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The influence of the Female Athlete triad on bone quality in endurance athletes

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1.Abstract

The female athlete triad was defined in 1997 by the American College of Sports Medicine (ACSM) consisting of three components: Disordered Eating, Amenorrhea and Osteoporosis (Waldrop, 2005). Previous literature has identified a reduced bone health in athletes with altered menstrual cycles and/or those with eating disorders. For this masters, athletes were recruited from elite athletic databases then grouped according to their menstrual cycle irregularities and physical activity levels identified from a questionnaire completed prior to participation. The three groups identified were controls, eumonrrheic athletes or amenorrheic athletes. Each participant was subject to dual energy X-ray absorptiometry (DEXA), peripheral quantitative computed tomography (pQCT) scanning, a blood test, if willing, to assess hormonal status. A three day food diary was completed prior to testing.

Significant differences between both eumenorrehic and amenorheic athletes and controls were identified for total calorie, proteins, carbohydrate, potassium and phosphorous intake per day. pQCT scanning showed significantly lower cortical thickness in the amenorrheic athletes compared with eumenorrheic athletes (P=0.025). The amenorrehic athletes had a greater endochondrol circumference than both control and eumenorrheic athletes, indicating that the amenorrehic athletes had a diaphysis much wider and thinner than the control and eumenorrheic athletes (P=0.011). There was a significant difference at the tibia cortical area of the diaphysis between the control and amenorrheic athletes (P=0.017). DEXA results highlighted significant differences between control and amenorrheic athletes, with controls having a greater bone mineral density, at non weight bearing sites of the head (P=0.038), trunk (p=0.004), ribs (p=0.027), pelvis (p=0.015), spine (p=0.008) and L1-L4 (p=0.025). With further significant differences found between the amenorrehic and eumenorrheic group of athletes at the trunk (p=0.020) and pelvis (p=0.016), in this case the bone mineral density of the eumenorrheic athletes was higher than the amenorrheic athletes. Blood tests demonstrated a significantly higher level of the bone resoprtion marker Tartrate resistant- acid phosphatase (TRAP) in amenorrheic athletes than in the eumenorrheic and control groups (p=0.026). Overall results demonstrate that elite level endurance athletes with amenorrhea have lower bone mineral density in the radius and tibia compared with other eumenorrheic athletes and controls which was associated with increased circulating levels of TRAP.

2. Introduction

2.1 An Overview

The female athlete triad was defined in 1997 by the American College of Sports Medicine (ACSM) consisting of three components: Disordered Eating, Amenorrhea and Osteoporosis (Waldrop, 2005). Some studies have shown that the prevalence the triad can be as high as 62% of female endurance athletes (Waldrop, 2005; Rosen & Hough, 1988).

Of the three components of the triad, low energy availability seems to be the most common. Rosen et al (1986) demonstrated 32% of his studied female athletes practised weight control, of which 70% did not recognise the dangers or harmful effects of such behaviours. In another study of 562 female and 832 male athletes, 13% showed weight control behaviours (Johnson et al., 1999).There were 9.2 % of females identified to have had clinically significant problems with bulimia nervosa versus 0.1% of the males. There were 2.85% females who had problems with anorexia nervosa with zero for males; however, 10.85% females reported binge eating on a weekly or greater basis versus 13.02% for males. 5.52% of females reported purging behaviour with 2.04% for males, on a weekly or greater basis. Similarly 18% of a Norwegian athlete study displayed disordered eating behaviour (Johnson et al., 1999). Up to 62% of elite athletes have been shown to portray disordered eating with as little as 0.4-4% of the general adolescent population practising the weight control behaviours (Nichols et al., 2006). The disordered eating component of the triad can be subtle or small at first but can progress to serious disorders of anorexia and bulimia nervosa.

The early stages of the disordered eating habits can initiate a hypothalamic amenorrhea, secondary to the athlete's energy deficit. As many as 79% of the female athletes with disordered eating patterns experience a menstrual disturbance (Lloyd et al., 1985). This could be either irregular or total absence of menses, where a regular menstrual cycle has been defined as menses every 25-35 days or 10-13 menses/year (Lloyd et al., 1985). Not only inapproporiate eating patterns, but also vigorous exercise increases the rates of oligomennorhea and amenorrhea from 5% within the sedentary population to as much as 20% of those who regularly exercise on a regular basis at a high intensity (Lloyd et al., 1985). There are numerous types of specific irregularities that can arise and will be discussed later in the 'Menstrual disturbances' section.

Amenorrhea is not the only type of menstrual dysfunction associated with the female athlete triad. Oligomenorrhea is a shortened luteal phase as well as annovulation, cycles with no ovulation, which is also associated with a detriation in bone health (Lebrun, 2007). The menstrual disturbances cause hypo-oestrogen levels leading to low and inadequate absorption of dietary calcium with a poor incorporation into bone. The bone health then suffers as a consequence of the imbalances and can lead to osteopenia or early onset osteoporosis (Lebrun and Rumball, 2002). In fact a decreased bone density is seen in 10-20% of female athletes (Powell, 2011), but it is not clear whether this is exacerbated in female athletes with the traid.
The individual components of the triad can cause significant morbidity but in combination the aspects are extremely detrimental, reducing chances of conceiving offspring and increasing the risk of diseases- associated with malnutrition (Lebrun and Rumball, 2002). It is therefore important to characterise the progression of the triad and impact on the skeletal system. Such work will help to determine the underlying causes and to identify novel therapeutic strategies. The focus of the present work will be on characterising the bone characteristics of young healthy women and athletes at high risk of developing the triad.

2.2 Bone growth, development, composition and function

Bone makes up the skeletal system of vertebrates and is capable of adapting according to stresses it undergoes (Snell 2008). The vertebrate skeleton is formed by two processes; intramembranous ossification and endochondral ossification. Intramembranous ossification forms flat bones of the skull, including the calvarium and mandible, where mesenchymal stem cells differentiate directly into bone forming cells; the osteoblasts (Mackie et al., 2011). The same process is involved in increasing the diameter of long bones by addition of new bone at the osteogenic front. Endochondral ossification forms the majority of the skeleton in which cartilage is replaced by bone tissue. In the 5th week of human development the progenitor cells begin to condense and form unmineralised and vascular models of developing bones evolving into the mesenchymal cells. The mesenchymal cells then differentiate into chondrocytes producing type II collagen, making up components of the ECM (extracellular matrix) and proteoglycan aggrecan. These chondrocytes form the cartilage anlagen establishing the template of the future skeleton (Newman and Wallis 2003; Eerden et al., 2003). Ossification of the cartilage is preceeded by

hypertrophy of chondrocytes in the mid shaft of the bone as well as deposition of bone via osteoblasts that surround the mid shaft. The primary centre of ossification is formed when blood vessels, osteoclast and precursors for bone marrow and osteoblasts have invaded the cartilage model (Mackie et al., 2011). Osteoclasts and osteoblasts work antagonistically by removing cartilage ECM and depositing bone respectively. This results in expansion of the primary ossficiation centre towards the ends of the anlagen. In long bones a secondary ossification centre forms at the end of the cartilage model (Mackie et al., 2011). The growth plate is situated between the primary and secondary centre of ossification and is responsible for the longitudinal growth of long bones.

The growth plate is a highly organised cartilage structure within which chondrocytes are arranged into longitudinal columns. At the epiphyseal end the reserve zone contains resting chondrocytes that have only a few cells that undergo replication. Recently it has been identified that these cells secrete a growth factor morphogen that acts on proliferating cells determining their orientation. Subsequently resting cells differentiate in a longitudinal direction forming proliferative zone columns of chondrocytes (Abad et al., 2002). The pre hypertrophic zone is where the cells then enlarge and become spherical before the final differentiation of these cells. This last division is associated by a massive increase in cell volume forming the hypertrophic zone chondrocytes (Mackie et al., 2011). Hypertrophy is accompanied by an increase in intracellular calcium concentration that is vital for vesicle production. Vesicles secrete calcium phosphate, hydroxyapatite and matrix metalloproteinases (MMPs) which cause mineralisation of the vesicles and the surrounding matrix. Hypertrophic chondrocytes produce vascular endothelial growth factor (VEGF) which attracts blood vessels from the underlying spongiosum (Eeerden et al., 2003). The end fate of hypertrophic cells is under scrutiny but in general it is accepted that chondrocytes are subject to death by apoptosis. Skeletal maturity and cessation of bone growth arises at puberty when the primary and secondary centres of ossification meet and eliminate the growth plate replacing it with bone. This is usually linked to alterations in sex hormones (Newman and Wallis 2003).

Throughout life, the primary function of bones is to provide attachments for muscle tendons and ligaments, protect organs, act as mechanical levers and play a role in haematopoiesis (Wilmore & Costil, 2004; Horada & Rodan, 2003). They also help to

maintain calcium levels in the blood as well as supporting the soft tissues. Approximately 75% of bone is composed of compact bone mass.
The inner segment of the bone is made of interconnecting spicules whose organization is determined by stresses put through the bone. This type of bone is called cancellous bone, also known as trabecular bone, and makes up the remaining 25% of total bone mass. The structure of this section is adapted to its function by having a larger surface area allowing bone forming and resorbing cells to have greater chance to contact the bone surface more frequently (White & Porterfield, 2007).The mass of bone is maintained by the balances of action of two types of bone cells known as osteoclasts and osteoblasts. Osteoblasts are involved in formation of bone whilst osteoclasts are involved in bone resorption. Osteoclasts are derived from haemopoietic stem cells. The osteoblast cells are derived from mesenchymal stem cells along with stromal and bone lining cells. Bone resorption occurs at a much higher rate than does bone formation and so even a small increase, hence an imbalance of the action, in osteoclasts could then cause bone loss (Horada & Rodan, 2003). Bone resorption by osteoclasts takes just 30 days and the bone remodelling cycle takes 4 months (Agerbaek et al., 1991)

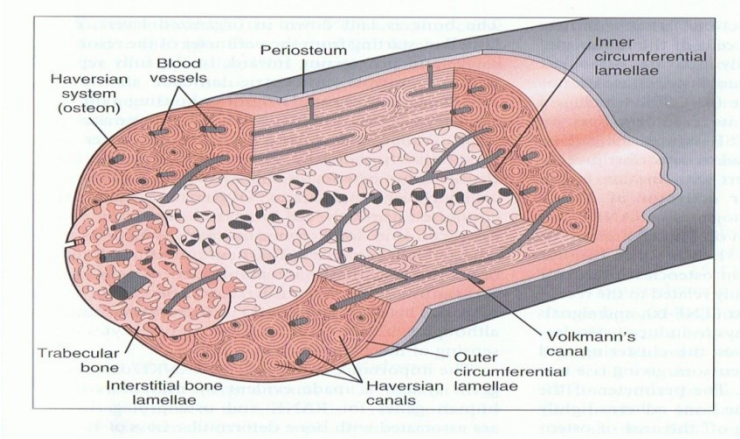

Figure1 : A typical long bone shaft (White & Porterfield, 2007).

The Haversian system indicated in figure 1 shows how osteoclasts are interconnected. The Haversian canals allow movement of the osteoclasts. Periosteum is the outer covering of the bone. The lamellae are the layers in which the bone is laid down in formation, starting with the outer lamellae, then the interstitial lamellae and finally the inner lamellae. The blood vessels provide blood supply to the bones and allow haematopoesis to be maintained. The Volkmann's

canal perpendicular to the Haversian canal and interconnect the Haversian canal's with the periosteum as well as further nerve fibres and a blood supply (Miller & Kasahara, 2005).

