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Differences in bone health and bone biomarkers between exercising male protein supplement users, and non-users.

by

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Declaration

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This is entirely my own work and has not been submitted to any other higher education establishment, or for any other academic award in this Institute. Where use has been made of the work of other people, it has been fully acknowledged and referenced.

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Abstract

Title: Differences in bone health and bone biomarkers between exercising male protein supplement users, and non-users.

By R. Warner and L. Doyle, *Department of Health, Sport and Exercise Science, Waterford Institute of Technology*

Background: Increasing protein intake through protein supplements is a growing practice amongst exercising individuals. However there are varying reports in terms of the detrimental[(1)] and beneficial[(2)] effects of protein on bone. The effect of high level protein supplementation on bone health in exercising individuals is relatively unexplored.

Aim: To investigate the effect of variations in protein intake on bone health and bone biomarkers in exercising males.

Subjects and Methods: 50 non-supplement users (25.9 ± 5.1y) and 52 supplement users (25.4 ± 4.9y) were recruited. The average length of time for supplement use was 33 months. All subjects completed a food diary for 3 days which was analyzed using Comp Eat^{TM}. Net endogenous acid production (NEAP) was calculated by the method described by Remer *et al*. (2003). Protein content of supplements consumed was obtained from product labels. Effect of exercise on bone health was calculated using osteogenic index (OI). Bone health (bone mineral density (BMD) and bone mineral content (BMC) and percent lean body mass (LBM) was measured using dual energy x-ray absorbtiometry (DEXA). Serum samples were analyzed for osetocalcin (S-OC) and crosslaps (S-CTx) using commercially available ELISA kits. Urine was measured for pH using a digital urine analyzer with urinary calcium (U-Ca) and creatinine (U-Cr) levels being measured spectrophotoemetrically. Independent samples t-test or Mann Whitney U test (depending on data normality) were used to test for any differences between supplement users and non-users.

Results: There were no significant differences in potential confounders of BMI, OI or percent LBM ($P > 0.05$) between users and non-users. Protein intake, NEAP and sulphur content of the diet was significantly greater in users than non-users. There were no significant differences in BMD, BMC, urine pH or calcium, serum osteocalcin or crosslaps between users and non-users. This study demonstrates protein supplementation of 33 months duration has no effect on bone health in exercising males.

(1)Abelow *et al.* (1992)
(2) Promislow *et al.* (2002)

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Glossary

AAA	Aromatic Amino Acids
BCAA	Branched-Chain Amino Acids
BGD	Bone gla Protein
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
CLAN	College Lifestyle and Attitudinal National Survey
CTx	Cross-linked C-terminal telopeptides
DEXA	Dual Energy X-ray Absorptiometry
DH	Department of Health
DRI	Dietary Reference Intake
ELISA	Enzyme Linked Immunoassay
GRF	Ground Reaction Force
IOM	Institute of Medicine
ISSN	International Society of Sports Nutrition
LBM	Lean Body Mass
MES	Minimum Effective Strain
NEAP	Net Endogenous Acid Production
OA_{est}	Estimated urinary organic anions
OI	Osteogenic Index
PRAL	Potential Renal Acid Load
PRI	Population Reference Intake
RDA	Recommended Dietary Allowance
SLÁN	Survey of Lifestyle, Attitudes and Nutrition in Ireland
S-OC	Serum Osteocalcin
U-Ca	Urinary Calcium
U-Cr	Urinary Creatinine
UI	Uncoupling Index
WADA	World Anti-Doping Association

Chapter One

Introduction

1.1 Background

Increasing protein intake through protein supplements is a growing practice amongst exercising individuals, particularly in young men. It is estimated that worldwide supplement use amongst athletes, on average, ranges between 40 and 60 percent (Petroczi & Naughton, 2007). According to the College Lifestyle and Attitudinal National (CLAN) Survey conducted in Ireland in 2002/2003, 28% of male students reported supplement use (Hope *et al.* 2005).

However there are varying reports in terms of the detrimental and beneficial effects of protein on bone. Protein is an essential nutrient for bone health. Several studies have observed a positive association between dietary protein intake and increased bone mineral content or decreased risk of fracture (Cooper *et al*. 1996; Munger *et al.* 1999; Hannan *et al.* 2000; Heaney & Layman, 2008). In contrast however, there are many contrasting studies suggesting that a high protein intake is associated with a higher risk of bone fracture and reduced bone mineral content (Abelow *et al.* 1992; Metz *et al.* 1993; Feskanich *et al.* 1996).

Osteoporosis in men is now recognized as a major public health issue, with one in 5 men over the age of 50 expected to develop the disease (Gannon *et al.* 2008). It represents a serious threat to the health and well-being of men but it is largely under-diagnosed and under-treated (Kaufman & Goemare, 2008). A recent report by the International Osteoporosis Foundation found that the lack of awareness of osteoporosis in men is similar to the lack of awareness in women 50 years ago (International Osteoporosis Foundation, 2010).

1.2 Rationale for the Study

The effect of high level protein supplementation on bone health in exercising individuals is relatively unexplored. Much of the literature examining protein and bone has been conducted in peri-menopausal women or in elderly populations. Only 2 studies have explored the effect of increased protein intake on bone health in young exercising subjects, (Mullins & Sinning, 2005; Ballard *et al.* 2005) and only 1 of these was in males (Ballard *et al.* 2005). Protein supplement usage is increasing and very little is known about the effects of marginally increased protein intakes in men. With an increasing prevalence of osteoporosis in men it is imperative that further investigations are made to examine whether or not these high protein diets are a contributing factor to reduced bone health or indeed a protective factor against bone loss.

1.3 Aim of the Study

The aim of this study is to investigate the effect of variations in protein intake on bone health and bone biomarkers in exercising males. The bone health of groups consuming varying levels of protein intake will be compared; firstly – between protein supplement users and non-users, secondly- between 4 groups; those who reported to have a normal diet, subjects who consumed extra dietary protein only in their diet, subjects who consumed extra protein through supplements only and subjects who took extra dietary protein and supplements to increase protein intake.

1.4 Limitations of the Study

50 non-supplement users and 52 supplement users were recruited. The original aim was to recruit 100 subjects for each group to make extrapolation of the results more significant but subject recruitment (especially of supplement users) was more difficult than originally foreseen. This may be due to the fact that some subjects could have been taking additional substances (such as anabolic steroids) in addition to protein supplements, and therefore feared giving a blood and urine sample. In one gym contacted not one person would take part for this reason. Even though subjects were assured the sample would be used for this study only, it may have hindered recruitment. It is also possible that some subjects may not have reported usage of other substances which would confound results obtained. However each subject was encouraged to take part only if they could answer all questions as truthfully as possible.

Whether subjects used vitamin and mineral supplements or not could not be controlled for when examining the realtionships between components of protein and indicators of bone health as all variables need to be scale values. This could influence the results as certain vitamins and minerals have a role in bone structure and maintenance.

Chapter Two

Literature Review

2.1 Osteoporosis

2.1.1 What is Osteoporosis?

Osteoporosis is defined as a systemic skeletal disorder characterized by low bone mass and micro architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (Cooper *et al.* 2006). The World Health Organization has defined osteoporosis as a bone mineral density (BMD) or bone mineral content (BMC) T-score value >2.5 SDs below the young adult mean for the population as measured by dual energy X-ray absorptiometry (DEXA) (World Health Organization, 1994). It is called a 'silent disease' because it progresses without symptoms until a fracture occurs. Osteoporosis most commonly affects the hip and the lumbar vertebrae, but other bones such as the radius, tibia and ribs may also fracture (Cauley, 2006).

2.1.2 Measurement of Bone Health

DEXA is the current gold standard for measurement of BMD. BMD corresponds to the ratio between bone mineral content (BMC; hydroxyapatite) and bone area scanned. Therefore BMD as measured by DEXA is not a volumetric (mass per volume) density but an areal density (mass per area) and the units of BMD are g/cm^2. However, information gained by BMD measurement combined with an assessment of clinical risk factors and bone biomarkers may be of more use in terms of therapeutic decision making (Kaufman & Goemaere, 2008). BMD cannot always predict fracture; it is sometimes the case that individuals with normal bone density will suffer fragility fractures due to external fractures independent of BMD, for example bone turnover rate (Garnero *et al.* 1996).

In this study we measured serum levels of osteocalcin (a bone formation marker) and C-telopeptide-2 (CTx) (resorption marker) to give us a better indicator of bone health. Osteocalcin is an indicator of bone formation. It is a bone gla protein (BGP) produced by osteoblasts and a by-product of bone matrix synthesis. It has a short half-life and can be detected in the blood stream approximately 20 minutes after the activation of osteoblasts. Approximately 90% of bone matrix is type I collagen. Cross-linked C-terminal (CTx) telopeptides are proteolytic fragments of Collagen I formed during bone resorption. Elevated serum levels of CTx indicate a higher rate of bone resorption.

2.1.3 Epidemiology

One in three women and one in five men over the age of 50 years may have osteoporosis in Ireland (Gannon *et al.* 2008). Traditionally thought of as a women's disease, recent epidemiological studies have confirmed that it is an increasing health problem in men and a growing public health issue. This development stems from an increased awareness of the problem in men and also an increase in longevity, the number of men above the age of 70 will continue to increase as life expectancy continues to rise (Gennari & Bilzekian, 2007). Osteoporosis develops less often in men because men usually have a greater peak bone mass, their bone loss starts later and they have no period of rapid hormonal change and bone loss. Men usually present with hip, vertebral body, or distal wrist fractures 10 years later than women (Cauley, 2006). The increase of incidence in hip fracture rises sharply at the age of 60-65 in women and at ~75 years of age in men (Cauley, 2006). Fracture incidence in men is higher than women below the age of 50 yr, it tends to peak in adolescence and again with advanced age (Khosla *et al.* 2008). The difference in fracture incidence observed between men and women is due not only to a difference in their bone

strength but also to the type and frequency of trauma experienced by men compared with women over life (Khosla *et al.* 2008). Men are more likely than women to sustain a fracture at younger ages, which is thought to be related to the greater frequency of severe trauma associated with their fractures, through sport related or workplace injuries or fights ((Khosla *et al.* 2008). After the age of 50, the trend reverses, with women tending to have a higher overall fracture incidence than men (Khosla *et al.* 2008). It is estimated that by 2025, the number of fractures in men will be similar to the number in women (International Osteoporosis Foundation, 2010).

2.1.4 Impact

Osteoporosis is well recognized as a major public health problem, it affects 4-6 million women and 1-2 million men in the United States (Schoen, 2008). It has a huge social and financial impact on society due to the increased risk of fractures, the most important consequence of the disease (Groothausen, 1997). Fractures can have life changing consequences and older people are most likely to suffer serious injuries, disability, psychological suffering and death. Fractures are associated with enormous costs and substantial morbidity and mortality. An economic assessment of falls and fractures shows that in 2008 these injuries in older people cost over 400 million euro to the Irish economy. If current trends continue it is estimated that costs will escalate to 1 billion euro by 2020 (Gannon *et al.* 2008).

Although fragility fractures are less common in men than in women, when they occur these fractures are associated with higher morbidity and death than in women (Center *et al.* 1999). In an American study of patients in Minnesota, the investigators found that hip fractures in men were associated with a 31% mortality rate within a year after the fracture;

this figure was 17% in women (Campion & Maricic, 2003). The associated increase in mortality in men may be due to the later onset of osteoporosis and a shorter life-span than women (Kaufman & Goemare, 2008). Osteoporosis represents a serious threat to the health and well-being of men but it is largely under-diagnosed and under-treated (Kaufman & Goemare, 2008).

2.1.5 Causes of Osteoporosis

Primary causes of osteoporosis in men are genetics, aging and idiopathic (Kaufman & Goemare, 2008). Aging is usually the cause of osteoporosis development in men, although some men develop it at a relatively young age, often for unexplained reasons (idiopathic osteoporosis). Approximately 40-50% of osteoporosis cases in men are idiopathic (Bilzezikian, 1999). Idiopathic osteoporosis in young men appears in most cases to be the consequence of some defect in acquisition of bone mass and size during growth, with a strong genetic component (Kaufman & Goemare, 2008).

Approximately half of all cases of osteoporosis in men are secondary, i.e. have a specific cause contributing to the disease (Cauley, 2006). Endocrine disorders (hypogonadism), alcoholism, immobilization, gastrointestinal diseases, smoking, deficient dietary calcium and medication-related side-effects (corticosteroids in particular) are the most common causes of osteoporosis (Kaufman & Goemare, 2008; Orwoll, 1998). The present study involves subjects involved in regular physical activity, which in itself could have an effect on bone. The effects of physical activity on bone are reviewed in the next section. In relation to dietary factors known to affect bone health, calcium is probably the most significant and extensively studied although the present review will specifically deal with protein and the possible effects it could have on bone.

2.2 Physical Activity & Bone Health

Regular weight-bearing exercise across the life span is recommended as an important component of primary osteoporosis prevention. Moderate to high impact exercise during growth has been shown to increase peak bone mass, size and strength by clinically important amounts, which if maintained in later life could delay the development of osteoporosis and reduce the risk of fracture (Daly & Bass, 2006). A maximal bone mass at skeletal maturity is considered the best protection against age-related bone loss and subsequent fracture risk (Groothausen *et al.* 1997). Although it is not certain at which age maximal bone mass is reached, it has been stated repeatedly that maximal bone mass is achieved before the end of the third decade of life (Groothausen *et al.* 1997). The maximum peak bone mass seems to be influenced by the level of physical activity previous to the age at which peak bone mass is achieved (Groothausen *et al.* 1997). Exercise during growth seems to increase the BMD peak by between 10-20% in the loading bones of active adolescents compared with sedentary controls (Bass *et al.* 1998). During early to mid-adulthood, exercise has been shown to have minimal effects on increasing areal bone mineral density (aBMD) (1-3%), (bones become less sensitive to loading after skeletal maturity is reached (age 18-25 years)) and thus it's primary role appears to be maintenance of bone mass (Turner & Robling, 2005; Daly & Bass, 2006). Studies have shown that both acute and chronic exercise can reduce bone resorption (Murphy & Carroll, 2003).

2.2.1 Mechanism by which Physical Activity Strengthens Bone

The skeleton possesses an inherent biological control system that directs bone formation in response to high mechanical stresses (or strains), thus strengthening the skeleton on highly stressed regions (Turner & Robling, 2005). For example, the humeri of professional tennis

players exhibit approximately 40% more cortical bone on the playing side (arm that holds the racquet) compared with the non-playing side (Turner & Robling, 2003). Bone responds to mechanical stimuli. Strain thresholds turn bone remodelling 'on' and 'off' at a local level by a mechanism known as the mechanostat (or minimum effective strain; MES) theory. Research has shown that optimal strains are dynamic, high in magnitude, high in rate and of abnormal distribution (Lanyon *et al.* 1982). Bone seems to adapt to the level of exercise intensity required depending on the mechanical stress generated by exercise. Therefore, the final effect of exercise on bones depends on the type, intensity, and duration of the stimulus. Although the most suitable sporting activities remain unknown, participation in weight-bearing activities generating high ground reaction forces, mainly if they include jumps, sprints and rapid changes of directions, seem to have the most evident osteogenic effect during growth (Vicente-Rodriguez *et al.* 2008).

Exercise may also aid in bone development by optimizing the effect of calcium supplementation on bone mass (Vicente-Rodriguez *et al*. 2008). The combination of physical activity and calcium intake may be more effective in increasing bone mass than either calcium intake or physical activity alone. A minimum requirement of 1000mg of calcium per day is required to make this combination effective as exercise without sufficient calcium would not increase bone mass in adolescents (Vicente-Rodriguez *et al*. 2008).

In the absence of weight-bearing activity nutritional or endocrine interventions cannot maintain bone mass (Murphy & Carroll, 2003). Bone mass is limited but not controlled by diet, but is regulated by mechanical loading (Heaney *et al*. 2000). Calcium has a permissive effect on bone mineral accrual and maintenance, while physical activity has a modifying

effect (Baxter-Jones *et al*. 2003). Specific to resistance-trained athletes, it is clear that the mechanical stimulus and/or blood flow changes induced by the exercise provides a strong stimulus for bone retention and anabolism (Specker & Vukovich, 2007). According to Specker and Vukovich (2007), exercise would appear to be more important than diet regarding bone strength because it has a direct effect (e.g. via loading) on bone mass and structural properties, whereas nutritional factors appear to have an indirect effect (e.g. via hormonal factors) on bone mass.

2.2.2 Osteogenic Index

The effect of exercise on bone can be estimated using an osteogenic index (OI). OI depends on the exercise intensity and the degree of desensitization. The OI for a single session of exercise is defined as *the intensity of skeletal exercise* ^{x} *ln (N+1),* where N is the number of loading cycles (Turner & Robling, 2003). The intensity is defined by the loads applied to the bone and should be proportional to the magnitude of the peak ground reaction force (GRF). A recent study has found that the lifetime OI is associated with bone size, quality and strength among older men, demonstrating that OI may be a useful indicator of the osteogenic potential of different human activities (Lau & Pang, 2008).

2.3 Protein

2.3.1 Function

Protein is a major component of body tissues and about half of the body's protein is present in structural tissues such as muscle, skin, intracellular matrices, hair and nails in the form of myosin, actin and collagen (somatic protein) (Thomas & Jefferson, 2001). Protein is thus

essential for growth and, because there is continuous turnover of body tissues, for maintenance of body structure throughout life. In addition to their structural role, proteins have a number of other diverse functions: all enzymes, transport molecules, antibodies and many hormones such as insulin and thyroxine are proteins (Food and Nutrition Board, 2005). Thus an adequate supply of dietary protein is essential to maintain cellular integrity and function, and for health and reproduction.

2.3.2 Structure

Proteins are macromolecules consisting of long chains of amino acid subunits. There are 20 amino acids found in nature. All amino acids are composed of a nitrogen-containing amino ($-NH_2$) group, a carboxyl (-COOH) acid group plus a third component – a distinctive side-chain which gives each amino acid its individual properties. The structure of an amino acid is shown in figure 2.1. The R group is the side chain which distinguishes each amino acid.

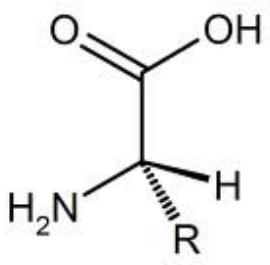

Figure 2.1: The general structure of an amino acid.

The amino group of one amino acid can form a link (a peptide bond) with the acid group of another amino acid to form a chain of amino acids, called a peptide. Interaction between reactive groups in the amino-acid side chains, within a polypeptide (>10 amino acids),

leads to formation of cross-links between parts of the chain or with other chains. These cross-links give the molecule it's particular shape and structure; for example, it may twist and fold to form a spherical globular structure (e.g. hemoglobin) or it may remain elongated and fibrous (e.g. collagen) (Food and Nutrition Board, 2005).

