

The Impact of Vitamin D Status upon Markers of Athlete Health

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Authors Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

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Date

I. Abstract

Introduction

At present there is a pandemic of low serum vitamin D (25[OH]D) concentration, partly due to a lack of sun exposure (the primary route for synthesis) and modern lifestyle choices. The bioactive form of vitamin D, 1,25-dihydroxyvitamin D (1,25[OH]₂D₃) exerts its biological activity by binding to the vitamin D receptor (VDR). These receptors play a central role in the biological actions of vitamin D and are expressed in nearly every tissue and cell type in the body (M. Holick, 2007).

Vitamin D deficiency is widespread within many general and athletic populations and associated with a number of detrimental health conditions, including a long-term impact on cardiovascular health (M. Holick, 2007; Larson-Meyer & Willis, 2010; Pittas, Lau, Hu, & Dawson-Hughes, 2007), and the aetiology of osteomalacia and osteoporosis (M. F. Holick, 2009). Given the prevalence and potential negative morbidity associated with deficiency (Larson-Meyer & Willis, 2010), regular vitamin D testing has been recommended as part of routine athlete screening.

Current literature shows inconsistent associations between vitamin D status and bone mineral density and cardiac health; (Bischoff-Ferrari, Kiel, et al., 2009; Marwaha et al., 2011) particularly in racial minorities and athletic populations. Whilst it is considered that athletes should have 'sufficient' vitamin D concentrations, the exact value to 'optimise' health is equivocal.

Finally, there appears to be a 'paradoxical relationship' between ethnicity and vitamin D concentration, that has largely been ignored, *i.e.* blacks generally present with the lowest vitamin D concentrations but the greatest bone mineral density (BMD) and reduced risk of fracture (Cauley et al., 2005). Vitamin D-binding protein (DBP) may account for observed racial differences in

manifestations of vitamin D (Powe et al., 2013). To date, research on vitamin D status in athletes has overlooked DBP. Whilst there are data that support the associations between vitamin D and markers of bone and cardiac health in the general population, definitive relationships in the athletic population are yet to be established. Therefore, the aim of this thesis was to examine the relationship between vitamin D and measures of bone mass and cardiac structure and function within a large, ethnically diverse cohort of healthy athletes, with a focus to the role of DBP in determining racial differences in bioavailable levels.

Studies

1. Oral vs. Intramuscular Vitamin D Supplementation for Treating Insufficient Athletes
2. No Association between Vitamin D Deficiency and Markers of Bone Mass in Athletes
3. No Association between Vitamin D Status and Markers of Bone Mass in Non-Weight Bearing Athletes
4. Why don't serum Vitamin D concentrations associate with BMD by DXA? A case of being 'bound' to the wrong assay? Implications for Vitamin D screening
5. Severely Vitamin D-Deficient Athletes Present Smaller Hearts than Sufficient Athletes

Methodical overview

Male athletes registered with the Qatar Olympic Committee (QOC) presented for pre-competition medical assessment at Aspetar Sports Medicine Hospital, Qatar. All athletes completed a vitamin D questionnaire that included questions specifically related to country of origin, sporting discipline, skin type, self-reported exposure to daily sunlight, use of sunscreen, dietary supplements and/or medication, and an assessment of skin colour.

All individuals undertook bone densitometry and body composition analysis by dual-energy x-ray absorptiometry (DXA; Osteocore III, Perols, France, version 5.22b). Venous blood samples were collected from athletes following an overnight fast and was analysed for PTH, calcium, albumin and serum 25[OH]D. Athletes were split into four 25[OH]D categories; severely deficient (<10 ng/mL), deficient (10–20 ng/mL), insufficient (20–30 ng/mL), or sufficient (>30 ng/mL). Serum vitamin D binding protein (DBP) concentrations ($\mu\text{g/mL}$) were determined using a commercially available kit (R&D Systems, UK). Free, bioavailable, and DBP-bound 25[OH]D were calculated using equations from supplementary material of (Powe et al., 2013). Lastly, all individuals assessed for family history of cardiovascular disease and personal symptoms, with a physical examination, 12-lead electrocardiogram and an echocardiogram.

Results

The key findings from the thesis are 1) serum 25[OH]D concentrations are not associated with markers of bone mass 2) bioavailable vitamin D is a better preceptor of BMD that serum 25[OH]D concentration and 3) severely 25[OH]D deficient athletes present with smaller cardiac structure that sufficient athletes.

Conclusion

In a healthy, ethnically diverse athletic population, there is no relationship between serum 25[OH]D concentration and makers of bone mass, regardless of sporting type and that bioavailable vitamin D is a better predictor of bone mineral density. Suggesting that our chosen method of assessment may not be appropriate to identify true deficiencies.

Systematic screening to determine 25[OH]D concentrations is expensive, and demonstrates a poor relationship to bone mass in an ethnically diverse athletic population. It can be argued that vitamin D testing should be reserved for the symptomatic athlete (i.e. musculoskeletal injury, REDs). In

turn, prophylactic vitamin D supplementation (2000IU/d D₃) to ‘correct’ insufficient athletes with normal bone health can be questioned, since supplementation recommendations are based on a measure that is not associated with bone health. Severely 25[OH]D deficient athletes present with smaller (<10 ng/ml) presented significantly smaller cardiac structures than insufficient (20–30 ng/ml) and sufficient (>30 ng/ml) athletes. The precise mechanism(s) causing this cardiac hypertrophy (or in our case, lack of hypertrophy) in the 25[OH]D-deficient state remains unclear. Clinically low vitamin D concentrations are detrimental to aspects of health that influence athletic performance. Therefore, the widespread prevalence of low serum 25[OH]D concentrations should not be ignored. However, vitamin D metabolism is a rapidly evolving field, with the prospect of a more complete picture of this complex endocrine system becoming ever so closer. The challenge for future research is to determine ethnically specific concentration ranges and evidenced based guidelines for the diagnosis and treatment of ‘true’ vitamin D deficiency and its impact on athlete health and performance.

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II. Dedication

For Amy, Ava, Hugo and Edward

III. Abbreviations

$1\alpha,25[\text{OH}]_2\text{D}_3$ - $1\alpha,25$ -dihydroxyvitamin D_3

$24,25[\text{OH}]\text{D}$ – $24,25$ -hydroxyvitamin D

$25[\text{OH}]\text{D}$ – 25 -hydroxyvitamin D

7 DHC- 7 -dehydrocholesterol

Alb – albumin

AMP – adenosine monophosphate

Ao – aortic root (mm)

BMD - bone mineral density (g/cm^2)

BMI – body mass index (kg/m^2)

Bio D - concentration of bioavailable D

BP – blood pressure (mmHg)

Ca - calcium

CHF - congestive heart failure

CI – confidence interval

CKD - chronic kidney disease

CSA – cross sectional area

CVD - cardiovascular disease

CV% - co-efficient of variation (%)

DAIb - concentration of albumin-bound vitamin D

DBP – vitamin D binding protein

DDBP - concentration of D-binding protein-bound 25 -hydroxyvitamin D

DFree - concentration of free (unbound) 25 -hydroxyvitamin D

DNA – deoxyribonucleic acid

DXA – Dual-energy x-ray absorptiometry

EAR – Estimated average requirement

ECG - Echocardiogram

ECM – extracellular matrix

EDTA - Ethylenediaminetetraacetic acid
EMS - electromyostimulation
EtOH - ethanol
FIFA - Fédération Internationale de Football Association
GC- group-specific component
IGF – insulin like growth factor
IM - intramuscular
IU – international units
IVSd - intraventricular septum diameter (mm)
Kalb - affinity constant between vitamin D and albumin = $6 \times 10^5 \text{ M}^{-1}$
KDBP - genotype-nonspecific affinity constant between 25-hydroxyvitamin D and DBP = $0.7 \times 10^9 \text{ M}^{-1}$
LA – left atrium (mm^2)
LVIDd - left ventricular internal diameter during diastole (mm)
LVM - left ventricular mass (g)
IVSd - intraventricular septum diameter during diastole (mm)
LVvolD - left ventricular volume during diastole (mL)
MI - myocardial infarction
mRNA - messenger ribonucleic acid
MSC - mesenchymal stem cell
PHE - periodic health examination
PTH - parathyroid hormone
PWTd - posterior wall thickness in diastole (mm)
RA - right atrium (mm^2)
RANKL- receptor activator of nuclear factor kappa-B ligand
RDA – recommended dietary allowance
SCD - sudden cardiac death
SD – standard deviation
SNP - single-nucleotide polymorphisms
Total DBP - concentration of serum DBP (g/L)

TUL - tolerable upper limit

UEFA - Union of European Football Associations

UV – ultraviolet

VDR – vitamin D receptor

Chapter 1:

General Introduction

1.1 Introduction

Vitamin D is a lipophilic pro-hormone created when adequate exposure to sunlight is available via synthesis in the skin. Produced in the earliest forms of life over 750 million years ago (M. F. Holick, 2003b), vitamin D synthesis became essential for land-dwelling vertebrates that required a mechanism to increase the efficiency of calcium absorption and maintain calcium and phosphate homeostasis. The natural selection hypothesis suggests that lighter skin colour evolved to optimise vitamin D synthesis for humans living at higher latitudes.

Fossil samples suggest the genus *Homo sapiens* evolved in equatorial Africa some 160,000 years ago, where the ultraviolet-B (UVB) rich insolation produced a deeply melaninised integument. The dark skin protected the dermis including the sweat glands from UV-induced damage, hence preserving thermoregulation function (Jablonski & Chaplin, 2000). Approximately, 40,000 years ago, hominids migrating into non-tropical latitudes faced the challenge of gaining sufficient vitamin D to meet their physiological requirements. As man's exposure to solar radiation lessened, so did the need for melanin; which absorbs ultraviolet radiation in the dermis, required for converting 7-dehydrocholesterol, a derivative of cholesterol, to pre-vitamin D under thermal isomerisation (Yuen & Jablonski, 2010). Depigmentation evolved to optimise vitamin D production at higher latitudes as the amount of UVB radiation is weaker and filtered by the more angled and longer path through the atmosphere (Loomis, 1967). Despite the evolutionary drive for skin depigmentation, vitamin D deficiency is pandemic in general and athletic populations, partly due to modern migration, a predominately indoor lifestyle alongside increased air pollution (Hosseinpanah et al., 2010) and increased use of sunscreen due to an increased awareness of skin melanomas (de Gruijl, 1999; Smedby et al., 2005).

In 1914, McCollum et al. successfully isolated and subsequently identified fat soluble vitamin D. This discovery together with a greater understanding of vitamin D led to treatment of the bone disease, rickets, with ultra-violet light (McCollum & Davis, 1914). In recent years the interest in vitamin D research has increased exponentially (Figure 1.1), partly due to the observation of an increased prevalence of deficiency in many populations (regardless of age, gender and ethnicity) and the association between vitamin D deficiency and a wide range of disease states (B. Hamilton, 2010). In athletes, vitamin D research currently goes beyond the exploration of bone health and disease, into athletic performance. Given the prevalence and potential negative morbidity associated with deficiency (Cannell & Hollis, 2008; Larson-Meyer & Willis, 2010), regular vitamin D testing has been recommended as part of routine athlete screening.

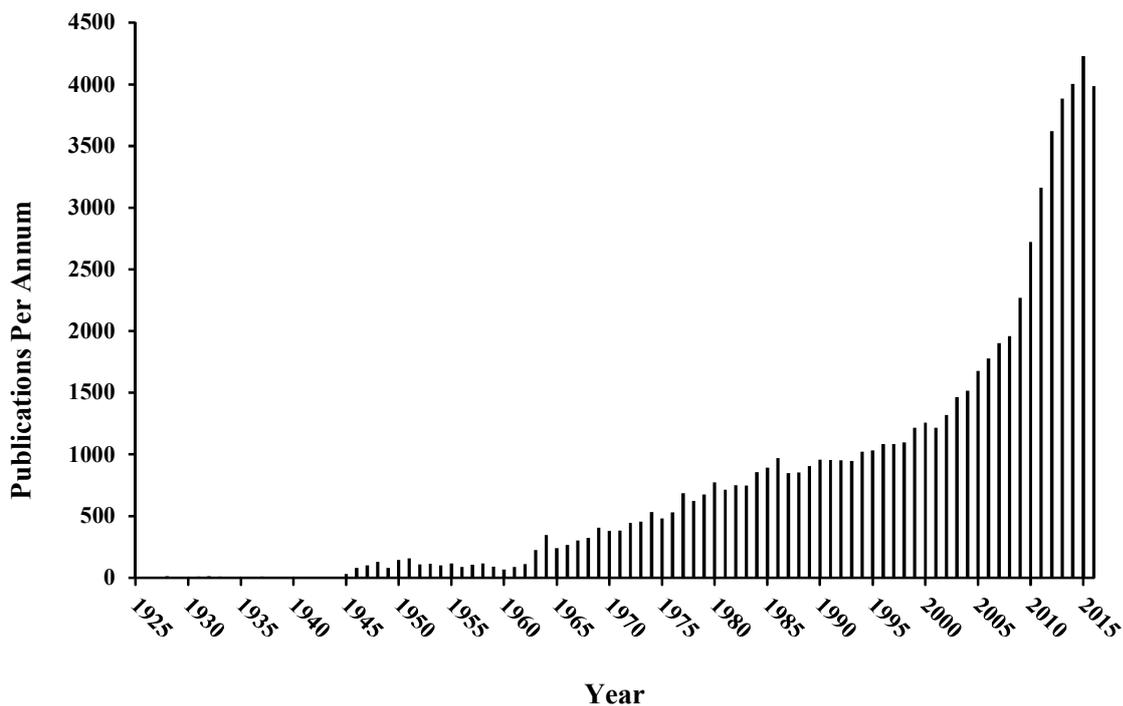


Figure 1.1 Research interest in vitamin D based on number of publication published per annum (PubMed)

1.2 Pre-competition medical assessment at Aspetar

The Aspetar Athlete Screening Programme is a multidisciplinary sports medicine initiative designed to optimise the health of athletes. Aspetar (Qatar Orthopaedic Sports Medicine Hospital) is a FIFA medical centre of excellence based in Doha, Qatar, built in 2007 to provide world class sports medicine services to athletes of the region. Unencumbered by historical biases and bureaucracy, Aspetar recognises the critical role pre-participation screening plays in the comprehensive care of the athlete and has set about establishing a preeminent screening service. To date, 13,000 plus athletes have presented for an annual athlete Periodic Health Examination (PHE); consisting of a number of standard components including but not limited to: Detailed medical and injury questionnaire, full blood work up, vitamin D assessment, DXA scan, dental X-ray, chest X-ray, musculoskeletal and cardiovascular examination, electrocardiogram, echocardiogram and resting lung function. Analysis of the data from PHE has enabled Aspetar to identify cardiac pathologies, dental disease, haemoglobinopathies and vitamin D and iron deficiencies that are particularly prevalent within the athletic population in Qatar. Ongoing analysis of PHE data via an established research programme is essential to enable the identification of any new injury or disease trends, and to assess the efficacy of any injury and illness prevention interventions with each sport.

1.3 Vitamin D deficiency

Current clinical ranges (Figure 1.2) (M. Holick, 2007) are based upon the association between 25[OH]D deficiency with osteomalacia and rickets (Heaney, 2011), and the approximate concentration at which parathyroid hormone (PTH) rises abruptly (Holick, 2009; Hollis, 2005; Zittermann, 2003). The cut off for insufficiency is the approximate concentration in which calcium absorption is maximized (Holick, 2009). Aspetar laboratory uses the conventional units for

25(OH)D (nanogram per milliliter [ng/mL]) whereas other laboratories use international system (SI) units (nanomole per liter [nmol/L]). The conversion factor to SI units is: 1 ng/mL = 2.496 nmol/L.

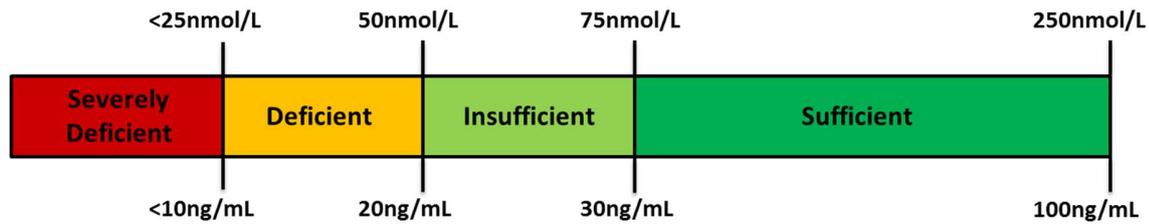


Figure 1.2 Vitamin D concentration classification (M. Holick, 2007)

1.4 Prevalence of vitamin D deficiency

Vitamin D deficiency is widespread within many athletic populations, with many severely deficient (Constantini, Arieli, Chodick, & Dubnov-Raz, 2010; Hamilton, Grantham, Racinais, & Chalabi, 2010; Magee et al., 2013), independent of sporting type or geographic location (Binkley et al., 2007; Hamilton et al., 2010; Lovell, 2008). This is reflected in the data from Aspetar pre-competition medical assessment (Table 1.0).

Table 1.0 Distribution of vitamin D status of athletes screened at Aspetar

<i>Vitamin D Status</i>	<i>Frequency</i>	<i>Percentage</i>
Severely deficient	1167	21.6
Deficient	2249	41.6
Inadequate	1357	25.1
Adequate	627	11.6
Total	5400	100

There is accumulating evidence suggesting the role of vitamin D in skeletal muscle and performance (Close, Leckey, et al., 2013; D. J. Owens et al., 2015; D. J. Owens et al., 2014). However, there are no studies examining the impact of vitamin D status on bone mass and cardiac structure in an ethnically diverse, dynamically loading population? Given the high prevalence of 25[OH]D deficiency in this cohort the relationship between vitamin D status and two main health parameters were identified for further examination; 1,) Bone mass and 2,) Cardiac structure and function, resulting in the production of this thesis.

1.5 Bone Health

Stress fractures are frequently observed in athletes, accounting for 0.7% to 20% of all sports medicine clinic injuries (Fredericson, Jennings, Beaulieu, & Matheson, 2006). Adequate vitamin D and calcium are required for bone development, growth and integrity. In the presence of low vitamin D concentrations and hypocalcaemia, levels of parathyroid hormone (PTH) are elevated to increase activation of bone resorption resulting in skeletal pathologies such as rickets, osteomalacia, or osteoporosis (B. Hamilton, 2010). However, current literature shows inconsistent associations between bone mineral density (BMD) and vitamin D status (Bischoff-Ferrari, Kiel, et al., 2009; Marwaha et al., 2011), particularly in racial minorities and athletic populations (Gerdhem, Ringsberg, Obrant, & Akesson, 2005; Hannan et al., 2008; Kremer, Campbell, Reinhardt, & Gilsanz, 2009). Athletes are a unique population, in that many are severely vitamin D deficient (Constantini et al., 2010; Hamilton et al., 2010; Magee et al., 2013), yet have the added stimulus of loading the musculoskeletal system through high-intensity dynamic sporting activity (Weidauer et al., 2014). Whilst it is considered that athletes should have 'sufficient' vitamin D

concentrations to optimise bone-mineral density (BMD), the exact value to ‘optimise’ bone health is equivocal.

1.6 Cardiac structure and function

Vitamin D deficiency is well known to have an adverse, long-term impact on cardiovascular function (M. F. Holick, 2006a; J. M. Lappe, Travers-Gustafson, Davies, Recker, & Heaney, 2007; Pittas et al., 2007). However, these studies were not deliberately designed to assess vitamin D status and established associations between vitamin D and cardiac structure and function only as a secondary marker. Yet the majority of these studies have only examined pathological conditions such as chronic kidney disease (Thadhani et al., 2012), thalassaemia-major (Noetzli, Carson, Coates, & Wood, 2011), hypertension (Kong et al., 2010), peripheral arterial disease (Fahrleitner et al., 2002) and congestive heart failure (Pilz, Marz, et al., 2008). Vitamin D receptors (VDR) are present throughout the heart and vascular system, specifically located in cardiac myocytes and fibroblasts (Chen et al., 2008). Furthermore, vitamin D deficiency is known to adversely affect cardiac contractility, vascular tone, cardiac collagen content, and cardiac tissue maturation (Achinger & Ayus, 2005).

Regular participation in intensive physical exercise is associated with several structural and electrophysiological adaptations of the heart. Such cardiac adaptations are collectively referred to as the “Athlete’s Heart” (Chandra, Bastiaenen, Papadakis, & Sharma, 2013). Numerous factors affect the adaptations of the athlete’s heart including; sporting modality, duration and intensity of training, age, ethnicity, gender, anthropometry and performance enhancing substance abuse. However, it is also well recognized that many professional athletes are Vitamin D deficient (Close, Russell, et al., 2013; Magee et al., 2013), and currently no studies have examined the association between vitamin D status and cardiac structure and function in healthy athletes.

1.7 Ethnicity

There appears to be a ‘paradoxical relationship’ between ethnicity and vitamin D concentration, that has largely been ignored, i.e. blacks generally have the lowest vitamin D but the greatest BMD, bigger hearts and reduced risk of fracture than Caucasians (Cauley et al., 2005; Hannan et al., 2008; Riding et al., 2012). Vitamin D-binding protein (DBP), may account for observed racial differences in manifestations of vitamin D (Powe et al., 2013). DBP is the primary vitamin D carrier, binding 85%–90% of total circulating vitamin D, thus altering the biologic activity of circulating vitamin D (Bikle et al., 1986). Polymorphisms in the DBP coding are known to differ between ethnic groups (Engelman et al., 2008). Furthermore, Black African athletes are known to present with larger cardiac dimensions than Caucasians. Given this, it is reasonable that ethnic variations in DBP and bioavailable vitamin D may be related to cardiac structure.

To date, research on vitamin D status in athletes has overlooked DBP, given the ethnic diversity of the athlete population screened at Aspetar (Figure 1.1), advice on treating vitamin D deficiency may be inaccurate.

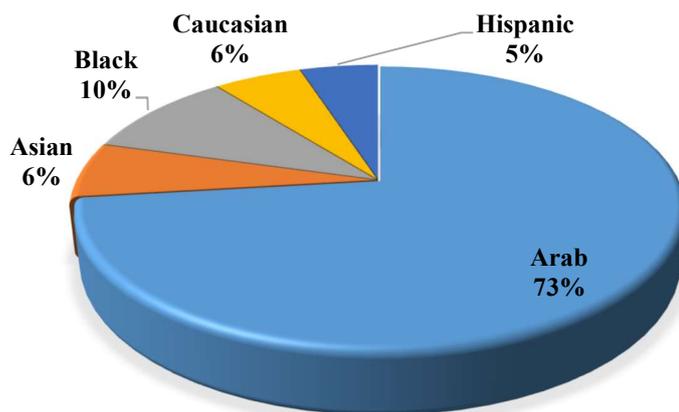


Figure 1.3 Ethnic distribution of athletes screened at Aspetar

1.8 Aims and Hypotheses

This thesis aims to examine the relationship between vitamin D and measures of bone mass, and cardiac structure and function within a large, ethnically diverse cohort of healthy athletes, with a particular focus the role of DBP in determining racial differences in bioavailable levels.

To achieve this, the following objectives were established:

1. To examine which modality of 25[OH]D supplementation was more efficacious at improving serum 25[OH]D status in an ethnically diverse athlete population (Chapter 4).
2. To investigate the relationship between serum 25[OH]D concentrations and measures of bone mass in weight-bearing athletes (Chapter 5).
3. To examine the relationship between serum 25[OH]D concentrations and markers of bone mass in non-weight bearing, Arabic athletes (Chapter 6).
4. To evaluate the role of bioavailable vitamin D as a predictor of bone mass within a large cohort of healthy athletes (Chapter 7).
5. To identify the associations between 25[OH]D status and cardiac structure and function in a large cohort of healthy athletes (Chapter 8).

It was hypothesised that 1,) serum bioavailable vitamin D would associate more accurately with markers of bone mass and 2,) cardiac structure than serum 25[OH]D concentration in an ethnically diverse athlete population.

Chapter 2:

Review of the Literature

2.1 Background

The beneficial effects of vitamin D are thought to have been known as far back as ancient Greece, when Hippocrates believed that living in areas with increased exposure to sunlight were advantageous to health. Centuries later, British medical missionary and epidemiologist, Theodore Palm, noted geographic differences in the prevalence of rickets, a disease that affected the young in epidemic proportions across Europe, America and North Africa. Observing that children living in equatorial countries did not develop the condition (Palm, 1890).

In 1906 Hopkins (Hopkins, 1906) postulated the existence of essential dietary factors necessary for the prevention of diseases such as scurvy or rickets. McCollum and his co-workers furthered this work. In 1914, they isolated a fat soluble, non-saponifiable factor from butterfat that was necessary for normal growth and prevention of the eye disease xerophthalmia in young rats. This was named vitamin A (McCollum & Davis, 1914).

By 1918, Sir Edward Mellanby, had conducted over 100 canine experiments investigating a cure for rickets. The experiment consisted of sunlight deprivation and a diet of oats; the combination in which induced rickets. Mellanby further observed that feeding rachitic dogs with cod liver oil cured rickets within a few of months (Mellanby, 1941). This led to additional experimentation on canine puppies, in which he was able induce rickets in the animals by feeding them milk and bread (Mellanby, 1989). It was noted that the dogs had a similar appearance to that of rachitic children. The addition of B vitamins (yeast) and Vitamin C (orange juice) failed to prevent the manifestation of rickets. However, it was prevented with the addition of butterfat or more effectively with cod-liver oil.

In 1920 Hopkins (Hopkins, 1920) discovered that 'vitamin A' was destroyed when subjected to heat or aeration and no longer prevented the development of xerophthalmia in rats. McCollum et

al. (McCollum, Simmonds, Kinney, Shipley, & Park, 1995) tested his hypothesis, that cod liver retained its properties with respect to night blindness and fractures post-heat treatment. They observed that cod liver oil still prevented fractures after heating and oxygenation, but it no longer prevented night blindness (McCollum et al., 1995); suggesting two separate compounds; one destroyed by heat and therefore responsible for preventing night blindness (vitamin A) and a second heat-stable component. Since water soluble factors had been termed “vitamin B” and anti-scurvy factor “vitamin C”, the new factor was named “vitamin D”.

This discovery and greater understanding of vitamin D was a major medical breakthrough and led to treatment of rickets with ultra-violet light. Furthermore, rickets could be cured not only by exposure of the host to sunlight, but also the ingestion of ultraviolet (UV) irradiated foods containing cholesterol (Goldblatt, 1923; Goldblatt & Soames, 1923; Hess, 1924; Steenbock, 1924). Initially the structure was named vitamin D₁. However, this structure was later proven to be an adduct of tachysterol and vitamin D₂, and thus vitamin D₁ was actually identified in error. Further work led to the isolation of different forms of vitamin D. Ergocalciferol (vitamin D₂) was derived from irradiation of plant sterols. The structure was confirmed by Windaus and Bock, who proceeded to induce the formation of vitamin D₃ by irradiating precursor 7-dehydrocholesterol (7-DHC) with ultraviolet radiation in animal skin (Goldblatt & Soames, 1923). Thus the structure of nutritional forms of vitamin D were identified (Windaus, Schenck, & von Werder, 1936).

Today, there has been a considerable increase in research attention towards vitamin D. This is in part due to the re-emergence of the preventable bone disorder rickets and a well-documented worldwide prevalence of vitamin D deficiency (discussed in section 2.3).

2.2 Vitamin D biochemistry and function

2.2.1 Forms of Vitamin D

The majority of vitamin D is obtained from exposure of skin to sunlight, which produces vitamin D₃ (cholecalciferol). Vitamin D can be obtained to a lesser extent from dietary intake in the form of vitamin D₂ (ergocalciferol) (Figure 2.1). Ergocalciferol is obtained from UV irradiation of the yeast sterol ergosterol found in plants such as mushrooms. Vitamin D₃ is produced in the skin on invertebrates and is present in oily fish such as mackerel, salmon and herring. Both undergo the same hydroxylation process and are transported in the blood, bound to vitamin binding protein (VDR) (Shinkyō, Sakaki, Kamakura, Ohta, & Inouye, 2004).

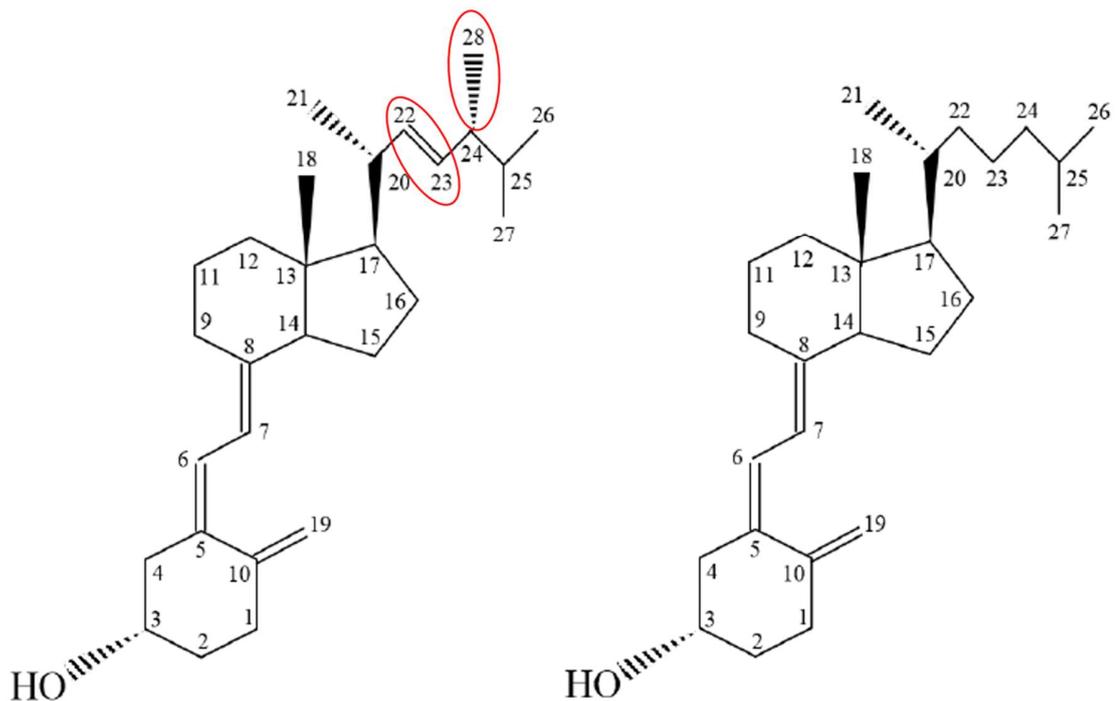


Figure 2.1 Nutritional forms of Vitamin D. Vitamin D₂ (left) and vitamin D₃ (right). The structural difference between vitamin D₂ and vitamin D₃ is in their side chains. The side chain of D₂ contains a double bond between carbons 22 and 23, and a methyl group on carbon 24.

2.2.2 Synthesis and Metabolism

Vitamin D is recognised as the ‘sunshine vitamin’, due to the production of Vitamin D₃ (cholecalciferol) in the skin via the photolytic process acting on 7-DHC, a derivative of cholesterol to pre-vitamin D₃ by 6-electron conrotatory electrocyclic reaction (DeLuca, 2004). During exposure of mammalian skin to sunlight, 7-DHC in the epidermis cells absorbs ultra-violet B (UVB) radiation, specifically wavelengths at 290-315nm. This absorbed radiation rearranges the 5, 7-diene in the B ring of the 7-DHC, causing the B-ring to form 9, 10-secoesterol, pre-vitamin D₃. Spontaneous isomerisation rearranges the double bonds of the thermodynamically unstable pre-vitamin D₃ to produce cholecalciferol (M. F. Holick, 2004). The thermal isomerisation process converts ~50 % of pre-Vitamin D₃ to Vitamin D₃ within 8 hours of exposure to UVB. The lower layers of the epidermis, stratum basale and stratum spinosum (Figure 2.2), have a greater capacity to produce pre-vitamin D₃ (due to higher concentrations of 7-dehydrocholesterol (Norman, 1998)).