There are different mechanisms and factors that influence the function of the bone. Calcium is needed for normal growth, development and maintenance of the skeleton; providing the strength and structure. Ninety-nine percent of the bodies' calcium is found in bone. It is one of the primary bone forming minerals and accounts for 1-2% of the adult human body weight (Flyn, 2003). A fall in serum calcium concentration is sensed by parathyroid principal cells know as calcium sensing receptors. There is then a triggered increased in secretion of parathyroid hormone that stimulates resorbtion from the bone's calcium stores allowing the serum calcium concentration to reach normal levels again. Once calcium levels have return to norm a rise in 1,25-dihydroxyvitamin D levels arise allowing absorption of calcium into the bone (Flyn, 2003). If the serum calcium levels become too high the hormone calcitonin is released from the thyroid gland. Calcitonin inhibits actions of osteoclasts, thus bone is no longer resorbed, and serum calcium levels return to normal (Plotkin et al., 1999). The calcium levels are important to be maintained so no detrimental effects occur on the bone, as in the case of low calcium levels. The homeostatic mechanism is additionally important as an excess of calcium can lead to vascular calcification. This can affect the heart and vasculature, impacting on blood flow around the body (Yang et al., 2004).

2.3 Bones adapt to loading

Bone strength is determined by its mass and density. Forces on the bone stimulate bone formation and a reduced load will stimulate bone resorbtion. The physical activity level of an individual is therefore an important factor in bone health and a vital reason to encourage participation in sport. Regular activity is linked with an increase in bone density (Nichols et al., 2007). The proportion of inactive people is rising in Western society leading to a growing incidence of bone disease, such as osteoporosis (Helge & Kanstup, 2002). Osteoporosis can be defined as '2.5 standard deviations below the average bone density value for young adults' (Birch, 2005). It is a characterized by low bone mass along with 'microarchitectural' deterioration of bone tissue that will increase the fragility of the skeleton and therefore increasing the likelihood of a fracture (Lebrun, 2007). Osteoporosis results in more than 1.3 million

osteoporotic fractures a year with an estimated cost of $13.8 billion in the US alone (Deng et al., 2000).

Besides activity, sex steroids also have an influence on the bone health. The hormone oestrogen is considered the main regulator of bone formation and resorption (Chiu et al., 1999). Oestrogen tends to reduce the osteoclast activity and thereby limits the resorbtion. A low concentration of oestrogen will have a negative effect on bone mineral density (Helge & Kanstrup, 2002). The factors described should be kept in positive balance for the bone to remain intact.

Premenarchal years have been shown to provide a 'window of opportunity' (Morris et al., 1997). This is a period in time in which bone remodelling and the remodelling process respond most strongly to mechanical loading. Exercise induced bone modelling is greater in the young skeleton corresponding to a stage of development when bone remodelling is most active. At this stage, concentrations of blood surrounding the bone are at their highest along with prostaglandins and growth hormone (Morris et al., 1997). The remodelling process is likely to be enhanced by further increase in oestrogen and testosterone. Morris et al (1997) showed an exercise group of premenarchal girls' accrued 5.5% greater total body BMC, 3.6% greater lumbar spine BMD and 10.3% greater femoral neck BMD than the controls. The exercise intervention programme incorporated a 30 minute physical activity session 3 times per week. The programme included a variety of high impact, vigorous aerobic workouts; these were such as modified soccer, step aerobics, skipping and weight training. Similar studies in women post menarchal aged 20-25 reported only 1.3% increase in lumbar spine BMD and no change in femoral neck. Similarly, premenopausal women only demonstrated an 0.8% increase in lumbar spine in response to a weight training intervention. Even less positive was the finding by Rockwell et al (1990) that showed a decrease of 4 % in the lumbar spine in response to a 9 month weight training programme. The women in this study were premenopausal aged 26.2 years +/- 1.3years with controls being 40.4 years +/- 1.6 years. None of the women had previously partaken in weight training and all ingested a 500mg calcium supplement each day throughout the study. At the post pubertal stage bone enhancing factors are less readily available and therefore bone remodelling occurs at a lesser rate (Mackelvie et al, 2002).Similar findings have also been found in veteran tennis players whereby exercise benefits are limited in adulthood due to the cessation of longitudinal skeletal growth (Ermin et al., 2012;

Ireland et al., 2014). The benefits of beginning physical activity are twice as great if the initial phases of training occur prior to menarche (Kannus et al., 1995; Ireland et al., 2014).

MacKelvie et al (2002) demonstrated a large increase in exercise in prepuberty elicits a bigger bone mineral gain over controls implementing exercise interventions within distinct maturity groups has provided evidence for the 'window of oppourtunity' for bone response in early puberty. By having knowledge of this time period it gives valid reason for implementing publicly funded exercise programmes to encourage young children to participate in activity and reduce the number of those that are less active and help to reduce bone disorders of later life (Eliakim and Beyth, 2003). It is known that 26% of final adult bone is accumulated during the two years surrounding peak bone growth velocity, which approximates the amount of bone lost during post menopausal years (MacKelvie et al., 2002; Eliakim and Beyth, 2003). The active boys and girls have been shown to accrue 10-40 % more bone in the two years during peak bone velocity than inactive children. In pre-pubertal children it has been established that the format or specificity of intervention may be less important than the magnitude of increase in weight bearing activity. Further demonstrating the benefits of exercise in pre puberty, a 5 week prospective endurance training programme was implemented causing an increase of around 15-39% of all bone formation markers compared to control subjects who had no changes in any of the bone markers. This study by Morris et al (1997) previously described further highlights the presence of this window of opportunity and its importance to the bone health in later life. However, it is important to note that there is not a linear dose-response relationship between physical activity and improved BMD as very strenuous activity could lead to 'athletic amenorrhea' which induces suppression of the hypothalamic gonadal axis causing deleterious effects on the bone health and positive benefits of weight bearing activity are no longer gained (Eliakim and Beyth, 2003). During athletic amenorrhea, oestrogen levels are reduced and therefore the final post puberty bone mineral density is lowered as peak bone mass is not achieved during the critical period of prepuberty. This athletic amenorrhea has been linked to the psychological stress of intense training as well as reduction in energy availability. It is estimated that a threshold calorie intake of 20-25kcal/kg of lean body mass must be met in order for menstrual cycles to occur regularly (Eliakim and Beyth, 2003), females below this threshold will have menstrual disturbances. This

indicates why some females undergoing the same high intensity physical training have no irregularities whereas some may. The factors of nutritional input and menstrual disturbances are discussed later. The low energy is further linked to a reduction in LH pulsatility and suppression of further hormones such as T3, insulin and IGF-1 (Eliakim and Beyth, 2003).

There are three main factors involved in bone remodelling; i) the hormonal status ii) weight bearing and physical activity iii) dietary intake. Athletes that are involved in consistent weight bearing activity, when either or both the hormonal and nutritional status become compromised may suffer from a reduction in bone mineral density. Females at greatest risk tend to be those involved in sports requiring a lean body mass or those that are judged for performance, such as gymnastics (Waldrop 2005; Nichols et al., 2007). Females involved in endurance sports are more likely to suffer from the disordered eating component of the female athlete triad. Generally men have a tendency to want to gain muscle mass for their sport in comparison to females (Rackoff & Honig, 2006). Within the general population, men exercise for fitness reasons only, leading to a positive self esteem and a lower prevalence of eating disorders. Women exercise more for a weight control and aesthetic reasons: this is more positively associated with eating disturbances (McDonald & Thompson, 1990).

2.4 Menstrual Cycle and Disturbances

One of the main factors that play a role in bone health of females involves the monthly menstrual cycle. The menstrual cycle is a natural process that occurs each month usually starting from the age of 12.8 years old (Wilmore & Costil, 2004). The cycles continue until the onset of menopause at around 49-52years old (Gold et al., 2001). The menstrual cycle is highly dependent on the functioning of the hypothalamic pituitary axis. There are two hormones that are mainly involved in the regulation of the menstrual cycle and are linked to this axis; lutenizing hormone (LH) and follicle stimulating hormone (FSH). LH requires a low rate of release of gonadotropin releasing hormones (GnRH), from the hypothalamus, whilst FSH responds to a high rate of release of GnRH (White and Porterfield, 2007). The basic menstrual cycle can be described in 9 stages. If fertilisation occurs, human chorionic gonadotropin will originate and remain for the 9 months of the pregnancy maintaining the corpus luteum. If fertilisation does not occur then the corpus luteum will degrade

and die due to a decline in progesterone levels. Negative feedback results and a rise in FSH levels 2 days before menstruation will occur. This FSH then causes antrial follicles to grow releasing oestrogen and inhibin B. The FSH secretion then declines, allowing an increased stimulation of the gonadotrophe and thus a rise in LH levels. A dominant follicle arises due to the decreased level of FSH. This follicle then produces increased amounts of estradiol 17β and inhibin B. The oestrogen stimulates a positive feedback response and results in a surge in LH levels mid cycle. The ovary will then stop dividing at metaphase of meisosis II. A new corpus letueum is derived from mural granulose cells and theca cells. In the LH surge the oestrogen levels decline whilst granulose cells secrete inhibin A. After the LH surge the oestrogen levels rise again and, combined with progesterone and inhibin A, exert a negative feedback on the gonadotrophe. The FSH and LH then return to their normal basal levels, bringing the cycle back to the beginning where the corpus luteum remains (White & Porterfield, 2007).

If the menstrual cycles are interrupted then a decline in bone health is one of the consequences. Reduction in bone health is usually a feature of post-menopausal older age. The peak bone mass is achieved in the first three decades of life and after this there is a greater chance for a decrease in bone mass and osteoporotic fractures (Nichols et al., 2007). In the elite female athlete the reduction in bone strength can be seen to occur at an earlier age associated with menstrual cycle disturbances. The disruption will cause depressed levels of oestrogen in the blood, which is the essential hormone for normal bone mineralization (Noakes, 2009). In athletes a decline in bone health increases the likelihood for bone related injuries.

There are different types of irregularities of the cycle. Torstviet and Sundgot-Burgen (2005) defined eumenorrhoea to be a regular menstrual cycle from 26 to 32 days. Luteal phase defects can arise resulting in no ovulation or oligomenorrhea; an irregular pattern of cycles (Birch, 2005). Amenorrhea is frequently described as the absence of menses for more than three cycles in a row. Primary amenorrhoea was defined as the absence of menarche by the age of 16 and secondary as the absence of three or more consecutive menstrual cycles after menarche outside of pregnancy, the prevalence being 15-66% in athletic women (Lebrun, 2007). Secondary amenorrhea is the cessation of menstrual bleeding after menarche has occurred for a period of time greater than or equal to 2 months, not due to pregnancy (Drew, 1961). Finally, oligomenorrhoea are known to be menstrual cycles of 35 days or

more and another separate disturbance is a short luteal phase with a menstrual cycle of less than 22 days. Collectively these are all classed as menstrual disturbances. Oestrogen is one of the sex steroids that regulate bone formation and resorption (Chiu et al., 1999). When the menstrual cycles are disturbed so are the levels of oestrogen and it has been observed that up 10- 44% of elite female athletes demonstrate amenorrhea (Birch, 2005) thus putting bone heath at risk. The protective effects of oestrogen are removed when the menses cease and so the women become more vulnerable to calcium loss resulting in a decline in BMD. The endocrine responses occurring due to menstrual cycle disturbance and low adipose tissue can outweigh the possible beneficial effects of physical activity on bone health. The hormone oestrogen is considered the main regulator of bone formation and resorption (Chiu et al., 1999). Oestrogen tends to have the effect of reducing the osteoclast number and so limiting the resorption of the bone. Oestrogen is vital for the closure of the epiphysis in formation of bone (Horada & Rodan, 2003). It is known that a low concentration of oestrogen will have a negative effect on bone mineral density (Helge & Kanstrup, 2002). Further pituitary hormones affected are growth hormone, Insulin growth factor-1 as well as triodothyronine, secreted from the thyroid gland, together these alterations affect bone mineralisation and therefore the development of bone mass (Stafford, 2005).

