplement 9, Supplement 11, Supplement 13, Supplement 14, and Supplement 15. This high standard deviation is most likely due to differences in enzymatic degradation since a high standard deviation was only seen with the protein supplements and not with the pure glutamine supplements, which do not require enzymatic degradation.

Using the HPLC method developed in this study, seven of the nine protein supplements that reported glutamine contents were found to contain less glutamine than the amount reported. These supplements included Supplement 2, Supplement 4, Supplement 11, Supplement 13, Supplement 16, Supplement 17, and Supplement 18.

One possibility to account for the decreased amount of glutamine detected using the HPLC analysis method compared to the amount reported on the labeling is that the protein supplement labels were inaccurate or unclear. The protein supplements may have ingredients not listed on the label or ingredients that are claimed but have unspecified amounts, such that the amount of glutamine in one serving is less than the amount of glutamine claimed to be in a serving. It is important to note that the method used in this study was not fully validated, so inaccuracy of labels is only one possibility for any discrepancies; this study did not prove in any way that the labels are inaccurate.

One way in which the labels were unclear is some of the protein supplement labels had very specific amounts of glutamine listed whereas other labels were more vague. For example, Supplement 11 listed its glutamine content as 3358 mg, while Supplement 18 only lists its glutamine content as over 4 g of glutamine precursors and

peptides. It is unclear to what exactly the phrase “glutamine precursors or peptides” refers. These precursors and peptides may not solely refer to glutamine content and therefore the actual amount of glutamine may be less than 4 g. This discrepancy in the wording of the labels would result in a difference between the amount of glutamine determined and that claimed on the label. A related issue is that some of the labels gave glutamine contents but listed glutamine and whey protein as separate ingredients. This could be misleading for consumers who do not realize that glutamine is included in whey protein, so the amount of glutamine in these products is actually more than the amount claimed on the label.

A second and perhaps more likely possibility for the low recoveries is that the protein samples were not completely degraded by the enzymes, and therefore, a different combination of enzymes may be needed for the degradation to be more effective. The literature is lacking with respect to enzymatic degradation of protein supplements. Although these enzymes, pronase E, leucine aminopeptidase, and prolidase, have been used previously for enzymatic degradation in quantifying glutamine by Baxter et al. (2004), these researchers were quantifying glutamine in commercial liquid nutritional products rather than protein supplements. Several of the protein supplements were tested in the current study both with and without the enzymatic degradation step in the method, and the use of enzymes resulted in an increase in glutamine recovery. However, glutamine recovery still was not increased to levels consistent with the labels.

A final possibility for the low recoveries is that the samples with high lipid contents did not completely dissolve in the ethanol. Hexane is often used to remove lipids

before quantification using HPLC (Yi, et al., 2009). Better data may have been obtained if any lipids were removed with hexane prior to quantification.

Although the accuracy of the current method may be held in question, two of the protein supplements were found to contain higher amounts of glutamine by the HPLC analysis than the amounts reported on the labels. Two protein supplements, Supplement 5 and Supplement 6, had glutamine recoveries higher than those claimed on their labels. The amounts of glutamine in the samples were about 1.5 times and 2.7 times those claimed on the labels, respectively.

These two protein samples had low standard deviations of less than 2% of the total areas for glutamine peaks between duplicate trials. Supplement 5 and Supplement 6 were tested along with Supplement 4 and Supplement 20 a second time in order to test the accuracy of the results since the amount of glutamine detected was higher than that claimed on the label. The new percentages of glutamine detected were all within 15% of the first set of trials. The detected glutamine contents for Supplement 5 and Supplement 6 were once again higher than the amounts claimed on the label. This second test was conducted over one month after the first set of trials and used the original calibration curve due to time constraints. Considering a calibration that was over a month old was used, this is reasonable variability, and it suggests that these two supplements do in fact have higher glutamine contents than is claimed on their labels. The data from the first set of trials was discussed above because the calibration used for these trials was more current than the calibration used for the second set of trials.

These results are especially surprising considering that the amounts of glutamine detected in the seven other supplements were much lower than the amounts claimed by

the manufacturers. When a higher amount of glutamine was determined than was claimed, it was most likely not due to a problem with the enzymatic degradation method. The enzymes alone were tested in ethanol to determine if there was glutamine in the enzymes that would add to the total glutamine content determined in the protein supplements. The enzymes did not add to the total glutamine content. Therefore, the only potential problem with the enzymatic degradation method is that the enzymes do not completely degrade the protein samples. However, this would lead to a decrease in the amount of glutamine detected by the HPLC method rather than an increase in the amount detected. The statistical significance of the results cannot be reported because there was not sufficient time to thoroughly validate the method. However, unlike the other supplements tested, the determined amount of glutamine in Supplement 5 and Supplement 6 were higher than the glutamine claims on the labels of these protein supplements. Therefore, it suggests that the glutamine contents reported by the manufacturers were in fact lower than the actual glutamine content for these two protein supplements.

It was hoped that the exact glutamine content of the protein supplements with unlisted glutamine contents could be reported. However, the protein supplements in this category could not be analyzed for their exact glutamine content because the accuracy of the method was not fully validated.

5.3.5 Implications of Research

The use of HPLC with PITC precolumn derivatization described in this thesis was proven to be an effective and reproducible method for quantifying glutamine in pure glutamine supplements. The pure glutamine products had as much glutamine as was

claimed on their labels. The manufacturers were accurate in their claims of glutamine content for these products. The reproducibility and accuracy of the current method for quantifying glutamine in protein supplements is unclear based on the data. For the majority of the protein supplements tested, the amount of glutamine determined by the HPLC analysis is much less than the amount of glutamine claimed by the manufacturers. This could be due to an enzymatic degradation methodology problem, solubility problem, or false manufacturing claims. However, it was shown that glutamine recovery increases when enzymes are used compared to when enzymes are not used. More method development would be beneficial in determining if the specific enzymes used are the best possible for protein supplement degradation. It also might be more effective to use other enzymes in addition to the ones used in this methodology.

The finding that two protein supplements had glutamine contents higher than those claimed on their labels is an important finding in this study. This is important information particularly for consumers of these supplements (Supplement 5 and Supplement 6) due to the possible risks associated with consuming more glutamine than anticipated. After validation of methods and further testing for confirmation, consumers may need to be informed of the dangers of excess glutamine use in combination with the knowledge that any specific products contain more glutamine than is reported on the labeling.

The results of the present study suggest that the biochemical analysis research pursuit in this study was justified as a valuable area of research and more research should be conducted in this area to verify the efficacy of the protein degradation method. This is important for ensuring that safe and accurately reported amounts of glutamine are present

in protein supplements. While the data presented here do not prove that any of the products tested had false manufacturing claims, they suggest that research with a fully validated method may show that certain protein supplements have more glutamine than claimed. Further method validation is also important to determine exactly how much glutamine is in protein supplements with no glutamine content claims.

The FDA is not able to regulate dietary supplements as stringently as products in the category of drugs, leaving consumers at risk until there is reason for the FDA to further investigate a particular supplement. Based on the results showing adverse effects of glutamine on mouse mesangial cells, product label glutamine claims that are lower than the actual glutamine content of the supplement are potentially dangerous to consumers. If consumers believe they are using safe amounts of glutamine based on false product claims, they could potentially be putting themselves at risk for detrimental effects to their kidneys.

5.3.6 Possible Limitations

There were a few important limiting factors in the glutamine quantification results obtained. Two related limitations are the use of enzymes for protein degradation and the instability of glutamine. Acid hydrolysis is the more commonly used method for protein degradation in HPLC analysis, but acid hydrolysis could not be used in this study because glutamine is unstable. A lack of literature on protein degradation in HPLC analysis exists, so the enzymes used by Baxter et al. (2004) were the enzymes used in this study even though they may not be the most effective enzyme combination. The instability of glutamine may have also led to the high standard deviations between duplicate trials for some of the results obtained.

Another major limitation was the time frame for this study. Extensive method development was required because there are few previous studies of glutamine quantification in the literature. This method development was a significant part of the results of the study. However, the amount of time spent on method development and the limited amount of time for research before the presentation of this thesis detracted from the quantification results obtained.

A final limitation was the expense of the chemicals used, particularly the enzymes. The enzymes were very expensive and their purchase was graciously funded by the FDA. However, a limited quantity of enzymes could be purchased due to the expense, so the quantity of enzymes was often a limiting factor in analyzing protein samples. In addition, according to Baxter et al. (2004), the enzymes work best when the enzyme solutions are prepared fresh. It was not always possible to use fresh enzymes in this study because the enzymes are very expensive, and it would not have been economically advantageous to discard old enzyme solutions that had not been used.

5.4 Urinalysis Discussion

5.4.1 Assumptions and Limitations

Given the nature of the study, some assumptions for the urinalysis follow-up study were made in order to generalize the results to a larger population. First, it was assumed that each particular spot urine sample given by voluntary student members was an accurate portrayal of the normal level of protein excretion in that particular person. Any random fluctuation in the person's levels of protein excretion at that time due to outside factors was not taken into consideration as to not skew the data. Secondly, it was assumed that the thermal conditions for the urine samples collected at the University Health Center were maintained at proper temperature and pH, so that the levels of protein excretion in the results would retain their accuracy during transport to the analysis facility and storage.

Similar to the survey component of the project, the follow-up urinalysis study was limited by a few uncontrollable factors. First, the study was limited by the last time of consumption of protein supplements by the participants relative to the time of day the sample was collected. If protein supplements were used that day or even a few days prior, or a meal was eaten with a significantly high amount of protein, the levels of protein represented in the urine may be significantly higher in that sample. These levels of protein excretion may not necessarily be reflective of normal protein supplement usage or of protein supplementation at all, which was why the first assumption was necessary. Second, the last time of exercise would inevitably affect the protein concentration in the urine as well. If the person had exercised recently, the protein excretion will be higher than if they had not exercised in a short while. Third, when the urine was actually tested

for protein excretion and analyzed, there would be limitations surrounding the sensitivity and specificity of the protein to creatinine test and kit assay. Each test varies in terms of the sensitivity and the specificity of the test and the kit assay and therefore, our results will reflect these variables.

Chapter 6: Conclusion

The three branches of study conducted by Team Juiced have important findings that, when integrated, highlight the potential for harm under the current state for consumers of protein supplements. The findings from kidney cell modeling conducted at USU show significant changes in cellular lipid uptake and pro-fibrotic behavior under chronic conditions of glutamine over-use. The survey results show that many college students are receiving far more than the recommended daily amount of protein and therefore are also likely to be receiving excess glutamine in their diets. Finally, the results from the biochemical analysis conducted at the CFSAN suggest that some supplements may have more glutamine than is claimed on their labels. Consumers could be unknowingly ingesting more glutamine than they realize.

The first significant finding from kidney cell modeling is that the expression of LDL-r in mouse mesangial cells more than doubled in response to supraphysiological glutamine in a time-dependent manner, increasing up to eight hours of exposure. This was important because many other genes take much longer to react to environmental conditions. LDL-r can significantly change its expression in mesangial cells in as little as two hours. This makes future *in vivo* studies that address the duration of elevated serum glutamine levels after ingestion critically important.

Second, kidney cell modeling showed that the expression of LDL-r in mouse mesangial cells did not respond to an increasing dose of glutamine at six hours of exposure. An increase in glutamine to physiological levels caused a threefold increase in LDL-r expression, but expression does not continue to increase with increasing glutamine concentrations. The level of glutamine only stimulated LDL-r up-regulation to a certain

threshold for the six hours time point. This threshold could either be a point of physiological saturation when the cells were producing as much LDL-r as possible or a state of homeostasis such that by negative feedback, the level of LDL-r up-regulation did not increase. The combination of elucidating the time- and dose-dependent behavior of LDL-r expression in response to glutamine was very important to the nephrology community. The American Society of Nephrology published an abstract of this data and the research was presented at the society's 2008 national conference in Philadelphia, PA.