2.3.3 Metabolism

Dietary proteins are broken down by digestive enzymes into their constituent amino acids. Some will directly enter the body's pool of amino acids and be used for protein synthesis; others may be converted to other amino acids by the process of transamination. Surplus amino acids will be deaminated, the amino group being converted to urea and excreted by the kidneys, and the remainder converted to glucose or used as a source of energy (Thomas & Jefferson, 2001).

2.3.4 Amino Acids

Some amino acids can be synthesized as needed by the body while others must be provided by the diet. The essential amino acids are those which cannot be synthesized to meet the body's needs and therefore must be provided in the diet. Conditionally essential amino acids are those which require a dietary source. Semi-essential amino acids can be synthesized from other amino acids provided that precursor amino acids are present in the diet in sufficient amounts. Non-essential amino acids can be readily synthesized by the body. In this study we are interested in examining the effect of amino acids which are related to calcium homeostasis and thus bone health. These include the sulphur-containing amino acids, aromatic amino acids (AAA's) and branched chain amino acids (BCAA's).

Table 2.1: Classification of amino acids

Requirement	Amino Acid	Chemical Features	RDA[1] (mg/kg body mass)	RDA[2] (mg/kg body mass)
Essential	Isoleucine	Branched-chain amino acid	19	20
	Leucine	Branched-chain amino acid	42	39
	Valine	Branched-chain amino acid	24	26
	Lysine		38	30
	Methionine	Sulphur-containing amino acid	19	10
	Phenylalanine	Aromatic amino acid	33	25
	Tryptophan	Aromatic amino acid	5	4
	Threonine		20	15
	Histidine	Aromatic amino acid	14	10
Semi-essential	Cysteine	Sulphur-containing amino acid made from methionine	19	
	Tyrosine	Aromatic amino acid, can be made from phenylalanine	33	
Non-essential	Glycine			
	Proline			
	Glutamic acid[3]			
	Aspartic acid[3]			
	Serine			
	Alanine			
	Arginine			

[1]Dietary Reference Intakes, Institute of Medicine (2005)

[2] World Health Organization, Food and Agriculture Organization of the United Nations, United Nations University (2002)

[3]May be conditionally essential in critically ill people (Thomas & Jefferson, 2001)

2.3.4.1 Sulphur-containing Amino Acids

There are two sulphur containing amino acids, methionine and cysteine, oxidiation of these amino acids generates sulphuric acid which affects calcium balance (Feskanich *et al.* 1996). This will be discussed in the section on mechanism of negative effects of protein on bone.

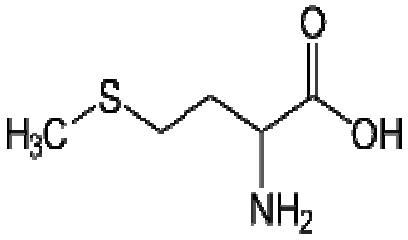

Figure 2.2: Sulphur-containing Amino Acid Methionine

2.3.4.2. Branched-chain and Aromatic Amino Acids

AAA's are those which contain a benzene ring as a side chain. These include phenylalanine, tryptophan, tryrosine and histidine. BCAA's refer to the amino acids having aliphatic side chains that are not linear; these are leucine, isoleucine and valine. AAA's in comparison to BCAA's may have an increased potential to affect calcium homeostasis (Dawson-Hughes *et al.* 2007). The impact of these amino acids on bone health will be discussed in the next section.

Figure 2.3: Aromatic Amino Acid; Phenylalanine and Branched Chain Amino Acid; Leucine

2.3.5 Protein Requirements

The Irish recommended dietary allowances (RDA) for protein are based on the EU population reference intake (PRI), which meets the dietary requirements of nearly all (97.5%) healthy people in a population. These recommendations are derived from nitrogen balance studies. The RDA for protein for adults is thus 0.75g/kg body weight/day. For children, pregnant and lactating women, an additional amount of protein for tissue growth or milk formation is required, up to 10g extra protein/day (Food Safety Authority of Ireland, 1999). The U.S. RDA for protein set by the Institute of Medicine is 0.8g/kg /d (Institute of Medicine, 2002). Dietary reference intakes (DRIs) for essential amino acids as per the US Food and Nutrition Board and World Health Organization recommendations for amino acids are illustrated in Table 1.

2.4 Protein and Bone Health

The impact of dietary protein on bone health is a controversial topic. Protein has been identified as being both detrimental (Avery Ince *et al.* 2004; Feskanich *et al.* 1996; Sellmeyer *et al.* 2001; Meyer *et al.* 1997; Metz *et al.* 1993) and beneficial (Whiting *et al.* 2002; Hannan *et al.* 2000; Munger *et al.* 1999; Bonjour, 2005; Cooper *et al.* 1996) to bone health, in relation to osteoporotic fractures, BMD, BMC and rates of bone loss, with evidence to support both sides of the debate. Tables 2.2 and 2.3 on pages 27 and 28, highlight some of the vast amounts of contradicting literature regarding dietary protein and bone health. These tables summarize what is contained in sections 2.4.1 and 2.4.2.

2.4.1 Negative Relationship between Protein and Bone

Abelow *et al.* (1992) reported that high animal protein intake was significantly and positively related to hip fracture incidence. In this study, cross-cultural variations on animal protein consumption and hip fracture incidence were examined. When female fracture rates derived from 34 published studies in 16 countries were regressed against estimates of dietary animal protein, a strong positive association was found. The highest rate of hip fracture was found to occur in industrialized Western countries, which had animal-protein intakes per capita between 60-80g/day, and the lowest incidence occurred in Asian and African populations in which animal protein intakes were considerably lower.

Frassetto *et al.* (2000) also found the cross-cultural relationship between hip fracture rates and dietary protein was positively related to animal protein intake and inversely related to vegetable protein intake. This was based on the relation of hip fracture incidence in women

aged 50 and over in 33 different countries to an indirect measurement of protein intake (country-specific data on per capita consumption of vegetable and animal foods as reported by the United Nations Food and Agricultural Organization). The countries with the highest rates of hip fracture had the highest animal protein consumption with animal protein intake exceeding vegetable protein intake. An argument against the validity of these cross-cultural studies is that the countries which have the highest incidence of fractures are those with the longest life-expectancy and thus the greatest life-time risk of fracture (Rizzoli & Bonjour, 2004). Also the method used to measure protein intake in both of these studies is an estimate based on population data which could be quite inaccurate at an individual level.

Metz *et al.* (1993) found dietary protein was significantly negatively associated with BMC of the distal and mid radius as well as distal BMD in a cross-sectional study of 38 young adult women. Consumption of protein was almost twice the RDA, close to 2g/kg body weight (72.9 +/- 22.7 g/day), but similar to other studies reporting protein intakes (Metz *et al.* 1993).

In a prospective study of 40,000 Norwegian men and women (aged 35-49 years at baseline) conducted over an average period of 11 years Meyer *et al.* (1997) found no association between non-dairy animal protein intake and hip fracture incidence. However, women in the lowest quartile of calcium intake and highest quartile of non-dairy animal protein intake had an elevated risk of fracture. Protein intake was measured using mailed food frequency questionnaires. Mean total protein intake was calculated to be 67.4 g/day in men and 49.5 g/day in women, non-dairy animal protein constituted eight ninths of the total protein intake in women. This study suggests that in the absence of an adequate calcium intake (below the RDA), a high intake of animal protein may be a risk factor for hip fractures.

Feskanich *et al.* (1996) as part of the Nurses Health Study found increased risk of forearm fracture in women consuming more than 90g protein/day (animal and vegetable protein) compared with those consuming less than 68g/day. This was a prospective 12 year study of 85,900 women aged 35-59 years. Dietary information was gathered using mailed FFQs and fracture incidence was self reported.

Additionally Kerstetter *et al.* (1999) also found changes in bone turnover in young women consuming different levels of dietary protein. In this study 16 healthy young women were maintained on 2 weeks of a well-balanced diet containing 1g protein/kg (adjustment period) followed by a 4-day experimental period containing one of three levels of protein; low (0.6g/kg), medium (1.0g/kg) or high (2.1g/kg). They found that the high protein group had significantly higher levels of bone resorption indicators (urinary N-telopeptide) than the low-protein group while there was no difference in bone formation indicators. This would suggest that a high protein diet increases bone resorption without a compensatory increase in bone formation. However the experimental period was only 4 days and not long enough to see if any compensatory mechanism would develop to maintain bone mass.

Sellmeyer *et al*. (2001) tested the hypothesis that a high dietary ratio of animal to vegetable foods, quantified by protein content, increases bone loss and the risk of fracture. Animal foods provide predominantly acid precursors whereas protein in vegetable foods is accompanied by base precursors not found in animal foods. This was a prospective study which examined protein intake and bone mineral density in a group of 1035 elderly women (aged > 65 years) over an average of seven years. They found that BMD was not significantly associated with the ratio of animal to vegetable protein intake. However, women with a high ratio had a significantly higher rate of bone loss at the femoral neck

than those with a low ratio and a greater risk of hip fracture. This led the authors to suggest that an increase in vegetable protein intake and a decrease in animal protein intake may decrease bone loss and the risk of hip fracture in contrast to Promislow *et al.'s* (2002) study which found a negative effect of vegetable rather than animal protein on bone.

2.4.2 Positive relationship between protein and bone

Several studies have observed a positive association between dietary protein intake and increased bone mineral content or decreased risk of fracture (Cooper *et al.* 1996; Munger *et al.* 1999; Hannan *et al.* 2000; Heaney & Layman, 2008).

Cooper *et al.* (1996) found that among 72 pre-menopausal women, there was a significant positive association between protein intake and bone mineral content, suggesting that dietary protein intake actually may be a determinant of the peak bone mass. Mean average protein intake in this group of women was 72g/day. However, there was a highly significant association between protein intake and calcium intake, and no separate analysis of animal protein was reported. Therefore it could be the combination of protein and calcium rather than protein itself which improved bone mineral content. They found no association between protein intake and bone mineral among the 218 postmenopausal women in the same study.

Munger *et al.* (1999) concluded that amongst postmenopausal women, intake of dietary protein, especially from animal sources may be associated with a reduced incidence of hip fractures. This was based on results which showed that the hip fracture group had a slightly lower mean daily intake of total protein that represented a lower intake of animal protein

but a higher intake of vegetable protein. The hip fracture group (n = 44) consumed 6.96g/MJ of animal protein/day compared to 7.96g/MJ in the non-hip fracture group (n = 32,006).

In the Framingham osteoporosis study, Hannan *et al.* (2000) examined the relation between baseline dietary protein and subsequent 4-year change in BMD in 391 women and 224 men, whose average age at baseline was 75y. Mean protein intake at baseline was 68g/day (+/- 24.0; range, 14-175g/day). Lower protein intake was significantly related to bone loss at femoral and spine sites with subjects with the lowest quartile of protein intake showing the greatest bone loss. Higher intakes of protein did not appear to affect the skeleton adversely in this elderly population.

Promislow *et al*. (2002) looked at the associations of total, animal and vegetable protein with BMD in an elderly population (55-92 years) of 572 women and 388 men over a 4 year period. They found a high animal-protein intake, assessed by food frequency questionnaires, had a positive association with BMD. For every 15g/day increase in animal protein intake, BMD increased significantly at the hip, femoral neck and total body. This association was statistically significant in women but not in men. A significant negative association between vegetable protein intake and BMD was observed. This was not expected as the greater alkaline content of vegetable foods should theoretically provide a protective buffering effect. However, a vegetarian diet could generate as much sulphuric acid as a meat based diet, which may account for the negative effect on bone.

In a 3-y, randomized, placebo-controlled study of 342 healthy men and women 65y of age and older, those who consumed the most protein and were supplemented with calcium

experienced the greatest improvement in bone mineral density (Dawson-Hughes & Harris, 2002). The mean protein intake of all subjects was 79.1 +/- 25.6g/day. Higher protein intake was significantly associated with a favorable change in total body BMD in the group supplemented with calcium and vitamin D but not in the placebo group. This suggests that increasing protein intake may have a beneficial effect on BMD in elderly subjects supplemented with calcium and vitamin D.

Heaney and Layman (2008) conducted a thorough review of the literature relating to dietary protein and bone health taking into account a number of confounding factors; the level of protein in the diet, the protein source, acid/base balance, calcium intake, weight loss and muscle mass. They came to the conclusion that higher protein diets are associated with greater bone mass and fewer fractures when calcium intake is adequate. They suggested that more concern should be focused on increasing the intake of alkalinizing fruits and vegetables rather than reducing protein sources and that more attention should be paid to increasing protein intake in the elderly to optimize bone health.

Many of the studies which have demonstrated a beneficial effect of increasing protein intake on bone have been conducted in an elderly population (Rapuri *et al.* 2003; Schürch *et al.* 1998; Delmi *et al.* 1990).

Protein energy malnutrition affects many elderly individuals (Price, 2008). A state of under-nutrition on admission to hospital is consistently documented in elderly patients with hip fracture (Jensen *et al.* 1982). Therefore in studies which have demonstrated improved BMD in elderly subjects supplemented with protein it is likely that their dietary protein intake before intervention was inadequate and not meeting their baseline protein

requirements of 0.8g/kg body weight/day. Therefore an increase in protein intake would obviously improve their overall nutritional status as well as bone health.

In one study a group of 59 elderly patients, hospitalized for femoral neck fractures and given an oral nutritional supplement providing 20g/day of protein, had significantly better clinical outcomes (lower rates of complications and shorter hospital stays) compared with those not receiving the supplement (Delmi *et al.* 1990). In this study however, most patients had nutritional deficiencies on admission to the hospital and nutritional requirements were not met during their stay. The benefits seen in the supplemented group may simply reflect an improvement in nutritional status from an increase in energy and protein intake. The supplemental protein may have helped them to meet their baseline daily requirements for protein rather than providing an additional benefit to bone health beyond that.

Rapuri *et al.* (2003) investigated the associations of dietary protein intake with baseline BMD and the rate of bone loss over 3y in 96 postmenopausal, elderly (65-77y) women. They found in this cross-sectional study that the quartile of women with the highest protein intake (~72g/d) had the highest BMD, only when calcium intake exceeded 408mg/d. In the longitudinal study no association was seen between protein intake and the rate of bone loss. This study reinforces the theory calcium and protein interact constructively to affect bone health.

Schürch *et al.* (1998) investigated the effect of protein supplementation of 20g/day on bone metabolism in 82 male and female elderly (~80yr) patients who had recently suffered a hip fracture, in comparison to an iso-caloric placebo. They found that the protein supplemented group had increased serum levels of insulin-like growth factor-1, attenuated proximal femur

bone loss and a shorter stay in hospital. Again, this study simply demonstrates the benefits of an improvement in nutritional status in elderly patients who are likely to be malnourished on admission, rather than demonstrating an additional benefit of protein beyond maintenance of bone at RDA levels.

In a systematic review and meta-analysis of the relation between dietary protein and bone health in healthy adults conducted by Darling *et al.* (2009) they found that the literature reviewed indicated a positive association between protein intake and BMD, BMC and a reduction in bone resorption markers. They found no clear relation between dietary protein and fracture risk in the qualitative review or in the meta-analysis. They did state, however, that 'in the absence of long-term intervention studies, the issue of whether protein intake does influence fracture risk must remain an open question' (p. 1690).

While there is a strong body of evidence to support the theory that increasing protein intake is beneficial to bone health there is still not enough evidence to rule it out as a potential risk factor for osteoporosis in young exercising men, the focus of our study. The majority of the studies examining the possible detrimental effects of elevated protein intake on bone have been conducted in pre or postmenopausal women. Protein intake in young exercising men who take protein supplements would obviously be significantly higher. Very little is known about the effect in men from adolescence to middle-age.

When comparing the literature which demonstrates positive and negative effects of dietary protein on bone there are many factors which need to be taken into consideration, and could confound study comparisons if not considered.

2.4.3. Confounding factors to bone health

(1) Subjects; age, gender, pre- or post-menopausal women (hormonal bone loss), ethnicity, all of these factors will impact on bone health.

(2) Type of study; cross-cultural, retrospective, prospective, intervention, in vitro v's clinical (some studies would argue that the physico-chemical dissolution of bone in response to a lower pH observed in vitro is not replicated in humans (Bonjour, 2005). Study duration and sample size also needs consideration in study comparisons.

(3) Different parameters used as indicators of bone health; fracture incidence, BMD, BMC, anatomic sites assessed, bone formation or resorption indicators and rates of bone turnover affect results purported.

(4) There may be marker variation in measurement of protein intake (food frequency questionnaires/ mailed responses / estimates based on protein available for a population minus the amount exported by a given country (Abelow *et al.* 1992; Frassetto *et al.* 2000)).

(5) Protein source needs to be considered; animal versus vegetable protein, milk versus meat; sulphur intake, purified forms of protein versus food sources.

(6) Level of protein intake; baseline and supplemental variations need consideration.

(6) Dietary acid/alkali balance of the diet could cause differing results.

(7) Protein balance could be affected by calcium intake.

(8) Effect of physical activity on bone.

(9) Muscle mass.

Table 2.2: Studies demonstrating a negative effect of protein on bone health.

(*High Intake → High Fracture Incidence/ reduced BMD/ increased rates of bone loss)*

Author(s)	Study Type	Subject Details	Protein intake/type	Measures
Abelow *et al.* (1992)	Cross-cultural	Women, 16 countries	60-80g/d	Fracture rate
Frassetto *et al.* (2000)	Cross-cultural	Women, ≥ 50y, 33 countries	animal > vegetable	Fracture rate
Metz *et al.* (1993)	Cross-sectional	38 premenopausal women	72.9±22.7g/d	Distal radius BMD
Meyer *et al.* (1997)	Prospective, 1y	39,787 men and women, 35-49y	animal >21.6g/d	Hip fracture[a]
Feskanich *et al.* (1996)	Longitudinal, 12y	85,900 women, 35-59y	>90g/d	Forearm fracture
Kerstetter *et al.* (1999)	Intervention	16 young women	2.1g/kg x 4 d	Bone loss
Sellmeyer *et al.* (2001)	Longitudinal, 7y	1035 women, >65y	animal > vegetable	Hip fracture

[a] In women only if calcium intake was low.

Table 2.3: Studies demonstrating a positive effect of protein on bone health.

(High Intake → Low Fracture Incidence/ increased BMD/ reduced rates of bone loss)

Author(s)	Study Type	Subject Details	Protein intake/type	Measures
Cooper *et al.* (1996)	Cross-sectional	72 postmenopausal women	72g/d[a]	BMD
Munger *et al.* (1999)	Prospective, 3y	32,050 postmenopausal women	7.96g/MJ (animal)	Hip fracture
Hannan *et al.* (2000)	Prospective, 4y	615 men and women, 75y	>82g/d	BMD
Promislow *et al.* (2002)	Prospective, 4y	960 men and women, >55y	15g/d ↑(animal)	BMD
Dawson-Hughes & Harris (2002)	Prospective, 3y	345 men and women ≥ 65y	79.1 ± 25.6 g/d	BMD[b]
Delmi *et al.* (1990)	Intervention	59 men and women with fractures, ~82y	+ 20g/d	Clinical
Rapuri *et al.* (2003)	Prospective, 3y	489 postmenopausal women, > 65y	> 72g/d	BMD[c]
Schürch *et al.* (1998)	Intervention	82 men and women with fractures, ~80y	+ 20g/d	Bone loss

[a]Protein intake was significantly associated with calcium intake in this group and no separate analysis of animal protein was reported.