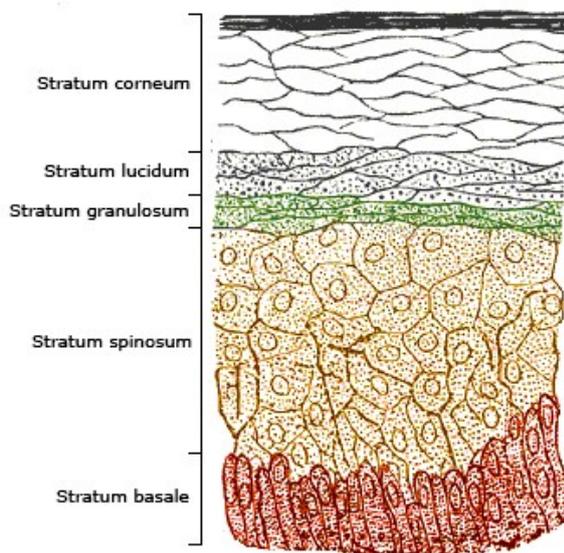


Figure 2.2 Layers of the human skin

Once vitamin D₃ enters the extracellular fluid space, it is attached to the vitamin D binding protein (DBP) in circulation, and thus enters the dermal capillary bed. Cholecalciferol is

subsequently converted to 25-hydroxyvitamin D (25[OH]D) in the liver (hepatocytes) under the control of a microsomal P450, CYP2R1 (Cheng, Levine, Bell, Mangelsdorf, & Russell, 2004; Shinkyo et al., 2004). Further hydroxylation of 25[OH]D to the active form of vitamin D 1,25 dihydroxyvitamin D (1, 25[OH]₂D) occurs in the kidney (Figure 2.3). The bioactive metabolite is transported in the blood and interacts with the VDR in the target tissue. When a sufficient quantity of vitamin D has been synthesised in the skin (at approximately 7-10% of the original 7-dehydrocholesterol content), pre-vitamin D₃ is converted to the biologically inactive photoproducts tachysterol and lumisterol (M. F. Holick, MacLaughlin, & Doppelt, 1981), serving as an endogenous toxicity regulator.

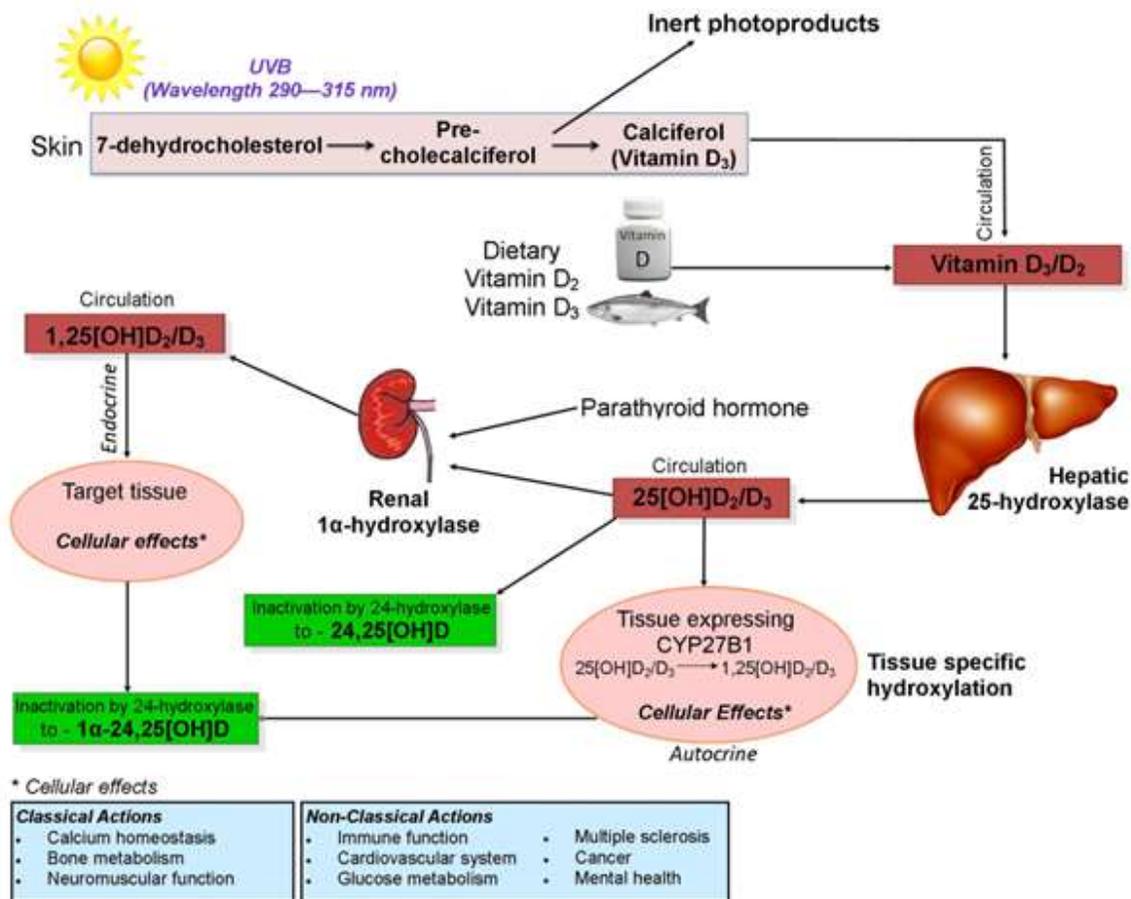


Figure 2.3 Schematic of the Vitamin D metabolic pathway. Vitamin D obtained from dietary sources or via ultraviolet B exposure undergoes hydroxylation at the liver to form 25[OH]D, the main marker of vitamin D status, and a further hydroxylation step in the kidney to form the biologically active 1,25[OH]₂D₃. The bioactive metabolite interacts with the VDR in the target tissue. When biological activity is no longer required, further hydroxylation at carbon 24 inactivates 1,25[OH]₂D₃ by acting as a blocking molecule at the VDR decreasing 1,25[OH]₂D₃ and forming 1,24,25[OH]D (Curtis, Aenlle, Roos, & Howard, 2014).

2.2.3 Vitamin D Receptor

Research during the past two decades has established that the diverse biological actions of 1,25[OH]₂D₃ are initiated through cell proliferation and specific gene expression, mediated by intracellular proteins termed vitamin D receptors (VDR) (Haussler et al., 1998; McDonnell, Mangelsdorf, Pike, Haussler, & O'Malley, 1987). Direct interaction between 1,25[OH]₂D₃ and the VDR prompts the receptor's rapid binding to regulatory regions of target genes, resulting with the formation of large protein complexes whose functional activities are essential for directed changes in transcription (Sutton & MacDonald, 2003). The expression of networks of target genes combine to orchestrate specific biological responses. These highly complex responses are tissue-specific and range from actions essential for homeostatic control of mineral metabolism to cell proliferation, differentiation and functional activity (Bouillon et al., 2008). Historically, it was assumed that VDR targeted gene expression were limited to the intestinal mucosa and bone. However, vitamin D affects the expression of as much as 3% of the transcribed genome in target cells including those of the immune system, skin, the pancreas and bone (M. Holick, 2007).

Of relevance to this thesis are the functional characteristics of the receptor in bone and cardiac tissue. In bone tissue 1,25[OH]₂D₃ binds and activates the VDR preventing the release of stored calcium in bone to serum by stimulating intestinal calcium absorption and renal reabsorption. This has been verified in genetically engineered VDR knock out mice (VDR-KO). Rachitic abnormalities could be recapitulated by ablation of the VDR gene. However, 1,25[OH]₂D₃ supplementation did not ameliorate these abnormalities (Bouillon et al., 2008). Reduced dietary phosphate intake and high mineral content increased bone growth in VDR-KO mice. Furthermore, bone resorption was reduced in osteoblast specific VDR-KO mice due to decreased expression of receptor activator of nuclear factor kappa-B ligand (RANKL), an essential molecule in

osteoclastogenesis. This demonstrates that the skeletal VDR play a role in bone regulation, specifically the VDR in osteoblasts are a negative regulator of bone mass (Yamamoto et al., 2013). Inactivation of intestine-specific VDR in mature osteoblast and the intestine decreases intestinal calcium absorption. An increase $1,25[\text{OH}]_2\text{D}_3$ levels stimulates bone turnover and suppress bone matrix mineralisation. Indicating that normocalcaemia has priority over skeletal integrity (Lieben et al., 2012).

The notion of vitamin D involvement in cardiac function began over 25 years ago, with the identification of VDR in rat cardiac myocytes; thus establishing a link between vitamin D deficiency and cardiovascular dysfunctions, such as pathological cardiac hypertrophy, fibrosis and hypertension (Simpson, Thomas, & Arnold, 1985). These early animal studies supported the direct action of $1,25[\text{OH}]_2\text{D}_3$ on cardiomyocyte VDR and indirect actions of circulating hormones. Furthermore, vitamin D deficient rats presented with enhanced ventricular muscle mass and contractile function (Weishaar, Kim, Saunders, & Simpson, 1990; Weishaar & Simpson, 1987a). Subsequently the VDR was identified in human heart cells (O'Connell & Simpson, 1996). A significant breakthrough came with research showing VDR-KO mice presented with hypertension, significantly increased heart weight/body weight ratios and markedly increased left ventricular myocytes (Li et al., 2002; Zhou et al., 2008).

These data strongly supported VDR involvement in the regulation of cardiovascular functions and bone health.

2.3 Vitamin D Deficiency

2.3.1 Measuring Vitamin D

Clinically, measuring serum 25[OH]D concentration provides the best estimate of vitamin D status (M. F. Holick, 1990; Iqbal, 1994) as both cholecalciferol and 1,25[OH]₂D have short half-lives, 24hrs and 4-6 hrs, respectively. Therefore, circulating levels provide limited information about vitamin D status. Additionally, cholecalciferol serum levels are affected by recent sunlight exposure and vitamin D ingestion and the assay is difficult to use due to the lipophilic nature of the molecule (Zerwekh, 2004). Whereas, 25[OH]D has a long serum half-life (3 weeks) and the 25-hydroxylation step is unregulated, thus reflecting substrate availability. During vitamin D deficiency, parathyroid hormone increases and drives the renal 1-alpha-hydroxylase, so that 1,25[OH]₂D levels increase. Only in severe deficiency when substrate is depleted, does the 1,25[OH]₂D become low. Partially treated vitamin D deficiency also results in marked elevations of 1,25[OH]₂D levels.

2.3.2 Prevalence of Vitamin D deficiency

Vitamin D deficiency is now recognised as one of the most common medical conditions in the world. Deficiency (<20ng/mL), is common in countries at Northern latitudes (>35° N) such as the UK (Bates et al., 2003), Ireland (Cashman et al., 2013), Denmark (Thuesen et al., 2012), France (Chapuy et al., 1997) and Germany (Hintzpeter, Mensink, Thierfelder, Muller, & Scheidt-Nave, 2008). However, a similar prevalence has also been observed even in areas with ample sunlight exposure, for example Australia (Daly et al., 2012), USA (Forrest & Stuhldreher, 2011) and Saudi Arabia (Elsammak, Al-Wosaibi, Al-Howeish, & Alsaeed, 2010). Vitamin D deficiency is also widespread within the global athlete population (Table 2.1).

As conferred in Chapter 1, serum 25[OH]D levels are categorised as being either severally deficient (<10 ng/mL), deficient (10-20 ng/mL), insufficient (20-30 ng/mL) or sufficient (>30 ng/mL) (M. F. Holick, 2009). However, the categorisation of what constitutes 25[OH]D deficiency is widely debated. Studies suggest that a 25[OH]D value >30ng/mL promotes bone health and reduces fracture risk in healthy young and older adults, whilst others suggest levels >40ng/mL(Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006) maybe required. More conservatively, the US Institute of Medicine (IOM) advises concentrations of ≥ 20 ng/ml (50 nmol/l) would meet the needs of 97.5% of the population (Ross et al., 2011).

Numerous endogenous factors and environmental influences can alter vitamin D production and contribute to deficiency, including altered vitamin D metabolism, mal-absorption and insufficient dietary intake of vitamin D. Due to the significant role sunlight plays in vitamin D synthesis any factor that promotes UV light insulation contributes to vitamin D deficiency, such as sunscreen use, skin melanin, concealing clothing, atmospheric dust particles, latitude or time of day (M. F. Holick, 2004).

The production of pre-vitamin D₃ is directly related to the number of UVB photons absorbed by 7-DHC. This process of production via photosynthesis is governed by a unique solar regulation to prevent intoxication from excessive sun exposure. Yet, UV radiation is a prominent and ubiquitous physical carcinogen. Epidemiological evidence demonstrates that over exposure to UVB radiation has been linked to skin cancer and malignant lymphomas (de Gruijl, 1999; Smedby et al., 2005), but is dependent on various factors, such as age, skin type ethnicity and duration of exposure (Armstrong & Krickler, 2001). This increased awareness of the link between sunlight and skin cancer has be attributed to the rise in vitamin D deficiency (Reichrath, 2006). Sunscreen used in the prevention of skin melanomas reduces vitamin D synthesis with factor 30 sunscreen reducing

the conversion of 7-DHC to pre-vitamin D₃ by more than 95% (Matsuoka, Ide, Wortsman, MacLaughlin, & Holick, 1987). The stratospheric ozone layer is efficient in absorbing UVB radiation above 290nm, which is responsible for producing pre-vitamin D₃ in the skin (M. F. Holick, 2004). Air pollution is one of the primary factors in determining the percentage of ground level UVB. The level of air pollution is inversely related to the extent of solar UVB that reaches earth surface, consequently, more polluted areas have less UVB passage and as a result, lowers vitamin D cutaneous synthesis (Hosseinpanah et al., 2010).

Table 2.1 Summary of studies assessing Vitamin D status in athletic populations

Study	Sample size	Country (latitude)	Type of sport	Vitamin D status
Allison et al. (2015)	750	Qatar (25 ⁰ N)	Soccer, handball, volleyball, basketball, sailing, archery, shooting, bowling	Deficient:n=398 (53%) Insufficient: 207 (n=28%)
Bescos Garcia and Rodriguez Guisado 2011	21	Spain (41 ⁰ N)	Basketball	Deficient: 12 Insufficient: 0
Close et al. (2013)	61 athletes, 30 controls	UK (50 ⁰ N)	Rugby, soccer, flat jockeys, jump jockeys	Deficient: 2 Insufficient: 18
(Close, Russell, et al., 2013)	30	UK (53 ⁰ N)	Various sports including soccer and rugby	Deficient: 6 Insufficient: 11
Constantini et al. (2010)	98	Israel (31 ⁰ N)	Dancing, basketball, swimming, Tae Kwon Do, judo, gymnastics, table tennis, tennis, soccer, running, triathlon, sailing	Deficient: 6 Insufficient: 66
Ducher et al. (2010)	18	Australia (31 ⁰ S)	Ballet	Deficient: 2 Insufficient: 7
Galan et al. (2012)	28	Spain (37 ⁰ N)	Soccer	Inadequate: fall: 2, winter: 18
Guillaume, Chappard and Audran (2012)	29	France (46 ⁰ N)	Cycling	Insufficient: 3

Halliday et al. (2011)	41	USA (41°N)	Soccer, football, cross-country, track and field, cheerleading, dance, wrestling, swimming, basketball	Deficient: fall: 1, winter: 1, spring: 1 Insufficient: fall: 4, winter: 20, spring: 4
Hamilton et al. (2010)	93	Middle East (29°N)	Football, handball, shooting, squash, cycling, martial arts, body building	Deficient: 85 Insufficient: 8
Hamilton et al. (2014)	342	Qatar (25°N)	Soccer	Deficient: 190 Insufficient: 98
He et al. (2013)	267	UK (53°N)	Running, cycling, swimming, triathlon, team games, racquet sports	Deficient: 18 Insufficient: 68
Lewis, Redzic and Thomas (2013)	45	USA (38°N)	Swimmers and divers	Deficient: 0 Insufficient: 0
Lovell (2008)	18	Australia (35°S)	Gymnastics	Deficient: 1 Insufficient: 5
Magee et al. (2013)	84	Ireland (53°N)	Gaelic games, boxers, Paralympians	Insufficient/deficient: 46
Peeling et al. (2013)	72	Australia (25°S)	Gymnastics, diving, sailing, field hockey, rowing, water polo, sprint cycling	Deficient: 3 Insufficient: 11
Pollock et al. (2012)	63	UK (53°N)	Track and field	Summer: deficient: 8; insufficient: 7 Winter: deficient: 8; insufficient: 6
Shanely et al. (2013)	50	USA (40°N)	Football, basketball, baseball, track, wrestling, lacrosse, tennis	Deficient: 13 Insufficient: 20
Shindle et al. (2011)	89	USA (40°N)	Football	Deficient: 27 Insufficient: 45
Storlie et al. (2011)	39	USA (47°N)	Football, cross-country, rugby, track and field	Deficient: 1 Insufficient: 10
Willis et al. (2012)	19	USA (30°N)	Running	Deficient: 2 Insufficient: 8
Wilson et al. (2013)	37	UK (53°N)	Jump and flat jockeys	Deficient: 17 Insufficient: 11
Wolman et al. (2013)	19	UK (52°N)	Ballet	Summer: deficient: 2; insufficient: 14 Winter: deficient: 5; insufficient: 14

2.3.3 Ethnicity

Skin pigmentation plays an essential role in the production of vitamin D from sunlight exposure. Melanin is the primary determinant of skin pigmentation and protects the body against UV radiation; however dermal synthesis of vitamin D is dependent of UV light. Highly pigmented skin, such as those of African origin, abrogates the majority of UV induced vitamin D synthesis (Shoenfeld, Amital, & Shoenfeld, 2009). The evolution of a more darkly pigmented complexion occurred early in the evolution of the human genus. Melanocytes produce the pigment melanin, which is deposited in vesicles termed melanosomes that exist in cutaneous epidermal cells. Melanosomes cover the cell nucleus and protect its components; in particular, nuclear DNA, from the deleterious effects of ultraviolet radiation (280–400 nm), which can lead to gene mutations. Melanin can also scavenge for reactive free radicals and oxidants (Shoenfeld et al., 2009). Unsurprisingly, people with dark skin require longer sunlight exposure to produce the equivalent amount of vitamin D as light skin individuals (M. F. Holick, 2003a). Therefore, dark skinned populations living at high latitudes typically have lower serum 25[OH]D concentrations than those living at lower latitudes (Kant & Graubard, 2008). Studies examining vitamin D in an ethnically diverse population, demonstrate strong evidence that certain ethnicities (Black, Hispanic and South Asian) living in the USA and UK present with vitamin D deficiency, but are at lower risk of osteoporosis, rapid bone loss and associated fractures compare to Caucasians (Giovannucci et al., 2006; Mersch, Middendorp, Bouhuys, Berrisma, & van der Hoofdakker, 1995; Patel et al., 2013; Powe et al., 2013)

The genetic factors that may be attributed to variations between vitamin D in ethnicity are discussed later in this chapter (2.4.3. Vitamin D binding protein and Bioavailable vitamin D).

2.4 Role of vitamin D in health and associated disease

2.4.1 Vitamin D and Cardiovascular disease

The heart and vascular system, like skeletal muscle, contain VDR (Bischoff-Ferrari et al., 2005) and the associated apparatus for 1,25[OH]₂D₃ production; with the VDR are specifically located in cardiac myocytes and fibroblasts (Chen et al., 2008). The association between serum 25[OH]D concentration and cardiovascular function was first observed 25 years ago in Sprague-Dawley rats. Subsequent research groups have established associations between serum 25[OH]D concentration and cardiac structure and function (in both human and animal models). Studies on rat models have shown that depletion of vitamin D₃ results in an increase in the contractile function of isolated hearts (Weishaar & Simpson, 1987b) and that 25[OH]D deficient rats present with significantly smaller ventricular myofibrils and increases in extracellular matrix proteins compared to 25[OH]D sufficient rats (Weishaar et al., 1990).

There is increasing body of evidence that vitamin D deficiency is associated with increased risk of cardiometabolic outcomes. Although, many of these studies are cross-sectional, thus limiting the strength of the conclusions, several longitudinal observational studies have examined vitamin D status and aspects of cardiovascular disease. The Framingham Offspring study found an association between lower serum 25[OH]D concentrations and increased risk of cardiovascular events. Still, the association was only among those participants that were hypertensive at baseline (Wang et al., 2008). Studies have also demonstrated a link between vitamin D status and fatal cardiovascular events. Two studies found similar associations between fatal stroke and vitamin D status (Kilkinen et al., 2009; Pilz, Dobnig, et al., 2008).

Analysis from the Health Professionals Follow-up Study (HPFS) found a significant association between lower 25[OH]D concentration and increased risk of myocardial infarction (MI). However,

MI cases in this study were generally unhealthier than the controls, e.g. higher BMI, family history of MI, diabetes, hypertension and hypercholesterolemia (Giovannucci, Liu, Hollis, & Rimm, 2008). Scragg et al, also demonstrated an inverse relationship between 25[OH]D levels and the incidence of MI in the general population. The authors acknowledge that this association may have been intermediated by physical activity (Scragg, Jackson, Holdaway, Lim, & Beaglehole, 1990). Data from the Third National Health and Nutrition Examination Survey (NHANES III) demonstrated a strong association between vitamin D deficiency and cardiovascular disease (CVD) risk factors (BP, diabetes, overweight, hypertriglyceridemia) (Melamed, Michos, Post, & Astor, 2008). A number of studies have examined the association between vitamin D deficiency and pathological conditions such as chronic kidney disease, thalassaemia-major, hypertension peripheral arterial disease and congestive heart failure (Table 2.2) (Dobnig et al., 2008; Fahrleitner et al., 2002; Giovannucci et al., 2008; Noetzli et al., 2011; Pilz, Marz, et al., 2008; Wang et al., 2008).

Studies examining the relationship between vitamin D and cardiac structure and function provide mixed results. In both the Tromsø and Hoorn studies serum 25[OH]D was not associated with LV structure and function. (Kamycheva, Johnsen, Wilsgaard, Jorde, & Mathiesen, 2013; Pilz et al., 2009). Whereas, a recent study showed serum levels of 25[OH]D were significantly associated with LV diastolic dysfunction (Akin et al., 2014). Moreover, 25[OH]D deficiency was shown to adversely affect cardiac contractility, vascular tone, cardiac collagen content, and cardiac tissue maturation (Achinger & Ayus, 2005). Lastly, supplementation with an active vitamin D compound, paricalcitol, over 48 weeks in patients with chronic kidney disease (CKD), had no impact on left ventricular mass (Thadhani et al., 2012) The heterogeneity of studies may be caused by different definitions of vitamin D status, age structures, definition and determination of

cardiovascular endpoints and other confounding factors. The relationship of vitamin D with cardiac function remains highly controversial and despite the growing body of evidence demonstrating a link between vitamin D deficiency and cardiovascular risk factors, to date, no studies have examined the association between 25[OH]D status and cardiac structure and function in healthy athletes; a population known to be vitamin D deficient.

Table 2.2 Summary of epidemiologic studies that have shown an association between hypovitaminosis D and mortality/cardiovascular events in the general population

Reference	N	Population	Key Findings
(Dobnig et al., 2008)	3258	Single center, referred for coronary angiography	Lower two quartiles of serum 25[OH]D with higher all cause and cardiovascular mortality
(Noetzli et al., 2011)	54	Patients with thalassemia major (TM)	While cardiac iron loading was more common in patients with vitamin D deficiency and secondary PTH elevation, values did not reach statistical significance.
(Fahrleitner et al., 2002)	161	Patients with peripheral arterial disease (PAD)	Patients with PAD IV showed significantly lower vitamin D ₃ and significantly higher PTH levels as compared to patients with PAD II.
(Giovannucci et al., 2008)	18255	Health professionals follow up study	Adjusted hazards for myocardial infarction compared with 25[OH]D levels
(Pilz, Marz, et al., 2008)	3316	Single center, referred for coronary angiography	Low vitamin D levels associated with increased fatal/nonfatal strokes
(Wang et al., 2008)	1739	Framingham Offspring Study	Low vitamin D levels associated with increased cardiovascular events
(Melamed et al., 2008)	13311	NHANES III	Lowest quartile of serum 25[OH]D with higher all-cause mortality
(Kilkkinen et al., 2009)	6219	Fatal cardiac event (national mortality registry)	Low vitamin D levels associated with higher risk of a fatal CVD event

PTH = parathyroid hormone, PAD = peripheral arterial disease, NHANES III = Third National Health and Nutrition Examination Survey

2.4.2 Vitamin D and Bone Mass

Vitamin D status is indicative of calcium absorption and bone mineralisation (Berry, Davies, & Mee, 2002), but observations of deficiency fail to universally affirm a proportionate susceptibility to bone loss, osteoporotic fractures or rickets (Hamson, Goh, Sheldon, & Samanta, 2003; Lowe, Mitra, Foster, Bhojani, & McCann, 2010).

Bone is a metabolically active tissue capable of adapting to mechanical stimuli and repairing structural damage (Frost, 1969). Bone remodeling is a dynamic physiological process in which the combined effect of bone formation and bone resorption occurs at a specific location of the bone architecture. This enables bone to adapt to mechanical stresses, to repair its microstructure and thus maintain the mechanical integrity of the skeleton, and to maintain mineral homeostasis. Bone remodeling consists of two main subprocesses, 1) creation and mineralisation of bone by osteoblasts and osteocytes, 2) reabsorption of bone by osteoclasts, followed by bone formation by osteoblasts without causing large changes in bone quantity, geometry, or size (Ashman et al., 1985; Cowin, 1993). An imbalance in the regulation of bone remodeling's two subprocesses results in many metabolic bone diseases, such as osteoporosis (Hsieh & Turner, 2001).

The regulation of osteoblast function is of greatest relevance in understanding the function of vitamin D in bone mineralisation. Bone tissue is broken down by osteoclasts and rebuilt by osteoblasts. Osteoclasts are nucleated cells that play a crucial role in bone homeostasis, through the liberation of minerals and other molecules stored in the bone matrix. Particularly, osteoclasts store phosphate and growth factors and regulate the release calcium from bone. Osteoblasts are responsible for building new tissue, by producing osteoid, the organic component of bone, composed of collagen. As osteoblasts form new bone tissue, many become embedded within the

matrix and differentiate into osteocytes. The strength of this new tissue and risk of fracture can be assessed using bone mineral density (BMD g/cm^2) by DXA.

The active form of vitamin D, $1,25[\text{OH}]_2\text{D}_3$ affects osteoblast function via different mechanisms. It controls remodeling via induction of receptor activator of NF- κ B ligand (RANKL) (Kim, Yamazaki, Zella, Shevde, & Pike, 2006), regulates phosphate homeostasis by increasing fibroblast growth factor 23 (FGF23) (Shimada et al., 2004) and may enhance the response of mechanical loads via stimulation of mitogen-activated protein kinase signaling (Robling, Castillo, & Turner, 2006). Lastly, $1,25[\text{OH}]_2\text{D}_3$ stimulates mesenchymal stem cell (MSC) differentiation to the osteoblast lineage and suppresses adipocyte formation. Although all cells in the osteoblast lineage express VDR, $1,25[\text{OH}]_2\text{D}_3$ may target different cells for different regulatory actions. The effect $1,25[\text{OH}]_2\text{D}_3$ on osteoblasts has been clearly shown in vitro models, however, there is poor agreement between in vitro and in vivo studies. Net bone growth ceases with the onset of adulthood, however, bone is constantly renewed throughout maturity by the action of osteoclasts that resorb bone and osteoblasts that replace bone lost by resorption. Vitamin D impacts on this osteoblast/osteocyte regulation in the process of bone remodeling and osteoblasts respond to a variety of resorptive signals including $1,25[\text{OH}]_2\text{D}_3$ and PTH. There is now evidence that bone cells are capable of producing $1,25[\text{OH}]_2\text{D}_3$ from the 25-hydroxyvitamin D_3 ($25[\text{OH}]\text{D}_3$) precursor, and that this activity is likely to account for the skeletal effects of circulating $25[\text{OH}]\text{D}_3$. This recent evidence suggests that bone is an intracrine organ of vitamin D metabolism (Figure 2.4)(Anderson & Atkins, 2008).

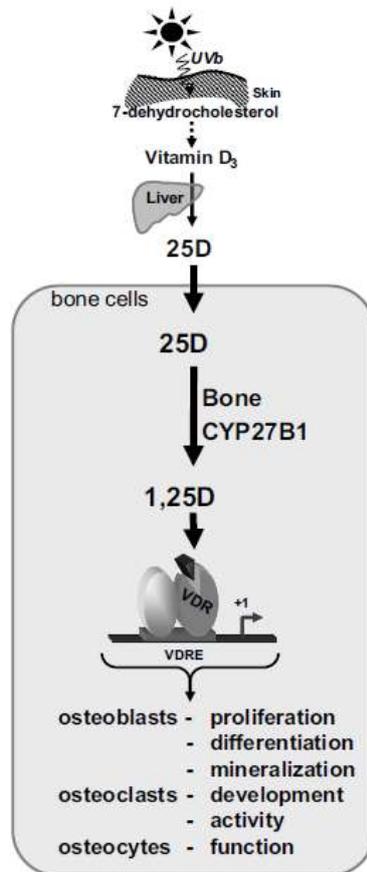


Figure 2.4 Bone cells are capable of producing 1,25[OH]₂D₃ from the 25-hydroxyvitamin D₃ (25[OH]D₃) precursor, and that this activity is likely to account for the skeletal effects of circulating 25[OH]D₃.

The active vitamin D metabolite, 1,25[OH]₂D₃, is known to have systematic effects on both calcium and phosphate homeostasis. Adequate concentrations of vitamin D and calcium are required for bone development, growth and integrity; with skeletal pathologies such as rickets and osteomalacia presenting when 25[OH]D concentrations are low (B. Hamilton, 2010). The role of vitamin D as a regulator of calcium and bone homeostasis is well established. 1, 25[OH]₂D₃, regulates intestinal calcium absorption, bone calcium resorption, and renal calcium reabsorption to maintain normal calcium levels in the blood (Lieben & Carmeliet, 2013). During periods of low

vitamin D concentration and hypocalcaemia, PTH secretion increases, resulting in an increased production of 1, 25[OH]₂D₃, that enhances mobilization of calcium from bone, when unable to acquire sufficient calcium from dietary absorption. Studies examining vitamin D deficiency and gene ablation, have demonstrated that 1,25[OH]₂D₃ also has a subtle, but direct action on osteoblast function, mediated by its interaction with the VDR.

Low 25[OH]D status is a worldwide issue and is particularly low in northern Europe during winter, with one study highlighting that one-third of adolescent girls have a value lower than 10ng/ml and nearly all below 20ng/ml (Andersen et al., 2005). Although, vitamin D and calcium are regarded as fundamental in bone health the vast majority of evidence supporting this relationship is derived from cohorts of elderly individuals or groups with existing skeletal disorders (Bischoff-Ferrari et al., 2005; Cauley et al., 2008; B. Dawson-Hughes, Harris, Krall, & Dallal, 1997; Vanderschueren et al., 2013). There is increasing evidence of low BMD as a result of 25[OH] D deficiency in today's youth (Franchi et al., 2014; Gordon, DePeter, Feldman, Grace, & Emans, 2004). Genetic, environmental and cultural factors associated with 25[OH]D deficiency (Kanis, 2002) impact on increase the risk of osteoporosis (Allali et al., 2006) and fracture risk (Dhesi, 2004). Low vitamin D concentration contributes to decreased BMD by elevating PTH, leading to increased calcium malabsorption, secondary hyperparathyroidism which sequentially increases osteoclast production (Breen et al., 2010; Cashman et al., 2008; Collins, Jasani, Fogelman, & Swaminathan, 1998; M. F. Holick, 2006b; Wöfl et al., 2013), resulting in increased incidence of stress fractures (Ruohola et al., 2006) and osteomalacia (Allali et al., 2006).

Studies examining supplementation and bone health suggest that a higher dose (>700IU/d D₃) may reduce hip and non-vertebral fractures by up to 20% (Bischoff-Ferrari et al., 2005; Boonen et al., 2007), while lower intakes (<400 IU/d) failed to offer benefit (Bischoff-Ferrari, Willett, et al.,

2009). Additionally, a study examining a mega oral dose of 500,000IU per year resulted in an increased risk of falls and fractures (Sanders et al., 2010). Conversely, improvements have been demonstrated with smaller doses (200IU/d and 400IU/d) in femoral and vertebral BMD (El-Hajj Fuleihan et al., 2006; Viljakainen et al., 2006). Vitamin D supplementation may increase calcium absorption, suppress intact PTH secretion, slow bone loss and reduce the number of skeletal fractures (Bischoff-Ferrari, Dietrich, Orav, & Dawson-Hughes, 2004; Bess Dawson-Hughes & Bischoff-Ferrari, 2007). A recent study demonstrated vitamin D supplementation reduces the incidence of fractures in older adults, partly mediated by the effect of vitamin D on neuromuscular function (Chapuy et al., 1997).