Third, at 48 hours of glutamine exposure at increasing concentrations, LDL-r did respond in a dose dependent manner. The expression of LDL-r for 10.0 mM glutamine was approximately double the observed expression at 0.5 and 2.0 mM glutamine. This suggested that one of the most significant variables in chronic glutamine over-use would be the level of glutamine exposure. It then becomes imperative to know how high the concentration of serum glutamine may reach *in vivo* after ingestion of glutamine.

Finally, collagen I and fibronectin, two matrix proteins considered to be pro-fibrotic and markers of glomerular sclerosis, were both significantly increased with 10.0 mM glutamine after 48 hours of exposure. Important follow-up research would attempt to elucidate the exact pathway by which collagen I and fibronectin were up-regulated. By knowing the exact biochemical pathway, it would be possible to develop new therapeutic strategies to prevent the progression of chronic kidney disease.

The survey results showed that the dangers of chronic glutamine use suggested by the *in vitro* studies on mouse mesangial cells are relevant to college students because many at the University of Maryland, College Park greatly exceed the recommended amount of protein in their diets through supplemental protein use. Survey research found

that over 40% of those surveyed were using protein supplements, and thirteen people were using L-glutamine. This proved that the targeted group was an effective sample for extracting information about the use of protein supplements.

The average protein user was taking twice the total recommended weekly amount of protein. It was suspected that people might have been taking so much protein because they were unaware of the effect that excess protein can have on the body. Male protein supplement users took more protein per week than women, possibly aspiring to a muscular body image portrayed in media. This could suggest more men are at risk for the negative effects of chronic glutamine over-use. It was surprising that there was no significant relationship between the amount of protein taken and the number of hours of exercise per week, as supported by literature.

The most significant finding of the survey research was that participants with risk factors for kidney disease were no less likely to take protein supplements than those with no risk factors. As discussed earlier, high levels of protein intake have been shown to be deleterious to kidney disease patients. Given the findings of the project that showed the ill effects of chronic glutamine over-use on kidney function, a large population of protein supplement users already at risk for kidney disease could have been putting their health at further risk by ingesting an excess of protein.

The survey research could have been improved by surveying more varsity athletes. Varsity athletes likely use more protein supplements than less competitive athletes or gym users. It would be very useful to study the pattern of protein supplement use by more varsity athletes in the future because these athletes may be at even higher risk for the negative effects of excess protein intake on the kidneys. Animal studies

following up with results from the current kidney cell modeling could also warrant further analysis of individuals at risk for kidney disease through survey research.

The final part of the team's research attempted to determine the accuracy of protein supplement label claims of glutamine content. All of the protein supplements tested and many of the pure glutamine products tested were listed as products taken by survey participants. A method for glutamine quantification was developed that was reproducible and effective for quantifying glutamine in pure glutamine supplements. All supplements listed as pure glutamine were found to contain at least 98% glutamine, leaving no reason to believe that the product label claims for glutamine content were inaccurate.

The reproducibility and the accuracy of the method for quantifying glutamine in protein supplements are unclear. The statistical significance of the determined glutamine content of protein supplements could not be determined. However, seven of the nine protein supplements with glutamine content claims had less glutamine than claimed based on the developed method. Two protein supplements, Supplement 6 and Supplement 5, were found to have glutamine contents much higher than the amount claimed. Determined glutamine contents higher than those claimed were unlikely due to a fault in the method. Because the general trend of the method was to undershoot the claimed glutamine concentration, seven out of nine times, it was even more important when two supplements showed higher glutamine concentrations than reported. This was significant because educated consumers, those who are aware of the data showing the possible adverse effects of excess glutamine on the kidneys yet want to use protein supplements for their positive effects, may wish choose a supplement with a lower listed

amount of glutamine. It is important that these consumers choose a supplement that actually has the amount of glutamine claimed. While statistical significance cannot be reported for protein supplements with higher glutamine concentrations because the method was not fully validated, it was still very important that the preliminary data suggest manufacturer's claims may not be accurate. This was the case with the weight loss supplement StarCaps, as recently brought to attention by *The New York Times*. Consumers should be well aware of the inherent risk involved with using supplements readily available in the market.

Overall, the research of Team Juiced showed that 1) glutamine changed the normal functioning of mesangial cells in a manner that could cause serious damage to the kidneys in conditions of chronic glutamine over-use, 2) the average protein supplement user consumed twice the recommended amount of protein per week, and the people at risk for kidney disease were no less likely to take excess protein than are those not at risk, and 3) all over-the-counter glutamine supplements tested had the amount of glutamine claimed on their labels while two protein supplements taken by survey participants may have contained more glutamine than was claimed by the labels. Together, these findings argue that college-aged student athletes who are predisposed to kidney disease by factors such as diabetes, hypertension, clinical obesity, or a family history of kidney disease are especially at risk under the current system of supplement regulation.

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Appendix

A.1 Survey Research Appendix

A.1.1 Distributed Survey

University of Maryland Protein Supplementation Survey

1) How did we find you?

(Circle one) Varsity athletic team Club/Intramural sport team Gym

2) Have you ever used protein supplements?

(Circle One) Yes No **If you answer “No” skip to Question 4.**

3) Provide the details of the product(s) and how you used them below. If you cannot remember the exact name of the product, refer to the LAST PAGE for a list of commonly used supplements.

Name of product

(fill in name)

Time of use (Circle One) I use it NOW I used to use it BEFORE

How

often?

Times per day

Times per week

(fill in number)

(Circle one)

How

much? Grams

Ounces

Scoops

Pills

(fill in number)

(Circle one)

For how

long? Weeks

Months

Years

(fill in number)

(Circle one)

Name of product

(fill in name)

Time of use

(Circle One)

I use it NOW

I used to use it BEFORE

How

often?

Times per day

Times per week

(fill in number)

(Circle one)

How

much? Grams

Ounces

Scoops

Pills

(fill in number)

(Circle one)

For how

long? Weeks

Months

Years

(fill in number)

(Circle one)

Name of product

(fill in name)

Time of use

(Circle One)

I use it NOW

I used to use it BEFORE

How

often?

Times per day

Times per week

(fill in number)

(Circle one)

How

much? Grams

Ounces

Scoops

Pills

(fill in number)

(Circle one)

For how

long? Weeks

Months

Years

(fill in number)

(Circle one)

4) What other supplements and/or prescription medication do you take or were taking at the time of protein use? (List below)

5) Do you have any of these health conditions? (Circle all that apply)

Diabetes
of these

Kidney Disease

Clinical Obesity

Hypertension

None

6) Do any of your blood relatives in your immediate family (siblings, parents, blood aunts/uncles, grandparents) have any of the following? (Circle all that apply)

Diabetes Kidney Disease Clinical Obesity Hypertension None
of these

7) Participant Info:

Sex (Circle one) Male Female

Age (Circle one) 18 19 20 21 22 23 24 25

Hours of physical training per week (Circle one) 1-5 6-10 >10

Please be sure all sections of the survey are complete. Thank you for your time and cooperation. Feel free to add any additional comments below:

A.1.2 Comparison Test

A.1.2.1 How did we find you? vs. Amount of protein used for product #s 1, 2, & 3

The first comparison of means assessed was entitled *How did we find you?* vs. Amount of protein used for product #s 1, 2, & 3. *How did we find you?* was the first question that the respondents answered in the team's survey. In this comparison of means, *How did we find you?* served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories that were not ordered by rank. The amount of protein taken for product #s 1, 2,

& 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that for varsity athletic teams, the number of varsity athletes using product #1 was 27 versus eight varsity athletes using product #2 versus three varsity athletes using product #3. The mean for varsity athletes using product #1 was 53.691 compared to a mean of 58.7888 for varsity athletes using product #2 compared to a mean of 116.6667 for varsity athletes using product #3. The standard deviation for varsity athletes using product #1 was 32.7443 versus a standard deviation of 68.19345 for varsity athletes using product #2 versus a standard deviation of 167.43158 for varsity athletes using product #3.

The number of club/intramural sport team athletes using product #1 was 113 versus ten club/intramural sport team athletes using product #2 versus two club/intramural sport team athletes using product #3. The mean for club/intramural sport team athletes using product #1 was 43.805 compared to a mean of 51.5500 for club/intramural sport team athletes using product #2 compared to a mean of 28.0000 for club/intramural sport team athletes using product #3. The standard deviation for club/intramural sport team athletes using product #1 was 30.9330 versus a standard deviation of 43.49422 for club/intramural sport team athletes using product #2 versus a standard deviation of 5.65685 for club/intramural sport team athletes using product #3.

The number of gym-goers using product #1 was 148 versus 23 gym-goers using product #2 versus five gym-goers using product #3. The mean for gym-goers using product #1 was 53.474 compared to a mean of 62.5691 for gym-goers using product #2 compared to a mean of 38.8000 for gym-goers using product #3. The standard deviation

for gym-goers using product #1 was 60.4108 versus a standard deviation of 81.16831 for gym-goers using product #2 versus a standard deviation of 22.40982 for gym-goers using product #3.

The number of ROTC members using product #1 was 3. No ROTC participant used a second or third product. The mean for ROTC members using product #1 was 30.000. The standard deviation for ROTC members using product #1 was 1.7321.

Combining the four groups surveyed, the total number of individuals using product #1 was 291 versus 41 individuals using product #2 versus ten individuals using product #3. The mean for individuals using product #1 was 49.498 compared to a mean of 59.1439 for individuals using product #2 compared to a mean of 60.0000 for individuals using product #3. The standard deviation for individuals using product #1 was 48.3928 versus a standard deviation of 69.88708 for individuals using product #2 versus a standard deviation of 89.46508 for individuals using product #3.

Figure 20: Sample Size Numbers, Means, and Standard Deviations for How did we find you? vs. Amount of protein used for product #s 1, 2, & 3

Report

How did we find you?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Varsity athletic team	Mean	53.691	58.7888	116.6667
	N	27	8	3
	Std. Deviation	32.7443	68.19345	167.43158
Club / Intramural sport team	Mean	43.805	51.5500	28.0000
	N	113	10	2
	Std. Deviation	30.9330	43.49422	5.65685
Gym	Mean	53.474	62.5691	38.8000
	N	148	23	5
	Std. Deviation	60.4108	81.16831	22.40982
ROTC	Mean	30.000		
	N	3		
	Std. Deviation	1.7321		
Total	Mean	49.498	59.1439	60.0000
	N	291	41	10
	Std. Deviation	48.3928	69.88708	89.46508

Sample numbers, means, and standard deviations for varsity athletes who used product #s 1, 2, & 3; sample numbers, means, and standard deviations for club/intramural sport team athletes who used product #s 1, 2, & 3; sample numbers, means, and standard deviations for gym-goers who used product #s 1, 2, & 3; sample numbers, means, and standard deviations for ROTC members who used product #s 1, 2, & 3; and sample numbers, means, and standard deviations for total number of respondents who used product #s 1, 2, & 3.

Since the independent variable in this comparison is nominal and has three or more categories (four categories to be exact) and the dependent variable is interval-ratio, the appropriate test to use was the One-Way ANOVA test. For individuals using product #1, the two-tailed significance p-value was 0.356 which was greater than 0.05 and therefore statistically insignificant indicating insufficient evidence to support a relationship between the independent and dependent variables. Similarly, for individuals using product #2, the two-tailed significance p-value was 0.921 which was greater than 0.05 and therefore statistically insignificant also demonstrating insufficient evidence to

support a relationship between the independent and dependent variables. Lastly, for individuals using product #3, the two-tailed significance p-value was 0.471 which was greater than 0.05 and therefore statistically insignificant as well portraying insufficient evidence to support a relationship between the independent and dependent variables.

Figure 21: One-Way ANOVA Test for How did we find you? vs. Amount of protein used for product #s 1, 2, & 3

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
How much Product #1 used (grams)	Between Groups	7617.098	3	2539.033	1.085	.356
	Within Groups	671521.9	287	2339.797		
	Total	679139.0	290			
How much Product #2 used (grams)	Between Groups	847.511	2	423.755	.083	.921
	Within Groups	194520.6	38	5118.964		
	Total	195368.2	40			
How much Product #3 used (grams)	Between Groups	13928.533	2	6964.267	.839	.471
	Within Groups	58107.467	7	8301.067		
	Total	72036.000	9			

A One-Way ANOVA test was used to determine whether a statistically significant relationship exists between the comparison of means entitled *How did we find you?* vs. Amount of protein used for product #s 1, 2, & 3.