Athletic amenorrhea, as previously mentioned, usually presents as secondary amenorrhea. In sports that require a lean physique and associated with a high level of competition and stress for the mind and body, menarche of an athlete may be delayed and is seen as primary amenorrhea. There is no one single factor that causes the delayed menarche (Waldrop, 2005). The intense physical activity undergone by elite athletes alters the levels of hormones, as does regular physical activity. There are numerous components involved with general athletics training, such as competition stress, diet and reduction of body fat and production of heat or hypoxia. The continued exercise induced hormonal alterations constantly undergone by elite females impair production of gonadotropins altering the luteal phase causing a deficiency in ovulation (Arena et al., 1995).

It is suggested that luteal phase defects and amenorrhoea are due to a decrease in availability of energy (Birch, 2005). There is no single nutritional deficiency that is linked to the menstrual disturbances associated with elite female runners. Certain traits, however, are often demonstrated between fellow athletes these maybe a low

fat and protein intake (Noakes, 2009). Weight and Noakes (1987) confirmed abnormal eating patterns are most common among competitive runners. In a group of American female collegiate runners Rosen et al1986 found that 47% demonstrated one or more weight controlling behaviours such as fasting, vomiting or taking appetite suppressants. To date the evidence suggests that menstrual dysfunction associated with restrictive eating patterns seen in elite female athletes originate from a disturbed psyche that underlies both the abnormalities (Noakes, 2009). Warren (1980) also supported the hypothesis of a negative energy balance induced suppression of reproductive function as a consequence of high frequency, intense exercise by identifying a close correlation between the onset of menarche with a reduction in training volume or during an injury or vacation (Williams et al., 2001). Further, Bullen et al., (1985) demonstrated that women who adhered to strenuous exercise and diet regimens for two menstrual cycles were more likely to have menstrual disturbances in comparison to females who only exercised.

The energy deficit that is often seen in female athletes, also previously highlighted in disordered eating section, is known to be linked to a reduction in Luteinizing hormone (LH) pulse frequency causing suppression in the release of gonadotropin releasing hormones in turn inducing the amenorrhea. The absence of the menstruation may also be another way that the body adapts to conserve energy (Waldrop, 2005). The energy imbalance disrupts the hypothalamic pituitary ovarian axis hence the reduction in LH release. The underlying mechanism inducing menstrual changes remains unclear. There have been links to the levels of leptin that may be associated with the menstrual cycle disturbances in elite female athletes. Leptin is secreted in proportion to adiposity providing a negative feedback signal in the regulation of body weight homeostasis. Gene expression and secretion of leptin is also affected by overall energy balance and reproductive hormone levels. In normal weight sedentary females an increase in leptin has been observed in the luteal phase of the menstrual cycle (Thong et al., 2000). Findings in the study by Thong et al., (2000) report lower levels of leptin in amenrroheic and annovulatory athletes along with the absence of its diurnal cycle in those that are amenorrheic suggesting the menstrual disturbances are linked to leptin levels. A study by Welt et al (2004) treated women with hypothalamic ammennorrhea with leptin. This led to normalization of levels of reproductive hormones, follicular development and menstrual cyclicity, thus demonstrating the importance of leptin. If there is a decline

in energy balance in such a way that amenorrhea is reached the overall hormone balance is affected. This imbalance is associated with oestrogen and IGF-1 deficiency which could also contribute to the bone loss associated with amenorrhea. Thong et al (2000) further studied a group of females and found lower plasma leptin levels in elite athletes than both active women taking the oral contraceptive pill or those normally cyclic. Lower body fat can account for the difference in leptin levels between elite, cyclic and recreational athletes. In those amenorrhic the plasma leptin levels remained lower suggesting there may be reasons other than adiposity that account for the lower leptin levels. The suppression of leptin gene expression and secretion from adipose tissue in amenorrhic athletes could be a mechanism that is triggered by the athletes negative energy balance (Farah et al., 2000). In the study by Welt et al (2004) markers of bone formation bone alkaline phosphatase (BAP) and osteocalcin both significantly increased after leptin administration. Insulin levels are also significantly lower in elite female athletes that are amenorrheic Insulin is released in the postprandial state and acts on cells, including adipocytes, to promote glucose uptake and anabolic status. Low insulin levels due to low calorie intake and high insulin sensitivity may provide a mechanism in which the adipose tissue will detect energy availability and up or down regulate the gene expression for leptin. Further studies are needed to finalise the influences that leptin has on reproductive hormones and signalling regarding the availability of energy

Overall, the major influence on menstrual disturbances in elite female athletes is their energy balance. The energy input to meet energy demands is vital to remain in balance so not to alter hormones levels to a state in which reproductive function is lost. If hormone balance is lost, general interbal physiological processes may also be diminished. However there is a genetic condition called polycystic ovarian syndrome (PCOS) that can also cause the menstrual cycle disturbances commonly seen within the female athlete triad. PCOS is the most common endocrinopathy in young women, it affects 6-10% of women of reproductive age (Stachenfield & Taylor, 2009; Barontini et al., 2001; Tsilchorozidou et al., 2004). Polsen et al., (1988) found 22% of just 257 females had PCOS. It can be defined as the association of clinical and/ or biochemical evidence of androgen excess with chronic anovulation, in which an oocyte is not released from the ovaries (Franks et al., 2005). The consequences of PCOS can include irregular menses, along with infertility and recurrent pregnancy loss (Stachenfield & Taylor, 2009). These disturbances in the menstrual cycle will

once again contribute to a decline in bone health due to the reduction in oestrogen that may occur (Chiu et al., 1999).

In athletes this condition can cause confusion. The diagnosis of PCOS can be missed and overlooked for components that revolve around the female athlete triad instead (Stachenfield & Taylor, 2009). The regular symptoms associated with PCOS are masked by certain levels of exercise making it hard to diagnose in the female athlete. One of the main risk factors of PCOS is obesity and female endurance athletes are not obese due to their high levels of physical activity. However, only 50% of women with PCOS are obese, but yet the condition seems to still be under diagnosed in women with a low or normal body mass index (Stachenfield & Taylor, 2009). The syndrome will still cause menstrual disturbances that are interlinked with a reduction in bone health. As the condition seems to be overlooked in athletes, this will prolong the time period with the menstrual irregularity.

Noting all these factors that may alter menstrual disturbances it is important that this aspect of the female athlete's physiology should be maintained.

2.5 Disordered eating

Female athletes are exposed to high psychological pressure relating to the expectation to perform to their best. There are also pressures to maintain a very lean physique, (with very low fat mass), in the belief it will enhance sporting performance. This leads athletes into a negative balance due to high energy expenditure during and after exercise and insufficient energy replenishment from food intake and a reduction in body weight will result (Raond- Braker et al., 2007). It has been reported female runners consume 30% less energy and carbohydrate per kilogram of body weight than male runners (Manore et al., 2007: Burket et al., 2001). Evidence suggests that women involved in lean sports, such as endurance running, gymnastics and cross country skiing, are at a greater risk for disordered eating than athletes in non lean sport (Reinking and Alexander, 2005).

The disordered eating component of the triad not only comprises of anorexia nervosa and bulimia nervosa but also a chronic reduction in energy intake through dietary restraint, all of which reduce energy availability. Dietary restraint is the most common behaviour that is associated with negative bone health (Barrack et al., 2008). Most females need a minimum of 5-6 g of carbohydrate per kilogram (kg) of body weight to maintain the glycogen stores that are needed to support

baseline/moderate training (Burket et al., 2004). Fatty acids stored in adipocytes and localised lipid droplets as well as glucose stored in liver and muscle glycogen stores provide the major sources of exercise fuel. Glucose sources are more limited in comparison to fat stores (Burke et al., 2004). The availability of glucose as a substrate for the muscle and central nervous system becomes a limiting factor in the performance of endurance sessions equal to or greater than 90 minutes (Burke et al., 2004) although higher intensity exercise will have a greater reliance on glucose as fuel and will decrease the exercise time that can be sustained by glucose metabolism. The use of glycogen stores during exercise will vary depending on intensity and duration of the exercise. The required amount will differ between trained and sedentary people for any given intensity and duration. In general Manore et al (2007) suggest prolonged high intensity exercise will require carbohydrate intake of 7-12g per kg of body weight. A significant amount more fibre has been found to be consumed by ammenorrheic athletes, this in turn reduces absorption of energy and nutrients. Although less data is currently available on protein requirements of the female athlete, it is generally advised to consume more protein in relation to the recommended daily allowance. In some elite athletes 1.0-1.6 g of protein per kg of body weight is needed, in comparison to 0.8 g for non athletic populations (Manore et al., 2007). When the energy intakes are low and/ or weight is being lost due to energy deficiency, the body will break down tissues, especially muscle, to liberate amino acids which can be used to synthesise glucose through gluconeogenesis to be used as energy substrates. Fat within diets of active females is generally lower than the recommended fat intake of 20-35 % of total energy intake. Athletes, particularly endurance trained athletes, have considerably higher fat metabolism during exercise compared with untrained people, but the fatty acids are not only used for beta-oxidation to make ATP, they also serve numerous metabolic, structural and endocrine processes (Manore et al., 2007).

Athletes may find themselves in a state of energy deficit unintentionally. The athlete may simply maintain the same food habits despite both volume and intensity of training increasing. Therefore the result is similar to that of a purposeful weight loss programme (Rackoff and Honig, 2006; Waldrop, 2005). In non-athletes, such an energy deficit would lead to mainly fat loss, where as the athlete has little fat to lose and would therefore degrade muscle mass instead. Another common behaviour is found by the restriction of certain food groups that may be high in fat and/ or protein.

As well as harming the bone health, the inadequate input of calories to meet the demands of exercise will result in the depletion of muscle glycogen stores, hypoglycaemia and electrolyte abnormalities, and consequently impair the physical performance of the athlete (Rackoff and Honig, 2006).

Anorexia athletica is a term that has been identified to distinguish from pathological anorexia and is characterised by a reduced energy intake and body mass despite high physical performance (Sudi et al., 2004). This category was introduced in the early 1990s as athletes constitute a unique population and the symptoms should be dealt with differently to non athletes (Sudi et al., 2004). Anorexia athletica presents with a specific criteria for diagnosis and is associated with training and sporting performance. The criteria, although seen in other people as well, includes perfectionism, compulsivness, competitiveness, high self motivation, menstrual disturbances along with one unhealthy method of weight control such as fasting, vomiting, the use of diet pills, laxatives or diuretics (Birch, 2005). In the category of anorexia athletic females clinically indicate a prevalence of 15-60% for disordered eating with 50% of these women compulsively over exercising (Birch, 2005).

Dietary intake that meets the energy demands of the athlete is a necessary component that is vital in order for the individual to reach their genetic potential with regards to peak bone mass. According to Wang et al (2003) the mid pubertal period is the one that is most highly influenced by nutritional input. A calcium intake of greater than 1000mg/day was associated with a higher young adult bone mass at mid puberty (Wang et al., 2003: Nichols et al., 2007), indicating that an adequate dietary intake reaching peak calcium intake allows peak bone mass to be achieved. Thus, young girls with sustained energy deficit are less likely to attain a high bone mass and may never reach the peak bone mass of their genetic predisposition. Thus, the negative energy balance in the teenage years could potentially have life-long consequences, whereby in early adulthood the increased risk of bone fracture could prematurely end the athletic career and in later life a bone fracture can impact on confidence and independence in older age (Nichols et al., 2007).