[b] In the group supplemented with calcium.

[c]Only when calcium exceeded 408mg/d.

2.4.3 Mechanism for Positive Effects of Protein on Bone

Protein is an essential nutrient for bone health; it plays a major role in the development and maintenance of bone structure.

Bone Structure

Protein provides the structural matrix of bone which undergoes continuous turnover and remodeling. 22% of bone tissue consists of protein, mainly type 1 collagen (Dawson-Hughes, 2003). Because cross-linking of collagen molecules in bone involves post-translational modifications of amino acids, many of the collagen fragments released during proteolysis as part of remodelling cannot be reutilized to build new bone matrix. Accordingly, a daily supply of dietary protein is required for bone maintenance (Heaney & Layman, 2008).

Insulin Like Growth Factor 1 (IGF-1)

Protein has also been shown to optimize IGF-1 levels, a growth hormone that stimulates bone formation. IGF-1 has been shown in vitro to increase osteoblast activity and production of type 1 collagen and to act as a coupling factor for bone resorption and bone formation (Ginty, 2003). Schürch *et al.* (1998) observed an increase in levels of (IGF-1) and subsequent attenuation of the decrease in proximal femur bone mineral density in elderly patients with hip fractures following 6-month supplementation of 20g protein/d. Dawson-Hughes *et al.* (2004) noticed a similar effect in 32 healthy elderly men and women. They found that the high protein group (0.75 g/kg) in comparison to the low protein group (0.04 g/kg) had higher levels of serum IGF-1 and lower levels of urinary N-telopeptide over a period of 35-63 days.

Calcium Absorption

Protein may increase intestinal Ca absorption (Kerstetter *et al.* 1998) thereby optimizing bone mass. Recent studies argue that the increase in urinary calcium observed in high protein diets is due to an increase in intestinal calcium absorption (Gaffney-Stomberg *et al.* 2010). Studies suggest that the aromatic amino acid components of protein, but not the branched-chain amino acids, may activate calcium sensor receptors in the gut and increase gastric acid production (Dawson-Hughes, 2007). The latter could enhance calcium absorption. Kerstetter *et al.* (1998) found that in healthy adults (20 women) with a fixed calcium intake at 20mmol/d, increasing dietary protein from 0.7 to 2.1g/kg was accompanied by a significant increase in intestinal calcium absorption as determined by dual stable isotopic methodology. The study consisted of 2 interventions, each of which consisted of a 2 wk well-balanced adjustment diet followed by an experimental period of 4 days at either a low protein intake (0.7g/kg), or a high protein intake (2.1g/kg). Fractional calcium absorption after the low protein diet was 0.19 ± 0.03, which was significantly lower than that after the high protein diet (0.26 ± 0.03, $P = 0.05$). However an experimental period of 4 days is not long enough to suggest that calcium absorption would remain elevated at higher protein intakes in the long-term. According to Kerstetter et al. (2003), 80% of the increase in urinary calcium observed in their study, was due to increased intestinal calcium absorption. This leaves the remaining 20% unaccounted for, which begs the question could this be of skeletal origin, and therefore protein may actually have a negative effect on bone?

Heaney (2000) found no effect of dietary protein on calcium absorption. His objective was to test whether variation in phosphorus and protein intakes is associated with variation in

calcium absorption. 191 nuns over the age of 48 were studied approximately 3 times each over a minimum 20 y period with a full metabolic balance regimen; controlled, chemically analyzed diets; and double tracer measurements of calcium absorption. The mean protein intake averaged at 1g/kg/d. The results showed no associations of either phosphorus or protein with calcium absorption. However, this was an observational, retrospective study which looked at associations of protein intake and intestinal calcium absorption in subjects consuming their typical diet rather than observing the effect of increasing protein intake.

Adverse effects of low protein intake on bone have been suggested. Intakes of less than 0.8g/kg/body weight/day have been associated with reduced intestinal calcium absorption and increased levels of parathyroid hormone increase, causing the release of calcium from bone (Kerstetter *et al.* 2003).

Effects on Muscle Mass and Strength

Protein may enhance BMD indirectly through its effect on muscle mass and strength (Geinoz *et al.* 1993). The mechanical stimulus and/or blood flow changes induced by muscle contraction provides a strong stimulus for bone retention and anabolism (Specker & Vukovich, 2007). Athletes competing in strength and power events, such as weight lifting and jumping have superior bone mass and structure than compared with their untrained counterparts in all age groups (Suominen, 2006). Increased muscle mass and strength may also reduce the risk of falls and the severity of fall-related injuries (Suominen, 2006).

2.4.4 Mechanism for Negative Effects of Protein on Bone

It is the common perception that the typical Western diet, high in protein and primarily animal based, is sufficient to evoke detrimental changes in calcium metabolism, which results in bone loss and subsequent osteoporotic fractures (Frassetto et al. 2002). This perception may be due to both human and animal studies, dating back as far as 80 years, demonstrating increased protein intake consistently resulted in marked, sustained increases in urinary calcium, over the entire range of protein intakes, from marginal to excess (Sherman, 1920; Kerstetter & Allen, 1990, 1994; Margen et al. 1974).

It has been estimated that there is a 1mg rise in urinary calcium for each 1g rise in dietary protein (Kerstetter & Allen, 1990). This observation has led to the hypothesis that excessive dietary protein consumption may have a negative effect on bone mass (Heaney & Recker, 1982). Studies suggest that there is an increased risk of fractures or osteoporosis as a result of the increased urinary calcium excretion with a high protein intake (Feskanich *et al.* 1996; Noakes *et al.* 2005). Kerstetter and Allen (1994) project that negative calcium balance of only 25-30 mg/d could reduce skeletal calcium by 10% per decade. This supports Wachman and Bernstein's (1968) assertion that 15% of skeletal calcium can be lost over a decade to buffer a mild metabolic acidosis resulting from different dietary practices.

The adverse effect of high protein intake on bone is thought to be due to the catabolism of the sulphur-containing amino acids, methionine and cysteine, to sulphuric acid. The increase in endogenous acid reduces blood pH level. In response to this reduction in pH it is theorized that bone is mobilized to neutralize the acid and to maintain blood calcium levels (Feskanich *et al.* 1996). Osteoclasts are stimulated to resorb bone at an acidic pH, being

most sensitive to changes at pH values of ~7.1 (Arnett, 1988). This is assumed to occur by physico-chemical release of alkaline bone mineral, hydroxide (OH^-) and phosphate (PO_4^{---}) anions along with calcium cations (Ca^{+++}) (Green & Kleeman, 1991). This assumption is consistent with Barzel's theory that the skeleton acts as a 'giant ion-exchange column' (Barzel, 1995). Wachman and Bernstein (1968) noted that "the increased incidence of osteoporosis with age may represent, in part, the results of a life-long utilization of the buffering capacity of the basic salts of bone for the constant assault against pH homeostasis." The process described is thought to be the primary mechanism by which bone resorption is increased and increased urinary Ca losses occur, in response to a higher dietary protein intake (Remer, 2000).

Kerstetter *et al*. (1999) observed that 16 healthy women (20-40 y) placed on high protein diets for a 4 day experimental period following an adjustment period of 2 weeks of a well balanced diet, had increased urinary calcium excretion and rises in N-telopeptide (bone resorption indice). This supports the theory that some of the increases in urinary calcium result from increased bone resorption. In addition, Avery Ince *et al.* (2004) found that reducing ad libitum protein intakes (67g/d) in 39 healthy pre-menopausal women (22 - 39 y) to RDA levels (47g/d) for 2 weeks reduced urinary calcium, increased urinary pH and reduced urine N-telopeptide levels, supporting this theory.

2.5 Protein Source and Bone

It has been suggested that animal proteins may adversely affect bone health whereas protein from plant sources does not adversely affect bone health (Heaney & Layman, 2008). Both

Sellmeyer et al. (2001) and Frassetto et al. (2000) observed a greater risk of fracture in subjects with a higher ratio of animal to vegetable protein as discussed previously. There is a misconception that animal protein (i.e. meat, eggs and dairy products) is the primary source of sulphur-containing amino acids, therefore generating a greater acid load. However, a vegetarian diet, with proteins derived from grains and legumes, would deliver as many millimoles of sulphur per gram proteins as would a purely meat-based diet (Rizzoli & Bonjour, 2004). There are additional properties of animal and vegetable proteins that influence acid/base balance which will be discussed in the section on dietary acid/base balance.

Roughead et al. (2003) studied healthy postmenopausal women and found consuming a high-meat diet (297 g/d of meat), providing 117g of protein, did not adversely affect urinary calcium excretion, or clinical indicators of bone formation and resorption compared with a low-meat diet (45g/d of meat and 68g of protein). The diet was maintained for 8 weeks in each case and calcium intake in both groups was the same at ~600mg/day and is contrary to the belief that animal protein is more detrimental to bone health than plant. Roughead et al. (2003) did however observe a higher initial renal acid excretion in the group on the high meat diet but this difference abated between 3 and 8 weeks.

Earlier studies testing the effect of protein on bone used purified proteins including lactalbumin, wheat gluten and casein (Kim & Linksweiler, 1979; Johnson et al. 1970). In contrast to meat protein, these were found to induce hypercalciuria and this effect did not adapt over time (Allen et al. 1979). Allen et al. (1979) saw no adaptation in renal acid

excretion or hypercalciuria in 6 adult men consuming 75g or 225g protein in the form of egg albumin and additional soy protein in the higher protein diet. This was not the case in studies in which food sources of protein were used (Roughead et al. 2003), in which hypercalciuria abated over time. This is thought to be due to the phosphorus content of common dietary protein sources which minimises the calciuric effect (Zamzam & Roughead, 2003). Phosphorus may reduce hypercalciuria by increasing renal tubular calcium reabsorption (Berkelhammer et al. 1998). This emphasizes that protein source may have a significant influence on calcium balance and therefore must be considered in any study involving the examination of protein in terms of bone health.

Thorpe et al. (2008) reported that the potential positive effect of protein on bone may be offset by the acidifying effect of its sulphur content. 161 postmenopausal women were assessed for areal bone mineral density (aBMD) of lumbar spine and total hip. Dietary intakes of protein, sulphur-containing amino acids and minerals were measured. The acid load of the diet was estimated using the ratio of protein:potassium intake, the potential renal acid load and intake of sulphate equivalents from protein. aBMD was regressed onto protein intake then protein was controlled for estimated dietary acid load. It was noted that protein alone did not predict areal BMD at the lumbar spine, but after accounting for the negative effect of sulphate the effect of the protein intake was positive. This study highlighted the need to evaluate sulphur contents of varying dietary protein sources rather than assuming a fixed ratio of sulphur to protein (Thorpe et al. 2008).

Dawson-Hughes et al. 2006 compared the effects of increased intake of aromatic amino acids (AAAs) versus branched chain amino acids (BCAAs) on calcium excretion, serum IGF-1, markers of bone turnover, and 4-h calcium excretion after an oral calcium load. In

contrast to BCAAs, AAAs bind to the calcium sensing receptor and thus have an increased potential to affect calcium homeostasis. After 2 weeks on low-protein metabolic diets, 30 healthy subjects were randomized to a five-fold increase in intake of AAAs or BCAAs for 2 weeks. They observed a significant increase in calcium excretion and IGF-1 in the AAA group relative to the BCAA group. The increase in calciuria did not appear to result from an increase in bone resorption and may occur by increase in calcium absorption. This suggests that AAAs may selectively influence calcium homeostasis through their interactions with the calcium sensing receptor.

2.6 Protein Supplements

Athletes are constantly striving to improve their performance, and as a result of this many fall victim to false or unsubstantiated claims concerning diet and nutritional supplements. Most athletes only see potential for enhancing performance and never consider the possibility of supplements detracting from performance or health.

Although the College Lifestyle and Attitudinal Survey conducted in 2002/2003, reported 28% of male students reported supplement use (Hope *et al.* 2005), the study does not define what type of supplements they were using but it is highly likely that many of them were taking protein supplements and this figure could well be higher at this point in time.

The marketing of nutritional supplements is an international multi-million euro business sustained by a motive to sell product rather than to encourage optimal nutrition through food. Emotive labeling on products promoted in gyms, sporting magazines, health food stores and internet sites ensures dietary supplements are very popular among athletes intending to increase muscle mass.

The industry targets young men in particular and millions are spent on successful advertising and promotion campaigns each year to lure them. Optimum Nutrition (a major sports supplement company) has become the latest sponsor for the Leinster rugby team which is likely to encourage young rugby players to take supplements.

Peers, coaches, parents, fitness instructors and occasionally doctors, pharmacists and non-registered nutritionists have all been cited as sources of advice to take supplements. It is understandable that many young men involved in sport may feel pressurized into taking supplements as it would appear to be the norm. The concern is that that these athletes may become so preoccupied with supplementing their diet with protein, vitamins and minerals that they disregard the overall balance and nutritional quality of their diet and possibly ignore the damaging effects of alcohol and smoking.

2.6.1 Is there a case for protein supplements?

An abundance of research indicates that those individuals who engage in physical activity/exercise require higher levels of protein intake than the current RDA of 0.8g/kg body weight/day. This amount of protein intake may be appropriate for non-exercising individuals, but it is likely to not be sufficient to offset the oxidation of protein/amino acids during exercise (approximately 1-5% of the total energy cost of exercise) nor is it sufficient to provide substrate for lean tissue accretion or for the repair of exercise-induced muscle damage (Campbell *et al.* 2007).

The International Society of Sport Nutrition (ISSN) recommends that exercising individuals ingest protein ranging from 1.4-2.0g/kg/day. Endurance athletes are recommended to ingest levels at the lower end of this range, individuals engaging in

intermittent activities should ingest levels in the middle of this range, and strength/power athletes should ingest levels at the upper end of this range (Campbell *et al.* 2007). Athletes in the early stage of strength training may have a higher demand for protein than athletes who routinely resistance train due to significant gains in muscle size at the onset of training (Rodriguez *et al.* 2000).

In the context of a carefully monitored situation (i.e. under the guidance of an appropriately qualified nutritionist/dietician), there is evidence that some supplements may be of benefit in improving athletic performance. Recent evidence has shown that high-quality proteins such as whey, casein or soy are effectively used for the maintenance of, and net gains in, skeletal muscle (Rodriguez *et al.* 2000). The ISSN states that while it is possible for physically active individuals to obtain their daily protein requirements through a varied, regular diet, supplemental protein in various forms are a practical way of ensuring adequate and quality protein intake for athletes (Campbell *et al.* 2007).

The known benefits of dietary protein supplementation include;

- Protein synthesis
- Athletic recovery
- Potential weight control (thermic effect, satiety)

Other potential benefits include;

- Accompanying nutrients
- Elevated antioxidant capacity

- Immune enhancement
- Overtraining amelioration (Lowery & Devia, 2009).

Whey protein is one of the most commonly used protein supplements. It is very popular in the sports nutrition market based on the alleged quality of the protein it provides. It has a high protein quality score and contains a high proportion of essential and branched chain amino acids (Campbell *et al.* 2007). Recent studies demonstrate the ability of whey proteins to promote whole body and muscle protein synthesis (Ha & Zemel, 2002). Further research explores health benefits of whey that extends beyond protein and basic nutrition. Many bioactive compounds derived from whey are under investigation for their ability to offer specific health benefits. The capacity of these compounds to modulate adiposity and to enhance immune function and antioxidant activity presents new applications potentially suited to the needs of active individuals (Ha & Zemel, 2002).

Athletes must be reminded that the core of a successful hypertrophy program is a suitably designed training program and well-structured meal plan (Burke & Deakin, 2006). There is no substitute for good nutritional advice and an over reliance on supplements may lead to neglect of proper nutrition. Indeed in the vast majority of cases, athletes putting into practice good nutritional advice will eliminate any need for supplements.

2.6.2 Are there any harmful side effects?

One of the main concerns regarding supplement use is that it up until recently it was a largely unregulated industry. Despite the entry into force in 2002 of EU regulations controlling supplements as foods, there are still a large number of sports supplements and sports foods which are not controlled (Irish Sports Council, 2009). It is a feature of the industry that unsubstantiated and or exaggerated claims have been made for the efficacy of many products. Due to the newness of regulation, there are variable industrial hygiene standards associated in the production of many supplements and there is often no guarantee of accuracy in relation to labeling of the ingredients of many supplements. This means that athletes cannot guarantee the content of what they are ingesting and may be putting their health at risk by taking substances that are in themselves harmful and may even be counter-productive in terms of performance. Supplements which claim to be muscle building or fat burning are more likely to be associated with contamination with anabolic steroids, stimulants and other contaminants (Irish Sports Council, 2009)). Geyer *et al.* (2004) found that on examination of supplements bought from various sources, up to 14.8% of the supplements were contaminated with undeclared substances which were on the World Anti-Doping Association (WADA) Prohibited List.

It is often reported that a chronically high protein intake is unhealthy and may result in unnecessary metabolic strain on the kidneys leading to impaired renal function. In studies examining this effect the evidence was generated from animal models and patients with co-existing renal disease (Brenner *et al*. 1982). Therefore the extension of this relationship to healthy individuals with normal renal function is not appropriate. Martin *et al.* (2005) stated that there is no evidence that athletes consuming high levels of dietary protein, even with

intakes in excess of 2.0g/kg/day are at greater risk of developing kidney disease or losses in renal function.

However the safety of high long-term protein supplement usage, with regard to the potential of protein to influence bone health, is an area which to date, has received little or no attention. In addition even though there is not sufficient evidence to suggest a tolerable upper level for dietary protein according to the Institute of Medicine (IOM) (2002), at present we do not know what the current dietary protein intake levels are amongst athletes who are and are not supplement users. Changing dietary practices amongst athletes which has resulted in increased protein supplement use, warrants an investigation as to whether current high level protein intake amongst athletes could influence bone health.

2.7 Dietary Acid/Base Balance

Acid-base homeostasis is absolutely critical to health. It is well documented that extracellular fluid pH remains between 7.35 and 7.45. Thus, it is a major requirement of our metabolic system to ensure that H^+ concentrations are maintained between 0.035 and 0.045 mEq/l (Green & Kleeman, 1991). Alveolar ventilation, renal acid-base regulatory activity, and the diet acid and base loads together, determine the set point at which the concentrations of blood hydrogen ion and plasma bicarbonate are regulated. The potential of dietary acid load to increase bone resorption and urinary calcium excretion depends in part on the dietary alkali load (potassium, sodium, calcium and magnesium), which has been shown to neutralize the pH lowering effects of a higher dietary acid load (Ginty, 2003).

Animal foods provide predominantly acid precursors because meat contains more chloride and fewer countering precursors of alkali than plants (Dawson-Hughes, 2003), whereas protein in vegetable foods is accompanied with base precursors not found in animal foods. The dietary intake of potassium occurs mainly as salts of weak organic acids and therefore has an alkalinizing effect. Fruit and vegetables are the major source of potassium and this may explain their reported benefit to bone health (Frassetto *et al.* 1998; New *et al.* 2000).