A.1.2.2 Hours of exercise per week vs. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of individuals who were using product #1 and exercising 1-5 hours per week was 62 compared to five individuals who were using product #2 and exercising 1-5 hours per week compared to three individuals who were using product #3 and exercising 1-5 hours per week. The mean for individuals who were using product #1 and exercising 1-5 hours per week was 49.089 versus a mean of 30.8577 for individuals who were using product #2 and exercising 1-5 hours per week versus a mean of 49.3333 for individuals who were using product #3 and exercising 1-5

hours per week. The standard deviation for individuals who were using product #1 and exercising 1-5 hours per week was 54.7520 compared to a standard deviation value of 31.89973 for individuals who were using product #2 and exercising 1-5 hours per week compared to a standard deviation value of 16.25833 for individuals who were using product #3 and exercising 1-5 hours per week.

The number of individuals who were using product #1 and exercising 6-10 hours per week was 136 compared to sixteen individuals who were using product #2 and exercising 6-10 hours per week. There were no individuals who were using a third product and exercising 6-10 hours per week. The mean for individuals who were using product #1 and exercising 6-10 hours per week was 46.573 versus a mean of 67.2375 for individuals who were using product #2 and exercising 6-10 hours per week. The standard deviation for individuals who were using product #1 and exercising 6-10 hours per week was 37.1459 compared to a standard deviation value of 92.83747 for individuals who were using product #2 and exercising 6-10 hours per week.

The number of individuals who were using product #1 and exercising greater than ten hours per week was 95 compared to 21 individuals who were using product #2 and exercising greater than ten hours per week compared to seven individuals who were using product #3 and exercising greater than ten hours per week. The mean for individuals who were using product #1 and exercising greater than ten hours per week was 53.930 versus a mean of 64.2767 for individuals who were using product #2 and exercising greater than ten hours per week versus a mean of 64.5714 for individuals who were using product #3 and exercising greater than ten hours per week. The standard deviation for individuals who were using product #1 and exercising greater than ten hours per week was 57.1930

compared to a standard deviation value of 57.27230 for individuals who were using product #2 and exercising greater than ten hours per week compared to a standard deviation value of 108.79623 for individuals who were using product #3 and exercising greater than ten hours per week.

Combining all three categories of hours, the total number of individuals who were exercising anywhere from one hour to greater than ten hours and using product #1 was 293 compared to a total number 42 individuals using product #2 compared to a total of ten individuals using product #3. The mean for the total number of individuals exercising and using product #1 was 49.491 versus a mean of 61.4261 for the individuals exercising and using product #2 versus a mean of 60.0000 for the individuals exercising and using product #3. The standard deviation for the total number of individuals exercising and using product #1 was 48.2451 compared to a standard deviation value of 70.59638 for the individuals exercising and using product #2 compared to a standard deviation value of 89.46508 for the individuals exercising and using product #3.

Figure 22: Sample Size Numbers, Means, and Standard Deviations for Hours of exercise per week vs. Amount of protein used for product #s 1, 2, & 3

Report

Hours of Exercise		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
1 => 5	Mean	49.089	30.8577	49.3333
	N	62	5	3
	Std. Deviation	54.7520	31.89973	16.25833
6 => 10	Mean	46.573	67.2375	
	N	136	16	
	Std. Deviation	37.1459	92.83747	
> 10	Mean	53.930	64.2767	64.5714
	N	95	21	7
	Std. Deviation	57.1930	57.27230	108.79623
Total	Mean	49.491	61.4261	60.0000
	N	293	42	10
	Std. Deviation	48.2451	70.59638	89.46508

Sample numbers, means, and standard deviations for individuals exercising 1-5 hours per week and using product #s 1, 2, & 3; sample numbers, means, and standard deviations for individuals exercising 6-10 hours per week and using product #s 1 and 2; sample numbers, means, and standard deviations for individuals exercising greater than ten hours per week and using product #s 1, 2, & 3; and sample numbers, means, and standard deviations for the total number of individuals exercising anywhere between one hour to greater than ten hours per week and using product #s 1, 2, & 3.

A.1.2.3 Age of the respondent vs. Amount of protein used for product #s 1, 2, & 3

The third comparison of means examined was entitled Age of the respondent vs. Amount of protein for product #s 1, 2, & 3. This was also one of the questions asked in the team’s survey. In this comparison of means, the age of the respondent served as the independent variable, and the level of measurement for this variable was ordinal. It was an ordinal variable since it involved categories that were ordered by rank. The amount of protein taken for product #s 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that the number of eighteen year-olds using product #1 was 58 compared to nine eighteen year-olds using product #2 compared to two eighteen year-olds using product #3. The mean for eighteen year-olds using product #1 was 53.229 versus a mean of 68.5344 for eighteen year-olds using product #2 versus a mean of 157.5000 for eighteen year-olds using product #3. The standard deviation for eighteen year-olds using product #1 was 53.4510 compared to a standard deviation value of 69.22417 for eighteen year-olds using product #2 compared to a standard deviation value of 215.66757 for eighteen year-olds using product #3.

The number of nineteen year-olds using product #1 was 63 compared to nine nineteen year-olds using product #2 compared to three nineteen year-olds using product #3. The mean for nineteen year-olds using product #1 was 45.463 versus a mean of 36.6667 for nineteen year-olds using product #2 versus a mean of 42.3333 for nineteen year-olds using product #3. The standard deviation for nineteen year-olds using product #1 was 25.5238 compared to a standard deviation value of 22.70325 for nineteen year-olds using product #2 compared to a standard deviation value of 12.05543 for nineteen year-olds using product #3.

The number of twenty year-olds using product #1 was 74 compared to twelve twenty year-olds using product #2 compared to two twenty year-olds using product #3. The mean for twenty year-olds using product #1 was 53.131 versus a mean of 53.7917 for twenty year-olds using product #2 versus a mean of 20.0000 for twenty year-olds using product #3. The standard deviation for twenty year-olds using product #1 was 60.6305 compared to a standard deviation value of 36.01985 for twenty year-olds using

product #2 compared to a standard deviation value of 0.00000 for twenty year-olds using product #3.

The number of twenty-one year-olds using product #1 was 38 compared to four twenty-one year-olds using product #2. There were no twenty-one year-old respondents using a third product. The mean for twenty-one year-olds using product #1 was 52.104 versus a mean of 99.1196 for twenty-one year-olds using product #2. The standard deviation for twenty-one year-olds using product #1 was 59.2100 compared to a standard deviation value of 193.93081 for twenty-one year-olds using product #2.

The number of twenty-two year-olds using product #1 was 26 compared to four twenty-two year-olds using product #2. There were no twenty-two year-old respondents using product #3. The mean for twenty-two year-olds using product #1 was 35.920 versus a mean of 63.7775 for twenty-two year-olds using product #2. The standard deviation for twenty-two year-olds using product #1 was 18.0605 compared to a standard deviation value of 45.70969 for twenty-two year-olds using product #2.

The number of twenty-three year-olds using product #1 was twelve compared to three twenty-three year-olds using product #2 compared to two twenty-three year-olds using product #3. The mean for twenty-three year-olds using product #1 was 62.321 versus a mean of 68.6667 for twenty-three year-olds using product #2 versus a mean of 28.0000 for twenty-three year-olds using product #3. The standard deviation for twenty-three year-olds using product #1 was 68.1558 compared to a standard deviation value of 75.08218 for twenty-three year-olds using product #2 compared to a standard deviation value of 5.65685 for twenty-three year-olds using product #3.

The number of twenty-four year-olds using product #1 was four. There were no twenty-four year-old respondents using product #s 2 & 3. The mean for twenty-four year-olds using product #1 was 39.375. The standard deviation for twenty-four year-olds using product #1 was 7.5650.

The number of twenty-five year-olds using product #1 was eight. There were no twenty-five year-old respondents using product #s 2 & 3. The mean for twenty-five year-olds using product #1 was 43.143. The standard deviation for twenty-five year-olds using product #1 was 22.7093.

The number of individual respondents over the age of 25 using product #1 was ten compared to one individual respondent over the age of 25 using product #2 compared to also one individual respondent over the age of 25 using product #3. The mean for the number of individual respondents over the age of 25 using product #1 was 45.330 versus a mean of 130.0000 for the individuals using product #2 versus a mean of 62.0000 for the individuals using product #3. The standard deviation for the number of individual respondents over the age of 25 using product #1 was 22.5469. Since the means and numbers of the individuals in this group taking product #s 2 & 3 were both of the value of one, standard deviation could not be assessed as a result of a lack of variation around the mean.

The total number of respondents from age eighteen onward using product #1 was 293 compared to 42 individuals using product #2 compared to ten individuals using product #3. The mean for these individuals using product #1 was 49.491 versus a mean of 61.4261 for the individuals using product #2 versus a mean of 60.0000 for the individuals using product #3. The standard deviation for the individuals using product #1 was

48.2451 compared to a standard deviation value of 70.59638 for the individuals using product #2 compared to a standard deviation value of 89.46508 for the individuals using product #3. This comparison of means for the total number of respondents from age eighteen onward vs. amount of protein taken for product #s 1, 2, & 3 is equivalent to the comparison of means for the total number of respondents exercising anywhere from one hour per week to greater than ten hours per week vs. amount of protein taken for product #s 1, 2, & 3.

Figure 23: Sample Size Numbers, Means, and Standard Deviations for Age of the respondent vs. Amount of protein used for product #s 1, 2, &

Report				
Age of Respondent (in years)		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
18	Mean	53.229	68.5344	157.5000
	N	58	9	2
	Std. Deviation	53.4510	69.22417	215.66757
19	Mean	45.463	36.6667	42.3333
	N	63	9	3
	Std. Deviation	25.5238	22.70325	12.05543
20	Mean	53.131	53.7917	20.0000
	N	74	12	2
	Std. Deviation	60.6305	36.01985	.00000
21	Mean	52.104	99.1196	
	N	38	4	
	Std. Deviation	59.2100	193.93081	
22	Mean	35.920	63.7775	
	N	26	4	
	Std. Deviation	18.0605	45.70969	
23	Mean	62.321	68.6667	28.0000
	N	12	3	2
	Std. Deviation	68.1558	75.08218	5.65685
24	Mean	39.375		
	N	4		
	Std. Deviation	7.5650		
25	Mean	43.143		
	N	8		
	Std. Deviation	22.7093		
Over 25	Mean	45.330	130.0000	62.0000
	N	10	1	1
	Std. Deviation	22.5469	.	.
Total	Mean	49.491	61.4261	60.0000
	N	293	42	10
	Std. Deviation	48.2451	70.59638	89.46508

Sample sizes, means, and standard deviations for individual respondents in various college-aged groups ranging from age eighteen to over 25 using product #s 1, 2, & 3.

Since the independent variable in this comparison is ordinal and the dependent variable is interval-ratio, the appropriate test to use was the Spearman's Rho test. For the individuals in the age range of eighteen to over 25 who were using product #1, the two-

tailed significance p –value was 0.603 which is greater than 0.05 and therefore statistically insignificant depicting insufficient evidence to support a relationship between the independent and dependent variables. For the individuals in the same age range taking product #2, the two-tailed significance p-value was 0.832 which is greater than 0.05 and therefore also statistically insignificant indicating insufficient evidence to support a relationship between the independent and dependent variables. Lastly, for the individuals in this age range taking product #3, the two-tailed significance p-value was 0.959 which is greater than 0.05 and is statistically insignificant thereby showing insufficient evidence to support a relationship between the independent and dependent variables.