Further complications may arise from a continued decrease in energy availability for the athlete. A hypoglycaemic state is one that will arise as a result of low glucose availability (Lebrun, 2007). Continued disordered eating patterns impair athletic performance and increase the risk of musculoskeletal injury. Further consequences could be depression, fluid and electrolyte imbalance and changes in cardiovascular,

endocrine, gastrointestinal and thermoregulatory systems (Lebrun and Rumball, 2002). Hypogylcaemia, hypoinsulinemia, hypothyroidism and hypercortisolism may all become apparent too. These adaptions could be ways in which the body attempts to conserve energy, hence why menses tend to halt (Laughlin and Yen, 1996). Longer term issues such as infertility, cardiac abnormalities and osteoporosis could ensure, and even result in fatalities (Lebrun and Rumball, 2002).

2.6 Bone Health and the Female Athlete Triad

In the US the number of women in particular who partake in vigorous exercise such as running has increased dramatically in the past decade. Now the amount of women participating is approximately 7 million with 80% being premenopausal. Those who exercise vigorously have an increased rate of amenorrhea of 20% (Lloyd et al., 1985).

In athletes a decline in bone health increases the likelihood for bone related injuries. A stress fracture, a common overuse injury, can be defined as the inability of the bone to repair itself via osteoblastic remodelling, at the same rate equal to that at which repetitive microtrauma is occurring. The osteoclasts work at a faster rate than the process of healing eventually leading the bone to fracture (Heyworth & Green, 2008). Women with a past history of menstrual disturbances are six times more likely to develop a stress fracture. The factors causing them are usually linked to low bone density, menstrual disturbances and dietary insufficiency along with excessive training (Daffner & Pavlov, 1992). In an athlete this will enforce a reduction in loading of the bone allowing it to heal and therefore will have implications on training and competitions. Overuse injury causes can be divided into 3 categories; training errors, such as excessive running intensity or a rapid increase in load, a biomechanical issue or anatomical issue. The first of the three can be linked to the female athlete triad in which excessive training will lead to an imbalance of hormones and the

likelihood to lose weight, due to an increase in training, leaving the bone and body susceptible to injury (Geraci and Brain, 2005). Fractures take from 8-12 weeks to heal, after which the process of loading again should be slow and gradual (Noakes, 2009).

A study involving 2248 female athletes, the 6.4% of endurance runners suffered stress injuries, which was a higher frequency than any other sport (Arendt et al., 2003). One study composing of 53 female and 58 male track and field athletes reported an incidence rate of stress fractures of 21.1 % within middle and long distance runners with most of their fractures located in the long bones and pelvis (Bennell et al., 1996). Women have up to 4 times higher risk for a bone stress injury (Lassus et al., 2002). The frequency of occurrence of such bone stress related injuries has also been investigated in relation to menstrual cycle irregularities. Using x-rays it was documented that 9% of women athletes with a regular menses suffered with a fracture whilst the rate was much higher at 24% for those who had absent or irregular menses. It was identified that women who had been injured during their running programme were more likely to have had or have absent and interrupted menses and would be less likely to use the oral contraceptive pill as well as participating in the sport for a longer length of time. (Lloyd et al.,1985). Such a study re iterates the importance of regular menses, and therefore can be linked back to the female athlete triad whereby the menses are affected by the level of physical activity in balance with nutritional input. The decreased oestrogen levels seen in those women who exercise frequently is linked to an increased risk of osteoporosis and fractures as well as hyperprolactinemia, premature ovarian failure and cessation, all documented in premenopausal athletes.

2.7 Summary of Findings

Numerous impacting factors can contribute to poor bone health in later life. Osteoporosis is a progressive loss of bone mass, primarily due to reduction in bone mineral density that mainly affects post-menopausal women. The loss of bone mineral density increases the risk of fractures. It is the most frequent bone remodelling disease (Takeda et al., 2002). In the United States there are currently 10

million people affected by osteoporosis and it is predicted to rise to affect approximately 14 million adults who are over the age of 50 by the year 2020 (Lane, 2005). Osteoporosis and osteoporotic fractures are major causes of morbidity and mortality. The National Osteoporosis Foundation has estimated 1.5 million fractures a year in the US due to osteoporosis. and it is found that 40-45-% of women aged over 50 will suffer an osteoporosis related fracture during their lifetime (Singer, 2006). At study of 823 caucasian women in their 7th decade revealed 24% had osteoporosis in their hip, spine or both, using the criteria defined by the world health organisation (Ballard et al., 1998). At the time of this study the annual cost of osteoporosis in England and Wales was approximately £742 million. The aetiology of osteoporosis is related to the peak bone mass attained during the lifetime usually in young adulthood. A person attaining a very high peak bone mass can delay the onset of osteoporosis despite progressively losing bone mass in later life. Conversely, someone who attains a low peak bone mass in early adulthood will more quickly deteriorate to a defined value of low bone mass that is clinically significant and, consequently, diagnosed as osteoporosis. Peak bone mass is influenced by both genetic and environmental factors (Slemenda et al., 1997), and while genetic susceptibility to low or high bone mass is difficult to control, the environmental factors can be targeted in an effort to promote skeletal growth. Environmental factors could include medication, nutritional status and physical activity levels. Physical activity levels and nutrition are easily modifiable and are therefore attractive targets. Athletes who engage in repeated, prolonged physical activity have been shown to have a 5-30% higher bone mineral density than sedentary individuals (Ackerman et al., 2012; Nichols et al., 2007, Wilks et al., 2000; Ireland et al., 2011). This increase in bone mineral density is linked to a 50-80% reduction in fracture risk (Nichols et al., 2007). As highlighted at the start of the review, there is a ‘window of opportunity’ in which bone is most responsive to physical activity (Nichols et al., 2007) which seems to be during adolescence and young adulthood with about 90% of peak bone mass being achieved by the age of 18 years old (Ackerman et al., 2012). During this opportunity the bone is particularly responsive to lifestyle factors. During puberty bone mineral mass more than doubles in skeletal sites such as the lumbar spine. This peak occurs 2 years later in males than females (Rizzoli and Bonjour 1999). An investigation using 156 healthy college aged women showed a median gain of bone mass in the third decade (measured as

a percentage per decade) 4.8% for the forearm, 5.9% for the lumbar BMC, 6.8% for lumbar BMD and 12.5% for total body bone mass.

It is clear to see that the bone health of female athletes is of major concern. There are several environmental factors along with genetic factors that are of such influence. There is a very fine line of balance that should be maintained in order for the athlete to remain at peak bone health. Pre pubertal stages are a major factor to play in the gain of peak bone mass, if this is not achieved to its full potential during the 'window of opportunity' then this could influence the ability to achieve for the athlete in the future. It is important to highlight these factors in order to reiterate the necessity of certain interventions are further investigations to help those athletes maintain their maximum bone health potential and succeed to their highest capabilities.

Over the years, a number of studies have been conducted into female athletes and their bone health. The majority of studies tend to look at separate entities that may affect the bone health rather than grouping the female athlete together as a whole and conducting an investigation from there. Bennell et al 1996 demonstrated that some of the significant risk factors in female athletes for stress fractures are a lower bone mineral density and menstrual disturbance history. This study aimed to identify specific factors in order for prevention methods to then be implemented. These significant factors were only established in females, of the measured variables no factors were deemed significant as a risk factor for male athletes. Overall bone mineral density was lower in the athletes with stress fractures but still significantly higher at the lower limb and of the lumbar spine than non active females. This therefore demonstrates some beneficial gains to weight bearing limbs from participation in weight bearing activity but overall these gains are lost due to other factors being the menstrual cycle disturbances. This leaves an open opportunity of research to investigate the comparative weight bearing sites in further detail to see if this is representative for other activities and other weight bearing sites.

Barrow and Saha (1988) identified stress fractures in 49% of their sample of female athletes that had very irregular menstrual cycles, 39% in those classed as irregular and just 29% had stress fractures with regular cycles. Separately 46% of the amenorrheal group, 7% of regular group and 7% of the regular group admitted to an eating behaviour disorder. This therefore identified two separate connections to bone health in that menstrual cycles could have an impact as well as eating behaviours;

leaving the two areas of research to be looked into depth further and whether the two can connect together and impact the bone health.

Mudd et al (2007)identified site specific BMD loss and gains in athletes in different sports. Runners had the lowest total body Bone mineral density and site specific locations except for the legs, demonstrating some gains are still seen in weight bearing limbs of certain activities ie legs in runners. Swimmers and divers were shown to have the lowest leg BMD, further showing there are site specific gains in BMD. However there are still gaps where research is needed to link the overall lower bone mineral density to other components of the triad. This study only focused on collegiate level athlete whether there still is a variety of ability. High end elite athletes are meant to be at the pinnacle of health, and whether this is demonstrated in their bone health is a matter to be investigated. Further studies into site specific gains have been shown by Eser et al (2009) who conducted a pQCT study into site specific gains of weight bearing activity using retired gymnasts. Specifically the BMC (bone mineral content) and CSA (cross-sectional area) were found to be greater at the distal radius, as well as muscle CSA showing 15-17.6% greater at the fore arm and upper arm of retired gymnasts. The findings showed that the retired athletes have site specific skeletal benefits with greater geometric adaption in the upper over the lower limbs. Theses site specific gains were identified in retired gymnasts' whether this benefit is still established during full training and competition is yet to be highlighted. Here there was little benefit identified in the legs as general controls exercise tend to involve some leg impact and so are seen to have some positive gains already, whereas gymnasts have the impact in increased use of the forearms.

Frisch et al (1981) tried to link training patterns to menstrual cycle disturbances which then in turn impact the bone health. It was identified 'Each year of training before menarche delayed menarche by 5 months'. Female athletes, runners and swimmers, were studied and whether they had started training pre or post menarche had an effect on the age of onset of the menstrual cycle. 18 pre menarche trained athletes had a mean menarcheal age of 15.1±0.5years whereas 20 post menarcheal trained athletes had a menarcheal age of 12.8±0.2years. Pre menarche trained athletes; 61% had irregular menstrual cycles with 22% amenorrhea in comparison 60% post menarche trained athletes had regular cycles with non demonstrating amenorrhea. Previously it has been established that a minimum weight for height and therefore a critical lean/fat ratio is necessary for menarche and the maintenance

of ovulatory cycles. This has been further highlighted from an individual case in which an athlete experienced a 2.3kg weight gain which induced menses. The athlete began at a height of 169.2cm and mass of 48.9kg which was 2.1kg below the stated weight needed to initiate menses. Once the athlete gained the 2.3kg menses began whilst continuing to train. After this she in turn lost the weight and menses ceased again. This study highlights the fact the high intensity training does have an effect on the menstrual cycle, menstrual cycle disturbances increased during training in both pre and post menarcheal groups of athletes. If this impact is known to occur then there needs to be an implementation in place or an aspect of control to allow menses and intense training to occur along side each other maintaining their necessary weight for their sport without negatively impacting upon the bone health. Further literature has then shown that the together the components of the female athlete triad make it a highly prevalent issue amongst young females. Hoch et al demonstrated this in 2009; 80 varsity athletes and 50 sedentary controls aged 13-18 years old were subject to a questionnaire that assessed eating behaviour, menstrual status, physical activity whilst also completing a three day food diary ad a serum hormonal, TSH and prolactin level test. BMD and body composition was also taken using a DEXA scanner. 54% of the athletes demonstrated menstrual irregularities in comparison to just 21 % of controls. Overall, 75% of the athletes were found to have one or more components of the triad, 65% were identified as the same in control group of subjects. This reiterates the high prevalence of the triad amongst female athletes but, as well, noting the high percentage still seen in controls; further indicating the major importance to prevent the situation within the young female population. If the issue is not addressed it leaves open the potential for the majority of young females to have a lower bone mineral density, not reaching their optimum peak bone mass during development, increasing the likelihood of problems at an older age and possibly hindering their athletic career. The affects of the triad on the bone health of young females has been further highlighted by Ackerman et al. In 2011 a group of aged 16-21 years amenhorrhiec athletes, eumenorrheic athletes and controls were assessed using DEXA and pQCT. At weight bearing sites the athletes tended to have a greater total trabecular area of bone compared to controls. However, looking at the bone micro architecture in finer detail it is seen that the amenorrhiec athletes had a lower cortical thickness than those eumenorrheic; they were also found to have a lowered trabecular number and higher separation. In bone