Imbalance between dietary acid and base precursors leads to a chronic net dietary acid load that may have adverse consequences on bone. Average net endogenous acid production of common mixed diets, measured as urinary net acid excretion (NAE), varies from ~40 to 80mEq/day (Remer *et al.* 2003). In studies which have observed a positive intake between protein intake and bone mineral content (Alexy *et al.* 2005; Thientz *et al*. 1992; Chevalley *et al.* 2005), the authors caution that the anabolic effect of dietary protein only occurs with an adequate intake of alkali equivalents, such as potassium and magnesium found in fruits and vegetables.

While some studies support the theory that a higher protein intake is beneficial to bone health, particularly in the elderly, concern should be focused on increasing fruit and vegetable intake to neutralize the acid production rather than reducing protein sources (Heaney & Layman, 2008).

2.8 Level of Protein in the Diet

The RDA for protein for the general population is 0.8g/kg body weight per day (IOM, 2002). Protein intake below this is said to be detrimental to bone health (Kerstetter *et al.* 2003). Gaffney-Stomberg *et al*. (2009) suggest that the RDA for elderly individuals should be increased to 1.0 -1.2g/kg /d to maintain bone health.

2g protein/kg is the upper limit of protein intake recommended for strength athletes. Many athletes habitually consume protein in excess of this (Chen *et al.* 1989). Dietary surveys of athletes, particularly strength and power-training athletes and bodybuilders, indicate that dietary protein intakes in the range of 2-2.5 g/kg and up to as high as 3 g/kg are not unusual (Phillips *et al.* 2007). In studies which have found a beneficial effect of protein on bone, protein intakes were as high as 175g/d (Hannan *et al.* 2000) and 104g/d (Dawson-Hughes & Harris, 2002), well over the RDA for protein intakes based on average weights of population. However some studies have shown a negative effect of protein on bone at intakes as high as 2g/kg/day (Metz *et al.* 1993), as described previously. It is a cause for concern that there is so little evidence looking at the effect of excessively high protein intakes on bone health in adult men as it could potentially have quite a detrimental effect.

In 2002 the Institute of Medicine (IOM) concluded that there was insufficient evidence to suggest a tolerable upper level for dietary protein. They reported that the implications of high dietary protein for bone metabolism were not sufficiently unambiguous to make recommendations (IOM, 2002).

2.9 Calcium and Protein Intake and Bone Health

By weight bone tissue is 70% bone mineral, 8% water and 22% protein with 99% of calcium contained in bone. Bone undergoes continuous remodeling and an adequate supply of mineral and amino acid substrate is needed to support the formation phase of bone remodeling. Calcium and protein interact constructively to affect bone health; intakes of both must be adequate to fully realize the benefit of each nutrient on bone.

It has been stated that adequate dietary calcium intake minimizes the hypercalciuric effect of excess dietary protein, limiting its adverse effect on bone (Massey *et al.* 2003; Weikert *et al.* 2005). Additionally Meyer *et al.* (1997) noted no association between protein intake and risk of hip fracture in most women, but among those with very low calcium intakes (400mg/d), a higher protein intake was associated with an increased risk of hip fracture (Meyer *et al.* 1997). Shapses *et al.* (1995) found that increased protein intake had no effect on bone resorption markers in subjects with calcium markers in the high-normal range. 15 healthy men and women participated in three 5-day diet periods. The three diets consisted of: 1)Low protein (0.5g/kg) and low calcium (430mg), 2) Low protein (0.45g/kg) and high calcium (1645mg), 3) High protein (2.71 g/kg) and high calcium (1590mg). The rate of bone resorption marker, pyridinium cross-links of collagen, did not vary with protein intake but was significantly lower during periods of high calcium intake compared with low calcium intake. This indicates that a short-term increase in calcium intake is accompanied by a reduced rate of bone resorption and that this effect is independent of protein intake. The potential anabolic effects of protein may be maximized by a higher Ca intake. Dawson-Hughes and Harris (2002) found that a higher protein intake had a positive effect on BMD in elderly subjects supplemented with calcium citrate malate and vitamin D. A calcium

intake of at least 20mg for every 1g protein has been suggested to protect bone (Massey, 1998). Similarly Vatanparast et al. (2007) noted that in young adults protein did not confer as much benefit to bone in the absence of calcium. It is evident that an adequate calcium intake is essential to maintain and protect bone from deterioration. This suggests that the potential damage that a high protein intake could impact on bone may be limited by an increase in dietary calcium.

2.10 High protein intake in athletes

Only two studies have examined the role of protein on bone health in exercising subjects. Mullins & Sinning (2005) examined whether resistance training on a protein intake at the recommended level (0.8g/Kg body weight/d) induced bone formation, and training at a higher level of protein intake (2.4g/Kg body weight/d) induced bone resorption in a group of 24 untrained, young women aged 18-29 years. Results found neither programme had any effects on bone (Mullins & Sinning, 2005). However when examining this study it can be seen that the length of time the study was carried out for may have been too short to observe significant bone changes, since the training was only 12 weeks duration and protein supplementation was only for 10 days. This study was conducted in young untrained women; the effects of protein supplementation in young male athletes could be quite different.

Ballard *et al.* (2005) conducted a study to determine if dietary protein supplementation (84g extra/d) in conjunction with a 6 month strength and conditioning training programme improved bone mineral density in 52 healthy men and women aged 18-25 yr. Interestingly

the results found the training programme itself increased bone mineral density, but protein supplementation had no additional beneficial or detrimental effect in comparison with an isocaloric placebo (Ballard *et al.* 2005). However, in the same study, they noticed an increase in IGF-1 in the protein supplemented group and a significantly higher concentration of urinary N-telopeptide (NTx) (resorption marker) compared with the placebo group. Men had higher concentrations of NTx than women. These findings are interesting but inconclusive, 6 months is not long enough to observe any significant changes in BMD. This study stated that longer duration studies are needed to determine the effect of increased dietary protein on bone in young adults (Ballard *et al.* 2005). Ballard *et al.* (2005, p898) state "the appropriate amount of dietary protein to maximize skeletal health is under constant debate".

Lowery and Devia (2009) conducted a review of the literature in this area and found no research has compared bone health in a group of resistance trainers who have or have not sought ample dietary protein over a multi-year period. They stated that 'well-controlled observational (cross sectional) studies in strength athletes, involving long-duration protein intakes are needed' (page numbers not available for citation). The ISSN Position Stand on protein and exercise (2007) also stated that there was not enough athlete specific data relative to exercise, skeletal muscle hypertrophy and protein intake and their cumulative effects on bone mass (Campbell *et al.* 2007). Kerstetter *et al*. (2003) noted that 20% of the increase in urinary calcium observed in high protein diets could be of skeletal origin, which is quite significant. As discussed previously osteoporosis is now recognized as a significant public health problem in men as well as women, warranting further investigations into high dietary protein intakes as a potential risk factor.

2.11 Conclusion

There is a strong body of evidence to support the theory that increasing levels of dietary protein is beneficial to bone health and reduces bone loss and fracture risk, particularly in the elderly or postmenopausal women (Delmi *et al.* 1990; Cooper *et al.* 1996). Many of these studies which demonstrated an improvement in bone health with higher levels of dietary protein were also associated with higher calcium intakes (Dawson-Hughes & Harris 2002; Rapuri *et al.* 2003) which are well established as anabolic factors for bone. The levels of protein consumed in these studies were not overly excessive, up to 80-90g/d, they may have been above the RDA level, but in the general population protein intakes are usually well in excess of requirements (Gregory *et al.* 1990). Average daily protein intakes in the UK are 85g/d for men and 62g/d in women (Gregory *et al.* 1990). These studies may simply be demonstrating the importance of protein as an essential nutrient for bone health rather than implying that consuming extra dietary protein above the RDA and normally consumed levels will benefit bone.

Perhaps concern should be focused on postmenopausal women and the elderly to ensure that they are at least meeting the RDA requirements for protein and calcium to maintain bone health and reduce fracture risk as they appear to be the population most at risk and have shown improvements with increased protein intakes.

The evidence for the negative effect of protein on bone is mixed and not quite so clear-cut. Assumptions are based on slightly out-dated theories (Wachman and Bernstein, 1968) and indirect data from cross-cultural (Abelow *et al.* 1992) or longitudinal prospective studies

(Feskanich *et al*. 1996). However there is still not enough evidence to rule out high protein intakes in excess of RDA levels as being detrimental to bone health. Increased levels of bone resorption markers have been observed in high protein diets (Kerstetter *et al.* 1999; Avery Ince *et al.* 2004) and 20% of the calciuria observed in high protein diets could be of skeletal origin (Kerstetter *et al*.) It has been reported that strength and power athletes regularly consume protein intakes in excess of 3g/kg (Phillips *et al.* 2007) and no studies to my knowledge have studied the effect that this level of protein intake may have on bone in the long term.

An examination of the relationship between bone health and NEAP, sulphur and aromatic amino acid intake of athletes consuming high levels of protein on bone will lend us more information on what specific qualities of protein intake have an effect on bone, if any.

Chapter Three

Methodology

3.1 Ethical Approval

Ethical approval for this study was obtained from the ethics committee in Waterford Institute of Technology (WIT). Data collected from volunteers remained confidential and was stored securely. Each volunteer signed a written consent form (Appendix A) which outlined the procedures involved and any potential health risks.

3.2 Subjects

3.2.1 Inclusion Criteria

Male

Aged 18-35 y

Must participate in regular exercise

If taking protein supplements must be taking them for at least 6 months in advance of testing.

Subjects completed a medical history screening form (Appendix B) to ascertain whether there were any other factors (diseases, injuries, medications) which may affect bone health.

3.2.2 Recruitment

Subjects were recruited through a variety of methods; posters in health-food shops, gyms, college campus; contact with local sports clubs and officials; advertising on club websites;

email; word-of-mouth. It was necessary to recruit an approximately even number of protein supplement users and non-users to compare the bone health of the two groups. A total of 127 volunteers were recruited, 62 supplement-users (age 25.44 ± 4.87 y, range 19 – 35 y) and 65 non-supplement users (age 25.89 ± 5.11 y, range 18 – 35 y). Of these, 50 users (age 25.8 ± 4.85 y, range 19 – 35 y) and 50 non-users (age 26.28 ± 3.24 y, range 18 – 35 y) completed all of the procedures and provided all the necessary data for the study.

3.3 Study Protocol

Once subjects were deemed suitable for the study they were given a DEXA scan to assess bone health and percentage muscle mass. Their height and weight were measured in order to calculate their BMI. Subjects completed a physical activity record to calculate their osteogenic index (Appendix C). This form was obtained from Daly & Bass, 2005. Subjects then completed a 3 day food diary (Appendix D) to assess dietary intake and a record of supplement use was also taken. During the 3 days that subjects recorded their dietary intake subjects provided a first morning urine sample (3 in total) which was measured for urinary pH. Several aliquots of urine were acidified with 3% HCL and then stored at -20°C. Calcium was subsequently measured on acidified urine. Unacidified urine stored at -20°C was subsequently measured for creatinine concentration. Blood samples were taken by a qualified phlebotomist, processed to serum and stored at -80°C until required for analysis. Serum samples were analyzed for serum osteocalcin and crosslap levels.

3.4 Anthropometry

Height was measured to the nearest cm by a free-standing stadiometer and weight was measured to the nearest 0.1 kg using an electronic balance. Body Mass Index (BMI) was calculated as: BMI = weight (kg)/[height (m)]^2.

3.5 Dietary assessment

Subjects completed a food diary for 3 days which was then analyzed using CompEat^{TM}. Subjects were instructed to maintain normal dietary habits and to estimate the food quantities as accurately as possible. Average daily intake of selected macro-nutrients (energy, protein, fat, carbohydrate), micro-nutrients (calcium, magnesium, potassium, phosphate, vitamin C, vitamin D, sodium) and essential and non-essential amino acids were measured in this way. Intake of protein from meat was extrapolated using CompEat^{TM}. Branched chain amino acids leucine, isoleucine and valine were added together to obtain total branched chain amino acids from diet. Similarly the aromatic amino acids, phenylalanine, tryptophan, tryrosine and histidine were added together to calculate total aromatic amino acid intake. Essential amino acids – histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were added together to calculate total essential amino acid intake. The non-essential amino acids – cysteine, tyrosine, arginine, alanine, aspartic acid, glutamic acid, glycine, serine and proline were added together to get total non-essential amino acid intake. Amino acid intakes were calculated separately from diet and from protein supplements.

3.5.1 Net endogenous acid production (NEAP)

NEAP was calculated by the following method described by Remer *et al.* 2003:

Estimated NEAP (mEq/d) = PRAL (mEq/d) + OA_{est} (mEq/d)

Whereby PRAL denotes potential renal acid load and OA_{est} denotes estimated urinary organic anions, with the 2 components calculated as follows:

PRAL (mEq/d) = 0.49 x protein (g/d) + 0.037 x phosphorus (mg/d) – 0.021 x potassium (mg/d) – 0.026 x magnesium (mg/d) – 0.013 x calcium (mg/d);

OA_{est} (mEq/d) = individual body surface area[1] x 41/1.73.

[1]Body surface area was calculated according to the formula of Du Bois and Du Bois (Wang *et al.* 1992) as follows: body surface area (m^2) = [0.007184 – height $(cm)^{0.725}$ – weight $(kg)^{0.425}$].

3.5.2 Sulphur Intake

The sulphur content of the diet was computed from the formula described by Sebastian *et al.* 1994 as follows:

Sulphur (mEq/diet) = 2 x [(mg methionine/149.2) + (2 x mg cysteine/240.3)].

3.6 Protein Supplements

Subjects provided details of supplements used and amount consumed. A list of the protein supplements subjects consumed is provided in appendix E. Protein intake and intake of essential and non-essential amino acids from supplements was subsequently calculated using nutritional information from product labels and through an internet search. Where essential and non-essential amino acid information was not available an email was sent to the product manufacturer seeking this information. However in several cases manufacturers failed to provide the amino acid levels of their supplements. On one manufacturers website (www.BSN.com), in response to the following frequently asked question; 'I cannot seem to find the amino acid profile for SYNTHA-6™ anywhere on the label. Can you supply this information?' the answer provided was as follows: 'Unfortunately, we cannot release SYNTHA-6™'s exact amino acid profile, owing to the fact that the blend is proprietary. By releasing this amino acid breakdown, we would be potentially opening the door to competitors copying the ratios.' (www.BSN.com). Of the 62 supplement types taken by the study participants it was only possible to obtain precise supplement information from 48 supplements.

3.7 Physical Activity Questionnaire and Osteogenic Index

Osteogenic index scores for each participant were calculated to account for the effect of physical activity on bone health. As physical activity is known to have a beneficial effect on bone (Specker & Vukovich. 2007), it was necessary to calculate this score to cancel out physical activity as a confounding factor when determining the effect of protein on bone health. Each subject completed a physical activity questionnaire, providing details of their

lifetime physical activity history. The physical activity questionnaire (Appendix D) was adapted from a questionnaire developed by Kriska *et al.* (1988). The questionnaire asks about physically active occupations and lifetime participation in sport and leisure physical activities to date. Using this information osteogenic index scores were calculated using the following formula described by Daly & Bass, (2005);

OI = GRF * [Ln(total minutes per week) +1)]*months/year*number years spent participating in each activity.

3.8 Bone measurements

Whole body bone mineral density (BMD), bone mineral content (BMC) and percent lean body mass were determined by dual-energy X-ray Absorptiometry (DEXA) using an Excell^{TM} DEXA scanner *(Norland Medical Systems, NY, USA).* Scans were undertaken by the author on completion of DEXA training and were performed at Waterford Institute of Technology. Whole body BMD and BMC were measured as opposed to site specific measurements. This was due to the wide variety of sports amongst study participants (e.g. rugby, football, athletics, tri-athletes, swimmers, weight lifters, martial arts).

3.9 Blood Samples

Fasting blood samples were drawn from a vein between 0800 and 1000 h. Within 1 hour of collection samples were centrifuged (CR422 Jouan Inc. VA, USA) at 4ºC for 10 minutes at 3,000rpm and serum was separated and stored at -80ºC until required for further analysis. Serum markers of bone turnover, osteocalcin (S-OC) and C-terminal peptide of collagen type-1 (S-CTx) were measured by enzyme-linked immunoassay (ELISA).

3.9.1 Serum Osteocalcin

Serum osteocalcin was measured using N-Mid® Osteocalcin ELISA by Immuno Diagnostic Systems. The intra- and inter- assay reproducibility is shown in Table 3.1.

Table 3.1: Precision of N-MID® Osteocalcin ELISA by Immuno Diagnostic Systems

		Intra-Assay Precision		**Inter-Assay Precision**	
Sample	**Mean**	**SD (ng/ml)**	**CV %**	**SD (ng/ml)**	**CV %**
1	6.7	0.1	1.3	0.2	5.1
2	26.2	0.4	1.8	0.7	2.7
3	53.9	1.2	2.2	2.3	4.2

3.9.2 Serum Crosslaps (CTx)

Serum CTx was measured using Serum Crosslaps® ELISA by Immuno Diagnostic Systems. The intra- and inter assay reproducibility is shown in Table 3.2.

Table 3.2: Precision of Serum Crosslaps® ELISA by Immuno Diagnostic Systems

		Intra-Assay Precision		**Inter-Assay Precision**	
Sample	**Mean**	**SD (ng/ml)**	**CV %**	**SD (ng/ml)**	**CV %**
1	0.121	0.004	3.0	0.013	10.9
2	0.444	0.007	1.7	0.043	9.7
3	1.967	0.035	1.8	0.050	2.5

3.9.3 Uncoupling Index (UI)

To further explore the effects of protein on serum markers of bone formation and resorption, an uncoupling index (UI) was calculated using a modification from the method of Eastell *et al.* (1993). The uncoupling index demonstrates whether more bone is being formed or lost in the subjects (i.e. bone turnover). The modified formula used involved the following calculation:

Uncoupling Index = Formation T Score – Resorption T Score

$$UI = \frac{OC - \text{mean (group)}}{SD\ (\text{group})} - \frac{CTx - \text{mean (group)}}{SD\ (\text{group})}$$

3.10 Urine Samples

Each participant collected 3 first-morning urine samples on 3 separate days and were instructed to store samples immediately at -4ºC. Freshly thawed samples were measured for pH using a digital urine analyzer. Samples were then acidified (1:20 dilution) using 3% HCL and stored at -20ºC until required for further analysis.

3.10.1 Urinary calcium (U-Ca)

U-Ca was measured spectrophotometrically [QuantiChrom^{TM} Calcium Assay Kit (DICA-500) by Bioassay Systems]. The intra- and inter- assay CV is shown in Table 3.3.