Figure 24: Spearman’s Rho Test for Age of the respondent vs. Amount of protein used for product #s 1, 2, & 3

Correlations

			Age of Respondent (in years)	How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Spearman's rho	Age of Respondent (in years)	Correlation Coefficient	1.000	-.030	.034	.019
		Sig. (2-tailed)	.	.603	.832	.959
		N	937	293	42	10
	How much Product #1 used (grams)	Correlation Coefficient	-.030	1.000	.194	-.060
		Sig. (2-tailed)	.603	.	.243	.887
		N	293	296	38	8
	How much Product #2 used (grams)	Correlation Coefficient	.034	.194	1.000	.847*
		Sig. (2-tailed)	.832	.243	.	.016
		N	42	38	42	7
	How much Product #3 used (grams)	Correlation Coefficient	.019	-.060	.847*	1.000
		Sig. (2-tailed)	.959	.887	.016	.
		N	10	8	7	10

*. Correlation is significant at the 0.05 level (2-tailed).

Spearman’s Rho test was used to determine whether there was a relationship in the comparison between the age of the respondent and how much product he or she was using.

A.1.2.4 Gender of the respondent vs. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of males using product #1 was 261 compared to 40 males using product #2 compared to ten males using product #3. The

mean for the males using product #1 was 51.496 versus a mean of 63.2647 for the males using product #2 versus a mean of 60.0000 for the males using product #3. The standard deviation value for males using product #1 was 50.4726 compared to a standard deviation value of 71.84933 for males using product #2 compared to a standard deviation value of 89.46508 for males using product #3.

The number of females using product #1 was 27 compared to two females using product #2. There were no female respondents using a third product. The mean for the females using product #1 was 31.976 versus a mean of 24.6550 for the females using product #2. The standard deviation value for the females using product #1 was 13.2412 compared to a standard deviation value of 12.94713 for the females using product #2.

Combining both the male and female groups surveyed, the total number of males and females using product #1 was 288 compared to 42 males and females using product #2 compared to ten males and females using product #3. The mean for the males and females using product #1 was 49.666 versus a mean of 61.4261 for the males and females using product #2 versus a mean of 60.0000 for the males and females using product #3. The standard deviation value for the males and females using product #1 was 48.5406 compared to a standard deviation value of 70.59638 for the males and females using product #2 compared to a standard deviation value of 89.46508 for the males and females using product #3.

Figure 25: Sample Size Numbers, Means, and Standard Deviations for Gender of the respondent vs. Amount of protein used for product #s 1, 2, & 3

Report

Gender of participant		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Male	Mean	51.496	63.2647	60.0000
	N	261	40	10
	Std. Deviation	50.4726	71.84933	89.46508
Female	Mean	31.976	24.6550	
	N	27	2	
	Std. Deviation	13.2412	12.94713	
Total	Mean	49.666	61.4261	60.0000
	N	288	42	10
	Std. Deviation	48.5406	70.59638	89.46508

Sample numbers, means, and standard deviations for the males using product #s 1, 2, & 3; sample numbers, means, and standard deviations for the females using product #s 1, 2, & 3; sample numbers, means, and standard deviations for the total number of males and females surveyed using product #s 1, 2, & 3.

A.1.2.5 Respondent with diabetes vs. Amount of protein used for product #s 1, 2, & 3

The fifth comparison of means attempted to be assessed was entitled Respondent with diabetes vs. Amount of protein used for product #s 1, 2, & 3. This was one of the questions asked in the team’s survey. In this comparison of means, Respondent with diabetes served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein taken for product #s 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that there were no individual respondents who had diabetes. Therefore, no sample numbers, means, and standard deviation values could be

determined since there were no diabetic individuals in the surveyed population and therefore no diabetic individuals using a first, second, or third product. In addition, since there were no diabetic individuals in the surveyed population, the independent variable in this comparison of means cannot be tested and therefore, a statistical test could not be executed.

A.1.2.6 Respondent with a family history of diabetes v. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of individuals with a family history of diabetes using product #1 was 93 compared to twenty individuals with a family history of diabetes using product #2 compared to five individuals with a family history of diabetes using product #3. The mean for the individuals with a family history of diabetes using product #1 was 55.770 versus a mean of 54.6549 for the individuals with a family history of diabetes using product #2 versus a mean of 82.4000 for the individuals with a family history of diabetes using product #3. The standard deviation for the individuals with a family history of diabetes using product #1 was 66.7734 compared to a standard deviation value of 51.71011 for the individuals with a family history of diabetes using product #2 compared to a standard deviation value of 127.92302 for the individuals with a family history of diabetes using product #3.

Figure 26: Sample Size Numbers, Means, and Standard Deviations for Respondent with a family history of diabetes vs. Amount of protein used for product #s 1, 2, & 3

Report

Diabetes (Fam.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	55.770	54.6549	82.4000
	N	93	20	5
	Std. Deviation	66.7734	51.71011	127.92302
No	Mean	46.341	69.2524	37.6000
	N	193	21	5
	Std. Deviation	36.3137	86.74476	19.75601
Total	Mean	49.407	62.1317	60.0000
	N	286	41	10
	Std. Deviation	48.4484	71.32332	89.46508

Numbers of individuals with a family history of diabetes, their associated means, and their associated standard deviations compared to the amount of protein they were using for product #s 1, 2, & 3.

A.1.2.7 Respondent with kidney disease vs. Amount of protein used for product #s 1, 2, & 3

The seventh comparison of means attempted to be assessed was entitled Respondent with kidney disease vs. Amount of protein used for product #s 1, 2, & 3. This was one of the questions asked in the team’s survey. In this comparison of means, Respondent with kidney disease served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein taken for product #s 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that there were no individual respondents who had kidney disease. Therefore, no sample numbers, means, and standard deviation values

could be determined since there were no individuals with kidney disease in the surveyed population and therefore no individuals using product #s 1, 2, and 3. In addition, since there were no individuals with kidney disease in the surveyed population, the independent variable in this comparison of means cannot be tested and therefore, a statistical test could not be executed.

A.1.2.8 Respondent with a family history of kidney disease vs. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of individuals with a family history of kidney disease using product #1 was three compared to one individual with a family history of kidney disease using product #2. There were no individuals with a family history of kidney disease using a third product. The mean for the individuals with a family history of kidney disease using product #1 was 36.333 versus a mean of 20.5000 for the individuals with a family history of kidney disease using product #2. The standard deviation for the individuals with a family history of kidney disease using product #1 was 23.4592. There was no standard deviation value available for individuals with a family history of kidney disease using product #2 since there was a lack of variation around the mean since there was only one individual with a family history of kidney disease using product #2.

Figure 27: Sample Size Numbers, Means, and Standard Deviations for Respondent with a family history of kidney disease vs. Amount of protein used for product #s 1, 2, & 3

Report

Kidney Disease (Fam.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	36.333	20.5000	
	N	3	1	
	Std. Deviation	23.4592	.	
No	Mean	49.546	63.1725	60.0000
	N	283	40	10
	Std. Deviation	48.6465	71.91593	89.46508
Total	Mean	49.407	62.1317	60.0000
	N	286	41	10
	Std. Deviation	48.4484	71.32332	89.46508

Numbers of individuals with a family history of kidney disease, their associated means, and their associated standard deviations compared to the amount of protein they were using for product #s 1, 2, & 3.

A.1.2.9 Respondent with clinical obesity vs. Amount of protein used for product #s 1, 2, & 3

The ninth comparison of means assessed was entitled Respondent with clinical obesity vs. Amount of protein used for product #s 1, 2, & 3. This was one of the questions asked in the team’s survey. In this comparison of means, Respondent with clinical obesity served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein taken for product #s 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that the number of individuals with clinical obesity using product #1 was 1. There were no individuals with clinical obesity using a second

product or a third product. The mean for the individuals with clinical obesity using product #1 was 31.000. There was no standard deviation value available for individuals with clinical obesity using product #1 since there was a lack of variation around the mean related to only one individual with clinical obesity using product #1.

Figure 28: Sample Size Numbers, Means, and Standard Deviations for Respondent with clinical obesity vs. Amount of protein used for product #s 1, 2, & 3

Report

Clinical Obesity (Ind.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	31.000		
	N	1		
	Std. Deviation	.		
No	Mean	49.354	60.6000	60.0000
	N	285	39	10
	Std. Deviation	48.5316	72.66305	89.46508
Total	Mean	49.290	60.6000	60.0000
	N	286	39	10
	Std. Deviation	48.4585	72.66305	89.46508

Number of individuals with clinical obesity using product #1 with its associated mean.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. For the individuals with clinical obesity using product #1, the two-tailed significance p-value was 0.706 which was greater than 0.05 and therefore statistically insignificant and demonstrating insufficient evidence to support a relationship between the independent and dependent variables. Since there were no individuals with clinical obesity using a second or third product, the independent variable in this comparison of means regarding a second or third product could not be tested

against the dependent variable and therefore, a statistical test could not be executed to determine a two-tailed significance p-value.

Figure 29: Independent Samples T-Test for Respondent with clinical obesity vs. Amount of protein used for product #s 1, 2, & 3

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
How much Product #1 used (grams)	Equal variances assumed			-.378	284	.706	-18.3538	48.6167	-114.0485	77.3409
	Equal variances not assumed						-18.3538			

An Independent Samples t-test was used to determine whether a statistically significant relationship exists between whether an individual with clinical obesity would be consuming greater quantities of product #1.

A.1.2.10 Respondent with a family history of clinical obesity vs. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of individuals with a family history of clinical obesity using product #1 was twelve compared to two individuals with a family history of clinical obesity using product #2 compared to one individual with a family history of clinical obesity using product #3. The mean for the individuals with a family history of clinical obesity using product #1 was 60.167 versus a mean of 93.0000 for individuals with a family history of clinical obesity using product #2 versus a mean of 5.0000 for individuals with a family history of clinical obesity using product #3. The standard deviation for the individuals with a family history of clinical obesity using product #1 was 64.5978 compared to a standard deviation value of 87.68124 for individuals with a family history of clinical obesity using product #2. There was no standard deviation value available for individuals with a family history of clinical obesity

using product #3 since there was a lack of variation around the mean related to only one individual with a family history of clinical obesity using product #3.

Figure 30: Sample Size Numbers, Means, and Standard Deviations for Respondent with a family history of clinical obesity vs. Amount of protein used for product #s 1, 2, & 3

Report

Clinical Obesity (Fam.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	60.167	93.0000	5.0000
	N	12	2	1
	Std. Deviation	64.5978	87.68124	.
No	Mean	48.936	60.5487	66.1111
	N	274	39	9
	Std. Deviation	47.7177	71.41230	92.65183
Total	Mean	49.407	62.1317	60.0000
	N	286	41	10
	Std. Deviation	48.4484	71.32332	89.46508

Number of individuals with a family history of clinical obesity using product #s 1, 2, & 3 with their associated means and standard deviations.

A.1.2.11 Respondent with hypertension vs. Amount of protein used for products 1, 2, & 3

The eleventh comparison of means assessed was entitled Respondent with hypertension vs. Amount of protein used for product #s 1, 2, & 3. This was one of the questions asked in the team’s survey. In this comparison of means, Respondent with hypertension served as the independent variable, and the level of measurement for this variable was nominal. It was a nominal variable since it involved categories of “yes” and “no” that were not ordered by rank. The amount of protein taken for product #s 1, 2, and 3 served as the dependent variable, and the level of measurement for this variable was interval-ratio.

Descriptive statistics revealed that the number of individuals with hypertension using product #1 was five. There were no individuals with hypertension using a second or third product. The mean for the individuals with hypertension using product #1 was 41.380. The standard deviation for the individuals with hypertension using product #1 was 17.5306.

Figure 31: Sample Size Numbers, Means, and Standard Deviations for Respondent with hypertension vs. Amount of protein used for product #s 1, 2, & 3

		Report		
Hypertension (Ind.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	41.380		
	N	5		
	Std. Deviation	17.5306		
No	Mean	49.430	60.6000	60.0000
	N	281	39	10
	Std. Deviation	48.8327	72.66305	89.46508
Total	Mean	49.290	60.6000	60.0000
	N	286	39	10
	Std. Deviation	48.4585	72.66305	89.46508

Number of individuals with hypertension using product #1 with its associated mean and standard deviation.

Since the independent variable in this comparison is nominal with only two categories and the dependent variable is interval-ratio, the appropriate test to use was the Independent Samples t-test. For the individuals with hypertension using product #1, the two-tailed significance p value was 0.713 which is greater than 0.05 and therefore statistically insignificant demonstrating insufficient evidence to support a relationship between the independent and dependent variables.