mineral density findings the lumbar spine was lowered in amenorrheic athletes whilst eumeonrrheic athletes had the highest BMD at the hip/ femoral neck. The study demonstrated the varying affects on the bones from the components of the female athletes triad, especially indicating the importance to maintain a regular menstrual cycle. In 2012; Ackerman further studied the bone in those eumonorrheic and amennorrheic using finite element analysis (FEA); this was conducted using HR-pQCT to estimate bone strength. Endurance athletes aged 14-21 years old partaking in impact sports were analysed. Findings showed that amenorrheic athletes had a reduced FEA estimated bone strength at the distal radius and the advantage of partaking in weight bearing activity is lost at the tibia compared to the same regions in eumenorrheic athletes. Not only is there a high prevalence and a great amount of damage that can occur to the bone health of these athletes; the different degrees of reduced BMD vary according to the sites whether they are weight bearing or non weight bearing. It is known that physical activity is meant to lead to a gain in bone mineral density, however when athletes are subject to one or more components of the triad, the benefits are somewhat diminished at differing sites of the skeleton. Young et al studied a group of 44 female ballet dancers aged approximately 17 years old, 18 sedentary subjects ammenorheic with anorexia and 23 subjects of comparable age with regular cycles. At weight bearing sites BMD is found to be normal or elevated in those with a regular menstrual cycle but normal or reduced in those with anorexia. Non weight bearing sites demonstrated a reduced BMD in both groups. This highlights that some weight bearing activity may be able to offset the impact of hypogonadism at weigh bearing sites. At sites with a greater percentage of trabecular bone, such as the lumbar spine, there is a severe adverse affect on the bone health despite it being an area of high impact. Deficits at non weight bearing sites are found to be maintained by body weight. However, the future is not all negative as some research has shown that bone health can be restored; one case study demonstrated that BMD was able to be regained well into the third decade of life by making alterations to body weight and nutrition, as demonstrated by Fredericson and Kent (2005). Gains of 25.5% were made in the spin and 19.5% in the hip allowing total BMD to reach within normal levels.

2.8 Finalisation

From the literature overview it is clear to see that the female athlete triad has various influences upon the bone health of female athletes as well as their hormonal functionality, all affecting their potential future athletic career. Prevention at a young age is a clear area that needs to be addressed in order to stop such implications arising. In order for such a prevention method to be implemented further research needs to be conducted to ensure that the preventative method is one in which is sure to stop such findings occurring.

Whilst previous literature has shown to identify differences in BMD and pQCT measures of both weight bearing and non weight bearing sites in athletes, no literature has introduced the wide variety of contributing factors toward female athlete bone health. Research has normally been broken down and just focused on one of many possible components. Other literature has also focused on 'general' athletes or those with a high level of activity, some focus on one sport others group into categories such as endurance activities or high impact. All of the groups have not used elite level athletes of one kind, tending to use a very wide range of ability, most likely due to availability.

In an ideal world it would be good to be able to group athletes according to whether they are within the triad or not. However, to do so three components would need to be accurately taken into consideration. Bone health is an objective measure and could be done via the DEXA, reporting menstrual cycle history for irregularities is also easy to measure via self reporting but there is a potential for bias. The nutritional aspect is hard to measure accurately using self reporting along with calculating the energy expenditure vs intake. Daily activities can be taken into consideration but this would vary again according to basal metabolic rate.

For this masters study the athletes were grouped according to their menstrual cycle irregularities. The nutritional aspect was then looked at as an additional component by a three day food diary and assess whether it has a significant impact on the used sample.

2.9 Aims and objectives

The main aim of this study is to understand the impact of menstrual irregularities in elite female distance runners on bone health. This will be achieved through the following objectives:

1. To recruit female distance runners into the study and characterise the dietary, physical activity and hormonal status that are determinants of the female athlete triad.
2. To compare bone characteristics of those amenorrheic and eumenorrheic using pQCT to measure tibia and radius bone parameters, DEXA to assess total body, lumbar and dual femur bone mineral density.
3. Biochemical blood markers and VO_2max testing were conducted and analysed additionally to identify any further possible relationships of those athletes with deteriorating bone health

3. Materials and Methods

3.1 Participants

Female non-athletes, and triad and non-triad elite athletes were recruited. See table 1 for participant characteristics. Elite athletes in this study are those who 'participate in long term vigorous exercise and training' and have qualified for a national team at the junior or senior level (Torstveit & Sundgot-Birgen, 1994). The elite athletes were defined as eumenorrheic or amenorrheic athletes using information gained from questionnaire and a food diary. The questionnaires were used to identify menstrual cycle disturbances and the food diary to assess the participant's consumption of the daily diet requirements. The food diary was assessed using Diet Plan 6 software (see later section for further detail). If the athlete had experienced menstrual irregularities either secondary or primary amenorrhea they were classed as amenorrheic (AA) if the athletes had regular menstrual cycles over the past year they were classed as eumenorrheic (EA). All controls(C) had regular menstrual cycles. All women were 18-40 years old. Subjects were recruited through advertising by the researchers to known elite female runners and fellow coaches. Non athletic controls had a low level of physical activity and did not participate in organized competitive team sports.

3.2 Experimental Protocol

Height of the subjects was measured on a stadiometer. Body mass was measured using electronic scales with the participant in light clothing.

Training history, past success and achievements and involvement in the sport was obtained by a questionnaire (Appendix-food dairy/questionnaire).

The participants were asked to complete a three day food diary on any three consecutive days prior to the testing, specifying both type and amount of food and drink consumed. This information was analysed using Diet Plan 6 software (Company, Cty, Nation) that calculates the intake of macronutrients and micronutrients over the three days and a total calculation for each day.

Menstrual history was assessed using a questionnaire. Specifically, the participants were asked about the phase of cycle at the date of testing, any past irregularities and their training loads at this time along with any medication they may be on to help regulate their cycles. The questionnaire also contained questions on smoking and alcohol consumption.

3.3 DXA

The DXA works on the basis that as x-rays pass through the body. The exiting attenuated signal is exponentially related to the path length, tissue density and energy of the X-ray. The X-ray source mounted beneath the patient generates a narrow tightly collimated beam of X-rays that pass through the patient at rapidly switched energies of 70 and 140 kVp (kilovolt peak). The transmitted intensity at each energy level is measured by a radiation detector mounted on a C frame directly above the X-ray source. During a scan the C arm oscillates rapidly in transverse direction while moving slowly longitudinally. The attenuation of the two energy peaks by soft tissue is quantified. Results include areal bone mineral density (BMD) ($g \cdot cm^{-2}$), which is derived by dividing the bone mineral content by projected area of the region that is scanned. DXA reports give the actual values in $g \cdot cm^{-2}$ as well as T and Z scores that reflect the number of standard deviations by which a patient's value differs from the mean of a group of young normal or age and sex matched controls, respectively (Lane N 2006-Osteo papers); a Z-score of -2.5 represents a bone mass value 2.5 standard deviations (SDs) below the mean of a young healthy adult population.

The bone mineral density was measured at the lumbar spine (L1-L4), and each of the two femurs. A whole body scan was taken to determine body fat percentage. For a full body DXA the participants lay supine on the scanning bed and were scanned from head to toe in approximately 10 minutes. A lumbar spine measure requires the patient to lie supine with their legs on a box at a 90^0 hip angle. The beam was aligned approximately 5 cm below their navel and the scan moved in a superior direction. The scan of the femurs requires the patient to lie supine with their feet strapped either side to a wide triangular box, turning the feet inward, opening the hips allowing ease of measure. The beam was aligned to one side just below the line of the inferior pubic ramus bone. The scan took a measure of one side and then repositioned for the second femur in line with the start position of the first femur measure that was taken.

Scanning the proximal femur also gives measures of the total hip, the femoral neck and the trochanter. The lowest value of all tends to be used for diagnostic purposes. For this study the average total BMD for the total hip was taken.

3.4 pQCT

Bone characteristics were measured at the non dominant distal radius and dominant tibia was assessed using an XCT-2000 pQCT scanner (Stratec Medizintechnik GnbH, Pforzheim, Germany). Density and size of the bone measures were taken at the epiphyseal site and gross architecture, density and strength and the diaphyseal site. Scans were acquired according to manufacturer's protocols. pQCT scanning involves some radiation exposure, each scout and full scan will give a dose of 1 and 0.9 µSievert, respectively. As we conduct 2 scout scans and 4 full scans this comprises a total does of approximately 5.6 µSievert. To put this dosage of radiation into context, people in the UK receive around 5 – 6 µSievert of background radiation .per day equalling around 2000 µSievert per year, and one hour of jet flight gives around 10 µSievert. Both scan operators have received training on the relevant regulations.

pQCT scans were calibrated with a European forearm phantom. During the study the pQCT scanners underwent daily quality assessment. Radius length was measured as the distance between the olecraneon process and the radial styloid process. The length of the tibia was assessed as the distance between the palpated medial knee joint cleft and medial malleolus. To asses epiphyseal and diaphyseal bone measures, scans were taken at 4 and 60% of the radius length and 4 and 66% of the tibia length, where 0% indicates the most distal part of the respective bones. Data were then exported using the Automated Analysis Tools in Version 6.00 of the software supplied with the machine. A peeling threshold of 650 $mg \cdot cm^{-3}$ was set for diaphyseal sections of bone, and a threshold of 180 $mg \cdot cm^{-3}$ set for the epiphyseal 4% slice. Given the lack of a standard nomenclature for pQCT results, the suggestions for reporting bone structure and density results (http://www.asbmr.org/StandardizationofBoneStructureandDensityNomenclature.aspx) and those of a recent publication (25-pQCT methods paper) have been followed. The parameters examined in the 4% epiphyseal slice were total bone area (Ar.tot, mm^2), total bone mineral content (vBMC.tot, $mg.mm^{-1}$) and trabecular bone mineral density (vBMD.tb, $mg \cdot cm^{-3}$). In diaphyseal sites, Ar.tot, vBMC.tot, cortical area (Ar.ct, mm^2) and cortical density (vBMD.ct, $mg \cdot cm^{-3}$) were examined, with adjustments made to the cortical density values (due to partial volume effect) by equations established in an earlier publication (28-pQCT methods paper). *In-vivo* precision of

the laboratory's pQCT measurements has been reported elsewhere (26-pQCTmethods paper) – precision is < 0.5% for vBMC.tot, Ar.tot and Ar.ct.

3.5 Biochemistry Blood Analysis

Resting 5-mL blood samples were obtained at 12 noon after a minimum of 4 hours of fasting, from the antecubital vein. The samples were collected into EDTA collection tubes. After half an hour they were centrifuged for 12 min at 2,000 g at 4 ^{0}C. The plasma was collected and stored at – 80 º Celcius until analysis. Finger prick blood samples were used to measure Haemglobin concentration (Haemocue) and haematocrit.

Estradiol 17-beta- concentration in the blood was measured with ELISA (Abcam, UK), following the instructions of the manufacturer. Briefly, 25µL of standard and sample were dispensed into their respective wells, and 200µl reagens added. .The plate was then covered with foil and incubated at 37ºC for 2 hours. After incubation the content of each well was aspirated and washed three times with 300 µL of diluted washing solution. Then 100 µL TMB substrate solution was dispensed into all the wells. The well of samples was then incubated in the dark at room temperature for 30 min. After 30 min 100 µL stop solution was dispensed into all the wells and the absorbance measured at 450nm.