Table 3.3: Precision of Calcium Assay by Bioassay Systems.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	**Calcium Conc.**	**CV %**	**Calcium Conc.**	**CV %**
1	4.01	2.4	4.78	3.15

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3.10.2 Urinary creatinine (U-Cr)

U-Cr was measured spectrophotometrically [Creatinine Colormetric Detection Kit by Assay Designs Inc.]. The intra- and inter- assay CV is shown in Table 3.4.

Table 3.4: Precision of Creatinine assay by Assay Designs, Inc.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	**Creatinine**	**CV %**	**Creatinine**	**CV %**
1	8.92	2.8	9.04	2.3
2	4.08	1.3	4.18	2.7
3	1.94	2.5	2.03	3.9
4	1.11	3.0	1.18	3.7

3.11 Statistical Analysis

All data was entered into Statistical Package for Social Sciences (SPSS) version 17.0. Data was first checked for normality. For tests involving a comparison between supplement users and non-users an independent samples t-test was utilized for normally distributed data and Mann Whitney U test for data not normally distributed. For analysis of differences

between 4 groups; normal diet subjects, subjects who consumed extra dietary protein only, subjects who consumed extra protein through supplements only and subjects who took extra dietary protein and supplements to increase protein intake, a 1 way ANOVA with Tukey's post hoc test was used for normally distributed data. For data which was not normally distributed a Kruskal-Wallis H test was used. Partial correlations were used to explore the relationship between protein and indicators of bone health while controlling for any potential confounding factors.

Chapter Four

Results

4.1 Introduction

The results documented in this chapter demonstrate the differences in physical characteristics, nutrient intake, protein intake and bone health between the supplement-user group and the non-supplement user group. Results are first presented as a comparison between supplement users and non-users (Section 4.2). In section 4.3 results comparing the following four groups are presented; those who reported to have a normal diet, subjects who consumed extra dietary protein only in their diet, subjects who consumed extra protein through supplements only and subjects who took extra dietary protein and supplements to increase protein intake. Finally relationships between certain components of protein intake and specific indicators of bone health are illustrated in section 4.4.

4.2 Protein Supplement Users versus Non-Users

4.2.1 Demographics

The mean values and standard deviations for selected demographic and physical characteristics for protein supplement users and non-users are shown in Table 4.1. There were no significant differences for any of the characteristics between the two groups. The mean values for BMI and OI 19+ yrs were notably higher in supplement-users than non-users but these differences were not significant (P = 0.085 and P = 0.076). When the same results were calculated using the subjects for which there was no missing data (n = 52 and n = 50) for both supplement users and non-users, there was a significant difference in BMI (P = 0.03) and OI 19+ yrs (P = 0.023). Both were significantly higher in the supplement user group (BMI; 26.68 ± 3.53 kg/m^2, OI 19+ y; 2334 ± 2107) than the non-user group (BMI; 25.32 ± 2.54 kg/m^2, OI 19+ y; 1529.33 ± 1106).

Table 4.1: Age, physical characteristics and OI of protein supplement users and non users.

	Protein Group				
	Non-users (*n* 65)		Supplement-users (*n* 62)		
	Mean	SD	Mean	SD	*P*
Age (y)	25.89	5.11	25.44	4.87	0.546
BMI (kg/m^2)	25.32	2.72	26.46	3.47	0.085
% LBM	78.18	6.25	79.67	6.19	0.184
Lifetime OI	3760.39	1843.93	4460.10	2846.40	0.140
OI 5-18yrs	2160.37	1385.39	1809.40	1411.33	0.204
OI 19+yrs	1517.94	1081.54	2375.96	1279.23	0.076

4.2.2 Nutritional Profile

Table 4.2 reports the mean daily intake of selected macro- and micro-nutrients for protein supplement users and non-users. Intakes of magnesium (P = 0.000), potassium (P = 0.037), phosphorus (P = 0.000) and vitamin D (P = 0.016) were significantly higher in supplement users than non-users. There were no other significant differences in nutrient intake between supplement users and non users.

Table 4.2: Macro- and micro nutrient intake per day in protein supplement users and non-users.

	Protein Group				
	Non-users (*n* 53)		Supplement-users (*n* 54)		
	Mean	SD	Mean	SD	*P*
Energy (kcals)	2615.33	663.68	2907.22	751.53	0.622
Fat (g)	108.24	34.17	110.46	32.79	0.733
Carbohydrate (g)	309.03	91.84	282.24	119.67	0.197
Calcium (mg)	1213.04	546.47	1258.92	592.47	0.678
Magnesium (mg)	341.18[a]	117.50	432.6[b]	118.94	0.000
Potassium (mg)	3659.54[a]	1119.06	4153.63[b]	1291.30	0.037
Phosphate (mg)	1719.69[a]	552.31	2180.80[b]	681.93	0.000
Sodium (mg)	3573.72	1210.29	3340.34	1170.68	0.313
Vitamin C (mg)	111.74	85.97	272.06	1153.31	0.786
Vitamin D (µg)	3.83[a]	7.94	4.43[b]	3.80	0.016

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$).

4.2.3 Amino Acid Profile

The mean values and standard deviations for non-essential, essential, branched-chain and aromatic amino acids for supplement users and non-users are illustrated in table 4.3. There were significant differences ($P < 0.05$) in intakes of non-essential, essential, branched-chain and aromatic amino acids between the two groups. All intakes were significantly higher in supplement users.

Table 4.3: Amino Acid Profile of Supplement-users and Non-users.

	Protein Group				
	Non-users (*n* 53)		Supplement-users (*n* 54)		
	Mean	SD	Mean	SD	*P*
Non-essential AA (g)	27.81[a]	19.84	59.33[b]	27.95	0.000
Essential AA (g)	14.96[a]	8.40	43.53[b]	23.76	0.000
BCAA (g)	7.42[a]	4.07	20.76[b]	11.16	0.000
AAA (g)	5.03[a]	2.72	11.04[b]	6.08	0.000

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$).

Abbreviations: AA – amino acid; BCAA – branched chain amino acids; AAA – aromatic amino acids

4.2.4 Total Protein and Essential Amino Acid Intakes compared to RDAs

Table 4.4 outlines the mean RDA, the mean intake and the mean percentage of RDA consumed for protein for supplement users and non-users. The RDA for protein for each subject was determined using the recommended level of protein intake for the type of exercise each subject is involved in as recommended by the ISSN; i.e. 1.4g/kg for endurance athletes, 1.7g/kg for athletes involved in intermittent activities and 2g/kg for strength athletes (Campbell *et al.* 2007). This value was then applied to lean body mass as opposed to total body mass to provide a more accurate measurement of individual protein requirements (Sears 1995).

In the non-user group the mean percentage RDA achieved for protein consumption was 106% suggesting that the majority of this group were meeting their individual protein

requirements. In the supplement user group the mean percentage RDA for protein was 151%, suggesting that protein intake in this group is marginally higher than requirements. There are significant differences in total protein intake, protein RDA and %RDA achieved between protein supplement users and non-users.

Table 4.4: Protein Intake and %RDA achieved of Supplement-users and Non-users.

	Protein Group				
	Non-users (*n* 53)		Supplement-users (*n* 54)		
	Mean	SD	Mean	SD	*P*
Total Protein Intake (g)	104.7[a]	31.21	193.53[b]	70.82	0.000
RDA[1] (g)	101.07[a]	17.96	118.25[b]	26.65	0.000
% RDA achieved	105.60[a]	30.30	151.60[b]	65.44	0.000

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$).

1 Protein RDA based on individual physical activity levels (Campbell *et al.* 2007) and lean body mass.

Table 4.5 presents the mean RDA, intake and % RDA consumed for essential amino acids. The RDA's for the individual essential amino acids are based on recommended DRIs as established by the IOM (2005) and calculated using the mean weight of each group.

Mean intake of all the essential amino acids are close to RDA levels, with intakes of tryptophan and isoleucine slightly greater than the RDA; at 127% and 120% respectively.

Mean intake of all the essential amino acids in supplement users are significantly greater than in non-users and far in excess of RDA levels. Intakes of some essential amino acids are almost 3-4 times the RDA level with the highest intakes observed in tryptophan and isoleucine, 374% and 362% of the RDA respectively.

Table 4.5: Essential Amino Acid Intakes compared to RDAs in supplement users and non-users.

	Protein Group					
	Non-users (*n* 53) 82 kg			Supplement-users (*n* 54) 85 kg		
	RDA	Mean	% RDA	RDA	Mean	% RDA
Isoleucine[1] (g)	1.56	1.87	120	1.62	5.86	362
Leucine[1] (g)	3.44	3.26	95	3.57	9.09	255
Valine[1] (g)	1.97	2.30	117	2.04	5.87	288
Phenylalanine[1] (g)	2.71	1.95	72	2.81	5.14	183
Tryptophan[1] (g)	0.41	0.52	127	0.43	1.61	374
Histidine[1] (g)	1.15	1.12	97	1.19	2.31	194
Methionine[1] (g)	1.56	0.92	59	1.62	2.30	142
Lysine[1] (g)	3.12	2.48	79	3.23	7.20	223
Threonine[1] (g)	1.64	1.62	99	1.70	5.28	311

[1] RDA's for individual essential amino acids based on Dietary Reference Intakes (DRIs) for Macronutrients and Energy (Institute of Medicine (IOM) 2005).

4.2.5 Protein Intake and Bone Health

Table 4.5 presents the differences in protein intake, bone biomarkers and bone health between the protein supplement users and non-users. Protein from meat ($P = 0.008$), NEAP ($P < 0.001$) and sulphur content of the diet ($P = 0.031$) were significantly greater in users than non-users. There were no significant differences ($P > 0.05$) in BMD, BMC, urine pH or calcium, serum osteocalcin, crosslaps or uncoupling index between supplement users and non-users.

Table 4.5: Protein intake and indicators of bone health in protein supplement users and non-users.

	Protein Group				
	Non-users (*n* 50)		Supplement-users (*n* 52)		
	Mean	SD	Mean	SD	*P*
NEAP (mEq/d)	14.45[a]	25.13	33.33[b]	34.75	<0.001
Protein from meat (g/d)	44.14[a]	20.71	66.40[b]	43.88	0.008
Dietary sulphur (mEq/d)	22.45[a]	12.37	28.61[b]	16.06	0.031
BMD (g/cm^2)	1.16	0.10	1.19	0.15	0.179
BMC (kg)	3.47	0.39	3.55	0.55	0.336
Urinary pH	6.30	0.55	6.21	0.37	0.421
U-Ca (mmol Ca/mmol Cr)	0.30	0.15	0.31	0.17	0.745
S-OC (ng/ml)	30.39	15.80	32.96	13.75	0.246
S-CTx (ng/ml)	1.00	0.38	0.95	0.35	0.579
Uncoupling Index	-0.12	1.56	0.13	0.79	0.151

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$).

4.3 Comparisons between 4 diet types

4.3.1 Demographics

The mean values and standard deviations for selected demographic and physical characteristics for subjects who reported to have a normal diet, subjects who consumed extra dietary protein only in their diet, subjects who consumed extra protein through supplements only and subjects who took extra dietary protein and supplements to increase protein intake are shown in Table 4.6. There was a significant difference observed in the BMI between the four groups (P = 0.005). BMI was highest in the group who took extra dietary protein as well as supplements (26.93 kg/m^2) and lowest in the group who consumed a normal diet only (24.89 kg/m^2). There were no other significant differences amongst the groups in age, percentage lean body mass or OI.

Table 4.6: Age and physical characteristics of the 4 diet types.

	Protein Group								
	Normal diet (*n* 46)		Extra dietary protein (*n* 19)		Extra protein supplements (*n* 14)		Extra protein supplements and dietary protein (*n* 48)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
Age (y)	26.52	5.44	25.67	4.98	24.5	5.33	25.71	4.75	0.231
BMI (kg/m^2)	24.89^{a}	2.61	$25.87^{a,b}$	3.15	$24.78^{a,b}$	2.55	26.93^{b}	3.57	0.005
% LBM	78.72	5.96	78.91	6.24	78.51	5.85	80.00	6.31	0.323
Lifetime OI	3687.06	1821.76	4110.25	2412.21	3836.58	2944.26	4647.16	2827.44	0.350
OI 5-18yrs	2083.36	1204.87	1984.89	1402.75	1714.73	1872.34	1837.80	1269.79	0.585
OI 19+ yrs	1548.6	1153.43	1946.95	1765.21	1872.35	1943.22	2514.45	2242.56	0.263

^{a,b} Means with unlike superscript letters were significantly different ($P < 0.05$).

4.3.2 Nutritional Profile

Table 4.7 reports the mean daily intake of selected macro- and micro-nutrients for the four protein groups. Intakes of magnesium (P = 0.001), phosphorus (P = 0.001) and vitamin D (P = 0.008) were significantly different amongst the groups. Potassium intake was almost significantly different amongst the groups (P = 0.051). Intake of magnesium and phosphorus was highest in the 'supplements plus extra dietary protein' group. Intake of vitamin D was highest in the "extra dietary protein' group.

Tukey's post hoc analysis revealed that magnesium and phosphorus intake was significantly higher in the 'supplements plus extra dietary protein' group than the 'normal diet' group and also significantly higher in the 'supplements plus extra dietary protein' group than the 'normal diet plus extra dietary protein' group.

Table 4.7: Macro- and micro nutrient intake per day of 4 diet types.

	Protein Group								
	Normal diet (*n* 46)		Extra dietary protein (*n* 19)		Extra protein supplements (*n* 14)		Extra protein supplements and dietary protein (*n* 48)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
Energy (kcals)	2676.88	620.03	2505.19	740.08	2515.55	555.12	2720.84	800.56	0.668
Fat (g)	110.33	36.28	112.12	38.56	112.43	34.72	109.9	32.64	0.912
Carbohydrate (g)	321.86	76.04	286.07	113.56	280.93	51.72	282.62	133.40	0.391
Calcium (mg)	1226.70	518.81	1188.60	606.85	1342.90	368.64	1234.92	643.93	0.907
Magnesium (mg)	339.73[a]	92.07	343.77[a]	156.01	360.36[a,b]	105.65	441.67[b]	117.41	0.001
Potassium (mg)	3659.74	940.21	3658.62	1413.18	3581.37	955.65	4317.13	1337.13	0.051
Phosphorus (mg)	1703.30[a]	472.12	1749.03[a]	686.42	1909.07[a,b]	587.99	2258.43[b]	693.25	0.001
Sodium (mg)	3633.04	1092.45	3467.57	1423.06	3395.93	597.72	3324.45	1294.06	0.735
Vitamin C (mg)	95.88	65.88	140.13	109.85	83.76	78.07	325.86	1305.54	0.182
Vitamin D (mg)	2.51	2.20	6.20	12.80	2.75	1.96	4.91	4.06	0.008

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$)

4.3.3 Amino Acid Profile.

The mean values and standard deviations for non-essential, essential, branched-chain and aromatic amino acids for each protein group 1) normal diet only, 2) normal diet plus extra food sources of protein, 3) protein supplements, 4) protein supplements and also extra food sources of protein) are illustrated in table 4.8 below.

There were significant differences ($P < 0.05$) in intakes of non-essential, essential, branched-chain and aromatic amino acids between the four groups with highest intakes of all amino acids in the ‘supplements plus extra dietary protein’ group and the lowest in the ‘normal diet only’ group.

Table 4.8: Amino Acid profile of 4 diet groups.

	Protein Group								
	Normal diet (*n* 46)		Extra dietary protein (*n* 19)		Extra protein supplements (*n* 14)		Extra protein supplements and dietary protein (*n* 48)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
Non-essential AA (g)	25.79	15.44	31.54	26.19	52.42	20.25	60.81	29.43	0.000
Essential AA (g)	14.85	9.67	15.15	5.71	40.21	19.89	44.49	24.98	0.000
BCAA (g)	7.39	4.66	7.48	2.82	18.72	7.52	21.36	12.02	0.000
AAA (g)	4.98	3.12	5.11	1.87	11.28	7.71	10.97	5.67	0.000

Abbreviations; AA - amino acids; BCAA - branched-chain amino acids; AAA = aromatic amino acids.

4.3.4 Total Protein and Essential Amino Acid Intakes compared to RDAs.

Table 4.10 outlines the mean RDA, the mean intake and the mean percentage of RDA achieved for protein by the four protein groups. RDA levels for protein were calculated by the same method as described in section 4.2.4. Each group achieved a mean of at least 100% of the RDA. The two groups who take protein supplements and supplements plus extra dietary protein had mean intakes of well over the RDA at 137% and 156% respectively.

Table 4.10: Total Protein Intake, RDA and % RDA achieved of 4 diet types.

	Protein Group								
	Normal diet (*n* 46)		Extra dietary protein (*n* 19)		Extra protein supplements (*n* 14)		Extra protein supplements and dietary protein (*n* 48)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
Total Protein (g)	100.56	25.98	112.12	38.56	161.25	51.09	202.75	73.43	0.000
RDA (g)	99.10	16.82	105.73	20.13	104.25	18.96	122.33	27.34	0.000
% RDA achieved (g)	104.48	27.21	107.39	35.63	136.77	57.81	155.96	65.51	0.000

[1] Protein RDA based on individual physical activity levels (Campbell *et al.* 2007) and lean body mass.

Table 4.11 presents the mean essential amino acid intake, mean RDA and mean % RDA for each of the four groups. The RDAs for the amino acids were calculated as described in section 4.2.4.

Intake of essential amino acids in the ‘diet only’ group and the ‘diet plus extra protein’ group are close to RDA levels. Essential amino acid intake in the ‘supplements only’ and the ‘supplements plus extra dietary protein’ groups are significantly higher than the groups who do not use supplements and far in excess of RDA levels, with intakes of essential amino acids up to 3 or 4 times the RDA.

Table 4.9: Total Protein and Essential Amino Acid intakes compared to RDAs of 4 diet types.

	Protein Group											
	Normal diet (*n* 46) 81kg			Extra dietary protein (*n* 19) 83kg			Extra protein supplements (*n* 14) 79kg			Extra protein supplements and dietary protein (*n* 48) 87kg		
	RDA	Mean	%RDA	RDA	Mean	%RDA	RDA	Mean	%RDA	RDA	Mean	%RDA
Total Protein (g)[1]	113.40	100.56	88	116.2 0	112.12	96	110.60	161.25	146	121.80	202.75	166
Isoleucine[2] (g)	1.62	1.85	114	1.66	1.90	114	1.58	6.20	392	1.74	5.75	330
Leucine[2] (g)	3.16	3.26	103	3.24	3.25	100	3.08	8.04	261	3.39	9.37	276
Valine[2] (g)	2.11	2.28	108	2.16	2.33	108	2.05	5.48	267	2.26	5.97	264
Phenylalanine[2] (g)	2.03	1.93	95	2.08	1.97	95	1.98	3.70	187	2.18	3.55	163
Tryptophan[2] (g)	0.32	0.52	163	0.33	0.52	158	0.32	1.25	384	0.35	1.40	400
Histidine[2] (g)	0.81	1.11	137	0.83	1.15	138	0.79	2.10	266	0.87	2.36	271
Methionine[2] (g)	0.81	0.92	114	0.83	0.94	113	0.79	1.89	239	0.87	2.42	278
Lysine[2] (g)	2.43	2.43	100	2.49	2.58	104	2.37	6.45	272	2.61	7.41	284
Threonine[2] (g)	1.22	1.60	131	1.25	1.66	133	1.19	4.47	376	1.31	5.51	421

[1]Recommended protein intake of 1.4g/kg based on mean of lower and upper recommended protein intake as subjects ranged between endurance and strength athletes.