Figure 32: Independent Samples T-Test for Respondent with hypertension vs. Amount of protein used for product #s 1, 2, & 3

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
How much Product #1 used (grams)	Equal variances assumed	.429	.513	-.368	284	.713	-8.0504	21.8965	-51.1504	35.0497
	Equal variances not assumed			-.963	5.179	.379	-8.0504	8.3636	-29.3278	13.2270

Independent Samples t-test was used to determine whether a statistically significant relationship exists between whether an individual with hypertension uses greater quantities of product #1.

A.1.2.12 Respondent with a family history of hypertension vs. Amount of protein used for product #s 1, 2, & 3

Descriptive statistics revealed that the number of individuals with a family history of hypertension using product #1 was 46 compared to three individuals with a family history of hypertension using product #2. There were no individuals with a family history of hypertension using a third product. The mean for the individuals with a family history of hypertension using product #1 was 44.565 versus a mean of 47.0000 for the individuals with a family history of hypertension using product #2. The standard deviation for the individuals with a family history of hypertension using product #1 was 36.9253 compared to a standard deviation value of 59.35487 for individuals with a family history of hypertension using product #2.

Figure 33: Sample Size Numbers, Means, and Standard Deviations for Respondent with a family history of hypertension vs. Amount of protein used for product #s 1, 2, & 3

Report

Hypertension (Fam.): Do you have this health condition?		How much Product #1 used (grams)	How much Product #2 used (grams)	How much Product #3 used (grams)
Yes	Mean	44.565	47.0000	
	N	46	3	
	Std. Deviation	36.9253	59.35487	
No	Mean	50.383	63.5783	64.0000
	N	239	37	9
	Std. Deviation	50.4682	73.71194	93.93881
22.00	Mean	39.000	54.0000	24.0000
	N	1	1	1
	Std. Deviation	.	.	.
Total	Mean	49.407	62.1317	60.0000
	N	286	41	10
	Std. Deviation	48.4484	71.32332	89.46508

Number of individuals with a family history of hypertension using product #s 1 and 2 with their associated means and standard deviations.

A.1.3 Inferential Statistical Procedure (Courtesy of Dr. Lehman)

Now it is time to consider which inferential statistical procedure you need to perform. All of these statistical procedures are found under the **Analyze** command. Below is a list of possible combinations of level of measurement and independent and dependent variables that you can use as a guide to finding the right statistical test. Note that Independent Variable is abbreviated IV and that Dependent Variable is abbreviated DV.

If Your:

IV is Nominal and DV is Nominal: **Analyze...Descriptive Statistics...Crosstabs**

* Put your IV in the box designated as “Column (s)” and your DV in the box designated as “Row (s).” For “Statistics,” choose “Chi-Square.” For “Cells,” in the box designated as “Counts”, check “Expected” and in the box designated as “Percentages”, check “Column.”

IV is Nominal and DV is Ordinal: **Analyze...Descriptive Statistics...Crosstabs**

* Put your IV in the box designated as “Column (s)” and your DV in the box designated as “Row (s).” For “Statistics,” choose “Chi-Square.” For “Cells,” in the box designated as “Counts,” check “Expected” and in the box designated as “Percentages,” check “Column.”

IV is Nominal with only two categories (e.g. Gender) and DV is Interval, Ratio, or Interval-Ratio: **Analyze...Compare Means...Independent Samples T-Test**

* Put your IV in the box designated as “Grouping Variable” and then specify the values of your groups in the box designated as “Define Groups” (Hint: check your frequency distribution if necessary to find the actual values of the variable that goes in the “Define Groups” box). Put your DV in the box designated as “Test Variable (s).”

IV is Nominal and has three or more categories (e.g. Race) and DV is Interval, Ratio, or Interval-Ratio: **Analyze...Compare Means...One-Way ANOVA**

* Put your IV in the box designated as “Factor” and your DV in the box designated as “Dependent List.” In the “Options” button under Statistics, check “Descriptives.”

IV is Ordinal and DV is Nominal: **Analyze...Descriptive Statistics...Crosstabs**

* Put your IV in the box designated as “Column (s)” and your DV in the box designated as “Row (s).” For Statistics, choose “Chi-Square.”

IV is Ordinal and DV is Ordinal: **Analyze...Correlate...Bivariate**

* Put both your IV and your DV in the box designated as “Variables.” Under “Correlation Coefficients,” *uncheck* the box designated as “Pearson,” and check the box designated as “Spearman.”

IV is Ordinal and DV is Interval, Ratio, or Interval-Ratio: **Analyze...Correlate...**

Bivariate

* Put both your IV and your DV in the box designated as “Variables.” Under “Correlation Coefficients,” *uncheck* the box designated as “Pearson,” and check the box designated as “Spearman.”

IV is Interval, Ratio, or Interval-Ratio and DV is Nominal: **Please avoid this combination.**

* The correct procedure is called “Logistic Regression” but is beyond the scope of the team’s project.

IV is Interval, Ratio, or Interval-Ratio and DV is Ordinal:

Analyze...Correlate...Bivariate

* Put both your IV and your DV in the box designated as “Variables.” Under “Correlation Coefficients,” *uncheck* the box designated as “Pearson,” and check the box designated as “Spearman.”

IV is Interval, Ratio, or Interval-Ratio and DV is Interval, Ratio, or Interval-Ratio:

Analyze...Correlate...Bivariate

Put both your IV and your DV in the box designated as “Variables.” Under “Correlation Coefficients,” leave the box designated as “Pearson” checked.

A.1.4 Product Codes

Name of Product	Code	Serving Size	Total amount of PROTEIN per serving	Total amount of GLUTAMINE per serving (if info available)
4Ever Fit L-Glutamine	29	1 scoop = 5 g	5 g of L-Glutamine in this product. This is a purely glutamine product.	5 g of L- Glutamine in product. This is a purely glutamine product.
All the Whey Opti-Blend	99	1 scoop = 30 g	20 g	3.203 g
American Whey	87	1 scoop = 27 g	20g	
AST GL3 L-Glutamine	25	2 teaspoon = 10 g	10 g of L-Glutamine in this product. This is a purely glutamine product.	10 g of L-Glutamine in this product. This is a purely glutamine product.
Beverly International Ultimate Muscle Protein	82	1 scoop = 31 g	20 g	
Big100 Protein Bar (Met-Rx)	60	1 bar = 100 g	28 g	
Bioplex L-Glutamine	31	1 tablespoon = 10 g	10 g of L- Glutamine in product. This is a purely glutamine product.	10 g of L-Glutamine in product. This is a purely glutamine product.
BioQuest MyoZene	10	3 scoops = 118 g	25 g	
Boost Nutritional Energy Drink	66	8 fl. oz	24 g	
Boost High Protein	68	8 fl. oz	15 g	

BrainQuicken Body Quick	12	2 capsules = 1.2682 g	.805 g	
BSNCellMass	1	1 scoop = 16g	2 g of glutamine AKG.	2 g of glutamine AKG.
BSN Syntha-6	79	1 scoop = 44g	22 g	
Champion Whey Protein and Soy Protein	46	½ packet = 34.708 g	23 g	
Cytogainer by Cytosport	71	4 scoops = 150 g	54 g	
Cytosport 100% Whey	64	1 scoop = 22 g	18 g	
CytoSport EvoPro	9	1 scoop = 32 g	26 g	
CytoSport Muscle Milk Light	3	8.5 fl. oz	20 g	
CytoSport Muscle Milk (powder)	63	2 scoops = 70 g	32 g	
CytoSport Muscle Milk RTD (liquid)	94	14 fl. oz	25 g	
Designer Whey	83	24 g	18 g	
Dymatize Xpand	13	18.5 g	1 g of glutamine fusion	1 g of glutamine fusion
Dymatize Elite Whey Protein	92	1 scoop = 31 g	24 g	
EAS Betagen HP	11	2 scoops = 10.57 g	no total protein amount in grams on label	2 g
EAS L- Glutamine	21	1.5 teaspoons = 5 g	5 g of glutamine	5 g

EAS Muscle Armor	16	30 g	no total protein amount in grams on label	15.5 g of JUVEN TECHNOLOGY of which an unstated amount of glutamine is part of.
EAS Whey Protein	55	1 scoop = 30 g	23 g	
GNC 50 gram slam	73	1 can = 15 fl. oz	50 g	
GNC Mega MRP Advanced Sports Meal	100	1 packet = 82 g	40 g	5 g
GNC MassXXX	39	1 scoop = 50g	8.877 g of glutamine	8.877 g
GNC ProPerformance Amino Burst 3000	84	3 tablets = 3.015 g	3 g	
GNC Pro Performance L-Glutamine	38	2 capsules = 1.5 g	1.5 g of L- Glutamine in this product. This is a purely glutamine product.	1.5 g of L-Glutamine in this product. This is a purely glutamine product.
GNC Pro Performance L-Glutamine (powder)	38	1 scoop = 5 g	5 g of L-Glutamine in this product. This is a purely glutamine product.	5 g of L-Glutamine in this product. This is a purely glutamine product.
GNC ProPerformance Mega Whey	85	1/3 cup = 35 g	24 g	
GNC Pro Performance Weight Gainer	37	3 cups = 390 g	74 g	
GNC Pro Performance Whey Protein aka GNC 100% Whey Protein	35	1 scoop = 31 g	20 g	

GNC Soy Protein	77	1 scoop = 33.81 g	25 g	
Gold Standard Whey Protein	69	29.4 g	24 g	
Higher Power L-Glutamine (same product as Higher Power Micronized L-Glutamine)	20	1 teaspoon = 4.5 g	4.5 g of L-Glutamine in this product. This is a purely glutamine product.	4.5 g of L-Glutamine in this product. This is a purely glutamine product.
Higher Power Micronized Glutamine	23	1 teaspoon = 4.5 g	4.5 g of L-Glutamine in this product. Pure.	4.5 g of L-Glutamine in this product. Pure.
IDS L-Glutamine with MSM	19	1 scoop = 5.5 g	no total protein amount in grams on label	5 g
IDS Smart Gainer	18	2 scoops = 165g	51 g	
Isopure	75	2 scoops = 65 g	50 g	
LA Weightloss Protein Bar	78	1 bar = 47.14 g	8 g	
Met-Rx protein booster	57	1 scoop = 31 g	8 g	
Met-Rx protein plus bar	93	1 bar = 85 g	32 g	
Muscle Tech Mass Tech	47	5 scoops = 227g	40 g	
Muscle Tech Nighttime Protein	44	1 scoop = 31.965 g	20 g	
Muscle Tech Nitro-Tech Hardcore	4	1 scoop = 28.5 g	20 g	

MuscleTech Mass Tech	14	5 scoops = 227 g	40 g	
Myoplex Light	61	1 packet = 54 g	25 g	
Naked Juice Protein Zone	52	8 fl. oz	17 g	
Nature's Best Isopure	8	87 g	50 g	
NOW L- Glutamine	26	1 capsule = 1g	1 g of L-Glutamine in this product. This is a purely glutamine product.	1 g of L-Glutamine in this product. This is a purely glutamine product.
NxLabs Plasmavol	7	2 tablespoons = 40 g		
Optimum Glutamine	24	1 teaspoon= 5 g	5 g of L-Glutamine	5 g of L-Glutamine
Optimum NATURAL 100% Whey	86	1 scoop = 32.4 g	24 g	
Optimum Nutrition Gold Standard	53	1 scoop = 32 g	24 g	
Optimum Nutrition Whey Protein	40	1 scoop = 32 g	24 g	
Optimum Pro- Complex	96	1 scoop = 37 g	30 g	
PermaLean	90	1 scoop = 30 g	20 g	
Power Bar Protein Plus	59	1 bar = 78 g	23 g	
Prolab N-Large II	72	4 scoops = 152g	52 g	