Optimum concentrations of serum 25[OH]D for the best possible skeletal health are still debated. Many investigators define the threshold for vitamin D sufficiency as the lowest serum 25[OH]D concentration that maximally suppresses PTH secretion and/or optimises BMD (Bischoff-Ferrari et al., 2006; Chapuy et al., 1997; Malabanan, Veronikis, & Holick, 1998). Based upon these criteria, most experts suggest that 25[OH]D levels of 21 to 30 ng/ml are indicative of relative vitamin D insufficiency, while levels ≤ 20 ng/ml constitute vitamin D deficiency. The US Institute of Medicine (IOM) states a serum 25[OH]D concentration of 20ng/mL is sufficient to maintain normal bone health in 97.5% of the population (Ross et al., 2011). Observational studies show inconsistent associations between bone mineral density (BMD) and serum 25[OH]D status, (Bischoff-Ferrari, Kiel, et al., 2009; Marwaha et al., 2011) particularly in racial minorities and athletic populations (Gerdhem et al., 2005; Hannan et al., 2008; Kremer et al., 2009).

2.4.3 Vitamin D binding protein and Bioavailable Vitamin D

Darker skin pigmentation has a photo-protective effect (Matsuoka, Wortsman, Haddad, Kolm, & Hollis, 1991), reducing the capacity of the skin to synthesise vitamin D₃ (Clemens, Adams,

Henderson, & Holick, 1982). When examining 25[OH]D deficiency in ethnically diverse populations, studies demonstrate that Black and Hispanic men are at elevated risk of 25[OH]D deficiency but at lower risk of osteoporosis, rapid bone loss and associated fractures compare to Caucasians (Engelman et al., 2008). In Caucasians, BMD significantly decreases as serum 25[OH]D declines but this is not observed in Black adults (Gutierrez, Farwell, Kermah, & Taylor, 2011).

Vitamin D-binding protein (DBP) provides insight into why certain ethnic groups may have distinct 25[OH]D and BMD relationships (Powe et al., 2013). Originally identified in 1959 (Hirschfeld, 1959), its function as a binding protein for all vitamin D metabolites in serum was discovered in 1975 (Daiger, Schanfield, & Cavalli-Sforza, 1975). DBP is the primary vitamin D carrier, binding 85–90 % of circulating 25[OH]D and 1,25-dihydroxyvitamin D₃ [1,25[OH]₂D₃], the biologically active form of vitamin D, with the remaining unbound 25[OH]D is considered to be bioavailable. Bioavailable vitamin D is defined as 25[OH]D that is either free or bound to albumin, (10-15% of total 25[OH]D is bound to albumin) in contrast to free 25[OH]D, which accounts for less than 1% of total circulating vitamin D (Bikle et al., 1986). Since the affinity of albumin to 25[OH]D or 1,25[OH]₂D₃ is weaker than that of DBP, the loosely bound fraction and the free fraction consist of bioavailable 25[OH]D (Brown & Coyne, 2012).

DBP is a 51–58 kDa multifunctional and highly polymorphic glycoprotein, synthesised primarily by the hepatic parenchymal cells. Originally known as the Group-specific component (Gc-globulin), DBP is a member of a multigene family that includes albumin (Alb) and is a monomeric peptide of 458 residues and three disulfide-bonded, structural domains (Haddad et al., 1992). Recently, two binding regions have been localized, 1) vitamin D binding domain located between residues 35–49 at the N-terminal, and 2) actin binding domain positioned between residues 350–

403 at the C-terminal. These are necessary to mediate DBP cellular functions (Zhang, Habel, Ramadass, & Kew, 2010) (Figure 2.5).

DBP has many physiologically important functions including transporting vitamin D₃ metabolites, binding/sequestering globular actin and binding fatty acids to possible roles in inflammation and the immune system. Genotyping has identified two common single-nucleotide polymorphisms (SNP) in the coding region of the DBP gene (rs4588 and rs7041). These variants change the amino acid sequence and alter the protein function and are common enough to generate population-wide constitutive differences in vitamin D status (Malik et al., 2013). Combinations of these two SNPs, produce three major polymorphic forms of DBP (Gc1F, Gc1S, and Gc2) which differ substantially in their binding affinity for 25[OH]D, circulating concentration, and variation between ethnic groups and are in turn linked to DBP function. The frequencies of which differ globally, with Gc1F alleles more common in populations of African descent. (Powe et al., 2013). Bioavailable vitamin D is calculated from the concentrations of total 25[OH]D, DBP, and albumin, with or without a factor accounting for DBP genotype-specific binding affinities (Equation shown 3.6). Variants of DBP have been associated with adverse health outcomes including diabetes (Cho et al., 2007), cancer (Bijian et al., 2009), epilepsy (Xiao et al., 2009) and Alzheimer's and Parkinson disease (Zhang et al., 2008). However, these correlations have been difficult to interpret, as the biology of DBP that is currently not well understood.

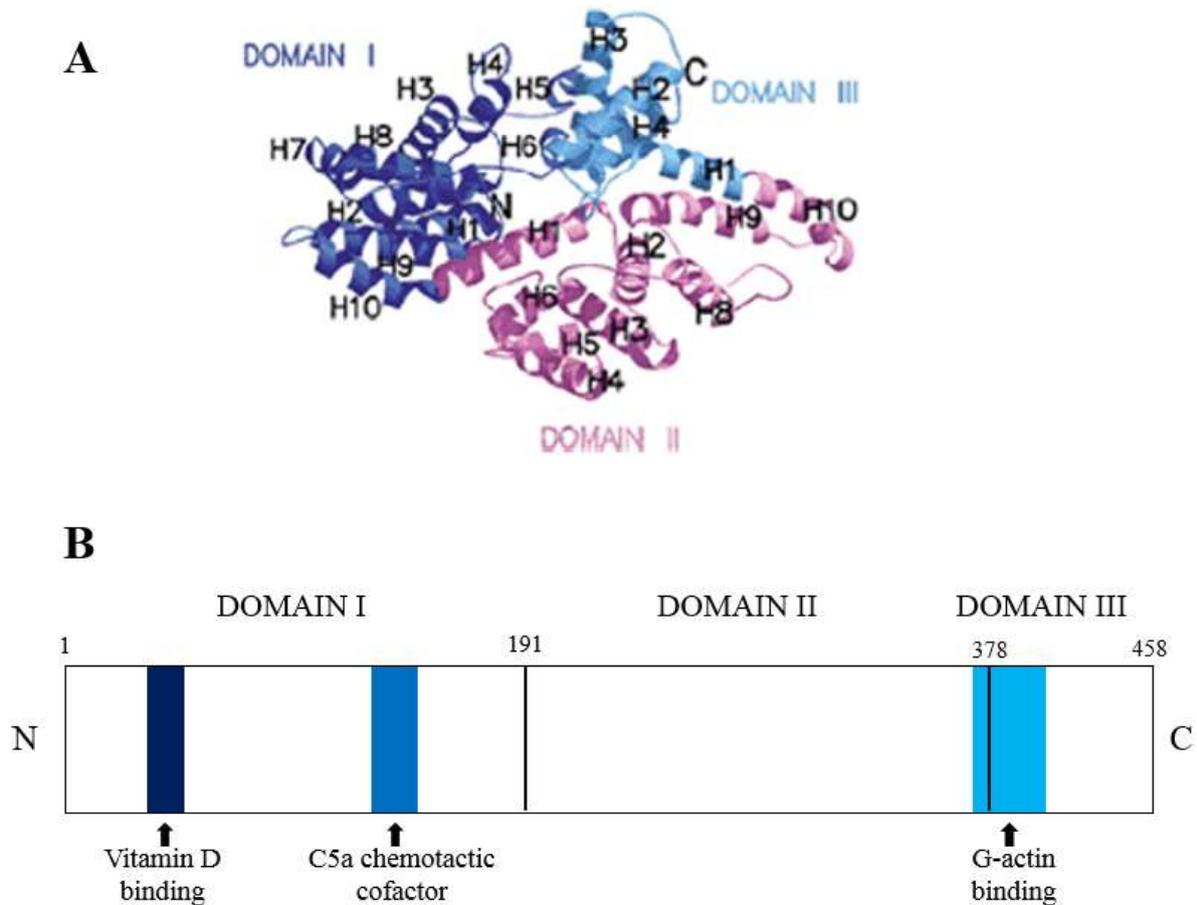


Figure 2.5 (A) 3D structure of DBP with 3 domains. (B) Schematic representation of the DBP domain structure. The 458 amino acid sequence of human DBP with the three structural domains and known functional regions indicated. Domain I: amino acids 1–191; domain II: 192–378; domain III: 379–458; vitamin D binding: 35–49; C5a chemotactic cofactor: 130–149; G-actin binding: 373–403. The domain and functional regions are drawn approximately to scale. The N-terminus refers to the start of a protein or polypeptide terminated by an amino acid with a free amine group ($-NH_2$). By convention, peptide sequences are written N-terminus to C-terminus (Malik et al., 2013).

DBP is not included in the majority of studies examining vitamin D deficiency and measures of health, however, the racial differences in DBP have been explored. A recent study demonstrated that community-dwelling blacks had lower levels of DBP and serum 25[OH]D compared to Caucasians. The authors hypothesised that blacks have similar levels of bioavailable 25[OH]D which may explain why blacks present with consistently low serum 25[OH]D compared with

whites but higher BMD (Powe et al., 2013). This questions the validity of the commonly used lab test for 25[OH]D levels in assessing vitamin D deficiency. DBP may provide insight into why currently no universally accepted consensus for vitamin D levels exist. Consistent with the ‘free hormone’ hypothesis, several recent studies have shown that some functions of vitamin D may be more closely related to the free or bioavailable fraction of vitamin D than to total serum 25[OH]D concentrations. For instance, the free or bioavailable fraction of circulating 25[OH]D was more strongly associated with bone mineral density than the total levels in healthy adults (Powe et al., 2011). Therefore, serum bioavailable vitamin D may be more accurate for assessing bone mass in a healthy ethnicity diverse athletic population.

2.5 Effect of vitamin D supplementation on health

2.5.1 Treatment/Supplementation

In the early part of the 20th Century, UVB radiation was used as an ergogenic aid (Cannell, Hollis, Sorenson, Taft, & Anderson, 2009) and research over that period suggested that both cardiovascular fitness and muscular endurance were enhanced with increased exposure to ultraviolet radiation (B. Hamilton, 2012). However, there remains little direct evidence for this and these findings are yet to be reproduced (Racinais, Hamilton, Li, & Grantham, 2010).

2.5.2 Ergocalciferol vs. Cholecalciferol

Two forms of vitamin D supplementation are commonly used, cholecalciferol (D₃) and ergocalciferol (D₂) (M. Holick, 2007). Both cholecalciferol, the most common form of Oral supplementation and ergocalciferol, its intramuscular (IM) counterpart, appear to improve

serum 25[OH]D levels in deficient individuals (Biancuzzo, Clarke, Reitz, Trivison, & Holick, 2013; M. F. Holick et al., 2008). In turn, there is evidence to suggest that D₂ has poorer bioefficacy than D₃, to increase serum 25[OH]D (Binkley et al., 2011) and thus may have limited use as a treatment supplement (Houghton & Vieth, 2006).

2.5.3 Mode of Supplementation (Oral vs. Intramuscular)

A single IM injection removes potential gut malabsorption and compliance issues, however a 6-month wait is required to ascertain if the deficiency has been corrected. Previous studies have attempted to examine the efficacy of oral vs. IM modalities, but with variable dosages and durations in many of these investigations, the clinical effectiveness of both modalities at correcting 25[OH]D deficiency remains unclear (Billoo et al., 2009; Zabihyeganeh, Jahed, & Nojomi, 2013). Furthermore, the majority of these studies have only examined individuals with known pathologies or health related issues, such as rickets, chronic kidney disease hyperparathyroidism, diabetes, cancer, osteoporosis and CVD, and not otherwise healthy athletes.

Zabihyeganeh et al, examined the efficacy of 300,000IU/D₃ given orally or intramuscularly in 92 adults with hypovitaminosis D. Both regimens were considered effective in treating hypovitaminosis D. Although the oral route produced significantly higher serum 25[OH]D levels at the 3 month point, although was not advantageous at 6 months (Zabihyeganeh et al., 2013).

Conversely, in a study involving paediatric patients with rickets, both oral and injectable forms of cholecalciferol were observed to be effective at increasing 25[OH]D levels, but the injectable

form was shown to be more efficacious. The authors suggest these findings are due to poor adherence to oral supplementation (Billoo et al., 2009).

Despite worldwide deficiency and increased public awareness of 25[OH]D deficiency, there are unanswered questions around which pharmaceutical form of vitamin D supplementation (oral or injection) is the most effective at correcting deficiency (Hashemipour et al., 2004).

The recommended maximum intake of vitamin D varying, dependent on age and vitamin D status. The US institute of medicine (IOM) recommends a daily intake from 400 to 4000IU, and state that toxicity is unlikely to be observed with intakes as high as 10,000IU/d. However, high dose, (50,000IU/d) vitamin D are readily available. These ‘mega dose’ capsules are seen to improve adherence, i.e. one 50,000IU capsule per month vs. 1000IU/d over a one-month period. However, research shows high dose vitamin D₃ supplementation (70,000 IU/wk) may be detrimental for its intended purposes due to increasing 24,25[OH]₂D production. Furthermore, withdrawal from high dose supplementation may inhibit the bioactivity of 1,25[OH]₂D₃ as a consequence of sustained increases in 24,25[OH]₂D that persist as 25[OH]D and 1,25[OH]₂D concentrations decrease. These data infer that smaller daily doses (1000-2000IU) may be most appropriate and gradual withdrawal from supplementation as opposed to rapid withdrawal may be favorable (D. Owens et al., 2016).

2.6 Effect of vitamin D on Athletic Performance

Athletic performance is determined by many genetic and environmental factors (MacArthur & North, 2005). Recently, vitamin D has been proposed as both performance limiting and enhancing when in deficiency and abundance (Cannell et al., 2009; B. Hamilton, 2012). The suggestion that vitamin D deficiency may act as a performance inhibitor has potentially widespread ramifications for athletes. Indeed, vitamin D deficiency may impact on athletic performance as a cause of

reduced training quality, increased injury/illness frequency and duration (Halliday et al., 2011). Despite variation in sun like exposure due to higher latitude and seasonal variation, athletes that train and compete indoors are at risk of vitamin deficiency all year round (M. Holick, 2007). Numerous studies have examined the association between serum 25[OH]D and factors that may impact athletic performance, such as maximal oxygen uptake (Ardestani et al., 2011), cardiovascular system (Allison, Close, et al., 2015), immune function (He et al., 2013) and neuromuscular coordination (Bischoff-Ferrari, Dietrich, Orav, Hu, et al., 2004), but currently evidence is inconclusive.

2.6.1 Bone

Whilst numerous studies have examined the relationship between 25[OH]D deficiency and bone health in the general population, little is known about athletes, a population where stress fractures are frequently observed (Johnson, Weiss, & Wheeler, 1994).

Mechanical loading from training or competition is associated with higher BMD even when adjusting for weight. Nikander et al., (Nikander, Sievanen, Heinonen, & Kannus, 2005) identified weight modality as a determinant of the structure and strength of femoral neck; observing that athletes competing in high-impact (basketball and volleyball) and odd-impact sports (football) demonstrated greater BMD scores than low impact sports such as swimming and cycling, whilst increased body mass contributes to the process of bone remodeling, and forms mechanically appropriate bone structure (Nikander et al., 2005). These findings are supported by studies examining different athlete groups such as, senior athletes (mean 65.9 years old; male and female) (Leigey, Irrgang, Francis, Cohen, & Wright, 2009), female adult athletes (Narra, Nikander, Viik, Hyttinen, & Sievanen, 2013), male combat sports (Andreoli et al., 2001), world-class skiers

(Nikander, Sievanen, Heinonen, Karstila, & Kannus, 2008) and in adolescent boys (Dias Quiterio, Carnero, Baptista, & Sardinha, 2011).

However, non-weight bearing athletes forgo the same skeletal loads that produce greater BMD scores (Fredericson et al., 2007; Nikander et al., 2005) and are at higher risk for low BMD (Guillaume, Chappard, & Audran, 2012; Rector, Rogers, Ruebel, & Hinton, 2008; Smathers, Bemben, & Bemben, 2009). Exercise is associated with an increase in BMD (Nikander, Sievanen, Uusi-Rasi, Heinonen, & Kannus, 2006; Rantalainen, Nikander, Daly, Heinonen, & Sievanen, 2011), whilst increased body mass contributes to the process of bone remodeling, and forms mechanically appropriate bone structure (Nikander et al., 2005).

The stimulus of loading the musculoskeletal system through high-intensity dynamic sporting activity is believed to compensate for 25[OH]D deficiency with the absence of poor bone health in athletes (Nikander et al., 2006; Weidauer et al., 2014). However, Hamilton et al, demonstrated that BMD and 25[OH]D status were not statistically linked in a sample of Middle Eastern male athletes. Suggesting that genetic polymorphism in the 25[OH]D/1,25[OH]₂D pathway may potentially account for some of these differences (Hamilton et al., 2010; Rabon-Stith et al., 2005).

2.6.2 Athlete's Heart

Research examining the athlete's heart started more than 100 years ago, when Henschen documented that Nordic skiers presented with larger hearts than sedentary individuals (Henschen, 1899). Since then research has shown that regular and prolonged participation in intensive physical exercise is associated with several structural and electrophysiological cardiac adaptations that enhance diastolic filling and facilitate a sustained increase in the cardiac output that is fundamental

to athletic excellence. Such cardiac adaptations are collectively referred as the “Athlete’s Heart” and are frequently reflected on the 12-lead ECG and imaging studies (Papadakis et al., 2012).

Pre-participation screening is becoming a global phenomenon, involving athletes of diverse ethnic groups. Main sporting bodies including FIFA, UEFA and the International Olympic Committee have endorsed pre-participation screening recommendations, despite limited understanding of the impact of ethnicity upon the 12-Lead ECG and cardiac structure.

The vast majority of athletes exhibit relatively mild structural and electrical changes, which are considered to be within conventionally defined normal limits. A small proportion of athletes develop pronounced changes, which overlap with phenotypic expressions of cardiac diseases implicated in exercise-associated sudden cardiac death (SCD). In such circumstances, the differentiation between physiological adaptation and cardiac pathology is challenging but an erroneous diagnosis has the potential for grave consequences.

Emerging studies suggest that ethnicity is an important determinant of cardiovascular adaptation to exercise, which should always be considered during assessment of an athlete. It is well established that ethnicity is one of the several factors that influence manifestations of athlete’s heart (Figure 2.6). The exponential increase in the number of elite athletes from varying ethnic backgrounds has driven the researchers to investigate the effect of exercise in those ethnicities.

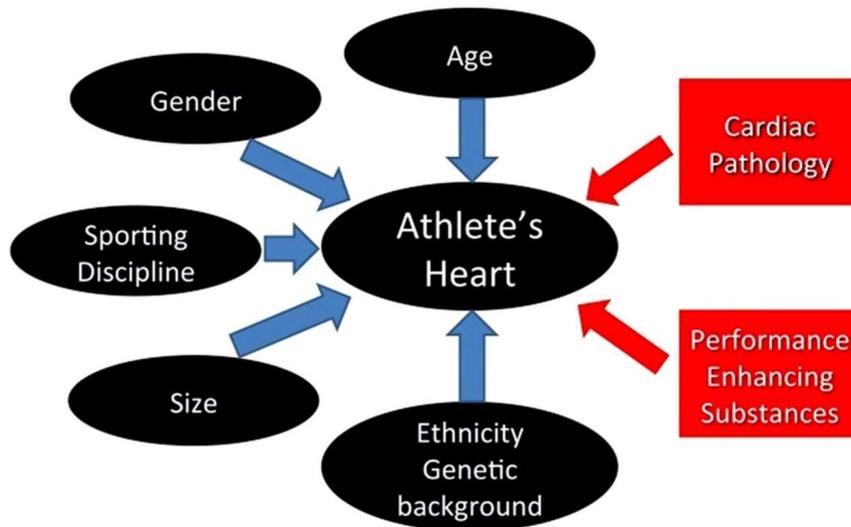


Figure 2.6 Schematic representation of demographic and pathological factors, which may influence the cardiovascular adaptation to exercise (Papadakis et al., 2012).

Studies demonstrate ethnic differences in cardiac structure and function. Papadakis et al., revealed that in elite male athletes African/Afro-Caribbean athletes exhibit an left ventricular (LV) cavity size of similar magnitude to white athletes (52.6 ± 4.4 vs 52.6 ± 4.3 mm, $p > 0.05$) but demonstrate slightly larger left atria (35.4 ± 4.5 vs 34.7 ± 4.7 mm, $p = 0.002$) and aortic root (30.2 ± 3.3 vs 29.5 ± 3.3 mm, $p < 0.001$) diameters (Papadakis et al., 2011). These findings are supported by the findings of Basavarajaiah et al, and Riding et al., who demonstrated that Black African athletes present significantly greater wall thicknesses and resultant LV masses than Arabic and Caucasian athletes (Basavarajaiah et al., 2008; Riding et al., 2012). Furthermore, a recent study examining athletes from Asia demonstrated that Japanese soccer players presented with a high end diastolic diameter compared to Black or Caucasian players (55.2 ± 3.3 vs 52.2 ± 3.8 vs 53.9 ± 3.7 mm, respectively, $p < 0.01$) (Kervio et al., 2013).

Recent data has demonstrated a high prevalence of vitamin D deficiency across ethnicities, particularly within Arabic athletes (Hamilton et al., 2010). Whilst vitamin D deficiency is associated with hypertension, MI, and stroke, as well as other cardiovascular-related diseases

(Brøndum-Jacobsen, Benn, Jensen, & Nordestgaard, 2012; Hsia et al., 2007). To date no studies have investigated the association between vitamin D status, ethnicity and cardiovascular function in athletic populations.

Chapter 3:

General Methodology

3.1 Ethical approval

Ethical approval for the research studies described in Chapter 4 and Chapter 8 were obtained from the Shafallah Medical Genetics Centre Ethics Committee, with all athletes completing informed consent in either Arabic or English. For subsequent studies (Chapters 5,6 & 7) ethical approval was obtained from Qatar Anti-Doping Laboratory IRB, with all athletes completing informed consent. All procedures conformed to the standards of the Declaration of Helsinki.

3.2 General Screening & Inclusion Criteria

Male athletes registered with the Qatar Olympic Committee presented for pre-competition medical assessment at Aspetar Sports Medicine Hospital, Qatar. All athletes completed a vitamin D questionnaire in collaboration with an Arabic, French or English-speaking nurse. This included questions specifically related to country of origin, sporting discipline, skin type, self-reported exposure to daily sunlight (nil or <30 min, 30–60 min, 60–120 min, >120 min), use of sunscreen, dietary supplements and/or medication, and an assessment of skin colour (dark, olive, fair).

Inclusion criteria for all studies were:

- Male athletes (No female athletes were included in the study because of low participation rates in Qatar)
- Aged 18-30 yrs.
- Free from any known comorbidities or family history of osteoporosis or bone disease
- Serum parathyroid hormone, calcium within normal range.
- Not taking supplemental Vitamin D₂ or D₃, fish oils or multi vitamins

3.3 Assessment of Bone Mineral Density

Dual-energy x-ray absorptiometry (DXA; Osteocore III, Perols, France, version 5.22b) scanning was used to assess hip and spine bone mineral density (BMD). A certified technologist from the International Society of Clinical Densitometry performed all calibrations and measurements. Quality assurance was performed each morning before testing. The coefficient of variation for these records is <1.01% in Aspetar. BMD was calculated in g/cm² for spine (L2–L4), hip-neck and hip-total. In addition, the clinical age-matched and gender-specific Z-score index was used to classify the BMD. T-scores were calculated for those athletes older than 20 years as per WHO recommendations ("Prevention and management of osteoporosis," 2003). Participants under 20 years were not included in the sub-analysis for (T-score) osteoporosis and osteopenia.

3.4 Laboratory analyses

3.4.1 Blood sampling

Venous blood samples were collected from athletes following an overnight fast. For the biochemical measures, samples (10 mL) were drawn from an antecubital vein using a tourniquet. The tourniquet has been applied for less than one minute (~20-30 seconds), in order to avoid local hemoconcentration that may result in erroneous laboratory findings (Nikolac et al. 2013). Serum was collected using blood collection tubes from BD vacutainer from the UK that were purple (spray-coated K2EDTA) and gold (acrylic based gel and a spray-dried clot activator coating). The blood sample was separated into two aliquots (5mL SST tubes), one aliquot for the immediate analysis of PTH, calcium and albumin and one aliquot for 25[OH]D analysis. Blood samples were centrifuged ~30 second after collection in a swinging bucket rotor of the Multifuge® 1S/1S-R for

10 minutes at 3000-rpm. The resulting serum was decanted and stored in Eppendorf tubes (Eppendorf, Hamburg, Germany).

3.4.2 25[OH]D

Serum samples for 25[OH]D were stored at -80°C to be analyzed later by Enzyme-linked immunosorbent assay (ELISA). Serum 25[OH]D was analysed utilizing chemiluminescent immunoassay technology (Liaison® 25-OH Vitamin D Total Assay; DiaSorin Inc., Saluggia, Italy). Serum is incubated with antivitamin-D coated microparticles and isoluminol derivative-conjugated 25OHD before measurement of the chemiluminescent signal.²⁸ The Liaison 25(OH)D assay is co-specific for 25(OH)D₂ and 25(OH)D₃, so it reports a total 25(OH)D concentration with sensitivity set at 7 ng/mL. The intra- and inter-assay coefficient of variation (CV) was 7.6–9.4% and 9.8–13.4%, respectively. The dynamic range was 4.0 – 150 ng/mL and functional sensitivity: ≤ 4.0 ng/mL. An automatic ELISA microplate reader (Infinite® 200 PRO NanoQuant, Switzerland) and Magellan Standard software (version 7.1) were used. Based upon the serum 25[OH]D results, athletes were split into four 25[OH]D categories; severely deficient (<10 ng/mL), deficient (10–20 ng/mL), insufficient (20–30 ng/mL), or sufficient (>30 ng/mL). Serum 25(OH)D levels have been demonstrated to be stable in spite of multiple freeze-thaw cycles

3.4.3 PTH, calcium and albumin

PTH: Levels of intact PTH were plasma samples were analysed (*within ~15 minutes after collection*) on Architect I1000 machine with chemiluminescence microparticle immunoassay (CMIA). The inter-assay CV was 2.5%. Calcium and albumin measures: serum samples were analyzed (*within ~15 minutes after collection*) on Dimension Vista® System with bi-chromatic

endpoint technique. Calcium reacts with o-cresolphthalein complexone to form a purple complex. The amount of complex thus formed is proportional to the calcium concentration and is measured using a bichromatic (577, 540 nm) endpoint technique. Analytical Measurement Range (AMR): 5.0–15.0 mg/dL [1.25–3.75 mmol/L]. Albumin: In the presence of a solubilizing agent, bromocresol purple (BCP binds to albumin at pH 4.9. The amount of albumin-BCP complex is directly proportional to the albumin concentration. The complex absorbs at 600 nm and is measured using a polychromatic (600, 540, 700 nm) endpoint technique. Analytical Measurement Range (AMR): 0.0–8.0 g/dL [0–80 g/L].

All the biochemical measures and analyzes were completed at the Aspetar Hospital laboratory, which is accredited by the College of American Pathologists (CAP).

3.4.4 Conversion Factor

Aspetar laboratory uses the conventional units for 25(OH)D (nanogram per milliliter [ng/mL]) whereas other laboratories use international system (SI) units (nanomole per liter [nmol/L]). The conversion factor to SI units is: 1 ng/mL = 2.496 nmol/L.

$$\text{Concentration (ng/mL)} = \frac{\text{Concentration (pmol/mL)} \times \text{Molar Mass}}{1000}$$

3.4.5 Vitamin D-Binding Protein

Serum vitamin D binding protein (DBP) concentrations ($\mu\text{g/mL}$) were determined using a commercially available kit (R&D Systems, UK). The limit of sensitivity was ≤ 0.65 ng/mL and an inter-assay coefficient of variation was 7.2%. An automatic enzyme-linked immunosorbent assay

(ELISA) microplate reader (Infinite® 200 PRO NanoQuant, Switzerland) and computer software Magellan Standard (version 7.1) were used to analyse DBP.

3.5 Estimation of Bioavailable Vitamin D

Free, bioavailable, and DBP-bound 25[OH]D were calculated using equations from supplementary material (Powe et al., 2013) adapted from those described by Vermeulen and colleagues (Vermeulen, Verdonck, & Kaufman, 1999). These methods use the free hormone hypothesis to define bioavailable hormone as the fraction that is both free and albumin-bound, that is, the fraction not bound to circulating binding proteins such as DBP.

Free levels of 25[OH]D were calculated using the following equation:

$$[D_{\text{free}}] = \frac{\frac{[D_{\text{DBP}}]}{K_{\text{DBP}}}}{[Total\ DBP] - [D_{\text{DBP}}]}$$

After calculating free 25-hydroxyvitamin D, equation 2 was used to calculate the concentration of bioavailable (non-DBP bound vitamin):

$$[Bio\ D] = [D_{\text{free}}] + [D_{\text{Alb}}] = (K_{\text{alb}} * [Alb] + 1) * [D_{\text{Free}}]$$

Definitions

- $[D_{\text{Free}}]$ = concentration of free (unbound) 25-hydroxyvitamin D
- $[D_{\text{DBP}}]$ = concentration of D-binding protein-bound 25-hydroxyvitamin D
- K_{DBP} = genotype-nonspecific affinity constant between 25-hydroxyvitamin D and DBP = 0.7×10^9 M⁻¹

- [Total DBP] = concentration of serum DBP g/L
- [Bio D] = concentration of bioavailable D
- [D_{Alb}] = concentration of albumin-bound vitamin D
- Kalb = affinity constant between vitamin D and albumin = $6 \times 10^5 \text{ M}^{-1}$
- Alb = albumin

3.6 Pre-participation cardiovascular screening

All individuals were screened using a precompetition medical assessment form, examining family history of cardiovascular disease and personal symptoms, with a physical examination undertaken by a sports medicine physician. A standard 12-Lead ECG was obtained using a GE Mac 5500 (New York, USA) after a 5-min rest in the supine position.

Echocardiographic examination was performed using a commercially available ultrasound system (Philips, USA) by an experienced sports cardiologist. Images of the heart were obtained in the standard planes, using previously published criteria (Riding et al., 2013). The left ventricular (LV) wall thickness was measured from two-dimensional short-axis views in end-diastole, with the greatest measurement within the LV wall defined as the maximal LV wall thickness (mLVWT). M-mode echocardiograms derived from two-dimensional images in the parasternal long axis were used for the measurement of LV end-diastolic diameter (LVED), left atrial (LA) and aortic root (Ao) diameter according to American Society of Echocardiography standards (Sahn, DeMaria, Kisslo, & Weyman, 1978) with LV diastolic volume (LV volD) derived using Simpson's biplane methodology. LV mass (LVM) was calculated using the formula of Devereux (Devereux & Reichek, 1977) but was also scaled for body surface area (BSA). LV diastolic function was assessed using pulsed-wave Doppler recordings from apical four-chamber orientations. All data

were analysed offline and a minimum of three cardiac cycles was averaged for all indices. For the tissue Doppler assessment of E', the apical four-chamber orientation was utilized and a 2 mm sample volume was positioned at both the septal and lateral wall aspect of the mitral valve annulus ensuring the best alignment between wall motion and the ultrasound beam. The Nyquist limit was set between 10 and 35 cm/s. Peak early diastolic (E0) tissue myocardial velocity was recorded and E/E0 was calculated.

3.7 Further exclusion

Athletes reporting symptoms (*i.e.* Discomfort, pressure, heaviness, or pain in the chest or arm), a family history of sudden cardiac death (SCD), and echocardiographic and/or ECG abnormalities considered to represent potential pathology were investigated further with 24 h ECG, maximal exercise testing and cardiac MRI to evaluate the broader phenotype of common cardiomyopathic processes such as hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC).