[2]RDA's for individual essential amino acids based on WHO/FAO/UNU recommendations (World Health Organization, 2002).

4.3.5. Difference in bone health between the 4 diets.

Table 4.10 presents the differences in protein intake, bone biomarkers and bone health between the four protein groups. Protein from meat ($P = 0.008$) and NEAP ($P < 0.001$) were significantly different amongst the groups. The greatest differences were observed between the ‘supplements plus extra protein’ group and the ‘diet only’ group, with levels signifcantly higher in the ‘supplements plus extra protein’ group. There were no significant differences ($P > 0.05$) in any of the indicators of bone health between the four groups.

Even though the sample size of subjects in each of the 4 groups was low (which is indicated as a potential limitation of the study in section 1.4) using a sample size calculator to determine the actual sample size needed to produce a significant difference in the results found 175 subjects per group would be needed to increase the power of the research to 0.8, which is beyond the scope of this research.

Table 4.10: Difference in bone health between the four diets.

	Protein Group								
	Normal diet (*n* 46)		Extra dietary protein (*n* 19)		Extra protein supplements (*n* 14)		Extra protein supplements and dietary protein (*n* 48)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
NEAP (mEq/d)	10.37[a]	25.57	18.27[a]	16.68	26.01[a,b]	20.63	40.15[b]	38.63	0.001
Protein from meat (g)	40.25[a]	15.62	51.10[a,b]	26.69	48.35[a,b]	32.50	71.61[b]	45.26	0.008
Sulphur (mEq/d)	22.24	13.49	22.81	8.85	23.08	8.99	31.24	17.9	0.087
BMD (g/cm^2)	1.16	0.10	1.17	0.13	1.15	0.10	1.19	0.16	0.548
BMC (kg)	3.49	0.38	3.49	0.05	3.44	0.43	3.58	0.56	0.703
Urinary pH	6.32	0.41	6.34	0.41	6.31	0.38	6.16	0.38	0.338
U-Ca (mmol Ca/mmol Cr)	0.29	0.14	0.31	0.41	0.33	0.20	0.31	0.18	0.924
S-OC (ng/ml)	29.49	16.50	34.26	15.06	33.60	15.17	32.37	13.27	0.225
S-CTx (ng/ml)	1.45	3.37	0.97	0.30	1.06	0.46	0.99	0.37	0.815
Uncoupling Index	0.11	0.76	-0.50	0.74	-0.10	1.05	-0.07	1.81	0.705

[a,b] Means with unlike superscript letters were significantly different ($P < 0.05$).

4.4 Relationship between Components of Protein Intake and Bone Health

Partial correlations were used to explore the relationship between protein and indicators of bone health while controlling for factors including age, BMI, % lean body mass, OI 19+ yrs, and calcium, magnesium, potassium, phosphorus, sodium, vitamin C and vitamin D intake. Table 4.11 presents the *P* values and table 4.12 presents the r values for these correlations, to enable the direction of the relationship to be observed.

As expected total protein (diet plus supplements), was significantly related to dietary protein, BCAA, AAA, essential AA and non-essential AA ($P < 0.000$), all having positive correlations. It was also significantly related to NEAP ($P = 0.048$), this was also a positive correlation (r = 0.210).

Dietary protein, as expected, was significantly related to meat protein ($P < 0.000$), a positive correlation. There was no significant relationship between dietary protein and BCAA, AAA, essential AA and non-essential AA ($P > 0.050$). There was a strong positive correlation (r = 0.505) between dietary protein and NEAP ($P < 0.000$). Dietary protein was also significantly related to sulphur acid load ($P = 0.003$), this was a positive correlation (r = 0.314). There were no relationships between dietary protein and any measurements of bone health ($P > 0.05$).

Protein from meat was significantly related NEAP ($P < 0.000$) having a strong positive correlation (r = 0.418). Protein from meat was also significantly related to serum crosslaps (CTx) ($P = 0.015$). This was a positive correlation (r = 0.259)

indicating that as protein from meat increased levels of CTX increased, a bone resorption marker.

BCAA were significantly related to AAA ($P < 0.000$), essential AA ($P < 0.000$), non-essential AA ($P < 0.000$), and sulphur ($P < 0.000$). These were all strong, positive correlations..

Similarly, AAA were significantly related to essential AA ($P < 0.000$), non-essential AA ($P < 0.000$) and sulphur ($P < 0.000$) with all relationships being positively correlated.

As expected essential AA were significantly related to non-essential AA ($P < 0.000$) and sulphur ($P < 0.000$), again these were significant positive correlations

Non-essential AA were also significantly related to sulphur ($P = 0.000$), a positive correlation.

No relationship was found between urine pH and any dietary component or indicator of bone health ($P > 0.050$).

NEAP and sulphur are significantly positively correlated ($P = 0.002$).

BMD, as expected was significantly related to BMC ($P < 0.000$), this was a positive correlation. BMD was also significantly related to serum osteocalcin (S-OC) ($P = 0.004$). This was a negative correlation ($r = -0.304$), indicating that in individuals

with higher BMD, osteocalcin levels were lower. BMD had no significant relationship with any dietary component ($P > 0.050$).

BMC was also significantly related to S-OC ($P = 0.009$). This was also a negative correlation (r = -0.278) indicating that a higher BMC was associated with lower levels of S-OC. BMC was not significantly related to any dietary component ($P > 0.050$).

As expected, serum osteocalcin was significantly related to serum crosslaps ($P = 0.000$) and this was a positive correlation (r = 0.457), indicating higher osteocalcin levels are associated with higher crosslap levels.

Serum crosslaps was not significantly related to any dietary component or other indicator of bone health ($P > 0.05$).

No relationship was found between the uncoupling (UI) and any dietary component or indicator of bone health ($P > 0.05$).

Table 11: Protein and bone health correlations – *P* values

	Total Protein	Dietary Protein	Meat Protein	BCAA	AAA	Ess AA	Non-ess AA	Urine pH
Total protein	0.000[a]							
Dietary Protein	0.001[a]	0.000[a]						
Meat protein	0.070	0.000[a]	0.000[a]					
BCAA	0.000[a]	0.340	0.487	0.000[a]				
AAA	0.000[a]	0.170	0.439	0.000[a]	0.000[a]			
Ess AA	0.000[a]	0.288	0.436	0.000[a]	0.000[a]	0.000[a]		
Non-ess AA	0.000[a]	0.460	0.671	0.000[a]	0.000[a]	0.000[a]	0.000[a]	
Urine pH	0.701	0.281	0.121	0.666	0.942	0.639	0.713	0.000[a]
BMD	0.857	0.758	0.479	0.780	0.548	0.703	0.741	0.692
BMC	0.327	0.985	0.735	0.925	0.844	0.938	0.947	0.737
S-OC	0.109	0.682	0.119	0.931	0.982	0.964	0.339	0.508
S-CTx	0.465	0.161	0.015[a]	0.712	0.442	0.517	0.940	0.751
UI	0.674	0.921	0.325	0.881	0.899	0.887	0.605	0.419
NEAP	0.048[a]	0.000[a]	0.000[a]	0.395	0.329	0.346	0.476	0.068
Sulphur	0.456	0.003[a]	0.098	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.230
U-Ca	0.726	0.270	0.550	0.654	0.725	0.708	0.412	0.376

Table 11 continued

	BMD	BMC	S-OC	S-CTx	UI	NEAP	Sulphur	U-Ca
Total protein								
Dietary Protein								
Meat protein								
BCAA								
AAA								
Ess AA								
Non-ess AA								
Urine pH								
BMD	0.000 [a]							
BMC	0.000 [a]	0.000 [a]						
S-OC	0.004[a]	0.009[a]	0.000 [a]					
S-CTx	0.733	0.858	0.000 [a]	0.000 [a]				
UI	0.912	0.891	0.579	0.916	0.000 [a]			
NEAP	0.271	0.164	0.844	0.903	0.096	0.000 [a]		
Sulphur	0.720	0.517	0.057	0.336	0.499	0.109	0.000 [a]	
U-Ca	0.631	0.384	0.330	0.753	0.359	0.324	0.738	0.000 [a]

[a] indicates a significant correlation ($P < 0.05$).

Table 12: Protein and bone health correlations – r values

	Total Protein	Dietary Protein	Meat Protein	BCAA	AAA	Ess AA	Non-ess AA	Urine pH
Total Protein	1.000 [a]							
Dietary Protein	0.345[a]	1.000 [a]						
Meat protein	0.193	0.557[a]	1.000 [a]					
BCAA	0.629[a]	0.111	0.081	1.000 [a]				
AAA	0.579[a]	0.160	0.110	0.904 [a]	1.000 [a]			
Ess AA	0.648[a]	0.124	0.091	0.990 [a]	0.940 [a]	1.000 [a]		
Non-ess AA	0.512[a]	0.090	0.052	0.819 [a]	0.837 [a]	0.822 [a]	1.000 [a]	
Urine pH	0.044	0.123	0.176	-0.054	0.009	0.059	0.048	1.000 [a]
BMD	-0.019	0.033	-0.076	0.033	0.070	0.045	0.040	-0.045
BMC	-0.105	-0.002	0.036	-0.011	-0.023	-0.009	-0.008	-0.038
S-OC	0.173	0.044	0.168	-0.010	-0.003	0.005	-0.119	0.077
S-CTx	0.079	0.151	0.259[a]	0.044	0.091	0.077	-0.009	-0.037
UI	-0.049	0.012	-0.115	-0.019	-0.016	-0.018	-0.068	-0.101
NEAP	0.210[a]	0.505[a]	0.418[a]	0.099	0.114	0.110	0.087	0.206
Sulphur	0.080	0.314 [a]	0.177	0.504 [a]	0.571 [a]	0.494 [a]	0.503 [a]	0.137
U-Ca	-0.040	-0.126	-0.068	0.056	0.044	0.047	0.107	0.101

Table 12 continued

	BMD	BMC	S-OC	S-CTx	UI	NEAP	Sulphur	U-Ca
Total Protein								
Dietary Protein								
Meat protein								
BCAA								
AAA								
Ess AA								
Non-ess AA								
Urine pH								
BMD	1.000 [a]							
BMC	0.901 [a]	1.000 [a]						
S-OC	-0.304[a]	-0.278[a]	1.000 [a]					
S-CTx	-0.037	-0.019	0.457[a]	1.000 [a]				
UI	-0.013	-0.016	0.066	0.013	1.000 [a]			
NEAP	0.118	0.149	-0.021	0.014	-0.193	1.000 [a]		
Sulphur	0.039	-0.070	-0.205	0.104	-0.079	0.172	1.000 [a]	
U-Ca	-0.055	-0.099	-0.113	0.036	-0.115	-0.112	-0.038	1.000 [a]

[a] indicates significant correlation values.

Chapter Five

Discussion

5.1 Introduction

This chapter offers a discussion of the differences in physical characteristics, nutrient intake, protein intake, and bone health observed amongst young exercising males consuming variable levels of protein in this study. Section 5.2 discusses the differences observed between two groups, protein supplement users and non-users. Section 5.3 discusses the differences amongst the 4 groups of subjects consuming different levels of protein as outlined in the results section. The correlations observed between different components of protein intake and specific indicators of bone health are discussed in section 5.4.

5.2 Protein Supplement Users versus Non-Users

5.2.1 Demographics

It is important to note that there were no significant differences between supplement users and non-users in age or physical attributes such as weight, BMI, percentage lean body mass or OI, all of which contribute to bone health and could have confounded results when comparing the bone health of the two groups. It is also noteworthy that the group who consume extra protein supplements presumably in the attempt to gain increases in muscle mass did not have a significant difference in percentage lean body mass than those who do not consume supplements. This in one way validates the recommendation by nutritionists that extra protein supplementation is not necessary to enhance muscle mass and it is possible by dietary means to enhance muscle mass (van Loon *et al.* 2007).

5.2.2 Nutritional Profile

Macronutrients

Energy intakes for both groups were lower than recommended levels. An estimation of energy requirements for each group was calculated using the Schofield equation (Department of Health (DH), 1991) and a physical activity level (PAL) of 1.7 for moderately active men (DH, 1991). This gave an average requirement of 3281 kcals for non-supplement users and 3358 kcals for supplement users, marginally higher than the estimated intake of both groups (2615 kcals and 2907 kcals). However under-reporting of true energy intake is very common in dietary records (Black *et al.* 1997) and therefore it is not of remarkable concern.

Fat intake for both groups comprises 37% of reported energy intake, which is greater than the recommended intake of 35% energy from total fat (DH, 1991). The Survey of Lifestyle, Attitudes and Nutrition in Ireland (SLÁN) 2008 reported percentage of energy from fat as 38% in men aged 18-29 y (Harrington *et al.* 2008), which is higher than levels seen in this research.

Carbohydrate intake comprises 44% of total energy intake in non-supplement users and 39% in supplement users. Both are lower than the recommended intake of 50% of energy from carbohydrate (DH, 1991). This indicates that a greater proportion of energy is derived from fat and protein in particular in supplement users. This may be of concern as diets which contain a high percentage of energy from protein and fat are significantly related to cardiovascular disease (DH, 1994).

Micronutrients

Intake of magnesium, potassium, phosphorus, vitamin D, vitamin C and sodium in both supplement users and non-users met or exceeded RDA levels and were similar to reported intakes in Irish men of a similar age group according to the SLÁN survey 2008 (Harrington *et al.* 2008). Intakes of magnesium, potassium, phosphate and vitamin D were significantly higher in the protein supplement users than non-users.

There was no significant difference in energy intake between the two groups. If overall energy intake had been significantly higher in the supplement users it may have explained the higher intake of these nutrients purely by consuming a larger quantity and variety of foods, meaning there is a greater intake of all nutrients. However the lack of significant difference in energy intake means supplements users were consuming diets higher in micro-nutrient density. High protein foods such as meat, eggs, and dairy products are all significant sources of micronutrients, which could explain the higher intake of these in the supplement user group, as the majority of this group reported that as well as supplements they also try to consume extra protein through food sources.

Calcium intake at 1213 mg/d in non-supplement users and 1258 mg/d in supplement users was higher than the RDA of 800mg/d (Food Safety Authority of Ireland, 1999), and similar to reported calcium intakes in Irish men aged 18-29 of 1231 mg/d (Harrington *et al.* 2008). Meyer *et al.* (1997) and Shapses *et al.* (1995) observed that an adequate dietary calcium intake minimizes the hypercalciuric effect of excess dietary protein, limiting its adverse effect on bone. Dawson-Hughes and Harris (2002) observed that the potential anabolic

effects of protein may be maximized by a higher Ca intake. This suggests that the high level of calcium intake in supplement users may have protected the bone from the potential detrimental effects of a high protein intake. It is also possible that the higher protein intake in combination with an increased calcium intake may have had an anabolic effect on bone.

5.2.3 Amino Acid Profile

Intakes of all types of amino acids; non-essential, essential, branched-chain and aromatic amino acids were significantly higher in supplement users than non-users. There is little or no data reporting regular amino acid intake in individuals who do or do not consume protein supplements to compare with amino acid intakes in this study. Studies on amino acid intakes in athletes are mostly intervention studies which control amino acid intake rather than observing regular intake. The Life Science Research Office (LSRO) of the Federation of American Societies on investigation of the benefits of protein and amino acid supplements found limited data that documented the extent to which these supplements are used (Anderson & Raiten, 1991).

There is some evidence to suggest that amino acid supplementation; particularly BCAA may be beneficial in terms of reducing the net rate of protein degradation in response to exercise (Campbell *et al.* 2007). However some nutritionists suggest that resistance-type exercise training does not necessarily increase protein requirements because protein metabolism becomes more efficient in a trained state (van Loon *et al.* 2007). Other studies have shown minimal beneficial effects in lean tissue mass and strength in adults who use protein supplements in conjunction with resistance training (Candow *et al.* 2006). As there

was no significant difference in percentage lean body mass between supplement users and non-users in this research there was no indication that significantly higher intakes of amino acids improved muscle mass.

5.2.4 Total Protein and Essential Amino Acid Intakes compared to RDA levels.

Protein

There were significant differences in total protein intake, protein requirements and % RDA between the two groups. All were significantly higher in the supplement users. Both the supplement users and non-users had an ample dietary protein intake meeting where individual requirements were met. The non-supplement users had a mean percentage RDA of 106% while the supplement users had a mean of 152%, far in excess of requirements.

This emphasises the fact that dietary protein intake in the general population is ample and usually in excess of minimum requirements (Gregory *et al.* 1990). Mean protein intake in non-supplement users was 105 g/d which is slightly lower than the reported mean protein intake of 119 g/d in Irish men aged 18-29 y, according to the SLÁN survey (Harrington *et al.* 2008).

The supplement users had a mean protein intake of 194g protein/day which averages at 2.3g/kg based on the average weight of the group. This is higher than the upper limit of 2g/kg recommended for strength athletes. On further investigation of the data it was observed that 34/54 supplement users had intakes greater than 2g/kg and the maximum level observed was 4g/kg, double the upper recommended limit. This is in line with other

studies which have shown that strength and power athletes regularly consume protein intakes in excess of 3g/kg (Phillips *et al.* 2007; Faber & Benade, 1987; Keith *et al.* 1996). There is some concern that protein intakes at this level may damage renal function (DH, 1991) however, Martin *et al.* (2005) stated that there is no evidence that athletes consuming high levels of dietary protein, even with intakes in excess of 2.0g/kg/day are at greater risk of developing kidney disease or losses in renal function.

Protein intake comprises 16% of total energy intake in non-supplement users and 29% in supplement users. In the SLÁN survey (2008), protein intake comprised 17% of energy in men aged 18-29 y, similar to the level observed in the non-supplement users. Protein intake in supplement-users is much greater than the recommended 15-20% of energy intake for the general population (DH, 1991) and a safe intake of 25% for athletes to maintain or improve lean body mass as suggested by Bilsborough and Mann (2006).
However, according to the Institute of Medicine (2005) 20-40% of energy from protein for strength athletes is an 'Acceptable Macronutrient Distribution Ranges (AMDR) and as Viewed by Endurance and Strength Athletes as Sufficient' (IOM, 2005), therefore a protein intake of 29% as seen in the supplement users would be considered acceptable provided that they are involved in resistance training.

The main concern regarding excessive protein intakes in this research is the potential detrimental effect on bone health; results describing the effects of protein on bone health are discussed in section 5.2.5.