PrimaForce GlutaForm	30	1 scoop = 5 g	5 g of L-Glutamine. This is a purely glutamine product.	5 g of L-Glutamine in this product. This is a purely glutamine product.
Prolab Glutamine Powder	22	1 teaspoon = 4.5 g	4.5 grams of L- Glutamine in this product. This is a purely glutamine product.	4.5 grams of L- Glutamine in this product. This is a purely glutamine product.
Prolab N-Large II	5	4 scoops = 152 g	52 g	
Protein Blitz	56	20 fl. oz	30 g	
Protein Booster C from the Smoothie Shop	41	1 serving size = 28.4 g	14 g	
Pure Protein Bar	62	1 bar = 78 g	32 g	
PureForm L- Glutamine Pro	32	1 serving size = 5 g	5 g of L-Glutamine in this product. This is a purely glutamine product.	5 g of L-Glutamine in this product. This is a purely glutamine product.
Pure Whey (Generic)	74	1 scoop = 31 g	20 g	
Ripped Fuel (powder)	81	.47825 g	.005 g	
Russian Bear 5000 weight gainer	95	292 g	36.8 g	
S.A.N. V-12 Turbo	15	12.5g		1 g

SciVation Xtend	6	2 scoops = 13 g		2.5 g
Smoothie King Gladiator	91	1 serving size = 47.151 g	45 g	
South Beach Living Protein Bar	80	1 bar = 35 g	10 g	
Textrolok Protein Shake	65	1 scoop = 37.5 g	32 g	
Twinlab Glutamine Fuel	33	2 capsules = 1.5 g	1.5 g of L-Glutamine in this product. This is a purely glutamine product.	1.5 g of L-Glutamine in this product. This is a purely glutamine product.
Ultimate Nutrition Glutapure	34	5 g	5 g of L-Glutamine in this product. This is a purely glutamine product.	5 g of L-Glutamine in this product. This is a purely glutamine product.
Universal Real Gains	88	3.5 scoops = 155 g	52 g	
Vitamin World Whey Protein	50	1 scoop = 24 g	18 g	
VP2	76	1 scoop = 28 g	23.5 g	
Weider L-Glutamine	28	1 teaspoon = 1.5 g	1.5 g of L-Glutamine in this product. Pure.	1.5 g of L-Glutamine in this product. Pure.
Xyience XM2	17	2 scoops = 78.003 g	32 g	
Xyience NOX-CG3	2	1 scoop = 13 g		1.5 g of Tri-Glutamine complex.
Zone Bar	89	1 bar = 50g	14 g	

A.1.5 Recommended Daily Allowance Calculations

*Assume our participants have the average body mass found in literature (Johnson, 1999):

72.1 kg

*Assume our participants have the average American dietary protein intake found in literature (Eisenstein et. al., 2002):

1.2 g protein/kg of body mass/day

*Use recommended daily allowance found in literature (Eisenstein et. al., 2002):

0.8 g protein/kg of body mass/day

Average weekly RDA:

$(0.8 \text{ g/kg/d}) * (72.1 \text{ kg}) * (7 \text{ d/wk}) = 403.76 \text{ g/wk}$ for a 72.1 kg individual

Average participant dietary intake:

$(1.2 \text{ g/kg/d}) * (72.1 \text{ kg}) * (7 \text{ d/wk}) = 605.64 \text{ g/wk}$ for a 72.1 kg individual

Average participant supplementary intake:

183.4 g/wk

Total weekly participant intake:

$$605.64 \text{ g/wk} + 183.4 \text{ g/wk} = 789.04 \text{ g/wk for a 72.1 kg participant taking protein}$$

% RDA participant protein intake:

$$(789.04 \text{ g/wk}) / (403.76 \text{ g/wk}) * 100 = 195.4 \rightarrow 200\% \text{ RDA}$$

% increase in protein intake from supplementation (assuming average American dietary intake):

$$(183.4 \text{ g/wk}) / (605.64 \text{ g/wk}) * 100 = 30.28 \rightarrow 30\% \text{ increase in protein intake}$$

A.2 Biochemical Analysis Appendix

A.2.1 Data From Biochemical Analysis

A.2.1.1: Measured glutamine concentration in pure glutamine and protein supplements.

Sample w/Enz	Amt. Weighed (mg)	[Sample] (mg/mL)	Expected [Gln] (mg/mL)	RT (min)	Area (uV*sec)	Avg of Duplicate Areas	Standard Deviation	% St Dev	[Gln] (mg/mL)	[Gln] Y-int=0 (mg/mL)	% Rec (w/Y-int)	% Rec (Y-int=0)
JU16-7A	52.51	1.0502	0.02431	13.90728	26194.55	26005.1	267.9135	1.030234	0.045399	0.047175	4.322878	4.491956
JU16-7B	52.51	1.0502	0.02431	13.89617	25815.66							
JU16-8A	55.37	1.1074	0.025634	13.9029	28216.74	28120.68	135.8487	0.483092	0.049246	0.051012	4.447008	4.606491
JU16-8B	55.37	1.1074	0.025634	13.90485	28024.62							
JU16-9A	66.28	1.3256	0.030685	13.90324	103380.3	102885.6	699.5848	0.679964	0.185211	0.18664	13.97186	14.07963
JU16-9B	66.28	1.3256	0.030685	13.88344	102391							
JU16-10A	52.27	1.0454	0.024199	13.91345	20437.37	20491.93	77.14839	0.376482	0.035373	0.037173	3.383661	3.555898
JU16-	52.27	1.0454	0.024199	13.90923	20546.48							

10B

JU16-11A	51.48	1.0296	0.023833	13.91467	25349.04	24797.84	779.5137	3.143474	0.043203	0.044984	4.196132	4.369123
JU16-11B	51.48	1.0296	0.023833	13.9101	24246.64							
JU16-12A	49.73	0.9946	0.023023	13.83493	20689.06	20785.37	136.1979	0.655258	0.035906	0.037706	3.610138	3.791038
JU16-12B	49.73	0.9946	0.023023	13.84832	20881.67							
JU16-13A	51.08	1.0216	0.023648	13.90469	30428.87	30353.15	107.0796	0.352779	0.053306	0.055062	5.2179	5.38979
JU16-13B	51.08	1.0216	0.023648	13.90044	30277.44							
JU16-14A	50.49	1.0098	0.023375	13.90269	26012.64	25999.91	18.00102	0.069235	0.045389	0.047165	4.494892	4.670738
JU16-14B	50.49	1.0098	0.023375	13.90017	25987.19							
JU16-15A	51.32	1.0264	0.023759	13.86154	16819.41	16766.92	74.2284	0.442707	0.028599	0.030416	2.786305	2.963368
JU16-15B	51.32	1.0264	0.023759	13.83344	16714.43							
JU16-	51.106	1.02212	0.02366	14.03077	558302.2	560387.2	2948.57	0.526167	1.017207	1.01657	99.51931	99.45698

16A

JU16-
16B

51.106 1.02212 0.02366 14.02676 562472.1

JU16-
17A

50.59 1.0118 0.023421 13.81088 2885.863 3185.961 424.4016 13.32099 0.003901 0.005779 0.38553 0.571209

JU16-
17B

50.59 1.0118 0.023421 13.81424 3486.058

JU16-
18A

102.263 2.04526 0.047344 14.03736 **10660.58** 10527.94 187.5892 1.781823 0.017253 0.019098 0.843543 0.933778

JU16-
18B

102.263 2.04526 0.047344 14.03311 10395.29

JU16-
19A

52.98 1.0596 0.024528 13.81742 8600.695 9846.491 1761.822 17.89289 0.016013 0.017862 1.511268 1.685732

JU16-
19B

52.98 1.0596 0.024528 13.82508 11092.29

JU16-
20A

50.44 1.0088 0.023352 13.80114 19601.44 24099.08 6360.627 26.39365 0.041933 0.043717 4.156684 4.333555

JU16-
20B

51.185 1.0237 0.023697 14.02412 28596.72

JU16-
21A

49.6 0.992 0.022963 13.81353 8190.296 9320.399 1598.207 17.14741 0.015057 0.016908 1.517808 1.704401

JU16-

49.6 0.992 0.022963 13.82854 10450.5

21B

JU16-22A	53.19	1.0638	0.024625	13.81064	9117.352	9765.263	916.2849	9.383105	0.015866	0.017715	1.491415	1.665225
JU16-22B	53.19	1.0638	0.024625	13.82777	10413.17							
JU16-23A	51.104	1.02208	0.023659	14.02969	547221.3	557161.4	14057.52	2.523061	1.011341	1.010718	98.94925	98.88835
JU16-23B	51.104	1.02208	0.023659	14.03774	567101.6							
JU19-1A	50.158	1.00316	0.023221	14.04874	549727.8	564132	20370.61	3.610965	1.024017	1.023363	102.0791	102.0139
JU19-1B	50.158	1.00316	0.023221	14.04205	578536.2							
JU19-2A	51.75	1.035	0.023958	13.83453	327682.9	304118.3	33325.37	10.95803	0.551165	0.551685	53.2527	53.30294
JU19-2B	51.75	1.035	0.023958	13.83711	280553.7							
JU19-3A	52	1.04	0.024074	13.83895	277528.7	276889.6	903.7727	0.326402	0.501648	0.502291	48.23542	48.29724
JU19-3B	52	1.04	0.024074	13.83385	276250.6							

A.2.1.2: Repeated measurements of glutamine concentration in a select few pure glutamine and protein supplements.

Sample	Amt. Weighed (mg)	[Sample] (mg/mL)	[Sample] in vial (mg/mL)	RT (min)	Area (uV*sec)	Avg of Duplicate Areas	Standard Deviation	% St Dev	[Gln] (mg/mL)	[Gln] Y-int=0 (mg/mL)	% GLN Detected (w/Y-int)	% GLN Detected (Y-int=0)	% GLN Detected 1st trial
JU19-2A	51.2	1.024	0.023704	14.16448	564240.2	554835.5	13300.24	2.397151	1.007111	1.006499	98.35065	98.29088	53.30294
JU19-2B	51.2	1.024	0.023704	14.15224	545430.8								
JU19-3A	51.03	1.0206	0.023625	14.16953	568480.5	570559	2939.422	0.515183	1.035705	1.035022	101.48	101.4131	48.29724
JU19-3B	51.03	1.0206	0.023625	14.16171	572637.5								
JU16-7A	26.27	1.0508	0.024324	14.15023	22043.78	22265.72	313.8664	1.40964	0.038599	0.040391	3.673253	3.843842	4.491956
JU16-7B	26.27	1.0508	0.024324	14.16306	22487.65								
JU16-8A	25.5	1.02	0.023611	14.15409	22854.63	22983.73	182.5785	0.794381	0.039904	0.041694	3.912187	4.087609	4.606491
JU16-8B	25.5	1.02	0.023611	14.14901	23112.84								
JU16-	26.24	1.0496	0.024296	14.16696	75775.21	76339.96	798.6772	1.046211	0.136936	0.138484	13.0465	13.19402	14.07963

9A													
JU16-9B	26.24	1.0496	0.024296	14.15687	76904.72								
JU16-10A	25.04	1.0016	0.023185	14.14513	17106.39	17512.21	573.9212	3.277262	0.029954	0.031768	2.990615	3.171726	3.555898
JU16-10B	25.04	1.0016	0.023185	14.15684	17918.04								
JU16-17A	24.95	0.998	0.023102	14.15892	22202.92	22063.99	196.4866	0.890531	0.038232	0.040025	3.83083	4.010536	0.571209
JU16-17B	24.95	0.998	0.023102	14.14792	21925.05								
JU16-22A	25.42	1.0168	0.023537	14.16078	23052.66	23095.83	61.05299	0.264346	0.040108	0.041897	3.944548	4.120473	1.665225
JU16-22B	25.42	1.0168	0.023537	14.1583	23139								

A.3 IRB Approval Forms

A.3.1 Initial Application



UNIVERSITY OF MARYLAND

INSTITUTIONAL REVIEW BOARD

MEMORANDUM

Application Approval Notification

2100 Blair Lee Building
College Park, Maryland 20742-5121
301.405.4212 TEL 301.314.1475 FAX
irb@deans.umd.edu
www.umresearch.umd.edu/IRB
June 28, 2007

To: Dr. Marc A Rogers
Sarah Tostanoski
Gemstone Program

From: Roslyn Edson, M.S., CIP *RE*
IRB Manager
University of Maryland, College Park