Human tartrate-resistant acid phosphatise 5b (TRACP-5b) was measured with ELISA (Cusabio biotree co., Ltd) according to the instructions of the manufacturer. Briefly, 100 µL of standard and sample were added to each well. An adhesive strip was then added to cover the wells and then incubated for 2 hours at 37ºC. After incubation period, the liquid of each well was removed and 100 µL of Biotinylated-antibody pipetted into each well. This was incubated for another hour and then each well is aspirated and washed with 200 µL wash buffer. Then 100 µL of HRP-avidin was added and incubated for another hour. The wash step was repeated after which 90 µL TMB substrate was added to each well and incubated for 15-30 mins at 37ºC protected from the light. Stop solution (50 µL) was then be added to each well. Tapping the plate will ensure thorough mixing. The optical density of each well was determined within 5 mins after adding the stop solution. A microplate reader was used and absorption measured at 450 nm and 540 nm. The reading at 540 nm was subtracted from the reading at 450 nm to correct for optical imperfections in the plate.

Adrenocorticotrophic hormone (ACTH), Osteoprotegerin (OPG), Osteocalcin (OC), Osteoponsin (OPN), Parathyroid hormone (PTH), Fibroblastic growth factor (FGF 23) and SOST were analysed using EMD Milipore Milliplex human bone magnetic bead panel. Briefly, standards were prepared according to instructions of the manufacturer. Reagents were warmed to room temperature (20-2 degrees Celsius). 200 µL of assay buffer was added to each well of the plate. The plate was then sealed and mixed on a plate shaker for 10 minutes at room temperature. The assay buffer was then decanted ensuring any residual was also removed. Then 25 µL of each standard or control was added into their appropriate wells. Then 25 µL of assay buffer was added to the sample wells. A further 25 µL of matrix solution was added to the background, standard and control wells. 25 µL of 1:2 diluted sample was then added into the appropriate wells. Finally 25 µL of mixed beads was then added to each well. The plate was then sealed overnight on a plate shaker at 4 ºC. The following day the plate was washed 3 times thoroughly. 50 µL of detection antibodies were added to each well. The plate was sealed again on a plate shaker for 30 minutes at room temperature. 50 µL of Streptavidin-Phycoerythrin was then added to each well and then the same previous incubation procedure was followed for another 30 minutes. After 30 minutes, the content of each well was removed and the pate washed three times. 100 µL of sheath fluid was added to all wells and the beads re suspended on the plate shaker for 5 minutes. The plate was then run on Luminex 200TM, HTS, FLEXMAP 3DTM or MAGPIX® with xPONENT software and the median fluorescent intensity data was analysed using a 5 parameter logistic or spline curve-fitting method.

3.6 VO_2max

VO_2max was assessed using a standard incremental running treadmill protocol. At the end of the treadmill a safety mat was provided. The test lasted approximately 20 minutes from warm up to cooling down. The heart rate was measured at the end of every minute using a polar heart rate monitor. Oxygen and CO_2 exchange were measured with a Cortex metalyzer.

The test began with the participant standing on the treadmill breathing as normal to collect baseline data. After 2 min the speed of the treadmill was set at 4 $km \cdot hr^{-1}$ and a 0% gradient. After 2 min speed was increased to 8 $km \cdot hr^{-1}$, still maintaining the 0% gradient. After another 2 min speed was increased to 11 $km \cdot hr^{-1}$ still with the 0%

gradient. After running 2 min at 11 km·hr^{-1} the speed was finally increased to 15 km·hr^{-1} and this speed was maintained for the remainder of the test. The incline on the treadmill was increased by 1% every min until the participant was exhausted. The participant received verbal encouragement throughout the test.

3.7 Statistical Analysis

Data were analysed using SPSSv19 (IBM, USA). A one-way ANOVA was used to asses any significant differences between control, eumenorrheic athletes and amenorrheic athletes. Where data was non-normally distributed, data were ^{10}log transformed and a one-way ANOVA done on the transformed data. Differences between groups were considered significant at $P<0.05$. When significance was established overall, to then identify where the differences were located between groups the Tukey test was conducted which gave the p value for comparison between groups.

4.Results

4.1 Participant Characteristics

Table 1 shows the participant characteristics. It can be seen that there were no significant differences in age, body mass and height between the control, amenorrheic athletes (AA) and eumenorrheic athletes (EA). The controls had a significantly lower lean mass than the eumenorrheic athletes, but not amenorrheic athletes (P=0.017). Fat mass was significantly lower in both amenorrheic and eumenorrrheic athletes compared with controls (P<0.0005).

Table 1. **Participant characteristics.**

	Control (C)	Eumenorrheic Athletes (EA)	Amenorrheic Athletes (AA)
n	10	8	7
Age (Years)	23.5±0.58	24.6±2.39	24.3±1.41
Height (m)	1.63±0.02	1.67±0.04	1.65±0.02
Mass (kg)	59.9±2.22	54.9±5.65	50.0±1.93
Body Mass Index (BMI)	22.4±2.30	19.8±2.01 [a]	18.5±1.46 [a]
Lean (kg)	3.78±2.14	4.59±1.72[a]	4.31±1.53
Fat (kg)	19.82±1.71	7.51±0.71[a]	4.81±0.74 [a]
%Fat	32.99±8.05	13.68±3.44[a]	9.38±3.33[a]
%Lean	63.14±8.76	63.91±9.00	66.29±10.89 [a]
VO_2 max(ml-¹kg-¹min-¹) n- NT=7 T=4		64.4±2.95(7)	64.0±4.86(4)
Age Graded Performance %		83.5±9.17	91.0±2.95

Values are presented as mean ± SD

Between brackets n if different from overall

[a] different from C at p<0.05

[b] different from NT at p<0.05

Some athletes volunteered to partake in a VO_2 max test. There was no significant difference between VO_2 max results of amenorrheic and eumenorrheic athletes. Both eumonrrheic and amenorrheic athletes had an age-graded performance higher than 80% for their best ranked running performance, with non significant difference between the two groups of athletes.

4.2 DEXA

In general, the amenorrheic tended to have a lower bone mineral density value in comparison with controls and eumenorrheic athletes. Eumenorrheic athletes tended to have similar bone mineral density values as controls. Interestingly, no significant difference between groups was found in the leg bones, where the runners experience direct impact on their bones.

Table 2: **Comparison between athletes and controls for DEXA (g/cm²).**

	Control (C)	Eumenorrheic Athletes (EA)	Amenorrheic Athletes (AA)
n	10	8	7
Head	2.31±0.08	2.30±0.07	1.95±0.09[a]
Left Total	1.16±0.03	1.94±0.02	1.08±0.03
Right Total	1.16±0.04	1.21±0.02	1.07±0.02
Arms	0.81±0.02	0.842±0.01	0.765±0.03
Legs	1.24±0.05	1.35±0.024	1.20±0.04
Trunk	0.917±0.02	0.898±0.020	0.771±0.02[a][b]
Ribs	0.688±0.02	0.664±0.02	0.602±0.01[a]
Pelvis	1.11±0.04	1.11±0.03	0.924±0.03[a][b]
Spine	1.06±0.03	1.00±0.03	0.847±0.04[a]
Total Body	1.16±0.03	1.20±0.02	1.07±0.03[b]
Right Femur (7)	1.05±0.06	1.15±0.03	0.924±0.05
Left Femur (7)	1.05±0.06	1.15±0.03	0.943±0.06
L1-L4	1.17±0.03	1.15±0.03	0.962±0.05[a]

Data are mean ± SEM

[a] different from C at $p<0.05$

[b] different from NT at $p<0.05$

4.3 pQCT

pQCT analysis was carried out at the 4% epiphyseal and 60% of the diaphysis of both the non weight bearing radius and weight bearing tibia. At the non weight bearing radius there was a significantly lower cortical thickness in the amenorrheic athletes compared with eumenorrheic athletes, figure 2a ($p=0.025$). However, the triad athletes had an endochondrol circumference that was greater than both control and eumenorrheic athletes, meaning that the amoenorrheic athletes had a diaphysis much wider and thinner than the control and eumenorrheic athletes, figure 2b ($p=0.011$). There was also a significant difference at the weight bearing tibia cortical area of the diaphysis between the control and amenorrheic athletes but not the control and eumenorrheic athletes, shown in figure 2c ($p=0.017$). For detailed definition of pQCT abbreviations see Appendix 7.4.

Table 3: **Comparison between athletes and controls for pQCT.**

		Control (C)	Eumenorrheic Athletes (EA)	Amenorrheic Athletes (AA)
n		10	8	6
Radius Diaphysis	Ar.tot (mm^2)	101.0±3.51	103.0±3.48	108.0±4.46
	$_v$BMDct ($mg.mm^{-3}$)	1185.0±8.3	1192.0±4.76	1152.0±17.30
	Ar.ct (mm^2)	76.0±2.88	80.0±2.28	71.0±4.8
	$Ct.Th_{der}$ (mm)	2.90±0.12	3.02±0.08	2.47±0.19[b]
	PsC (mm)	36.0±0.62	36.0±0.60	37.0±0.76
	EcC (mm)	17.3±0.85	17.1±0.76	21.3±1.19[ab]
Radius Epiphysis	$_v$BMC.tot ($mg.mm^{-1}$)	107.0±5.88	116.0±5.76	94.0±2.78
	$_v$BMD.tb ($mg.mm^{-3}$)	196.0±13.30	204.0±16.65	154.0±9.00
	Ar.tot (mm^2)	329.0±16.9	356.0±23.44	359.0±9.67
Tibia Diaphysis	Ar.tot (mm^2)	448.0±22.2	493.0±14.58	526.0±27.76
	vBMDct ($mg.mm^{-3}$)	1182.0±9.57	1176.0±9.29	1155.0±15.38
	Ar.ct (mm^2)	284.6±12.1	334.6±12.43[a]	291.6±11.46
	$Ct.Th_{der}$ (mm)	4.85±0.29	5.60±0.32	4.38±0.31
	PsC (mm)	74.8±1.77	76.7±1.16	81.1±2.13
	EcC (mm)	44.3±3.02	44.1±2.64	53.6±3.69
Tibia Epiphysis	vBMC.tot ($mg.mm^{-1}$)	311.0±16.48	320.0±33.8	317.0±13.7
	vBMD.tb ($mg.mm^{-3}$)	241.0±14.12	257.0±15.3	249.0±11.0
	Ar.tot (mm^2)	986.0±30.29	950.0±89.3	1091.0±22.2

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pQCT measurement abbreviations; Ar.tot (mm^2) - Total area, vBMDct ($mg.mm^{-3}$) - Cortical bone mineral density, Ar.ct (mm^2) - Cortical Area, $Ct.Th_{der}$ (mm) - Cortical Thickness, PsC (mm) - Perichondral circumference, EcC (mm) - Endochondral circumference, vBMC.tot ($mg.mm^{-1}$) - Total bone mineral content, vBMD.tb ($mg.mm^{-3}$) - Trabecular bone mineral density

Data are presented as mean ± SEM

[a] different from C at $p<0.05$

[b] different from NT at $p<0.05$

Figures 2: **Bone measurements form pQCT in athletes and controls. p<0.05 C vs NT *, p<0.05 C vs T **, p<0.05 T vs NT *****