Amino Acids

In the non-supplement users, mean intakes of all the essential amino acids were close to RDA levels, with intakes of tryptophan and isoleucine greater than the RDA at 127% and 120% respectively. In the supplement-users, mean intake of all the essential amino acids were significantly greater than non-users and are far in excess of RDA levels. Intakes of all essential amino acids were at least 1.5 times the RDA level with intakes close to 4 times the RDA observed in tryptophan and isoleucine. As previously mentioned there is very little data with which to compare amino acid intakes. However as it has been reported that protein intake in athletes generally exceeds even the highest requirements (Phillips *et al.* 2007; Faber & Benade, 1987; Keith *et al.* 1996), presumably amino acid intake is also much greater than requirements which would coincide with the present results.

Excessive amino acids may have adverse effects; however, there is very little data to confirm or deny this position (Pencharz *et al.* 2008). For some amino acids considerable literature exists from human and animal studies; in particular, glutamate, aspartate and phenylalanine because of their use as food-flavouring agents, whereas there is much less data on other amino acids, particularly on adverse effects in humans (Garlick, 2004).

The most toxic essential amino acids for both humans and animals appear to be methionine and histidine (Garlick, 2004). Human studies involving methionine have revealed significant adverse effects. In a study by Commor *et al*. (1978) 8g methionine/d given over 4 days resulted in decreased serum folate and increased white cell count Methionine intake in this study was 2.3g/d in supplement users. If 8g methionine over 4 days can reduce

serum folate and increased white cell could it would suggest that regular intakes of at least 2g methionine/d over long periods of time could have equally adverse effects.

High dietary histidine levels have been shown to result in potentially serious adverse effects in both animals and humans (Garlick, 2004). In human studies, when four overweight/obese subjects were given 24-64 g/d of histidine, increases in urinary zinc, headache, weakness, drowsiness, nausea, anorexia, painful eyes, changes in visual acuity, mental confusion, poor memory and depression occurred (Geliebter *et al.* 1981). However, there were no overt side effects when up to 4.5 g/d of histidine was given as treatment for obesity, rheumatoid arthritis and chronic uraemia (Garlick, 2004). Mean intake of histidine was 1.12g in non-users and 2.31 g in supplement users which would appear to be a safe level.

There is no evidence of serious adverse effects attributable directly to high intakes of tryptophan in humans (Garlick 2004). Tryptophan is a precursor for serotonin, a brain neurotransmitter theorized to suppress pain (Williams 2005). It is widely sold as a sleep aid. In animals fed low-protein diets high intakes of tryptophan were found to depress food intake and growth but not in those fed higher protein diets (Harper *et al.* 1970).

Threonine has been studied extensively but appears to be one of the least toxic of the amino acids (Garlick, 2004). In human studies no serious side effects were reported when up to 6g of threonine was given daily for 2 weeks to patients with spasticity (Growdon *et al.* 1991). In this research threonine intake was 1.62 g/d in non-users and 5.28 g/d in users.

In general, there is little evidence of serious adverse effects in humans from most amino acid supplements (Garlick, 2004) however this does not mean that there is no potential for

adverse effects resulting from high intakes of amino acids, particularly as data related to humans is very limited. There have been no upper limits established for amino acid intake as existing dose-response data is inadequate (IOM, 2002), as such, unanticipated adverse consequences of consuming large amounts cannot be ruled out. Use of amino acid supplements is not prohibited by the World Anti-Doping Agency (WADA) (Williams, 2005). It is also worrying that a number of protein supplement manufacturers would not supply the amino acid information on their products, which suggests that something may be amiss.

5.2.5 Protein Intake and Bone Health

Protein and dietary acid load

Total protein intake ($P < 0.001$), protein from meat ($P = 0.008$), NEAP ($P < 0.001$) and the sulphur acid load of the diet ($P = 0.031$) were all significantly higher in the supplement users than the non-users. All of these have been cited as contributing factors to the theorized dissolution of bone in high protein diets (Feskanich *et al.* 1996; Thorpe *et al.* 2008; Remer *et al.* 2003).

Total protein intake in this study, 104.7 g/d in non-users and 193.53 g/d in users, is higher than in some studies which have shown a negative intake of dietary protein on bone health; Metz *et al.* (1993) observed that intakes of ~73g protein/d was negatively associated with BMD and BMC in young adult women; Abelow *et al.* (1992) found that 60-80 g protein/day was associated with greater risk of hip fracture in women. There appears to be no studies which have shown a negative impact of dietary protein intake on men, most of

these studies are conducted in peri-menopausal women. Females consistently report to not meet the RDA for calcium (Gregory *et al.* 1990; Harrington *et al.* 2008) while males consistently consume the RDA for calcium (Harrington *et al.* 2008), it may be the lack of calcium intake in studies involving females causes a disparity in results.

Protein from meat has been reported to have a more damaging effect on bone than vegetable protein (Sellmeyer *et al.* 2001). Sellmeyer *et al.* (2001) observed that a high ratio of animal protein to vegetable protein containing ~ 48 g of animal protein/d was associated with a high rate of hip fracture and bone loss in elderly women. In this research protein from meat was 44 g/d in non-users and 66 g/d in users, significantly higher than levels in Sellmeyer *et al.*'s study (2001). However, Roughead *et al.* (2003) found no adverse effects of a high-meat diet (117 g protein/d from meat) on bone health in healthy postmenopausal women. In addition, Promislow *et al*. (2002) found a high animal-protein intake had a positive association with BMD in elderly men as well as women. For every 15g/day increase in animal protein intake, BMD increased significantly at the hip, femoral neck and total body. However this association was not statistically significant in men.

No studies have examined net endogenous acid production and sulphur acid load in relation to bone health in young exercising males. Most of the literature regarding these dietary components in relation to bone health is conducted in peri-menopausal women (Macdonald *et al.* 2005; Thorpe *et al.* 2008).

One study examined the relationship among dietary estimates of net endogenous acid production, bone mineral density and biochemical markers of bone turnover in 1028 healthy men and women aged 20-72 years (Rahbar *et al.* 2009). Mean estimate of energy-

adjusted rate of NEAP was measured by the ratio of protein to potassium intake normalized to a diet of mean calories (Frassetto *et al.* 1998). In men, this averaged 109.69 mEq/d. This was much higher than the mean NEAP observed in this research which was calculated using a function of protein, calcium, potassium, magnesium and phosphorus intake known as the potential renal acid load (PRAL) as well as an anthropometry-based estimate for organic acid excretion (Remer *et al.* 2003), (see section 3.5.1). Mean NEAP in the current study was 14.45 mEq in non-users and 33.33 mEq/d, in supplement users. In a study by Macdonald *et al.* (2004) which looked at estimates of NEAP in association with low bone mineral density in perimenopausal women, the method by (Remer *et al.* 2003) was also used to calculate NEAP. NEAP in Macdonald *et al.*'s study (2004) ranged from 30-50mEq/d, closer to the range observed in our results. The women with the lowest quartile of NEAP (30 mEq/d) had greater axial and peripheral bone mass than the women with the highest quartile of NEAP (50 mEq/d).

In Thorpe *et al.*'s study (2008) mean sulphur acid load in 161 postmenopausal women was 28.4 mEq/d, using the same method used in this study (Sebastian *et al.* 1994). This was similar to levels found in our study, 22.45 mEq/d in non-users and 28.61 mEq/d in supplement users. At this level Thorpe *et al.* (2008) found that dietary protein was positively associated with BMD but benefit at the lumbar spine was offset by a negative impact of the protein sulphur acid load.

Indicators of Bone Health

There were no significant differences observed between the supplement users and the non-users in any of the indicators of bone health including BMD, BMC, S-OC, S-CTx, urine pH and urinary calcium or uncoupling index, which would suggest that protein supplementation of 33 months duration has no effect on bone health in exercising males.

Again, studies with which to compare the results found in this research are limited as most studies which have examined the effects of protein supplementation on bone health are conducted in peri-menopausal women or elderly subjects rather than young men, and different parameters of bone health are measured. There appears to be no studies which have demonstrated a negative effect of protein on bone health in men. Meyer *et al.* (1997) found no association between non-dairy animal protein intake and hip fracture incidence in men aged 35-49 consuming an average of 67.4 g protein/day over an 11 y period. Hannan *et al.* (2000) found a protein intake of > 82 g/d had a positive effect on BMD in elderly men > 75 y in a 4 y prospective study. In this research protein intake averaged at 105 g/d in non-supplement users and 194 g/d in supplement users, these levels are much higher than those observed in studies examining the relationship between protein and bone.

Ballard *et al.*'s study (2005) was the only study which looked at increasing protein supplementation on bone health in exercising men. Unfortunately comparisons cannot be made for BMD results as Ballard *et al.* used volumetric and areal BMD measurements as indicators of bone health rather than whole body BMD which was used in this research. In Ballard *et al.*'s study (2005) mean BMC at baseline for both men and women was 2.82 kg and there were no significant differences after the 6 month strength training and protein

supplementation. Mean BMC in this research was 3.47 kg in non-supplement users and 3.55 kg in supplement users with no significant difference between the two groups. The higher BMC in this research may be due to the fact that they are all men rather than a mixture of men and women as in Ballard *et al.*'s study which would reduce the mean as women tend to have a lower BMC than men (Cauley, 2006). The lack of any difference in BMC after the 6 month protein supplementation and training period in Ballard *et al.*'s study reflects the results observed in this research, in which we found no difference in BMC between protein supplement users and non-users. This suggests that high protein intakes do not affect BMC.

In a study by Ratamess *et al.* (2007), mean whole body BMD in 33 male, healthy resistance-trained football players (mean age 20 y) was 1.4g/cm^2. This is higher than the BMD observed in this study, 1.16g/cm^2 in non-users and 1.19 g/cm^2 in supplement users. However the sample in this study are a mix of all types of athletes, from intermittent team sport players to endurance and strength athletes rather than purely strength athletes which may account for differences observed in BMD, as resistance training is known to have an anabolic effect on bone (Suominen, 2006).

Mullins & Sinning's study (2005) looked at the effect of resistance training and protein supplementation in young healthy women. Serum osteocalcin was measured as an indicator of bone formation and urinary calcium was measured as an indicator of bone resorption. Mullins & Sinning (2005) observed a higher level of urinary calcium excretion in the protein supplemented group while in this research there was no significant difference in urinary calcium between supplement users and non-users. This may be explained by a gender difference and by the much shorter duration of protein supplementation in Mullins

& Sinning's (2005) study (10 days) compared to this study, in which average length of supplement use was 33 months. Roughead *et al*. (2003) observed an adaption in renal acid excretion and hypercalciuria over time in response to high protein diets. This would also explain why there was no significant difference observed in urine pH between supplement users and non-users.

Serum osteocalcin levels at baseline were 9-10 ng/ml in Mullins & Sinning's (2005) study and there were no significant changes with protein supplementation. Similiarly in this research there were no significant differences in osteocalcin between supplement users and non-users, however osteocalcin levels were a lot higher, 30.39 ng/ml in non-supplement users and 32.96 ng/ml in supplement-users. This is presumably due to differences in male and female bone homeostasis. Ratamess *et al.* (2007) reported similar osteocalcin levels in 33 male, healthy resistance-trained football players of 25-34 ng/ml at baseline (before resistance training period).

In most studies examining indicators of bone resorption in relation to dietary protein, urinary N-telopeptide is used (Ince *et al*. 2004; Kerstetter *et al.* 1999) rather than serum crosslaps as in this research. In addition these studies measure levels of resorption markers after a short-term experimental period rather than habitual levels so comparisons cannot be made due to adaptation differences.

To my knowledge no study has included uncoupling index as an indicator of bone health in relation to dietary protein to compare with results from this study.

It is interesting that despite significant differences in NEAP and dietary sulphur content, there were no differences observed in indicators of bone health between the two groups.

However, in Thorpe *et al.*'s study (2008) it was observed that neither PRAL or NEAP using the protein:potassium ratio contributed to the prediction of aBMD. In addition, the negative impact of the protein sulphur acid load on BMD was only observed at one of the two sites measured which implies that it may not have a significant effect on whole body BMD.

It is possible that other factors were involved which may have negated the negative acidifying effects of protein. Potassium, magnesium, phosphorus and vitamin D were all significantly higher in the supplement group. All of these nutrients are well established as having important roles in the maintenance of bone health. Potassium and magnesium are major contributors to the dietary alkali load (Prentice *et al.* 2006), found as alkali salts in fruit and vegetables, which would help to neutralize the dietary acid load and minimize any potential negative effect that might have on bone. Phosphorus is essential for bone structure; bone mineral is predominantly calcium phosphate (Heaney, 2004). Vitamin D may have a direct effect on bone synthesis through its involvement in calcium homeostasis (Arens & Thomas, 2001). A higher intake of these nutrients which have a positive effect on bone may have negated the potential negative effects of the higher protein intake.

While there was no evidence for a negative effect of protein supplementation on bone health observed in this research, there was also no evidence that protein had a positive effect on bone. This suggests that protein intake exceeding RDA levels has no additional benefit on bone health.

5.3 Comparisons between 4 diet types

The subjects were divided into four groups as follows based on their protein intakes; 1) protein in normal diet only, 2) reported extra dietary protein 3) supplement users, 4) supplement users plus reported extra dietary protein.

5.3.1 Demographics

BMI was the only significantly different physical characteristic amongst the four groups. It was highest in the group who took extra dietary protein as well as supplements (26.93 kg/m^2) and lowest in the group who consumed a normal diet only (24.89 kg/m^2). Although there was no significant difference in percentage lean body mass between the four groups, it was lowest in the diet only group who also had the lowest BMI, and percent lean body mass was highest in the high protein diet and supplements group. This would suggest that the greater BMI observed in the group taking extra dietary protein may be due to a slightly greater muscle mass.

5.3.2 Nutritional Profile

Macronutrients

Protein intake was significantly higher in the ‘supplements plus extra dietary protein group’ than in the supplements plus extra dietary protein group’. Protein comprised 30% of energy intake in the ‘supplements plus extra dietary protein group’ compared to 15% of energy in the ‘normal diet’ group. This is a huge difference. As explained above 30% of energy from

protein is far in excess of recommendations for general population (15-20%) (DH, 1991) but 20-40% of energy from protein is recognised by the IOM as an acceptable range for strength athletes (IOM, 2005).

Percentage of energy from fat is higher than the recommended < 35% in all groups, with the highest percentages of 40% observed in the 'normal diet plus extra food sources of protein' and the 'protein supplements' groups.

Percentage of energy from carbohydrate is lowest in the 'supplements plus extra dietary protein group' at 39%. This is marginally lower than the recommended 50% (DH, 1991). Carbohydrate intake is presumably spared at the expense of a higher protein intake. Total carbohydrate intake in this group was 283 g/d, which averaged at 3 g carbohydrate/kg. This would not be sufficient for athletes with a moderate training program who require 6-10 g carbohydrate/kg depending on type, length and intensity of the training (Crosland, 2001). Therefore these athletes are likely to have low muscle glycogen stores which would reduce energy levels, limit performance and reduce resistance to infection (Sports Dietitians Australia, 2009). Low carbohydrate diets are also not recommended due to a lower intake of dietary fibre and reduced intake of vitamins and minerals found in carbohydrate foods. In addition, as mentioned previously, a higher percentage of energy from protein and fat is significantly related to cardiovascular disease (DH, 1994).

Micronutrients

Intakes of magnesium, potassium, phosphorus and vitamin D were significantly different amongst the groups with the highest intakes of all the nutrients in the 'supplements plus

extra dietary protein group' and the lowest intakes in the groups that don't take supplements. Again, as explained in section 5.2.3 high protein foods such as meat and dairy products are significant sources of magnesium, potassium, phosphorus and vitamin D which explains the marginally higher intake of these nutrients in the 'supplements plus extra dietary protein group'.

All of the four diets meet the RDA for vitamins and minerals. Athletes may require greater intakes of micronutrients as exercise stresses many of the metabolic pathways where micronutrients are required (Rodriguez *et al.* 2009) therefore the groups consuming additional protein may be at an advantage in terms of a greater intake of micronutrients consumed through protein rich foods.

The group who consumed a normal diet without any additional protein met requirements for all macro- and micronutrients and protein intake was averaged at 1.24g/kg which is well above the minimum requirement for protein (0.8 g/kg) and may only be slightly insufficient to meet the requirements of endurance and strength athletes which require 1.4 g/kg and 2g/kg respectively (Campbell *et al.* 2007). This questions the need for excessive protein intake through diet and supplements if both protein and the additional vitamins and minerals provided by food sources are in excess of requirements and have no additional benefit.

5.3.3 Amino Acid Intake

There were significant differences in intakes of non-essential, essential, branched-chain and aromatic amino acids between the four groups with highest intakes of all amino acids in the

'supplements plus extra dietary protein' group and the lowest in the 'normal diet only' group. It is difficult to say whether those who have a higher intake of amino acids are gaining any benefit in terms of increased strength or muscle mass or if in fact they are actually damaging their health due to potentially toxic levels of amino acids. Based on percentage lean body mass there were no differences amongst the groups, suggesting that amino acid intake has no beneficial effect on muscle mass. However, perhaps further measures of strength would indicate differences amongst the groups.

5.3.4 Total protein and Essential Amino Acid Intakes compared to RDAs.

Total protein intake in the 'supplements plus extra dietary protein' group (202.75 g) was double the protein intake in the 'normal diet only' group (100.56 g). In the 'normal diet only' group protein supplementation averaged at 1.2 g/kg which is more than ample for most of the population. The 'supplements plus extra dietary protein' group consume on average 2.3 g protein/kg, exceeding upper recommended limits. Each group achieved a mean of at least 100% of the RDA. The two groups who take protein supplements and supplements plus extra dietary protein had mean intakes of well over the RDA at137% and 156% respectively.

These results amplify the vast differences in protein intake between athletes who consume a normal diet and athletes who are preoccupied with protein and consume vast amounts through food sources and supplements. There is no evidence that consuming protein in excess of requirements offers any advantage to athletes (Rodriguez *et al.* 2009), yet athletes

are either unaware or choose to ignore this evidence and continue in vain to consume vast quantities of protein.

Up to 3 or 4 times the RDA of essential amino acids are consumed in both the group who consume ‘supplements only’ and in the group who consume ‘supplements plus extra dietary protein’. Intakes of amino acids in both groups that do not take any supplements have intakes close to RDA levels.

As discussed in section 5.2.4 the groups consuming high levels of amino acids are potentially putting their health at risk. There is insufficient data to suggest a safe level for amino acid intake so it is unknown if long term supplement use will have serious adverse effects on health or not.

5.3.5 Protein Intake and Bone Health

Total protein, protein from meat and NEAP were significantly different amongst the groups. There were no significant differences ($P > 0.05$) in any of the indicators of bone health between the four groups. These results reflect those observed when comparing protein and bone health between supplement users and non-users as discussed in section 5.2.5. Dividing the subjects into 4 subgroups which amplified differences in protein intake did not reveal any other noteworthy observations in terms of bone health.

5.4 Relationship between specific components of protein intake and Bone Health.

There was a strong positive correlation between total dietary protein and all types of amino acids. Interestingly there was no relationship between protein from diet only and any of the amino acid types. This is perhaps due to protein supplements being purified sources of amino acids whereas amino acids in food sources are not as concentrated. This demonstrates that protein supplements make a significant contribution to amino acid intake.

Total protein, dietary protein and protein from meat were all significantly positively correlated with NEAP. This confirms that protein is a significant contributor to endogenous acid production. However there was no correlation found between NEAP and any indicator of bone health which suggests that NEAP itself does not significantly affect bone resorption or formation. This supports results from Thorpe *et al.*'s study (2008) which observed that neither PRAL or NEAP using the protein:potassium ratio contributed to the prediction of aBMD. This also contradicts MacDonald *et al.*'s study (2004) which found that postmenopausal women with the highest quartile of NEAP (50 mEq/d) had the lowest axial and peripheral bone mass, suggesting a negative effect of NEAP on bone. Again, differences in male and female hormonal status and bone structure make it difficult to compare studies.

Dietary protein, BCAA, AAA, essential and non-essential AA were all significantly related to dietary sulphur acid load. This indicates that if sulphur content has a significant negative effect on bone health as Thorpe *et al*.'s study (2008) would suggest, it would be very difficult to ascertain what sources of protein should be avoided as all types of amino acids were significantly related to sulphur and sulphur-containing amino acids are found in nearly

all food sources of protein (Rizzoli & Bonjour, 2004). However total protein, (from diet and supplements), was not significantly related to sulphur, which suggests that perhaps dietary sources of protein rather than purified protein have higher sulphur contents. Total protein was significantly related to NEAP however, which suggests that compounds other than sulphur found in protein supplements contribute to the dietary acid load. However no relationship was observed between sulphur and any indicator of bone health. This does not support Thorpe *et al.*'s (2008) theory that sulphur acid load has a negative effect on bone health, however perhaps if measurements of BMD had been made at specific sites as in Thorpe *et al.*'s study we may have observed a relationship.

One of the most interesting results we found from the correlations was that protein from meat was significantly related to serum crosslaps, an indicator of bone resorption. This was a positive correlation which indicates that as protein from meat increases, CTx levels are increased, suggesting that protein from meat may induce bone resorption. Protein from meat has been suggested as having a more negative effect on bone health than vegetable protein due to a greater content of acid precursors (chloride), also found in meat (Heaney & Layman, 2008). However Roughead *et al.* (2003) found no indication of any detrimental effect of a high meat protein intake (117 g/d) on bone health in postmenopausal women. As protein from meat in this research was not related to BMD or BMC there is not enough evidence to suggest that it might have significant detrimental effects on bone.

Urine pH and urinary calcium were not significantly related any component of protein or indicator of bone health. As studies have shown that higher initial renal acid excretion and hypercalciuria observed in high protein diets tends to abate over time (Roughead *et al.* 2003) it is not surprising that no relationships were observed with urinary pH and urinary

calcium as the higher protein diets were consumed over long periods of time, at least 6 months before testing.

Both BMD and BMC had a significant negative correlation with serum osteocalcin, indicating higher BMD and BMC is associated with lower levels of osteocalcin. This may be due to lower rates of bone turnover in individuals with greater BMD and BMC, although there was no association observed between BMD and BMC with serum crosslaps. However osteocalcin and crosslaps are significantly related so this may support the theory that the negative correlation observed between BMD, BMC and osteocalcin is due to lower rates of bone turnover.

BMD and BMC did not have any relationship with any components of protein or dietary acid load suggesting that protein supplementation in young exercising males does not have a negative or positive effect on bone. This is similar to results found in Ballard *et al*'s study (2005) which saw no effect of protein supplementation on bone in young, exercising males and females over 6 months. It is also similar to results in Mullins & Sinning's study (2005) which found no effect of a high protein intake for 10 days on bone metabolism in healthy exercising young women.

5.5 Concluding Remarks and Recommendations

Protein intake in young exercising men consuming protein supplements is far in excess of the upper recommended limits of protein intake of 2 g/kg/d and may even be doubling this level. There is no tolerable upper limit established for protein intake (IOM, 2002) and no significant evidence of any detrimental effects on bone health or renal function of excessive

protein intake in young, healthy individuals. However there are no benefits to be gained by consuming protein far in excess of requirements, particularly at the expense of carbohydrate intake. In addition, protein supplements can be harmful as they are still widely unregulated in terms of contamination and containing banned substances.

Intakes of essential amino acids in supplement users are exorbitantly higher than RDA levels, with intakes of up to 3 or 4 times the RDA. While there is no established tolerable upper limit for amino acid intake (IOM, 2002) there is still a strong possibility of serious adverse effects on health with long term excessive intakes.

The aim of this study was to assess whether a high protein intake in those consuming extra quantities of protein through diet and supplements had a negative on bone. We found no difference in bone health between subjects consuming large quantities of protein through diet and supplements and those consuming normal quantities of protein through diet only. This suggests that protein supplementation of an average of 33 months has no detrimental or beneficial effect on bone.

Supplement users had significantly higher intakes of magnesium, potassium, phosphorus and vitamin D which all have a positive effect on bone. It is possible that the alkalizing effect potassium and magnesium salts may have negated the acidifying effects of the elevated protein intake, thus minimizing any detrimental effect on bone. In addition vitamin D and phosphorus have important roles in the structure and maintenance of bone and this may have offset the potentially damaging effect of protein.

Recommendations

There appears to be no limit to the amount of protein through diet and supplements that athletes of all levels will take to achieve their desired goal. Athletes need to be aware of the risks involved in consuming large quantities of protein and amino acid supplements. While there is no tolerable upper limit established for either protein or amino acids, information regarding the minimal level of supplementation that is required to confer any benefits should be available to athletes and that intake of amino acids above this level does not confer any benefit and may be potentially damaging to health. As the supplement industry is largely unregulated and cannot be relied upon to issue any health warnings it is the responsibility of national bodies such as the Food and Safety Authority of Ireland, the Irish Nutrition and Dietetic Institute and the Irish Sports Council to relay this information to athletes. Athletes should at least be aware of the potential risks involved in consuming excessive amino acids and can then make informed decisions regarding supplement use.

The age limit of our study was 35 as after this age bone begins to deteriorate and this would confound results when comparing bone health with younger subjects. While this study showed no effect of high levels of protein on bone health literature suggests that it cannot be ruled out as a potential risk factor in the development of osteoporosis, 20% of the calciuria observed in high protein diets could be of skeletal origin (Kerstetter *et al.* 1999)

Future studies in this area should examine the effects of high levels of protein intake over long periods of time in middle-aged men over the age of 35. Men of this age involved in strength training and bodybuilding regularly consume excessive protein through diet and supplements. As bone health in this age group of men will have already begun to

deteriorate as a result of the aging process it is important that they are not potentially confounding this by consuming large quantities of protein which may be having a negative effect on bone.

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Appendix A

Appendix A: Consent Form

Waterford Institute *of* Technology
INSTITIÚID TEICNEOLAÍOCHTA PHORT LÁIRGE

Bone Health Study
Consent by Subject for Participation in Research Protocol

Subject Number:_____ Name of Volunteer:_____

Title of Protocol: Protein and bone health in young exercising males.

Researcher: Rebecca Warner
Phone: 087 9499136
Email: rebeccarosewarner@gmail.com

Supervisor: Dr. Lorna Doyle
Email: lmdoyle@wit.ie

You are being asked to participate in a research study. The researchers at Waterford Institute of Technology study the impact of exercise and dietary practices on possible disease development in an attempt to reduce further disease incidence. In order to decide whether or not you want to be part of this research study, you should understand enough about its risks and benefits to make an informed judgement. This process is known as informed consent. This consent form gives detailed information about the research study which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Osteoporosis and bone fragility affects one in three women and one in five men in Ireland. The incidence of osteoporosis is increasing among males. Protein supplement use is also increasing amongst male athletes. Increased protein intake increases urine acidity, calcium excretion, bone resorption, and ultimately reduces bone health. Studies have demonstrated the association between reduced bone health and increased protein consumption in normal populations, but few have examined increased use of protein supplements in athletes, or components of protein intake, and their potential effect on bone health. Due to increasing osteoporosis incidence in males this study investigates the influence of protein supplement use and components of protein intake (meat, sulphur-containing amino acids, high acid/alkali load), on urine acidity, calcium excretion and bone health.

What does it involve?

Each subject will be screened to ensure they have no factors which could affect bone health and are well enough to take part in the study. After screening each subject will complete a physical activity questionnaire (osteogenic index measurement) to assess their physical activity level, since this could have a beneficial effect on bone health. Each subject will then complete a food diary for 3 days (2 weekdays and 1 weekend day) which will be analyzed on the dietary analysis programme CompEat. While the 3 day diary is being completed each subject will collect 3 first morning urine samples for measurement of urine pH. A blood samplewill be taken from each subject by a trained phlebotomist. This sample will be used to analyze bone formation and resorption indicators. Bone density in each subject will be measured with use of DEXA.

How inconvenient will this study be to you?

Taking blood sometimes may cause bruising. Very rarely it may cause inflammation of the vein and possible infection. The phlebotomist makes every effort to avoid these situations. You will be asked to fast overnight on occasions that you give blood samples, this entails not eating from approximately 9.00 pm the night before and delaying breakfast until after the blood sample (between ~8.00 - 9.00 a.m.) which will be taken here in Waterford Institute of Technology.

We will be glad to provide you with the results of this study including your dietary intakes. The information that we collect is only for our research and will be confidential. This information will be stored in a secure place and in any publications that arise from this research; volunteers will be identified by number codes only.

The DEXA scanner used to measure bone density emits a very small dose of radiation, about 0.01 mSv, which is about the same as the average person receives from background radiation in one day, so the potential carcinogenic effect of exposure to radiation is minimal.

We consider this study to involve only "minimal risk", that is we think the worst thing to happen would be minor bruising after the taking of blood.

Benefits to the volunteer

- As a result of taking part in this research volunteers can get feedback on:
- Dietary intake and advice
- Bone health status
- Body fat and protein content

Your decision to take part in this study is entirely voluntary. You may leave the study at any time. If you have any questions concerning the study, you may contact Ms. Rebecca Warner at 087 9499136 who will deal with any queries you have.

Agreement to Consent

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no

guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and I may withdraw my consent at any time. Agreement to consent to take part in this study adheres to the regulations of the Data Protection Act. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. No subject in this research will be referred to and will be assigned a code (subject number) when dealing with result presentation, in order to ensure confidentiality. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I, the undersigned, hereby consent to participate as a subject in the above-described project conducted at the Department of Sport and Exercise Science, Waterford Institute of Technology. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the researchers listed above.

After reading the entire consent form, if you have no further questions about given consent, please sign where indicated.

Researcher:_____

Signature of Subject:_____

Witness:_____

Date:_____ Time:_____ am/pm (circle)

Appendix B

Appendix B: Medical Screening Form

Waterford Institute *of* Technology
INSTITIÚID TEICNEOLAÍOCHTA PHORT LÁIRGE

Bone Health Study
Screening Questionnaire

All information provided will remain confidential

Personal Details

Name:_____

Address:_____

Phone:_____Email: _____

Height: _____Weight: _____

Year of birth:_____

GP's name:_____

GP's address:_____

GP's number:_____

Medical History

1. Have you ever broken any bones or experienced stress fractures?
   ☐Yes ☐No
   If yes please give details
   .....
2. Have you ever been immobilized for more than two weeks? ☐Yes ☐No
   If yes please give details
   .....
   .....
3. Has anyone in your family suffered from osteoporosis? ☐Yes ☐No
   If yes please give details
   .....
4. Do you, or have you ever, suffered from any problems concerning your bones or joints (i.e. osteoarthritis, rheumatism, lower back pain, metabolic bone diseases)?
   ☐Yes ☐No
   If yes please give details

.....

5. Are you suffering from any of the following conditions?

Thyroid or parthyroid disorder	☐Yes ☐No
Kidney disease	☐Yes ☐No
Digestive/Hormonal Disorder	☐Yes ☐No
Diabetes	☐Yes ☐No

Drug History

6. Are you now, or have you ever taken any of the following medications and if so for how long and at what age?

   Diuretics ☐Yes ☐No

   .....

   Antibiotics ☐Yes ☐No

   .....

   Antacids ☐Yes ☐No

   .....

   Digoxin ☐Yes ☐No

   .....

   Steroids ☐Yes ☐No

   .....

   Sleeping tablets ☐Yes ☐No

   .....

7. Are you currently taking any other medication? ☐Yes ☐No

   If yes please give details

   .....

8. Do you smoke? ☐Yes ☐No

   If YES;

   How old were you when you started? .....

   How many do you smoke per day on average over the last year? .....

   If NO;

   Have you ever smoked? ☐Yes ☐No

   How old were you when you started? .....

   How old were you when you stopped?.....

   How many did you smoke/day on average?.....

9. Do you drink alcohol? ☐Yes ☐No

   If YES;

   How many days per week.....

   What do you drink and how much on an average day/night

   During week .....

At weekend .....

10. Do you or have you ever taken any protein supplements (e.g. creatine, whey/casein protein powder, protein bars, etc.) ? □Yes □No

If YES;

Type of supplement and brand name	**When did you start taking it?**	**When did you stop?**	**How much**	**How often**

11. Do you try to take extra protein in your diet? □Yes □No
If YES;
What foods/drinks do you eat more of and how much?.....

12. Do you take any vitamin or mineral supplements? □Yes □No
If YES;

Type of supplement and brand name	**When did you start taking it?**	**When did you stop?**	**How much**	**How often**

Appendix C

Appendix C: Physical Activity Questionnaire

Waterford Institute *of* Technology
INSTITIÚID TEICNEOLAÍOCHTA PHORT LÁIRGE

Bone Health Study - Physical Activity Questionnaire

Section 1. Occupation

1. What is your current occupation? If you are a student please state this as your occupation and also list any part time work during term-time.

_____

2. If you have or have ever had a physically active job please list in the table below.

Job	When did you start	When did you finish	Average number of hours per week physically active on the job.

Section 2. Sport and Leisure

1. Please circle all of the activities below that you have participated in on a regular basis throughout your lifetime.

00 Aerobics
01 Athletics
02 Badminton
03 Basketball
04 Boxing
05 Canoeing
06 Circuit training
07 Cycling
08 Dancing
09 Diving
10 Fishing
11 Gaelic Football
12 Golf
13 Gymnastics
14 Hiking
15 Hockey
16 Horseriding
17 Hurling
18 Jogging
19 Martial Arts
20 Orienteering
21 Racquetball
22 Rugby
23 Rockclimbing
24 Rowing
25 Sailing
26 Skating
27 Skiing
28 Soccer
29 Softball
30 Squash
31 Surfing
32 Swimming
33 Table-tennis
34 Tennis
35 Volleyball
36 Walking (for exercise)
37 Walking (occupational)
38 Waterpolo
39 Waterskiing
40 Weightlifting
41 Yoga/Pilates

For all the activities circled please fill out the table below using the numbers given to each sport as above. We need to know....

a. The number of years the activity was performed in that age period.
b. The average number of months per year that the activity was performed during those years.
c. The average number of hours per week that the activity was performed during that time.

Sport Code	**Age 6-18**			**Age 19-30**		
	No. of yrs	Mths/yr	Hr/wk	No. of yrs	Mths/yr	Hr/wk

Section 3. Exercise Diary

This is to be filled out the same week that you are keeping the food diary and collecting the urine samples. Please list any physical activity performed and the duration.

Monday Date_____	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday

Appendix D

Appendix D: Food Diary

Waterford Institute *of* Technology
INSTITIÚID TEICNEOLAÍOCHTA PHORT LÁIRGE

Bone Health Study

Food Diary

PLEASE KEEP A RECORD FOR THREE DAYS, ONE OF WHICH MUST BE A WEEKEND DAY AND WRITE THE DATE FOR EACH DAY

PLEASE BE AS ACCURATE AND DETAILED AS POSSIBLE.

- Try and estimate the portion size using household measures e.g. cups, spoons or give estimate of actual weight or volume.
- Record everything, food, drinks, snacks, supplements, no matter how small the portion.
- Don't forget to include things like, tea, coffee, water, alcohol, gravy, sugar, salt.
- Try and record method of cooking e.g. grilled/fried/roasted.
- Please specify type of milk e.g. low-fat or full-fat and brand of spread used.
- Try not to change your normal eating habits while you are keeping the diary, please be as honest and accurate as possible.

THANK YOU!

ID NO. DATE:

	FOOD / DRINK / SUPPLEMENT	PORTION SIZE
BREAKFAST		
SNACK		
MID-DAY MEAL		
SNACK		
EVENING MEAL		
SNACK		

Appendix E

Apppendix E: List of Protein Supplements Consumed

Brand Name	Product Name/Details	Frequency	Amino Acid Composition Provided
Optimum Nutrition	Whey/Casein 56% casein, 16% whey	1	Yes
Optimum Nutrition	100% Whey	8	Yes
Optimum Nutrition	Serious Mass	1	Yes
Not supplied	Whey protein	5	Yes (general brand)
Not supplied	Glutamine	1	Yes (general brand)
Myprotein.co.uk	Whey	3	Yes
Myprotein.co.uk	Casein	1	Yes
Myprotein.co.uk	BCAA	1	Yes
Myprotein.co.uk	Pitstop all in one	1	Yes
Myprotein.co.uk	Hurricane X	1	No
Biosynergy	Whey protein	1	Yes
Nutrition Connection	Whey	1	Yes
BSN	True Mass	1	No
BSN	Protein	1	No
BSN	Syntha-6	3	No
BSN	BCAA	1	No
BSN	Muscle TechAMP	1	No
Cytosport	Whey Protein	1	Yes
Maximuscle	Whey Protein	1	No
Activita	Whey Isolate	1	Yes
Cytogainer	Weight Gainer	1	No
USN	Pure Protein	2	Yes
Ultimate Nutrition	Prostar Whey Protein	2	Yes
Trec Nutrition	Anabolic BCAA	2	Yes
Trec Nutrition	XXL Mass Gainer	1	Yes
Trec Nutrition	Night Protein, Perfect Whey	1	Yes
Trec Nutrition	Hard Mass	1	Yes
Trec Nutrition	Amino Max	1	Yes
Reflex	Instant Mass	1	No
PVL	Mutant Mass	2	Yes
Provon Revive		2	Yes
Powerbar	Protein Plus	1	No
Complete Supplements	Whey	2	Yes
Proform	Ultimate Body Fuel	1	No
Pox Protein	Pox Explode II (whey protein conc.)	1	No
Biotest	Metabolic Drive Complete	2	No
Nutrition X	Big Whey Protein	1	Yes
Nutrition X	Mass X	1	No
Nutrition X	RAM	1	Yes
Maximuscle	Promax Diet	1	Estimated from promax
Maximuscle	Promax Extreme	2	Estimated from

			promax
Maximuscle	Promax	1	Yes
Maximuscle	Progain (whey)	2	No
Casilan 90	Whey protein	1	Yes
Marathon	Protein bar	1	No
Inner Armour	Whey Protein	1	Yes
GMAX	Whey Protein	1	Yes
EAS	100% Whey Protein	1	No
Cytosport	Monster Mass	1	No
Bulk Powders	Whey Protein	2	Yes