Re: **IRB Application Number: 07-0265**
Project Title: "Glutamine Supplementation on the University of Maryland"

Approval Date: June 25, 2007

Expiration Date: June 25, 2008

Type of Application: Initial

Type of Research: Non-exempt

Type of Review For Application: Expedited

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with 45 CFR 46, the Federal Policy for the Protection of Human Subjects, and the University's IRB policies and procedures. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you intend to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you would like to modify the approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting a request are posted on the IRB web site at: http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm. *(continued)*

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

Consent Form

Project Title	<i>Glutamine Supplementation on the University of Maryland</i>
Why is this research being done?	<i>This is a research project being conducted by Team JUICED, a Gemstone team. You have been selected for this survey because you are an "active" member at the University of Maryland (UMD) which means that you either participate in a varsity, club, or intramural sport and/or you frequently work out.</i>
What is the purpose of this project?	<i>This study aims to examine the use of supplements containing glutamine (a key amino acid component of many protein supplements) by active members of the UMD campus. Other supplements/ medication you may be using that can predispose you to health risks that lead to the development of chronic kidney disease will also be addressed in the study.</i>
What will I be asked to do?	<i>After being approached by a member of Team JUICED, you will fill out a simple, five-minute survey consisting of seven short questions on protein supplementation. Such questions are: Have you ever used protein supplements? What product(s) do you use? When did you use them? How often do you or did you use them? How much did you use? For how long did you use them?</i>
How will my confidentiality be protected?	<i>Your survey will be detached from your consent form and contact information, which will ensure the anonymity of your responses. Contact information will only be used for participation in a follow-up study and raffle. Your confidentiality will be protected by a four-digit number that appears on the top of your survey and will allow us to link your answer to the follow-up question to your contact information if needed. You will be asked on the on a separate sheet of paper if you would be interested in participating in a follow-up study in which we will take urine samples of users and non-users of glutamine and test it for the presence of protein concentration. Your information may be shared with representatives of the University of Maryland, College Park or governmental authorities if you or someone else is in danger or if we are required to do so by law.</i>
What are the risks of this project?	<i>There are no foreseeable risks associated with completing this survey.</i>
What are the benefits of this project?	<i>Although there is no direct benefit to you for participating in this study, your participation will likely benefit the UMD community and young adults, like yourself, in the future. Your participation will help us as researchers better understand the use of glutamine among active individuals on this campus. We hope that in the future others will also be able to benefit from the findings about how to safely consume glutamine.</i>

Consent Form

Do I have to be in this research? Can I stop participating at any time?	<i>Your participation in this study is completely voluntary. After agreeing to participate, you reserve the right to drop out at any time. There are no penalties or consequences of any kind if you decide to drop out. You can refuse to answer any question.</i>	
Project Title	<i>Glutamine Supplementation on the University of Maryland</i>	
What if I have questions?	<i>This research is being conducted by the Gemstone Team JUICED on the UMD campus. If you have any questions about this study, members of the research team will be happy to answer them now. If you have any questions in the future, please contact us at TeamJuiced@gmail.com. If you would like to contact our faculty mentor, please direct your inquiry to this location: Dr. Marc Rogers, 2140 Health and Human Performance building; (email) mrogers1@umd.edu; (telephone) 301-405-2482. If you have any questions about your rights as a research subject or wish to report a research-related injury, please contact: Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742; (email) irb@deans.umd.edu; (telephone) 301-405-0678.</i>	
Statement of Age of Subject and Consent	<i>Your signature indicates that: You are at least 18 years of age; The research has been explained to you; Your questions have been answered; and You freely and voluntarily choose to participate in this research project</i>	
Signature and Date	NAME OF SUBJECT	
	SIGNATURE OF SUBJECT	
	DATE	



1) How did we find you?

(Circle one) Varsity athletic team Club/Intramural sport team Gym

2) Have you ever used protein supplements?

(Circle One) Yes No If you answer "No" skip to Question 5.

3) Provide the details of the product(s) and how you used them below. If you cannot remember the exact name of the product, refer to the LAST PAGE for a list of commonly used supplements. REPEAT for additional supplements.

Ai) Name of product

(Fill in name)

ii) Time of use (Circle One) I use it **NOW** I used to use it **IN THE PAST**, but not currently

iii) How often?

(fill in number)

Times per day

Times per week

(Circle one)

iv) How much?

(fill in number)

Grams

Ounces

Scoops

Pills

(Circle one)

v) For how long?

(fill in number)

Weeks

Months

Years

(Circle one)

Bi) Name of product

(Fill in name)

ii) Time of use (Circle One) I use it **NOW** I used to use it **IN THE PAST**, but not currently

iii) How often?

(fill in number)

Times per day

Times per week

(Circle one)

iv) How much?

(fill in number)

Grams

Ounces

Scoops

Pills

(Circle one)

v) For how long?

(fill in number)

Weeks

Months

Years

(Circle one)

Ci) Name of product

(Fill in name)

ii) Time of use (Circle One)

I use it **NOW**

I used to use it **IN THE PAST**, but not currently

iii) How often?

(fill in number)

Times per day

Times per week

(Circle one)

iv) How much?

(fill in number)

Grams

Ounces

Scoops

Pills

(Circle one)

v) For how long?

(fill in number)

Weeks

Months

Years

(Circle one)

4) What other supplements and/or prescription medication do you take or were taking at the time of protein use? (List below)

5) Do you have any of these health conditions? (Circle all that apply)

Diabetes

Kidney Disease

Clinical Obesity

Hypertension

None of these

6) Do any of your blood relatives in your immediate family (siblings, parents, blood aunts/uncles, grandparents) have any of the following? (Circle all that apply)

Diabetes

Kidney Disease

Clinical Obesity

Hypertension

None of these

7) Participant Info:

Sex (Circle one)

Male

Female

Age (Circle one)

18

19

20

21

22

23

24

25

Hours of exercise AND sport practices per week (Circle one) 1-5 6-10 >10

Please be sure all sections of the survey are complete. Thank you for your time and cooperation. Feel free to add any additional comments below:

APPENDIX) Refer to this list of commonly used supplements to help you recall the exact names of the ones you used.

BSN CellMass
Xyience NOX-CG3
CytoSport Muscle Milk RTD/Light
MuscleTech Nitro-Tech Hardcore
Prolab N-Large II
SciVation Xtend
NxLabs Plasmavol
Nature's Best Isopure
CytoSport EvoPro
BioQuest MyoZene
EAS Betagen HP
BrainQuicken Body Quick
Dymatize Xpand
MuscleTech Mass Tech
S.A.N. V-12 Turbo
EAS Muscle Armor
Xyience XM2
IDS Smart Gainer
IDS L-Glutamine with MSM
Higher Power L-Glutamine
EAS L-Glutamine
Prolab Glutamine Powder
Higher Power Micronized Glutamine
Optimum Glutamine
AST GL3 L-Glutamine
NOW L-Glutamine
Optimum Glutamine Product
Weider L-Glutamine
4Ever Fit L-Glutamine
PrimaForce GlutaForm
Bioplex L-Glutamine
PureForm L-Glutamine Pro
Twinlab Glutamine Fuel
Ultimate Nutrition Glutapure
GNC Pro Performance Whey Protein
MuscleTech Cell-Tech Fruit Punch
GNC Pro Performance Weight Gainer
GNC Pro Performance L-Glutamine

Team JUICED is also conducting another study to test the level of a protein in the urine from both users and non-users of glutamine. For this research, we will need participants to simply provide a urine sample for us to test the presence of a particular protein. We will not be testing for anything else. Participants in this study will have the chance to win many prizes.

Are you interested in participating?

YES

NO

If you are interested, how can we contact you?

E-mail address:

Phone number:

Please note that this sheet will not be associated with your survey in anyway. It will be detached from the survey and placed in a separate location. The information you provide will be used solely to contact you in the future should you be interested in participating in our follow-up study. Your confidentiality is ensured.

A.3.2 Addendum October 19, 2007



UNIVERSITY OF
MARYLAND

INSTITUTIONAL REVIEW BOARD

2100 Blair Lee Building
College Park, Maryland 20742-5125
301.405.4212 TEL 301.314.1475 FAX
irb@deans.umd.edu
www.umresearch.umd.edu/IRB

October 19, 2007

MEMORANDUM

Addendum Approval Notification

To: Dr. Marc A. Rogers
Sarah Tostanoski
Department of Gemstone

From: Roslyn Edson, M.S., CIP, IRB Manager *face*
University of Maryland, College Park

Re: **IRB Application Number: 07-0265**
Project Title: "Glutamine Supplementation on the University of Maryland."

Approval Date Of Addendum: October 18, 2007

Expiration Date of IRB Project Approval: June 25, 2008

Application Type: *Addendum/Modification;* Approval of request, received on October 12, 2007, to pass out a flier and send a short advertisement to campus listservs to attract students to take the survey throughout the year.

Type of Review of Addendum: Non-exempt

Type of Research: Expedited

The University of Maryland, College Park Institutional Review Board (IRB) Office approved your IRB application. The research was approved in accordance with the University's IRB policies and procedures and 45 CFR 46, the Federal Policy for the Protection of Human Subjects. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you want to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

(continued)

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate an apparent immediate hazard to the subjects. If you would like to modify an approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting an addendum are posted at: http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

A.3.3 Addendum March 31, 2008



UNIVERSITY OF
MARYLAND

INSTITUTIONAL REVIEW BOARD

2100 Lee Building
College Park, Maryland 20742-5121
301.405.4212 TEL 301.314.1475 FAX
irb@dcans.umd.edu
www.umresearch.umd.edu/IRB

March 31, 2008

MEMORANDUM

Application Approval Notification

To: Dr. Marc A. Rogers
Sarah Tostanoski
Department of Gemstone

From: Roslyn Edson, M.S., CIP *RAE*
IRB Manager
University of Maryland, College Park

Re: **IRB Application Number: # 07-0265**
Project Title: "Glutamine Supplementation on the University of Maryland"

Approval Date of Addendum: **March 31, 2008**

Expiration Date of IRB Project Approval: **June 25, 2008**

Application Type: Addendum / Modification: Approval of request, submitted to the IRB office on March 10, 2008, to (1) collect urine samples from a small subset of the survey respondents (2) use the same survey instrument in an electronic form that would be sent to varsity athletes via Survey Monkey

Type of Review of Addendum: **Expedited**

Type of Research: Non-Exempt

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with 45 CFR 46, the Federal Policy for the Protection of Human Subjects, and the University's IRB policies and procedures. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you intend to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you would like to modify the approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting a request are posted on the IRB web site at:

http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm.

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

Consent Form

Project Title	<i>Glutamine Supplementation on the University of Maryland</i>
Why is this research being done?	<i>This is a research project being conducted by Team JUICED, a Gemstone team. You have been selected for this follow-up study because you have indicated on our previous survey that you were interested in being contacted in the future to help us continue our research.</i>
What is the purpose of this project?	<i>This study aims to examine the use of supplements containing glutamine (a key amino acid component of many protein supplements) by active members of the UMD campus. Its purpose is to measure protein and creatinine levels in urine samples to determine if there is statistically significant difference in protein content of control and experimental groups. Creatinine is a waste product used to standardize protein content.</i>
What will I be asked to do?	<i>After being contacted by a member of team JUICED, you will be asked to come to the University Health Center at a specific time of your choosing to give at least a 10 μL urine sample in a sealed container. The sample will then be refrigerated and sent to be tested for protein content in Bethesda, MD. The test should take no longer than 5 minutes. You will have the opportunity to see your results if you like. You will also be entered into a raffle to win a prize for participating.</i>
How will my confidentiality be protected?	<i>Your confidentiality will be protected by a four-digit number that will appear on the top survey that you previously filled out for team JUICED that allowed us to link your affirmative answer to the urine sample. This particular informed consent form will be kept in a confidential location separate from your previous survey that you filled out and subsequently provided your contact information on. This urine data will be accessible only to members of team JUICED and faculty mentor Dr. Marc Rogers. The urine sample will be destroyed in the urinalysis lab at the Uniformed Services University of the Health Sciences lab after the test is done.</i>
What are the risks of this project?	<i>There are no foreseeable risks associated with donating a small urine sample. This is a non-invasive procedure/study.</i>
What are the benefits of this project?	<i>Although there is no direct benefit to you for participating in this study, your participation will likely benefit the UMD community and young adults, like yourself, in the future. Your participation will help us as researchers better understand the implications of glutamine use among active individuals on this campus. We hope that in the future others will also be able to benefit from the findings about how to safely consume glutamine.</i>

Consent Form

Do I have to be in this research? Can I stop participating at any time?	<i>Your participation in this study is completely voluntary. After agreeing to participate, you reserve the right to drop out at any time. There are no penalties or consequences of any kind if you decide to drop out. You can refuse to participate at any time.</i>	
Project Title	<i>Glutamine Supplementation on the University of Maryland</i>	
What if I have questions?	<i>This research is being conducted by the Gemstone Team JUICED on the UMD campus. If you have any questions about this study, members of the research team will be happy to answer them now. If you have any questions in the future, please contact us at TeamJuiced@gmail.com. If you would like to contact our faculty mentor, please direct your inquiry to this location: Dr. Marc Rogers, 2140 Health and Human Performance building; (email) mrogers1@umd.edu; (telephone) 301-405-2482. If you wish to contact one of the student coordinators for Team JUICED, please direct your inquiry to Devang Sharma at dsharma1@umd.edu or 301-908-2575. If you have any questions about your rights as a research subject or wish to report a research-related injury, please contact: Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742; (email) irb@deans.umd.edu; (telephone) 301-405-0678.</i>	
Statement of Age of Subject and Consent	<i>Your signature indicates that: You are at least 18 years of age; The research has been explained to you; Your questions have been answered; and You freely and voluntarily choose to participate in this research project</i>	
Signature and Date	NAME OF SUBJECT	
	SIGNATURE OF SUBJECT	
	DATE	



Script for Team JUICED's Online Survey for Varsity Student Athletes
"Glutamine Supplementation on the University of Maryland"

Team JUICED is an undergraduate student research team working in the Gemstone Program. We are studying the social, physiological, and commercial effects of an amino acid called glutamine that is found in most protein supplements. Of the many types of legal, over the counter supplements, protein supplementation has proven to be the most popular among the college-aged student community, ages 18 through 25. Because of protein supplementation's direct impact on the student community and the fact that literature has suggested that it can contribute to kidney disease and ultimately kidney failure, especially among individuals that show certain risk factors including diabetes, high blood pressure (hypertension), obesity, and/or a family history of kidney disease, we have decided to investigate its prevalence on campus.

We have targeted individuals on campus who go to either the Eppley Recreation Center or Richie Coliseum, the ROTC programs, and those who participate in varsity, club, and intramural athletics. The purpose of this is to maximize the amount of responses of people who use protein supplements. As a member of a varsity team, Team JUICED asks for a few minutes of your time to complete this short survey which will take you no longer than 5-10 minutes to complete. The questions you will be asked relate to how much, how often, and for how long you have taken **protein** supplements, as well as what specific supplement or supplements you take. We provide you with a list of common protein supplements that are sold over the counter if you cannot remember the name of what you are taking. We also will ask your age, gender, how often you exercise, and if you have any family or personal history of diabetes, high blood pressure (hypertension), obesity, and/or a family history of kidney disease. All of these questions require you to either fill in the blank or circle one of several options. Because this survey distribution will occur via email and the internet, we cannot guarantee anonymity, however, Team JUICED will **NOT** be matching survey responses with names, instead we will assign you a subject #. Your participation in this study and your data will not be available to your coach or the athletic department.

At the very end of the survey, we will ask if you would be interested in a follow up study, in which we will take urine samples and **test them for protein ONLY**. If you indicate that you are interested, you must provide us with your contact information so that we can reach you after you have completed the survey. **Nothing else will be tested in the urine sample.** The only people that will have access to your survey question data will be the members of Team JUICED and our faculty mentor, Dr. Marc Rogers. If you have any questions, please feel free to direct them to our faculty mentor Dr. Rogers at:

2140 Health and Human Performance building
mrogers1@umd.edu
301-405-2482.

You may also contact team member Devang Sharma at:
dsharma1@umd.edu
301-908-2575

Thank you for your cooperation.

Pressing the following button signifies that you have read the above information and agree to participate in the survey on protein supplementation use.



If you have questions about your rights as a research subject or wish to report a research-related injury, please contact: Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742;
(e-mail) irb@deans.umd.edu; (telephone) 301-405-0678



A.3.4 Addendum May 2, 2008



UNIVERSITY OF
MARYLAND

INSTITUTIONAL REVIEW BOARD

2100 Lee Building
College Park, Maryland 20742-5121
301.405.4212 TEL 301.314.1475 FAX
irb@deans.umd.edu
www.umresearch.umd.edu/IRB

May 2, 2008

MEMORANDUM

Application Approval Notification

To: Dr. Marc A. Rogers
Sarah Tostanoski
Department of Gemstone

From: Roslyn Edson, M.S., CIP *REL*
IRB Manager
University of Maryland, College Park

Re: **IRB Application Number: # 07-0265**
Project Title: "Glutamine Supplementation on the University of Maryland"

Approval Date of Addendum: May 1, 2008

Expiration Date of IRB Project Approval: June 25, 2008

Application Type: Addendum / Modification; Approval of request, submitted to the IRB office on April 30, 2008, to (1) use a revised survey which includes a question regarding oral contraceptive use for female subjects and (2) record the height and weight of the subjects.

Type of Review of Addendum: Expedited

Type of Research: Non-Exempt

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with 45 CFR 46, the Federal Policy for the Protection of Human Subjects, and the University's IRB policies and procedures. Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you intend to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you would like to modify the approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting a request are posted on the IRB web site at:

http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm.

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or

redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

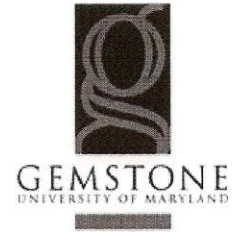
Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

Urinalysis Questions

Team JUICED

Date: _____

Survey Number: _____



1. Name: _____

2. Email Address: _____

3. Are you currently on University of Maryland payroll? YES NO

4. Gender: MALE FEMALE

5. Are you taking any oral contraceptives? YES NO

-----Do not fill out below this line-----

4. Height: _____

5. Weight: _____



A.3.5 IRB Renewal August 4, 2008



UNIVERSITY OF
MARYLAND

INSTITUTIONAL REVIEW BOARD

2100 Lee Building
College Park, Maryland 20742-5121
301.405.4212 TEL 301.314.1475 FAX
irb@deans.umd.edu
www.umresearch.umd.edu/IRB

August 4, 2008

MEMORANDUM

Application Approval Notification

To: Dr. Marc A. Rogers
Sarah Tostanoski
Gemstone Program

From: Roslyn Edson, M.S., CIP *RE*
IRB Manager
University of Maryland, College Park

Re: **IRB Application Number: # 07-0265**
Project Title: "Glutamine Supplementation at the University of Maryland"

Approval Date: August 4, 2008

Expiration Date: August 4, 2009

Type of Application: Renewal

Type of Research: Non-Exempt

Type of Review For Application: Expedited

The University of Maryland, College Park Institutional Review Board (IRB) approved your IRB application. The research was approved in accordance with 45 CFR 46, the Federal Policy for the Protection of Human Subjects, and the University's IRB policies and procedures. The IRB approves waiver of signed informed consent as per criteria in 45 CFR 46.116(d). Please reference the above-cited IRB application number in any future communications with our office regarding this research.

Recruitment/Consent: For research requiring written informed consent, the IRB-approved and stamped informed consent document is enclosed. The IRB approval expiration date has been stamped on the informed consent document. Please keep copies of the consent forms used for this research for three years after the completion of the research.

Continuing Review: If you intend to continue to collect data from human subjects or to analyze private, identifiable data collected from human subjects, after the expiration date for this approval (indicated above), you must submit a renewal application to the IRB Office at least 30 days before the approval expiration date.

Modifications: Any changes to the approved protocol must be approved by the IRB before the change is implemented, except when a change is necessary to eliminate apparent immediate hazards to the subjects. If you would like to modify the approved protocol, please submit an addendum request to the IRB Office. The instructions for submitting a request are posted on the IRB web site at:

http://www.umresearch.umd.edu/IRB/irb_Addendum%20Protocol.htm.

Unanticipated Problems Involving Risks: You must promptly report any unanticipated problems involving risks to subjects or others to the IRB Manager at 301-405-0678 or redson@umresearch.umd.edu.

Student Researchers: Unless otherwise requested, this IRB approval document was sent to the Principal Investigator (PI). The PI should pass on the approval document or a copy to the student researchers. This IRB approval document may be a requirement for student researchers applying for graduation. The IRB may not be able to provide copies of the approval documents if several years have passed since the date of the original approval.

Additional Information: Please contact the IRB Office at 301-405-4212 if you have any IRB-related questions or concerns.

Consent Form

Project Title	<i>Glutamine Supplementation on the University of Maryland</i>
Why is this research being done?	<i>This is a research project being conducted by Team JUICED, a Gemstone team. You have been selected for this survey because you are an "active" member at the University of Maryland (UMD) which means that you either participate in a varsity, club, or intramural sport and/or you frequently work out.</i>
What is the purpose of this project?	<i>This study aims to examine the use of supplements containing glutamine (a key amino acid component of many protein supplements) by active members of the UMD campus. Other supplements/ medication you may be using that can predispose you to health risks that lead to the development of chronic kidney disease will also be addressed in the study.</i>
What will I be asked to do?	<i>After being approached by a member of Team JUICED, you will fill out a simple, five-minute survey consisting of seven short questions on protein supplementation. Such questions are: Have you ever used protein supplements? What product(s) do you use? When did you use them? How often do you or did you use them? How much did you use? For how long did you use them?</i>
How will my confidentiality be protected?	<i>Your survey will be detached from your consent form and contact information, which will ensure the anonymity of your responses. Contact information will only be used for participation in a follow-up study and raffle. Your confidentiality will be protected by a four-digit number that appears on the top of your survey and will allow us to link your answer to the follow-up question to your contact information if needed. You will be asked on the on a separate sheet of paper if you would be interested in participating in a follow-up study in which we will take urine samples of users and non-users of glutamine and test it for the presence of protein concentration. Your information may be shared with representatives of the University of Maryland, College Park or governmental authorities if you or someone else is in danger or if we are required to do so by law.</i>
What are the risks of this project?	<i>There are no foreseeable risks associated with completing this survey.</i>
What are the benefits of this project?	<i>Although there is no direct benefit to you for participating in this study, your participation will likely benefit the UMD community and young adults, like yourself, in the future. Your participation will help us as researchers better understand the use of glutamine among active individuals on this campus. We hope that in the future others will also be able to benefit from the findings about how to safely consume glutamine.</i>

Consent Form

Do I have to be in this research? Can I stop participating at any time?	<i>Your participation in this study is completely voluntary. After agreeing to participate, you reserve the right to drop out at any time. There are no penalties or consequences of any kind if you decide to drop out. You can refuse to answer any question.</i>						
Project Title	<i>Glutamine Supplementation on the University of Maryland</i>						
What if I have questions?	<i>This research is being conducted by the Gemstone Team JUICED on the UMD campus. If you have any questions about this study, members of the research team will be happy to answer them now. If you have any questions in the future, please contact us at TeamJuiced@gmail.com. If you would like to contact our faculty mentor, please direct your inquiry to this location: Dr. Marc Rogers, 2140 Health and Human Performance building; (email) mr Rogers1@umd.edu; (telephone) 301-405-2482. If you have any questions about your rights as a research subject or wish to report a research-related injury, please contact: Institutional Review Board Office, University of Maryland, College Park, Maryland, 20742; (email) irb@deans.umd.edu; (telephone) 301-405-0678.</i>						
Statement of Age of Subject and Consent	<i>Your signature indicates that: You are at least 18 years of age; The research has been explained to you; Your questions have been answered; and You freely and voluntarily choose to participate in this research project</i>						
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