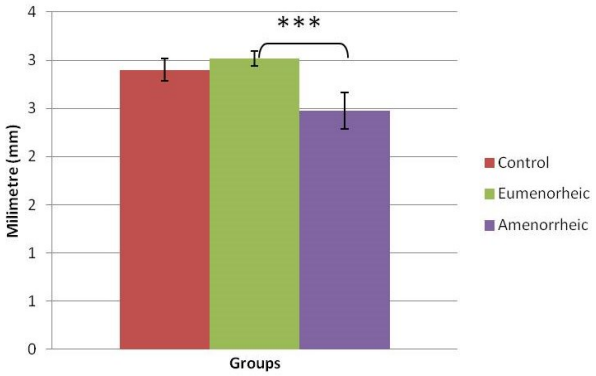

Figure 2a: **Cortical Thickness of Radius Diaphyseal Section**

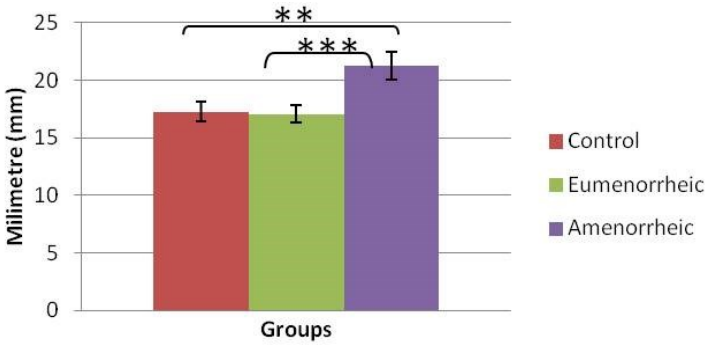

Figure 2b: **Endochondral circumference of radial Diaphyseal section**

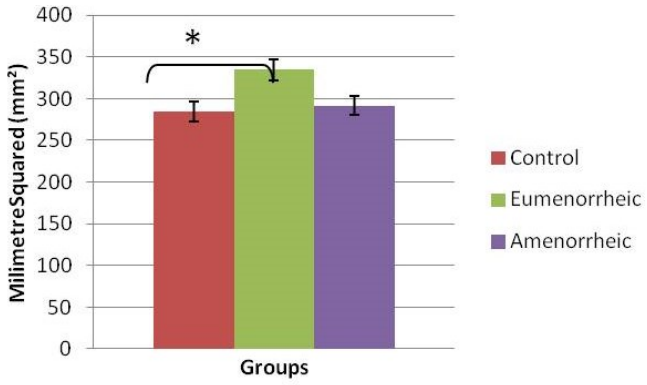

Figure 2c: **Cortical Area of Tibia Diaphyseal Section**

4.4 Circulating Hormones and bone markers

In blood samples the following proteins were measured: Oestrogen, Tartrate resistant- acid phosphatase (TRAP), Adrenocorticotrophic hormone (ACTH), Osteoprotegerin (OPG), Osteocalcin (OC), Osteoponsin (OPN), Parathyroid hormone (PTH), Fibroblastic growth factor (FGF 23) and SOST. Groups did not differ in any of the analytes, except for TRAP, which was significantly higher in amenorrheic athletes compared with control (P=0.026).

Table 4: **Circulating markers of bone remodelling**

	Control (C)	Eumenorrheic Athletes (EA)	Amenorrheic Athletes (AA)
n	6	7	6
TRAP(U/ml)	0.048±0.041	0.074±0.0278	0.43±0.22[a]
Oestrogen (pg/ml) n- C=6, NT=7, T=5	1.545±0.00 (6)	1.545±0.00 (7)	1.545±0.00 (5)
ACTH (pg/ml)	2.50±2.50	0.65±0.65	4.65±4.02
DKK1 (pg/ml)	1215±107	1038±104	865±124
OPG (pg/ml)	225±27.6	247±23.1	197±15.0
OC (pg/ml)	12145±1962	12619±825	8471±990
OPN (pg/ml)	18072.75±2928	26623±3042	19100±3740
SOST(pg/ml)	3137±598	3107±233	2741±283
PTH (pg/ml)	85.9±16.8	87.1±12.5	64.8±7.39
FGF23 (pg/ml)	296±195	120±59.0	420±101

Data are mean ± SEM

Between brackets n if different from overall

[a] different from C at $p<0.05$

[b] different from NT at $p<0.05$

4.5 Food Intake

Significant differences between groups were identified for all food groups, except for the total calcium intake per day. For total calorie, proteins, carbohydrate, potassium and phosphorous intake per day, both groups of athletes consumed higher amounts compared with controls but the amenorrheic athletes had a lower intake than the eumenorrheic athletes. For total fat and magnesium intake per day, the eumenorrheic athletes consumed higher amounts compared with controls.

Table 5: **Food intake measurements for athletes and controls**

	Control (C)	Eumenorrheic Athletes (EA)	Amenorrheic Athletes (AA)
n	8	7	6
Total Calorie Intake/ day ($kcal \cdot day^{-1}$)	1264±166	2725±228.0[a]	2382±330[a]
Total Protein/day ($g \cdot day^{-1}$)	53.9±5.21	95.2±10.67[a]	88.8±10.2[a]
Total Carbohydrate/day ($g \cdot day^{-1}$)	168±31.4	437±40.8[a]	378±57.5[a]
Total Fat/day ($g \cdot day^{-1}$)	41.0±7.95	78.0±6.30[a]	68.0±11.0
Total Potassium/day ($mg \cdot day^{-1}$)	2387±334	4132±613.0[a]	4385±647[a]
Total Calcium/day ($mg \cdot day^{-1}$)	545±84.0	1403±343.0	1178±140
Total Magnesium/day ($mg \cdot day^{-1}$)	228±28.0	444±106.0[a]	392.0±52.1
Total Phosphorous/day ($mg \cdot day^{-1}$)	1015±110.0	1822±293[a]	1708±185[a]

Data are shown mean ± SEM

[a] different from C at $p<0.05$

5.Discussion

The Female athlete triad is defined as disordered eating, amenorrhea and osteoporosis and in some cases has reached up to a prevalence of 62% of female athletes (Waldrop, 2005; Rosen & Hough, 1988). The main aim of this study was to examine total body bone mineral density and to examine in closer detail the radial and tibial bone characteristics of elite female endurance runners with, and without, menstrual irregularities. The results showed that amenorrhoeic athletes (AA) had lower bone mineral density in the lumbar spine, pelvis, ribs, trunk and head compared with age-matched non-athletic young women. Compared with eumenorrhoeic athletes (EA), the AA had lower bone mineral density of the total body, pelvis and trunk. The data from pQCT showed the endochondrial circumference of the radius diaphysis to be higher in AA compared with controls and EA, while the cortical thickness of the radius diaphysis was smaller in AA compared with EA. The bone differences of AA were accompanied by elevated circulating levels of TRAP.

Previous literature has identified the influence of different components, individually, upon the female athlete triad. In general, the female athletes train to a high level succumbing their bone to a lot of strenuous exercise. This causes an imbalance of hormones in turn having a negative impact on their menstrual cycle. This alteration of hormone balance then influences the bone health where net bone resorption occurs.

More and more female athletes suffer from the female athlete triad as the up and coming generations arise. The way in which the problem can be counteracted is going to be prevention. This prevention could involve exercise or supplementation intervention or simply better and more education programmes involving governing bodies that can provide a monitoring system for their vulnerable athletes from a young age. This education is a vital necessity that must be informed and this study has been a step toward getting that informed information. For the masters study we chose to group the elite athletes according to their menstrual cycle irregularities, as this is a major problem and influence on the bone health, and measured their bone characteristics using pQCT and DEXA as well as looking at the other components and influences of the triad as separate measures of the individual groups of athletes. The other components are nutritional input, hormonal balance via blood draw and general bodily characteristics. By conducting such comparisons, differences between

the athletes can be detected with findings from previous literature using general athletes rather than elite and identify where the balance seems to be lost.
The main observation of this study was that elite level endurance athletes with amenorrhea had lower bone mineral density compared with eumenorrheic athletes and controls. This was seen in both the radius and the tibia and was associated with increased circulating levels of TRAP.
Numerous bone health parameters were measured to get an overall impression of the possible effects of amenorrhoeia on bone health. pQCT scanning was carried out at 4% and 60% of the non dominant non weight bearing radius and the dominant weight bearing tibia. At the non weight bearing radius the amenorrheic athletes had a significantly lower cortical thickness than eumenorrheic athletes (P=0.025). However, the amenorrheic athletes had an endochondrol circumference that was greater than both control and eumenorrheic athletes, meaning that the amenorrheic athletes had a diaphysis much wider and thinner than the control and eumenorrheic athletes (P=0.011). Statistical analysis also demonstrated a significant difference at the weight bearing tibia, the cortical area of the diaphysis between the control and amenorrheic athletes but not the control and eumenorrheic athetes, (P=0.017).
These observations confirm previous findings, already highlighted, that demonstrate the detrimental effects on the bone health from menstrual cycle irregularities and components of the female athlete triad. Our findings, demonstrate that the amenorrheic athletes do experience some bone benefits from the impact of their physical activity in the lower limbs. Although compromised, lower bone health in AA than EA, AA have a better bone health than controls indicating some benefit of impact exercise. In the upper limbs, where little loading occurs, they have a lower bone strength which could be pinpointed to their potential lack of nutrition or their hormonal status. Other studies using pQCT have demonstrated similar findings. Ackerman et al (2011) studied a group of aged 16-21 years amenhorrhiec athletes, eumenorrheic athletes and controls were assed using DEXA and pQCT. At weight bearing sites the athletes tended to have a greater total trabecular area of bone compared to controls. However, looking at the bone micro architecture in finer detail it is seen that the amenorrhiec athletes had a lower cortical thickness than those eumenorrheic; they were also found to have a lowered trabecular number and higher separation. These findings agree with what we have demonstrated in this study, that those athletes with amenorrhea or have components of the triad show a deterioration

in bone health at non weight bearing sites, such as pQCT findings of the radius, despite high levels of physical activity.

DEXA results also support this finding; showing that the amenorrheic athletes have a reduction in bone health in comparison to the gains shown by eumenorrehic athletes over controls. The limitation on significant findings is due to the limited numbers. DEXA revealed significant findings that demonstrated that amenorrehic athletes tended to portray a lower bone mineral density in comparison with controls than the eumenorrheic athletes. Interestingly, these significant findings were not on the direct weight bearing areas in elite runners such as the legs. Thus, suggesting that, even though the athlete is within the triad, not all the positive effects are lost from their impact through physical activity to the bone at the weight bearing sites. Previous studies by Young et al (1994) using ballet dancers demonstrated that at weight bearing sites the BMD is found to be normal or elevated in those with a regular menstrual cycle (similar to the non triad athletes in this study) but normal or reduced in those with anorexia (similar to the triad athletes). However, from table 2 it is clear to see that there is a clear pattern that is followed, in all cases the BMD value of the EA is at the same level or in most cases elevated in comparison to controls, in all cases the AA BMD values are less than both the control group and non triad athletes. The limited significant findings could be linked with the varied numbers in groups and the small overall sample size, which is invariably a limitation in studies of elite-level athletes.

TRAP is a marker for increased bone resorption and may provide a mechanistic insight to explain the reduced bone mineral density of AA measured from the DEXA and pQCT. Amenorrheic athletes had levels of 0.43 U/ml with controls just 0.048 U/ml and eumenorrheic athletes 0.074 U/ml; thus demonstrating a much higher level of TRAP in the amenorrheic athletes. This result further follows the similar pattern of previous findings (REF). However, no significant findings were found in any of the other markers of bone remodelling in this study. This may be due to the high inter-individual variability of the markers for each hormone or due to the fact that the low sample size was not enough to identify significant differences. TRAP has not regularly been examined in elite athletes of such young age as it is normally used to identify bone resorption in the diagnosis of osteoporosis (Bikle, 1997; Garnero and Dalmas, 1997). Biochemical analysis of TRAP has also been progressed to use as an early identification of rheumatoid arthritis (Garnero et al., 2002). Our results

suggest TRAP maybe a marker in young females for susceptibility or detection of the triad. This then may help to initiate early interventions to rectify bone health while the athlete has not reached the end of the 'window of opportunity' in terms of bone health.