3.8 Statistical analysis

All data was coded and analysed using SPSS Predictive Analytics Software (v.20, IBM Corporation, New York, US). Descriptive statistics were presented as mean and standard deviation (SD) for continuous variables. For categorical variables, frequency and percentage were reported. For the comparison of two group means, a t-test was used and where comparison of multiple groups of means was required an analysis of variance (ANOVA) was used. Data sets were first checked for normal distribution and where data violated the assumption of normality, a natural log-transformation was applied prior to analysis. Post-hoc analyses with Bonferroni correction

were performed where appropriate for further comparisons in the event of significance. To determine the relationship of bone mass parameters with serum 25[OH]D, DBP and bioavailable 25[OH]D, simple regression without covariates and multiple linear regression analysis including covariates such as age and ethnicity was performed with bone mass as a dependent variable, with age and ethnicity covariates. Parameter estimates along with standard error were reported. A p-value<0.05 was used as a cut-off for statistical significance.

Chapter 4:

Oral vs. Intramuscular Vitamin D Supplementation for Treating Insufficient Athletes

4.1 Abstract

Purpose: To correct vitamin D (25[OH]D) insufficiency in athletes, the physician may decide to supplement. However, there is no consensus as to the most efficacious form of treatment to correct insufficiencies. The present study examined which modality of supplementation was more efficacious (Oral vs. single intramuscular injection IM) at improving serum 25[OH]D concentration in insufficient (<30 ng/mL) athletes.

Methods: In a non-randomised patient-choice approach, 355 athletes presenting a serum 25[OH]D level ≤ 30 ng/mL were treated with either Oral Cholecalciferol D₃ [2000IU/d for 60 days] (n = 123) or a single IM Ergocalciferol D₂ injection [300,000 IU] (n = 232), and reassessed at 2-months.

Results: There were no significant differences in baseline serum 25[OH]D concentration between Oral vs. IM groups (13.2 ± 0.5 vs. 14.2 ± 0.4 ng/mL). Following 2-months of treatment, both groups significantly improved serum 25[OH]D concentration (25.8 ± 0.9 vs. 24.7 ± 0.6 ng/mL); with no significant difference between treatments at 2-months. However, a significantly greater number of athletes became sufficient (>30 ng/mL) at 2-months following Oral vs. IM supplementation (37.4% vs. 21.6%, $p < 0.001$). A greater percentage of athletes presenting with severe 25[OH]D deficiency (<10 ng/mL) at baseline moved to sufficiency (>30 ng/mL) after 2-months with Oral vs. IM (27.5% vs. 10.0%). Oral supplementation also moved a greater number of insufficient athletes to sufficiency [(>30 ng/mL); 86.7% vs. 50%].

Conclusion: With appropriate education and adherence, 2000IU/d Oral 25[OH]D₃ supplementation may be more efficacious at improving 25[OH]D insufficiencies than IM injections of 25[OH]D₂.

4.2 Introduction

Vitamin D (25[OH]D) deficiency (<20 ng/mL) is associated with a number of detrimental health conditions including cardiovascular disease, diabetes mellitus, cancer, autoimmune disease, poor musculoskeletal health and depression (Brøndum-Jacobsen et al., 2012; Deeb, Trump, & Johnson, 2007; Hyppönen, Läärä, Reunanen, Järvelin, & Virtanen, 2001; Kanis, 2002). Recent evidence has demonstrated that many athletes are 25[OH]D deficient (Constantini et al., 2010; Hamilton et al., 2010; Magee et al., 2013), with some authors suggesting that correcting serum 25[OH]D concentrations in deficient individuals may in turn improve athletic performance (Close, Russell, et al., 2013; Wyon, Koutedakis, Wolman, Nevill, & Allen, 2013); however this is not universally supported (B. Hamilton, Whiteley, Farooq, & Chalabi, 2014). Nevertheless, given the high prevalence of deficiency across many athletic populations, the implication that deficiency may be detrimental to overall health, as well as being a possible athletic performance inhibitor has significant implications for the sports medicine physician.

The categorisation of what constitutes vitamin D insufficiency is widely debated. Current ranges are based upon the association between 25[OH]D deficiency with osteomalacia and rickets (Robert P. Heaney, 2011). Currently, serum 25[OH]D concentrations are categorised as deficient (<10 ng/mL), deficient (10-20 ng/mL), insufficient (20-30 ng/mL) or sufficient (>30 ng/mL) (M. Holick, 2007). Whilst it is considered that individuals should have 'sufficient' 25[OH]D concentrations, there is limited evidence to suggest that >30 ng/mL assists in the maintenance of 'good' health or improves athletic performance.

Nevertheless, in order to improve 25[OH]D concentrations in deficient athletes, the sports medicine physician may decide to 1) provide sun exposure and dietary education, and/or 2) supplement. Even with the best nutrition advice, achieving an increase in serum 25[OH]D concentration can be problematic due to a lack of foods naturally containing vitamin D (M. Holick, 2007). The method of supplementation may be achieved orally or via an intramuscular (IM) injection; both of which are available in a range of dosages and durations, and in two different forms (D₂ or D₃).

Athlete's were offered the choice between oral cholecalciferol or intramuscular (IM) ergocalciferol, as was current practice in Aspetar. Both cholecalciferol (D₃ – the most common form of Oral supplementation) and ergocalciferol (D₂ – its IM counterpart) appear to improve serum 25[OH]D concentrations in deficient individuals (Biancuzzo et al., 2013; M. F. Holick et al., 2008), yet there is limited evidence to demonstrate which modality is more efficacious in improving overall 25[OH]D status in athletes (Zabihyeganeh et al., 2013). A single IM injection of ergocalciferol (D₂) may remove the potential gut malabsorption and athlete compliance issues, however; it is unacceptable for the treating physician to wait 6-months to ascertain if the deficiency has been corrected when the athlete is competing weekly, as was current practice in Aspetar. In turn, there is evidence to suggest that D₂ has poorer bioefficacy than D₃, and thus may have limited use as a treatment supplement (Houghton & Vieth, 2006).

Previous studies have attempted to examine the efficacy of oral vs. IM modalities, but with variable dosages and durations in many of these investigations, the clinical effectiveness of both modalities at correcting 25[OH]D deficiency remains unclear (Billoo et al., 2009; Zabihyeganeh et al., 2013).

Furthermore, the majority of these studies have only examined individuals with known pathologies or health related issues, such as rickets, chronic kidney disease hyperparathyroidism, diabetes, cancer, osteoporosis and CVD, and not otherwise healthy athletes.

4.3 Aims and Hypotheses

The aim of the present investigation was to examine which modality of 25[OH]D supplementation (Oral Cholecalciferol D₃ [2000IU/d] vs. a single IM Ergocalciferol D₂ injection [300,000 IU]) was more efficacious at improving overall serum 25[OH]D status in severely deficient (<10 ng/mL), deficient (10-20 ng/mL) and insufficient (<30 ng/mL) athletes, following 2-months of treatment. It was hypothesised that oral vitamin D₃ supplementation would be more efficacious at improving serum 25[OH]D concentrations than a single IM Ergocalciferol D₂ injection.

4.4 Methods

Ethical approval was obtained from the Shafallah Medical Genetics Centre Ethics Committee, with all athletes completing informed consent in either Arabic or English

4.4.1 Participants

Three hundred and fifty-five athletes demonstrating serum 25[OH]D concentration of ≤ 30 ng/mL when presenting for pre-competition medical assessment in our facility, were invited to take part in the study. All 355 athletes completed a pre-assessment survey.

4.4.2 Vitamin D supplementation

A non-randomised protocol was chosen, as our facility follows a patient choice approach to 25[OH]D supplementation. After discussion with a sports medicine physician, athletes selected either an oral modality (2000IU/d) for 2-months [Oral Cholecalciferol D₃, 2x 1000IU; Jarrow Formulas, Los Angeles, USA] or a single IM (300,000 IU) injection [IM Ergocalciferol D₂, Biotika, Solvenska Lupca, Slovak Republic]. All athletes were given a 2-month follow-up appointment with a sports medicine physician to re-assess 25[OH]D status. One hundred and twenty three athletes eventually chose Oral supplementation vs. 232 who chose IM. Statistical analysis was carried out independently of the treating sports medicine physician(s), in order to rule out study bias. No athlete was supplemented with calcium, in order to independently ascertain the effects of the 25[OH]D treatment.

4.4.3 Laboratory analyses

As described in the general methodology chapter

4.4.4 Statistical Analysis

As described in the general methodology chapter

4.5 Results

4.5.1 Athlete Demographics

The 355 athletes participating in the study came from 33 countries (Table 4.1). However, there were no significant differences in height, body mass, body surface area, body mass index, exposure

time to sunlight, use of sunscreen, athlete skin colour or the use of calcium supplements between Oral vs. IM groups at baseline.

Table 4.1 Origin of screened athletes

<i>Region</i>	<i>Number of nations from region (n)</i>	<i>Total athletes (n)</i>
Gulf states	7	191
Middle Eastern	5	86
Africa	9	40
Persia	1	13
Western/European	6	15
Asian	5	10
Total	33	355

4.5.2 25[OH]D and PTH

No significant differences were observed at baseline in the distribution of serum 25[OH]D concentration in the severely deficient (<10 ng/mL) or deficient (10-20 ng/mL) groups between Oral vs. IM supplementation modalities (Table 4.2), whilst there were more insufficient (20-30 ng/mL) athletes in the IM treatment group (p=0.05). However, there was no significant difference (p>0.05) in serum 25[OH]D or calcium values between Oral vs. IM groups at baseline, whilst PTH was significantly higher in the Oral vs. IM group at baseline (p=0.019; Table 4.3).

Table 4.2 Baseline characteristics of athletes, together with the distribution of serum 25[OH]D concentrations between Oral vs. IM supplementation modalities at baseline

<i>Variables</i>	<i>Oral N=123</i>	<i>IM N=232</i>	<i>Oral vs. IM P value</i>
Height (m)	1.75± 9.6	1.75 ± 8.5	0.691
Body Mass (kg)	72.1 ± 15.6	76.7 ± 17.6	0.086
Body surface area(m²)	1.87 ± 0.23	1.92 ± 0.22	0.136
Body Mass Index (kg/m²)	23.3 ± 3.8	24.8 ± 5.0	0.052
Ethnicities n (%)			
Caucasian	6 (4.9)	9 (3.9)	
Asian	2 (1.6)	8 (3.4)	
African	5 (4.1)	35 (15.1)	
Middle-Eastern	34 (27.6)	52 (22.4)	0.028
GCC	73 (59.3)	118 (50.9)	
Persian	3 (2.4)	10 (4.3)	
Exposure to sunlight n (%)			
No sunlight exposure	35 (28.5)	65 (28.0)	
30-60 minutes	44 (35.8)	125 (35.2)	
60-120 minutes	23 (18.7)	61 (17.2)	0.846
>120 minutes	21 (17.1)	69 (19.4)	
Use of sunscreen n (%)			
Yes	8 (6.5)	11 (4.7)	
No	115 (93.5)	221 (95.3)	0.483
Skin color n (%)			
Dark	25 (20.3)	63 (27.2)	
Olive	70 (56.9)	126 (54.3)	0.312
Fair	28 (22.8)	43 (18.5)	
Calcium supplements n (%)			
Yes	5 (4.1)	18 (7.8)	
No	118 (95.9)	214 (92.2)	0.179
Serum 25[OH]D			
<10 (ng/mL) (%)	40 (32.5)	60 (25.9)	
10-20 (ng/mL) (%)	68 (55.3)	124 (53.4)	0.101
20-30 (ng/mL) (%)	15 (12.2)	48 (20.7)	

4.5.3 Post-supplementation serum 25[OH]D concentration

Both Oral and IM modalities significantly increased serum 25[OH]D concentration following 2-months of supplementation (Table 4.3) with no significant difference in mean 25[OH]D status between treatments at 2-months. However, a significantly greater number of athletes became sufficient (>30 ng/mL) at 2-months following Oral vs. IM supplementation (37.4% vs. 21.6%, $p < 0.001$). This result remained significant after adjusting for athlete ethnicity.

A greater percentage of athletes presenting with severe 25[OH]D deficiency (<10 ng/mL) at baseline moved to sufficiency (>30 ng/mL) after 2-months when undertaking Oral vs. IM supplementation (27.5% vs. 10.0%; Table 4.4). Oral supplementation also moved a greater number of insufficient (20-30 ng/mL) athletes to sufficiency [(>30 ng/mL); 86.7% vs. 50%]. However, a similar number of athletes had reductions in 25[OH]D concentrations, moving from insufficiency (20-30 ng/mL) to deficiency (10-20 ng/mL) in 6.7% (Oral) vs. 8.3% (IM) after 2-months, respectively.

Table 4.3 Comparison of mean serum measures at baseline and following 2-months of vitamin D supplementation

<i>Variable</i>	<i>Oral</i>	<i>IM</i>	<i>Oral vs. IM same time point</i>	<i>Oral Baseline vs. 2-month</i>	<i>IM Baseline vs. 2-month</i>
25[OH]D (ng/mL)					
Baseline	13.2 ± 0.5	14.2 ± 0.4	0.107	<0.001	<0.001
2 Month	25.8 ± 0.9	24.7 ± 0.6	0.075		
PTH (pg/mL)					
Baseline	53.9 ± 2.7	49.6 ± 1.5	0.019	0.009	0.821
2 Month	47.2 ± 2.2	50.0 ± 1.7	0.239		
Ca (mmol/L)					
Baseline	2.26 ± 0.01	2.26 ± 0.01	0.456	0.179	0.084
2 Month	2.28 ± 0.01	2.25 ± 0.01	<0.001		

PTH, parathyroid hormone, Ca; calcium.

4.5.4 Post-supplementation serum parathyroid hormone concentration

All athletes presented with clinically normal PTH values at baseline (range 14-72 pg/mL). Baseline PTH levels were significantly higher in the Oral group vs. IM group (53.9 ± 2.7 vs. 49.6 ± 1.5 pg/mL, $p < 0.05$), which was removed following 2-months of 25[OH]D supplementation (Table 4.3). A significant decrease in PTH was observed after 2-months following Oral supplementation ($p = 0.009$), whereas PTH remained unchanged in the IM treatment modality ($p = 0.821$).

Table 4.4 Inter-group improvements in serum 25[OH]D concentration from baseline and following 2-months of vitamin D₂/D₃ supplementation

<i>Treatment</i>		<i>Serum 25[OH]D after 2-months of supplementation (ng/mL)</i>			
		<10	10-20	20-30	>30
Baseline Group (ng/mL)					
Oral (D₃)	<10 (%)	11 (27.5)	8 (20.0)	10 (25)	11 (27.5)
	10-20 (%)	0 (0.0)	17 (25.0)	29 (42.6)	22 (32.4)
	20-30 (%)	0 (0.0)	1 (6.7)	1 (6.7)	13 (86.7)
Baseline Group (ng/mL)					
IM (D₂)	<10 (%)	5 (8.3)	18 (30.0)	31 (51.7)	6 (10.0)
	10-20 (%)	2 (1.6)	29 (23.4)	72 (58.1)	21 (16.9)
	20-30 (%)	0 (0.0)	4 (8.3)	20 (41.7)	24 (50.0)

4.5.5 Post-supplementation serum calcium concentration

Baseline serum Ca levels were within the normal range (2.12-2.52 mmol/L), with no significant differences at baseline between Oral vs. IM groups. Following 2 months of 25[OH]D supplementation, Ca levels were significantly lower following IM supplementation vs. Oral (2.25 ± 0.01 vs. 2.28 ± 0.01 , $p < 0.001$).

4.6 Discussion

The present study aimed to examine which modality of vitamin D supplementation (Oral Cholecalciferol D₃ [2000IU/d] vs. a single IM Ergocalciferol D₂ injection [300,000 IU]) was more efficacious at improving serum 25[OH]D status in severely deficient (<10 ng/mL), deficient (10-20 ng/mL) and insufficient (<30 ng/mL) athletes, following 2-months of treatment. We were motivated by the fact that to date, there is no consensus amongst the sports medicine community when treating insufficient athletes, as to the optimum mode, dose or type of vitamin D supplementation.

4.6.1 Vitamin D (25[OH]D)

The main finding of this study is that, in athletes presenting at baseline as either severally deficient, deficient or insufficient, both Oral and IM supplementation significantly improved mean serum 25[OH]D ($p < 0.001$). Although there was no difference in the group mean serum 25[OH]D concentrations between treatment modalities following 2-months of vitamin D supplementation, a significantly greater percentage of athletes shifted to sufficiency (>30 ng/mL) with Oral vs. IM supplementation (45.37% vs. 50.21.6%, $p < 0.001$). The precise mechanism(s) explaining this result remain unclear. One possible explanation is the type of vitamin D supplementation used in the present study. We used Oral cholecalciferol D₃ [1000IU/d] vs. a single IM ergocalciferol D₂ injection [300,000 IU]. Recent studies have demonstrated no significant difference in serum 25[OH]D concentrations after ingestion of 1,000 IU 25[OH]D₂ or 25[OH]D₃ (Biancuzzo et al., 2013; M. F. Holick et al., 2008), implying that 25[OH]D¹-hydroxylase recognises both secosteroids equally. However, it is known that there is a small molecular structural difference between D₃ and D₂. This minor variation in the side-chain chemistry between the two forms of

25[OH]D may result in differences at the site of hydroxylation, leading to differing biologically active metabolites (Houghton & Vieth, 2006). These differences do not appear to affect activation, but deactivation. It may be possible that the supplement given in a sustained manner (2000IU/day) provides a constant supply for conversion to 25[OH]D. In contrast, a singular IM (300,000IU) may cause an initial increase in 25[OH]D which ultimately cannot be sustained over time.

Trang et al. (Trang et al., 1998) observed that both D₂ and D₃ increased serum 25[OH]D concentrations, yet the increase in 25[OH]D was found to be 1.70 times greater (70%) with D₃ than D₂. This data is supported by Logan et al. (Logan, Gray, Peddie, Harper, & Houghton, 2013) who recently observed that daily supplementation with vitamin D₃ was more effective than D₂ at improving serum 25[OH]D concentrations. Even in individuals with pathology, Mastaglia et al. (Mastaglia, Mautalen, Parisi, & Oliveri, 2006) observed that in a 3-month supplementation study using osteoporotic women, a 2.5-times higher dose of D₂ (250 µg D₂/d) was required to match similar serum 25[OH]D values when compared to a similar study using D₃ (100 µg D₃/d). Whilst in patients with chronic kidney disease, Daroux et al. (Daroux et al., 2013) found that D₃ was more effective than D₂ in providing adequate 25[OH]D serum concentrations in hemodialysis patients.

In summary, it may not necessarily be the modality of supplementation (Oral vs. IM) that causes the observed differences in the percentage of athletes that became 25[OH]D sufficient after 2-months of supplementation, but the type of 25[OH]D (D₂ vs. D₃) provided to the athlete. Although, it is impossible to differentiate between mode entirely, this study demonstrates that Oral D₃ supplementation has a greater efficacy at increasing serum 25[OH]D compared to IM D₂.

4.6.2 Parathyroid Hormone (PTH)

In order to exert its effects on bone metabolism and Ca absorption, 25[OH]D must first be converted to 1,25[OH]₂D₃, which occurs through the action of PTH. This is a self-regulating process, for which evidence of 25[OH]D deficiency may also manifest itself through high levels of PTH, as observed in this study. PTH has also been shown to directly regulate 1 α -hydroxylase activity and mRNA in renal proximal tubular cells via changes in cyclic AMP through stimulation of 1 α -hydroxylase gene transcription (Khan et al., 2012). The mechanism(s) explaining why PTH was significantly reduced following 2-months of Oral supplementation remain unclear. It is possible that the variance in PTH was random; however, in keeping with previous studies from non-athletic populations, PTH levels tended to be inversely related to 25[OH]D (Bjorkman, Sorva, & Tilvis, 2008; Khan et al., 2012). Soliman et al. (Soliman, Adel, Wagdy, Alali, & Aziz Bedair, 2011) also observed a significant inverse relationship between serum 25[OH]D and PTH concentrations, following a single IM dose of cholecalciferol in 100 vitamin D deficient Arabic adolescents. This may be explained because PTH increases CYP27B1 activity, the enzyme responsible for the hydroxylation 25[OH]D to 1,25[OH]₂D. Therefore, when CYP27B1 becomes saturated and adequate 1,25[OH]₂D is synthesised, PTH is suppressed. Therefore, the stimulation of CYP27B1 reduced comparable to when serum 25[OH]D in deficiency.

4.6.3 Treatment

Our facility follows a patient choice approach to 25[OH]D supplementation. Prior to treatment, athletes consult with a sports medicine physician and make an informed choice between taking either an Oral (2000IU/d) supplement for 2-months or a single intramuscular injection (300,000IU), with reassessment at 2 months unless otherwise indicated. Our data demonstrates

that a greater proportion of severely deficient (<10 ng/mL) athletes became sufficient (>30ng/mL) with Oral supplementation vs. IM (11 (27.5%) vs. 6 (10.0%)). However, 27.5% of athletes participating in the Oral supplementation pathway remained severely deficient after 2-months, compared to 8.5% of the IM cohort (Table 4.4). Adherence to any form of Oral treatment/supplementation is a concern for any sports medicine physician. Thus, from a clinical prospective, a strong argument can be made, that for those severely deficient athletes (regardless of adherence or compliancy), there is a 91.7% chance of moving out of the severe deficient category with a single IM injection (300,000IU) of 25[OH]D₂.

4.6.4 Alternative vitamin D dosages

Whilst the ergocalciferol dosage of 300,000IU provided in this study is considered to be the standard dose, some clinicians consider the Oral dose of 2000IU/d of 25[OH]D₃ for 2-months to be inadequate at correcting 25[OH]D deficiencies. (Biancuzzo et al., 2013; M. F. Holick et al., 2008). Yet, 8.3% of the IM cohort had reductions in serum 25[OH]D values at 2-months post injection; moving from insufficiency (20-30 ng/mL) to deficiency (10-20 ng/mL), respectively. Thus, whether our findings of serum 25[OH]D reductions in these 19 athletes are a true reflection of 25[OH]D status reduction, or is a consequence of the athletes being supplemented with D₂ and is thus, a metabolism and/or an assay error remains unclear. These findings have potential clinical implications for routine supplementation of vitamin D. Although both modes of supplementation significantly improved mean serum 25[OH]D after 2-months, 1.6% of deficient athletes became severely deficient and 8.3% of insufficient athletes became deficient at 2-months following IM. These reductions in concentration following IM injection cannot be attributed to poor adherence. Furthermore, Oral supplementation significantly increased a greater percentage of insufficient (20-

30 ng/mL) athletes to sufficiency (>30 ng/mL) at 2-months [86.7% vs. 50%]. Therefore, our data strongly suggest Oral cholecalciferol should be the modality of choice in the compliant insufficient athletes.

4.7 Limitations

Whilst the prescribing sports medicine physician was unbiased in providing counsel to the athletes in the decision of which modality of supplementation to take (the physician was not privy to recruitment or statistical analysis), significantly more Africans chose IM supplementation over Oral. Additionally, the assay used for 25[OH]D analysis (Diasorin Liaison), is known to recognise 25OHD₂ more than 25OHD₃, an anomaly that remains to be explained. Adherence to the Oral treatment was also not recorded, as our experience with athlete-led training/nutrition diaries are particularly unreliable. No measurement of UVB exposure during the 2-months after supplementation was undertaken. We accept that the treating physician provided dietary and sun exposure education before supplementation took place. Consequently, the interpretation of the dose–response for 25[OH]D supplementation, for both Oral and IM, may have been affected by the athlete exposing themselves to UVB radiation. There is a current lack of solid evidence concerning the validity of serum 25[OH]D measure as a biomarker of effect. In order to make definitive conclusions about the efficacy of mode of supplementation, future work should aim to examine the impact of oral cholecalciferol vs. IM cholecalciferol, controlling for adherence and the effect of supplementation on bioavailable vitamin D should also be addressed. Finally, recent evidence showing that rapid withdrawal from high dose vitamin D supplementation may be insufficient at correcting deficiencies. These data should be considered in future supplementation studies and supplementation protocols.

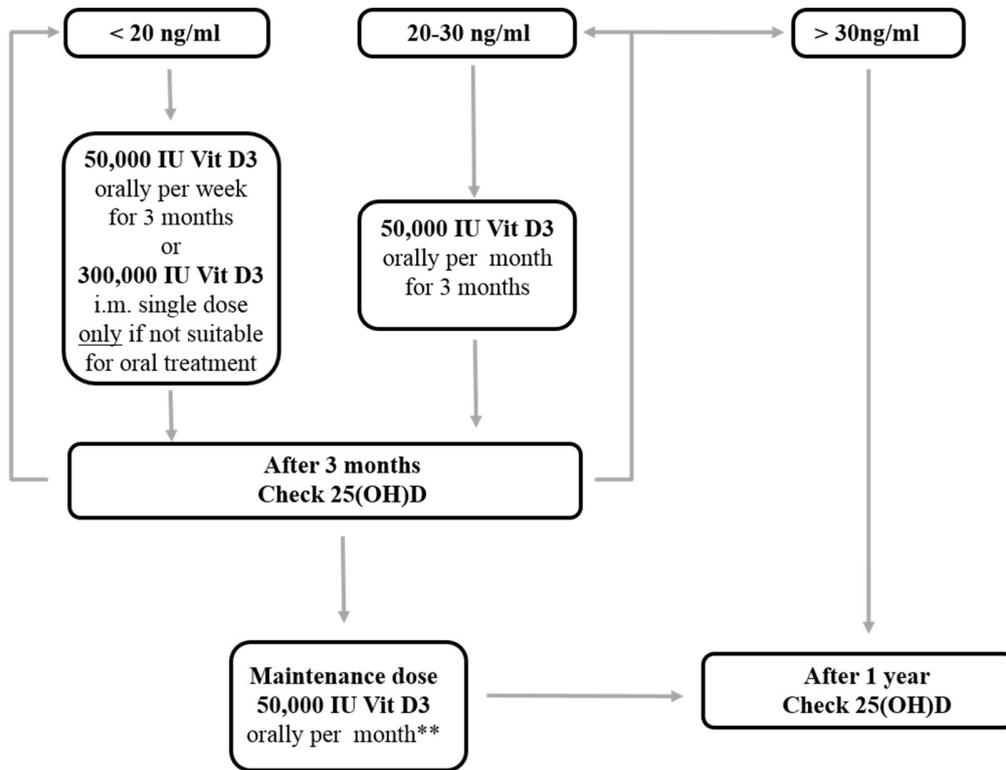
4.8 Conclusion

Whilst the optimal dosages for either Oral or IM 25[OH]D supplementation have yet to be established, our data demonstrates that both Oral (Cholecalciferol D₃ [2000IU/d]) and IM (Ergocalciferol D₂ [300,000 IU]) injection significantly improve serum 25[OH]D concentrations following 2-months of supplementation. However, a significantly greater number of athletes became sufficient (>30 ng/mL) at 2-months following Oral vs. IM supplementation. Our data supports that with appropriate athlete education and compliance, Oral 25[OH]D₃ supplementation may be more efficacious at improving 25[OH]D insufficiencies than IM injections of 25[OH]D₂. Lastly, these data demonstrate a high prevalence of 25[OH]D insufficiency in professional athletes, yet the majority present with no symptoms of deficiency. Future studies should aim to examine associations between 25[OH]D status and markers of athlete health.

4.8.1 Practical outcomes

The findings from this study had direct clinical applications on the treatment of vitamin D deficiency at our institution, culminating with the implication of updated vitamin D supplementation pathway for use by the treating physician (Figure 4.1). Future work should assess the efficacy of this pathway at correcting vitamin D deficiencies.

Vitamin D 25(OH)D
(Calcium within the normal range)



* Children under 16: 50 000 IU Vit D3 orally per month for 3 months

** Children under 16: 50 000 IU Vit D3 orally every 2 months



Figure 4.1 Aspetar's Vitamin D supplementation pathway based on 25[OH]D concentrations

Chapter 5:

No Association between Vitamin D Deficiency and Markers of Bone Mass in Athletes

*Publications resulted from this chapter; Allison, R. J., A. Farooq, B. Hamilton, G. L. Close, and M. G. Wilson. No Association between Vitamin D Deficiency and Markers of Bone Mass in Athletes. *Med. Sci. Sports Exerc.*, Vol. 47, No. 4, pp. 782–788, 2015*

5.1 Abstract

Purpose: Adequate vitamin D (25[OH]D) is required to maintain good bone mass, yet many athletes are 25[OH]D deficient. This study sought to examine the relation between serum 25[OH]D and measures of bone mass in an ethnically diverse athletic population.

Methods: Nine hundred and fifty male athletes presented for pre-competition medical assessment in our facility. An additional 436 individuals registered with a Qatari sporting federation (such as sailing, archery, shooting, bowling) but exercising $<2\text{h}\cdot\text{wk}^{-1}$ were used as control population. There were 30 Asian, 242 Black African, 235 Caucasian, 491 from Gulf Cooperation Countries, 336 Middle Eastern, and 52 Persian participants. All individuals undertook bone densitometry and body composition analysis by dual-energy x-ray absorptiometry and serum 25[OH]D evaluation.

Results: From 950 athletes, 17.5% demonstrated severe deficiency, 39.2% demonstrated deficiency, 24.5% demonstrated insufficiency, and 18.8% demonstrated sufficiency, compared with 436 controls, 25.9% of whom demonstrated severe deficiency, 46.3% demonstrated deficiency, 19.0% demonstrated insufficiency, and 8.7% demonstrated sufficiency. No athlete presented with a T-score suggestive of osteoporosis (-2.5 SD) or osteopenia (-1.0 SD) at hip total. After adjustment for age, anthropometry, ethnicity, and athletic participation, there was no association between 25[OH]D and any BMD and T-score at any site within athletes. African and Caucasian athletes present with greater BMD and T-scores at the spine, neck, and hip total than those of Asian, Gulf Cooperation Countries, Middle Eastern, and Persian ethnicities. Athletes participating in high-impact sports present with higher measures of bone mass than control participants regardless of 25[OH]D status.

Conclusions: There is no association between 25[OH]D and BMD and T-score for any site within male athletes after adjusting for age, ethnicity, and sporting participation.

5.2 Introduction

Adequate levels of both calcium and 25[OH]D are required to maintain good bone health. 25[OH]D deficiency results in reduced absorption of dietary calcium by 85-90% in the small intestine. Current clinical ranges (M. Holick, 2007) of what constitutes 25[OH]D sufficiency [severely deficient (<10 ng/mL), deficient (10-20 ng/mL), insufficient (20-30 ng/mL) or sufficient (>30 ng/mL)] are based upon the association between 25[OH]D deficiency with osteomalacia and the approximate concentration at which parathyroid hormone (PTH) rises abruptly (M. F. Holick, 2009; Hollis, 2005; Zittermann, 2003); whereas the cut off for insufficiency is the approximate concentration in which calcium absorption is maximized (M. F. Holick, 2009). Whilst sufficient levels of serum 25[OH]D are important in optimising bone-mineral density (BMD) in active (J. Lappe et al., 2008; Ruohola et al., 2006) and inactive individuals (Bischoff-Ferrari et al., 2006), the exact 25[OH]D value to 'optimise' bone health for the regulation of calcium homeostasis (calciotropic properties) is still debated (M. F. Holick, 1996). Studies suggest that a 25[OH]D value >30ng/mL promotes good bone health and reduces fracture risk in both healthy young and older adults (Bischoff-Ferrari, Dietrich, Orav, & Dawson-Hughes, 2004; Bischoff-Ferrari et al., 2006), while some authors suggest levels>40ng/mL maybe required (Bischoff-Ferrari et al., 2006). Although studies have demonstrated a significant association between serum 25[OH]D and bone health (Collins et al., 1998; Gutierrez et al., 2011; Sadat-Ali, Al Elq, Al-Turki, Al-Mulhim, & Al-Ali, 2011), there are no studies examining the relationship between serum 25[OH]D concentration and measures of bone health in a large ethnically diverse athletic population. Athletes are a unique population, in that many are known to be severely 25[OH]D deficient (Constantini et al., 2010; Hamilton et al., 2010; Magee et al., 2013), but have the added stimulus of loading the

musculoskeletal system through high-intensity dynamic sporting activity resulting in greater bone mass at loaded sites compared with non-athletes (Nikander et al., 2006; Weidauer et al., 2014).

5.3 Aim and Hypothesis

A high prevalence of 25[OH]D insufficiency is observed in many athletic populations (range, 67%–91%) (Constantini et al., 2010) including athletes screened at Aspetar (Chapter 1, Figure 1.1). and negatively associated with bone mass. Chapter 4 highlights that vitamin D supplementation improves mean 25[OH]D status (13.7ng/mL to 25.3ng/mL) regardless of modality (IM or Oral). Correcting vitamin D insufficiencies is the duty of sports medicine physician, but what the impact of vitamin D status on and bone mass in an ethnically diverse population is unknown.

This study sought to examine the relation between serum 25[OH]D levels against markers of bone mass (BMD and T-scores) in a large and ethnically diverse athletic population.

5.4 Methods

5.4.1 Participants

Nine hundred and fifty male athletes registered with the Qatar Olympic Committee (QOC) Asian (n=18), Black African (n=218), Caucasian (n=206), GCC (n=222), Middle Eastern (n=251) and Persian (n=35) exercising ≥ 6 h/week presented for pre-competition medical assessment in our facility; with a further 436 individuals registered with the QOC but exercising < 2 h/week (such as sailing, archery, shooting and bowling) were used as control participants, Asian (n=12), Black African (n=24), Caucasian (n=29), GCC (n=269), Middle Eastern (n=85) and Persian (n=17).

Participants were screened for inclusion as described in the General Methods chapter of this thesis. After meeting this initial inclusion criteria, participants provided a venous blood sample, completed vitamin D questionnaire and undertook bone mineral density analysis as described in of the General Methods chapter of this thesis.

5.4.2 Laboratory analyses

As described in the general methodology chapter

5.4.3 Assessment of Bone Mineral Density

As described in the general methodology chapter

5.4.4 Statistics

Statistical analyses were performed as described in Chapter 3 with the additional calculations. Anthropometric comparisons between athletes and controls were performed using a Student's t-test. A one-way ANOVA was performed to assess anthropometric differences between the four 25[OH]D groups (<10 ng/mL, 10-20 ng/mL, 20-30 ng/mL and >30 ng/mL). A *post-hoc* analysis with bonferroni correction was used for further comparisons in the event of significance. To compare osteopenia and osteoporosis DXA scores between athletes and controls, non-parametric exact tests were performed since expected count in cells was <5. To determine the relationship of bone mass parameters with 25[OH]D, multiple linear regression analysis was performed with bone mass as a dependent variable with athletic participation as fixed factor of interest and age, body composition and ethnicity as covariates. Beta-coefficients ($\beta \pm SE$) were reported. A P-value <0.05 was used as a cut-off for statistical significance.

5.5 Results

5.5.1 25[OH]D status and anthropometry

From 950 athletes, 17.5% (n = 166) demonstrated severe deficiency, 39.2% (n = 372) demonstrated deficiency, 24.5% (n = 233) demonstrated insufficiency, and 18.8% (n = 179) demonstrated sufficiency, compared with 436 controls, 25.9% (n = 113) of whom demonstrated severe deficiency, 46.3% (n = 202) demonstrated deficiency, 19.0% (n = 83) demonstrated insufficiency, and 8.7% (n = 38) demonstrated sufficiency. Compared with control participants, athletes were significantly ($P < 0.003$) younger (24.3 ± 4.6 vs 25.9 ± 7.3 yr), taller (183.2 ± 10.6 vs 173.5 ± 6.6 cm), and heavier (81.2 ± 14.3 vs 74.1 ± 15.6 kg) and had reduced body mass index (24.0 ± 2.9 vs 24.6 ± 4.7 kg/m²), larger body surface area (2.0 ± 0.2 vs 1.9 ± 0.2 m²), lower percentile body fat ($16.3\% \pm 5.5\%$ vs $22.2\% \pm 8.4\%$), increased lean mass (64.5 ± 10.3 vs 54.5 ± 8.9 kg), and reduced fat mass (13.0 ± 6.5 vs 16.5 ± 9.6 kg). The majority of these anthropometric parameters remained significant when athletes and controls were compared in their respective 25[OH]D group (Table 5.1). In both athletes and controls, a significant association was observed between serum 25[OH]D and skin exposure ($p > 0.05$), sun exposure ($p < 0.002$) and ethnic origin ($p < 0.001$).

Table 5.1 Physical characteristics of athletes and controls based upon 25[OH]D status

Variables		Vitamin D category (ng/mL)			
		<10	10-20	20-30	>30
		(n=279)	(n=574)	(n=316)	(n=217)
Age (yr)	A	23.89±5.0 [15.7-38.0]	23.90±4.4* [18.0-36.0]	24.65±4.6* [16.9-38.0]	25.24±4.6 ^{ab} [16.9-39.0]
	C	24.19±5.8 [13.7-46.0]	25.84±7.6 [13.9-53.0]	28.17±8.1 ^a [18.0-57.0]	25.82±6.0 25.5[18.0-42.0]
Height (m)	A	1.80±1* [1.43-2.10]	1.82±1* [1.58-2.11]	1.84±1* ^a [1.62-2.16]	1.86±1* ^{ab} [1.57.0-2.14]
	C	1.73±0.6 [158.8-197.0]	1.73.07±6.5 [151.2-190.6]	174.44±7.3 [161.2-208.0]	173.70±6.0 [164.0-186.0]
Weight (kg)	A	77.85±15.3 [50.8-129.8]	80.11±14.9* [46.0-158.0]	82.81±14.1* ^a [52.8-156.1]	84.56±11.4* ^{ab} [56.0-113.3]
	C	75.51±20.0 [42.7-157.7]	74.09±14.9 [31.5-141.0]	74.15±12.1 [52.6-115.2]	69.56±9.2 [51.1-88.5]
Body mass index (kg/m²)	A	23.82±3.2* [17.0-37.6]	23.86±3.2* [15.4-53.8]	24.09±2.5 [17.7-33.1]	24.08±2.3 [18.0-29.5]
	C	25.05±5.9 [15.8-47.0]	24.69±4.7 [13.8-41.0]	24.28±3.1 [17.8-33.0]	22.93±2.4 [18.5-27.5]
Body Surface Area (m²)	A	1.96±0.2* [1.2-2.7]	2.01±0.2* [1.5-2.6]	2.06±0.2* ^{ab} [1.6-3.0]	2.09±0.2* ^{ab} [1.6-2.6]
	C	1.88±0.2 [1.4-2.7]	1.87±0.2 [1.2-2.6]	1.89±0.2 [1.6-2.5]	1.83±0.1 [1.6-2.1]
Tissue % Fat	A	17.18±6.1* [7.6-38.9]	16.40±5.9* [6.9-52.5]	16.05±5.1* [7.0-36.7]	15.64±4.7* [6.9-33.8]
	C	24.45±9.0 ^{cd} [10.2-49.7]	22.12±8.3 [9.0-46.2]	20.74±7.9 [5.7-39.4]	18.63±6.4 [9.2-38.1]
Lean Mass (kg)	A	61.06±11.3* [18.9-102.6]	63.49±10.3* [38.9-92.9]	65.99±9.7* ^{ab} [42.3-95.3]	67.81±8.4* ^{ab} [41.7-91.0]
	C	53.70±10.5 [34.7-99.2]	54.59±8.6 [23.7-90.8]	55.81±7.7 [41.7-77.1]	53.93±7.1 [41.3-71.4]
Fat mass (kg)	A	13.28±7.1* [5.2-46.2]	12.98±7.1* [4.3-81.3]	13.04±6.1* [4.6-55.3]	12.83±4.9 [4.5-35.9]
	C	18.90±12.0 ^{cd} [5.7-76.6]	16.49±9.2 [4.8-58.4]	15.24±7.6 [4.1-38.2]	12.60±5.2 [5.2-28.2]

A, athletes; C, controls. *significant difference between A vs. C, p<0.05. a: significantly greater than <10ng/mL, b: significantly greater than 10-20ng/mL, c: significantly greater than 20/30ng/mL and d: significantly greater than >30ng/mL

5.5.2 25[OH]D and markers of bone mass

Caucasian athletes demonstrated significantly greater ($p < 0.001$) serum 25[OH]D concentrations than all other ethnicities. Athletes of GCC origin presented significantly lower serum 25[OH]D values than Middle Eastern, African and Caucasian athletes. African and Caucasian athletes demonstrated significantly greater ($p < 0.05$) BMD and T-scores across all sites (spine, neck and hip) compared to Asian, GCC, Middle East and Persian athletes (Table 5.2).

Table 5.2 Bone mass parameters and serum 25[OH]D by ethnicity

	<i>Asian</i>	<i>GCC</i>	<i>Middle East</i>	<i>Persian</i>	<i>Africa</i>	<i>Caucasian</i>
Spine BMD	1.30±0.2* [1.0-1.7]	1.27±0.2* [0.7-1.7]	1.37±0.2* [0.8-1.8]	1.31±0.1* [1.0-1.5]	1.46±0.2 [0.9-2.0]	1.48±0.1 [1.1-1.9]
Neck BMD	1.29±0.2* [0.9-1.6]	1.24±0.2* [0.7-1.8]	1.34±0.2* [0.7-1.9]	1.35±0.2* [1.0-1.8]	1.46±0.2 [0.9-2.0]	1.42±0.2 [0.9-1.9]
Hip Total BMD	1.28±0.2* [0.9-1.6]	1.25±0.2* [0.4-1.8]	1.34±0.2* [0.7-1.9]	1.35±0.1* [1.1-1.6]	1.45±0.2 [1.0-1.9]	1.41±0.1 [1.0-1.8]
Spine T-score	0.55±1.4* [-1.8-3.3]	0.51±1.4* [-2.9-4.2]	1.28±1.3* [-3.7-5.0]	0.69±1.2* [-1.9-2.6]	2.03±1.3 [-2.7-6.3]	2.14±1.2 [-1.0-5.7]
Neck T-score	1.59±1.9* [-1.5-4.1]	1.32±1.5* [-2.5-5.3]	2.11±1.3* [-3.2-6.0]	2.02±1.1* [-0.2-4.3]	2.92±1.3 [-1.4-7.0]	2.72±1.2 [-1.0-5.9]
Hip T-score	1.11±1.5* [-1.4-3.3]	1.09±1.3* [-2.4-4.9]	1.66±1.1* [-3.1-5.2]	1.58±0.9* [-0.3-3.4]	2.38±1.1 [-0.7-5.5]	2.14±1.0 [-0.5-4.9]
Mean serum 25[OH]D	17.84±9.7 [6.4-55.6]	13.82±7.6‡ [4.0-53.0]	20.44±10.1 [4.0-74.9]	17.19±11.0 [4.0-54.2]	19.85±10.7 [4.0-62.7]	28.16±12.3† [5.7-87.4]

*Significantly lower compared with values for Africans and Caucasians.

†Significantly higher than Asians, GCC, Middle Easterns, Persians, and Africans.

‡Significantly lower than Middle Easterns, Africans, and Caucasians

Athletes had significantly greater ($p < 0.05$) bone mineral density (BMD) and T-scores across all sites (spine, neck and hip) compared to control participants. Athletes presenting with either 25[OH]D insufficiency (20-30 ng/mL) or sufficiency (>30 ng/mL) demonstrated significantly

greater ($p < 0.05$) BMD scores for spine, neck and total hip than severely deficient (< 10 ng/mL) athletes (Table 5.3). For control subjects, there were no significant differences in any BMD or T-scores across the four 25[OH]D categories.

Table 5.3 Bone mineral density and T-scores for athletes and controls based upon 25[OH]D status

<i>Variables</i>		<i>Vitamin D category (ng/mL)</i>			
		<i><10</i>	<i>10-20</i>	<i>20-30</i>	<i>>30</i>
Spine BMD	A	1.38±0.2* [1.0-1.7]	1.42±0.2* [0.8-1.8]	1.44±0.1* ^a [1.0-2.0]	1.46±0.1* ^{ab} [1.2-1.9]
	C	1.20±0.2 [0.7-1.6]	1.23±0.2 [0.7-1.7]	1.25±0.2 [0.8-1.6]	1.27±0.2 [0.9-1.5]
Neck BMD	A	1.37±0.2* [1.0-1.8]	1.40±0.2* [0.8-2.0]	1.43±0.2* ^a [1.0-2.0]	1.42±0.2* ^a [1.1-1.9]
	C	1.17±0.2 [0.8-1.6]	1.20±0.2 [0.8-1.8]	1.19±0.2 [0.7-1.8]	1.23±0.2 [0.9-1.7]
Hip Total BMD	A	1.36±0.2* [0.4-1.8]	1.40±0.2* ^a [0.8-1.9]	1.41±0.2* ^a [1.1-1.8]	1.41±0.1* ^a [1.1-1.8]
	C	1.17±0.2 [0.8-1.5]	1.21±0.2 [0.8-1.6]	1.20±0.2 [0.7-1.6]	1.24±0.2 [0.9-1.6]
Spine T-score	A	1.54±1.2* [-1.5-4.2]	1.78±1.2* [-1.3-5.2]	1.85±1.2* [-1.5-6.3]	1.93±1.1* ^a [-0.6-5.1]
	C	0.02±1.3 [-2.8-3.1]	0.17±1.3 [-2.9-3.8]	0.14±1.4 [-3.7-3.2]	0.45±1.4 [-2.9-2.5]
Neck T-score	A	2.44±1.2* [-0.4-5.5]	2.60±1.3* [-1.2-6.8]	2.75±1.2* [-0.3-7.0]	2.60±1.2* [0.1-5.7]
	C	0.82±1.4 [-2.3-3.7]	0.94±1.4 [-2.1-4.6]	0.90±1.4 [-3.2-5.2]	1.31±1.5 [-1.4-4.5]
Hip T-score	A	1.93±1.0* [-0.5-4.7]	2.14±1.1* [-0.8-5.5]	2.19±1.1* [-0.3-5.0]	2.09±0.9* [0.1-4.3]
	C	0.61±1.3 [-2.1-3.0]	0.72±1.1 [-1.8-3.8]	0.67±1.2 [-3.1-3.1]	1.12±1.4 [-1.6-3.4]

A, athletes; C, controls. *significant difference between A vs. C, $p < 0.05$. a: significantly greater than < 10 ng/mL and b: significantly greater than 10-20 ng/mL

No athlete had a T-score consistent with osteoporosis (-2.5 SD) at one of the three sites (neck, spine and hip), Osteopenia (-1 to -2.5 SD) was noted in 1 (0.1%) athlete at the neck and in 6 (0.8%)

athletes at the spine (Table 5.4). No athlete presented with a T-score suggestive of osteopenia at the hip.

Table 5.4 Prevalence of osteopenia and osteoporosis based upon T-scores for athletes and controls

<i>Variables</i>	<i>Status</i>	<i>Athletes</i>	<i>Controls</i>	<i>p-value</i>	<i>Athletes Mean 25[OH]D</i>	<i>Controls Mean 25[OH]D</i>
Neck T-score	Normal	750(99.9)	317(90.1)	<0.001	21.0±11.7	16.6±9.1
	Osteopenia	1(0.1)	33(9.4)		11.5±0.0	17.2±12.2
	Osteoporosis	0(0.0)	2(0.6)		0.0±0.0	23.5±1.3
Hip T-score	Normal	751(100.0)	323(91.5)	<0.001	21.0±11.7	16.7±9.1
	Osteopenia	0(0.0)	29(8.2)		-	17.0±12.6
	Osteoporosis	0(0.0)	1(0.3)		-	
Spine T-score	Normal	745(99.2)	283(80.6)	<0.001	21.0±11.7	17.0±9.6
	Osteopenia	6(0.8)	59(16.8)		13.9±6.3	15.0±7.6
	Osteoporosis	0(0.0)	9(2.6)		-	20.7±13.3

Control participants, however, presented with significantly ($p < 0.001$) lower ‘normal’ T-scores than athletes across all sites (neck, hip and spine). Furthermore, control participants presented a significantly ($p < 0.001$) higher prevalence of osteopenia at all sites compared to athletes. Although the prevalence of control participants who presented with T-scores consistent with osteoporosis was higher than the prevalence among athletes in all sites, the difference was not significant.

5.5.3 Indoor versus outdoor sports

Outdoor athletes presenting with severe 25[OH]D deficiency demonstrated significantly ($p < 0.05$) lower spine, neck and total hip BMD than insufficient (20-30 ng/mL) and sufficient (>30 ng/mL) outdoor athletes; with outdoor athletes presenting with severe 25[OH]D deficiency also demonstrating significantly ($p < 0.05$) lower neck and hip T-scores than sufficient (>30 ng/mL) outdoor athletes. For athletes who compete indoors, there was no significant difference in any

BMD or T-score at either the neck, spine of hip across all four 25[OH] D categories (Figure 5.1).

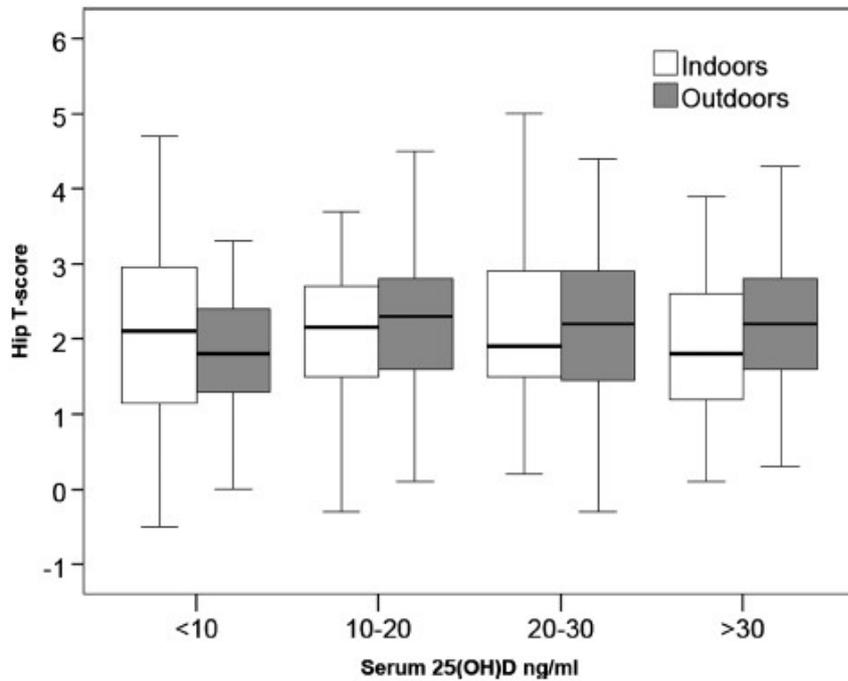


Figure 5.1 BMD and T-scores for indoor versus outdoor athletes based on 25[OH]D status

5.5.4 Logarithmic transformation

After logarithmic transformation adjusting 25[OH]D for age, anthropometry and ethnicity athletes had a significantly greater BMD and T-scores across all sites than control participants. However after further adjustment for athletic participation, there was no association between 25[OH]D and any BMD and T-score for any site within athletes.

5.6 Discussion

There is a high prevalence of 25[OH]D deficiency across many athletic populations, correcting these deficiencies may in turn improve athlete health (Close, Russell, et al., 2013; Constantini et

al., 2010; B. Hamilton et al., 2014) and we found 57% of athletes presenting with either 25[OH]D deficiency or severe deficiency. We observed, however, that despite this inadequate concentration of 25[OH]D, after adjusting 25[OH]D for age, anthropometry, ethnicity and athletic participation, there was no association between 25[OH]D and BMD and any T-score (spine, neck or hip). Control participants exercising less than 2 h / wk presented with significantly ($p < 0.001$) lower 'normal' T-scores than athletes across all sites (neck, hip and spine).

5.6.1 Effect of ethnicity on 25[OH]D and markers of bone mass

Our data demonstrate that across three key skeletal sites (spine, neck and hip), African and Caucasian athletes present with significantly greater ($p < 0.05$) bone mineral density (BMD) and T-scores than those of Asian, GCC, Middle East and Persian ethnicity. However, for the vast majority of athletes regardless of ethnicity, BMD and T-scores were within the normal clinical range set out by the World Health Organisation (Kanis, 2002). Caucasians present significantly greater mean serum 25[OH]D values compared to all other ethnicities, with participants originating from the GCC by contrast presenting the lowest mean serum 25[OH]D score.

Ethnicity is an important factor when considering serum 25[OH]D status and its potential impact upon BMD. Previous research has shown that among Caucasian adults, BMD significantly decreases ($p < 0.01$) as serum 25[OH]D declines but this is not observed in Black adults ($p = 0.2$) (Gutierrez et al., 2011). Darker skin pigmentation has a photo-protective effect (Matsuoka et al., 1991), reducing the capacity of the skin to synthesise vitamin D₃ (Clemens et al., 1982).

Vitamin D-binding protein provides insight as to why certain ethnic groups may have distinct 25[OH]D and BMD relationships (Powe et al., 2013) Vitamin D-binding protein is the primary

vitamin D carrier, binding 85-90% of total circulating 25[OH]D with the remaining non-binding 25[OH]D considered to be bioavailable (Bikle et al., 1986). Vitamin D-binding protein is therefore believed to inhibit certain actions of vitamin D because the bound fraction is unavailable to act on target cells. Polymorphisms in the vitamin D-binding protein however, produce proteins that differ in affinity for 25[OH]D, and it is these polymorphisms that are known to differ between ethnic groups (Engelman et al., 2012). Consequently, genotyping of common single-nucleotide polymorphisms in the coding region of the vitamin D-binding protein gene (rs4588 and rs7041) demonstrate variation between race and are in turn linked to vitamin D-binding protein function (Powe et al., 2013). Whilst controversy exists surrounding the role of vitamin D-binding protein and its effects on bioavailable 25[OH]D (Weintraub, 2014), our data demonstrates higher BMD and lower 25[OH]D scores in Black athletes compared to Caucasians, similar to result observed in the studies above (Powe, Karumanchi, & Thadhani, 2014).

5.6.2 Lifestyle

Numerous lifestyle factors contribute to 25[OH]D deficiency, including sunlight exposure, sun-block use, insufficient dietary vitamin D consumption, refraction and/or non-absorption of UVB light due to atmospheric dust particles, and the wearing of concealing clothing, particularly relevant in the Middle East (Fonseca, Tongia, el-Hazmi, & Abu-Aisha, 1984; Hamilton et al., 2010; Hatun et al., 2005). Our data oppose previous research by Hamilton et al. who observed no association between serum 25[OH]D concentrations in male athletes and use of sunscreen, sunlight exposure or skin exposure. Furthermore, there was no significant difference in mean serum 25[OH]D concentration between athletes that competed and trained indoors (volleyball, basketball and handball) and athletes that competed outdoors (football). This may be explained by the fact

that the majority of football training and competition in Qatar is performed after sunset due to several cultural, social and environmental factors.

5.6.3 Sports participation and bone loading

Exercise is associated with an increase in BMD (Nikander et al., 2006; Rantalainen et al., 2011). Physical loading contributes to the process of bone remodeling, and forms mechanically appropriate bone structure (Nikander et al., 2005). Indeed, numerous studies in adolescent female (Narra et al., 2013; Weidauer et al., 2014) and male athletes (Dias Quiterio et al., 2011; Silva, Goldberg, Teixeira, & Dalmas, 2011), adult athletes (Nikander et al., 2005; Nikander et al., 2006; Rantalainen et al., 2011) and senior athletes (Leigey et al., 2009) have all demonstrated the beneficial effect of load bearing physical activity upon bone mass. Athletes engaged in high-impact sports, present significantly greater total BMD scores than low-impact sports (Fredericson et al., 2007).

5.6.4 Future research

Sport type is an important contributing factor to high peak bone mass. Nikander et al., observed that following adjustment for anthropometry, the sport (or loading modality) was the determinant of the structure and strength of femoral neck; observing that in athletes competing in high-impact (basketball and volleyball) and odd-impact sports (football) demonstrated greater BMD scores than low impact sports such as swimming and cycling (Nikander et al., 2005). Although unlike the current study, these studies did not factor serum 25[OH]D status or other lifestyle factors such as UVB exposure into their analysis.

We found that 25[OH]D insufficient or sufficient athletes present significantly greater ($p < 0.05$) BMD scores than severely deficient athletes. However, after adjusting 25[OH]D for age, anthropometry, ethnicity and athletic participation, there was no association between 25[OH]D and BMD, and any T-score (spine, neck or hip). The fact that no athlete presented a T-score suggestive of osteoporosis despite a prevalence rate of 57% for 25[OH]D deficiency and severe deficiency, suggests that in this cohort, the osteogenic effect of impactful weight-bearing exercise is sufficient to maintain markers of bone mass, irrespective of 25[OH]D status in adults.

5.6.5 Indoor versus outdoor athletes

All athletes recruited in the present study competed in weight bearing sports (football, basketball, volleyball and handball), yet an association between serum 25[OH]D and bone mass (BMD and T-scores) was only observed for the outdoor and not the indoor athlete. Sports such as basketball, volleyball and handball appear to produce more site specific osteogenic effects with greater BMD scores compared to ‘odd-impact’ sports such as football (Fredericson et al., 2007). The rapid accelerating and decelerating movements associated with football, are often in directions that the body and hip region are not accustomed to, therefore eliciting a different effect on bone remodeling (Nikander et al., 2005).

5.6.6 Do athletes require 25[OH]D supplementation?

This study demonstrates that adjusting 25[OH]D for age, anthropometry, ethnicity and athletic participation, there was no association between 25[OH]D and BMD and any T-score; and thus questions the notion that sports medicine physicians should be supplementing athletes insufficient

in 25[OH]D on the basis of bone mass. Recent studies have suggested that correcting serum 25[OH]D in deficient individuals may in turn improve athletic performance (Close, Russell, et al., 2013); however this is universally supported (B. Hamilton et al., 2014). Ethical rules dictate that the physician should 'do no harm', yet several ethical and methodological issues regarding 25[OH]D supplementation remain unanswered. For athletes, these may include potential supplement contamination, the effects of toxicity such as hypercalcemia and what exactly is an 'optimal' 25[OH]D status.

5.7 Limitations

Whilst we acknowledge that training volume and intensity were not recorded; athletes were only included in the study if they were registered with the Qatar Olympic Committee, competed at national or international level and trained for more than 6 h / week. We also acknowledge that serum 25[OH]D measurement only offered a snapshot of current status. Thus it is possible for severely deficient (<10 ng/mL) and deficient (10-20 ng/mL) athletes in the present study to have spent many years sufficient (>30 ng/mL) prior to recruitment.

5.8 Conclusion

In summary, our data questions 25[OH]D supplementation programs for 25[OH]D deficient athletes purely on the basis of bone mass. Despite 57% presenting with either 25[OH]D deficiency or severe deficiency, after adjusting 25[OH]D for age, anthropometry, ethnicity and athletic participation, there was no association between 25[OH]D and BMD and any T-score (spine, neck or hip). We conclude that no athlete presented a T-score indicative of osteoporosis (-2.5 SD) in the

neck, spine or hip, or a T-score indicative of osteopenia (-1 to -2.5 SD) in the hip. These data suggest that the osteogenic effect of impactful weight-bearing exercise is sufficient to maintain markers of bone mass, irrespective of 25[OH]D status in adults. Finally, African and Caucasian athletes present with significantly greater ($p<0.05$) bone mineral density (BMD) and T-scores across all sites (spine, neck and hip) than those of Asian, GCC, Middle East and Persian ethnicity. Sports medicine physicians working with athletes who are presented with a 25[OH]D deficient athlete must factor in ethnicity, as athletes of Black African origin are known to present with lower serum 25[OH]D concentrations, alongside other factors such as diet and UVB exposure, in the decision to supplement that athlete.

Chapter 6:

No Association between Vitamin D Status and Markers of Bone Mass in Non-Weight Bearing Athletes

6.1 Abstract

Objectives: To examine the relationship between serum 25[OH]D concentrations and markers of bone mass [bone mineral density (BMD) & T-score] in non-weight bearing, Arabic athletes.

Design: One hundred and two male athletes registered with the Qatar Olympic Committee in a non-weight bearing sport and originating from the Gulf Cooperation Council (GCC) (n=93) and Middle East (n=9), presented for pre-competition medical assessment in Aspetar.

Methods: All participants undertook bone densitometry (neck, hip, spine and total), body composition via Dual-energy X-ray absorptiometry and serum 25[OH]D evaluation.

Results: From 102 athletes, 29.4% (n=30) demonstrated severe vitamin D deficiency (<10ng/mL), 40.2% (n=41) deficiency [10-20ng/mL], 23.57% (n=24) insufficiency [20-30ng/ml] and 6.8% (n=7) sufficiency [$>30\text{ng/mL}$]. No difference in serum 25[OH]D concentrations were observed between athletes with clinically normal T-score (n=76) and those presenting with osteopenia (-1 to -2.5 SD) (n=20) or osteoporosis (-2.5 SD) (n=6) in any location (neck, hip, spine or total). There were no associations observed between serum 25[OH]D concentrations and age, body mass index, body fat %, calcium, parathyroid hormone and skin exposure (surface area and time) to sunlight. Cyclists had higher serum 25[OH]D concentrations compared with indoor athletes ($P\leq 0.006$). Mean body mass was lower ($p<0.05$) in athletes with a T-score suggestive of osteopenia/osteoporosis.

Conclusions: No association was observed between serum 25[OH]D concentrations and markers of bone mass in non-weight bearing Arabic athletes. Lower body weight was associated with osteopenia and/or osteoporosis regardless of serum 25[OH]D status. This study suggests 25[OH]D concentration is inappropriate in predicting bone mass in non-weight bearing athletes.

6.2 Introduction

Exercise (loading) is associated with an increase in BMD (Nikander et al., 2006), whilst increased body mass contributes to the process of bone remodeling, forming mechanically appropriate bone structure(s) (Nikander et al., 2005). Studies show that exercise and sporting type (or weight modality) effect BMD; with athletes engaging in high-impact sports (football, basketball, and handball) presenting with significantly greater total BMD scores than low-impact sports (swimming and cycling) (Fredericson et al., 2007). Although numerous studies have demonstrated the impact of weight bearing exercise in a variety of populations [adolescent females (Narra et al., 2013), male athletes (Dias Quiterio et al., 2011) and senior athletes (Leigey et al., 2009)], no studies have examined the relationship between serum 25[OH]D and measures of bone mass in non-weight bearing athletes. Further, this concept has not been studied in Arabic athletes, a population known to have a high prevalence of severe deficiency (Hamilton et al., 2010; B. Hamilton et al., 2014).

Vitamin D deficiency is endemic across the GCC and Middle-East, given that 90% of Arabic athletes are 25[OH]D insufficient (Hamilton et al., 2010). It appears that the stimulus of loading the musculoskeletal system through high-intensity dynamic sporting activity has an osteogenic effect, regardless of serum 25[OH]D status (Nikander et al., 2006). However, popular sports in the region include archery, billiards bowling, cycling, diving, equestrian, racing, rowing, sailing, shooting, snooker, swimming and water polo; all of which are non-dynamic, non-weight bearing sports. The findings from Chapter 5 demonstrate no association between serum 25[OH]D concentrations and BMD or T-score within athletes regardless of 25[OH]D statuses, even after adjusting for age, ethnicity and sporting participation (Allison, Farooq, Hamilton, Close, &

Wilson, 2015). We postulated that the osteogenic effect of weight bearing exercise may be sufficient to maintain markers of bone mass, irrespective of 25[OH]D status in adults.

6.3 Aims and Hypotheses

Accordingly, the study described in Chapter 6 aimed to examine the relationship between serum 25[OH]D concentrations against markers of bone mass (BMD and T-score) and anthropometrical measures in an Arabic athletic population participating in non-weight bearing sports. We hypothesize that 25[OH]D concentrations were not associated with markers of bone mass (BMD) in non-weight bearing Arabic athletes.

6.4 Methods

6.4.1 Participants

One hundred and two male athletes registered with the Qatar Olympic Committee (QOC) in a non-weight bearing sport (archery, billiards bowling, cycling, diving, equestrian, motor-racing, rowing, sailing, shooting, snooker, swimming and water polo) and originating from the Gulf Cooperation Council (n=93) and Middle East (n=9), presented for pre-competition medical assessment in Aspetar.

Participants were screened for inclusion as described in the General Methods chapter of this thesis. After meeting this initial inclusion criteria, participants provided a venous blood sample, completed vitamin D questionnaire and undertook bone mineral density analysis as in of the General Methods chapter of this thesis.

6.4.2 Laboratory analyses

Venous blood samples were collected from athletes following an overnight fast and analysed for serum 25-hydroxyvitamin D (25[OH]D) as described in section 3.4 of General Methods Chapter.

6.4.3 Assessment of Bone Mineral Density

Dual-energy x-ray absorptiometry (DXA; Osteocore III, Perols, France, version 5.22b) scanning was used to assess hip and spine BMD. T-scores were calculated for those athletes older than 20 years. Twenty-three participants under 20 years were not included in the sub-analysis for (T-score) osteoporosis and osteopenia.

6.4.4 Statistical analysis

Statistical analyses were performed as described in Chapter 5.

6.5 Results

6.5.1 25[OH]D status and anthropometry

From 102 athletes, 29.4% (n=30) demonstrated severe vitamin D deficiency, 40.2% (n=41) deficiency, 23.6% (n=24) insufficiency and 6.8% (n=7) sufficiency. No significant associations were observed between anthropometrical measures (BMI, body surface area, height and weight) and serum 25[OH]D, except for body mass index ($p=0.046$) and body fat (%) ($p=0.022$). Post-hoc analysis however, revealed no significant differences in anthropometric measures between the four different 25(OHD) status categories (Table 6.1).

Table 6.1 Physical characteristics based on 25[OH]D status (Mean±SD)

<i>Variables</i>	<i>Vitamin D category (ng/mL)</i>				p-value
	<10	10-20	20-30	>30	
	n=30	n=41	n=24	n=7	
Age (Yr)	24.1±6.7	30.0±9.8	30.0±10.3	27.4±8.9	0.056
Height (m)	1.72±6.6	1.72±5.8	1.76±6.4	1.72±4.2	0.079
Weight (kg)	74.0±23.1	82.2±18.0	77.9±14.9	67.7±8.6	0.139
Body mass index (kg/m²)	24.9±7.1	27.7±5.6	24.9±3.6	22.9±2.8	0.046
Body Surface Area (m²)	1.9±0.2	1.9±0.2	1.9±0.2	1.8±0.1	0.124
Body fat (%)	27.3±9.8	30.5±8.9	25.0±8.2	21.1±8.0	0.022
Lean mass (kg)	50.1±8.6	54.0±7.8	55.5±8.8	51.1±5.8	0.073
Fat mass (kg)	21.3±15.5	25.3±11.8	19.4±8.9	14.1±6.8	0.074

*P-values based on One way ANOVA, † P-value based on Kruskal Wallis test.

6.5.2 25[OH]D and markers of bone mass

Cyclists and rowers had the greatest serum 25[OH]D concentrations, with sporting type significantly associated with serum 25[OH]D status. Cyclists had greater serum 25[OH]D compared to equestrian, and all other indoor sport athletes ($p \leq 0.006$; Figure 6.1). No significant associations were observed between serum 25[OH]D and skin exposure area ($p=0.133$), but increased sun exposure (minutes) was associated with higher serum 25[OH]D concentration ($p < 0.001$). Post-hoc pairwise comparisons revealed significantly higher serum 25[OH]D concentration only in those athletes exposed to ≥ 120 minutes per day, compared to <30 sunlight or less <60 minutes per day ($p < 0.004$).

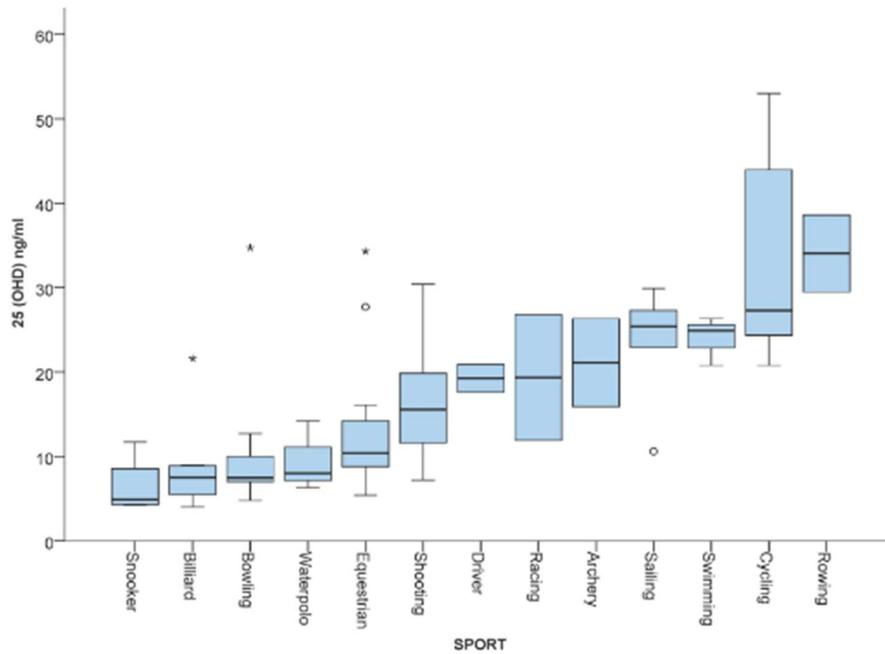


Figure 6.1 Serum 25[OH]D by sport

There were no significant differences in T-score or total BMD between any of the four 25[OH]D categories. However, athletes presenting with 25[OH]D insufficiency (20-30ng/mL) demonstrated greater spine BMD than sufficient athletes (>30 ng/mL; p=0.029; Table 6.2).

Table 6.2 Bone mineral density and T-score‡ based upon 25(OH)D status

<i>Variables</i>	<i>Vitamin D category (ng/mL)</i>				<i>p-value</i>
	<i><10</i>	<i>10-20</i>	<i>20-30</i>	<i>>30</i>	
	<i>n=30</i>	<i>n=41</i>	<i>n=24</i>	<i>n=7</i>	
Spine BMD	1.1±0.1	1.1±0.1	1.2±0.2*	1.0±0.1	0.017
Neck BMD	1.1±0.1	1.0±0.2	1.1±0.2	1.0±0.1	0.725
Hip Total BMD	1.1±0.2	1.1±0.1	1.1±0.2	1.0±0.1	0.386
Total BMD	1.1±0.1	1.2±0.1	1.2±0.1	1.1±0.1	0.030
Neck T-score	-0.3±1.1	-0.3±1.1	-0.3±1.4	-0.8±0.8	0.805
Hip T-score	-0.2±1.2	-0.3±0.9	-0.4±1.4	-1.1±0.5	0.419
Total T-score	-0.7±0.8	-0.3±1.0	-0.5±1.4	-1.2±0.4	0.254
Spine T-score	-0.8±0.9	-0.6±1.0	-0.5±1.3	-1.8±1.0	0.121
Total BMC	2659.0±359.7	2936.4±402.3†	2979.8±516.2†	2564.9±190.9	0.004

‡ T-score was not available for 23 athletes less than age 20. * Significantly higher than >30 Serum 25(OH)D group; † Significantly higher than <10 and >30 Serum 25(OH)D group

Overall, there were no differences between the prevalence of osteoporosis and osteopenia at neck, hip or total in any of the four 25(OH)D categories (Table 6.3).

Table 6.3 Prevalence of osteopenia and osteoporosis based upon 25[OH]D status (Mean±SD)

<i>Variables</i>	<i>Vitamin D category (ng/mL)</i>				p-value	
	<10 n=20	10-20 n=33	20-30 n=20	>30 n=5		
Neck T-score	Normal	14(70.0)	23(69.7)	14(70.0)	2(40.0)	0.22
	Osteopenia	6(30.0)	10(30.3)	4(20.0)	3(60.0)	
	Osteoporosis	0(0.0)	0(0.0)	2(10.0)	0(0.0)	
Hip T-score	Normal	15(75.0)	26(78.8)	14(70.0)	1(20.0)	0.103
	Osteopenia	5(25.0)	7(21.2)	5(25.0)	4(80.0)	
	Osteoporosis	0(0.0)	0(0.0)	1(5.0)	0(0.0)	
Spine T-score	Normal	12(60.0)	24(72.7)	14(70.0)	2(40.0)	0.163
	Osteopenia	7(35.0)	7(21.2)	5(25.0)	1(20.0)	
	Osteoporosis	1(5.0)	2(6.1)	1(5.0)	2(40.0)	
Total T-score	Normal	13(65.0)	24(72.7)	13(65.0)	1(20.0)	0.294
	Osteopenia	6(30.0)	9(27.3)	6(30.0)	4(80.0)	
	Osteoporosis	1(5.0)	0(0.0)	1(5.0)	0(0.0)	

No difference in serum 25[OH]D was observed between athletes with normal T-score and those presenting with osteopenia or osteoporosis (overall T-score<1.0) in any location (neck, hip, total) (p=0.383). Furthermore, 66% of severely deficient (<10ng/mL) and deficient (20-10ng/mL) athletes presented with T-score indicative of normal bone density (+1 to -1 SD) at neck, hip, spine and total. Finally, mean body mass was significantly lower in athletes with a T-score indicating osteopenia/osteoporosis compared to those athletes with a normal T-score (70.9kg vs. 87.3kg, p<0.05; Table 6.4).

Table 6.4 Prevalence of osteopenia and osteoporosis based upon 25[OH]D, body weight and body fat percentage(Mean±SD)

<i>Variables</i>		<i>25 [OH]D</i>	<i>Body weight (kg)</i>	<i>Body fat (%)</i>
Neck T-score	Normal	15.8±7.4	84.1±20.1*	30.4±8.2
	Osteopenia	18.3±13.3	68.8±12.4	26.2±7.2
	Osteoporosis	23.5±1.3	68.6±6.4	24.3±4.5
Hip T-score	Normal	15.4±7.2	84.3±19.6*	23.9±6.3
	Osteopenia	19.9±13.7	65.7±9.6	27.5±0.0
	Osteoporosis	22.6±0.0	73.1±0.0	29.6±8.9
Spine T-score	Normal	16.7±8.7	83.5±20.8*	27.3±5.8
	Osteopenia	14.8±9.0	70.1±12.2	28.7±7.3
	Osteoporosis	23.6±15.0	71.9±10.4	23.9±6.3

6.6 Discussion

6.6.1 Sports participation and serum 25[OH]D

The present study hypothesised that 25[OH]D concentration was not associated with BMD in non-weight bearing Arabic athletes. Indeed, we observed no association between serum 25[OH]D concentration and BMD, but did observe that a lower body mass was associated with T-score suggestive of osteopenia or osteoporosis.

In the present study, 93% athletes presented with 25[OH]D insufficiency with 29.4% (n=30) demonstrating severe vitamin D deficiency. The majority of 25[OH]D is generated de novo by the basal layers of the epidermis via ultraviolet-B radiation. Athletes who train and compete outdoors would be expected to present with sufficient vitamin D concentrations. Our data supports this notion, with athletes competing in cycling and rowing (outdoor sports) presenting with higher 25[OH]D concentration than those athletes competing indoors ($p \leq 0.006$). Our group previously reported no difference in mean serum 25[OH]D between athletes that competed and trained indoors (volleyball, basketball, and handball) and athletes that competed outdoors (football). This

may be explained by the fact that the majority of football training and competition in Qatar is performed after sunset, whereas rowing and cycling are performed during daylight hours due to safety reasons. Furthermore, irrespective of sporting type, athletes that reported greater daily sunlight exposure >120minutes per day presented greater serum 25[OH]D concentrations than athletes <60 minutes per day ($p \leq 0.004$). This contradicts research by Hamilton et al. 2010 who observed no association between serum 25[OH]D concentrations in male athletes and sunlight exposure, but conversely supports our previous research (Allison, Close, et al., 2015; Allison, Farooq, et al., 2015).

6.6.2 25[OH]D and markers of bone mass

Despite a combined 25[OH]D deficiency and severe deficiency prevalence rate of 69%, there were no significant differences in T-score at any site (neck, hip, spine or total) across all four 25[OH]D categories ($p < 0.05$); suggesting that for this cohort, markers of bone mass are independent of serum 25[OH]D status. Indeed, athletes presenting with 25[OH]D insufficiency (20-30 ng/mL) demonstrated greater spine BMD than sufficient athletes (>30 ng/mL) ($p = 0.029$; Table 6.2).

6.6.3 Bone Loading

Mechanical loading from athletic training or competition is associated with higher BMD even when adjusting for weight. Nikander et al., 2005 identified weight modality as a determinant of the structure and strength of femoral neck; observing that athletes competing in high-impact (basketball and volleyball) and odd-impact sports (football) demonstrated greater BMD scores than low impact sports such as swimming and cycling. However, non-weight bearing athletes forgo the same skeletal loads that produce greater BMD scores (Fredericson et al., 2007) and are at

higher risk for low BMD (Guillaume et al., 2012; Smathers et al., 2009). One third of athletes in our cohort presented with a T-score indicative of osteopenia (-1 to -2.5 SD) or osteoporosis (-2.5 SD), in at least one site (neck, hip, spine and total). However, this observation was independent of serum 25[OH]D concentration, suggesting that the osteogenic effect of weight-bearing exercise is more essential for good bone mass in athletes than 25[OH]D sufficiency. Furthermore, athletes with normal T-score presented with significantly greater mean body masses than athletes with T-score indicative of osteopenia and/or osteoporosis (87.3kg vs. 70.9kg, respectively). Our data demonstrate that even in non-weight bearing athletes, a greater body mass contributes to a healthier BMD.

6.6.4 Calcium and PTH

Vitamin D and calcium are regarded as fundamental in bone mass, however observational studies show inconsistent associations between BMD and 25[OH]D status (Bischoff-Ferrari, Kiel, et al., 2009), with optimum concentrations of serum 25[OH]D for optimal skeletal health greatly debated. According to the US Institute of Medicine, a serum 25[OH]D concentration of 20ng/mL is sufficient to maintain normal bone mass in approximately 97% of the population (Ross et al., 2011). Yet 66% of athletes in this study had serum 25[OH]D concentrations below this value, but presented had T-score indicative of normal bone density (+1 to -1 SD) across all sites. Our data confirm that poor bone mass is not mediated by serum 25[OH]D deficiency alone. Bone is a dynamic tissue that undergoes continual adaptations to different physiological mechanical stimuli, in order to attain and persevere skeletal size, shape and structural integrity (Raggatt & Partridge, 2010). Although the positive link between weight bearing exercise (or loading) and bone mass

have been established, there are still multiple mechanisms that regulate mechanosensation in bone are inextricably interdependent.

The known beneficial effects of 25[OH]D on bone health are potentiated by the presence of adequate dietary calcium intake (Tenforde, Sayres, Sainani, & Fredericson, 2010). It is difficult however, to distinguish between the effects of calcium versus those of vitamin D on skeletal integrity, because the main mechanism of action for vitamin D is promotion of calcium absorption in the gut and not direct incorporation of calcium in bone (Xue & Fleet, 2009). Calcium homeostasis maintains serum calcium levels within a narrow physiological range. Hypocalcaemia increases PTH secretion, that enhances the production of the active form of vitamin D, 1,25[OH]₂D₃. The role of vitamin D as a regulator of calcium and bone homeostasis is well established. 1,25[OH]₂D₃ regulates intestinal calcium absorption, bone calcium resorption and renal calcium reabsorption, to maintain normal calcium levels in the blood (Lieben & Carmeliet, 2013). Essentially, 1,25[OH]₂D₃ will mobilise calcium from bone, when unable to acquire sufficient calcium from dietary absorption.

In a study where specific VDR inactivation was induced in both the intestine and in mature osteoblasts in order to interfere with calcium and bone homeostasis, resulting in decreased intestinal calcium absorption. Increased 1,25[OH]₂D levels not only stimulated bone turnover, leading to osteopenia, but also suppressed bone matrix mineralization. These findings indicate that maintaining normocalcaemia is a greater priority than skeletal integrity, and that to minimise skeletal calcium storage, 1,25[OH]₂D not only increases calcium release from bone but also inhibits calcium incorporation within bone (Lieben et al., 2012). In the present study, all athletes

presented with normal serum calcium and PTH, confirming that role of 25[OH]D in bone adaptation in healthy, weight bearing active people is inconsequential.

6.6.5 Energy availability

The association between lower body mass and markers of poor bone mass may be attributed to the osteogenic effects on weight. Yet, sports such as cycling (Vogt et al., 2005) and rowing where athletes routinely restrict energy intake for competition needs, may have a negative impact upon bone mass. Low energy availability is defined as dietary energy intake minus exercise energy expenditure, and is the amount of dietary energy remaining for other body functions (Loucks, 2004). Low energy availability affects bone mass due to decreased IgF-1 and bone formation markers. Historically, low energy availability and poor bone mass was considered to be a component of the female athlete triad. However, it has also been reported to occur in male athletes, hence guidelines have been recently updated (Mountjoy et al., 2014). Whilst nutritional history was not assessed in the current study (and therefore energy intake could not be calculated), dietary insufficiencies increase the risk of stress fractures in both sexes (McCabe, Smyth, & Richardson, 2012).

6.6.6 Lifestyle

Numerous lifestyle factors contribute to 25[OH]D deficiency, including lack of sunlight exposure, sun-block use, insufficient dietary vitamin D consumption, refraction and/or non-absorption of UVB light due to atmospheric dust particles, and particularly relevant in the Middle East, the wearing of concealing clothing for cultural reasons (Hatun et al., 2005). Our data opposes previous

research by Hamilton et al.(Hamilton et al., 2010) who demonstrated no association between serum 25[OH]D concentrations in male athletes and use of sunscreen, sunlight exposure or skin exposure.

6.6.7 Do athletes require 25[OH]D supplementation?

Should non-weight bearing athletes take 25[OH]D supplementation or load their bones? We have previously demonstrated no association between 25[OH]D and measures of bone mass (BMD and T-score) in weight bearing athletes (Allison, Farooq, et al., 2015). This study further supports that serum 25[OH]D levels are not associated with BMD and T-score in male Arabic non-weight bearing athletes. In the current study, none of the athletes were taking vitamin D supplementation at the time of screening. The impact of vitamin supplementation on athletic performance is still debated, with recent evidence reporting that vitamin D deficiencies may actually impair muscle regeneration (D. J. Owens et al., 2015), whilst others have shown that correction of serum 25[OH]D in deficient individuals may improve athletic performance. Emerging work from Close et al.(Close, Hamilton, Philp, Burke, & Morton, 2016) suggests that in non-deficient individuals, high dose supplementation may have negative consequences related to the function of the vitamin D endocrine system, and that oral doses should not exceed 4000IU/d. In conclusion, blanket vitamin D supplementation for athletes without specific risk factors should be considered questionable. Male athletes in non-weight bearing sports such as cycling are at high risk for low BMD (Guillaume et al., 2012; Smathers et al., 2009). Steps to improve overall bone health in these cohorts should include, 1) avoiding unnecessary periods of low energy availability and 2) where possible, incorporate loading of the musculoskeletal system via strength training (Nikander et al., 2006). Programs of high-impact loading and resistance training should be implemented at least 2–

3 days/week for athletes in non-weight bearing sports and/or those with decreased BMD (Nattiv et al., 2007). Finally, reassess BMD via DXA every 12 months to ensure that long term bone mass is not being compromised by the athletes chosen sporting career.

6.7 Limitations

Whilst we acknowledge that training volume and intensity were not recorded; athletes were only included in the study if they were registered with the QOC, competed at national or international level and trained or competed for more than 6 h / week in their chosen sport. We also acknowledge that serum 25[OH]D measurement only offered a snapshot of current status. Thus, it is possible for severely deficient (<10 ng/mL) and deficient (10-20 ng/mL) athletes in the present study to have spent many years sufficient (>30 ng/mL) prior to recruitment. Finally, as previously mentioned, is the absence of robust diet history it is impossible to assess the athlete's overall energy and dietary calcium intake.

6.8 Conclusion

This study demonstrated that in non-weight bearing Arabic athletes, there was no association between serum 25[OH]D concentrations and markers of bone mass (BMD and T-score), but that lower body mass was associated with osteopenia or osteoporosis regardless of serum 25[OH]D concentration. One third of athletes presented with a T-score indicative of osteopenia (-1 to -2.5 SD) or osteoporosis (-2.5 SD), in at least one site (neck, hip, spine and total). This was independent of serum 25[OH]D concentration, suggesting that the osteogenic effect of weight-bearing exercise is more essential for good bone mass in athletes than 25[OH]D sufficiency.

6.9 Practical implications

- Avoid unnecessary periods of low energy availability, rate of bone mineralisation declines as energy availability declines
- Incorporate loading of the musculoskeletal system via strength training at least 2–3 days/week for athletes in non-weight bearing sports and/or those with decreased BMD.
- Reassess BMD via DXA every 12 months to ensure that long term bone mass

Chapter 7:

Why don't serum Vitamin D concentrations associate with BMD by DXA? A case of being 'bound' to the wrong assay? Implications for Vitamin D screening

*Publications resulted from this chapter; Allison, R. J., A. Farooq, Cherif, A., B. Hamilton, G. L. Close, and M. G. Wilson. Why don't serum vitamin D concentrations associate with BMD by DXA? A case of being 'bound' to the wrong assay? Implications for vitamin D screening. *Br J Sports Med* Published Online First: 10 August 2017. doi: 10.1136/bjsports-2016-097130*

7.1 Abstract

Background: The association between bone mineral density (BMD) and serum 25[OH]D concentration is weak, particularly in certain races (e.g. Black African vs. Caucasian) and in athletic populations. We aimed to examine if bioavailable vitamin D rather than serum 25[OH]D was related to markers of bone mass within a racially diverse athletic population.

Methods: In 604 male athletes [Arab (n=327), Asian (n=48), Black (n=108), Caucasian (n=53) & Hispanic (n=68)], we measured total 25-hydroxyvitamin D (25[OH]D), vitamin D-binding protein, and bone mineral density (BMD) by (DXA). Bioavailable vitamin D was calculated using the free hormone hypothesis.

Results: From 604 athletes, 21.5% (n=130) demonstrated severe 25[OH]D deficiency, 37.1% (n=224) deficiency, 26% (n=157) insufficiency and 15.4% (n=93) sufficiency. Serum 25[OH]D concentrations were not associated with BMD at any site. After adjusting for age and race, bioavailable vitamin D was associated with BMD (spine, neck and hip). Mean serum vitamin D binding protein concentration was not associated with 25[OH]D concentrations (p=0.392).

Conclusion: Regardless of age or race, bioavailable vitamin D and not serum 25[OH]D was associated with BMD in a racially diverse athletic population. If vitamin D screening is warranted, clinicians should use appropriate assays to calculate vitamin D binding protein and bioavailable vitamin D concentration rather than serum 25[OH]D. In turn, prophylactic vitamin D supplementation to 'correct' insufficient athletes should not be based upon serum 25[OH]D measures.

7.2 Introduction

Vitamin D₃ (cholecalciferol) is a lipophilic pro-hormone produced in the skin from exposure to sunlight. Cholecalciferol is transported bound to vitamin D-binding protein (DBP), and is hydroxylated in the liver to form 25-hydroxyvitamin D (25[OH]D). 25[OH]D undergoes further hydroxylation in the kidney, to form the active hormone 1, 25-dihydroxyvitamin D [1,25(OH)₂D]. This bioactive metabolite, regulates intestinal calcium absorption, bone calcium resorption and renal calcium reabsorption in order to maintain calcium homeostasis, and promote skeletal mineralisation (M. F. Holick, 1996; Lieben & Carmeliet, 2013). Accordingly, skeletal pathologies such as rickets and osteomalacia often present when 25[OH]D levels are consistently deficient (B. Hamilton, 2010).

Clinically to date, measuring serum 25[OH]D concentration provides the best estimate of vitamin D status as both circulating levels of cholecalciferol and 1,25(OH)₂D have short half-lives, approximately 24hrs and 4-6hrs, respectively (M. F. Holick, 1990; Iqbal, 1994). Serum cholecalciferol levels can also be affected by recent sunlight exposure, and is difficult to measure due to the lipophilic nature of the molecule. Optimum concentrations of serum 25[OH]D for skeletal health are still debated (7). Many clinicians define vitamin D sufficiency as the lowest serum 25[OH]D concentration that maximally suppresses parathyroid hormone (PTH) secretion and/or optimises bone mineral density (BMD) (Bischoff-Ferrari et al., 2006; Chapuy et al., 1997; Malabanan et al., 1998). The generally accepted serum 25[OH]D concentration ranges are: severely deficient (<10 ng/mL), deficient (10–20 ng/mL), insufficient (20–30 ng/mL), or sufficient (>30 ng/mL); although the vast majority of evidence supporting these categories are derived from elderly cohorts or groups with existing skeletal disorders (Bischoff-Ferrari et al., 2005; Cauley et al., 2008; B. Dawson-Hughes et al., 1997; Vanderschueren et al., 2013).

Yet, the association between bone mineral density (BMD) and serum 25[OH]D concentration is weak (Bischoff-Ferrari, Kiel, et al., 2009; Gerdhem et al., 2005; Hannan et al., 2008; Kremer et al., 2009; Marwaha et al., 2011). Our group previously demonstrated no association between serum 25[OH]D and markers of bone mass in weight bearing athletes of different racial background, suggesting that markers of bone mass are independent of serum 25[OH]D concentrations (Allison, Close, et al., 2015). It appears there is a ‘paradoxical relationship’ between race and vitamin D concentration, that has largely been ignored, i.e. black individuals generally have the lowest serum 25[OH]D concentrations but the greatest BMD and reduced risk of fracture (Cauley et al., 2005; Hannan et al., 2008). DBP may provide an insight as to why certain racial groups have distinct 25[OH]D and BMD relationships (Powe et al., 2013). DBP is the primary vitamin D carrier, binding 85%–90% of total circulating 25[OH]D, with the remaining unbound 25[OH]D considered to be bioavailable (Bikle et al., 1986). Polymorphisms in the DBP coding genes (specifically rs4588 and rs7041) produce proteins that differ in affinity for 25[OH]D, and it is these polymorphisms that are known to differ between racial groups (Engelman et al., 2008). Differentiating between total vitamin D (measured as 25[OH]D) and bioavailable vitamin D is crucial, given that the latest evidence suggests that DBP inhibits certain actions of vitamin D, since the bound fraction is unavailable to act on target cells. (Bikle et al., 1986; Powe et al., 2013; Safadi et al., 1999). Consequently, serum bioavailable vitamin D may represent a better reflection of bone mass than serum 25[OH]D concentration (Powe et al., 2013). To date, research on vitamin D status in athletes has focused solely upon serum 25[OH]D and as a consequence, the current advice and guidelines given to athletes and clinicians treating vitamin D deficiency may be inaccurate.

7.3 Aims and Hypotheses

Therefore, the present study set out to examine if bioavailable vitamin D is related to markers of bone mass within a racially diverse athletic population. It was hypothesised that bioavailable vitamin D and not serum vitamin D would be associated with markers of bone mass in a racially diverse athlete population.

7.4 Methods

7.4.1 Participants

Six hundred and four male athletes registered with the Qatar Olympic Committee [Arab (n=328), Asian (n=48), Black (n=108), Caucasian (n=53) and Hispanic (n=35)], exercising ≥ 6 h/week presented for pre-competition medical assessment at Aspetar Sports Medicine Hospital, Qatar. Participants were screened for inclusion as described in the General Methods chapter of this thesis. After meeting this initial inclusion criteria, participants provided a venous blood sample, completed vitamin D questionnaire and undertook bone mineral density analysis as described in of the General Methods chapter of this thesis.

7.4.2 Laboratory Analyses

Venous blood samples were collected from athletes following an overnight fast and analysed for serum 25-hydroxyvitamin D (25[OH]D) as described in section 3.4.2, of General Methods Chapter.

7.4.3 Vitamin D-Binding Protein

Serum vitamin D binding protein (DBP) concentrations ($\mu\text{g/mL}$) were determined using a commercially available kit (R&D Systems, UK). The limit of sensitivity was $\leq 0.65 \text{ ng/mL}$ and an inter-assay coefficient of variation was 7.2%. An automatic enzyme-linked immunosorbent assay (ELISA) microplate reader (Infinite® 200 PRO NanoQuant, Switzerland) and computer software Magellan Standard (version 7.1) were used to analyse DBP.

7.4.4 Assessment of Bone Mineral Density

As described in the general methodology chapter

7.4.5 Calculation of Bioavailable 25-hydroxyvitamin

Bioavailable and DBP-bound 25[OH]D were calculated using equations described in Chapter 3.

7.4.6 Statistical analysis

Statistical analyses were performed as described in Chapter 4 with the additional calculations to determine the relationship of bone mass parameters with serum 25[OH]D, DBP and bioavailable 25[OH]D, simple regression without covariates and multiple linear regression analysis including covariates such as age and ethnicity was performed with bone mass as a dependent variable, with age and ethnicity covariates. Parameter estimates along with standard error were reported. A $p\text{-value} < 0.05$ was used as a cut-off for statistical significance. Furthermore, the statistical methods underwent external assessment by BJSM

7.5 Results

7.5.1 Participants

From 604 athletes, 21.5% (n=130) demonstrated severe 25[OH]D deficiency, 37.1% (n=224) deficiency, 26% (n=157) insufficiency and 15.4% (n=93) sufficiency. There was no difference in athlete age or body mass index across the four vitamin D status categories. However, 25[OH]D sufficient (>30ng/mL) athletes presented with significantly lower body fat % compared to insufficient (P=0.024), deficient (P=0.002) and severely deficient (P=0.001) athletes. Markers of bone mass were normal for all athletes across all sites.

7.5.2 Vitamin D–Binding Protein, Bioavailable Vitamin D and Markers of Vitamin D Status

Whilst there was a positive linear association between 25[OH]D status and bioavailable vitamin D ($r=0.702$; $p<0.001$), DBP concentration was not associated with 25[OH]D concentrations ($r=-0.035$, $p=0.392$). PTH was significantly greater in severely 25[OH]D deficient athletes compared to insufficient (P=0.010), deficient (P=0.005) and sufficient (P<0.001) athletes. Albumin was significantly greater in sufficient athletes compared to 25[OH]D deficient athletes (P=0.029) (Table 7.1). Bioavailable vitamin D showed a strong negative association with DBP ($r=-0.733$; P<0.001) and PTH ($r=-0.310$; P<0.001) but not with 1,25[OH]₂D ($r=-0.023$, P=0.738).

Table 7.1 Measures of vitamin D, albumin and parathyroid hormone against 25[OH]D status categories

	Vitamin D category (ng/mL)			
	<10 (n=130)	10-20 (n=224)	20-30 (n=157)	>30 (n=93)
Age Mean (SD)	23.7 (5.4)	24.6 (5.6)	25.4 (5.6)	25.0 (4.5)
Body mass index Mean (SD)	23.6 (3.9)	24.7 (3.4)	24.2 (3.5)	24.0(3.6)
Race n (%)				
Arab (n=327)	96 (29.4)	146 (44.6)	65 (19.9)	20 (6.1)
Asian (n=48)	10 (20.8)	17 (35.4)	14 (29.2)	7 (14.6)
Black (n=108)	17 (15.7)	43 (39.8)	32 (29.6)	16 (14.8)
Caucasian (n=53)	4 (7.5)	13 (24.5)	20 (37.7)	16 (30.2)
Hispanic (n=68)	3 (4.4)	5 (7.4)	26 (38.2)	34 (50.0)
Blood markers (mean±SD)				
1,25(OH) ₂ D	39.3 (14.3)	39.4 (14.9)	39.9(14.0)	34.9(9.9)
ALB (g/L)	42.1(2.4)	41.8 (2.4)	41.9(2.5)	42.6(2.8) ^b
PTH (pg/ml)	67.2 (50.8) ^{bcd}	52.9(16.2)	50.1(14.0)	41.3(8.3)
DBP (µg/ml)	478.5 (410.4)	385.1(304.0)	416.6(298.4)	370.5(327.1)
Bioavailable 25[OH]D (ng/mL)	0.7 (0.5)	1.5(0.9) ^a	2.6(1.7) ^{ab}	4.7(3.0) ^{abc}

ALB; albumin, PTH; parathyroid hormone, DBP; vitamin D binding protein. a: significantly greater than <10ng/mL, b: significantly greater than 10-20ng/mL, c: significantly greater than 20-30ng/mL and d: significantly greater than >30ng/mL.

7.5.3 Impact of Race upon Serum 25[OH]D, Bioavailable Vitamin D and BMD

Mean serum 25[OH]D concentrations significantly differed between race, with Caucasians and Hispanics presenting greater serum 25[OH]D levels than Arabs, Asians and Blacks (Table 7.2). Whilst 1,25(OH)₂D and PTH were not significantly different between racial groups, DBP was significantly lower in Black athletes compared to in Arabs (P<0.001), Caucasian (P<0.001) and Hispanic (P<0.001) athletes. Arab athletes presented with significantly lower bioavailable 25[OH]D concentrations compared to Black (P<0.001), Caucasian (P<0.001) and Hispanic (P<0.001) athletes. Arab athletes demonstrated significantly lower BMD scores across all sites (spine (P<0.001), neck (P<0.001) and total hip (P<0.001) compared to Caucasians, Blacks and

Hispanic athletes. There was no difference in BMD across all sites between Black, Caucasian and Hispanic athletes. Finally, 25[OH]D sufficient Arab athletes presented with higher spine BMD than severely deficient Arabs athletes ($p=0.036$; 1.39 vs. 1.24 g/cm³ respectively); this was not observed in any other race.

Table 7.2 Measures of vitamin D, BMD, albumin and parathyroid hormone by athlete race (Mean±SD)

	Athlete Race				
	Arab [A] (n=328)	Asian [B] (n=48)	Black [C] (n=108)	Caucasian [D] (n=53)	Hispanic [E] (n=35)
25[OH]D	15.9(8.8)	19.3 (11.8)	19.9 (10.4) ^a	26.2 (12.9) ^{abc}	32.1 (13.5) ^{abcd}
1,25(OH)₂D	39.6(14.7)	39.8 (14.4)	39.3 (14.6)	38.8 (10.4)	33.0 (11.7)
ALB (g/L)	41.8(2.32)	42.2 (2.1)	41.2 (2.5)	43.3 (2.6) ^{ac}	43.1 (2.7) ^{ac}
PTH (pg/ml)	60.7(39.5)	47.7 (11.1)	52.1 (15.4)	47.2 (14.9)	41.2 (8.2)
DBP (µg/ml)	449.8(314.6) ^c	376 (176.0)	288.2 (336.1)	422.2 (372.8) ^c	434.2 (416.4) ^c
Bioavailable 25[OH]D (ng/mL)	1.5(1.3)	1.7 (1.2)	3.3(2.7) ^{ab}	2.5 (1.7) ^a	3.3 (2.8) ^{ab}
Spine BMD	1.29 (0.21)	1.32 (0.16)	1.48(0.16) ^{ab}	1.50 (0.13) ^{ab}	1.45 (0.14) ^{ab}
Neck BMD	1.25 (0.21)	1.31 (0.19)	1.48 (0.17) ^{ab}	1.43 (0.14) ^{ab}	1.41 (0.17) ^{ab}
Hip BMD	1.26 (0.20)	1.31 (0.18)	1.47 (0.17) ^{ab}	1.40 (0.13) ^{ab}	1.40 (0.15) ^{ab}
Total BMD	1.26 (0.16)	1.28 (0.10)	1.43 (0.13) ^{ab}	1.42 (0.10) ^{ab}	1.38 (0.10) ^{ab}
Spine T-score	0.61 (1.20)	0.64 (1.19)	1.85 (1.43) ^{ab}	1.98 (1.33) ^{ab}	1.74 (1.16) ^{ab}
Neck T-score	1.22 (1.42)	1.53 (1.49)	2.72 (1.65) ^{ab}	2.25 (1.37) ^a	2.44 (1.42) ^{ab}
Hip T-score	1.02 (1.20)	1.20 (1.23)	2.18 (1.41) ^{ab}	1.68 (1.07) ^a	2.00 (1.12) ^{ab}
Total T-score	0.63 (1.21)	0.67 (0.96)	2.06 (1.39) ^{ab}	1.78 (1.24) ^{ab}	1.72 (1.07) ^{ab}

ALB; albumin, PTH; parathyroid hormone, DBP; vitamin D binding protein, BMD; bone mineral density. a: significantly greater than Arabs, b: significantly greater than Asian, c: significantly greater than Blacks and d: significantly greater than Caucasians.

7.5.4 Relationship between Markers of Bone Mass, Serum 25[OH]D, PTH and Bioavailable Vitamin D

Serum 25[OH]D, DBP and bioavailable vitamin D showed skewed distributions and were logarithmic transformed in order to meet the assumptions of the parametric statistical techniques. P-values and parameter estimates presented are based on log-transformed data. The age and ethnicity adjusted spine BMD increased [$B=0.032$ 95% CI (0.012 to 0.051); $P=0.001$] with each one log unit change in bioavailable vitamin D.

Similarly, bioavailable vitamin D was also associated with spine [$B=0.032$ 95% CI (0.012 to 0.051), $P=0.001$], neck [$B=0.037$ 95% CI (0.016 to 0.057), $P<0.001$] and hip [$B=0.035$ 95% CI (0.016 to 0.055), $P<0.001$] BMD (Table 7.3). DBP was negatively associated with spine [$B=-0.035$ 95% CI (-0.059 to -0.012), $P=0.004$], neck [$B=-0.040$ 95% CI (-0.065 to -0.016), $P=0.001$] and hip [$B=-0.037$ 95% CI (-0.061 to -0.014), $P=0.002$] BMD. Serum 25[OH]D concentrations were not associated with BMD at any site. DBP was positively associated with neck ($P=0.034$) and hip T-score ($P=0.040$) but not spine ($P=0.067$). Serum 25[OH]D and bioavailable 25[OH]D were not associated with T-score at any site. PTH was not associated with BMD across all sites and T-scores of Spine ($r=-0.029$, $P=0.641$), Hip ($r=-0.093$, $P=0.134$) and Neck ($R=-0.117$, $P=0.061$).

Table 7.3 Parameter estimates (β 95% CI) of association of log transformed vitamin D parameters against independent bone mass variables (spine, neck and hip BMD and T-scores) after adjusting for age and race

Dependent variables	Vitamin D parameters*					
	Serum 25[OH]D <i>B (95% CI)</i>	P value	DBP <i>B (95% CI)</i>	P value	Bioavailable 25[OH]D <i>B (95% CI)</i>	P value
Spine BMD	0.022 (-0.007 to 0.052)	0.141	-0.035 (-0.059 to -0.012)	0.004	0.032 (0.012 to 0.051)	0.001
Neck BMD	0.028 (-0.002 to 0.059)	0.071	-0.040 (-0.065 to -0.016)	0.001	0.037 (0.016 to 0.057)	<0.001
Hip BMD	0.029 (0.000 to 0.059)	0.051	-0.037 (-0.061 to -0.014)	0.002	0.035 (0.016 to 0.055)	<0.001
Spine T-score	-0.030 (-0.229 to 0.170)	0.770	-0.151 (-0.313 to 0.011)	0.067	0.078 (-0.055 to 0.211)	0.250
Neck T-score	-0.006 (-0.244 to 0.231)	0.957	-0.209 (-0.401 to -0.016)	0.034	0.119 (-0.038 to 0.277)	0.138
Hip T-score	0.000 (-0.198 to 0.198)	1.000	-0.169 (-0.329 to -0.008)	0.040	0.098 (-0.034 to 0.230)	0.144

* log transformed covariates and adjusted for age and ethnicity *B: Unstandardised coefficient and (95 % confidence intervals.)*

7.6 Discussion

The aim of the present study was to examine if bioavailable vitamin D was related to markers of bone mass within a racially diverse athlete population. It was observed that after adjusting for age and race, bioavailable vitamin D was closely associated with BMD (spine, neck and hip), whilst there was no association between serum 25[OH]D concentration and BMD at any site. DBP was positively associated with neck and hip T-score but not at spine. Serum 25[OH]D and bioavailable 25[OH]D were not associated with T-score at any site. Furthermore, mean serum DBP concentrations were not associated with 25[OH]D concentrations. For clinicians treating vitamin D insufficiency, our data suggests that the current choice of assay is not fit for practice when examining the relationship between bone mass and vitamin D concentrations.

7.6.1 The role of bioavailable vitamin D upon Markers of Bone Mass

Vitamin D is just one of many factors including energy availability and weight bearing exercise that can impact upon bone mass. The role of vitamin D and calcium in bone development, growth and integrity have been well documented (M. F. Holick, 1996). Previous studies however, demonstrate inconsistent associations between serum 25[OH]D concentrations and BMD in general (Bischoff-Ferrari, Kiel, et al., 2009; Marwaha et al., 2011) and athletic populations (Allison, Farooq, et al., 2015). It has been postulated that the osteogenic effect of weight-bearing exercise (i.e. loading the bones) may be sufficient to maintain markers of bone health, irrespective of 25[OH]D status in healthy adults. Though the present data also suggests that the clinical utilisation of serum 25[OH]D could be an additional factor for this inconsistency, given that bioavailable vitamin D and not serum 25[OH]D was associated with BMD at all sites in a racially diverse athletic population. Using the free hormone hypothesis, the unbound fraction (or bioavailable vitamin D) may exert a stronger biological effect on BMD than total 25[OH]D (Bikle et al., 1986; Safadi et al., 1999). Since serum 25[OH]D is generally used in previous observational and interventional studies, it may explain such inconsistencies in the poor association between serum 25[OH]D and BMD (Johnsen et al., 2014; Powe et al., 2011). Accordingly, our data supports the notion that current 25[OH]D reference ranges provide an inaccurate representation of true vitamin D status in athletes. In contrast to previous findings, DBP concentration was not associated with 25[OH]D concentrations ($p=0.392$) (Powe et al., 2011). Whilst there was a positive linear association between 25[OH]D status and bioavailable vitamin D, PTH levels were not associated with 25[OH]D levels, BMD or T-scores, suggesting that the association between bioavailable 25[OH]D levels and BMD is not mediated via PTH.

7.6.2 Impact of Race

Studies support that racial differences or a ‘paradox’ exists in the relationship between serum 25[OH]D concentrations and markers of bone mass (Gutierrez et al., 2011; Hannan et al., 2008; Powe et al., 2014). Specifically, recent studies in the general population have demonstrated that polymorphisms in the DBP gene were associated with corresponding changes in total 25[OH]D levels and the risk of osteoporosis (Engelman et al., 2008; Fang et al., 2009). Aggarwal et al. demonstrated that bioavailable 25[OH]D was a better measure of vitamin D status with respect of BMD in patients with nephrotic syndrome (Aggarwal et al., 2016). To our knowledge, this is the first study to examine DBP and bioavailable vitamin D alongside bone mass as assessed by DXA in athletes. Our data supports these previous observations, with Hispanic athletes presenting with greater mean serum 25[OH]D levels (32.1 ng/mL) compared to Caucasian (26.2ng/mL) and Black (19.9ng/mL) athletes, despite no difference in bioavailable vitamin D or BMD at any site. Bioavailable vitamin D is determined by vitamin D-binding protein concentration, which is encoded by the group-specific component (GC) gene (Malik et al., 2013). Polymorphisms in the GC gene, produce proteins that differ in affinity for 25[OH]D, and it is these polymorphisms that are known to differ between racial groups. Racial differences in the prevalence of common genetic polymorphisms provide a likely explanation for this observation. Consequently, our data have implications for the wider general population, with further research warranted on adolescent and female athletes.

7.6.3 Financial considerations for treating clinicians

Screening athlete for vitamin D insufficiency is expensive. In our facility, 25[OH]D assessment costs \$255USD per athlete. Since Aspetar screens approximately 1500 elite athletes per year,

systematic 25[OH]D screening costs our facility \$382,500USD per year. Since our data clinically questions the value of routine 25[OH]D screening, the financial burden of vitamin D testing only adds weight to the debate of whether clinicians should be testing for vitamin D insufficiency, since all athletes in the present study demonstrated normal bone mass. Our data also questions the value of prophylactic vitamin D supplementation (typically 2000IU/d oral cholecalciferol) in insufficient athletes to ‘correct’ 25[OH]D status, but who show no symptoms of relative energy deficiency syndrome, poor bone mass or musculoskeletal injury. Perhaps targeted vitamin D screening and supplementation should be reserved for those athletes presenting with stress fracture related injuries, or those competing in non-weight bearing sports such as cycling and swimming where the osteogenic effect of bone loading is sub-optimal. If testing is warranted however, clinicians should use the appropriate assays to calculate DBP and bioavailable vitamin D status rather than serum 25[OH]D.

7.7 Limitations

The primary limitation of work is that the equation adopted to calculate bioavailable D used of the generic affinity constant of the Gc1F allele due to lack of genetic analysis. The equations used to estimate free and bioavailable 25[OH]D in this study used the affinity constants of the Gc1F allele, as did the equations in Powe et al. (Powe et al., 2011). Genotyping has identified two common SNPs in the coding region of the DBP gene (rs4588 and rs7041). Combinations of these SNPs, produce three major polymorphic forms of DBP (Gc1F, Gc1S, and Gc2 phenotypes) which differ substantially in their binding affinity for 25[OH]D and proportionally vary between ethnicities, e.g. Blacks homozygotes present with a greater percentage of Gc1F and Caucasian homozygotes present with a greater percentage of Gc1S phenotype (92.7% vs 76.0% respectively). Furthermore,

a recent study has highlighted the potential discrepancies between monoclonal versus polyclonal ELISA (Jemielita et al., 2016). The monoclonal antibody (R&D ELISA) used in this study binds to a single peptide fragment of DBP and may bind differently to Gc1F and Gc1S variants, yielding underestimated concentrations in black individuals (Bouillon, Jones, & Schoenmakers, 2014; Hollis & Bikle, 2014). Prior studies have shown no racial differences in circulating DBP using polyclonal assays (Bouillon et al., 2014; Winters, Chennubhatla, Wang, & Miller, 2009). While there may be cross-reactivity of polyclonal antibodies with other proteins (Christiansen et al., 2007; Powe et al., 2014) which likely explains the systematically higher DBP results with the polyclonal assay, this is non-differential by race. Although, not significant in this study, Black athletes presented with higher mean bioavailable vitamin D, and lower DBP than Caucasian athletes, so the variation within the testing techniques cannot be ignored. Finally, the impact of training load, dietary intake data to assess total energy and calcium intake and the assessment of steroid hormones were not accounted for in this study. Future, longitudinal trials including genotype-specific binding affinity constants in the calculation for bioavailable vitamin D, training, dietary and hormone analysis on ethnically diverse populations are warranted. We also acknowledge that serum 25[OH]D measurement only offered a snapshot of current status. Thus, it is entirely possible for severely deficient (<10 ng/mL) and deficient (10-20 ng/mL) athletes in the present study to have spent many years sufficient (>30 ng/mL) prior to recruitment. Deuterium enrichment protocol, bone biopsy

7.8 Conclusion

In conclusion, regardless of age or race, bioavailable vitamin D and not serum 25[OH]D was associated with BMD in a racially diverse athletic population. Our data questions the value of routine 25[OH]D screening in athletes, since all athletes in the present study demonstrated

clinically normal bone mass, despite 85% of athletes presenting with either serum 25[OH]D insufficiency, deficiency or severe deficiency respectively. Our data suggest that bioavailable vitamin D, rather than the current standard of 25[OH]D is a better measure of vitamin D status with respect to BMD.

What are the findings?

- This study demonstrated that in a racially diverse athletic population, bioavailable vitamin D was closely associated with bone mineral density (BMD; spine, neck and hip; $p < 0.002$).
- No association was observed between serum 25[OH]D concentration and BMD at any site ($p > 0.05$).
- Mean serum DBP concentration were not correlated with 25[OH]D concentrations ($p = 0.392$).

How might it impact on clinical practice in the near future?

- Determining bone mass is multifactorial. Systematic screening to determine 25[OH]D concentrations in isolation is expensive, and demonstrates a poor relationship to bone mass in a racially diverse athletic population.
- If testing is warranted, clinicians should use the appropriate assays to calculate DBP and bioavailable vitamin D concentration rather than total serum 25[OH]D.

Chapter 8:

Severely Vitamin D-Deficient Athletes Present Smaller Hearts than Sufficient Athletes

*Publications resulted from this chapter; Allison, R. J., G. L. Close, A. Farooq, N. Riding, O. Salah, B. Hamilton, and M. G. Wilson. Severely vitamin D-deficient athletes present smaller hearts than sufficient athletes. *Eur J Prev Cardiol.*, Vol. 22, No. 4, pp. 535-542, 2015*

8.1 Abstract

Background: Vitamin D (25[OH]D) deficiency has associations with bowel/colon cancer, arthritis, diabetes, and cardiovascular disease. Many athletes are vitamin D deficient, yet no studies have examined the association between 25[OH]D status and cardiac structure and function in healthy athletes.

Methods: A total of 506 national-level athletes [football (50%), handball (23%), volleyball (16%), and basketball (11%)] and 244 control participants presented for precompetition medical assessment. Controls were healthy individuals registered with a sporting federation undertaking <2 h of exercise per week. All individuals undertook a physical examination, 12-lead electrocardiogram, echocardiogram, and serum 25[OH]D evaluation.

Results: From 506 athletes and 244 controls, 23 and 12.3% demonstrated 25[OH]D sufficiency (>30 ng/ml), 30 and 23.4% insufficiency (20–30 ng/ml), 37.2 and 48.8% deficiency (10–20 ng/ml), and 11 and 15.6% severe deficiency (<10 ng/ml). Severely 25[OH]D-deficient athletes present significantly ($p<0.05$) smaller aortic root and left atria diameters, intraventricular septum diameter (IVSd), left ventricular diameter during diastole (LVIDd), left ventricular mass (LVM), left ventricular volume during diastole (LVvoid), and right atrial (RA) area than insufficient and sufficient athletes. Furthermore, following logarithmic transformation adjusting 25[OH]D for age, body surface area, ethnicity, and athletic participation, positive associations were observed between 25[OH]D and IVSd, LVIDd, posterior wall thickness during diastole, LVM, and LVvoid in athletes but not in the control participants.

Conclusions: Severely 25[OH]D-deficient athletes present significantly smaller cardiac structural parameters than insufficient and sufficient athletes. Future research should investigate the precise mechanism(s) causing cardiac hypertrophy with increases in serum 25[OH]D in healthy athletes.

8.2 Introduction

Ever since Weishaar et al. observed an association between 25[OH]D and cardiovascular function using 25[OH]D deficient rats 25 years ago, several research groups have established associations between 25[OH]D and cardiac structure and function (in both human and animal models) (Weishaar & Simpson, 1987b). Yet the majority of these studies have only examined pathological conditions such as chronic kidney disease (Thadhani et al., 2012), thalassaemia-major (Noetzli et al., 2011), hypertension (Kong et al., 2010), peripheral arterial disease (Fahrleitner et al., 2002) and congestive heart failure (CHF) (Pilz, Marz, et al., 2008).

It is known that the vitamin D receptor and the associated apparatus for 1,25-dihydroxyvitamin D production are present throughout the heart and vascular system, with the vitamin D receptor specifically located in cardiac myocytes and fibroblasts (Chen et al., 2008). Further, 25[OH]D deficiency is known to adversely affect cardiac contractility, vascular tone, cardiac collagen content, and cardiac tissue maturation (Achinger & Ayus, 2005). Histological analysis of 25[OH]D deficient rats demonstrate significantly smaller ventricular myofibrils and increases in extracellular matrix proteins compared to 25[OH]D sufficient rats (Weishaar et al., 1990).

Regular participation in intensive physical exercise is associated with several structural and electrophysiological cardiac adaptations that enhance diastolic filling and facilitate a sustained increase in cardiac output that is fundamental to athletic excellence. Such cardiac adaptations are collectively referred as the “Athlete’s Heart” and are frequently reflected on the 12-lead electrocardiogram (ECG) and imaging studies (Chandra et al., 2013). Numerous factors affect the adaptations of the athletes heart including; sporting modality, duration and intensity, age, ethnicity, sex, anthropometry and performance enhancing substance abuse. However, it is also well recognised that many professional athletes are vitamin D deficient (Close, Russell, et al., 2013),

and no studies have examined the association between 25[OH]D status and cardiac structure and function in healthy athletes.

8.3 Aims and Hypotheses

The present study aimed to examine the relationship between serum 25[OH]D and bioavailable vitamin D with cardiac structure and function within a large, ethnicity diverse cohort of healthy athletes for pre-competition medical assessment at our institution.

8.4 Methods

8.4.1 Participants

Between November 2010 and June 2012, 521 male athletes (exercising ≥ 6 h/week) presented at our institution for pre-competition medical assessment [football (n=269, 51%), handball (n= 115, 22%), volleyball (n = 82, 16%), basketball (n=55, 11%)]. A further 244 individuals registered with a Qatari sporting federation (such as sailing, archery, shooting, bowling) but exercising ≤ 2 h/week were used as a control population. Arabs (n=427) came from seven Gulf States and Five Middle-Eastern countries; Black Africans (n=207) came from nine African countries; Caucasians (n=116) came from six European/North American countries.

8.4.2 Pre-participation cardiovascular screening

All individuals were screened using a precompetition medical assessment form, examining family history of cardiovascular disease and personal symptoms, with a physical examination undertaken by a sports medicine physician. A standard 12-Lead ECG was obtained using a GE Mac 5500 (New York, USA) after a 5-min rest in the supine position.

Echocardiographic examination was performed using a commercially available ultrasound system (Philips, USA) by an experienced sports cardiologist. Images of the heart were obtained in the standard planes, using previously published criteria (Riding et al., 2013).

8.4.3 Athlete exclusion and further evaluation

Fifteen athletes demonstrated ECG and/or echocardiographic abnormalities suspicious of a disease associated with sudden cardiac death, and were excluded from further analysis.

8.4.4 Vitamin D assessment

The remaining 506 athletes and 244 controls undertook serum 25[OH]D evaluation via chemiluminescent immunoassay technology (Liaison® 25-OH Vitamin D Total Assay; DiaSorin Inc., Saluggia (Vercelli), Italy). Sensitivity for 25[OH]D was 7 ng/ml, below which levels were recorded as <7 ng/ml. The intra- and interassay CV was 7.6–9.4% and 9.8–13.4%, respectively. In addition, athletes completed a vitamin D questionnaire that addressed specific areas relevant to vitamin D status as described in Chapter 3.

8.4.5 Statistical analysis

Statistical analyses were performed as described in Chapter 3 with the additional calculations. Two-way ANOVA was used to compare athlete physical and lifestyle habits and cardiac structure and function parameters between four levels of serum 25[OH]D groups within and between the subject groups (athletes vs. controls). Post-hoc Bonferroni adjustments were performed where appropriate. Mean, standard deviation, and ranges were reported for the continuous variables. The Chi-squared test was used to test the association between lifestyle habits and serum 25[OH]D

groups. Multiple linear regression was used to determine the association of ECG and echocardiographic parameters against serum 25[OH]D. Natural log transformations were applied to serum 25[OH]D concentrations to remove positive skewness, adjusting for age, ethnicity, body surface area, and athletic participation (athlete vs. controls). To determine the relationship of cardiac structure parameters with bioavailable vitamin D, multiple liner regression analysis including covariates such as age and BSA with manual backwards-stepwise deletion was performed. Parameter estimates along with standard error were reported. A p-value<0.05 was for statistical significance.

8.5 Results

Of the 506 athletes, 23% demonstrated 25[OH]D sufficiency, 30% insufficiency, 37.2% deficiency, and 11% severe deficiency. Of the controls, 12.3% demonstrated 25[OH]D sufficiency, 23.4% insufficiency, 48.8% deficiency, and 15.6% severe deficiency (Table 8.1). Athletes were significantly ($p<0.05$) taller, heavier, and had a larger body surface area than controls in their respective 25[OH]D groups. 25[OH]D severely deficient athletes were significantly ($p<0.05$) shorter and lighter compared to insufficient and sufficient athletes. The incidence of severe 25[OH]D deficiency or deficiency in Caucasian athletes was significantly lower compared to Arabic and Black African athletes, while significantly more Caucasian athletes were 25[OH]D sufficient compared to Arabic and Black African athletes. Of both athletes and controls, 48% did not expose themselves to sunlight, and 17 and 18%, respectively, exposed themselves to >2 h of sunlight per week (Table 8.2). Of those individuals that received no UVB radiation, 16.9 and 9.4%, respectively, were sufficient in 25[OH]D, and of those that received >2 h of sunlight per week, 5.8 and 6.8%, respectively, were severely deficient in 25[OH]D. For those individuals that exposed

themselves to sunlight, 86 and 69.3%, respectively, uncovered more than their hands and face.

Finally, 8.2 and 5.3%, respectively, used sunscreen.

Table 8.1 Physical characteristics of individuals severely deficient, deficient, insufficient and sufficient vitamin D

<i>Variables</i>	<i>Vitamin D (ng/mL)</i>				<i>p-value</i>
	<i><10</i>	<i>10 - 20</i>	<i>20-30</i>	<i>>30</i>	
Numbers and percentage					
Athletes	55 (11)	186 (37.2)	150 (30)	115 (23)	-
Controls	38 (15.6)	119 (48.8)	57 (23.4)	30 (12.3)	-
Age (years)					
Athletes	21.7±4.8	22.3±5.2	24.0±5.5 ^{*a,b}	23.3±5.3	0.008
Controls	21.7±6.5	23.7±7.3	26.5±10.1 ^a	27.0±9.0	0.013
Height (m)					
Athletes	1.77±13.7 [*]	1.81±11.8 [*]	1.84±11.6 ^{*,a}	1.84±11.1 ^{*,a}	<0.001
Controls	1.72±6.5	1.73±6.6	1.72±9.1	1.72±6.4	0.88
Body mass (kg)					
Athletes	75.3±20.8 [*]	80.7±17.2 [*]	84.2±14.5 ^{*,a}	81.9±13.5 ^{*,a}	0.005
Controls	68.2±15.6	71.6±14.7	73.3±16.0	68.9±11.7	0.312
Body surface area (m²)					
Athletes	1.9±0.3 [*]	2.0±0.3 [*]	2.1±0.2 ^{*,a}	2.0±0.2 [*]	<0.001
Controls	1.8±0.2	1.8±0.2	1.9±0.2	1.8±0.2	0.403
Resting systolic BP (mmHg)					
Athletes	122.7±10.6	125.7±9.9 [*]	125.2±11.7 [*]	126.8±8.8	0.255
Controls	118.0±9.6	122.4±13.0	120.0±11.1	122.3±9.1	0.403
Ethnicity (%)					
Athletes					
Arabic	13.4	44.6	28.1	13.8	<0.001
Black African	12.3	36.9	29.6	21.2	
Caucasian	2.9	19.4	33	44.7	
Controls					
Arabic	14.8	52.7	22.7	9.9	<0.001
Black African	28.6	39.3	28.6	3.6	
Caucasian	0	7.7	23.1	69.2	

Values are Mean±SD or %.; *Significant difference between athletes vs. controls in their respective 25[OH]D groups.;

^a Significant difference compared to the <10 ng/ml cohort.; ^b Significant difference compared to the 10–20 ng/ml cohort.

Table 8.2 Lifestyle characteristics of individuals severely deficient, deficient, insufficient and sufficient vitamin D

<i>Variables</i>	<i>Vitamin D (ng/mL)</i>				Total	p-value
	<10	10 - 20	20-30	>30		
Exposure time to sunlight (min)						
Athletes						
No sunlight exposure	27 (11.2)	99 (40.9)	75 (31.0)	41 (16.9)	242	0.004
30-60 minutes	10 (10.5)	43 (45.3)	22 (23.2)	20 (21.1)	95	
60-120 minutes	13 (15.7)	21 (25.3)	23 (27.7)	26 (31.3)	83	
>120 minutes	5 (5.8)	23 (26.7)	30 (34.9)	28 (32.6)	86	
Controls						
No sunlight exposure	21 (17.9)	57 (48.7)	28 (23.9)	11 (9.4)	117	0.568
30-60 minutes	11 (20.8)	22 (41.5)	12 (22.6)	8 (15.1)	53	
60-120 minutes	3 (10.0)	17 (56.7)	7 (23.3)	3 (10.0)	30	
>120 minutes	3 (6.8)	23 (52.3)	10 (22.7)	8 (18.2)	44	
Skin exposure to the sun						
Athletes						
None	1 (25.0)	1 (25.0)	2 (50.0)	0 (0.0)	4	0.012
Hands and feet only	14 (19.4)	34 (47.2)	13 (18.1)	11 (15.3)	72	
More than hands and feet	40 (9.3)	151 (35.1)	135 (31.4)	104 (24.2)	430	
Controls						
None	0 (0.0)	2 (40.0)	3 (60.0)	0 (0.0)	5	0.091
Hands and feet only	12 (17.1)	37 (52.9)	18 (25.7)	3 (4.3)	70	
More than hands and feet	26 (15.4)	80 (47.3)	36 (21.3)	27 (16.0)	169	
Use of sunscreen						
Athletes						
Yes	0 (0.0)	8 (19.5)	10 (24.4)	23 (56.1)	41	<0.001
No	55 (11.8)	178 (38.3)	140 (30.1)	92 (19.8)	465	
Controls						
Yes	0 (0.0)	4 (30.8)	5 (38.5)	4 (30.8)	13	0.038
No	38 (16.5)	115 (49.8)	52 (22.5)	26 (11.3)	231	

Values are n (%) or n.

8.5.1 Cardiac structure

All athletes and control participants presented with cardiac morphological parameters within clinically accepted limits. Deficient, insufficient, and sufficient athletes had significantly ($p < 0.05$) greater absolute aortic root (Ao) diameter, left atrium (LA) diameter, intraventricular septum

during diastole (IVSd), left ventricular diameter during diastole (LVIDd), left ventricular mass (LVM), left ventricular volume during diastole (LVvolD), and right atrial (RA) area than their respective 25[OH]D control participants (Table 8.3). However, severely deficient athletes had significantly ($p < 0.05$) smaller Ao diameter, IVSd, LVIDd, LVM, LVvolD, and RA area than insufficient and sufficient athletes. Furthermore, LVvolD and LVM were significantly ($p < 0.05$) smaller in deficient athletes compared to insufficient and sufficient athletes. LVIDd and LVM were significantly ($p < 0.05$) smaller in severely deficient controls compared to insufficient and sufficient control participants, with no further cardiac structural parameter different between the respective 25[OH]D control participants.

Table 8.3 Cardiac structure of individuals severely deficient, deficient, insufficient, and sufficient in vitamin D

<i>Variables</i>	<i>Vitamin D (ng/mL)</i>				<i>P value</i>
	<i><10</i>	<i>10 - 20</i>	<i>20-30</i>	<i>>30</i>	
Ao (mm)					
Athlete	26.3±3.1 (20–33)*	27.3±3.0 (19–35)*	28.0±2.6 (21–34)* ^a	27.7±2.4 (22–34)* ^a	0.001
Control	24.9±2.6 (21–30)	26.4±2.5 (21–33)	26.3±3.2 (21–34)	26.2±2.0 (22–29)	0.145
LA (mm)					
Athlete	32.9±5.1 (22–47)*	33.9±3.9 (22–43)*	34.8±3.6 (26–48)* ^a	33.8±3.6 (24–45)*	0.013
Control	30.6±4.0 (24–39)	32.3±4.1 (21–43)	32.7±4.8 (18–43)	32.0±3.4 (24–39)	0.109
IVSd (mm)					
Athlete	8.6±1.4 (6–11)	8.9±1.2 (6–14)*	9.1±1.0 (6–13)* ^a	9.1±1.1 (6–13)* ^a	0.014
Control	8.1±1.1 (6–10)	8.5±1.1 (6–12)	8.3±1.1 (6–11)	8.5±1.0 (6–11)	0.172
LVIDd (mm)					
Athlete	51.2±5.5 (39–61)*	53.5±4.7 (40–62)*	54.5±5.7 (45–64)* ^a	54.6±4.2 (42–65)* ^a	<0.001
Control	48.6±3.7 (41–57)	49.8±4.0 (42–59)	51.3±5.1a (41–60)	51.5±4.4a (42–58)	0.006
PWTd (mm)					
Athlete	8.0±1.2 (6–10)	8.3±1.1 (6–13)	8.7±2.9 (6–13)	8.6±2.2 (6–10)	0.072
Control	7.7±0.9 (6–9)	8.4±4.4 (6–14)	8.1±0.8 (6–10)	8.2±0.9 (6–10)	0.607
LVM (g)					
Athlete	157.7±52.7 (63–285)*	173.9±42.6 (65–291)*	184.8±36.3 (80–271)* ^{a,b}	184.0±36.2 (82–266)* ^{a,b}	<0.001
Control	131.9±27.8 (81–205)	147.2±34.9 (75–286)	152.7±38.0a (68–237)	157.3±34.1a (79–218)	0.011
LV vol D (ml)					
Athlete	117.1±34.1 (54–195)	131.8±29.9 (55–212)* ^a	142.7±27.3 (70–245)* ^{a,b}	140.3±30.9 (58–271)* ^{a,b}	<0.001
Control	106.2±26.8 (64–159)	108.0±21.3 (53–176)	112.5±25.0 (63–175)	110.2±23.2 (66–161)	0.54
LA area (mm²)					
Athlete	17.3±4.5 (9–27)	17.7±3.8 (8–29)	18.5±3.5 (11–31)	18.1±3.6 (8–33)	0.113
Control	15.6±3.3 (10–24)	15.4±3.1 (9–23)	19.1±22.3 (10–183)	16.4±3.3 (11–25)	0.223
RA area (mm²)					
Athlete	15.0±4.6 (7–28)	16.5±3.6 (8–28)*	17.3±3.1 (11–29)* ^a	16.7±3.1 (7–24)* ^a	0.001
Control	13.6±2.8 (9–19)	14.9±7.0 (8–21)	14.7±3.1 (9–22)	14.9±3.5 (8–23)	0.643

Values are Mean±SD (range).; *Significant difference between athletes vs. controls in their respective 25[OH]D groups.; ^a Significant difference compared to the <10 ng/ml cohort.; ^b Significant difference compared to the 10–20 ng/ml cohort.; Ao, aortic root diameter; IVSd, intraventricular septum during diastole; LA area, left atrial area; LA, left atrial diameter; LVIDd, left ventricular internal diameter during diastole; LVM, left ventricular mass; LVvolD, left ventricular volume in diastole; PWTd, posterior wall thickness in diastole; RA area, right atrial area.

However, when adjusted for body surface area deficient, insufficient, and sufficient controls had significantly ($p < 0.05$) greater (LA/BSA) diameter than athletes of the same 25[OH]D status (Table 8.4). Deficient and sufficient controls had greater (IVSd/BSA) than athletes of the same 25[OH]D status. Insufficient and sufficient controls had greater (LVIDd) than athletes of the same 25[OH]D status, was greater in sufficient controls than insufficient controls. Deficient controls had greater PWTd/BSA but smaller LA/BSA than deficient athletes. Athletes have larger (LVM/BSA) than controls across all vitamin D groups, whereas both severely deficient athletes and controls had significantly smaller LVM/BSA than deficient, insufficient and sufficient counterparts. Deficient, insufficient and sufficient athletes had significantly ($p < 0.05$) greater (LVvolD/BSA), than their respective 25[OH]D control participants and severely deficient athletes.

Table 8.4 Cardiac structures of individuals adjusted for BSA compared to vitamin D status

<i>Variables</i>	<i>Vitamin D (ng/mL)</i>			
	<10	10-20	20-30	>30
Ao (mm)/BSA				
Athlete	13.43±3.06	12.5±4.09	12.26±4.32	12.45±4.1
Control	12.61±4.62	13.09±4.38	12.05±5.37	11.5±6.0
LA (mm)/BSA				
Athlete	17.35±2.00	17.0±2.03	16.88±1.93	16.62±1.8
Control	17.08±1.75	17.54±1.89*	17.66±2.28*	17.7±1.4*
IVSd (mm)/BSA				
Athlete	4.51±0.53	4.44±0.58	4.40±0.54	4.47±0.58
Control	4.53±0.51	4.64±0.59*	4.48±0.54	4.74±0.50*
LVIDd (mm)/BSA				
Athlete	27.15±3.03	26.8±2.55	26.46±3.26	26.82±2.30
Control	27.24±2.83	27.17±2.52	27.75±2.69*	28.6±2.4* ^b
PWTd (mm)/BSA				
Athlete	4.22±0.50	4.15±0.48	4.25±1.42	4.24±1.30
Control	4.29±0.46	4.60±2.40*	4.38±0.49	4.55±0.56
LVM (g)/BSA				
Athlete	80.95±19.4*	85.5±16.8* ^a	88.36±16.4* ^a	88.61±16.0 ^a
Control	73.29±13.53	78.99±18.1	81.48±15.9	86.8±17.0 ^a
LVvoid (ml)/BSA				
Athlete	60.32±10.9	65.2±11.0* ^a	67.22±13.7* ^a	68.09±11.0* ^a
Control	58.92±12.86	58.23±11.6	60.40±10.4	60.8±11.0
LAarea(mm²)/BSA				
Athlete	8.97±1.55	8.81±1.58*	8.85±1.92	8.87±1.60
Control	8.71±1.73	8.34±1.72	10.22±11.2	9.05±1.5
RAarea (mm²)/BSA				
Athlete	7.77±1.69	8.17±1.45	8.26±1.79	8.17±1.4
Control	7.58±1.36	8.08±4.54	7.92±1.51	8.23±1.7

Values are Mean±SD (range).; *Significant difference between athletes vs. controls in their respective 25[OH]D groups.; ^a Significant difference compared to the <10 ng/ml cohort.; ^b Significant difference compared to the 10–20 ng/ml cohort.; Ao, aortic root diameter; IVSd, intraventricular septum during diastole; LA area, left atrial area; LA, left atrial diameter; LVIDd, left ventricular internal diameter during diastole; LVM, left ventricular mass; LVvoid, left ventricular volume in diastole; PWTd, posterior wall thickness in diastole; RA area, right atrial area.

8.5.2 Logarithmic transformation

Following logarithmic transformation adjusting 25[OH]D for age, body surface area, and ethnicity (Table 8.5), athletes had significantly larger LA, IVSd, LVIDd, LVM, and LVvolD compared to control participants. When athletes and controls were grouped together, there were positive associations between 25[OH]D and LVIDd, PWTd, LVM, and LVvolD. However, after further adjustment for athletic participation, positive associations between 25[OH]D and IVSd, LVIDd, PWTd, LVM, and LVvolD were observed in athletes, that was not maintained in the control participants.

Table 8.5 Logarithmic transformation adjusting 25[OH]D against cardiac morphology for age, body surface area, ethnicity and athletic participation in athletes and/or controls

<i>Variables</i>	<i>Athletes vs. controls</i>	<i>Athletes plus controls</i>	<i>Athletes only</i>	<i>Controls only</i>
Ao (mm)	0.3±0.2 (0.148)	0.2±0.2 (0.315)	0.2±0.2 (0.336)	0.0±0.3 (0.976)
LA (mm)	0.8±0.3 (0.010)	0.1±0.2 (0.787)	0.1±0.3 (0.834)	0.1±0.4 (0.790)
IVSd (mm)	0.2±0.1 (0.013)	0.1±0.1 (0.063)	0.1±0.1 (0.011)	-0.1±0.1 (0.536)
LVIDd (mm)	1.6±0.4 (<0.001)	0.9±0.3 (0.002)	0.9±0.4 (0.016)	0.8±0.5 (0.120)
PWTd (mm)	-0.2±0.2 (0.479)	0.4±0.2 (0.037)	0.4±0.2 (0.016)	0.3±0.4 (0.466)
LVM (g)	11.1±2.6 (<0.001)	6.7±2.1 (0.002)	5.9±2.6 (0.024)	4.8±3.6 (0.185)
LV vol D (ml)	10.6±1.9 (<0.001)	3.8±1.5 (0.013)	5.6±1.9 (0.003)	-0.5±2.7 (0.860)
LA area (mm ²)	0.4±0.6 (0.471)	0.3±0.5 (0.558)	0.2±0.3 (0.464)	0.0±1.6 (0.986)
RA area (mm ²)	0.5±0.3 (0.198)	0.2±0.3 (0.388)	0.3±0.3 (0.239)	0.0±0.7 (0.976)

Values are $\beta \pm SE$ (p-value).; Ao, aortic root diameter; IVSd, intraventricular septum during diastole; LA area, left atrial area; LA, left atrial diameter; LVIDd, left ventricular internal diameter during diastole; LVM, left ventricular mass; LVvolD, left ventricular volume in diastole; PWTd, posterior wall thickness in diastole; RA area, right atrial area.

8.5.3 Cardiac function

Regardless of serum 25[OH]D status, all athletes and control participants displayed normal cardiac functional parameters. There was no significant difference in any cardiac functional parameter between athletes and control participants, or within athletes or control 25[OH]D participants.

8.6 Discussion

It is well recognized that 25[OH]D is necessary for optimal bone mass. 25[OH]D-deficient athletes may be at an increased risk for potential problems such as stress fractures, respiratory infections, frequent illness, and muscle injuries. Yet, to our knowledge, this is the first study to investigate the association between 25[OH]D status and cardiac structure and function in young healthy athletes.

The main finding of the study is that severely 25[OH]D-deficient athletes (<10 ng/ml) presented significantly smaller ($p<0.05$) Ao and LA diameter, IVSd, LVIDd, LVM, LVvold, and RA area than insufficient (20–30 ng/ml) and sufficient (>30 ng/ml) athletes. Adjust for BSA severely deficient athletes had smaller LVM/BSA and LVvold only. Whereas, deficient, insufficient, and sufficient controls had significantly ($p<0.05$) greater (LA/BSA) diameter than athletes of the same 25[OH]D status. Athletes had greater developed cardiac structure than controls, but also greater BSA.

Furthermore, following logarithmic transformation adjusting 25[OH]D for age, body surface area, ethnicity, and athletic participation, positive associations were between 25[OH]D and IVSd, LVIDd, PWTd, LVM, and LVvold in athletes but not in the control participants.

In the present study, 23% of athletes demonstrated 25[OH]D sufficiency, with near half demonstrating either 25[OH]D deficiency (37.2%) or severe deficiency (11%). This high

prevalence of deficiency has been noted previously (B. Hamilton et al., 2014). It is likely that this high deficiency prevalence reflects the cultural tendency to train and compete after sunset in Qatar, due to the high daily temperatures. However, it should be noted 16.9% of athletes and 9.4% of controls who did not expose themselves to sunlight were sufficient in 25[OH]D. The majority of 25[OH]D is generated de novo by the basal layers of the epidermis via ultraviolet-B radiation and/or, to a lesser extent, consumed within the diet. While we did not measure nutritional intake prior to assessment, it is possible for individuals to become 25[OH]D sufficient with appropriate alimentation with foodstuffs as fish, milk, and cereals. Secondly, significantly more Caucasian athletes were 25[OH]D sufficient compared to Arabic and Black African athletes. Consideration for ethnicity and its impact upon 25[OH]D status is important, as a recent study by Powe et al. reported that Black individuals (n=1191) present significantly ($p<0.001$) lower serum 25[OH]D levels than Caucasians (n=904), despite being comparable in age, sex, body mass index, and menopausal status (15.6 ± 0.2 vs. 25.8 ± 0.4 ng/ml, respectively). A unique feature of this investigation was the examination of vitamin D-binding protein (the primary vitamin D carrier protein). The authors observed a higher prevalence among Black individuals of a polymorphism in the vitamin D-binding protein gene that was associated with low levels of vitamin D binding protein, resulting in bioavailable 25[OH]D levels similar to Caucasian individuals despite lower levels of total 25[OH]D. The authors suggest that low levels of vitamin D-binding protein in Black individuals may offer protection against the consequences of 25[OH]D deficiency, such as for example, a higher bone mineral density scores in Black individuals compared to Caucasians (Powe et al., 2013).

8.6.1 25[OH]D and cardiac structure and function; healthy vs. chronic disease states

Few studies have examined 25[OH]D status in association with cardiac structure and function in the general population without significant comorbidities. In an older female population (mean±SD age 73.9±4.9 years, 69.7% female), Van Ballegooijen et al. measured serum 25[OH]D together with ECG and echocardiographic parameters in 2312 individuals free of cardiovascular disease at baseline (van Ballegooijen et al., 2013). While mean serum 25[OH]D demonstrated populational insufficiency (25.2±10.2 ng/ml), serum 25[OH]D was not associated with any ECG or echocardiographic parameter. However, the vast majority of evidence examining 25[OH]D deficiency against cardiac morphology is in individuals with established chronic disease, and suggests that those individuals who are 25[OH]D deficient display significant increases in LV hypertrophy. Ky et al. observed a significant negative interaction between serum 25[OH]D and LV mass, end-diastolic and end-systolic volumes in 1431 individuals with chronic renal insufficiency (Ky et al., 2013). This association between 25[OH]D deficiency and increased LV mass is also observed in paediatric patients with chronic kidney disease (Patange, Valentini, Gothe, Du, & Pettersen, 2013) and in individuals with essential hypertension, (Kulah et al., 2007) CHF (Bozic et al., 2011) and dilated cardiomyopathy (Al Azkawi & Al Mutair, 2012).

The precise mechanism(s) causing this cardiac hypertrophy (or in our case, lack of hypertrophy) in the 25[OH]D-deficient state remains unclear. What is understood, however, is that the remodeling mechanisms associated with cardiac disease and chronic overloading (such as long-standing mitral insufficiency, essential hypertension, CHF, kidney disease and dilated cardiomyopathy) differ considerably from the physiological adaptations seen in athletes induced through prolonged and intensive exercise. Furthermore, the observation that 25[OH]D deficiency is associated with, for example LVH in diseased individuals, may be a simple reflection of lack

UVB exposure due to severe limitations in their functional capacity to exercise; meaning that they do not walk outside and are thus 25[OH]D deficient.

8.6.2 25[OH]D, cardiac structure and function in Murine models

Unlike models using humans with chronic disease, murine models offer information regarding 25[OH]D deficiency and cardiac structure at the cellular and myofibril level in apparently healthy hearts at baseline. However, unlike the present study which observed LV hypertrophy (wall thickness, mass and volumes) in 25[OH]D-sufficient athletes compared to severely deficient athletes, Assalin et al. recently reported that male weaning Wistar rats fed a 25[OH]D-deficient diet for 4 months compared to rats fed a diet with 1,000 IU of vitamin D/kg, demonstrated LV hypertrophy together with lower fractional shortening and ejection fraction (Assalin et al., 2013). Furthermore, biochemical analyses showed lower beta-hydroxyacyl coenzyme-A dehydrogenase activity and higher lactate dehydrogenase activity in 25[OH]D-deficient rats; with 25[OH]D deficiency significantly related to increased cytokines release, oxidative stress, apoptosis and fibrosis. These observations are supported by Weishaar et al. who found a significant increase in cardiac hypertrophy (an increase in heart to body mass ratio) in rats feed a 25[OH]D deficient diet after both 9 and 18 weeks (Weishaar et al., 1990).

Yet the precise mechanism(s) causing cardiac hypertrophy in the 25[OH]D-deficient rat is unclear, with murine models demonstrating conflicting results. Histological analysis of Weishaar et al. rats observed significantly smaller ventricular myofibrils and increased extracellular matrix proteins compared to 25[OH]D-sufficient rats. However, Gezmish et al. examined the effects of a 25[OH]D-deficient diet upon the hearts of 4-week-old Sprague Dawley rats whose mothers were feed a 25[OH]D-depleted diet from 6 weeks prior to pregnancy until 4 weeks of lactation. The

authors demonstrated that maternal 25[OH]D deficiency leads to an increase in LV volume, accompanied by an increase in both cardiomyocyte hypertrophy and number (proliferation), in the hearts of 4-week-old rats (Gezmish et al., 2010).

8.6.3 To supplement with 25[OH]D or not

No studies have examined the effect of supplementation upon cardiac structure and function in healthy hearts, but a few have examined those with known cardiac comorbidities. Shedeed evaluated the effect of 25[OH]D₃ supplementation in infants with CHF. In this double-blind placebo-controlled intervention study of 80 infants with CHF, the intervention consisted of either giving vitamin D₃ or placebo. After 12 weeks of 25[OH]D₃ supplementation, significant improvements in LV end-diastolic and systolic diameters, ejection fraction, and myocardial performance index together with significant improvements in serum 25[OH]D status were observed compared to the placebo group (Shedeed, 2012). In the current study, none of the athletes were taking vitamin D supplementation at the time of screening. While the aim of this investigation was not to examine whether athletes should supplement with 25[OH]D to augment cardiac structure, future double-blind placebo-controlled studies should look to examine if 25[OH]D supplementation increases cardiac size in healthy athletes, especially in severely deficient and deficient cohorts.

8.7 Limitations

While we acknowledge that cardiorespiratory fitness, training volume, and intensity were not recorded, athletes were only included in the study if they competed at national level and trained for more than 6 h/week. It is felt that the logarithmic transformation adjustments of 25[OH]D for

age, body surface area, ethnicity, and, importantly, athletic participation were satisfactory to distinguish cardiac morphological differences between and within athletes and controls.

8.8 Conclusion

Severely 25[OH]D-deficient athletes (<10 ng/ml) present significantly ($p < 0.05$) smaller Ao diameter, LAd, IVSd, LVIDd, LVM, LVvolD, and RA area than insufficient (20–30 ng/ml) and sufficient (>30 ng/ml) athletes. Furthermore, following logarithmic transformation adjusting 25[OH]D for age, body surface area, ethnicity, and athletic participation, positive associations between 25[OH]D and IVSd, LVIDd, PWTd, LVM, LVvolD were observed in athletes but not in control participants. Future research should look to identify the precise mechanism(s) causing cardiac hypertrophy with increases in serum 25[OH]D in healthy athletes.

Chapter 9:

General Discussion

9.1 Realisation of Aims

9.1.1 Aim 1 - *Examine which modality of 25[OH]D supplementation was more efficacious at improving serum 25[OH]D status in an ethnically diverse athlete population.*

This aim was addressed in Chapter 4. Athletes presenting with total serum 25[OH]D ≤ 30 ng/mL were supplemented with either oral Cholecalciferol D3 [2000IU/d] or a single intramuscular Ergocalciferol D2 injection [300,000 IU] and reassessed at 2-months. Both modes of supplementation increased serum 25[OH]D concentrations (Oral; 25.8 ± 0.9 vs. IM; 24.7 ± 0.6 ng/mL); from baseline with no significant difference in mean 25[OH]D status between treatments at 2-months. However, a significantly greater number of athletes became sufficient (>30 ng/mL) at 2-months following Oral vs. IM supplementation. Suggestive that supplementation with oral Cholecalciferol D3 may be more efficacious at improving 25[OH]D insufficiencies than IM injections of 25[OH]D2. The findings from this study had clinical implications that resulted in the production and implementation of a new vitamin D supplementation pathway (Chapter 4, Figure 4.1).

9.1.2 Aim 2 - *Investigate the relationship between serum 25[OH]D concentrations and measures of bone mass weight-bearing athletes*

Aim 2 was addressed in Chapter 5. Nine hundred and fifty weight bearing athletes and 436 controls undertook bone densitometry and body composition analysis by dual-energy x-ray absorptiometry and serum 25[OH]D evaluation. After adjustment for age, anthropometry, ethnicity, and athletic participation, there was no association between 25[OH]D and BMD and T-score at any site (spine, neck, or hip total). African and Caucasian athletes presented with greater ($p < 0.05$) BMD and T-scores at the spine, neck, and hip total than those of Asian, GCC, Middle Eastern, and Persian

ethnicities. Although 57% were considered to be vitamin D deficient or severely deficient (<20ng/mL), no athletes presented with a T-score suggestive of osteoporosis (-2.5 SD) at any site or osteopenia (-1.0 SD) at hip total. These findings suggest that the osteogenic effect of impactful weight-bearing exercise is sufficient to maintain markers of bone mass, irrespective of 25[OH]D status. Therefore, it was necessary to explore these associations in non-weight bearing athletes.

9.1.3 Aim 3 - *Examine the relationship between serum 25[OH]D concentrations and markers of bone mass in non-weight bearing, Arabic athletes.*

This aim was addressed in Chapter 6. Findings from the previous study demonstrated no association between markers of bone mass and serum 25[OH]D concentration. We postulated that the osteogenic effect of weight bearing exercise may be sufficient to maintain markers of bone mass, irrespective of serum 25[OH]D concentration in adults. Exercise (loading) is associated with increased BMD, whilst increased body mass contributes to the process of bone remodeling, forming mechanically appropriate bone structure(s). Therefore Aim 3 sought to examine the relationship between serum 25[OH]D concentrations against markers of bone mass (BMD and T-score) in a non-weight bearing Arabic athletic population.

Despite a combined 25[OH]D deficiency and severe deficiency prevalence rate of 69%, no association was observed in serum 25[OH]D concentrations between athletes with clinically normal T-score (n=76) and those presenting with osteopenia (-1 to -2.5 SD) (n=20) or osteoporosis (-2.5 SD) (n=6) at any location (neck, hip, spine or total). However, mean body mass was lower ($p<0.05$) in athletes with a T-score suggestive of osteopenia/osteoporosis. Thus supporting the hypothesis that 25[OH]D concentrations were not associated with markers of bone mass (BMD) in non-weight bearing Arabic athletes. Together with the findings from Chapter 5, there is strong

evidence to support the notion that multiple mechanisms regulate mechanosensation in bone that are inextricably interdependent of serum 25[OH]D concentration.

9.1.4 *Aim 4 - Evaluate the role of bioavailable vitamin D as a predictor of bone mass within a large cohort of healthy athletes*

This aim was addressed in Chapter 7. Six hundred and four male athletes [Arab (n=328), Asian (n=48), Black (n=108), Caucasian (n=53) & Hispanic (n=35)] undertook measures of total 25-hydroxyvitamin D, vitamin D-binding protein, and bone mineral density (BMD) by (DXA). Bioavailable vitamin D was calculated using free hormone hypothesis. After adjusting for age and ethnicity, bioavailable vitamin D was associated with spine (P=0.001), neck (p<0.001) and hip BMD (p<0.001), with no association observed between serum 25[OH]D concentration and BMD at any site (p>0.05). These findings allow acceptance of the hypothesis that bioavailable vitamin D is a better predictor of bone mass than serum 25[OH]D.

9.1.5 *Aim 5- Identify the associations between 25[OH]D status and cardiac structure and function within a large cohort of healthy athletes.*

This aim was addressed in Chapter 8. Five hundred and six athletes and 224 controls undertook cardiac structure and function assessment via physical examination, 12-lead electrocardiogram and echocardiogram in addition to serum 25[OH]D concentration. Severely 25[OH]D-deficient athletes present significantly (p<0.05) smaller cardiac structure (aortic root, left atria diameters, intraventricular septum diameter (IVSd), left ventricular diameter during diastole (LVIDd), left ventricular mass (LVM), left ventricular volume during diastole (LVvold), and right atrial (RA) area) than insufficient and sufficient athletes. Following logarithmic transformation adjustment for

25[OH]D, age, body surface area, ethnicity, and athletic participation, positive associations were observed between 25[OH]D and IVSd, LVIDd, posterior wall thickness during diastole, LVM, and LVvolD in athletes but not in the control participants. However, there was no significant difference in any cardiac function was observed between athletes and control participants, or within athletes or control 25[OH]D participants. Although Aim 5 was addressed the precise mechanism for lack of hypertrophy in the 25[OH]D deficient remains unclear.

9.2 Overview of the experimental studies in this thesis

Pre-Competition Medical Assessment

Annual medical assessment, consisting of several components; including cardiac screening, DXA, and vitamin D evaluation.

Chapter 1.2.1: A high prevalence of vitamin D (25[OH]D) deficiency was identified



Chapter 4: Oral Vs. Intramuscular Vitamin D Supplementation for Treating Insufficient Athletes

Vitamin D supplementation improves mean 25[OH]D status (13.7ng/mL to 25.3ng/mL) regardless of modality (IM or Oral). The subsequent studies sought to examine the relationship between serum 25[OH]D concentrations and markers of bone mass in a large and ethnically diverse athletic population.



Chapter 5: No Association between Vitamin D Deficiency and Markers of Bone Mass in Athletes

25[OH]D status was not associated with markers of bone mass (BMD and T-score) at any site. The osteogenic effect of weight bearing exercise may be sufficient to maintain markers of bone mass, irrespective of 25[OH]D status in adults. Chapter 5 aimed to examine the relationship between serum 25[OH]D and measures of bone mass in non-weight bearing athletes.



Chapter 6: No Association between Vitamin D Status and Markers of Bone Mass in Non-Weight Bearing Athletes

No association was observed between serum 25[OH]D concentrations and markers of bone mass in non-weight bearing Arabic athletes. This study suggests 25[OH]D concentration is irrelevant in predicting bone mass in non-weight bearing athletes.



Chapter 7: Systematic serum 25[OH]D screening in athletes is pointless; We have been 'Bound' to the wrong assay all along!

Chapters 5 & 6 propose that serum 25[OH]D concentration is an inappropriate measure for predicting bone mass in an ethnically diverse athletic population, irrespective of exercise type (weight/non-weight bearing). Research suggests racial differences in manifestations for vitamin D and markers of bone mass do exist.

Chapter 7 aimed to elucidate if bioavailable vitamin D is a better predictor bone mass than total serum 25[OH]D in an athletic cohort of mixed ethnicity.

Bioavailable vitamin D was closely associated with bone mineral density, whereas no association was observed between serum 25[OH]D concentration and BMD at any site.



Chapter 8: Severely vitamin D-deficient athletes present smaller hearts than sufficient athletes

Aimed to examine the relationship between 25[OH]D status and cardiac structure and function within a large cohort of healthy athletes presenting for pre-competition medical assessment at our institution.

Severely 25[OH]D-deficient athletes present significantly) smaller cardiac structures than insufficient (20–30 ng/ml) and sufficient (>30 ng/ml) athletes.

9.3 General Discussion

Although vitamin D is strongly implicated in bone health, these papers contributed to the growing body of evidence that suggest serum 25[OH]D concentration is not associated with bone mineral density or T-scores in an ethnically diverse athletic population. It is speculated that vitamin D binding protein may be responsible for ethnic variations in serum 25[OH]D concentrations in the absence of any associated disease, such as osteoporosis. As discussed, observational studies demonstrate varying results between markers of bone health and vitamin D status. Serum 25[OH]D concentration is a commonly used measure and understood to be an accurate representation of vitamin D status, due to the difficult nature of measuring the active metabolite 1,25[OH]₂D. To date, research on vitamin D status in athletes has focused solely upon serum 25[OH]D and consequently, the current advice and guidelines given to athletes and clinicians treating vitamin D deficiency should be consider inaccurate. There increasing evidence to support the notion that DBP concentrations varying between ethnicities, which in turn impact on the quantity of bioavailable vitamin D (Goździk et al., 2011; Powe et al., 2013). Additionally, these data show no clinical relationship between bioavailable vitamin D and markers of cardiac structure, whereas a correlation between serum 25[OH]D was observed. It may be the case that these ethnic variations support the case for measuring DBP and bioavailable vitamin D.

Vitamin D deficiency is a pandemic and the classification for vitamin D status is based on current concentration ranges. The ranges used throughout this thesis are based the inverse relationship between serum 25[OH]D concentration and PTH, when 25[OH]D reaches 30-40ng/mL PTH levels plateau (Chapuy et al., 1997). Additionally, intestinal calcium transport increases by 45 to 65% when 25[OH]D rises from 20 to 32 ng/mL(R. P. Heaney, Dowell, Hale, & Bendich, 2003),

suggestive that 25[OH]D of 21 to 29ng/mL can be considered to indicate insufficiency (B. Dawson-Hughes et al., 2005).

It should be acknowledged that the latest evidence from the growing body of vitamin D research contributes to the revision of vitamin D status. Recently, Manson et al, highlighted that the vitamin D reference values set out by the US Institute of Medicine (IOM) (Ross et al., 2011) could be open to misinterpretation (Manson, Brannon, Rosen, & Taylor, 2016). The majority of vitamin D research uses serum 25[OH]D concentration ranges <20ng/mL as a cut-off for defining insufficiency. These references values are based on dietary reference intakes (DRIs) that comprise a series of estimates of a given nutrients needed by different groups of healthy people within a population. These may vary from country to country, (e.g. dietary reference values, (DRVs) in the UK vs. DRI in USA). The DRI is broken down into two separate values:

- Estimated Average Requirement (EAR), which is the median of the distribution of human requirements, reflects the most likely requirement for the population.
- Recommended Dietary Allowance (RDA), reflects the estimated requirement for people the top at the highest end of the distribution, 97.5% of the population requirements are below the RDA.

The EAR is 400IU/d D₃ corresponding to a serum 25[OH]D level of 16ng/mL(40nmol/L), whereas the RDA is 600-800IU/D₃ corresponding to 20ng/mL(50nmol/L) The RDA is only required by 2.5% of the population and this misconception may contribute to exaggerated prevalence of vitamin D deficiency. The goal of treatment for the clear majority of the population is to present above the EAR not the RDA. Additionally, the Tolerable Upper limit (TUL, the level at which there may be adverse effects) is >50ng/mL. If our institution used the EAR set by the IOM, 48.5% of the athlete population would be considered vitamin D deficient (Table 9.1).

Table 9.1 Distribution of vitamin D status of athletes screened at Aspetar based IOM guidelines for EAR, DRI and TUL

<i>25[OH]D concentration</i>	<i>N=</i>	<i>Percentage (%)</i>
<16 ng/mL	2619	48.5
16-20 ng/mL	775	14.4
20-50 ng/mL	1954	36.2
>50 ng/mL	52	1.0
	5400	100.0

Misdiagnosis of true vitamin D status may have adverse financial and healthcare consequences, due to the over use of screening and unnecessary use of supplementation. However, these updated concentration ranges would not alter the findings from this thesis as serum 25[OH]D concentration was not associated with bone mass (BMD or T-score) at any site, and athletes presenting with smaller hearts are would still be vitamin D deficient. However, these findings contribute to the debate that current concentration ranges are not applicable to all ethnicities.

9.4 Limitations

The primary limitation of this thesis is the use of dual energy X-ray absorptiometry (DXA), t-scores and z-scores in Chapters 5, 6 and 7 to markers of bone health. DXA uses a two-dimensional model to measure areal bone mineral density (aBMD, g/cm²), that accounts for about 70% of the variance in compressive bone strength. Thus, disregarding volumetric BMD, bone geometry and bone microarchitecture. The nature of this modality makes the measurement of true volumetric bone mineral density (BMD, g/cm³) of the cortical and trabecular compartments impossible (Rantalainen, Nikander, Heinonen, Suominen, & Sievanen, 2010). Additionally, BMD values are

based of comparison to the BMD of young, gender matched, healthy individuals to generate a T score. BMD of age-matched individuals with normal bone mass generates a Z score, that are mostly utilised in cases of severe osteoporosis. The World Health Organisation (WHO) categorisations (-1 and -2.5 indicates osteopenia, -2.5 or below signifies osteoporosis) were established in Caucasian, postmenopausal women. Therefore, may be limited in their application to a racially diverse, male athletic population.

Variations in the 3D microarchitecture of bone tissue compromise its structural integrity and strength. The measure of bone strength would be markedly improved by using high-resolution, peripheral quantitative, computed tomography (HR-pQCT) that provides three-dimensional volumetric analysis of bone microarchitecture (Macneil & Boyd, 2008). Allowing the analysis of bone mass, BMD, bone geometry, and estimated bone strength Therefore, these chapters are examining bone mass an element of, but not a complete measure of bone health.

A secondary limitation of this work is monoclonal antibody assay (R&D ELISA) and the equation adopted to calculate bioavailable D in Chapter 8. Future studies should address these limitations.

9.5 Practical recommendations

There are several practical recommendations that can be drawn from the findings of this thesis:

- Systematic screening for vitamin D status in an asymptotic, racially diverse athlete population is unnecessary.
- If testing is warranted, clinicians should use appropriate assays to calculate DBP and bioavailable vitamin D status rather than serum 25[OH]D.

- Prophylactic vitamin D supplementation (Oral 1000-2000IU/d D₃) is advised. All supplements must be batch tested by a recognised quality assurance program, such as LGC's world-class sports anti-doping laboratory.

9.6 Future Studies

The data presented here disprove the use of serum 25[OH]D concentration as a marker of bone mass and cardiac structure in a racially diverse athletic population. Thus, questioning which markers of vitamin D status are a true indicator or predictor for normal skeletal and cardiac health. Future studies should examine the association between bone health including aBMD, volumetric BMD, bone geometry and bone microarchitecture against bioavailable vitamin D concentrations, using monoclonal and polyclonal antibodies. Longitudinal, prospective trials over 10 years with 6 monthly follow ups are required to examine the association between bioavailable vitamin D and markers of bone health in both male and female participants of varying ethnicity. These trials should examine both healthy and pathologic athletes with the aim of establishing racially, specific concentration ranges for both bioavailable and serum 25[OH]D concentrations.

A final consideration for this field of research is whether bioavailable vitamin D is associated with markers of athletic performance such as, skeletal muscle function. Recent research demonstrates that current vitamin D classifications (IOM) are not meaningful for skeletal muscle function, at the whole tissue and single fiber scale of analysis. Defining new classification based on bioavailable vitamin D may provide a 'true' vitamin D status that is applicable to all ethnicities. However, conducting large scale studies to exam bioavailable vitamin D and markers of bone health, cardiac function and skeletal muscle function in ethnically diverse populations would be costly and complex.

9.7 Conclusion

The key findings from the thesis are; firstly, serum 25[OH]D concentrations are not associated with markers of bone health. Secondly, bioavailable vitamin D is a better preceptor of BMD than serum 25[OH]D concentration. Therefore, an important message from this paper from is that if testing is warranted, clinicians should use the appropriate assays to calculate DBP and bioavailable vitamin D concentration rather than total serum 25[OH]D in racially diverse athletic populations. Thirdly, severely 25[OH]D deficient athletes present with smaller cardiac structure than sufficient athletes. After further adjustment for athletic participation, positive associations between 25[OH]D and IVSd, LVIDd, PWTd, LVM, and LVvolD were observed in athletes, that was not maintained in the control participants.

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