Interestingly, PTH showed a similar, though inverse, pattern to TRAP, although it did not reach significance; Amenorrheic athletes demonstrate the lowest level of PTH at 64.8±7.39pg/ml, controls and eumenorrheic athletes have similar levels at 85.9±16.8pg/ml and 87.1±12.5pg/ml, respectively. PTH hormone regulates calcium levels in the blood and is high when calcium levels in the blood are low. The lower levels of PTH in the blood could indicate that the response mechanism of the eumenorrheic athletes is altered due to their hormonal menstrual status and so cannot respond to lower levels of calcium appropriately and therefore the consequence will be resorption of the bone to restore correct levels of calcium.

Osteocalcin measures also demonstrate a pattern supporting the findings of this study. Osteocalcin is a marker found where bone building arises by the action of osteoblasts. The amenorrheic values are much lower than both the eumenorrheic and controls in this case, although not significant; it may indicate a tendency for bone resorption arising in AA.

Finally from the food diary a number of significant differences were identified between groupings. For total Calorie, proteins, carbohydrate, potassium and phosphorous intake per day a significant difference was found between the both groups of athletes and the control groups, as demonstrated by table 5. In each case both the amenorrheic and eumenorrheic athletes had a larger intake than controls, however, the total calorie intake of the controls was only 1264 ± 166 kCal/day, which is considerably lower than the expected total calorie intake of healthy young women and raises the possibility of under-reporting of food intake by the control group. For total fat and magnesium intake per day there was only a significant difference between the eumenorrheic athletes and control group, with the eumenorrheic athletes consuming a greater amount. The food diary findings do not show a direct link to the bone health as did the DEXA, pQCT and TRAP bone marker result, however, it does suggest a further possible factor that may contribute to the bone health of the athlete. In each case reported the amenorrheic athlete consumed less than that of the eumenorrheic athlete, In all cases the AA and EA consumed more than controls but the AA eating less than EA. These findings combined with bone

characteristic results would suggest the AA would be in a state of energy deficit. However, the use of food diaries does entail the risk of misreporting as shown by Posluna et al (2009). In the case there were some abnormal readings, one from each group of which were omitted from analysis and all analysis was carried out using the same procedure and software eliminating as far as possible bias due to wrong reporting and subjective analysis. Again there was still a small sample size and ideally larger numbers of participants are needed to ensure findings are consistent. A further limitation is that food diaries only reveal half the picture for energy balance, the other half coming from energy expenditure. In this study, it was not possible to monitor habitual energy expenditure so we cannot conclude on whether any of the groups were in a negative energy state.

The main limitation of the present study was the small sample size. Small sample sizes are inherent in studies of elite athletes. Furthermore, the main interest in this study was to examine differences between triad athletes and non-triad, however, it was not possible to make such grouping distinctions a-priori because food intake and bone density needed to be measured during the testing session. Thus, groupings were made on the menstrual status.

Overall the findings from this study suggest elite female endurance athletes who report menstrual cycle disturbances were significantly more likely to have lower bone mineral density in several sites across their body as well as elevated levels of TRAP which indicate greater resorption. Further studies should be conducted on a greater scale that will aid in the understanding if the female athlete triad and then in turn highlight areas that can be targeted as an approach for early intervention and possible areas that can be studied for therapies that might limit the triad from establishing itself initially.

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7.Appendix

7.1 Food diary

As part of your study participation, you will be asked to keep a Diet Diary of *everything* you eat and drink for 3days.

Begin with the first food or beverage in the morning and write down what you eat as you go throughout the day. The dietitian will review your completed Diet Diary. Please bring your Diet Diary at your study visit

Diet Diary

GENERAL INSTRUCTIONS

1. Write legibly.
2. Record each meal/snack as soon as possible after it is eaten.
3. Record one food item per line and leave a blank line between meals/snacks.
4. Start each new day on a new page. Additional pages are provided as you may need more than one page per day.
5. Please record the time.
6. When eating out or eating take-out food please record the name of the restaurant.
7. *Details, details, details!!!* Remember to describe everything you can about a food. List contents of mixed dishes (i.e. casseroles and soups), and include brand names when possible. Some descriptions may include fresh, in juice, in syrup, in water, battered, frosted, fat-free, reduced fat, sugar-free, decaffeinated, flavor (chocolate, strawberry, etc), with or without skin, cut of meat, % fat, lean, type of grain, milk-skim,1%, 2%, whole.
8. For each food, record the preparation method (i.e. baked, boiled, steamed, pan-fried, deep-fried, stir-fried, broiled, grilled, roasted, microwaved). This is extremely important, especially for meats.
9. Record and describe any additions to a food (i.e. salad dressings, sugar, margarine, butter, catsup, relish, pickles, mayonnaise, mustard, gravies, cream, etc.) that were added in cooking or eaten with the food. Also don't forget to record beverages, candy, gum, "bites" or "tastes" of foods, etc.
10. Record the amount of each food item (and any addition) using common household measurements. Add raw / prepared for amounts of meat. Common measures may include teaspoon, tablespoon, cup, ounces, pounds, and fluid ounces. Dimensions may also be used for example 1/8th of a 9 inch pie or 2" X 3" X ½". Refer to the Visualize Your Portion Size to help estimate portion sizes. Record only the amount of food you actually consumed.

VISUALIZE YOUR PORTION SIZE

Can you visualize 3 ounces of meat or tell how many ounces of mashed potatoes you ate for dinner? Good knowledge of your portion sizes is important for you to record your diet correctly and for us to calculate your nutrient intake accurately.
Here are some useful tips to help you estimate how much you eat.

Visual hints-

- ¼ cup is about the size of a golf ball or ping-pong ball.
- ½ cup is about the size of a cupcake or muffin paper liner.
- 1 cup is about the size of a baseball or tennis ball.
- An inch diameter sauce cup will hold one ounce.
- A 'pint' deli container holds 16 oz or about 2 cups.

¼ C =  Golf ball

1 C =  Tennis ball

Meats

- 3 oz serving is similar in size to a deck of playing cards or an audiotape.
- A 1-inch meatball is about an ounce.
- 4 oz of raw, lean meat is about 3 ounces after cooking.
- 3 oz of grilled fish is the size of a checkbook.

3 oz  = deck of cards

3 oz  = a  checkbook

Fruits and Veggies-

- A medium apple, peach, or orange is about the size of a tennis ball.
- 3 medium pieces of fruit will weigh about one pound.
- 1 cup of vegetables or mashed potatoes is about the size
- of your fist.
- A cup of lettuce is about four leaves.

1 medium  =  = 

3 medium pieces  = 1 pound

Cereal and pastas

- A handful of cereal, snacks, or chips is ½ - 1 cup, depending on the size of your hand. Grab a handful of cereal or chips and measure it, to determine what "a handful" for you really is.
- 1-cup pasta is about a tennis ball size.
- An average bagel is the size of a hockey puck.

1 cup pasta  = 

1 bagel  =  hockey puck

Cheese, butter and spreads

- 1 oz of cheese is about 1 inch square or about the size of your thumb or four stacked dice.
- 1 Tbsp of peanut butter/ butter is about the size of your thumb.
- 1 tsp of peanut butter is about the area of your thumbnail.
- A typical salad dressing ladle in a restaurant will hold 3-4 Tbsp of dressing.

1 oz  Cheese =  your thumb

1 tsp  butter/spread = tip of thumb

Date: ☐☐/☐☐/☐☐☐☐ (mm/dd/yyyy)

Entered on ☐☐/☐☐/☐☐☐☐ (mm/dd/yyyy)

Please fill in all foods and beverages you take during the day.

Time	Food / Beverage & preparation method	Amount	Notes

7.2 Patient Information Sheet and Questionnaire

Training and Menstrual history

Participant Surname:	Participant First Name:	Participant ID:
Gender:	D.O.B.:	Height:
Weight:	Contact email address:	

Training

At what age did you first begin to partake in endurance running at an elite level?

Please list some of the highest level achievements that you have reached since starting elite endurance running and at what age these were achieved?

How many times would you compete on average in one month?

In brief please could you provide an outline of your weekly training? Not only include running but anything additional such as cross training/ gym work/drills etc and how long each one would normally take.

Please see the example below:

Day	AM	PM
Monday	Run 50 mins Gym work 1 hour	20 min run Rehab 20 mins
Tuesday	Running session lasting about 2 hours in total	
Wednesday	As Monday	
Thursday	Running session lasting about 2 hours in total	
Friday	20 min run 1 hour gym work	
Saturday	Running session lasting about 2 hours in total	
Sunday	Long run 60-90 mins	45 min gym work

Day	AM	PM
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		

Sunday		

Menstrual History:

At what age(including months), if any, did you first begin menstruating?

If not begun menstruation please state

Are you taking any medication to help regulate your menstrual cycle such as the oral contraceptive pill? Please state if so and briefly state when you started to take the medication. (Please state if taking the pill for other reasons and when started to take)

Has there since been any disruption to your regular cycle? If so please can you state approximately when this occurred and how long it lasted? Please list all disruptions if multiple.

Menstrual cycle Irregularities Summary to be filled in:

Time of Irregularity e.g. Feb 2001	**How long irregularity lasted? Or is it still ongoing?**	**Training load at time of irregularity?**	**Any other info that may be linked? Change in environment/ eating habits etc?**

At what stage of your menstrual cycle are you today? And what date was the start and end of your last menstrual cycle?

Do you smoke? (please circle)

- YES
- NO

If yes how many on average per week do you smoke?

Do you consume alcohol? (please circle)

- YES
- NO

If so please state the frequency that suits you the best (please circle)

- Everyday
- 2-3 times per week
- Monthly
- Odd occasion

Please state any other relevant info that you may think will be helpful:

7.3 Patient Information Sheet and Questionnaire- Control

Training and Menstrual history

Participant Surname:	Participant First Name:	Participant ID:
Gender:	D.O.B.:	Height:
Weight:	Contact email address:	

Exercise

Do you partake in any physical activity? If so please state the type.

Please list if any the amount of physical activity you would partake in on average in a week.

In brief please could you provide an outline of you usual week including work/ exercise/social and timings etc

Please see the example below:

Day	AM	PM
Monday	University work	20 min run Rehab 20 mins
Tuesday	Work 9-5	Aerobics 30 min
Wednesday	Work 9-5	
Thursday	University	30 min cycle
Friday	Rest	

Saturday		Out at night for 3 course meal and drinks
Sunday	rest	45 min gym work

Day	AM	PM
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		
Sunday		

Menstrual History:

At what age(including months), if any, did you first begin menstruating?

If not begun menstruation please state

Are you taking any medication to help regulate your menstrual cycle such as the oral contraceptive pill? Please state if so and briefly state when you started to take the medication. (Please state if taking the pill for other reasons and when started to take)

Has there since been any disruption to your regular cycle? If so please can you state approximately when this occurred and how long it lasted? Please list all disruptions if multiple.

Menstrual cycle Irregularities Summary to be filled in:

Time of Irregularity e.g. Feb 2001	**How long irregularity lasted? Or is it still ongoing?**	**Training load at time of irregularity?**	**Any other info that may be linked? Change in environment/ eating habits etc?**

At what stage of your menstrual cycle are you today? And what date was the start and end of your last menstrual cycle?

Do you smoke? (please circle)

- YES
- NO

If yes how many on average per week do you smoke?

Do you consume alcohol? (please circle)

- YES
- NO

If so please state the frequency that suits you the best (please circle)

- Everyday
- 2-3 times per week
- Monthly
- Odd occasion

Please state any other relevant info that you may think will be helpful: