Abstract

In Wales nearly 5% (160,000) of the population have been diagnosed with diabetes, and it is thought that there is another 66,000 undiagnosed cases (Diabetes UK). Vitamin D deficiency is also increasing in prevalence, and there is emerging evidence linking vitamin D deficiency to impaired β-cell function, insulin resistance, and glucose intolerance, all of which are central to the pathogenesis of type 2 diabetes (Song & Manson, 2010). Following NHS and University ethical approval Welsh domiciled participants (n = 116) with varying levels of glucose control attended the Department of Sport and Exercise laboratory three times over a twelve-month period to examine the relationship between vitamin D and glucose homeostasis. Significant associations were observed between 25(OH)D and measures of glycaemia, and a seasonal variation was observed in 25 hydroxyvitamin D (25(OH)D) in this Welsh population (summer 71.3 ± 23.8; winter 42.6 ± 23.8 nmol/l). Participants with normal glucose control (≤6 mmol/l) had significantly (U = 884.00, p = 0.03) higher 25(OH)D concentrations than those with abnormal glucose control (≥ 6.1 mmol/l). A randomised control trial failed to find an effect of vitamin D supplementation (2000 IU/day) and a fifteen-week cycling programme on measures of glycaemia and body composition in 36 healthy participants. There was a significant (23%) increase in vitamin D status in participants in the supplementation groups demonstrating the effectiveness of the dose administered. Combined with the body of evidence in this area (Pittas et al., 2007) the findings from this thesis provide some support for the potential role of vitamin D supplementation in the management and prevention of type 2 diabetes.
Chapter 1: Literature Review

1.1 Context

Worldwide it was estimated that in 2000 there were 171 million cases of type 2 diabetes (T2D), this figure is predicted to rise to 366 million by 2030 (Wild et al., 2004). T2D accounts for 90-95% of all cases of diabetes (Albright et al., 2000) and while it does have genetic determinants, it is estimated that nine out of ten cases are attributable to lifestyle choices such as participation in physical activity and diet (Hu et al., 2001). Vitamin D deficiency and T2D share many of the same demographic characteristics (elderly, dark skin, obesity) as well as many of the same risk factors (age, increased adiposity, and physical inactivity) (Saintonge et al., 2009). The prevalence of vitamin D deficiency is high in many parts of the world (Prentice, 2008) and there is evidence to suggest a resurgence of vitamin D deficiency in the UK (Callaghan et al., 2006; Prentice, 2008). Once only recognised for its role in bone health, vitamin D has now been linked to numerous health outcomes including T2D (Alfonso et al., 2009) with numerous observational studies having reported associations between vitamin D status and measures of glycaemia (Baynes et al., 1997; Chiu et al., 2004; Scragg et al., 2004). Furthermore, while supplementation studies have reported inconsistent findings do date, there is some evidence to suggest that correction of vitamin D deficiency results in improved glucose tolerance (Nikooyeh et al., 2010).

Increasing physical activity levels and weight loss are main the components of T2D prevention and management programs, however weight loss is difficult to achieve and maintain, it is therefore critical to identify more easily modifiable risk factors for the primary prevention of T2D (Peechakara & Pittas, 2008). Vitamin D
supplementation is a possible candidate as a preventative and protective therapy in relation to T2D.
1.2 Vitamin D

The molecule vitamin D was discovered in 1920 and was recognised as a nutritionally essential vitamin, at that time primarily for its role in calcium homeostasis in the intestine and bone. It was in 1932 that vitamin D's chemical structure was revealed and it was identified as a steroid hormone (Norman, 2006). Vitamin D is however only the raw material needed for the synthesis of its active metabolite 1,25 dihydroxyvitamin D$_3$ (1,25(OH)D) (Vieth, 2006). There are two forms of vitamin D: vitamin D$_3$ (cholecalciferol) and vitamin D$_2$ (ergocalciferol). Vitamin D$_2$ is present in some dietary plants and is produced commercially by irradiation of yeast to be used in the fortification and supplementation of foodstuffs (Thatcher and Clarke, 2011). Vitamin D$_3$ may be obtained from dietary sources (i.e. animal sources such as deep sea fatty fish, liver or egg yolk) although relatively few natural food stuffs contain vitamin D$_3$ so it is primarily formed endogenously in the skin via exposure to sunlight Ultraviolet (UV B 290-315 nm) through the photochemical conversion of 7-dehydrocholesterol (Zella & DeLucia, 2003).

7-dehydrocholesterol resides in the plasma membrane of the epidermal cells (Holick, 2003) and absorbs the UV B photons which results in the re-arrangement of double bonds causing the B ring to open forming pre-vitamin D$_3$ (Holick, 2002). This is then isomerised to vitamin D$_3$, at which point it is no longer sterically compatible to reside within the cells plasma membrane and is released into the extra cellular space (Holick, Tian, & Allen, 1995). Dietary vitamin D is incorporated into chylomicrons and then absorbed into the lymphatic system prior to being deposited into the venous circulation (Holick, 2002). Once vitamin D has entered the blood circulation it is bound to the vitamin D binding protein for transportation. Only two thirds of
dietary vitamin D binds to vitamin D binding protein compared to 100% of vitamin D formed in the skin, giving cutaneous vitamin D₃ a longer half-life in the circulation (Holick, 2002). The remaining vitamin D is deposited in the adipose tissue, although not in sufficient quantities to prevent seasonal variations (Levis et al., 2005).

Figure 1.1: Metabolic pathway of vitamin D (Zitterman, 2003).

Whether orally ingested or obtained via sunlight, vitamin D is biologically inert and requires two consecutive hydroxylations to form it’s biologically active form; 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃). Only a small quantity of lipophylic vitamin D is stored in adipose tissue (Wortsman et al., 2000) with the majority being directed to the liver (Holick, 2002) (by the enzyme 25-hydroxylase, or CYP2R1) for the first hydroxylation process forming 25 hydroxyvitamin D₃ (25(OH)D₃) (at carbon 25). The
second hydroxylation occurs predominantly in the kidney (carried out by the enzyme 1-a hydroxylase or CYB27B1) producing the final active metabolite; 1,25(OH)$_2$D$_3$ (at α position of carbon 1) and also 24,25 dihydroxyvitamin D an inactivated form (Mathieu & Badenhoop, 2005) (Figure 1.1). The renal hydroxylation process is tightly regulated being enhanced by parathyroid hormone (PTH), hypophosphataemia and hypocalcaemia and inhibited by 1,25(OH)$_2$D. The effects of vitamin D hormone, 1,25 (OH)$_2$D$_3$ are mainly exerted via the activation of the nuclear vitamin D receptor, which is part of the nuclear receptor super-family of ligand activated transcription factors (Mathieu & Badenhoop, 2005). 1,25(OH)D has a central role in maintaining calcium and phosphorus homeostasis and is an important mediator of bone mineralisation (Armas et al., 2004).

Emerging Roles of Vitamin D

Until the late 1970s vitamin D’s role in bone mineralisation was believed to be its only biological function; however vitamin D receptors have now been identified in a wide variety of other tissues including bone and kidney cells, skeletal, heart, and smooth muscle, intestinal epithelial cells, stomach, liver, skin keratinocytes and hair follicular cells, breast, pancreatic islets (β-cells), thyroid, parathyroid, adrenal and pituitary glands, immune cells, brain, prostate, ovaries and testes (Danescu et al., 2009). Vitamin D deficiency is associated with several diseases including cancer, cardiovascular disease (CVD), hypertension, stroke, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, periodontal disease, macular degeneration, mental illness, propensity to fall, chronic pain and diabetes (Cannell et al., 2008). In addition to the nuclear vitamin D receptor identified in numerous cells and tissues,
the presence of the enzymes (i.e. 1α-hydroxylase) required for its synthesis have been discovered (Overbergh et al., 2000) indicative of alternative non classical actions (Palomer et al., 2008). In addition to the effects of vitamin D on insulin secretion, it is also believed to have other roles in human biology including modulation of the inflammatory response, cell maturation and cell differentiation (Holick, 2004; Nagpal et al., 2005). The discovery of vitamin D receptor’s in genes with key roles throughout the immune system, and in genes with important functions in the pancreatic β-cells, underlines the importance of exploring vitamin D in the context of diabetes (Christakos et al., 2003; Mathieu & Gysemans, 2006).

**Vitamin D Status and Intake**

There is no consensus as to the optimal levels of 25(OH)D, although many experts (Ross et al., 2011) would define vitamin D deficiency by a 25(OH)D concentration of below 50 nmol/l (Table 2.1) (Holick, 2007). In 2011 the Institute of Medicine Committee reported that serum 25(OH)D concentrations of 40 nmol/l would cover the requirements of approximately half the population, and 50 nmol/l would cover requirements of 97.5% of the population (Ross et al., 2011).
Table 1.1: Serum 25-Hydroxyvitamin D [25(OH)D] Concentrations and Health.

<table>
<thead>
<tr>
<th>nmol/l</th>
<th>Ng/ml</th>
<th>Health status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 30</td>
<td>&lt; 12</td>
<td>Associated with vitamin D deficiency, leading to rickets in infants and children and osteomalacia in adults</td>
</tr>
<tr>
<td>30-50</td>
<td>12-20</td>
<td>Generally considered inadequate for bone and overall health in healthy individuals</td>
</tr>
<tr>
<td>≥ 50</td>
<td>≥ 20</td>
<td>Generally considered adequate for bone and overall health in healthy individuals</td>
</tr>
<tr>
<td>&gt; 125</td>
<td>&gt; 50</td>
<td>Emerging evidence links potential adverse effects to such high levels, particularly &gt;150 nmol/L (&gt;60 ng/mL)</td>
</tr>
</tbody>
</table>

Serum concentrations of 25(OH)D are reported in both nanomoles per liter (nmol/l) and nanograms per milliliter (ng/ml). 1 nmol/l = 0.4 ng/mL (Ross et al., 2011). In this thesis 25(OH)D concentration will be reported as nmol/l.

Many have argued that these values are underestimates and fail to consider the health benefits of vitamin D beyond those related to bone health (Heany & Holick, 2011). From an evolutionary, epidemiology, molecular and cellular biology perspective all the evidence indicates that serum concentrations of 25(OH)D of 70 nmol/l or more as being natural and necessary for any benefits to human health (Vieth, 2004; Bischoff-Ferrari et al., 2006). The Institute of Medicine (Ross et al., 2011) report concluded that there was adequate evidence in relation to the role of vitamin D (and calcium) in skeletal health (consistent with a cause-and-effect relationship), however evidence in relation to non-skeletal health outcomes (including cancer, CVD, autoimmune diseases and diabetes) was considered too inconsistent to provide a basis for determination of intake requirements.

Based on bone health the report concluded that the Recommended Dietary Allowance (RDA) for vitamin D should be 600 International Units per day (IU/day) for
ages 1 – 70 years, and 800 IU/day for those over the age of 71 years. The report concluded that there was a need for more randomised controlled trials (RCT). However Heany and Holick (2011) commented that the type of trials called for would be infeasible as they would require such low intake contrast groups with 25(OH)D concentrations below the Institute of Medicine recommendation which many researchers in the field still consider to be too low. Heany & Holick (2011) concluded that this purported “need” for RCTs would be simply unethical and leaves nutritional policy issues in permanent limbo. Consequently there is an on-going debate in relation to what should be the clinical cut-off point for vitamin D insufficiency, with a 25(OH)D concentration of 50 nmol or less being the most widely accepted value for when an individual should receive treatment (Need, 2006). There have however been calls to define vitamin D sufficiency based on numerous different 25(OH)D values (Table 2.2).

Table 1.2: Recommended cut-off values to define vitamin D sufficiency.

<table>
<thead>
<tr>
<th>Source</th>
<th>25(OH)D (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holick, 2007</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Bischoff-Ferrari et al., 2006</td>
<td>&gt; 75</td>
</tr>
<tr>
<td>Hollis &amp; Wagner, 2006</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>Vieth, 1999</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

When 25(OH)D levels fall below an optimum level there is a compensatory increase in parathyroid hormone (PTH) and calcium homeostasis is altered (Tai et al., 2008b). This seems to occur most predictably when the plasma 25(OH)D concentrations are less than 50 nmol/l (Need, 2006; Tai et al., 2008b). There are however limitations
when relying on PTH concentrations as they fluctuate in relation to time of day, renal function, physical activity, and diet (Bischoff-Ferrari et al., 2006). The other approach when exploring vitamin D status in relation to various bone outcomes has been to identify the concentration of serum 25(OH)D that maximises calcium absorption (Heaney, 2005; 2007). It has been shown that the calcium absorption efficiency from a standard meal improves once serum 25(OH)D levels increase from 50 nmol/l to 80 nmol/l (a plateau occurs above this level) (Heaney, 2005). This indicates that whilst below 80 nmol/l serum 25(OH)D may be rate limiting with regards to its classic physiological effect (absorption), above this value it appears circulating 25(OH)D is adequate and allows the system to regulate itself through other controls. Consequently it is reported that hyperabsorption of calcium is avoided despite high levels of 25(OH)D (Heaney, 2005).

However with the growing number of roles of vitamin D being recognised there is a need to identify different criterion by which to determine optimum 25(OH)D concentrations in relation to different conditions such as diabetes. With only limited data available to date, Hyppönen & Power (2006) identified a breaking point in the relationship between 25(OH)D and HbA1c. A steep linear increase in HbA1c was observed alongside decreasing 25(OH)D concentrations until 25(OH)D reached 65 nmol/l, at this point the relationship weakened. In one of the first studies designed to specifically try and identify a threshold effect of 25(OH)D on glucose metabolism Sorkin et al. (2014) also reported a significant association between 25(OH)D and measures of glycaemia when 25(OH)D concentrations were below 65 nmol/l but not above.
To provide clarity and assist care providers in Wales, the Welsh Endocrine and Diabetes Society have developed guidelines for the diagnosis and management of vitamin D deficiency (Table 2.3).

**Table 1.3:** Serum 25-hydroxyvitamin D concentrations, status and management.

<table>
<thead>
<tr>
<th>Serum 25-hydroxyvitamin D concentrations (nmol/l)</th>
<th>Status</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25 nmol/l (&lt;10 mcg/l)</td>
<td>Deficiency:</td>
<td>High dose treatment initially (3200 IU daily for 8-12 weeks), then long term maintenance treatment required (1600 IU/day).</td>
</tr>
<tr>
<td>25-50 nmol/l (10-20 mcg/l)</td>
<td>Insufficiency:</td>
<td>long term maintenance treatment (1600 IU/day)</td>
</tr>
<tr>
<td>50-75 nmol/l (20 – 30 mcg/l)</td>
<td>Healthy:</td>
<td>give lifestyle advice</td>
</tr>
<tr>
<td>&gt;75 nmol/l (&gt;30 mcg/l)</td>
<td>Optimal</td>
<td></td>
</tr>
</tbody>
</table>

It has been shown that vitamin D consumption in quantities up to 4000 IU/day are safe for adults (Vieth et al., 2004) with very few physicians having ever seen a case of vitamin D toxicity (asymptomatic hypercalcaemia) as it is exceedingly rare (Vieth, 1999). It has been suggested that most adults would have to take > 10,000 IU/day for several months or even years to induce toxicity. True toxicity occurs when hypercalcaemia goes undetected and calcifies the internal organs; in particular the kidneys. The majority of individuals with vitamin D toxicity will recover by simply stopping/reducing their intake (Cannell et al., 2008).
Sunlight and Vitamin D

Short regular exposure to unprotected sunlight is considered the best way to increase vitamin D status, although UV B from other sources such as tanning beds is also effective (Penckofer et al., 2010). Twenty minutes sun exposure (arms, legs and face) can produce the equivalent of 15,000 - 20,000 IU of vitamin D₃. However, there is individual variation as demonstrated in a recent study of Hawaiian surfers who were all exposed to at least 15 hours of sun per week. Their serum 25(OH)D concentrations varied from 27 up to 177 nmol/l (Hollis et al., 2007). Sub cutaneous synthesis of D₃ is influenced by several factors including the use of sun screen, skin pigmentation and the solar zenith angle (the angle between the local vertical and the position of the sun in the sky) (Webb, 2006). When the solar zenith angle is small all the photons in a beam fall on a small area as the solar radiation has a relatively short path length through the atmosphere with less chance of attenuation. Summer, noon and low latitudes are associated with smaller solar zenith angles. When the sun is low in the sky there is a larger solar zenith angle and consequently the radiation traverses a longer path through the atmosphere and the photons when they reach their destination surface are spread over a larger area. Early morning/late afternoon, winter and high latitudes are features of large solar zenith angles. Factors controlling the solar zenith angle are the motion of the earth rotating around the sun, and about its own axis in combination with position on earth’s surface, i.e. latitude, time of day and season (Web, 2006).

The majority of healthy young adults will have marked seasonal fluctuations in serum 25(OH)D levels with higher levels in the summer compared to winter measurements (Zittermann, 2003). At the latitude of 52˚N, Aberystwyth in Wales is 52.4˚N, UV B
radiation of the sunlight is negligible from October to April (Zittermann, 2003). There is a limit to the amount of pre-vitamin D$_3$ that forms in the skin and once this limit is reached only inert isomers increase. Vitamin D$_3$ is photolabile and may be broken down by sunlight (UV B and UV A wavelengths) and as such continued exposure may be counterproductive. It takes several hours for pre-vitamin D$_3$ to be isomerised to vitamin D$_3$ (Webb, 2006).

Adipose tissue may also effect the availability of circulating 25(OH)D. When exposed to an identical dose of UV B radiation both obese subjects and none obese subjects had an increase in blood vitamin D$_3$ levels. Whilst obese subjects had a larger surface area, and so may be expected to have a larger increase in blood vitamin D$_3$ levels, the increase in obese subjects was actually 75% less than that of non-obese subjects (Wortsman et al., 2000). The content of 7-dehydrocholesterol and the percentage conversion of pre-vitamin D$_3$ and vitamin D$_3$ were similar between obese and non-obese subjects, and as such it seems plausible that the increased subcutaneous fat in the obese subjects may have sequestered more of the cutaneous synthesised vitamin D$_3$ (Wortsman et al., 2000). However, it has been suggested that some vitamin D stored in the adipose tissue may be released when required at a later date (Penckofer et al., 2010).

Prevalence of vitamin D deficiency

A European multicentre study measured 25(OH)D concentrations in 824 elderly people (December 1888 – March 1989) and observed that 47% of females and 36% of males have 25(OH)D concentrations below 30 nmol/l (Wielen et al., 1995). This
study is of value as it explores 25(OH)D concentrations in participants from 11 different countries (*i.e.* different latitudes) with standardisation of population, methodology and season. Whilst a previous review paper has reported (with a few exceptions) an inverse association between latitude and 25(OH)D concentrations, drawing parallels between the different studies is restricted due to lack of standardisation (Mckenna & Muldowney, 1985). For example comparisons were made between infirm populations living in care homes and consequently spending majority of time indoors (*i.e.* no sun exposure) (UK) and healthy (Finland) populations. Relatively few countries have nationally representative vitamin D data for their populations as estimated by 25(OH)D concentrations (Prentice, 2008). There is limited data available in relation to vitamin D status of people living in Britain (Stathopulu, 2002), although there is some evidence to suggest a resurgence of vitamin D deficiency (Callaghan et al., 2006; Prentice, 2008).

The elderly have been identified as a group at risk of vitamin D deficiency and there is emerging evidence that young adults and children are also at risk of vitamin D deficiency (Holick, 2007). Since 1992 The National Diet and Nutrition Surveys of the UK has included 25(OH)D measurements. The prevalence of vitamin D deficiency during 1992 and 2001, when defined as a 25(OH)D concentration of < 25 nmol/l, ranged between 5% and 20% in most age groups. This increased to 20 – 40% in specific sub-groups including: both males and females aged 19 – 14 years, females over 85 years old, children of British Asians and care home residents (Prentice, 2008). When > 50 nmol/l was used as the threshold for sufficiency the prevalence was 20 – 60% throughout the population, but exceeded 75% in British Asian children, young adults and the elderly. When 80 nmol/l or above was used approximately 90% of the UK population fell below this level (Prentice, 2008).
1.3 Type 2 Diabetes (Pathogenesis, Diagnosis and Treatment)

Globally there is an increasing number of cases of T2D and worldwide it is now one of the most common chronic diseases, affecting 285 million adults (Shaw, Sicree, & Zimmet, 2010). The term diabetes describes a metabolic disorder that is characterised by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism and is caused by defective insulin secretion, insulin action or both. The condition may present with symptoms such as intense thirst, polyuria and blurred vision. Diabetes is a complex metabolic disorder affecting numerous organs and systems within the body and can devastate the lives of affected individuals (Khuwaja et al., 2010). Extreme symptoms, if left untreated, include ketoacidosis or a non-ketotic hyperosmolar state that may lead to coma or death. The long term effects of diabetes can include the progressive development of several conditions including; CVD, specific complications of retinopathy with potential blindness, neuropathy with risk of foot ulcers, amputation, charcot joints, and features of autonomic dysfunction including sexual dysfunction and nephropathy which can lead to renal failure (Alberti & Zimmet, 1998).

Pathophysiology of T2D

Initially in the development of T2D insulin resistance may be well established whilst glucose tolerance remains normal due to the compensatory increase in insulin secretion. Further on in the development of T2D, β-cell dysfunction will however prevent the compensatory increase in insulin secretion resulting in hyperglycaemia (Kahn, 2003). In a non-diabetic state, insulin secretion from the pancreas would reduce glucose output by the liver, enhance glucose uptake by skeletal muscle, and
suppress fatty acid release from fat tissue. Once β-cell dysfunction has developed, and insulin secretion compromised, hepatic glucose output is maintained while uptake by skeletal muscle is reduced. In diabetes, hyperglycaemia during a fasted state is directly related to hepatic glucose output while in the postprandial state the dictating factors are ineffective suppression of glucose output and defective stimulation by insulin of target tissues, primarily skeletal muscle (Inzucchi et al., 2012). Figure 2.2 shows the various factors that contribute to the pathogenesis of T2D as a result of compromised secretion and action of insulin, leading to increased circulating fatty acids and hyperglycaemia (Stumvoll, Goldstein, & van Haeften, 2010).

In T2D the main determinant of hyperglycaemia is this functional incompetence of the β-cell. A further abnormality is the pancreatic α-cells hypersecrete glucagon, which further promotes hepatic glucose production. β-cell dysfunction is not necessarily irreversible and enhancing insulin action by an intervention that improves glycaemia, relieves β-cell secretory burden thus ameliorating β-cell dysfunction to a certain extent (Inzucchi et al., 2012). It has been suggested that the deterioration in β-cell function may be evident up to 10-12 years prior to diagnosis with T2D, with a 50% loss of pancreatic β-cell function observed in individuals upon diagnosis (Piero Marchetti et al., 2009).
In addition to β-cell dysfunction, a prominent feature in the many of T2D patients, particularly the obese, is insulin resistance in target tissues (myocardium, adipose tissue, muscle and liver) which results in glucose over production and underutilisation. The increased delivery to the liver of fatty acids then favours their oxidation contributing to increased gluconeogenesis, with the absolute overabundance of lipids promoting hepatosteatosis (Inzucchi et al., 2012).

Whilst β-cell dysfunction and insulin resistance are central abnormalities of T2D, the underlying aetiology in many cases is unclear. Many lines of research have been directed towards the role of the inflammatory response in the development of T2D.
(Pickup et al., 1997). A number of biochemical and clinical features are commonly observed simultaneously in patients with T2D; obesity (particularly central or abdominal obesity), accelerated atherosclerosis, hypertension and dyslipidaemia characterised by an elevation in serum total, and very low density lipoprotein (VLDL) cholesterol and a reduction in high-density lipoprotein (HDL) cholesterol concentrations. One of the factors proposed to mediate an increase in insulin resistance is inflammation such as that seen in obesity (Teegarden & Donkin, 2009). This clustering of CVD risk factors has been referred to as the metabolic syndrome X, the metabolic syndrome or the insulin resistance syndrome (Pickup et al., 1997).

**Diagnosis**

The accepted method of diagnosing diabetes has been by oral glucose tolerance test (OGTT) (previously a FPG of ≥ 7.0 mmol/l or a two hour post glucose load value of ≥ 11.1 mmol/l). Recently, however glycated haemoglobin (HbA1c) has been recommended as a tool for the initial diagnosis of diabetes. Diagnosis should always be confirmed with a second test unless plasma glucose concentrations of ≥ 11 mmol/l and clinical symptoms are present (World Health Organisation, 2011). The proposed HbA1c value for T2D diagnosis is 48 mmol/mol (6.5%). HbA1c reflects average glucose control over the previous eight to twelve weeks and can be performed at any time of the day with no requirement for fasting. Both HbA1c and fasting plasma glucose (established diagnostic measure) have a similar relationship with the prevalence of retinopathy (McCance et al., 1994).
Treatment

In treating patients with T2D one of the primary goals is to reduce the incidence of microvascular disease, to do this the American Diabetes Association (ADA) ‘Standards of Medical Care in Diabetes’ recommends lowering HbA1c to less than 53 mmol/mol (< 7.0%) (Inzucchi et al., 2012). Guidelines for achieving this in most patients focus on attaining a mean plasma glucose concentration of 8.3 - 8.9 mmol/l, with fasting and pre meal values being maintained at approximately 7.2 mmol/l or less, and postprandial less than 10 mmol/l. With some of the more complex T2D cases less stringent goals (HbA1c 58 - 64 mmol/mol) are often considered by clinicians to be more appropriate and realistic.

Lifestyle interventions addressing food intake and physical activity levels are critical components of T2D management (Klein et al., 2004). Even modest weight reduction (5 - 10%) can contribute to improved glycaemic control and positively affect other cardiovascular risk factors. Tokmakidis et al. (2004) did however observe significant reductions in glucose (8.1%) and insulin (20.7%) areas under the curve following 4 weeks of training without any significant alterations in body mass, or body mass index (BMI).

Patients with T2D should aim for a minimum of 150 min/week of moderate activity (aerobic, flexibility and resistance training) (Boulé et al., 2001). It is recommended that at diagnosis where possible patients should be given the opportunity to engage in lifestyle changes prior to pharmacotherapy (usually metformin) (Inzucchi et al., 2012).
The most widely used first-line T2D treatment drug is the biguanide; metformin which works primarily by reducing hepatic glucose production. Sulphonylurea insulin secretagogues are the oldest oral agents and their actions are exerted by stimulating insulin release through the closure of ATP-sensitive potassium channels on the β-cell. Whilst often effective in controlling glucose levels, their use is associated with several side effects (weight gain and risk of hypoglycaemia). There are shorter acting secretagogues that work through similar mechanisms but require more frequent dosing. Thiazolidinediones (TDZs) are peroxisome proliferator-activated receptor γ activators, which reduce hepatic glucose production and improve insulin sensitivity in skeletal muscle. Again they have associated side effects (including weight gain, fluid retention and increased risk of bone fracture) but are more durable in their effect than metformin and sulfonylureas. More recently drugs focusing on the incretin system have been introduced; the injectable GLP-1 receptor agonists that work by mimicking the effects of endogenous GLP-1 (stimulating pancreatic insulin secretion in a glucose dependant fashion, suppressing pancreatic glucagon output, slowing gastric emptying and deceasing appetite) (Inzucchi et al., 2012).

1.4 Vitamin D and Glucose Control

The evidence suggests that the role of vitamin D in preserving glucose tolerance is exerted via its effects on insulin secretion and insulin sensitivity (Alvarez & Ashraf, 2010). As discussed in Section 2.2, insulin resistance and β-cell dysfunction are both central to the development of T2D. Figure 2.3 illustrates the proposed mechanisms by which vitamin D may affect the pathophysiology of T2D via its actions on insulin resistance and β-cell dysfunction.
Figure 1.3: Hypothesized mechanisms underlying the interrelationships among vitamin D deficiency, insulin homeostasis and T2D (Song & Manson, 2010).

It is now recognised that there are vitamin D receptors in the pancreatic β–cells and vitamin D dependant calcium binding proteins in pancreatic tissues (Palomer et al., 2008). Numerous animal and in vitro studies have demonstrated the functional role of vitamin D in relation to insulin secretion and insulin sensitivity. For example in rat pancreatic β-cells an in vitro study demonstrated how 1,25(OH)₂D induced the biosynthesis of insulin (Bourlon & Billaudel, 1999). Vitamin D may directly affect β-cell function via 1,25(OH)D binding to vitamin D receptors in β-cells (Norman et al., 1980) and it may also have an indirect effect on calcium dependant insulin secretion through the regulation of calcium transport through the β-cells (Norman, 2008).
There is evidence of a vitamin D dependant rise in intracellular calcium concentration through non-selective voltage dependant calcium channels (Palomer et al., 2008). Consequently one of the major mechanisms facilitating the effect of vitamin D on insulin synthesis and secretion could involve the β–cell calcium dependant endopeptidases producing the cleavage that enables the conversion of proinsulin to insulin 24, (Boucher, 1997; Chiu et al., 2004).

Chiu et al. (2004) explored the relationship between 25(OH)D concentration, β-cell function and insulin sensitivity accessed via the hyperglycaemic clamp technique in glucose tolerant participants. Using glucose tolerant subjects can be advantageous as abnormal glucose tolerance could adversely affect β-cell function and insulin sensitivity (Rossetti, Giaccari, & DeFronzo, 1990). Whilst Chiu et al. (2004) observed no independent effect of measured β-cell function, a subtle effect of 25(OH)D concentration on β-cell function was indicated by the inverted, and independent, relationship between 25(OH)D and plasma glucose concentrations. Chiu et al. (2004) suggested that by extrapolating from the findings of the study increasing 25(OH)D from 25 to 75 nmol/l insulin sensitivity may be improved by 60%. This improvement in insulin sensitivity could potentially eliminate the burden on the β-cells with a reversal of abnormal glucose tolerance. In glucose tolerant subjects β-cells compensate for insulin resistance in an attempt to maintain plasma glucose concentrations within a relatively narrow range. Subjects with the lower 25(OH)D concentrations had decompensated β–cell function accessed through higher plasma glucose concentrations compared to subjects with higher 25(OH)D concentrations. It has also been identified that the effect of 25(OH)D concentrations on β-cells is continuous; the lower the 25(OH)D concentration, the more adverse the effect on β-cell function. In addition to the role of calcium in insulin exocytosis it is necessary for
β–cell glycolysis, which is involved in signalling circulating glucose concentration (Boucher, 1997). The 60% improvement in insulin sensitivity reported by (Chiu et al., 2004) is indicative of the potential potency of vitamin D, if compared to available anti-diabetic drugs; troglitazone (54%) or meformin (13%).

Insulin resistance is increased by systemic inflammation, and T2D and obesity are conditions of increased inflammatory reaction. Through its immunomodulatory and anti-inflammatory effects, it is suggested that vitamin D may reduce the insulin resistance in these conditions. Both 1,25(OH)$_2$D and its less calcemic analog 1,24(OH)$_2$D have been shown to suppress TNFα expression in macrophages by increasing IkBα and decreasing NFkB activity (Cohen-Lahav et al., 2007). The immunomodulatory properties of 1,25 (OH)$_2$D$_3$ include the enhancement of function such as chemotaxis and phagocytosis, and reduction of T cell activation, MHC class II expression, and reduction of surface expression of co-stimulatory molecules (Xu et al., 1993). In a T2D population when monocytes from T2D patients were stimulated with IFN-g in the presence of 1,25(OH)$_2$D$_3$, there was a down regulation in the expression of IL-6, IL-1, IL-8 and TNF-α (Giulietti et al., 2007). It has been suggested that elevated cytokines may have a role to play in β–cell dysfunction by triggering β–cell apoptosis (Pittas et al., 2007).

There is an inverse relationship between 25(OH)D and PTH, and it is well documented that individuals with hyperparathyroidism have an increased prevalence of insulin resistance and diabetes (Boucher, 1997; Scragg et al., 2004). A correction in abnormal glucose tolerance and insulin resistance has frequently been observed following parathyroidectomy (Palomer et al., 2008). One of the consequences of increased PTH is a disproportionate increase in calcium (The ‘calcium paradox’).
which is one of the mechanisms proposed by which PTH may increase insulin resistance (Palomer et al., 2008). Therefore, vitamin D may have a beneficial effect on insulin action either directly, by stimulating the expression of insulin receptors and thereby enhancing insulin responsiveness for glucose transport, or indirectly via its role in regulating extracellular calcium and ensuring normal calcium influx through cell membranes and adequate intracellular cytosolic calcium pool. Calcium is also essential for insulin mediated intracellular processes in insulin responsive tissues such as skeletal muscle and adipose tissue (Ojuka, 2004; Wright et al., 2004). Changes to calcium levels in primary insulin target tissues may contribute to peripheral insulin resistance (Draznin et al., 1987; Zemel, 1998), via impaired insulin signalling transduction leading to decreased glucose transporter 4 (GLUT-4) activity (Zemel, 1998).

Evidence from Cross Sectional Studies; Vitamin D and Glycaemia

A number of cross sectional studies have shown inverse associations between serum 25(OH)D concentrations and the presence of T2D or measurements of glycaemia in a variety of different populations (Baynes et al., 1997; Chiu et al., 2004; Scragg et al., 2004; Ford et al., 2005; Need et al., 2005; Forouhi et al., 2008; Hyppönen & Power 2006; Cigolini et al., 2006; Scragg et al., 1995). This relationship has not been consistent (Wareham et al., 1997; Snijder et al., 2006). The National Health and Nutrition Examination Survey (NHANES) provided the first large data set in relation to serum 25(OH)D concentrations in a non-institutionalised U.S population (Looker et al., 2002). When reporting on data from the NHANES Ford et al. (2005) observed an inverse association between 25(OH)D and metabolic syndrome; waist
circumference > 102 cm males, > 88 cm in females; hypertriglyceridemia: ≥ 1.69 mmol/l; low HDL cholesterol: < 1.04 mmol/l males, < 1.29 mmol/l females; blood pressure (BP): ≥ 130/85 mmHg; and FPG ≥ 6.1 mmol/l in 8,421 U.S adults (age ≥ 20 years). This association did not differ among three major racial or ethnic groups (white, African American, Mexican American) (p = 0.37) or between males and females (p = 0.72).

When reporting on the same data set, though this time exploring vitamin D and diabetes (FPG ≥ 7 mmol/l or 2 hour ≥ 11 mmol/l) Scragg et al. (2004) excluded participants with diagnosed diabetes, missing 25(OH)D data and/or glucose values, BMI data or were of ‘other’ nationality (not non-Hispanic White, non-Hispanic black, and Mexican-American adults). They reported an inverse association between quartiles of 25(OH)D and odds of diabetes in non-Hispanic whites and Mexican Americans but not in non-Hispanic blacks. A decreased sensitivity to vitamin D, and/or related hormones such as PTH, has been suggested as a reason for the absence of a relationship between 25(OH)D and diabetes in non-Hispanic blacks. Snijder et al. (2006) suggested that ethnic variations could account for some of the inconsistencies reported in relation to 25(OH)D and glucose tolerance. Many studies that include different ethnic groups have not reported on separate analyses and there remains uncertainty.

In patients with impaired glucose tolerance and newly diagnosed diabetes serum 25(OH)D was lower than in a control group (n = 5677) (Scragg et al., 1995). Targher et al. (2006) also observed lower 25(OH)D concentrations in participants with T2D when compared with controls (390 per group). Whilst these studies add to the body of literature in this area concerns have been raised (Scragg et al., 1995) in relation to
exploring vitamin D status in those with diagnosed T2D. As these studies provide information on 25(OH)D measured after diagnosis this could potentially be attributable to a reduced ability to metabolise vitamin D in T2D. However, Liu et al. (2009) has also observed an inverse association with fasting glucose and fasting measures of insulin resistance (HOMA-IR) in a healthy population. A positive correlation has also been reported between 25(OH)D concentration and insulin sensitivity in 126 glucose tolerant subjects living in California (Chui et al., 2004). It is still uncertain whether low levels of 25(OH)D cause impaired glucose tolerance and T2D or whether they are a consequence of the disease process (Scragg et al., 1995).

When exploring 25(OH)D concentrations in a 142 elderly Dutch men with varying levels of glucose control (13% IGT, 6% newly diagnosed T2D and 81% normoglycaemic) Baynes et al. (1997) observed an inverse relationship between 25(OH)D and 1 hour area under the curve (AUC) OGTT. They also reported increasing insulin concentrations with decreasing 25(OH)D concentrations during the OGTT, although this was only significant when cases with diabetes ($n = 8$) were removed. An inverse association may appear to contradict the hypothesis that optimum vitamin D concentrations are required for beta cell synthesis of insulin, however this is a complex area. These results suggest that those with higher levels of 25(OH)D secrete less insulin as they may be more sensitive to the actions of insulin.
Seasonal Variations in Vitamin D and Measures of Glycaemia

Seasonal variations have been observed in numerous human physiological and pathophysiological processes in healthy individuals and in those with chronic diseases (Tseng et al., 2005; Garde et al., 2000). It is well established that there are seasonal variations in 25(OH)D concentrations with lower levels reported during the winter months (Maxwell, 1994; Hyppönen & Power, 2007) (Figure 2.5). Seasonal variations in the incidence of T2D have been shown with several possible contributing factors including; infection, dietary changes, changes in physical activity patterns and climatic factors (Gamble & Taylor, 1969). Seasonal variation in 25(OH)D is now also considered as a possible contributory factor.

In 1985 Mortensen reported seasonal variations in HbA1c values, and since then several studies have observed seasonal fluctuations (higher levels being reported during the winter) in insulin and glucose values (Tseng et al., 2005; Suarez & Barrett-Connor, 1982; Macdonald et al., 1987; Behall et al., 1984). Numerous factors have been suggested such as the excess of food consumed in the US during the winter holidays. However when exploring risk factors for CVD, lipids demonstrated seasonal variation independently of physical activity, BMI, diet, age or gender (Hentschel & Rassoul, 2001). The seasonal variation that has been frequently reported in glycaemic control (control being worse during the winter months) supports the association of T2D (Ishii et al., 2008; Zittermann, 2003).

Tseng et al. (2005) suggested that in their population of 285,705 US diabetic veterans the seasonal fluctuations in HbA1c could have been influenced by temperature (25(OH)D was not measured). There is some uncertainty in relation to the long term effects of seasonal variations on glycaemic control and it has been
suggested that this may not be fully reversible (Chen et al., 2004). This would mean that the cumulative effect of increased HbA1c values during the winter is likely to contribute to the increased HBA1c values in participants with T2D over the years. Although in contrast to this Tseng et al. (2005) reported a linear decrease in HbA1c values over a two year period (Figure 2.4). This could potentially be due to numerous contributing factors including differences in quality of health care services or lifestyle factors. Another potential contributory factor for the decrease in HbA1c illustrated below (Figure 2.4) could be as a consequence of study participation, which is known to affect participant behaviour (Knekt et al., 2008).

The seasonal fluctuations in HbA1c illustrated below (Figure 2.4) are the mirror image of the seasonal variation observed in vitamin D status (25(OH)D (Figure 2.5); with higher levels of vitamin D during the summer and lower during the winter and lower HbA1c values in the summer and higher in the winter.
Figure 1.4: seasonal fluctuations in HbA1c in 285,705 US diabetic veterans over 2 years (Tseng et al., 2005).

Figure 1.5: Seasonal variation in vitamin D over 1.5 years in the 1958 British cohort (males $n = 3725$, females $n = 3712$) (Hyppönen & Power, 2007).
Temperature is one of the factors proposed to cause seasonal variations in HbA1c. Although the direct mechanisms by which temperature would affect HbA1c are unclear; it may be a physiological response to cold reflected by alterations in heart rate variability and BP (Kristal-Boneh et al., 2000; Sega et al., 1998). Tseng et al. (2005) reported on data from participants living in different parts of the US, whilst all had warm summers and cold winters, they had varying average temperatures. Interestingly they found the greatest HbA1c seasonal variations in participants living in moderately cold temperatures as opposed to those living in areas experiencing extreme cold winters and the greatest summer and winter variations. This suggests that it is not just local climate that affects health outcomes, but also people’s behavioural patterns in response to different climates. One of the reasons for their findings could be due to patients with diabetes remaining indoors in extremely cold climates and then being more likely to spend time outdoors in milder climates and thus experiencing greater exposure to cold temperatures (Tseng et al., 2005).

Simon et al. (1989) observed no seasonal fluctuation in HbA1c in 3,240 healthy non-diabetic adults living in the Paris area. Potentially this could be explained by the fact that the population studied were predominately office workers with low levels of physical activity and as such would not have spent adequate time outdoors being exposed to seasonal variations in temperature and sun exposure. Whilst seasonal variation may be partly attributable to decreased physical activity and increased calorie intake during the winter months (Ishi et al., 2008), there is emerging evidence suggesting a vitamin D effect on glycaemic control (Pittas et al., 2007). An individual’s vitamin D levels will vary depending on numerous factors, but the primary influence is sun exposure (Levis et al., 2005). When exploring seasonal variations in
vitamin D much of the work has been conducted at higher latitudes, which would be associated with weaker sun light during the winter months. Although not as large, seasonal variations in 25(OH)D concentrations have also been observed at low latitudes (Florida 25˚N) (Levis et al., 2005; Bolland et al., 2007).

Suarez et al. (1982) demonstrated a significant seasonal variation in fasting plasma glucose; 0.6 mmol/l higher during the winter months (4,541 participants). Fasting plasma glucose levels correlated with possible sun exposure \( (p = 0.03) \), and inversely with temperature \( (p = 0.04) \), these relationships were not altered by measured obesity or sex. Contradictory to the majority of evidence in this area, Bunout et al. (2003) conducted a 12 month physical activity and nutritional intervention study in a healthy elderly population, and reported increased insulin resistance during the warm summer months when compared to winter time measurements. The reasons for these contradictory findings are unclear, although the authors speculated that it may be linked to increased activity of adipose tissue lipoprotein lipase (indicated by lower triacylglycerol) during the winter months, which is dependent on insulin action and therefore may be associated with lower insulin resistance (Shirai et al., 1999).

Fat mass and 25(OH)D concentrations have previously been shown to be inversely correlated, and participants with greater fat mass demonstrate smaller seasonal variations in 25(OH)D (Bolland et al., 2007). This could be due to vitamin D being fat-soluble and as such is sequestered in the adipose tissue (Wortsman et al., 2000; Bell et al., 1985). An additional contributory factor may be because overweight participants wear clothing that covers more of their skin and are less physically active which would translate to less time outdoors (Bolland et al., 2007).
examining the relationship between seasonal variations in HbA1c levels and body fat

Sohmiya et al. (2004) concluded that the most plausible explanation for seasonal variations in HbA1c concentrations may be due to the increase in insulin resistance during the winter months. They observed seasonal fluctuations in HbA1c values and body fat, but no change in body mass in 11 males (age 60 ± 10.1 years) with T2D.

Gerstl et al. (2008) reported a modest seasonal variation in HbA1c values in a combined longitudinal analysis on data from 27,035 children from 207 centres across Germany and Austria. They observed marginally better HbA1c values in August and September with a median HbA1c of 7.86% compared to January where HbA1c was 8.08% ($p < 0.0001$).

Many of the studies that have observed a seasonal variation in HbA1c have been conducted in locations with significant differences in temperatures between summer and winter. Higgins et al. (2009) explored HbA1c values in five locations (Melbourne, Singapore, Calgary, Edmonton, and Marshfield) in the southern hemisphere where there are only minimal differences between temperatures in the summer and winter. A seasonal variation was observed only for the Marshfield data, which out of the five countries is the one that has the greatest temperature difference between summer and winter.

Sakura et al. (2010) demonstrated seasonal variations in HbA1c values in 78.3% of 2,511 diabetic patients who visited an outpatient clinic in Tokyo over a ten-year period. An inverse association was observed between HbA1c levels and mean temperature with the lowest HbA1c levels in August (7.46%) and highest levels in March (7.69%; $p < 0.001$). Ishii et al. (2008) observed seasonal fluctuations in HbA1c in Japan (Fukushima) where they have very warm summers and cold winters. In 39 T2D patients (12 males and 27 females) mean HbA1c values were
approximately 5% lower in the summer than in the winter (6.42 ± 0.65% in July; 6.96 ± 0.90% in March, \( p < 0.01 \)).

While there are several potential mechanisms (e.g. changes in lifestyle and temperature) by which the seasons could affect glycaemic control in different populations (Sakura et al., 2010; Tseng et al., 2005). There is now mounting evidence of a vitamin D related mechanism exerted through sub-cutaneous synthesis of vitamin D as a consequence of time spent in sunlight. (Pittas et al., 2007; Ishii et al., 2008; Zittermann, 2003). One of the difficulties to date is that several of the studies exploring seasonal variations in glycaemic control have been examining the role of temperature or lifestyle (Sakura et al., 2010; Tseng et al., 2005) rather than sun exposure and/or other measures of vitamin D status. It has been suggested that physical activity could be a surrogate marker of sun exposure (Ardestani et al., 2011), and whilst seasonal changes in temperature will affect many health outcomes, it is also an indicator of sun exposure as warmer temperatures are usually associated lower latitudes and longer sunlight hours.

In Wales nearly 5% (160,000) of the population have been diagnosed with diabetes, and it is thought there’s another 2% (66,000) undiagnosed cases (Diabetes UK). There is a limited amount of data available in relation to seasonal variations in vitamin D and glucose control in Wales. Hyppönen & Power. (2007) did report a seasonal variation in vitamin D status in Wales, however their data for Wales were in fact combined with the data for the Midlands. Demonstrating evidence for seasonal variations in 25(OH)D in Wales Chan (2000) reported a seasonal variation in tuberculosis in a Welsh population, and an inverse association has been reported between tuberculosis and vitamin D concentrations. Alfaham et al. (1995) reported
that a significant proportion of Asian women living in Cardiff could be sub clinically deficient in vitamin D, however they failed to report the time of year and this population are an at risk group in relation to vitamin D deficiency.

*The Long-term Relationship Between Vitamin D and Glycaemic Control*

In a prospective longitudinal study that measured 25(OH)D concentrations at baseline, it was observed that 25(OH)D was inversely associated with glucose status and insulin resistance at the ten year follow up (Forouhi et al., 2008). Knekt et al. (2008) conducted a pooled analysis on two nested case-control studies (Aromaa et al., 1989; Reunanen et al., 1983) and reported similar findings (average follow up 22 years). They explored 25(OH)D concentrations in males and females separately, and they reported that males had higher 25(OH)D concentrations than females. In males the relative odds between the highest and lowest quartiles of 25(OH)D concentrations was 0.28 (95% CI = 0.10 - 0.81) (females; 1.14 (0.60 - 2.17) with a 82% lower risk of diabetes when compared to those in the lowest vitamin D quartile. Of note, many of the participants in this study had low baseline 25(OH)D concentrations and the study was conducted in Finland which has short periods of sunshine limiting the ability to generalise findings to populations with high levels of vitamin D longer sunlight exposure.

When Liu et al. (2010) developed a regression model to predict 25(OH)D in a subsample of the Framingham Offspring Study, they concluded that higher concentrations of 25(OH)D were associated with a reduced risk of developing T2D. Over a 7 year follow up individuals in the middle and highest tertile had a 30% and
40% reduced risk of developing T2D when compared with those in the lowest tertile of the predicted 25(OH)D value ($p = 0.03$). A limitation of this study was the use of a predicted serum 25(OH)D score, which only explained 26% of the variance in 25(OH)D in measured serum 25OHD levels. Mattila et al. (2007) observed that higher 25(OH)D concentrations were associated with reduced risk of T2D (17 year follow up) in 4,097 participants from the Mini Finland Health Survey. However this association was attenuated and was non-significant in the multivariate analyses. Whilst Mattila et al. (2007) did measure 25(OH)D concentrations they did not have confirmed diagnosis of T2D, instead their follow up of incidence of T2D was based on a nationwide registry of patients receiving medication reimbursement. Not all patients with T2D would receive reimbursement and some whilst diagnosed, would not be prescribed medication. Whilst several authors have reported that baseline 25(OH)D can be predictive of future insulin resistance and glycaemic status (Forouhi et al., 2008), these findings have not always been consistent. A post hoc analyses of a three nested case-control studies of breast cancer, colon cancer and fractures revealed no significant association between 25(OH)D and incidence of diabetes in 5,140 women (Robinson et al., 2011).

**Vitamin D Supplementation**

A number of analyses from large data sets provide support of an association between dietary vitamin D intake and abnormal glucose homeostasis, although they also indicate that dietary calcium may enhance the effects of dietary vitamin D (Teegarden & Donkin, 2009). Calcium and vitamin D supplementation may attenuate the development of insulin resistance and the progression of abnormal glucose
metabolism toward insulin resistance and type T2D (Teegarden & Donkin, 2009). In a post hoc analysis of double blind randomised controlled trial, vitamin D (700 IU) and calcium (500 mg/day) supplementation had no effect when fasting glucose concentrations were within normal limits (< 5.6 mmol/l; n = 222). However in those with fasting glucose concentrations above normal (5.6 - 6.9 mmol/l; n = 92) there was a reduction in fasting plasma glucose at 3 years compared with placebo (0.02 mmol/l v 0.34 mmol/l, respectively; p = 0.04), with a lower increase in HOMA-IR (Pittas et al., 2007). The effect of combined vitamin D and calcium supplementation in this study were similar in magnitude to the progression of fasting glycaemia seen in the Diabetes Prevention Program (Knowler et al., 2002) with intensive lifestyle intervention or metformin (Pittas et al., 2007).

Several cohort studies have reported associations between dietary calcium, vitamin D intake and reduced incidence of T2D (Liu et al., 2006). In 5,292 participants age ≥ 70 years, Avenell et al. (2009) found no evidence to confirm that vitamin D (800 IU alone or in combination with 1000 mg of calcium) could delay the development of T2D or reduce the need for medication in individuals with T2D. They did however conclude that with the observed confidence intervals the protective effects of vitamin D in relation to T2D could not be ruled out. 2.2% of participants supplementing with vitamin D reported developing T2D compared with 2.5% from the placebo group (adjusted odds ratio 1.11, 95% CI 0.77 – 1.62, p = 0.57).

Some of the earlier findings in relation to the effect of vitamin D supplementation in this area have been focused on participants undergoing dialysis as 1,25(OH)_{2}D_{3} deficiency is a common complication in patients undergoing dialyses, and evidence suggests that it is this deficiency and/or secondary hyperparathyroidism that results
in insulin resistance in this population (Kautzky-willer, 1995). Intravenous 1,25(OH)₂D₃ has been shown to improve insulin resistance in participants with chronic renal failure (Gunal et al., 1997; Kautzky-Willer et al., 1995). It has been difficult to differentiate between the effects of 25(OH)D and a reduction in PTH in some studies. However Mak (1998) observed a correction between vitamin D deficiency, glucose intolerance, insulin resistance and hypoinsulinemia in the absence of PTH suppression in 8 participants with uraemia.

To date vitamin D supplementation studies have reported mixed findings in relation to glucose homeostasis, with some reporting no effect (Fliser et al., 1997; De Boer et al., 2008), some a positive effect (Hurst et al., 2010; Asemi et al., 2013) and some even a negative effect. Beukel et al. (2008) reported an adverse effect of vitamin D supplementation on insulin sensitivity in HIV patients over six months. Interestingly, however when the dose was decreased from 2000 IU to 1000 IU/day, insulin sensitivity improved (returned to baseline values) over the subsequent 6 months. Orwoll et al. (1994) found no significant effect of vitamin D (200 IU/day) on their primary outcomes (Table 2.4), in 35 participants with T2D. They did however report a significant correlation between responses to 1,25(OH)₂D treatment and duration of diabetes in maximal insulin secretion (partial r² + 0.29, p = 0.05) and in integrated insulin secretion (partial r² + 0.28, p = 0.05 after stimulation), in that those with shorter term diabetes had the greatest response. These data support the notion that once the β-cells are exhausted vitamin D supplementation would no longer be useful. When Orwoll et al. (1994) supplemented with 1, 25(OH)₂D and failed to find an effect they discussed the form of supplementation as a potential reason for the absence of effect, as the pancreatic beta cell is able to produce 1,25(OH)D by an autocrine mechanism. However, based on more recent evidence in this area neither
the dose nor duration of supplementation were adequate to induce any changes in measures of glycaemia.

Patel et al. (2010) observed no significant effect of vitamin D on measures of glycaemia, serum adiponectin and lipid levels when pre values were compared to post intervention values in the experimental group or when compared to placebo group. Several potential reasons for this were highlighted, again the dose of vitamin D was considered too low; 400 IU/day. Additionally, there were only 24 participants and the participants had excellent glucose and lipid values at base line and as such it would have been difficult to improve them further. They also speculated that the absence of co-supplementation of calcium may have contributed to the null effect, as it has been suggested that the effects of vitamin D and calcium are additive. A recent study (Nikooyeh et al., 2011) exploring the effect of a yogurt drink fortified with vitamin D and/or calcium reported that the vitamin D fortified drink improved glycemic status in subjects with type 2 diabetes. Ninety diabetic subjects were randomly allocated to 1 of 3 groups; yogurt drink containing no vitamin D and 150 mg Ca/250 ml, vitamin D–fortified yogurt drink containing 500 IU vitamin D₃ and 150 mg Ca/250 ml, or vitamin D + calcium–fortified yogurt drink containing 500 IU vitamin D₃ and 250 mg Ca/250 ml. Participants receiving the vitamin D fortified yogurt drink and the vitamin D + calcium fortified yogurt drinks had significantly decreased fasting serum glucose \([-12.9 \pm 33.7 \text{ mg/dl} (p = 0.01) \text{ and } -9.6 \pm 46.9 \text{ mg/dl} (p = 0.03)]\), HbA1c \([-0.4 \pm 1.2\% (p = < 0.001) \text{ and } -0.4 \pm 1.9\% (p = < 0.001)]\), HOMA-IR \([-0.6 \pm 1.4 (p = 0.001) \text{ and } -0.6 \pm 3.2 (p = < 0.001)]\), waist circumference \((-3.6 \pm 2.7 \text{ and } -2.9 \pm 3.3; p < 0.001 \text{ for both})\), and BMI \([\text{kg/m}^2]: -0.9 \pm 0.6 (p = < 0.001) \text{ and } -0.4 \pm 0.7 (p = 0.005)]\) when compared to the calcium yogurt group. An inverse correlation was observed between changes in serum 25(OH)D₃ and fasting serum glucose (r =
-0.208, \( p = 0.04 \)), fat mass (\( r = -0.219, \ p = 0.03 \)), and HOMA-IR (\( r = -0.219, \ p = 0.01 \)). It has previously been difficult to differentiate between the effects of vitamin D and calcium. The results of this study however, demonstrate an effect of vitamin D supplementation, not only in the presence of co-supplementation with calcium but also alone. This is not to say that any vitamin D effects are not exerted through a calcium related physiological mechanism, but it is indicative of the importance of vitamin D intake.

Hurst et al. (2010) reported significant improvements in insulin sensitivity following vitamin D supplementation of 4000 IU/day for 6 months with no differences in insulin secretion (C-peptide). They observed the greatest improvements in insulin resistance when endpoint 25(OH)D was 80 nmol/l or greater and reported an optimum concentration of 80-119 nmol/l was required for glucose homeostasis. When supplementing with an identical dose (4000 IU/day) Harris et al. (2012) found no effect of vitamin D on measures of glycaemia (OGTT), and divergent effect on insulin secretion and insulin sensitivity. It is possible that the adverse effect on insulin sensitivity could be a spurious finding as a consequence of the difference between groups in that the effect was caused by the increase in the placebo group as opposed to the actual effect in the treatment group. Insulin secretion was increased in the supplementation group, although not enough to overcome the reduction in insulin sensitivity as evidenced by the neutral effect on glycaemia. Whilst the supplementation with 4000 IU/day did correct vitamin D deficiency potentially the 12-week duration may not have been an adequate period for any changes in glycaemia to become apparent. In addition, only 51% of the supplementation group attained 25(OH)D concentrations of 75 nmol/l or above and, Hurst et al. (2010) reported the greatest effect on glycaemia when levels were above 80 nmol/l.
Borissova et al. (2003) reported a significant 34% increase in first phase insulin secretion and a significant relationship between changes in first phase insulin secretion and 25(OH)D following one month of vitamin D supplementation with only 1332 IU/day. Whilst there was also a 21.4% decrease in insulin resistance, the change was non-significant, there were however only 27 female participants in the study. Considering that the main defects of T2D are insulin resistance and reduced first phase insulin secretion, the favourable effect of vitamin D on these outcomes has clinical relevance in relation to this condition.

A twelve month RCT (Davidson et al., 2013) supplementing vitamin D \((100 - \text{baseline serum 25-OHD}) \times \text{kg body wt} \times 15.7 = \text{IU/week}\) with the goal of raising 25(OH)D concentrations to 65-90 nmol/l observed no significant differences between groups (53 placebo; 56 vitamin D) in several measures of glucose control at 3, 6, 9 and 12 months. Whilst the sample size was relatively small, power calculations for this study showed there was a >95% power to detect a 1-SD change in BMI, fasting plasma glucose, 2-h glucose, or HbA1c levels between groups. A strength of this study includes the supplementation strategy, which focused on attaining a given concentration of 25(OH)D based on baseline measurements and it was also relatively long in duration. The authors do discuss that 12 months may not be adequate duration when exploring the development of T2D, however the Diabetes Prevention Program reported significant differences in development of diabetes between groups after one year. While Strobel et al. (2014) also observed no significant effect of vitamin D supplementation on measures of glycaemia they did report the lowest HbA1c values were observed when 25(OH)D was 50 nmol/l or less which is consistent with research in the area.
Table 1.4: Summary of research studies that have investigated the effect of vitamin D on glycaemia.

<table>
<thead>
<tr>
<th>Study</th>
<th>Population &amp; group numbers</th>
<th>Measures</th>
<th>Vitamin D: Dose &amp; study duration</th>
<th>Baseline 25(OH)D (nmol/l)</th>
<th>Sig results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strobel et al., 2014</td>
<td>T2D Vit D = 43 Pla = 43</td>
<td>FBG, FPI, PTH, HbA1c, HOMA</td>
<td>1904 IU/day 6 months</td>
<td>Mdn Vit D = 87 Pla = 50</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Asemi et al., 2013</td>
<td>Gestational diabetes</td>
<td>FPG, serum insulin, HOMA, Quantitative Insulin Sensitivity Checker Index</td>
<td>400 IU/day 9 weeks</td>
<td>Vit D = 44.4±3.2 Pla = 36.1±2.9</td>
<td>FPG ↓ in D – 0.65 &amp; ↓ pla -0.02 (interaction p =0.01)</td>
</tr>
<tr>
<td></td>
<td>Vit D = 27 Placebo = 27</td>
<td></td>
<td></td>
<td></td>
<td>Quantitative Insulin Sensitivity Checker Index ↑ in D 0.02 &amp; ↓ pla -0.02 (interaction p = 0.01)</td>
</tr>
<tr>
<td>Breslavsky et al., 2013</td>
<td>T2D Vit D = 24 Pla = 23</td>
<td>FPG, lipids, HbA1c, hs-CRP, adiponectin, leptins, HOMA-IR</td>
<td>1000 IU/day 12 months</td>
<td>Vit D = 32.1±26.4 Pla = 26.7±16.2</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Davidson et al., 2013</td>
<td>Prediabetes: Placebo n=53 Vit D n=56</td>
<td>OGTT, Glucose insulin; HOMA, AUC, FPIS, SPI, Insulinogenic index, HbA1c</td>
<td>(100 − baseline serum 25-OHD) × kg body wt × 15.7 = IU/week over 1 year. To raise serum 25(OH)D to 65-80 nmol/l. mean weekly dose of 88,865 IU</td>
<td>&lt;75</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Harris, Pittas, &amp; Palermo, 2012</td>
<td>Obese African American, Pre-early 2DM Placebo = 46 Vit D = 43</td>
<td>Insulin secretion, insulin sensitivity, OGTT</td>
<td>4000 IU 12 wks</td>
<td></td>
<td>Insulin sensitivity ↓ 4% in D &amp; ↑ 12% in pla (p =0.03). insulin secretion ↑ 12% in D &amp; 2% in pla (p =0.02)</td>
</tr>
<tr>
<td>Study</td>
<td>Population &amp; group numbers</td>
<td>Measures</td>
<td>Vitamin D: Dose &amp; study duration</td>
<td>Baseline 25(OH)D (nmol/l)</td>
<td>Sig results</td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Witham et al., 2010</td>
<td>2DM Placebo = 20 22 = low dose 19 = high dose</td>
<td>Endothelial function, BP B-type Natriuretic peptide, insulin resistance, HbA1c</td>
<td>Single dose 100 000 IU or 200 000 IU, or placebo. 16 weeks</td>
<td>&lt;50</td>
<td>B-type Natriuretic peptide ↓ in 200,000 IU group.</td>
</tr>
<tr>
<td>Hurst, Stonehouse, &amp; Coad, 2010</td>
<td>Insulin resistant south Asian women Placebo = 39 Vit D = 42</td>
<td>25(OH)D HOMA2, C peptide</td>
<td>4000 IU/day Placebo 6 months</td>
<td>&lt;50</td>
<td>insulin sensitivity ↑ (p=0.03), insulin resistance ↓ (p=0.02) &amp; fasting insulin ↓(p = 0.02). HOMA ↓ in D and ↑ in pla (p = 0.03).</td>
</tr>
<tr>
<td>Patel et al., 2010</td>
<td>2DM Vit D low =13 Vit D high = 11</td>
<td>FPG, HbA1c, QUICKI, serum lipid, adiponectin</td>
<td>400 IU 1200 IU 4 months</td>
<td>&lt;60</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Jorde &amp; Figenschau, 2009</td>
<td>T2D (metformin and bedtime insulins) Pla = 16 Vit D = 16</td>
<td>FPG, serum ins, HbA1c, calcium, PTH, 25(OH)D, 1,25(OH)D, total cholesterol, triglycerides, HDL, LDL</td>
<td>Cholecalciferol 40,000 IU/wk (2 x 20,000 IU capsules per week) 6 month</td>
<td>D = 60.0±14.0 Pla = 58.5±21.0</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Nagpal, Pande, &amp; Bhartia, 2009</td>
<td>Healthy males Vit D = 36 Pla = 35</td>
<td>HOMA, QUICKI, OGIS, lipids, BP</td>
<td>120 000 IU/2 wks 6 week</td>
<td>D = 36.5±14.5 Pla = 30.0±12.5</td>
<td>↑ OGTT insulin secretion (p = 0.03)</td>
</tr>
<tr>
<td>Beukel et al., 2008</td>
<td>20 HIV patients No placebo</td>
<td>25(OH)D, 1,25(OH)D, FPG, FPI, adiponectin, leptin, IL6, TNF-α</td>
<td>2000 IU 24 wks + 1000 IU 48 wks</td>
<td>MdN(IQR) 26.4(19.0-26.4)</td>
<td>24 wks ↑ insulin sensitivity (p = 0.01) No sig effect at 48 weeks (return to baseline)</td>
</tr>
<tr>
<td>Tai et al., 2008</td>
<td>33 vit d insufficiency No control</td>
<td>OGGT, PG, serum insulin,QUICKI, PTH, HOMA</td>
<td>100 000 IU twice 2 wks apart</td>
<td>&lt;50</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Study</td>
<td>Population &amp; group numbers</td>
<td>Measures</td>
<td>Vitamin D: Dose &amp; study duration</td>
<td>Baseline 25(OH)D (nmol/l)</td>
<td>Sig results</td>
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</tr>
<tr>
<td>Borissova et al., 2003</td>
<td>Females 2DM = 10, Control = 17</td>
<td>FPIS, SPIS, peripheral IR</td>
<td>Cholecalciferol 1332 IU/day 1 month</td>
<td>35.3±15.1</td>
<td>FPIS increased sig 35% Sig correlation between change in 25(OH)D and FPIS (p = 0.01)</td>
</tr>
<tr>
<td>Mak, 1998</td>
<td>Hemodialysis group 1 = 8, group 2 = 8</td>
<td>PTH, 1,25(OH)2D3, FG, ins, lipids, ca+, albumin</td>
<td>Intravenous 25(OH)D (cont group received dihydrotachysterol)</td>
<td>72±12</td>
<td>FPG &amp; lipid ↓, FPI ↑ (p = 0.05)</td>
</tr>
<tr>
<td>Boucher et al., 1995</td>
<td>22 high risk of T2D</td>
<td>Specific insulin, c-peptide, blood glucose, proinsulin, 32,33 proinsulin</td>
<td>Intravenous 100 000 IU 8-12 weeks</td>
<td>8.9±4.4</td>
<td>↑ c-peptide &amp; specific insulin (30 min OGTT) c-peptide sig correlation with 25(OH)D (r =0.12, p = 0.04)</td>
</tr>
<tr>
<td>Kautzky-willer et al., 1995</td>
<td>Hemodialysis vit D = 10</td>
<td>OGTT, ins, c-peptide, PTH</td>
<td>1,25 dihydroxycholecalciferol</td>
<td>Not reported</td>
<td>↓ PTH, insulin sensitivity index reached a value similar to control</td>
</tr>
<tr>
<td>Orwoll et al., 1994</td>
<td>35 (23 male) 2DM</td>
<td>FG, insulin, C-peptide, glucagon</td>
<td>200 IU 4 days</td>
<td>35±7</td>
<td>No sig effect</td>
</tr>
<tr>
<td>Raghuramulu et al., 1992</td>
<td>2DM = 42</td>
<td>OGTT, insulin, calcium, phosphorus, cholesterol</td>
<td>Intramuscular 300 000 IU 4 weeks</td>
<td>Not reported</td>
<td>↓ OGTT blood glucose &amp; insulin</td>
</tr>
<tr>
<td>Inomata et al., 1986</td>
<td>14 2DM</td>
<td>BG(OGTT), ca, phosphorus, lipids, ins, 1α(OH)D₃ 80 IU/day</td>
<td>Not reported</td>
<td></td>
<td>↑ Insulin response &amp; calcium, ↓ FFA.</td>
</tr>
</tbody>
</table>
Whilst daily oral doses of vitamin D are considered more effective, adherence can be a concern and an alternative can be single, weekly or monthly high doses of vitamin D. A single intramuscular dose of vitamin D (300 000 IU) resulted in improvements in the oral glucose tolerance and glucose stimulated increase in serum insulin levels after 4 weeks when compared to a placebo group in participants with T2D (Raghuramulu et al., 1992). Nagpal et al. (2009) and Tai et al. (2008a) drew inconclusive results, Nagpal et al. (2009) administered three 120 000 IU doses of vitamin D at two week intervals and reported that whilst there was an improvement in postprandial insulin sensitivity there were no significant changes in any of their secondary measures. Tai et al. (2008a) supplemented 100 000 IU of vitamin D twice 2 weeks apart also found no effect on glycaemia.

However when Boucher et al. (1995) administered a single vitamin D dose of 100 000 IU they reported a significant relationship between 25(OH)D and c-peptide and 25(OH)D and insulin at 30 minutes during an OGTT. When exploring individual responses to supplementation the greatest increases in insulin secretion (C peptide & specific insulin) were observed in those who already had highest concentrations at baseline (Figure 1.6). These findings highlight the importance of exploring vitamin D in participants with healthy glucose control and pre-diabetes as an early stage preventative therapy prior to severe β-cell dysfunction and impaired insulin secretion.
Figure 1.6: Individual changes in C-peptide and specific insulin (30 minute OGTT concentrations) pre and post vitamin D supplementation in 22 participants with no diabetes but considered high risk for diabetes (Boucher et al., 1995).

Jorde & Figenschau (2009) failed to find an effect of high doses (40,000 IU/week) of vitamin D over a six month period. There were potentially several factors that could have influenced these findings. Whilst the dose and duration were both adequate, none of the participants were deficient in vitamin D (< 25 nmol/l) at baseline, also participants were taking metformin and bed time insulin which may have masked the effects of the vitamin D. Whilst a dose response effect has been discussed in relation to vitamin D, there could be a cut-off point at which effects on glucose control may no longer be favourable. This was suggested by Beukel et al. (2008) who in the short term (first 6 months) found a higher dose of vitamin D (2000 IU/day) was associated
with an adverse effect on markers of glucose control, however when this dose was subsequently reduced to 1000 IU/day insulin sensitivity improved.

When exploring vitamin D in gestational diabetes a significant effect was observed on several measures of glycaemia with an improvement in plasma antioxidant capacity (Asemi et al., 2013). This study was only 9 weeks long with a relatively low dose of vitamin D (400 IU/day), and possibly there were significant effects on measures as the changes observed in gestational diabetes are transient and consequently more readily reversible. Oxidative stress is involved in the pathophysiology of several chronic conditions including T2D and gestational diabetes and whilst there are only limited data available in relation to vitamin D and oxidative stress it is believed to have antioxidant properties (Hamden et al., 2009).

There have been mixed findings in relation to vitamin D supplementation in relation to glycaemic control. To date inconsistent findings may be attributable to heterogeneous cohorts, unaddressed potential confounders and varying doses and durations of supplementation programs (Strobel et al., 2014). A recent review concluded that there was insufficient evidence to support a recommendation for vitamin D supplementation for improving glucose metabolism or insulin action in patients with pre-diabetes or established diabetes (George et al., 2012).

1.5 Physical Activity and Glucose Control

While the evidence in relation to vitamin D and T2D remains inconclusive (George et al., 2012), the role of physical activity in the prevention and management of T2D is well established (Kelley & Goodpaster, 2001). The Diabetes Prevention Program research group (Knowler et al., 2002) conducted a RCT involving 3,234 adults who
were considered a high risk group for developing diabetes. Participants were randomly allocated to either placebo, metformin (850 mg twice daily), or a lifestyle modification group, the goals of which included a minimum of a 7% weight loss and at least 150 minutes of physical activity a week. The average follow up was 2.8 years and the incidence of diabetes was reported to be 11.0, 7.4 and 4.8 cases per 100 participants in the placebo, metformin (31% reduction compared to placebo) and lifestyle modification (58% reduction compared to placebo) group respectively.

The exercise induced increase in insulin sensitivity has been extensively researched since it was first described by Ruderman’s group in 1982. Rogers et al. (1988) observed changes in insulin action after just one week of physical training, although this could be due to the acute effects of physical activity rather a training effect per se (Angelopoulos et al., 1998;). Exercise is thought to decrease insulin resistance in the peripheral tissues and alleviate the defect in insulin-stimulated glycogen metabolism in skeletal muscle (Perseghin et al., 1996). Ishii et al. (1998) observed a 48% improvement in insulin sensitivity following 4-6 weeks of moderate intensity, high volume resistance training in previously untrained non-obese individuals. Aerobic exercise training results in an increase in the conversion of low oxidative type IIb fibres to moderate oxidative type IIa fibres. Type IIa have a higher GLUT4 concentration, a greater capillary density, and they exhibit an increased response to insulin compared to type IIb fibres (Lillioja et al., 1998). While the resistance training intervention carried out by Ishii et al. (1998) had a positive effect it consisted of a relatively low resistance with a high volume of repetitions and, as such, had an aerobic element that could have contributed to the slight increases in capillary-to-muscle ratio observed in the study.
In 2007 Burgomaster et al. compared the effect of six weeks of high intensity training (HIT) and traditional endurance training and reported similar metabolic adaptations. McCoy, Proietto, & Hargreves (2013) have also reported that while HIT results in modest increases in GLUT4, 25% above baseline compared to > 100% observed following a traditional endurance training, the concentrations remained elevated for longer, 6 weeks compared to the 1-2 weeks after cessation of endurance training. One of the advantages of HIT is that it is considered by some to be a time effective mode of enabling the general public to meet physical activity guidelines for health (Babraj et al., 2009). Positive correlations have been observed between transient improvements in insulin sensitivity and exercise intensity in young people. This has been less explored in an older population, potentially because it is considered unlikely that an older population would be capable of performing higher intensity exercise due to the loss of aerobic capacity, fear of injury and existing chronic disease (ACSM, 2006). As such moderate intensity is considered a safe and more suitable option for an older population for numerous reasons including factors associated with adherence (DiPietro et al., 2006). However, when comparing a moderate intensity (65% \( \dot{V}O_2\text{peak} \)) and a high intensity (80% \( \dot{V}O_2\text{peak} \)) exercise program DiPietro et al. (2006) reported a greater effect on insulin sensitivity in older women following nine months of the higher intensity program; exercise volume was matched (300 kcals) between groups. Babraj et al. (2009) reported similar effects of HIT involving 250 kcals of work in healthy young males. The authors concluded that HIT was an effective way to combat metabolic risk factors in young and middle aged populations who may not otherwise adhere to more time consuming exercise programs.
While exercise interventions for the management of diabetes are well supported by the evidence (Knowler et al., 2002), there are numerous different packages delivered to patients and a paucity in the evidence comparing the effectiveness between the different intervention programs. Backx et al. (2011) compared the effectiveness of a standard care programme and a 12-week exercise intervention in participants with newly diagnosed T2D. The standard care group were advised to exercise at moderate to high intensity five times a week for 30 minutes, and whilst a significant reduction in waist circumference was observed there were no changes in any of the other measures. The supported exercise program consisted of three 60 minute sessions a week at varying intensities (moderate to high) and resulted in significant improvements in several measures including waist circumference (101.4 vs. 97.2 cm; \( p = 0.02 \)), body mass (91.7 vs. 87.9 kg; \( p = 0.01 \)), HbA1c (6.4 vs. 6%; \( p = 0.01 \)), and insulin resistance (3.0 vs. 2.1; \( p = 0.049 \)). This work highlights the necessity for research into supported and unsupported exercise programs to establish comprehensive lifestyle advice for patients with T2D.

### 1.6 Physical Activity, Vitamin D and Glucose Control

Significant positive correlations have been observed between 25(OH)D and physical activity and/or fitness level (Ardestani et al., 2011; Gerdhem et al., 2005; Chomistek et al., 2011). Cardiorespiratory fitness is a surrogate marker of an individual’s daily physical activity, and it may simply be that the increased serum 25(OH)D levels seen in those who are more physically active are a reflection of time spent outdoors i.e. sun exposure. There have however been several mechanisms proposed by which
25(OH)D, physical activity and fitness may interact. Exercise training results in increased muscle mass and induces increases in mitochondrial mass, volume, and mitochondrial enzymes (Baar et al., 2002). It has been proposed that in addition to enhancing \( \dot{V}O_2 \text{max} \) any alterations in mitochondrial volume and enzymatic actions could affect vitamin D metabolism and status as the mitochondria are essential sites for the biosynthesis of steroid hormones such as vitamin D (Miller, 2013).

The limiting factors in relation to \( \dot{V}O_2 \text{max} \) are cardiac output, arterial oxygen content, shunting of blood to the active muscles and oxygen extraction from the blood. Since low levels of 25(OH)D have been shown to cause myocardial hypertrophy, increased blood pressure and endothelial dysfunction (Zittermann et al., 2003), potentially low 25(OH)D may decrease cardiac output and increase peripheral resistance thereby decreasing \( \dot{V}O_2 \text{max} \). Ardestani et al. (2011) identified a positive correlation \((r = .29, p = 0.0001)\) between serum 25(OH)D and \( \dot{V}O_2 \text{max} \) in 200 healthy participants which was more prominent in those who were less physically active. It was suggested that 25(OH)D may have a greater effect on cardiac remodelling in those who engage in a low volume of moderate to vigorous activity as opposed to those who partake in a high volume of activity. There was also a significant interaction between self-reported moderate to vigorous physical activity and serum 25(OH)D.

Muscle fibre type may also be involved, lower levels of physical activity (Bischoff-Ferrari et al., 2004; Andersen et al., 1977) and serum 25(OH)D (Bischoff-Ferrari et al., 2004; Sorensen et al., 1976) can result in a shift from fast twitch type IIa to IIb muscle fibres. When 11 elderly patients were supplemented with a vitamin D analog (1-alpha-hydroxycholecalciferol) and calcium, a decrease in type IIb and an increase in type IIa fibres was observed (Sorensen et al., 1976). Even a modest increase in
type Ila muscle fibre type resulted in increased glucose uptake in rats (Izumiya et al., 2008). In addition to this, low levels of lower body muscle mass could provoke the development of insulin resistance (Olsen et al., 2005). Vitamin D supplementation has been shown to contribute towards lean mass accumulation and improved muscle function (El-Hajj Fuleihan et al., 2006).

To explore factors that are conducive to increasing muscle mass and function, Carrillo et al. (2013) conducted a study exploring the effect of vitamin D supplementation (4000 IU/day for 12 weeks) during a resistance training intervention on body composition, muscle function and glucose tolerance. It was hypothesised that vitamin D supplementation would result in greater improvements in the above than exercise alone. There was a significant increase in peak power in the vitamin D group at 4 weeks and a significant correlation between the change in 25(OH)D and waist to hip ratio ($R^2 = 0.205$, $p = 0.02$), but no other significant effects were observed. The inverse relationship between 25(OH)D and waist to hip ratio is important as abdominal fats have been implicated as an important factor in the development of T2D. Whilst there is still much work to be done in this area it is becoming apparent that the higher levels of 25(OH)D observed in those who are more physically active may not simply be due to increased sun exposure alone. Several plausible mechanisms have been suggested (Miller, 2003; Zittermann et al., 2003) by which increasing physical activity and increasing 25(OH)D status could have additive effects working together to increase cardiorespiratory fitness and circulating 25(OH)D concentrations.
1.7 Thesis aims and objectives

Study 1: The relationship between vitamin D and blood glucose control in a cross section of adults living in Mid Wales.

Aim:

To characterise, statistically and clinically, the relationship between vitamin D status and glycaemic control in adults domiciled in Wales with varying levels of glucose control.

Objectives:

a) To recruit a sample of Welsh domiciled adults with varying levels of glycaemic control;

b) To assess whether vitamin D status differs between groups when classified by glucose control;

c) To determine any association between vitamin D status and glycaemic control.

Study 2: Seasonal Variation in Vitamin D Status and Measures of Glycaemia in a Mid-Wales Population.

Aim:

To identify if there is a seasonal variation in vitamin D status in individuals living in and around the Aberystwyth area and if there is a seasonal variation in vitamin D status if this is then reflected in measures of glycaemic control.
Objectives:

a) Does vitamin D status vary across the seasons (winter/summer/winter)?

b) Are seasonal variations in vitamin D status reflected in glycaemic control?

c) Is baseline vitamin D status associated with future glycaemic control?

Study 3: The effect of vitamin D supplementation and physical activity on vitamin D status and glucose control in healthy participants.

Aim:

To explore the effect of vitamin D supplementation and/or a 15-week cycling exercise intervention on glycaemic control in healthy adults.

Objectives:

a) Examine the effect of vitamin D on glycaemic control;

b) Examine the effect of physical activity on glycaemic control;

c) To examine the effect of exercise added to vitamin D on glycaemic control.
1.8 Rationale for Methods

Following the review of the literature the variables identified to explore the area further include a measure of vitamin D status and measures of glycaemic control. The following section describes the rationale for methods used to measure the selected variables.

Measurement of Vitamin D

Researchers have used sunlight exposure questionnaires as a proxy measure of vitamin D status. However there is no validated, routinely used questionnaire to quantify sun exposure (McCarty, 2008). Personal UV dosimetry and observed sun exposure have both been used to validate sunlight exposure questionnaires, although neither method is considered the ‘gold standard’ (McCarty, 2008). Significant correlations have been observed between various measures of self-reported sun exposure and serum 25 (OH)D concentrations (Brot et al., 2007; Mei et al., 2006). Although the largest correlation coefficient was 0.39 which meant that the majority of the variation in serum 25 (OH)D concentration could not be accounted for within the self-report questionnaire (Mei et al., 2006). It has also been shown that the contribution of sunlight to serum 25 (OH)D concentrations is more important in the summer than the winter months (McCarty, 2008). As such there is potential for misclassification of vitamin D status relying on sun exposure measures and more objective methods such as the measurement of serum 25(OH)D are needed. Similarly to other exposure assessments, sun exposure questionnaires are also susceptible to recall bias, and as such the shorter the recall period the more accurate the recall may be (McCarty, 2008).
Sun Exposure Questionnaire

The Hanwell et al. (2010) Sun Exposure Questionnaire is a simple questionnaire which has been shown to offer a good prediction of 25(OH)D concentrations in healthy Caucasian adults. Similar to other exposure assessments Sun Exposure Questionnaires are also susceptible to recall bias (McCarty, 2008). The questionnaire includes an appropriate time frame to minimise recall bias, and involves minimum subject burden. The maximum time outdoors that may be recorded is > 30 which is adequate duration as in excess of would not result in any additional synthesis of vitamin D$_3$ (Webb, 2006).

There is general agreement that serum 25(OH)D$_3$ is a better indicator of vitamin D status as it has a slower clearance rate than 1,25 (OH)$_2$D$_3$ (Ogunkolade et al., 2002; Zittermann, 2003). The biological half-life of 25(OH)D is approximately 19-20 days (Zittermann, 2003), D$_3$ approximately 24 hours and 1,25(OH)$_2$D$_3$ approximately 4-6 hours (Danescu et al., 2009). Also plasma concentrations of 25(OH)D are 100 times higher than 1,25(OH)D, and most peripheral tissues have the capacity to convert circulating 25(OH)D to 1,25(OH)D the biologically active metabolite (Mosekilde, 2005).

One of the reported issues of using 25(OH)D to describe vitamin status is whether vitamin D deficiency or insufficiency should be defined based on a reference range from a ‘normal’ population, or whether a predetermined cut-off or threshold value should be applied. By using the method based on a reference range from a normal population, unmanageable consequences are incorporated, as plasma 25(OH)D
concentrations vary due to several ecological and individual factors (latitude, skin pigmentation). Whilst research in this area is lacking, there are concerns over the predictive value of a point measurement of plasma 25(OH)D for previous and future vitamin D status. When using 25(OH)D to determine vitamin D status the accuracy of the method used is dependent upon the cross calibration and standardisation (Lips, 2001). The measurement of PTH can also be useful to confirm 25(OH)D levels as it mirrors 25(OH)D concentrations with peak values late winter due to secondary hyperparathyroidism and lower values in late summer (Mosekilde, 2005). Thus, despite potential limitations of using 25(OH)D concentrations as a method to assess vitamin D levels, it is still considered the best method for describing vitamin D status in at risk groups.

25(OH)D will be used to measure vitamin D status within this thesis, and a sun exposure questionnaire will also be included to examine if it correlates with actual vitamin D status.

**Measurement of Insulin resistance and/or sensitivity**

There are various methods, both direct and indirect, for the quantification of insulin sensitivity and resistance. The hyperinsulinemic euglycemic glucose clamp is considered the gold standard for quantifying insulin sensitivity as it directly measures the effects of glucose utilisation under steady state conditions (Katz et al., 2000). This method is, however, not suitable in many circumstances as it requires an intravenous infusion of insulin and frequent blood sampling over a three-hour period with adjustment of glucose required for each participant. A well accepted, slightly less labour intensive, and less expensive alternative, involves minimal model analysis of a frequently sampled intravenous glucose tolerance test (FSIGTT).
However, as it requires approximately 30 blood samples over a three-hour period it is again unsuitable for larger cohorts. In addition, whilst it correlates well with glucose clamp measurements, the identification of the minimal model index of insulin sensitivity is problematic in patients with insulin resistance (Saad et al., 1994).

There are several indirect methods for estimating insulin sensitivity from fasting insulin and glucose values. The fasting condition represents a basal steady state in which glucose is homeostatically maintained within a normal range in healthy humans. Insulin levels are fairly stable and hepatic glucose production is constant with basal insulin secretion from the β-cell maintaining a relatively constant level of insulinemia determined by level of insulin sensitivity/resistance. These indirect simple surrogate measures of insulin sensitivity/resistance can however be easily applied in most settings (i.e. clinical research investigations, epidemiological studies and large clinical trials) and are relatively inexpensive and useful when insulin sensitivity is of secondary interest (Muniyappa et al., 2008). However, due to lack of standardisation of insulin assays, it is not possible to determine a universal cut off point for insulin sensitivity from surrogate indexes of insulin sensitivity/resistance (Muniyappa et al., 2008).

In healthy individuals 1/(fasting insulin) is a useful proxy for insulin sensitivity as elevations in fasting insulin correspond to increased insulin resistance. However this method does not take into account the inappropriately low insulin levels seen in hyperglycaemia with diabetic or glucose intolerant participants, and as such its use is limited (Laakso, 1993). In addition, due to the non-normal distributions of insulin concentrations, linear correlations between 1/(fasting insulin) and insulin sensitivity from the glucose clamp are not that strong (Muniyappa et al., 2008). An indirect
method that correlates well the euglycemic glucose clamp ($r = 0.57$) is the Quantitative Insulin Sensitivity Check Index (QUICKI), which is an empirically derived mathematical transformation of fasting glucose and insulin samples that provides a reproducible, reliable and accurate index of insulin sensitivity (Katz et al., 2000). However again the correlation is weakened in diabetic participants with fasting hyperglycaemia and impaired β-cell function (Muniyappa et al., 2008).

HOMA-IR is another method used for assessing β-cell function and insulin sensitivity from fasting glucose and insulin concentrations (Wallace, Levy, & Matthews, 2004). Whilst HOMA may not give appropriate results in participants with severely impaired or absent β-cell function, it is useful to evaluate insulin resistance in participants with glucose intolerance and mild to moderate diabetes, such is frequently employed in clinical trials and research studies (Haffner, Miettinen, & Stern, 1997; Muniyappa et al., 2008). HOMA-IR will be used within this thesis as an indirect measure of insulin sensitivity. This will require the measurement of glucose and insulin.

*Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)*

This method is advantageous as it only requires a single plasma sample to determine glucose and insulin values, and it compares favourably with other methods. The euglycemic clamp has been referred to as the gold standard for assessing insulin sensitivity and β-cell function, and strong correlations have been derived from HOMA-IR and the euglycemic clamp ($R^2 = 0.88$, $p < 0.0001$) (Matthews et al., 1985). HOMA-IR has been used to examine insulin sensitivity and β-cell function in participants of different ethnic origins with varying degrees of glucose
tolerance. When using single pairs of insulin/glucose samples baseline HOMA-IR was associated with the development of diabetes in 1,449 Mexicans with normal or IGT (Haffner et al. 1996). HOMA-IR can be used at the individual level to identify whether β-cell failure or insulin sensitivity predominates, although it is suggested for this purpose that triplicate insulin samples are used to improve the CV (Wallace et al., 2004). HOMA-IR is not recommended for use in participants on insulin, however it can be used in participants on insulin secretagogues, although results need to be interpreted with caution (Wallace et al., 2004).

When reporting HOMA-IR, both insulin sensitivity and β-cell need to be reported as normal glucose levels may indicate 100% insulin sensitivity and 100% β-cell function, or in some cases 200% insulin sensitivity and 50% β-cell function. If 50% β-cell function was reported in isolation one could assume failing β-cells, where in fact it is more probable that insulin secretion is low due to high insulin sensitivity (as indicated by 200% IS) (Wallace et al., 2004).

Reporting of HbA1c

HbA1c has previously been reported as (%): Diabetes Control and Complications Trial (DCCT) units, since then it was recommended that HbA1c be reported as International Federation of Clinical Chemistry (IFCC) units (mmol/mol). In appreciation of the complexity of a global change in units dual reporting of both units was recommended for a transition period (2009 – 2011), with medical journals also encouraged to report both units (Levy, 2013). In the UK the IFCC units alone should have been reported from June 2011; however this date was deferred by three
months until October 2011. Part of the reason for this was that it was thought the new units were not well understood and would cause confusion for patients and clinicians. Whilst many countries are still reporting both units (Levy, 2013) results will be reported only in the new IFCC units for this thesis, however Table 2.5 shows conversion from old to new units for HbA1c.

**Table 1.5:** HbA1c in old units (%) and new (mmol/mol).

<table>
<thead>
<tr>
<th>HbA1c (%)</th>
<th>HbA1c (mmol/mol)</th>
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<tbody>
<tr>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>6.5</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
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<tr>
<td>7.5</td>
<td>58</td>
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<td>8</td>
<td>64</td>
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<tr>
<td>14</td>
<td>130</td>
</tr>
</tbody>
</table>

**Assessment of Physical Activity**

There are numerous physical activity assessment tools available to use for the measurement of various dimensions and attributes of physical activity, the majority of which have focused on energy expenditure. In identifying which method to use it is important to focus on the dimension of physical activity associated with the outcome of interest (Kriska and Caspersen, 1997). For example, while 100 calories expended
during walking or swimming would be beneficial for cardiovascular health and other health related outcomes, for a favourable effect on bone mass or osteoporosis risk the 100 calories would have to be expended during some form of weight bearing exercise (Kriska and Caspersen, 1997). In population studies in the field of chronic diseases (i.e. diabetes) it is the amount of energy expended during all activities associated with day to day living, not just planned and structured exercise that is of interest. Due to the absence of a readily available, inexpensive, relatively non-invasive, valid and reliable technological method for assessing activity related energy expenditure, it is necessary to rely on estimates of energy expenditure from physical activity questionnaires (Neilson et al., 2008) and physical activity logs (Kriska et al., 1997).

Researchers using subjective methods to quantify physical activity will often use an objective method to validate the subjective method in a given population (Kriska and Caspersen, 1997). Doubly labelled water technique is considered by many as the ‘gold standard’ measure of energy expenditure and is commonly accepted as the optimum in construct validation (Gibney, 2000). Correlation coefficients between doubly labelled water and various physical activity questionnaires vary; correlations between doubly labelled water and the Beake total activity index were 0.68, 0.64 for the Tecumesh estimate of total energy expenditure and 0.68 in women and 0.79 in men for the physical activity scale in elderly (Schuit et al., 1997). The analyses for the doubly labelled water method is however expensive and it has been reported that the energy cost in diseased populations may be further complicated by altered (increased) basal metabolism (Gibney, 2000).
Motion sensors such as pedometers and accelerometers have provided a cheaper second method. Again these methods are not without limitations, the accelerometer has been shown to underestimate several sources of physical activity (Johnson-kozlow et al., 2006). Cardiovascular fitness and physical activity Diaries have also been used as validation measures (Bonnefoy et al., 2001). The physical activity log involves the self-reporting of activity, intensity and duration, which are related to a fixed list of activities to minimise misinterpretation of questions (Schmidt et al. 2003). However physical activity logs do place a relatively high demand on the individuals recording their physical activity levels.

In population studies questionnaires are typically chosen as they poses the characteristics of non-reactiveness (doesn't alter behaviour), practicality, applicability and accuracy (LaPorte et al., 1985; Kriska and Caspersen, 1997). The correlation between $\dot{V}O_2$max and physical activity questionnaires is stronger for heavy as opposed to light activity measures as significant correlations with aerobic fitness would only be observed for vigorous sweat producing activities (Shephard, 2003)

*Physical Activity Questionnaire*

Reliability of different physical activity questionnaires has been shown to diminish with the length of the recall period (Shephard, 2003). The international physical activity questionnaire (IPAQ – long), asks about specific activities within four domains over the previous seven days; leisure time physical activity, domestic and gardening activities, work related physical activity and transport related physical activity. It also includes questions on sitting as an indicator of sedentary behaviour
(Hagströmer et al., 2007). Whilst it is possible to get both categorical and continuous indicators of physical activity from the IPAQ, given the non-normal distribution of energy expenditure in many populations it is suggested that the continuous indicator be presented not as means but as median minutes/week or median MET minutes a week.

The IPAQ has previously been used in a diabetic population to categorise patients into a low, moderate or high intensity group (Jiang et al., 2009). The use of a single estimate of the energy cost of a specific activity derived from a published compendium and applied to all individuals is an acknowledged limitation to self-report instruments. Quantifying energy expenditure in this way cannot account for inter-individual variation in energy expenditure for a given intensity or through variations in mechanical and metabolic efficiency. However, published estimates of costs of different types of physical activity are the only such data available to date (Hagströmer et al., 2007). A study comparing the IPAQ and accelerometer method reported that the accelerometer under reported total physical activity by 247%, with 71% sensitivity and 59% specificity. This was attributable to the inability of the accelerometer to capture the full range of physical activity domains recorded by IPAQ (Johnson-kozlow et al., 2006).

Several studies have demonstrated that IPAQ is a valid and reliable method for collecting physical activity data (Craig et al., 2003; Boon et al., 2010; Hagströmer et al., 2007). The IPAQ is easy to administer, as and there is interview IPAQ and a self-administered long and short versions of the IPAQ (Johnson-kozlow et al., 2006). Other additional benefits of the IPAQ are it provides a measure of sedentary behaviour, and has been translated into several languages so provides a standard
instrument for the measure of physical activity internationally (Johnson-kozlow et al., 2006). In addition to providing total energy expenditure it is suggested that the IPAQ long version be used for research purposes, or potentially in studies requiring more detail on the separate domains or dimensions (Hagströmer et al., 2007). The IPAQ short will be used within this thesis.

Submaximal exercise test

Maximal exercise testing is considered the gold standard for assessing maximal aerobic capacity. In populations were maximal exercise tests are contraindicated however, submaximal exercise tests are frequently used. A predictive submaximal exercise test uses measured HR or \( \dot{V}O_2 \) at two or more work loads, then a predicted \( \dot{V}O_2 \)max may be obtained by extrapolating the relationship between HR and \( \dot{V}O_2 \) to age predicted maximum HR (Noonan & Dean, 2000). A cycle ergometer is the chosen exercise mode as it offers the convenience of a stable sitting position. Also when compared to the treadmill it has the added advantage of the knowledge of the exact amount of external work performed, allowing the evaluation of the work rate-\( \dot{V}O_2 \) relationship (Vanhees et al., 2005).
Chapter 2: General Methods and Recruitment

2.1 General Methods

The current thesis comprises of three individual studies, all which have aspects of the methods in common. Studies include; Study 1: The relationship between vitamin D and blood glucose control in a sample of adults living in Mid Wales, Study 2: Seasonal variation in vitamin D status and measures of glycaemia in a Mid-Wales population, Study 3: The effect of vitamin D supplementation and physical activity on vitamin D status and glucose control in healthy participants. Unless stated otherwise the following sections apply to all 3 studies, additional measures were taken for Study 3, which are detailed in Chapter 5.

2.2 Ethics

All three studies were reviewed by the Aberystwyth University Ethics Committee; this involved two applications with Studies 1 and 2 being submitted via a single application and Study 3 a separate application. As Studies 1 and 2 involved recruitment of individuals with diabetes they were also reviewed by the Dyfed Powys Research Ethics Committee (DPREC); submitted through the Integrated Research Application System (IRAS). IRAS is an online application process that creates application forms for relevant parties, in this case National Health Service (NHS) REC, NHS/Health and Social Care, and Hywel Dda University Health Board Research and Development office. The application was reviewed in accordance with the Streamlined NHS Permissions Approach to Research Cymru (SPARC) operating guidelines in order to obtain permission from participating NHS organisations.
The 'Involving People Network' was consulted during the development of both the protocol and all of the recruitment documentation, including participant information, for Studies 1 and 2 (Appendix 2).

2.3 Procedures

For all visits to the laboratory, with the exception of training sessions during Study 3, participants attended following an overnight fast. On each occasion stature (Harpenden Stadiometer: Holtain Ltd, Crymych, UK) and body mass (Seca 899, Seca GmbH & Co, Hamburg, Germany) were recorded before blood sampling procedures were completed (Section 3.4). Following blood sampling participants completed two questionnaires; The Sun Exposure Questionnaire (Hanwell et al. 2010) and the International Physical Activity Questionnaire (IPAQ), and the Participant Data Sheets (Appendix 1). The experimenter was available during participant's entire time spent in laboratory to answer any questions. All questionnaires were checked, ID coded and stored in the participant packs for later analyses.

2.4 Blood sampling

When collecting blood samples experimenters wore puncture resistant nitrile gloves. Prior to any venous blood samples being drawn participants were seated for 10 minutes to account for plasma volume changes associated with posture shift. The antecubital area was cleaned with an alcohol swab (70% Alcohol; Robinson Healthcare Group, Worksop, UK) and a tourniquet applied to the upper arm. Blood
was drawn from an antecubital vein using a 0.8 x 38 mm needle (BD Vacutainer Precision Glide Multi Sample Needle, Plymouth, UK). Once blood flow was established the tourniquet was loosened and blood collected in to one 6 ml BD 367873 plastic serum tube (red top) and one 6 ml K2EDTA plasma tube (lavender top). When the needle was withdrawn blood flow was stemmed by applying pressure to the area with a medical wipe tissue (Kimberly-Clark, Surrey, UK). All contaminated materials were disposed of immediately.

*Plasma (K2EDTA: lavender tube)*

**Whole blood**

A 0.5 ml sample of whole blood was pipetted from the lavender plasma tube into an Eppendorf and refrigerated for no longer than 4 days before being analysed for HbA1c (Section 2.5).

*Plasma*

The remaining blood in the lavender plasma tube was then centrifuged (Heraeus Multifuge 3SR, Kendro Laboratory Products GmbH, Hanau, Germany) for 10 minutes at 1500 g to separate plasma. Plasma was removed and stored in 1.5 ml Eppendorf tubes at minus 80°C for later analyses of insulin and glucose (Section 2.5).

*Serum (BD 367837: red tube)*

The red serum tube was left to stand for one hour and 30 minutes at room temperature before being centrifuged at 4°C for 10 minutes at 1300 g to separate the
serum. Serum was removed and stored in 1.5 ml Eppendorf tubes at minus 80°C for later analyses of 25(OH)D (Section 2.5).

2.5 Analyses

*Glycated haemoglobin (HbA1c)*

The 0.5 ml whole blood samples were analysed for HbA1c (D-10 HbA1c analyser, Bio-Rad Laboratories Ltd) at the Diabetes Research Network Wales (DRNW) Laboratory in Swansea University. The first set of samples were transported and analysed by the experimenter with subsequent samples transported via the royal mail in pre-labelled boxes (provided by DRNW) and analysed by DRNW staff.

The D-10 HbA1c analyser is an automated system providing an integrated sample preparation, separation, and determination of the relative percentage of specific haemoglobins in whole blood. A buffer solution was delivered to the analytic cartridge and detector via the high performance liquid chromatography pump and proportioning valve. The whole sample then underwent a two-step dilution process prior to delivery to the analytical flow path. Sample probes were rinsed between samples to reduce the risk of carry over. Increasing in ionic strength a programmed buffer gradient delivered samples to the analytical cartridge where haemoglobins are separated based upon ionic interactions with the cartridge material. Separated haemoglobins then passed through the filter photometer flow cell where changes in absorbance were measured at 415 nm.
Quality assurance was conducted across an assay range of 3.8 – 18.5% with both inter (Table 2.1) and intra (Table 2.2) assay precision assessed both internally (Internal QC Scheme: Biorad Lyphocheck Diabetes Control 1 and 2, Bio-Rad Ltd, Hemel Hempstead, Herts, UK) and externally (External QC Scheme: WEQAS Quality Laboratory, The Quadrant Centre Cardiff, Business Park Llanishen, Cardiff, CF14 5WF).

**Table 2.1**: D-10 HbA1c analyser inter-assay precision:

<table>
<thead>
<tr>
<th>Mean (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>2.4</td>
</tr>
<tr>
<td>10.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Table 2.2**: HbA1c analyser intra-assay precision:

<table>
<thead>
<tr>
<th>Mean (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>0.81</td>
</tr>
<tr>
<td>10.3</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*Quantification of plasma insulin*

The frozen 0.5 ml plasma samples were transported on dry ice for analysis for insulin at the DRNW Laboratory in Swansea University using an Invitron Insulin Assay (IV2-001; Invitron Ltd, Monmouth, UK). The experimenter transported the samples and assisted with all analyses. The Invitron Insulin Assay is a two-site immunoassay with a solid phase antibody immobilised on microtitre wells and a soluble antibody labelled with a chemiluminescent acridinium ester which recognises all circulating forms of insulin. Plasma was incubated in microtitre wells, alongside the buffer, then
preceding the wash an antibody solution was added. The plate was then washed removing any unbound labelled antibody and measured using a microtitre plate luminometer (Berthold Plate Luminometer: LB96P and Centro). The assay has a sensitivity of 0.25 μM/L and assay precision across a range of values are reported in Table 2.3.

**Table 2.3:** Insulin inter-assay precision:

<table>
<thead>
<tr>
<th>Mean mU/L</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.8</td>
<td>7.2</td>
</tr>
<tr>
<td>37.3</td>
<td>7.8</td>
</tr>
<tr>
<td>88.7</td>
<td>8.1</td>
</tr>
</tbody>
</table>

*Quantification of fasting plasma glucose*

Plasma glucose was measured using the IL Test Glucose Oxidase Assay in conjunction with the ILab 300 plus analyser, the assay has a range of 0 – 24.8 mmol/l. Inter-assay precision is reported in Table 3.4.
Table 2.4: Glucose inter-assay precision:

<table>
<thead>
<tr>
<th>Mean (mmol/l)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>2.5</td>
</tr>
<tr>
<td>15.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Calculation of β-cell function, IS% and HOMA-IR

Fasting plasma glucose (mmol/l) and insulin (pmol/l) values were used to calculate β-cell function, IS% and HOMA-IR. The HOMA-IR calculator was downloaded from Oxford Centre for Diabetes, Endocrinology and Metabolism website (http://www.dtu.ox.ac.uk/homacalculator/ (accessed 20/11/11))

25(OH)D Analysis

The frozen 0.5 ml serum samples were transported on dry ice for analysis of 25(OH)D at the Merthyr Tydfil Hospital Biochemistry Laboratory. Samples were thawed to room temperature and deproteinised prior to analysis (Agilent 6410 LC-MS/MS) by a precipitation procedure, which involves purification on a trap column prior to reverse phase chromatographic separation and injection in to the MS/MS.

Sample preparation

A 100 μL of sample/calibrator/control was pipetted into 1.5 mL reaction vials. To this 25 μL of the precipitation reagent and 200 uL of internal standard was added and was then vortexed for 20 seconds. Samples were incubated for 10 minutes at 4°C, and centrifuged for five minutes at 1500 g. 200 μL of the supernatant was then
transferred into the autosampler vials, which were then placed into the autosampler tray. Rinsing solutions were placed in positions 1 and 91, calibrators in positions 2-5 and QCs in positions 6-7, with additional QCs added after the last sample (Tables 3.5 and 3.6).

**Measurement**

The MS/MS is composed of 2 quadrupole mass spectrometers with a separating collision cell and operates in multiple reactions monitoring mode. Prior to entering the first MS the samples were first ionised by an atmospheric chemical ionisation (APCI) ion source. The LC eluent was then sprayed through a heated vaporiser at atmospheric pressure, producing a gas phase solvent. Molecules from the solvent were then ionised by an electron discharge from the corona needle, and these solvent ions then passed the electrons to the analyte molecules by chemical reactions, producing the parent (or precursor) ions. The parent ions of interest were isolated by MS1 ($m/z$ ratios without fragmentation) based on the $m/z$ ratio. The parent ions were then passed in to the collision cell where Nitrogen was used as a collision gas to break down the parent ions into daughter ions. MS2 (fragmentation of peptides) then selects the daughter ions of interest based on the $m/z$ ratio, which were then passed through the detector (an electron photomultiplier).

**Table 2.5:** 25(OH)D inter-assay precision:

<table>
<thead>
<tr>
<th>Mean (nmol/l)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.6</td>
<td>7.6</td>
</tr>
<tr>
<td>101.6</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table 2.6: 25(OH)D intra-assay precision:

<table>
<thead>
<tr>
<th>Mean (nmol/l)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.9</td>
<td>6.2</td>
</tr>
<tr>
<td>93.1</td>
<td>7.4</td>
</tr>
</tbody>
</table>

2.6 Recruitment Process

Recruitment

It was initially planned that data collection for Studies 1 and 2 would start during the summer of 2011. However, due to the unforeseen issues with participant recruitment the first data collection phase was put back six months, beginning winter 2012 (January – March). It had originally been proposed that GPs would play a role in identifying potential participants, however the larger practises in the area were unwilling to support the project as they felt it would be an additional burden on their already busy administrative staff. Following several letters, phone calls and meetings with GPs, diabetes nurses and practise managers some of the smaller practises agreed to support the project. Over 200 letters of invitation were written (in both English and Welsh), printed, packed into stamped envelopes and distributed to local practises (Llanilar, Borth and Aberaeron). The practices then forwarded the letters of invitation to patients on their registers who met the inclusion criteria of the study. The letter included details of the research project and contact details for staff involved with the project. Whilst this method of recruitment was developing, it was apparent that adequate numbers would not be attained, and alternative strategies were employed and the study was advertised.
Posters were placed around local medical centres and Aberystwyth University, the University weekly email, local paper, radio BBC Cymru and University Facebook, twitter and alumni newsletter were also used. The principal investigator for the study also attended the local Diabetes UK support group in Machynlleth, diabetes clinics in Bronglais Hospital and local GP practices informing patients of the study details.

The final recruitment strategy was the initiation of a series of Diabetes Information Evenings held in the Department of Sport and Exercise Science. Initially there was to be one meeting with the aim of informing local people, and potential participants, about the research being conducted at the University in collaboration with the NHS. In attendance to answer questions were the research team, clinicians from the NHS, a representative from Diabetes UK and members of the local Diabetes UK Support group. There were several participants recruited as a consequence of the evening (Figure 3.1). The evening was so successful in bringing together researchers, health professionals and members of the public that it was decided another would be scheduled for six months later. The evening proved an efficient method for feeding back to participants about the progress of the study and also provided support and information in relation to managing their condition. The Diabetes Information Evenings are now held at every six months in the Department of Sport and Exercise Science and are well attended. Guest speakers have included the Exercise Referral Coordinator for Wales, Diabetes Consultants, NHS dietician, nurse from the XPERT Program as well as patients who talk about their own experiences.

The majority of participants came from within the county of Ceredigion, although as a consequence of the wide range of recruitment strategies employed there were
participants from up to 56 miles (Welshpool) from Aberystwyth (Figure 2.1 and Table 2.7).
Figure 2.1: Geographical locations of participants recruited for Studies 1 and 2.

Table 2.7: Distance from participants home addresses to Aberystwyth University.

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>Distance travelled in miles</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>0-5</td>
</tr>
<tr>
<td>25</td>
<td>5-10</td>
</tr>
<tr>
<td>35</td>
<td>10-20</td>
</tr>
<tr>
<td>13</td>
<td>20-30</td>
</tr>
<tr>
<td>5</td>
<td>30-60</td>
</tr>
</tbody>
</table>
2.7 Development of analytical methods

*HbA1c, insulin and glucose*

When developing the methods for all three studies advice was sought from the DRNW group based in Swansea University. Following several meetings appropriate analytical methods were identified which were possible to perform in the DRNW laboratories. While it was possible to freeze, and batch, plasma for the analysis of insulin and glucose, the analysis of whole blood for HbA1c necessitated assays to be completed on a weekly basis. As such, while assays for insulin and glucose were completed by the principal researcher, only the initial batch of whole blood samples were assayed for HbA1c personally with all subsequent samples being posted to the DRNW laboratories and assays performed by the laboratory staff.

*25(OH)D*

Whilst there are many commercially available immunoassays for the quantification of 25(OH)D many of them will not detect 25(OH)D with adequate sensitivity for research purposes. Consequently it was decided that the Liquid Chromatography–Mass Spectrometry (LC-MS) method would be developed. Discussions began with Cardiff Metropolitan University (CMU) to develop a LC-MS method for the analysis of 25(OH)D based on the methods of Turpinen (2003). The LC system at CMU was an Agilent 1200 series, a LiChrospher column was employed with 50 uL injection volumes; temperature was manipulated between 35°C and 50°C, and flow rates between 1 ml/min and 2 ml/min. The mobile phase was methanol/water, which was manipulated between a range of 65/35 to 80/20 before settling on a mix of 76/24. UV
detection was set at 265 nm with a background correction of 360 nm. Twenty
different combinations of the above parameters were trialled with a run time of 60
minutes for each injection. After several attempts there was an improvement in
sensitivity of 25% from the first run. There was good linearity and reproducibility for
concentrations within the range of 250 nmol/l to 2000 nmol/l but unfortunately it was
not possible to achieve the desired sensitivity below this range. Solutions were
checked by the Prince Charles Hospital Biochemistry Laboratory who have an
established working method for the analyses of 25(OH)D and it was identified that
the method and instrument would not be able to go as low as required. We were
unable to determine the precise reason for this.

Unfortunately, after several weeks we were unable to develop this method with
adequate sensitivity and limits of detection in CMW. It was therefore decided to get
samples analysed by the Prince Charles Hospital Biochemistry Laboratory. The
researcher transported all samples to the biochemistry laboratory and assisted with
the analyses.
Chapter 3: Study 1

Study 1: The relationship between vitamin D and blood glucose control in a cross section of adults living in Mid Wales

3.1 Introduction

In 2003 5% of the Welsh population (16 years of age and above) were receiving treatment for diabetes, by 2012 this figure had risen to 7% (Welsh Health Survey, 2012). Of these, 69% reported treatment by tablet and 24% by injection, these medications in combination with the treatment of complications associated with diabetes are costing the NHS in Wales approximately £500 million each year (~10% of annual budget). Consequently, there is a great deal of interest in developing a safe and cost effective intervention for the prevention and management of glycaemic control (Peechakara & Pittas, 2008).

The global prevalence of both diabetes and vitamin D deficiency are increasing and inverse associations have frequently been reported between serum 25(OH)D concentration and measures of glycaemia in a variety of different populations (Baynes et al., 1997; Chiu et al., 2004; Scragg et al., 2004; Ford et al., 2005; Need et al., 2005; Forouhi et al., 2008; Hyppönen & Power, 2006; Cigoliniet al., 2006). Furthermore, results from a number of cross sectional studies have shown that participants with IGT or T2D have significantly lower concentrations of 25(OH)D when compared to healthy controls (Scragg et al., 1995; Targher et al., 2006). Emerging evidence suggests vitamin D supplementation is a promising candidate for a cost effective intervention for glycaemic control. However, vitamin D status is not only influenced by factors associated with lifestyle (influenced by culture and
religion), but also sunlight exposure. It is therefore important to establish region specific relationships between vitamin D status and glycaemic control prior to any recommendations in relation to vitamin D supplementation and diabetes.

**Aim:**

To characterise, statistically and clinically, the relationship between vitamin D status and glycaemic control in adults domiciled in Wales with varying levels of glucose control.

**Objectives:**

1. To recruit a sample of Welsh domiciled adults with varying levels of glycaemic control;
2. To assess whether vitamin D status differs between groups when classified by glucose control;
3. To determine any association between vitamin D status and glycaemic control.

**Null hypotheses:**

There will be no difference in vitamin D status in individuals with normal glucose control, impaired glucose control or T2D.

There will be no correlation between concentrations of serum 25(OH)D and measures of glucose.
3.2 Methods

Participant recruitment and retention

See section 2.2 for description of ethical approval. Individuals from the Aberystwyth area were invited to join the study if they were aged between 18 and 85 years (see section 2.6). Due to the potential effect some medications, including insulin, may have on glucose and vitamin D status exclusion criteria included treatment with insulin and the presence of pre-existing co morbidities including cardiovascular disease, type 1 diabetes and cancer.

See section 2.6 for description of recruitment method.

---

**Figure 3.1:** Flow diagram of participant recruitment and retention.
Prior to inclusion, all participants provided written informed consent (Appendix 2). Participants were given a minimum of 24 hours to read all information and consent forms, and the opportunity to ask questions and discuss any concerns with research staff, prior to providing consent.

**Procedures**

Ethical approval was granted by Dyfed Powys Research Ethics Committee (section 2.2). Following an overnight fast 116 participants (see table 3.1 for participant characteristics) attended the Department of Sport and Exercise Science.

**Table 3.1**: participant characteristics (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>56 males</th>
<th>60 females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>59.5 ± 10.3</td>
<td>55.1 ± 11.8</td>
</tr>
<tr>
<td><strong>Mass (kg)</strong></td>
<td>89.6 ± 21.9</td>
<td>75.8 ± 20.9</td>
</tr>
<tr>
<td><strong>Stature (cm)</strong></td>
<td>174.8 ± 7.8</td>
<td>158.6 ± 3.4</td>
</tr>
</tbody>
</table>

All participants lived within a 56-mile radius of Aberystwyth and of the 116, 57 reported a diagnosis of T2D. On arrival the study procedures were explained once more and participants had the opportunity to ask questions. They were also reminded of their right to withdraw at any time without having to provide a reason. Participants then remained seated for the study procedures to be completed (general methods sections 2.3 – 2.5). Laboratory visits took between 30 and 45 minutes. Before leaving the laboratory all participants were given information about the next biannual Diabetes Information Evening.
Statistical analyses

Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Data were checked for normal distribution using the Shapiro-Wilk test prior to statistical analysis. Data are presented as mean ± SD, or, in the case of non-normally distributed data, median and interquartile range. Participants were grouped based on FPG concentrations to explore differences between groups in 25(OH)D. Differences between groups were analysed using a One Way ANOVA or an Independent Samples T-test and where appropriate the non-parametric equivalents: A Kruskal – Wallis test or Mann-Whitney U test. Quartiles were created based on FPG values and further groups were formed based on clinical cut off points for normal glucose control (≤ 6 mmol/l), IGT (6.1 mmol/l – 6.9 mmol/l) and T2D (≥ 7 mmol/l) (WHO, 2006). Relationships between variables were assessed using Pearson’s product-moment correlation coefficient, if data were not normally distributed the equivalent non-parametric tests were performed; Spearman’s rank-order. Tests are considered statistically significant at the 5% alpha level (p < 0.05).
3.3 Results

Difficulty obtaining samples was the primary cause of missing data points (plasma sample: n = 10; serum: n = 11), also the HOMA-IR calculator required the insulin values to be within the range of 20-200 pmol/l which resulted in an additional six missing data points for β-cell function, insulin sensitivity and HOMA-IR values. Descriptive statistics for each variable can be seen in Table 3.2.

**Table 3.2:** Descriptive data for non-normally distributed statistics.

<table>
<thead>
<tr>
<th>variable</th>
<th>number</th>
<th>Median</th>
<th>Interquartile range (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D (nmol/l)</td>
<td>106</td>
<td>35</td>
<td>34.6</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>107</td>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>FPG (mmol/l)</td>
<td>105</td>
<td>5.7</td>
<td>2</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>105</td>
<td>44</td>
<td>18.5</td>
</tr>
<tr>
<td>FPI (pmol/l)</td>
<td>103</td>
<td>47.5</td>
<td>56</td>
</tr>
<tr>
<td>IS (%)</td>
<td>97</td>
<td>105.9</td>
<td>98</td>
</tr>
<tr>
<td>B-cell (%)</td>
<td>97</td>
<td>67.1</td>
<td>37</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>97</td>
<td>0.9</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Participants were grouped based on FPG concentrations: 57 participants had FPG ≤ 6 mmol/l (normal glucose control), 20 were between 6.1 mmol/l and 6.9 mmol/l (IGT) and 24 had ≥ 7 mmol/l, which is the recommended cut off point for the diagnosis of diabetes (WHO, 2006).

A Kruskal – Wallace H Test showed no significant differences in 25(OH)D between groups when classified by clinical thresholds ($X^2(2) = 4.929, p = 0.08$) with a mean rank of 55.49 (nmol/l) for the normal glucose control group (group 1), 41.86 (nmol/l)
for the IGT group (group 2) and 43.06 (nmol/l) for the T2D group (group 3), see figure 3.2.

Figure 3.2: 25(OH)D concentrations in the 3 groups; group 1: FPG ≤ 6 mmol/l, group 2: FPG ≥ 6.1 – 6.9 mmol/l and group 3: ≥ 7 mmol/l.

When the data for the two clinical groups were combined to form two groups (FPG ≤ 6 mmol/l vs ≥ 6.1 mmol/l) there was a significant difference between the groups, Mann-Whitney test (U = 884.000, p = 0.03) (Figure 3.3).
Figure 3.3: Difference between groups (FPG ≤ 6mmol/l vs ≥ 6.1mmol/l) in 25(OH)D concentrations

Quartiles

Participants were grouped based on quartiles of FPG values. A Kruskal – Wallace H Test showed no significant differences in 25(OH)D between groups ($X^2(3) = 4.402, p = 0.22$) (Table 3.3). The difference between the mean rank 25(OH)D (nmol/l) values between quartiles was 2.59 between the 1st and 2nd quartile, 9.95 between the 2nd and 3rd, and 1.64 between the 3rd and 4th quartile.
Table 3.3: Mean rank sum of 25(OH)D concentrations shown per FPG quartile.

<table>
<thead>
<tr>
<th>Quartile</th>
<th>Range FPG (mmol/l)</th>
<th>Mean rank 25(OH)D (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st quartile</td>
<td>3.77-5.01</td>
<td>58.28</td>
</tr>
<tr>
<td>2nd quartile</td>
<td>5.04-5.75</td>
<td>55.69</td>
</tr>
<tr>
<td>3rd quartile</td>
<td>5.77-6.94</td>
<td>45.74</td>
</tr>
<tr>
<td>4th quartile</td>
<td>7.07-16.0</td>
<td>44.1</td>
</tr>
</tbody>
</table>

Correlation analyses

Spearman's rank-order correlations revealed significant negative correlations between 25(OH)D; and FPG ($r = -0.224$, $n = 101$, $p = 0.02$, Figure 3.4) and FPI ($r = -0.242$, $n = 98$, $p = 0.01$, Figure 3.5). No significant relationship was observed between 25(OH)D and HbA1c (Table 3.4). The HOMA-IR method was used to calculate β-cell function and insulin sensitivity. There was no significant association between 25(OH)D and β-cell function (Table 3.4). Significant relationships were identified by Spearman's rank-order correlations between 25(OH)D and insulin sensitivity ($r = 0.256$, $n = 93$, $p = 0.01$, Figure 3.6), and HOMA score ($r = -0.233$, $n = 93$, $p = 0.02$, Figure 3.7).
Figure 3.4: The relationship between 25(OH)D and FPG ($r = -.224$, $n = 101$, $p = 0.02$).

Figure 3.5: The relationship between 25(OH)D and FPI ($r = -.242$, $n = 98$, $p = 0.01$).
Figure 3.6: The relationship between 25(OH)D and insulin sensitivity, \( r = .256, n = 93, p = 0.01 \).

Figure 3.7: The relationship between 25(OH)D and HOMA \( r = -.233, n = 93, p = 0.02 \).
Table 3.4: Non-significant relationships when exploring relationship between 25(OH)D and other variables.

<table>
<thead>
<tr>
<th>Relationship between 25(OH)D (nmol/l) and;</th>
<th>Correlation coefficient</th>
<th>Number of comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>-0.117</td>
<td>101</td>
<td>0.25</td>
</tr>
<tr>
<td>β-cell function (%)</td>
<td>-0.101</td>
<td>93</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Forty-two of the participants were receiving medication for diabetes.

Table 3.5: Medication list for participants with T2D.

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>T2D medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>metformin</td>
</tr>
<tr>
<td>2</td>
<td>metformin, diamicron</td>
</tr>
<tr>
<td>1</td>
<td>metformin, glucophage</td>
</tr>
<tr>
<td>2</td>
<td>metformin, januvia, statins</td>
</tr>
<tr>
<td>8</td>
<td>metformin, pioglitazone</td>
</tr>
<tr>
<td>1</td>
<td>metformin, simvastatin</td>
</tr>
<tr>
<td>2</td>
<td>metformin, sitagliptin (januvia)</td>
</tr>
</tbody>
</table>

When statistical tests were ran with those data points excluded the relationships did not alter greatly: a Spearman’s rank-order correlation indicated a significant relationship between 25(OH)D and FPG when tests were conducted on the full data set ($r = -0.224$, $n = 101$, $p = 0.02$), and also when a further test was conducted when
participants receiving medication for diabetes were removed ($r = -0.253$, $n = 66$, $p = 0.04$).

A Spearman's rank-order correlation showed that there was no significant relationship between total 25(OH)D and the self-reported lifestyle questionnaires; sun exposure questionnaire ($r = -0.064$, $p = 0.54$) and self-reported physical activity ($r = -0.005$, $p = 0.96$).
3.4 Discussion

The first null hypothesis was rejected as a significant difference was observed between groups; participants with normal glucose control had significantly higher 25(OH)D concentrations than those with fasting plasma glucose values of 6.1 mmol/l or above. However when participants were split into three groups based on clinical cut-off points (WHO 2006) for normal glucose control, impaired glucose tolerance and diabetes, or quartiles based on glucose values there were no significant differences in 25(OH)D. This could be attributed to loss of power due to smaller groups, or potentially there may be no clinically meaningful differences in vitamin D between groups above a certain threshold (i.e. the point at which glucose tolerance becomes disturbed). Whilst no significant differences were observed between the glucose defined quartiles of 25(OH)D, there was a point between the second and third quartile where the greatest difference in values for 25(OH)D were observed. This occurred between 5.75 mmol/l and 5.77 mmol/l which combined with the data showing the significant difference between the two groups (≤ 6 vs ≥ 6.1 mmol/l) implies that the tipping point in the relationship between vitamin D status and glycaemia may occur within the region between normal and abnormal glucose control. This would be in agreement with much of the previous work in this area that has reported significant differences in vitamin D status in participants with normal glucose control when compared to participants with impaired glucose tolerance and newly diagnosed diabetes (Scragg et al., 1995)

The second null hypothesis (There will be no association between concentrations of serum 25(OH)D and measures of glucose control) was also rejected as significant associations were observed between 25(OH)D and several of the measures of glycaemic control. The results in the current study reflect the findings of Baynes et al.
(1997) who reported similar values for 25(OH)D and also observed a significant relationship between 25(OH)D and fasting plasma glucose. Both the current study and the Baynes study were conducted at a latitude of 52°N. The negative relationship observed between 25(OH)D and fasting plasma insulin may seem to contradict the suggestion of vitamin D enhancing β-cell function and insulin secretion. However, the significant association between 25(OH)D and insulin sensitivity (%) (estimated by HOMA-IR), demonstrates the potential role of vitamin D in relation to glucose control, and this improved insulin sensitivity in participants would negate the necessity for increased insulin secretion.

Type 2 diabetes is primarily characterised by insulin resistance, as such the positive relationship identified between insulin sensitivity and 25(OH)D could have important implications in relation to this chronic condition. Laboratory studies support these findings as vitamin D receptors have been identified in numerous tissues including skeletal muscle, the primary site for insulin and calcium mediated glucose uptake (Danescu et al., 2009). There are several lines of research supporting the role of vitamin D in relation to pancreatic β-cell function including the presence of vitamin D receptors in pancreatic β-cells (Pittas et al., 2007), and potentially the absence of a significant relationship between 25(OH)D and β-cell function, could be due to methodological issues. Whilst single blood sampling has frequently been used when using HOMA-IR, insulin secretion is pulsatile and as such some recommend triple sampling over a 15 minute period (Wallace et al., 2004). However single sampling is the method most frequently utilised due to convenience.

The absence of a significant relationship between 25(OH)D and HbA1c was unexpected as significant associations have previously been observed between
these measures in different populations (Kositsawat et al., 2010; Hyppönen & Power 2006). Although Kositsawat et al. (2010) observed significance in participants aged 35 – 74 years ($p = 0.004$), and not those aged 18 – 34 years or over 75 years. Several possible reasons for this were suggested such as an age threshold for the effect of vitamin D status on glucose homeostasis, or the absence of abnormal HbA1c values in the younger population; although these would probably not be relevant to the current study as the participants were aged 29 – 79 years. One potential confound could be the possible disconnect of time. HbA1c represents average blood glucose control for the previous 8 -12 weeks and it is unclear how a single measure of 25(OH)D would represent average vitamin D status for the previous 8 – 12 weeks, (25(OH)D has a half-life of approximately 19 days) (Zittermann, 2003).

Whilst Baynes et al. (1997) reported physical activity to be a primary determinant of 25(OH)D concentration, no significant relationship was observed between 25(OH)D and self-reported (IPAQ) physical activity in the current study. In contrast to the findings of Wielen et al. (1995) who reported clothing to be a predictor of 25(OH)D concentrations no-significant association was observed between 25(OH)D and self-reported sun exposure (Hanwell et al. 2003). This is potentially attributable to questionnaire validity for participants living in Wales during the winter months as it has previously been used in sunnier climates (Southern Italy 43˚N). At the latitude of 52˚N the potential for vitamin D synthesis is negligible from October to April (Zittermann 2003), and participants would be more dependent on dietary intake.

The statistical correlations observed between some of the metabolic indices and 25(OH)D concentrations do not infer causality. It has been suggested that low
25(OH)D concentrations could be in response to the disease process and not the cause of impaired glucose tolerance and T2D (Autier et al. 2014). However, there is more evidence (Palomer et al., 2008) to support the role of low vitamin D status in the pathophysiology of these conditions. In the current study many of the participants had normal glucose control or very early stage impaired glucose tolerance which is potentially reversible. As such it is unlikely that the low vitamin D status observed in the current study would be as a result of the disease process (Scragg et al., 1995).

Additionally there are other factors that could be affecting this association. Hyperparathyroidism is often found in vitamin D depletion and evidence has shown that the insulin resistance observed in primary hyperparathyroidism improves after parathyroidectomy (Kahal et al., 2012). Administration of 1,25(OH)$_2$D$_3$ has been shown to improve impaired glucose tolerance, insulin secretion and insulin resistance developing in participants with renal failure. Whether this was a direct effect of increased circulating vitamin D or a decrease in circulating PTH was not identified (Gunal et al., 1997, Kautzky-Willer et al., 1995). In support of a vitamin D role, however, Mak (1998) observed a correction of vitamin D deficiency, glucose intolerance, insulin resistance and hypoinsulinemia in the absence of PTH suppression, although this was only in 8 participants (with uremia).

Whilst most of the vitamin D thresholds are based on bone health outcomes, 25(OH)D concentrations below 65 nmol/l (Hyppönen & Power, 2006) and 40 nmol/l (Need et al., 2005) have been associated with poor glucose control. In the current study the mean 25(OH)D concentration was 42.6 ± 23.8 nmol/l and there was a relatively high proportion (n = 57) of participants with fasting plasma glucose that would be classed above the normal range (WHO, 2006) who presented with
significantly lower concentrations of 25(OH)D. The low 25(OH)D concentrations observed in the study population, alongside the inverse association (and large proportion of participants with high FPG), demonstrates how adults living in Wales could be at an increased risk during the winter months. The low 25(OH)D concentrations in the current study would be attributable to the low solar radiation experienced during the winter months at this latitude (Zittermann, 2003). Whilst there are some data reporting seasonal variations in other health outcomes (Tuberculosis) (Chan, 2000), there are no available published data for diabetes in mid Wales. Seasonal variations have previously been observed in 25(OH)D and measures of glycaemia in different populations, consequently a useful line of research would be to investigate these measures post summer in the current study population.

With the majority of evidence used to determine vitamin D thresholds being based on bone health outcomes, more research is needed to identify the optimal 25(OH)D concentration for glucose control for the prevention and management of T2D. When exploring cross sectional data of vitamin D status in participants with impaired glucose tolerance or T2D, it is not possible to confirm if low levels of 25(OH)D are causal or a result of altered vitamin D metabolism in response to the disease process (Scragg et al., 1995). Thus, more longitudinal and experimental studies are required.
Chapter 4: Study 2

Study 2: Seasonal Variation in Vitamin D Status and Measures of Glycaemia in a Mid-Wales Population

4.1 Introduction

There are established seasonal variations in a number of disease states. The incidence of tuberculosis in Wales, for example, increases during the winter months. Tuberculosis in turn has been shown to have an inverse association with 25(OH)D concentrations (Chan, 2000). Furthermore, seasonal variations in measures of glycaemia have been reported in both healthy individuals (Suarez & Barrett-Connor, 1982) and those with diabetes (Tseng et al., 2005). These seasonal variations have potentially important implications for the prevention and management of T2D. Whilst the cause is undoubtedly multifactorial (Gamble & Taylor, 1969) there is evidence, as with tuberculosis, to suggest a role of vitamin D; as a consequence of reduced sun exposure during the winter months. It is difficult to determine the role of vitamin D in relation to glycaemic control as many studies reporting seasonal variations in glycaemia have not measured 25(OH)D or recorded time spent outdoors (Tseng et al., 2005); a surrogate measure of vitamin D status (Sakura et al., 2010).

Little is known about seasonal variation of vitamin D levels in Wales, and while comparison can be made to individuals living in countries of a similar latitude (Baynes et al., 1997), this would not account for cultural differences such as dietary intake and clothing. Data from study 1 indicated that during the winter months individuals living in Wales are at risk of low levels of vitamin D. Furthermore, it was shown that those participants with normal fasting plasma glucose (6 mmol/l or less)
had significantly higher 25(OH)D concentrations than those with higher fasting plasma glucose (6.1 mmol/l or above).

It is critical, both clinically and when conducting research studies, to understand the magnitude of regional, seasonal variations in 25(OH)D and the subsequent influence this may have on different health outcomes.

Aim:

To identify if there is a seasonal variation in vitamin D status in individuals living in and around the Aberystwyth area, and if there is to determine if measures of glycaemic control are similarly varied.

Objectives:

   d) Does vitamin D status vary across the seasons (winter/summer/winter)?
   e) Are seasonal variations in vitamin D status associated with variations in glycaemic control?
   f) Is baseline vitamin D status associated with future glycaemic control?

Null hypotheses:

   a) There will be no seasonal variation in vitamin D status
   b) There will be no association between changes in vitamin D status and glycaemic control in participants with varying levels of glucose control.
   c) Vitamin D status will not be associated with future glycaemic control in individuals with varying levels of glucose control.
4.2 Methods

Ethical approval was granted by Dyfed Powys Research Ethics Committee (section 2.2). The participants from the cross-sectional cohort (Study 1) were invited back to the Sport and Exercise Science laboratory on two further occasions (at 6 months and 12 months after the first data collection point). The first visit (Study 1/winter) took place during the months of February (average daylight hours in Wales 9 hours 35 minutes) and March (11 hours 45 minutes), the second visit (summer) during August (14 hours 32 minutes) and September (12 hours 37 minutes) and the third visit was February and March again. Where possible every participant attended the lab exactly six and 12 months after their first visit. Of the one hundred and sixteen participants one hundred and two responded to the invitation and attended for the second visit, and ninety nine attended for the third visit.

Table 4.1: Participant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Winter: 0 months</th>
<th>Summer: 6 months</th>
<th>Winter: 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>56 males (48.3%)</td>
<td>60 females (51.7%)</td>
<td>50 males (49.0%)</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>59.5 ± 10.3</td>
<td>55.1 ± 11.8</td>
<td>60.9 ± 10.4</td>
</tr>
<tr>
<td><strong>Mass (kg)</strong></td>
<td>89.6 ± 21.9</td>
<td>75.8 ± 20.9</td>
<td>88.8 ± 22.4</td>
</tr>
<tr>
<td><strong>Stature (cm)</strong></td>
<td>174.8 ± 7.8</td>
<td>158.6 ± 3.4</td>
<td>175.3 ± 7.8</td>
</tr>
</tbody>
</table>

On arrival at the laboratory, following an overnight fast, the study procedures were explained once more to the participants who had the opportunity to ask questions. They were also reminded of their right to withdraw from the study at anytime. Participants remained seated for study procedures to be completed (See general
methods for data collection procedures, section 2.3). Laboratory visits took between 30 and 45 minutes and before leaving all participants were given information about the next biannual Diabetes Information Evening.

**Statistical Analyses**

Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Data were checked for normal distribution using the Shapiro-Wilk test prior to statistical analysis. Data are presented as mean ± SD, or, in the case of non-normally distributed data, median and interquartile ranges (IQR). Differences were explored across the three time points using a Repeated Measures ANOVA and where appropriate the non-parametric equivalent: the Friedman test; posthoc pairwise comparisons were performed with Bonferroni correction for multiple comparisons. Participants were then grouped based on clinical cut-off points for normal glucose control using FPG (≤ 6 mmol/l vs ≥ 6.1 mmol/l) and HbA1c (≤ 47 mmol/mol vs ≥ 48 mmol/mol). Differences between groups in 25(OH)D were analysed with a 2-way mixed ANOVA with groups as the *between subjects factor* and the three time points as the *within subject factor*. Relationships between variables were evaluated using Pearson’s product-moment correlation coefficient or if data were not normally distributed a Spearman’s rank-order correlation. Tests are considered statistically significant at the 5% alpha level (*p* < 0.05).
4.3 Results

Descriptive statistics for each variable for all participants can be seen in table 4.2.

Table 4.2: Values for 25(OH)D concentration and measures of glycaemia in participants at all three time points.

<table>
<thead>
<tr>
<th>Variable</th>
<th>0 Months: Winter</th>
<th>6 Months: Summer</th>
<th>12 Months: Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D (nmol/l)</td>
<td>35.5 (35)</td>
<td>71.3 ± 21.2</td>
<td>41.6 (29)</td>
</tr>
<tr>
<td></td>
<td>(n = 106)</td>
<td>(n = 84)</td>
<td>(n = 89)</td>
</tr>
<tr>
<td>FPG (mmol/l)</td>
<td>5.8 (2)</td>
<td>5.5 (2)</td>
<td>5.7 (2.4)</td>
</tr>
<tr>
<td></td>
<td>(n = 105)</td>
<td>(n = 83)</td>
<td>(n = 82)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>44 (19)</td>
<td>43 (17)</td>
<td>41 (18)</td>
</tr>
<tr>
<td></td>
<td>(n = 105)</td>
<td>(n = 83)</td>
<td>(n = 79)</td>
</tr>
<tr>
<td>FPI (pmol/l)</td>
<td>49 (56)</td>
<td>57 (55)</td>
<td>48.5 (48)</td>
</tr>
<tr>
<td></td>
<td>(n = 103)</td>
<td>(n = 79)</td>
<td>(n = 84)</td>
</tr>
<tr>
<td>IS (%)</td>
<td>105.9 (98)</td>
<td>86.9 (83)</td>
<td>103.9 (99)</td>
</tr>
<tr>
<td></td>
<td>(n = 97)</td>
<td>(n = 69)</td>
<td>(n = 74)</td>
</tr>
<tr>
<td>B-cell (%)</td>
<td>67.1 (37)</td>
<td>83.1 (54)</td>
<td>61.5 (42)</td>
</tr>
<tr>
<td></td>
<td>(n = 97)</td>
<td>(n = 69)</td>
<td>(n = 74)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.9 (1.2)</td>
<td>1.2 (1.4)</td>
<td>1.0 (0.7)</td>
</tr>
<tr>
<td></td>
<td>(n = 97)</td>
<td>(n = 69)</td>
<td>(n = 74)</td>
</tr>
</tbody>
</table>

Values presented for all variables (Shapiro-Wilk normality test; median and inter-quartile range (Mdn(IQR)) for non-normally distributed variables and mean ± SD for normally distributed variables) at each time point and number of samples analysed (n = number of participants).

Friedman tests were conducted to explore differences in variables over time, a significant difference was identified in 25(OH)D concentration between the time points (χ²(2), = 81.913, p < 0.05 (n = 69)). Post hoc analyses revealed statistically significant differences in 25(OH)D concentrations from 0 months (Mdn = 43.2, IQR = 35) to 6 months (Mdn = 70.1, IQR = 27.4) (p < 0.05) and 6 months to 12 months (Mdn = 41.6, IQR = 25) (p < 0.05) but not 0 to 12 months (Figure 4.1). FPI was
significantly different across time points ($\chi^2(2), = 8.024, p < 0.05 (n = 65))$. Posthoc analysis revealed a significant difference in 25(OH)D between 6 months (Mdn 55, IQR = 62.3) and 12 months (Mdn 42, IQR = 42.5) ($p < 0.05$), but not 0 months (Mdn 43, IQR = 53.2) and 6 months or 0 months and 12 months (Figure 4.2).

A Friedman test identified a significant difference in β-cell function between time points ($\chi^2(2), = 22.314, p < 0.001 (n = 50))$. Post hoc analysis identified significant differences between 0 months (Mdn 66, IQR = 41) and 6 months (Mdn 80, IQR =53) and 6 months and 12 months (Mdn 61, IQR = 41) ($p < 0.05$) but not 0 months and 12 months (Figure 4.3). No significant differences were observed in FPG, HbA1c, insulin sensitivity or HOMA-IR (Table 4.3).
Figure 4.1: 25(OH)D concentration at the different time points: 0 months (winter), 6 months (summer) and 12 months (winter).

Friedman test: * significant difference from 0 months; ** significant difference from 6 months; *** significant difference from 12 months.
Figure 4.2: Fasting plasma insulin concentration at the different time points: 0 months (winter), 6 months (summer) and 12 months (winter).

Friedman test: ** significant difference from 6 months; *** significant difference from 12 months.
Figure 4.3: β-cell function at the different time points: 0 months (winter), 6 months (summer) and 12 months (winter).

Friedman test: * significant difference from 0 months; ** significant difference from 6 months; *** significant difference from 12 months.
Table 4.3: Non significant results from Friedman tests for complete data sets at the three different time points.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Friedman test statistic</th>
<th>Winter: 0 months</th>
<th>Summer: 6 months</th>
<th>Winter: 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG (mmol/l)</td>
<td>$X^2(2) = 2.537, p = 0.27$</td>
<td>5.7 (2.4)</td>
<td>5.6 (2.4)</td>
<td>5.7 (2.3)</td>
</tr>
<tr>
<td>($n = 68$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>$X^2(2) = 1.629, p = 0.44$</td>
<td>41 (17)</td>
<td>42 (18.5)</td>
<td>41.9 (25.2)</td>
</tr>
<tr>
<td>($n = 72$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS (%)</td>
<td>$X^2(2) = 5.792, p = 0.05$</td>
<td>111 (114)</td>
<td>93 (85)</td>
<td>101 (93)</td>
</tr>
<tr>
<td>($n = 50$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>$X^2(2) = 4.722, p = 0.09$</td>
<td>0.9 (1)</td>
<td>1.2 (1)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>($n = 50$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values presented as median and inter-quartile range: Mdn(IQR)

A 2-way mixed ANOVA was conducted with groups based on FPG (group one ≤ 6 mmol/l ($n = 49$) vs group two ≥ 6.1 mmol/l ($n = 14$)) as the between subjects factor and the three time points as the within subject factor to explore differences in 25(OH)D concentration. Prior to the test being conducted 7 participants were removed from analysis as they changed groups from 0 months to the other time points and 2 outliers were removed. 25(OH)D concentration was normally distributed for both groups at time point two and three, and in group two but not group one at time point one as assessed by Shapiro-Wilk’s test; the test was considered adequately robust to proceed. There was homogeneity of variances as assessed by Levene’s test ($p < 0.05$) and homogeneity of covariance as assessed by Box’s test of equality of covariances matrices ($p = 0.38$). There was a significant main effect of group in 25(OH)D concentration ($F(1,59) = 4.860, p < 0.05$, partial $r^2 = 0.076$) and a main effect of time point ($F(2,118) = 75.751, p < 0.05$, partial $r^2 = 0.562$). There was
no statistically significant group × time point interaction (F(2,118) = 0.680, \( p = 0.51 \), partial \( \eta^2 = 0.011 \)) (Figure 4.4).

Post hoc analysis with a Bonferroni adjustment revealed that 25(OH)D concentrations were significantly increased from 0 months to 6 months (23.9 (95% CI, 18.1 to 29) nmol/l, \( p < 0.05 \)), with a significant decrease from 6 months to 12 months (-24 (95% CI, -18.1 to -29.4) nmol/l, \( p < 0.05 \)), and no significant difference between 0 months and 12 months (-0.1 (95% CI, -5.2 to -5.5) nmol/l, \( p = 1.0 \)) (Figure 4.4).

**Figure 4.4:** 25(OH)D concentration at the different time points in normal and high FPG groups (FPG ≤ 6 mmol/l vs ≥ 6.1 mmol/l).

2-way mixed ANOVA: * significant difference from 0 months; ** significant difference from 6 months; *** significant difference from 12 months.
A 2-way mixed ANOVA was also conducted with groups, based on HbA1c (group one \((n = 37) < 48 \text{ mmol/mol}\) vs group two \((n = 17) \geq 48 \text{ mmol/mol}\)) as the between subjects factor, the three time points as the within subject factor to explore differences in 25(OH)D concentration. Prior to the test being conducted 3 outliers were removed. 25(OH)D concentration was normally distributed for both groups at all three time points as assessed by Shapiro-Wilk’s test. There was homogeneity of variances as assessed by Levene’s test \((p < 0.05)\) and there was homogeneity of covariance as assessed by Box’s test of equality of covariances matrices \((p = 0.38)\).

There was no main effect of group in 25(OH)D concentration \((F(1,52) = 2.108, p = 0.153, \text{ partial } n^2 = 0.039)\). There was however a main effect of time \((F(2,104, = 65.840, p < 0.05, \text{ partial } n^2 = 0.559)\) (Figure 4.5). There was no statistically significant group \(\times\) time point interaction \((F(2,104) = 0.85, p = 0.43, \text{ partial } n^2 = 0.018)\).

Post hoc analysis with a Bonferroni adjustment revealed that 25(OH)D concentrations were significantly increased from 0 months to 6 months \((25.5 \ (95\% \ Cl, 17.8 \ to \ 30.8) \text{ nmol/l, } p < 0.05)\), with a significant decrease from 6 months to 12 months \((-24.9 \ (95\% \ Cl, -17 \ to \ -30) \text{ nmol/l, } p < 0.05)\), and no significant difference between 0 months and 12 months \((-0.6 \ (95\% \ Cl, -4.9 \ to \ 5.5) \text{ nmol/l, } p = 1.0)\) (Figure 4.5).
Figure 4.5: 25(OH)D concentration (mean ± SD) in groups (< 48 mmol/mol vs ≥ 48 mmol/mol) at the three data collection points.

2-way mixed ANOVA: * significant difference from 0 months; ** significant difference from 6 months; *** significant difference from 12 months.

A Spearman's rank-order correlation revealed a significant relationship between 25(OH)D at 0 months and 25(OH)D at 12 months ($r = .729$, $n = 84$, $p < 0.001$) (Figure 5.6). Further significant correlations (Spearman's rank-order) were found between 25(OH)D at 0 months and HbA1c at 12 months ($r = -0.241$, $n = 75$, $p = 0.04$) (Figure 4.7) and FPI at 12 months ($r = -.225$, $n = 81$, $p = 0.04$) (Figure 4.8).

No significant relationships were observed between 25(OH)D at 0 months and FPG, IS%, β-cell function or HOMA-IR at 12 months (Table 4.4)
Figure 4.6: The relationship between 25(OH)D at 0 months (winter) and 25(OH)D at 12 months (winter).

Figure 4.7: The relationship between 25(OH)D at 0 months (winter) and HbA1c at 12 months (winter).
Figure 4.8: The relationship between 25(OH)D at 0 months (winter) and Fasting plasma insulin at 12 months (winter).

Table 4.4: Non-significant relationships between 25(OH)D at 0 months and other variables at 12 months

<table>
<thead>
<tr>
<th>Relationship between 25(OH)D and;</th>
<th>Correlation coefficient</th>
<th>Number of comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) FPG (mmol/l)</td>
<td>-.105</td>
<td>80</td>
<td>0.35</td>
</tr>
<tr>
<td>(S) Insulin sensitivity (%)</td>
<td>.191</td>
<td>72</td>
<td>0.11</td>
</tr>
<tr>
<td>(S) β-cell function (%)</td>
<td>-.050</td>
<td>72</td>
<td>0.67</td>
</tr>
<tr>
<td>(S) HOMA-IR</td>
<td>-.186</td>
<td>72</td>
<td>0.12</td>
</tr>
</tbody>
</table>

(P) = Pearson’s test, (S) = Spearman’s test
When the difference between values at 0 months and 6 months were calculated; values at 0 months minus values at 6 months no significant associations were observed between changes in 25(OH)D and FPG, HbA1c, FPI, β-cell function, insulin sensitivity or HOMA-IR (Table 4.5).

**Table 4.5:** Results for non-significant statistics when exploring the relationship between change in 25(OH)D and changes in all other variables over 6 months (winter to summer).

<table>
<thead>
<tr>
<th>Relationships between 25(OH)D (nmol/l) and:</th>
<th>Correlation coefficient</th>
<th>Number or comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) FPG (mmol/l)</td>
<td>-.032</td>
<td>80</td>
<td>0.78</td>
</tr>
<tr>
<td>(s) HbA1c (mmol/mol)</td>
<td>.197</td>
<td>76</td>
<td>0.09</td>
</tr>
<tr>
<td>(S) FPI (pmol/l)</td>
<td>-.118</td>
<td>75</td>
<td>0.32</td>
</tr>
<tr>
<td>(P) β-cell function (%)</td>
<td>-.187</td>
<td>64</td>
<td>0.13</td>
</tr>
<tr>
<td>(P) IS (%)</td>
<td>.074</td>
<td>63</td>
<td>0.56</td>
</tr>
<tr>
<td>(P) HOMA-IR</td>
<td>-.134</td>
<td>64</td>
<td>0.29</td>
</tr>
</tbody>
</table>

(P) = Pearson’s test, (S) = Spearman’s test

The difference between the values at 0 months and 12 months were calculated; values at 0 months minus values at 12 months. No significant relationships were observed between change in 25(OH)D and changes in HbA1c, FPG, FPI, β-cell function, insulin sensitivity, or HOMA-IR (Table 4.6).
**Table 4.6:** Non-significant results for relationships between changes in 25(OH)D and changes in all other measures (0 – 12 months).

<table>
<thead>
<tr>
<th>Relationship between 25(OH)D and;</th>
<th>Correlation coefficient</th>
<th>Number of comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(s) FPG (mmol/l)</td>
<td>-.054</td>
<td>78</td>
<td>0.64</td>
</tr>
<tr>
<td>(s) HbA1c (mmol/mol)</td>
<td>.079</td>
<td>78</td>
<td>0.50</td>
</tr>
<tr>
<td>(s) FPI (pmol/l)</td>
<td>-.001</td>
<td>78</td>
<td>0.99</td>
</tr>
<tr>
<td>(s) β-cell function (%)</td>
<td>-.089</td>
<td>65</td>
<td>0.48</td>
</tr>
<tr>
<td>(s) IS (%)</td>
<td>.067</td>
<td>65</td>
<td>0.59</td>
</tr>
<tr>
<td>(s) HOMA-IR</td>
<td>-.115</td>
<td>65</td>
<td>0.37</td>
</tr>
</tbody>
</table>

(P) = Pearson’s test, (S) = Spearman’s test

At the second test (6 months, summer) significant inverse associations (Spearman’s test) were observed between 25(OH)D and FPI ($r = -.297, n = 79, p = 0.01$) (Figure 4.10), and HOMA-IR ($r = -.258, n = 69, p = 0.04$) (Figure 4.11), and a positive association was observed between 25(OH)D and insulin sensitivity ($r = .258, n = 67, p = 0.03$) (Figure 4.12). There were no significant relationships between 25(OH)D and HbA1c, FPG or β-cell function. No correlation was observed between 25(OH)D and the IPAQ or the Sun Exposure Questionnaire (Table 4.7).
**Figure 4.9:** The relationship between 25(OH)D and FPI at 6 months (summer).

**Figure 4.10:** The relationship between 25(OH)D and HOMA-IR at 6 months (summer).
Figure 4.11: The relationship between 25(OH)D and insulin sensitivity at 6 months (summer).

Table 4.7: Non-significant relationships when exploring relationship between 25(OH)D and other variables at 6 months (summer).

<table>
<thead>
<tr>
<th>Relationships between 25(OH)D (nmol/l) and:</th>
<th>Correlation coefficient</th>
<th>Number of comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) FPG (mmol/l)</td>
<td>-0.150</td>
<td>77</td>
<td>0.19</td>
</tr>
<tr>
<td>(S) HbA1c (mmol/mol)</td>
<td>-0.120</td>
<td>78</td>
<td>0.29</td>
</tr>
<tr>
<td>(S) β-cell function (%)</td>
<td>-0.143</td>
<td>66</td>
<td>0.25</td>
</tr>
<tr>
<td>(S) Sun exposure questionnaire</td>
<td>0.135</td>
<td>76</td>
<td>0.24</td>
</tr>
<tr>
<td>(S) Physical activity questionnaire</td>
<td>0.085</td>
<td>75</td>
<td>0.45</td>
</tr>
</tbody>
</table>

(P) = Pearson’s test, (S) = Spearman’s test
4.4 Discussion

A seasonal variation was observed in 25(OH)D, as such the first null hypothesis was rejected. Vitamin D status was significantly increased during the summer when compared to the winter values and this was reflected in significant differences in fasting plasma insulin (decrease from 6 months to 12 months) and β-cell function (increase at 6 months compared to 0 and 12 months). There were no associations between changes in vitamin D status and measures of glycaemia; therefore the second null hypothesis was accepted. There were mixed results in relation to the third null hypothesis as there were associations between vitamin D status and some of the measures of future glycaemic control (HbA1c and insulin concentrations).

To examine differences in seasonal variations in 25(OH)D between clinically relevant sub-groups of the study population, participants were separated into two groups based on measures of glycaemia. Significant differences were observed between groups with normal glucose control compared to those with higher fasting glucose values (≥ 6.1 mmol/l). Whilst differences were observed in vitamin D status across the seasons, and there were differences between the groups, there was no interaction between the groups over time indicating that both groups responded over the course of the year in a comparable way.

Participants were also grouped based on HbA1c values and the cut-off point was set at the recommended diagnostic value for diabetes (48 mmol/mol). A significant effect of season was still observed on vitamin D status but there was no difference between the groups. These findings concur with those from study 1 in which the lack of power to detect differences due to smaller groups was suggested as a causal factor when no differences were observed between those with normal glucose
control, impaired fasting glucose and diabetes. The effect size was greater when
groups were separated by the fasting plasma glucose value of 6.1 mmol/l and this
combined with data from the baseline population would suggest that the tipping point
for differences in vitamin D status lies between normal and abnormal glucose
control.

There was agreement between the correlational analyses from baseline population
and those performed on the data at 6 months (summer) with significant inverse
associations between 25(OH)D and fasting plasma insulin, and 25(OH)D and
HOMA-IR, and a positive association between 25(OH)D and insulin sensitivity.
Participants with higher concentrations of 25(OH)D had increased insulin sensitivity
and lower insulin values compared to those with lower 25(OH)D concentrations at
both time points (0 months (winter) and 6 months (summer)). Whilst these findings
may indicate that higher concentrations of 25(OH)D are associated with increased
insulin sensitivity it is not possible to draw definitive mechanistic conclusions in
relation to 25(OH)D, insulin secretion and insulin sensitivity based on the methods
used in the current study. The relationship between insulin synthesis and insulin
sensitivity is complex (see section 2.2 for a description), and will vary within the
current study’s participants, whose glucose status ranged from normal control to
established diabetes.

There was a significant association between 25(OH)D at 0 months and 25(OH)D at
12 months with a large effect size of $r = 0.7$, (> 50%). This demonstrates how those
who are deficient in vitamin D are likely to continue to be at risk of deficiency long
term in the absence of an intervention (vitamin D supplementation). Little is known
about the stability of vitamin D status across the years (Forouhi et al., 2008) which is
of clinical relevance when considering monitoring at-risk groups and re-testing of vitamin D status. Vitamin D status at 0 months was associated with future glycaemic control with a significant inverse association observed between 25(OH)D at 0 months, and fasting plasma insulin and HbA1c at 12 months. This is in partial agreement with others (Forouhi et al., 2008 and Knekt et al., 2008) who have identified a significant association between vitamin D status and various measures of future glycaemic control including fasting glucose, fasting insulin and HOMA-IR. However, no significant association was observed between 25(OH)D at 0 months and fasting plasma glucose, insulin sensitivity, β-cell function or HOMA-IR at 12 months in the current study. The identification of the association between vitamin D and subsequent HbA1c is important as HbA1c is a clinically relevant measure being one of the recommended diagnostic tools for diabetes, and is also relied upon to guide management and adjust therapy (Gillett, 2009). HbA1c is also considered the more accurate measure of glycaemic control as it has substantially less biologic variability (< 2% day-to-day for HbA1c when compared to 12-15% for fasting plasma glucose).

No significant relationships were observed in any of the change data from 0 to 6 months, or 0 to 12 months. Also no significant relationship was found to exist between 25(OH)D and scores from the sun exposure questionnaires (Hanwell et al., 2010). This was unexpected during the summer months as the study population reside in the rural area around Aberystwyth, with many being retired or having occupations that meant they spent time outdoors during the day (farmers, labourers etc.) which would translate to more time spent exposed to the sun. Potential factors influencing this could be that despite spending time outdoors participants also reported wearing clothing that would cover arms and legs thereby reducing the
opportunity for subcutaneous synthesis of vitamin D. Previous research reporting a correlation between the Hanwell et al. (2010) questionnaire and 25(OH)D was conducted in Southern Italy (40°N). The location of the current study was at a latitude of 52.4°N and consequently there would be less opportunity for synthesis of vitamin D as a direct result of lower temperatures and therefore more clothing.

There is a body of evidence demonstrating the role of vitamin D in stimulating insulin secretion (Palomer et al., 2008) and significant increases were observed in 25(OH)D, β-cell function and insulin concentration during the summer in the current study, which would support this. The long term cumulative effects of seasonal variation on glucose control remains unclear, Tseng et al. (2005) reported a linear decrease in HbA1c over time and Chen et al. (2004) an increase. There was no significant difference in HbA1c values across the seasons in the current study although the median HbA1c value was less during the final winter visit. The differences observed by Tseng (US based study) and Chen (Taiwanese based study) may simply reflect differences in quality of care and lifestyle advice in the different populations.

There was a significant relationship between 25(OH)D and fasting plasma glucose during study 1 but not study 2, there are several possible explanations for why this relationship was no longer significant during the 6 month summer time point. Whilst there are no established thresholds for vitamin D status in relation to T2D, Need et al. (2005) suggested 40 nmol/l which is less than the mean 25(OH)D concentration for the current study participants. Hurst et al. (2010) suggested 50 nmol/l as a threshold and Hyppönen & Power (2006) observed stronger correlations between 25(OH)D and HbA1c when 25(OH)D concentrations were ≤ 65 nmol/l. The median
value for 25(OH)D was 71 nmol/l during the summer visit which is above the recommended thresholds, and above the 50 nmol/l threshold generally considered adequate for overall health (Ross et al. 2011a). There was a 15% ($n = 17$) reduction in participant numbers which would have resulted in less of power to identify significant relationships. Changes in dietary intake of vitamin D, or a change in lifestyle as a consequence of participation in the study, could have influenced the findings as described by Knekt et al. (2008) who reported similar observations and attributed them to increased awareness and increased fish intake (rich in vitamin D) during their study period.

The identification of a significant seasonal variation in vitamin D is important as aside from the tuberculosis data (Chan, 2000) there is very little information in relation to seasonal variation in vitamin D for a Welsh population (Looker et al., 2002). The effect of seasonal variations on 25(OH)D is something that has often been over looked when monitoring vitamin D status, and is evident in many countries even those lying at lower latitudes (Bolland et al., 2007). Seasonal variations in glucose control and 25(OH)D have important implications for healthcare, although more research is required into the long term cumulative effect of seasonal variation on markers of glucose control. The finding that over 50% of the variance in future vitamin D status can be accounted for by baseline 25(OH)D provides useful information in relation to long term monitoring of vitamin D status. These findings combined with the differences between groups and the inverse association between 25(OH)D and future glycaemic control indicate that those who have low levels of 25(OH)D are at long term risk of low vitamin D status, and may be at an increased risk of developing abnormal glucose control.
A potential limitation of the study was the absence of any measurement of calcium as vitamin D and calcium are metabolically related with vitamin D being required for the maintenance of intracellular calcium homeostasis. There are many unknowns in relation to the role of vitamin D and/or calcium in relation to T2D and whether they act independently or synergistically in lowering the risk of T2D (Gagnon et al., 2011). When Liu et al. (2005) reported an association between vitamin D intake and metabolic syndrome it was not independent of calcium intake. Additionally the 15% study dropout rate, and the bias introduced by study participation may have affected the results. Strengths of this study include its prospective design and the measurement of 25(OH)D as a marker of vitamin D status.

The findings from the current study support the proposed link between vitamin D and glucose control and risk of diabetes (Alvarez & Ashraf, 2010), however observational studies are unable to confirm if low levels of 25(OH)D are causal, or a result of, altered vitamin D metabolism in response to the disease process (Scragg et al., 1995). Randomised controlled trials supplementing with vitamin D are now needed to explore the role of vitamin D in relation to glycaemic control.
Chapter 5: Study 3

Study 3: The effect of vitamin D supplementation and physical activity on vitamin D status and glucose control in healthy participants

5.1 Introduction

Study 1 and 2 of this thesis identified significant associations between vitamin D status and measures of glycaemia. While there is mounting evidence linking vitamin D deficiency to abnormal glucose control (Song & Manson, 2010), the evidence overall is mixed, since some studies have reported null effects (Fliser et al., 1997; De Boer et al., 2008; Jorde & Figenschau, 2009; Patel et al., 2010). Null effects may reflect limitations in the design, conduct and analyses of these studies, including small case studies (Taylor & Wise, 1998), insufficient dose (Patel et al., 2010), and use of vitamin D dietary intake, as opposed to measurement of 25(OH)D, as an estimate of vitamin D status (Liu et al., 2006; Pittas et al., 2007). There remains uncertainty concerning the relationship between vitamin D and glucose control.

There is good evidence that increased physical activity has a beneficial effect on glucose control. Knowler et al. (2002), for example, demonstrated that 150 minutes of physical activity a week was more effective than metformin in reducing the incidence of T2D in 3,234 non-diabetic participants with elevated fasting and post load glucose (average follow up 2.8 years). Moreover, evidence indicates that physical activity has a dose-response effect on measures of glycaemic control, such that higher intensity exercise has a larger beneficial effect than low or moderate intensity exercise (Chomistek et al., 2011; DiPietro et al., 2006). The positive effect
of physical activity on insulin sensitivity and glucose metabolism has been well described, but the exact mechanisms are still not fully understood (DiPietro et al., 2006).

Positive correlations have frequently been observed between physical activity, physical fitness and vitamin D status (Constantini et al., 2010). Whilst this may simply be as a consequence of physically active individuals spending more time outdoors, there are several mechanistic links proposed (Constantini et al., 2010; Palomer et al., 2008). For example, increasing physical activity levels may result in body composition changes, such as decreased adipose storage (Chomistek et al., 2011). An inverse association has been observed between adiposity and 25(OH)D concentrations (Bolland et al., 2007). Vitamin D is sequestered in the adipose tissue where it is unavailable for biological processes within the body (Wortsman et al., 2000; Bell et al., 1985) and any reductions in adipose tissue could result in an increase in circulating 25(OH)D.

Another proposition is that exercise training results in increased muscle mass and induces increases in mitochondrial mass, volume, and mitochondrial enzymes (Baar et al., 2002). The mitochondria are essential sites for the biosynthesis of steroid hormones with the mitochondrial P450 enzymes being crucial for the activation and degradation of vitamin D (Miller, 2013), consequently any alterations in mitochondrial volume and enzymatic actions could affect vitamin D metabolism and status. Vitamin D has a well described role in muscle function as evidenced by the classic symptoms of vitamin D deficiency; altered gait, difficulty rising from a chair, decreased muscle strength and muscle pain (Pfeifere et al., 2002). It has been proposed that vitamin D may exert its effects on insulin sensitivity partly through an increase in muscle mass
which would improve overall body insulin sensitivity (Teegarden & Donkin, 2009). Thus, it is plausible that physical activity and vitamin D exert not only independent effects on measures of glycaemic control, but also potential interactive effects.

In addition to the significant associations observed between vitamin D status and glycaemia in the earlier studies of this thesis, significant differences were observed in 25(OH)D between groups when split on measures of glycaemia. These studies were observational and as such it is not possible to infer causality. There is now a need for randomised controlled trial evidence on the independent and interactive effects of vitamin D and physical activity on glycaemic control.

Aim and objectives:
The aim of this study is to explore the potential direct and interactive effects of vitamin D and exercise on measures of glycaemic control, such as FPG, HbA1c, FPI and OGTT. The specific objectives are:

a) To estimate the effect of vitamin D supplementation on glycaemic control;
b) To estimate the effect of physical activity on glycaemic control;
c) To estimate the effect of exercise added to vitamin D on glycaemic control.

Null hypothesis

There will be no effect of vitamin D and physical activity, either alone or combined, on measures of glycaemia in participants with normal glucose control.
5.2 Methods

The experimental protocol was approved by Aberystwyth University’s Ethics Committee and was performed in accordance with the Declaration of Helsinki.

Design

This was a randomised, placebo-controlled trial with four parallel groups. Individuals were randomised using a computer-generated random allocation sequence, which was held by the lead supervisor. After each participant was enrolled into the trial by the researcher, the supervisor revealed the group allocation. Thus, the allocation was concealed from the person responsible for participant enrolment. Whilst it is not possible to blind participants to the exercise intervention, a placebo ensured that participants were blind to vitamin D supplementation.

Participants

Participants were recruited via posters displayed around the University campus and in local community centres, and study information was included in the weekly University email to all staff and students.

Eligible participants were aged $\geq$18 years. Four exclusion criteria were applied: (1) current exercise training, (2) diagnosis of diabetes, (3) current use of a vitamin D supplement, and (4) any contraindication for moderate intensity exercise, as assessed by the Physical Activity Readiness Questionnaire. Of the 89 people who were assessed for eligibility, 51 were excluded for current use of vitamin D supplements ($n = 23$), diagnosis of diabetes ($n = 18$) and a health condition for which moderate intensity exercise was contraindicated ($n = 10$).
Figure 5.1: Participant recruitment process including details of study withdrawal rates.
Written consent was obtained from each subject after being informed of the procedures, risks and potential benefits associated with the study. Participants were also informed of their right to withdraw from the study at any time. Thirty-nine previously untrained individuals participated in the study (males: \(n=6\), age 45.2 ± 12.7 years, stature 178.3 ± 9.3 cm, mass 93.6 ± 22.9 kg, females: \(n=33\), age 41.7 ± 13.4 years, stature 164.8 ± 6.6 cm, mass 67.9 ± 12.6 kg).

**Experimental groups**

Participants were randomised to 1 of 4 trial groups: (1) vitamin D supplementation alone, (2) placebo alone, (3) vitamin D plus exercise, and (4) exercise plus placebo.

**Vitamin D (VitD+Pla):** Participants were required to take two Gelatine capsules (size 00; 100% HIDE gelatine derived from the skin; Blackburn Distributions LTD, Lancashire, UK) a day for a period of 15 weeks. One vitamin D tablet 25 mcg (1000 IU) (Healthspan, Gurnsey, UK) was broken in half and concealed within each capsule. Participants were given enough capsules to last 5 weeks (plus 3 days) during their first laboratory visit. They were invited back to the laboratory every 5 weeks to return used capsule pots and collect a new supply. This was an opportunity to count remaining capsules to assess adherence.

**Placebo (NoEx+Pla):** Participants were required to take two Gelatine capsules (size 00; 100% HIDE gelatine derived from the skin; Blackburn Distributions LTD, Lancashire, UK) a day for a period of 15 weeks. The placebo was matched to vitamin D supplementation for colour, size and frequency of consumption, but contained only microcrystalline cellulose powder 99.12% purity (Blackburn Distributions LTD, Lancashire, UK). Participants were given enough capsules to last
5 weeks (plus 3 days) during their first laboratory visit. They were invited back to the laboratory every 5 weeks to return used capsule pots and collect a new supply. This was an opportunity to count remaining capsules to assess adherence.

*Exercise + Placebo (Ex+Pla):* In addition to placebo, as described above, participants completed a 15-week cycling, exercise programme. Sessions were held at 8.30am and 1pm every weekday, and participants were advised to attend a minimum of two sessions a week (Figure 5.2). Participants completed an additional exercise test mid-way through the exercise program for the recalculation of exercise workloads and were progressed as required. All sessions consisted of 10 minutes warm up and a 5 minute cool down which were performed at 40-50% of the HR reserve. Exercise sessions were alternated between interval and continuous exercise.

During week one, the cardiorespiratory phase consisted of 10 minutes steady state cycling at 60-75% HR reserve, which increased by ten minutes in week two, and a further ten minutes in week three. Participants continued to cycle for 30 minutes at 60-75% HR reserve in these sessions for the remainder of intervention period.

The interval phase started at 10 minutes and increased by 5 minutes each week until 30 minutes was reached. The interval sessions began with a 2-minute low intensity phases at 40-50% HR reserve alternated with 2 minutes of high intensity periods at 80-90% HR reserve.

*Exercise + vitamin D (Ex+VitD):* Participants completed the exercise regimen and vitamin D supplementation as described above.
Figure 5.2: Exercise test schedule for all participants and exercise intervention protocol for the exercise groups (Ex+VitD and Ex+Pla)

Measures

All participants made separate visits to the Department of Sport and Exercise Science in order to complete test measurements. During the first visit participants attended the Department following an overnight fast for measures of vitamin D status and glycaemic control. During this visit they also completed two questionnaires; The Sun Exposure Questionnaire Hanwell et al. (2010) and the IPAQ (Appendix 2). On a separate visit the sub-maximal exercise test and measures of body composition
(Dual-Energy X-ray Absorptiometry) were performed. These two visits were completed at both baseline and 15 weeks.

**Vitamin D status:** Vitamin D status was assessed by measuring 25(OH)D, using the methods previously described (Section 2.4: Blood sampling and Section 2.5: Analyses).

**Glycaemic control:** Glycaemic control was assessed by measuring HbA1c, FPG, FPI and OGTT. Measures of HbA1c, FPG and FPI used the methods described previously (Section 2.4: Blood sampling and Section 2.5: Analyses).

To assess OGTT, finger prick capillary blood samples were taken at pre and 20, 40, 60, 80, 100, and 120 minutes following the ingestion of 75 grams of glucose in 300 ml of water. Participants remained seated for the two hour test. The fingertip was prepared using an alcohol swab (70% Alcohol; Robinson Healthcare Group, Worksop, UK) and pierced by an automated lancet (Accu-Check Safe-T-Pro Plus, Roche Diagnostics GmbH, Germany). The first drop of blood was wiped away. Blood was then drawn into a heparinised capillary tube and analysed for glucose using a YSI glucose and lactate analyser (2300 STAT PLUS, YSI, Yellow Springs, USA). Daily checks of the analyser function were performed in accordance with manufacturer’s guidelines. The blood analyser waste container was emptied and cleaned on a daily basis. Dual samples of standards and dual samples of clinical controls were tested and on each occasion samples were within 2% of their given criterion values. Sharps were disposed of in a sealed sharps bin and all contaminated disposables were disposed of immediately.
**Sub-maximal exercise test:** Participants were seated in the laboratory for 15 minutes to obtain resting HR. To establish each individual's HR - work rate relationship participants performed a sub-maximal exercise test on a cycle ergometer (Monark, 874E, Varberg, Sweden). The protocol consisted of four, four minute work stages at 60, 90, 120 and 150 watts. At the end of each stage HR and RPE (Borg, 1998) were recorded and a linear regression equation was established between workload and HR, from individualised exercise workloads were determined from specific percentages of HR reserve (Karvonen et al., 1957 in Backx et al., 2011).

\[
220 - \text{age} = \text{HR max} \\
\text{HR max} - \text{resting HR} = \text{HR reserve} \\
\text{Exercise HR} = (\text{HR reserve} \times \text{training %}) + \text{resting HR}
\]

**Figure 5.3:** Calculation for HR reserve.

**Body composition:** Dual-energy x-ray absorptiometry, a non-invasive method of assessing body composition, was utilised to measure changes in body fat over the study period. Scans were performed in express mode on a Hologic Discovery A machine (Hologic Inc., Massachusetts, USA). Radiation exposure for both the operator and the participant was monitored throughout, using personal dosimeter badges that were replaced every three months in accordance with the Ionising Radiation Regulations (1999), with a record kept by the University Health and Safety Office.

Prior to visiting the laboratory participants were instructed to wear loose fitting clothing that contained no buttons, zips, beads or any metal, upon arrival participants were instructed to remove any metal they were wearing (e.g. watches or jewellery).
In the rare cases where participants arrived in inappropriate clothing they were given loose fitting surgical apparel to wear during the scan.

Participants lay on the scan bed in a supine position, hands by their sides, palms facing each other. Polystyrene blocks (approximately 30 cm x 5 cm x 10 cm) were placed between the palms of the participants’ hands and the lateral part of the thigh to guarantee a fixed gap to improve the ease of analysis. Prior to the scan being conducted the participants spine and pelvis were aligned by depressing the shoulders and pulling down on the ankles. The participants’ legs and feet were internally rotated and then allowed to return to a natural, comfortable position. Analysis was completed using Apex v3.2.1 (Hologic, Massachusetts, USA) software.

**Statistical Analyses**

Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Data were checked for normal distribution using the Shapiro-Wilk test prior to statistical analysis. Data are presented as mean ± SD, or, in the case of non-normally distributed data, median and interquartile ranges (IQR). Statistical comparisons were also made using a 2-way mixed ANOVA in which the four groups were the between subjects factor and the time points the within subject factor, with paired t-test used for post-hoc tests where appropriate.

Change data were calculated (post – pre values) for all variables. To identify differences between change data for the four groups a one-way ANOVA was employed or for non-normally distributed data its non-parametric equivalent: the Kruskal-Wallis H test. Independent t-tests were conducted to explore differences
between change data for two groups, or in the case of non-parametric data a Mann-Whitney U test. Tests are considered statistically significant at the 5% alpha level ($p < 0.05$).
5.3 Results

When recruited all participants verbally reported partaking in a low level of physical activity; however when they completed the IPAQ the results identified 19% of participants as having low levels of physical activity, 53% moderate levels and 28% were categorised as having high physical activity levels at baseline. Two participants from the NoEx+VitD group withdrew from the study, and one from the NoEx+Pla group, their baseline data were removed from analyses.

Glycaemic control

There was no significant effect of vitamin D or exercise alone or in combination on any of the measures of glycaemia. There was no effect of vitamin D when combined with exercise on any of the measures of glycaemia. Pre and post values are reported in Table (5.1).

FPG

The 2 x 2 between subjects ANOVA failed to find a main effect of vitamin D supplementation on FPG ($F(1,26) = 0.617, MS_e = 0.315, p = 0.43$). The ANOVA revealed no main effect of exercise ($F(1,26) = 0.384, p = 0.54$) with no interaction of vitamin D and exercise ($F(1,26) = 0.496, p = 0.48$).
**HbA1c**

The 2 x 2 between subjects ANOVA failed to find a main effect of vitamin D on HbA1c ($F(1,28) = 0.623$, $MSe = 10.311$, $p = 0.44$) with no main effect of exercise ($F(1,28) = 1.721$, $p = 0.20$) and no interaction of vitamin D and exercise ($F(1,28) = 0.37$, $p = 0.85$).

**FPI**

The ANOVA failed to find a main effect of vitamin D on FPI ($F(1,26) = 1.049$, $MSe = 612.034$, $p = 0.31$) with no main effect of exercise ($F(1,26) = 0.326$, $p = 0.57$), and no interaction of 25(OH)D and exercise ($F(1,26) = 1.834$, $p = 0.18$).

**Table 5.1:** Pre and post intervention values for measures of glycaemia: FPG, HbA1c and FPI (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>Ex+VitD</th>
<th>NoEx+Vit D</th>
<th>Ex+Pla</th>
<th>NoEx+Pla</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FPG (mmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>5.5±0.4</td>
<td>5.9±0.8</td>
<td>5.7±0.7</td>
<td>5.4±0.4</td>
</tr>
<tr>
<td>Post</td>
<td>5.7±0.5</td>
<td>5.8±0.7</td>
<td>5.5±0.3</td>
<td>5.2±0.6</td>
</tr>
<tr>
<td><strong>HbA1c (mmol/mol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>36.9±4.8</td>
<td>36.2±4.6</td>
<td>36.8±2.1</td>
<td>35.0±6.1</td>
</tr>
<tr>
<td>Post</td>
<td>36.2±4.6</td>
<td>36.2±4.6</td>
<td>35.1±3.4</td>
<td>36.1±3.1</td>
</tr>
<tr>
<td><strong>FPI (pmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>43.9±34.1</td>
<td>35.1±18.7</td>
<td>29.7±12.5</td>
<td>43.4±27.6</td>
</tr>
<tr>
<td>Post</td>
<td>41.6±31.4</td>
<td>26.8±8.1</td>
<td>29.7±12.5</td>
<td>56.0±49.0</td>
</tr>
</tbody>
</table>
Oral glucose tolerance test

A series of 2 x 2 between subjects ANOVAs were conducted to examine the effect of vitamin D and exercise alone and in combination on the different time points of the OGTT and total area under the curve.

**Table 5.2:** Pre and post intervention values for measures of glycaemia (OGTT)

<table>
<thead>
<tr>
<th></th>
<th>Ex+Vit D</th>
<th>NoEx+VitD</th>
<th>Ex+Pla</th>
<th>NoEx+Pla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td><strong>AUC</strong> (mmol/l*min)</td>
<td>848.2±95.4</td>
<td>843.9±93.4</td>
<td>912.9±163.0</td>
<td>843.0±148.1</td>
</tr>
<tr>
<td>0 minute (mmol/l)</td>
<td>4.6±0.3</td>
<td>4.6±0.3</td>
<td>4.6±0.4</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td>20 minute (mmol/l)</td>
<td>7.3±1.0</td>
<td>7.5±1.2</td>
<td>7.6±1.0</td>
<td>7.4±1.0</td>
</tr>
<tr>
<td>40 minute (mmol/l)</td>
<td>8.4±1.2</td>
<td>7.5±1.2</td>
<td>9.1±1.5</td>
<td>7.4±1.0</td>
</tr>
<tr>
<td>60 minute (mmol/l)</td>
<td>7.9±1.3</td>
<td>7.6±1.5</td>
<td>8.4±1.8</td>
<td>7.7±1.6</td>
</tr>
<tr>
<td>80 minute (mmol/l)</td>
<td>6.7±1.1</td>
<td>6.7±0.8</td>
<td>7.7±2.1</td>
<td>6.9±1.6</td>
</tr>
<tr>
<td>100 minute (mmol/l)</td>
<td>6.2±0.9</td>
<td>6.6±0.8</td>
<td>7.0±1.7</td>
<td>6.7±1.3</td>
</tr>
<tr>
<td>120 minute (mmol/l)</td>
<td>6.2±1.0</td>
<td>5.7±1.1</td>
<td>6.4±1.4</td>
<td>6.2±1.4</td>
</tr>
</tbody>
</table>
The ANOVA revealed no main effect of exercise on blood glucose at 0 minutes ($F(1,32) = 0.794, p = 0.38$), 20 minutes ($F(1,32) = 0.014, p = 0.91$), 40 minutes ($F(1,32) = 0.835, p = 0.37$), 60 minutes ($F(1,32) = 1.686, p = 0.20$), 80 minutes ($F(1,32) = 1.783, p = 0.19$), 100 minutes ($F(1,32) = 1.874, p = 0.36$), or 120 minutes ($F(1,32) = 1.874, p = 0.36$).

There was no interaction effect of vitamin and exercise on blood glucose at 0 minutes ($F(1,32) = 1.279, p = 0.27$), 20 minutes ($F(1,32) = 1.727, p = 0.19$), 40 minutes ($F(1,32) = 0.001, p = 0.98$), 60 minutes ($F(1,32) = 0.294, p = 0.59$), 80 minutes ($F(1,32) = 0.004, p = 0.95$), 100 minutes ($F(1,32) = 0.508, p = 0.48$), or 120 minutes ($F(1,32) = 0.035, p = 0.85$).

The $2 \times 2$ between subjects ANOVA also failed to find a main effect of vitamin D on total area under the curve of the OGTT, ($F(1,32) = 1.036, MS_e = 9422.524, p = 0.32$), $\alpha = 0.05$. The ANOVA revealed no main effect of exercise, ($F(1,32) = 3.180, p = 0.84$) and no interaction of vitamin and exercise, ($F(1,32) = 0.053, p = 0.8$)

Additional Analyses

Sub maximal exercise test: HR

With the non-significant effect of exercise on measures of glycaemia further analyses were conducted to identify if the exercise programme was of adequate volume to induce any changes in HR or RPE.
Exercising HR at 60 watts

A 2-way mixed ANOVA was conducted with intervention groups as the between subjects factor and the two time points as the within subjects factor to explore differences in exercising HR at 60 watts. HR data were normally distributed for all groups except one (Group 2 pre) as assessed by Shapiro-Wilk’s test; the test was considered adequately robust to proceed. There was a significant group x time interaction (F(3,31) = 4.855, $p = 0.01$, partial $n^2 = 0.320$). There were no significant differences between groups pre intervention for HR at 60 watts (F(3,32) = 1.554, $p = 0.22$, partial $n^2 = 0.127$). However post intervention NoEx+VitD group had significantly higher HR compared to Ex+VitD group (M = 29, SE = 9 bpm, $p = 0.02$) and Ex+Pla group (M = 28, SE = 10 bpm, $p = 0.04$), with no significant difference compared to NoEx+Pla group (M = 11, SE = 9 bpm, $p = 0.64$). There was a significant reduction in exercising HR at post when compared to pre intervention in Ex+VitD group (F(1,9) = 6.533, $p = 0.03$, partial $n^2 = 0.421$), but no significant difference between pre and post HR at 60 watts in NoEx+VitD group (F(1,6) = 4.531, $p = 0.07$, partial $n^2 = 0.430$), Ex+Pla group (F(1,7) = 0.307, $p = 0.59$, partial $n^2 = 0.042$) or NoEx+Pla group (F(1,9) = 1.420, $p = 0.22$, partial $n^2 = 0.127$) (Table 5.3)

Exercising HR at 90 watts

A 2-way mixed ANOVA was conducted to explore differences in exercising HR at 90 watts. HR data were normally distributed for all groups as assessed by Shapiro-Wilk’s test. There was no statistical interaction between groups and time on exercising HR at 90 watts (F(3,29) = 1.506, $p = 0.21$, partial $n^2 = 0.054$). There was
no main effect of time ($F(1, 29) = 1.660, p = 0.21$, partial $\eta^2 = 0.054$) or group ($F(3,29) = 1.357, p = 0.28$, partial $\eta^2 = 0.0983$) (Table 5.3).

Analyses were not conducted on HR data at 120 or 150 watts as too few participants were able to complete these stages.

**Table 5.3:** Exercise test HR for participants in the four groups (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>HR (bpm) @ 60 watts</th>
<th>HR (bpm) @ 90 watts</th>
<th>HR (bpm) @ 120 watts</th>
<th>HR (bpm) @ 150 watts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex+VitD pre</td>
<td>114±17 (n = 10)</td>
<td>133±22 (n = 10)</td>
<td>155±20 (n = 7)</td>
<td>152±17 (n = 2)</td>
</tr>
<tr>
<td>Ex+VitD post</td>
<td>*107±15 (n = 10)</td>
<td>126±20 (n = 10)</td>
<td>143±24 (n = 9)</td>
<td>125±4 (n = 2)</td>
</tr>
<tr>
<td>NoEx+VitD pre</td>
<td>122±20 (n = 8)</td>
<td>149±23 (n = 8)</td>
<td>152±28 (n = 4)</td>
<td></td>
</tr>
<tr>
<td>NoEx+VitD post</td>
<td>122±23 (n = 7)</td>
<td>144±19 (n = 6)</td>
<td>140±21 (n = 2)</td>
<td></td>
</tr>
<tr>
<td>Ex+Pla pre</td>
<td>111±20 (n = 8)</td>
<td>133±26 (n = 8)</td>
<td>140±21 (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Ex+Pla post</td>
<td>109±12 (n = 8)</td>
<td>125±17 (n = 8)</td>
<td>144±19 (n = 8)</td>
<td></td>
</tr>
<tr>
<td>NoEx+Pla pre</td>
<td>128±16 (n = 10)</td>
<td>142±15 (n = 9)</td>
<td>162±11 (n = 6)</td>
<td></td>
</tr>
<tr>
<td>NoEx+Pla post</td>
<td>125±16 (n = 10)</td>
<td>146±18 (n = 10)</td>
<td>149±24 (n = 4)</td>
<td></td>
</tr>
</tbody>
</table>

*significantly different from pre intervention

**Sub maximal exercise RPE**

**Exercising RPE at 60 watts**

A Kruskal-Wallis test failed to find any differences between the four groups in changes in RPE when exercising at 60 watts ($x^2(3) = 6.844, p = 0.07$). A Mann-Whitney U test showed no significant difference in changes in RPE between exercise
(Groups Ex+VitD and Ex+Pla) and non-exercise group (Groups NoEx+VitD and NoEx+Pla) at 60 watts (U = 98.5, z = 98.5, p = 0.07).

**Exercising RPE at 90 watts**

A Kruskal-Wallis test was conducted to explore any differences in change in RPE between the 4 experimental groups from pre to post intervention. No significant differences between changes in RPE pre and post intervention at 90 watts ($X^2(3) = 6.844, p = 0.07$) (Table 5.4). A Mann-Whitney U test identified a significant difference in change in RPE scores between the exercise (Groups Ex+VitD and Ex+Pla) and non-exercise group (Groups NoEx+VitD) in pre and post 90 watts cycling (U = 65.0, z = 65.5, p = 0.006). Median RPE was unchanged (13) in the non-exercise groups, and reduced from 13 to 11.5 in the exercise groups post intervention.
Table 5.4: RPE values for participants in the 4 experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>RPE @ 60</th>
<th>RPE @ 90</th>
<th>RPE @ 120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>watts</td>
<td>watts</td>
<td>watts</td>
</tr>
<tr>
<td>Ex+VitD Pre</td>
<td>13(4)</td>
<td>13(2)</td>
<td>16(3)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 7)</td>
<td></td>
</tr>
<tr>
<td>Ex+VitD Post</td>
<td>9(2)</td>
<td>12(2)</td>
<td>15(5)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td></td>
</tr>
<tr>
<td>NoEx+VitD Pre</td>
<td>12.5(3)</td>
<td>12.5(4)</td>
<td>15(0)</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>NoEx+VitD Post</td>
<td>11(4)</td>
<td>13(4)</td>
<td>14(0)</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 6)</td>
<td>(n = 2)</td>
<td></td>
</tr>
<tr>
<td>Ex+Pla Pre</td>
<td>13.5(3)</td>
<td>13.5(5)</td>
<td>14(3)</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Ex+Pla Post</td>
<td>8(3)</td>
<td>11(1)</td>
<td>14(3)</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>NoEx+Pla Pre</td>
<td>13(6)</td>
<td>13(2)</td>
<td>15(3)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>NoEx+Pla Post</td>
<td>10(5)</td>
<td>12.5(3)</td>
<td>13(3)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 4)</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as median and inter quartile range (median(IQR))
Body composition

The 2 x 2 between subjects ANOVA failed to find a main effect of vitamin D supplementation on trunk fat ($F(1,29) = 0.748$, $MS_e = 1128278.436$, $p = 0.39$), total fat ($F(1,29) = 0.305$, $MS_e = 3448919.722$, $p = 0.58$), or lean mass ($F(1,29) = 2.086$, $MS_e = 2095500.801$, $p = 0.16$). The ANOVA revealed no main effect of exercise on trunk fat ($F(1,29) = 0.204$, $p = 0.65$), total fat ($F(1,29) = 0.126$, $p = 0.72$) or lean mass ($F(1,29) = 2.766$, $p = 0.11$) with no interaction of vitamin D and exercise on trunk fat ($F(1,28) = 0.099$, $p = 0.76$), total fat ($F(1,28) = 0.151$, $p = 0.70$) or lean mass ($F(1,28) = 0.232$, $p = 0.63$) (Table 5.5)

Table 5.5: Pre and Post values for body composition.

<table>
<thead>
<tr>
<th></th>
<th>Ex+Vit D Pre</th>
<th>Ex+Vit D Post</th>
<th>NoEx+Vit D Pre</th>
<th>NoEx+Vit D Post</th>
<th>Ex+Pla Pre</th>
<th>Ex+Pla Post</th>
<th>NoEx+Pla Pre</th>
<th>NoEx+Pla Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trunk fat (grams)</td>
<td>11652±4123</td>
<td>11399±3953</td>
<td>13366±4477</td>
<td>13408±5050</td>
<td>12632±3886</td>
<td>12101±3306</td>
<td>13847±7310</td>
<td>13435±2707</td>
</tr>
<tr>
<td>Total fat (grams)</td>
<td>22961±6768</td>
<td>22416±7142</td>
<td>25376±7647</td>
<td>25332±8413</td>
<td>24683±7398</td>
<td>23935±6432</td>
<td>27636±10829</td>
<td>26359±10808</td>
</tr>
<tr>
<td>Lean mass (grams)</td>
<td>44608±11291</td>
<td>43821±10520</td>
<td>46977±12818</td>
<td>45065±12390</td>
<td>43359±6119</td>
<td>43037±5943</td>
<td>47129±14974</td>
<td>46227±14483</td>
</tr>
</tbody>
</table>
The effect of vitamin D supplementation on vitamin D status

25(OH)D concentration increased in the supplementation groups (Groups Ex+VitD and NoEx+Pla) by 28% and decreased by 33% in the placebo groups (groups Ex+Pla and NoEx+Pla) over the 15-week study period; pre and post 25(OH)D values are reported in Table 5.6. Pre intervention 25(OH)D concentration were not normally distributed for one of the groups, however a sensitivity analyses was conducted and the decision was made to report the results from the one-way ANOVA. There was homogeneity of variance as assessed by Levene’s test ($p = 0.65$). There were no significant differences in 25(OH)D between the 4 groups at baseline ($F(3,30) = 0.086, p = 0.97$). A two-way mixed ANOVA revealed a significant main effect of vitamin D supplementation on 25(OH)D concentrations in the vitamin D supplementation groups ($F(1,30) = 36.276, MS_e = 617.520, p < 0.001$). The ANOVA revealed no main effect of exercise ($F(1,30) = 0.228, p = 0.64$) and no interaction of vitamin and exercise ($F(1,30) = 0.58, p = 0.81$). Dependant t tests identified significant differences in 25(OH)D concentration from pre to post intervention (Table 5.6).

Table 5.6: 25(OH)D concentrations in the vitamin D and placebo groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre 25(OH)D (nmol/l)</th>
<th>Post 25(OH)D (nmol/l)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex+VitD</td>
<td>67.6 ± 25.7</td>
<td>↑*96.9 ± 19.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NoEx+VitD</td>
<td>72.2 ± 25.1</td>
<td>↑*99.5 ± 20.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ex+Pla</td>
<td>68.8 ± 22.8</td>
<td>↓*48.2 ± 17.6</td>
<td>0.001</td>
</tr>
<tr>
<td>NoEx+Pla</td>
<td>72.5 ± 27</td>
<td>↓*46.2 ± 20.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*significantly different from pre intervention.
All of the participants in the exercise programme completed the 15-week intervention program. Adherence to the exercise sessions is reported below in Table 5.1, which shows the number of sessions attended by the participants over the 15-week exercise program. Only 6 of the 18 participants attended the minimum number (30) of exercise sessions required (2 sessions per week).

**Table 5.7: Adherence to exercise sessions over the 15-week cycling intervention**

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>1</th>
<th>3</th>
<th>2</th>
<th>5</th>
<th>1</th>
<th>4</th>
<th>2</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sessions attended</td>
<td>23</td>
<td>24</td>
<td>26</td>
<td>28</td>
<td>29</td>
<td>30</td>
<td>32</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

*Sub maximal exercise test*

One participant in the NoEx+VitD group was unable to complete the post intervention exercise test due to an ankle injury and one failed to complete the 90 watt stage post intervention having completed at the pre-test. Two participants failed to complete the 90 watt stage, and three failed to complete the 120 watt stage when they had had done so in the pre-test from group NoEx+Pla. Two participants from Ex+VitD, and three participants from the Ex+Pla group completed the 120 watt stage post intervention who were unable to do so at the pre intervention test.
Change data (post values minus pre values)

Additional analyses were performed on changes in measures of glycaemia and body composition, change data were calculated (post values – pre values) for all measures across the four experimental groups. A series of one-way ANOVA tests were conducted to explore differences between the groups in changes over the 15-week intervention. There were no significant differences between groups in any of the measures of glucose control: FPG (Figure 5.4), HbA1c (Figure 5.5), FPI (Figure 5.6), blood glucose for OGTT at 0, 20, 40, 60, 80, 100 or 120 minutes, or total area under the curve (Figures 5.7 – 5.14). No significant effects were observed on body composition; trunk fat DXA (Figure 5.15), total fat DXA (Figure 5.16), or lean mass DXA (Figure 5.17) in this population of healthy adults.

Differences between groups in change data for FPG, HbA1c and FPI

A one way ANOVA revealed no significant differences between the four groups for the FPG (F(3,26) = 0.639, p = 0.59) (Figure 5.3), HbA1c (F(3,28) = 0.781, p = 0.51) (Figure 5.4) or FPI (F(2,26) = 0.996, p = 0.53) (Figure 5.5) change data.
**Figure 5.4:** Change values from pre to post intervention for fasting plasma glucose.

**Figure 5.5:** Change values from pre to post intervention for HbA1c.
Figure 5.6: Change values from pre to post intervention for fasting plasma insulin.  

*Differences between groups in change data for OGTT*  

The one-way ANOVA found no differences between groups for changes blood glucose values during the AUC test at 0 minutes ($F(3,32) = 1.070, p = 0.37$) (Figure 5.7), 20 minutes ($F(3,32) = 1.488, p = 0.23$) (Figure 5.8), 40 minutes ($F(3,32) = 0.356, p = 0.78$) (Figure 5.9), 60 minutes ($F(3,32) = 1.042, p = 0.38$) (Figure 5.10), 80 minutes ($F(3,32) = 0.755, p = 0.52$) (Figure 5.11), 100 minutes ($F(3,32) = 0.463, p = 0.71$) (Figure 5.12) or 120 minutes ($F(3,32) = 0.621, p = 0.61$) (Figure 5.13). There were also no differences in change values for total AUC between groups ($F(3,32) = 1.305, p = 0.20$) (Figure 5.14).
Figure 5.7: Change values from pre to post intervention for OGTT glucose at 0 minutes.

Figure 5.8: Change values from pre to post intervention for OGTT glucose at 20 minutes.
Figure 5.9: Change values from pre to post intervention for OGTT glucose at 40 minutes.

Figure 5.10: Change values from pre to post intervention for OGTT glucose at 60 minutes.
Figure 5.11: Change values from pre to post intervention for OGTT glucose at 80 minutes.

Figure 5.12: Change values from pre to post intervention for OGTT glucose at 100 minutes.
Figure 5.13: Change values from pre to post intervention for OGTT glucose at 120 minutes.

Figure 5.14: Change values from pre to post intervention for total area under the curve.
Differences between groups in change data for insulin body composition measures (DXA)

There were no significant differences between groups for changes in body composition over the 15 week intervention. A one-way ANOVA revealed no differences between groups in changes in trunk fat ($F(3,29) = 0.280, p = 0.84$) (Figure 5.14), total fat ($F(3,29) = 0.147, p = 0.93$) (Figure 5.15) or lean mass ($F(3,29) = 1.316, p = 0.28$) (Figure 5.16).

![Figure 5.15: Change values from pre to post intervention for trunk fat (DXA).]
Figure 5.16: Change values from pre to post intervention for total fat.

Figure 5.17: Change values from pre to post intervention for lean mass.
5.4 Discussion

The null hypothesis was accepted as there was no significant effect of vitamin D supplementation or exercise on measures of glycaemic control or body composition in 36 healthy participants. There was a significant increase in 25(OH)D concentrations of 28% in the vitamin D supplementation groups, while there was a significant decrease of 33% in the placebo groups. This would suggest that the vitamin D dose administered in the current study not only negated the seasonal decrease observed during the winter months (Maxwell, 1994), but also maintained values at what would be considered by most as adequate levels (Vieth, 2004; Bischoff-Ferrari et al., 2006).

The non-significant effect of vitamin D supplementation on glucose control was not totally unexpected as much research in this area has indicated that 25(OH)D concentrations would need to be deficient (<50 nmol/l) for an effect of vitamin D to be observed (Wareham et al., 1997). Sorkin et al. (2014) have recently reported a threshold effect of 25(OH)D concentrations in relation to fasting glucose of 65 nmol/l. The study was conducted during the latter part of the summer and consequently participants were not vitamin D deficient at baseline, which is in agreement with the findings from Study 2 in which summer vitamin D status was optimal. While Patel (2010) observed no significant effect of vitamin D supplementation on measures of glycaemia despite baseline concentrations less than 25 nmol/l, this was attributed to sub optimal doses of vitamin D (400 IU and 1200 IU) and the dose in the current study is considered adequate and increased vitamin D status in the majority of participants. Jorde & Figenschau (2009) also found no significant effect of vitamin D supplementation, although it is difficult to make direct comparisons due to different study populations (normal glucose control vs. T2D).
The non-significant effect of exercise was unexpected as the majority of work in this area has demonstrated a positive effect of physical activity (DiPietro et al., 2006) on measurements of body composition and glycaemia (Chomistek et al., 2011). The exercise intervention was based on a 12-week programme (Backx et al., 2011) and had previously induced significant improvements in several measures of body composition and measures of glycaemia in newly diagnosed participants with T2D.

Adherence to the exercise programme may have been a limitation in the current study as not all of the participants completed the minimum requirement of two sessions a week despite much encouragement. However, the exercise intervention was of adequate volume to induce improved cardiorespiratory fitness, demonstrated by the significant reduction in exercising HR at 60 watts post intervention in Ex+VitD group. There was no significant reduction in HR for Ex+Pla group which implies an enhancing role of vitamin D alongside the exercise program in reducing HR. Vitamin D has previously been shown to reduce HR when co-supplemented with calcium for eight weeks more than calcium supplementation alone (Pfeifer et al., 2001).

Increased regular physical activity and cardiorespiratory fitness would result in improved heart rate recovery time, which is an independent predictor of CVD and all-cause mortality in diabetes (Duncan, 2006; Cheng et al., 2003).

Improvements in insulin sensitivity appear to follow a dose response trend in relation to exercise intensity, and the high intensity interval sessions in the current study would be considered adequate to induce improvements in insulin sensitivity (DiPietro et al., 2006). However, it has been observed that the effect of exercise on glycaemic control may decrease with age (DiPietro et al., 2006) and the participants in the current study would be classed as middle aged.
Potentially the absence of any significant changes in the exercise groups could be attributed to many of the participants already engaging in moderate or high levels of physical activity and their normal glucose control. Also the degree of insulin resistance at baseline may affect the responsiveness to exercise training (DiPietro et al., 2006), in that those with the greatest degree of insulin resistance would demonstrate the greatest improvement, and the participants in the current study had healthy glucose control at baseline.

Potential limitations of the current study are small sample size and the inability to measure baseline 25(OH)D concentrations at the participant recruitment phase as this would have enabled selection based on vitamin D status. Hurst et al. (2010) suggested that there will only be an effect of vitamin D supplementation on measures of glycaemic control when participant baseline values are < 50 nmol/l, and only eight of the participants in the present study had 25(OH)D concentrations < 50 nmol/l. Additionally, Hurst et al. (2010) reported the effect of time in that there were only significant changes after a duration of six months with no significant changes after three months of supplementation. These findings were despite the significant increase in 25(OH)D concentrations observed in the study participants (Hurst et al., 2010) and highlights the importance of long term maintenance of optimum vitamin D levels. The dose in the current study has been identified by some (Hurst et al., 2010) as adequate, consequently it seems plausible that the lack of effect of vitamin D supplementation could be attributable to duration and baseline 25(OH)D concentrations.

Multiple observational studies have shown significant associations between vitamin D status and measures of glycaemia in different populations (Forouhi et al., 2008;
Liu et al., 2009). These studies have been supported mechanistically by experimental studies (Palomer et al., 2008), and some have shown a positive effect of supplementation on measures of glycaemia (Hurst et al., 2010). However, no significant effect of vitamin D supplementation was observed in the current study. Plausible explanations lie within the characteristics of the group studied in that they had normal glucose control and adequate vitamin D status at baseline. The findings were also in conflict with much of the previous literature in the area exploring physical activity in relation to glycaemic control and body composition (Knowler et al., 2002; Backx et al., 2011), this may again be attributable to the study group characteristics in that they were relatively active at baseline, and also that many failed to attend the required number of exercise sessions.

The dose of vitamin D in the current study was adequate to significantly raise 25(OH)D concentrations, yet no effect was observed on measures of glycaemia in this healthy adult population. Future intervention studies should consider the same dose but over a longer period with a larger study population with low vitamin D status at baseline. It has also been suggested that vitamin D is unlikely to have an effect in healthy participants unless combined with calcium when it may have a role in the prevention of T2D (Pittas et al., 2007).
Chapter 6: Concluding Remarks

6.1 Concluding Remarks

The three studies within this thesis were designed to explore the role of vitamin D and physical activity in relation to glycaemic control in a Welsh population. Studies 1 and 2 were observational and the population studied ranged from those with normal glucose control through to impaired fasting glucose and T2D. The third study was a 15-week intervention study testing the effect of physical activity and vitamin D supplementation on measures of glycaemia in healthy participants.

Null Hypotheses:

Study 1:

- The first null hypothesis “There will be no difference in vitamin D status in individuals with normal glucose control, impaired glucose control or T2D” was rejected.

- The second null hypothesis “There will be no correlation between concentrations of serum 25(OH)D and measures of glucose control in individuals with varying levels of glucose control” was rejected.

Study 2:

- The first null hypothesis “There will be no seasonal variation in vitamin D status” was rejected.

- The second null hypothesis “There will be no association between changes in vitamin D status and glycaemic control in participants with varying levels of glucose control” was accepted.
• The third null hypothesis “Vitamin D status will not be associated with future glycaemic control in individuals with varying levels of glucose control” was rejected.

Study 3:
• The null hypothesis “There will be no effect of vitamin D and physical activity, either alone or combined, on measures of glycaemia in participants with normal glucose control” was accepted.

Glycaemic control was assessed via HbA1c, fasting plasma glucose and insulin, and β-cell function and insulin sensitivity as measured by HOMA-IR. Several associations were observed between vitamin D status (serum 25(OH)D) and measures of glycaemia in the observational studies (Figure 7.1). Figure 7.2 shows how the results from studies 1 and 2 support the proposed mechanisms by which vitamin D may affect glucose homeostasis and risk of developing T2D.
**Figure 7.1**: Significant results reported in Studies 1 and 2.
Figure 7.2: Proposed mechanistic support from studies 1 & 2. Original diagram Song, & Manson (2010).
There were significant correlations observed during both the winter and summer months between vitamin D status and measures of glycaemia (fasting plasma insulin, insulin sensitivity and HOMA-IR). These findings were consistent for fasting plasma insulin, insulin sensitivity and HOMA-IR, irrelevant of the seasonal fluctuation in vitamin D status. However, the relationship between 25(OH)D and fasting plasma glucose only reached significance during the winter months when sub-optimal vitamin D concentrations were evident. Whilst vitamin D only accounted for a small proportion of the variance (~4 - 8%) in the measures of glycaemia, the development of T2D is multifactorial and any easily modifiable risk factors are noteworthy. In addition to the correlational data significant differences in vitamin D concentrations were observed between participants with normal glucose control compared to those with impaired glucose tolerance or T2D. Whilst these results clearly show that poor glucose control is associated with lower vitamin D status, there is no evidence for cause and effect. It has been suggested that lower vitamin D status may be as a consequence rather than the cause of the disease process in that there may be a reduced ability to metabolise vitamin D in T2D (Scragg et al., 1995).

There was no significant association between 25(OH)D and HbA1c at either of the single time points (0 months: winter or 6 months: summer) which could have relevance in relation to the uncertainty of the stability of 25(OH)D as a measure of previous and future vitamin status (Lips, 2001). HbA1c provides the average value for blood glucose from the previous 8 – 12 weeks whilst 25(OH)D provides an immediate value for circulating 25(OH)D (biological half-life of 25(OH)D is approximately 19 - 20 days (Zittermann, 2003). However, the results from Study 2, a strong correlation \( r = .729 \) between baseline (0 months: winter) 25(OH)D and 25(OH)D concentrations at 12 months (winter), suggest that this measurement gives
a reasonable indication of future vitamin D status and therefore possibly previous status (in the absence of vitamin D therapy). A significant negative association was however observed between 25(OH)D at 0 months and HbA1c at 12 months. Even a slight reduction in HbA1c is clinically relevant as a 1% decrease is associated with a 15% reduction in the relative risk of non-fatal myocardial infarction (Inzucchi et al., 2012). There were significant negative correlations between baseline 25(OH)D and fasting plasma insulin at 12 months. Those with higher vitamin D concentrations at baseline may require less insulin for glucose clearance due to increased sensitivity in those with optimal vitamin D status. Whilst this is speculative as no significant relationship was observed between 25(OH)D and insulin sensitivity, there was a positive linear trend for higher insulin sensitivity in those with higher 25(OH)D.

Results from Seasonal Variations Analysis

Studies 1 and 2 demonstrated how Welsh adults are at risk of vitamin D deficiency during the winter months. A seasonal variation in vitamin D status was also evident which is important as there are only very sparse data in relation to geographically specific areas in the UK and in particular Wales. A nationwide survey of the UK has previously shown that seasonal variations in vitamin D are evident in the UK with a prevalence gradient evident across the country with the highest variations in Scotland and Northern England (Hyppönen, & Power, 2007) (Figure 7.3). When the data for Wales were reported they were combined with data from the Midlands (1238 participants) which covers a larger geographical area. Potentially this would also introduce some cultural and demographic variation, which could result in different behaviours that affect vitamin D status. The findings from the observational studies
(Studies 1 and 2) demonstrate the prevalence of 25(OH)D concentrations below 40 nmol/l to be 55% during the winter (January – March 2011) and 7% during the summer (July –September 2012) in participants living in Mid Wales (56 mile radius of Aberystwyth). Values were in agreement with Hyppönen and Power (2007) during the winter, however during the summer there was a lower percentage of individuals in the current studies with values below 40 nmol/l (7% compared to 10 - 19.9%). This may be due to the larger geographical area studied by Hyppönen and Power (2007) or it may be attributable to annual variations in solar radiation as data were collected 2002 – 2004 in the Hyppönen and Power (2007) study and 2011 – 2012 for Study 2.

**Figure 7.3:** Seasonal and geographical variation in the prevalence of hypovitaminosis in the UK (25(OH)D < 40 nmol/l).

The elderly and ethnic minority groups have previously been considered at risk groups (Saintonge et al., 2009), based on the current study and the work of
Hyppönen and Power (2007) it is now apparent that British white middle aged adults are also at risk for at least half the year. It is well established that low vitamin D levels have negative consequences in relation to several health outcomes (bone health, multiple sclerosis), although the long term implications of this in relation to glucose homeostasis remain unclear. It has been suggested that there would be a cumulative effect of poorer glucose control during winter which would cause a linear increase in glucose values over time (Chen et al., 2004).

Both β-cell function and insulin concentrations were elevated during the summer when vitamin D levels were at their highest, potentially due to a positive effect of increased vitamin D status in relation to insulin secretion. β-cell damage is thought to be irreversible so increasing vitamin D status would be ineffective once damage and fibrosis are established (Boucher et al., 1995). Increasing vitamin D concentrations prevents elevated cytokines which have a role to play in β–cell dysfunction by triggering β–cell apoptosis (Pittas et al., 2007). In the current study it appears that any β-cell dysfunction was minimal in this group as the seasonal correction of vitamin D deficiency resulted in enhanced β-cell function. This could be due to a high proportion of participants having normal glucose control or impaired glucose control and β-cell dysfunction may not be evident in these conditions. Pittas et al. (2007) reported that prolonged vitamin D depletion leads to failure of insulin secretion. Also in support of vitamin D supplementation as an early stage intervention, Orwoll et al. (1994) reported the greatest response (maximal insulin secretion and integrated insulin secretion) to 1,25(OH)2D supplementation in those with shorter term diabetes.
The Effect of Vitamin D and Exercise on Glucose Homeostasis, Body Composition and Sub-Maximal Exercising Heart Rate.

There was no effect of 15 weeks of exercise and vitamin D supplementation on any of the measures of glycaemia or body composition (Study 3). There was a significant increase in 25(OH)D concentrations in the supplementation groups, and a significant decrease in exercising HR at 90 watts post intervention in the Ex+VitD group. The absence of any effect on measures of glycaemia could be attributable to participants having optimum vitamin D status, and/or healthy glucose control at baseline (Tai et al., 2008). While the study was conducted during the summer the data from Study 2 (summer 25(OH)D concentrations for a Mid Wales population) were not available at this point and vitamin D status was unexpectedly high. It would have been useful to screen participant 25(OH)D concentrations as part of the eligibility assessment, but this was not possible. Based on emerging evidence in this area participants with 25(OH)D concentrations below 65 nmol/l would be the most relevant group to study. When considering glucose control Chui et al. (2005) have previously discussed the importance of using participants with healthy glucose control to provide a ‘clean data set’ to examine in relation to the effect of 25(OH)D. However, when exploring the role of 25(OH)D (700 IU/day + calcium 500 mg/day) in relation to glucose control, Pittas et al. (2007) only observed an effect in those with fasting glucose concentrations above normal (5.6-6.9 mmol/l).

Additional Measures for Consideration

Ideally hyperinsulinemic euglycemic glucose clamp would be used for quantifying insulin sensitivity as it directly measures the effects of glucose utilisation under steady state conditions (Katz et al., 2000). However, as discussed in Section 2.6,
this method is only used in a small proportion of studies and more often, less invasive, indirect methods are employed. With the significant associations observed between 25(OH)D and fasting plasma insulin within this thesis an additional measure of C-peptide would have added insight to the role of vitamin D in relation to insulin secretion (Hurst et al., 2010). C-peptide is secreted from the β-cell in equimolar concentration with insulin, but not extracted by the liver (as insulin is) to any significant degree, so consequently can be used as a more reliable marker of beta cell insulin secretion (Gottsäter et al., 1992).

The gold standard method for analysing 25(OH)D concentrations is LC-MS/MS (Toss, & Magnusson, 2012), however, the cost of the analyses is a barrier to a large scale screening programme. For example the cost for analyses was £10.50 per sample for 25(OH)D, compared to the cost of HPLC analyses of HbA1c which was £6.50, and this measure only requires whole blood which negates the requirement for serum (additional costs associated with preparation and storage). In addition, while 25(OH)D is considered the gold standard for vitamin D status, it is uncertain what a single point measurement indicates about previous and future vitamin D status (Lips, 2001). More research is required to explore the stability of 25(OH)D concentrations over time, and the bioavailability of circulating 25(OH)D and 1,25(OH)D in different conditions (i.e. obesity, IGT and T2D). PTH is often used as a confirmation marker of 25(OH)D due to its inverse association with 25(OH)D and as such PTH would be a useful additional measure (Mosekilde, 2005). Both 25(OH)D and PTH affect calcium regulation (Ojuka, 2004; Wright et al., 2004) and due to the established role of calcium in glucose homeostasis a measure of calcium status should be considered when exploring the role of vitamin D. This would enable a
greater understanding of whether the effects are predominately exerted through a direct effect of vitamin D or indirectly through a calcium mediated mechanism.

T2D is a condition of increased inflammation and it has been suggested that the effect of vitamin D in diabetes may be partially exerted through its immunomodulatory and anti-inflammatory properties (Cohen-Lahav et al., 2007). Including measures of inflammation such as IL-6, IL-1, IL-8 and TNF-α (Giulietti et al., 2007) would help to identify the specific mechanisms. Also adiponectin is a key regulator of insulin sensitivity and tissue inflammation and significant associations have been observed between 25(OH)D and adiponectin (Whitehead et al., 2006). Whilst not addressed in any detail within this thesis oxidative stress is increased in T2D and vitamin D supplementation has been observed to increase plasma oxidative capacity in T2D (Asemi et al., 2013).

**Future Directions**

Evidence is emerging in relation to a threshold effect of 25(OH)D concentration in relation to glycaemia, and this is an area that warrants further attention. However to determine cause and effect large scale RCTs are now required although it may be difficult to conduct the type of RCTs required. Much of the research into the role of vitamin D in relation to glucose control implies that the greatest effect of vitamin D would be in those who are deficient, and it would raise ethical concerns to have comparison groups with vitamin D deficiency maintained on a low vitamin D intake (Heany, & Holick, 2011). With the suggestion that vitamin D metabolism may be altered in the diseased state, future trials should explore the changes in 25(OH)D in response to supplementation in participants with healthy glucose control compared to those with impaired glucose tolerance or T2D. There is also a need for large
observational cohort studies to explore vitamin D status and glucose homeostasis over time (≥ 10 years).

Vitamin D deficiency has been linked to numerous health outcomes aside from diabetes including CVD, depression, bone health and multiple sclerosis (Cannell et al., 2008). With the cost associated with 25(OH)D analysis there is a need for research to explore cost effective methods for identifying those at risk of vitamin D deficiency. It has been suggested that physical activity and sun exposure questionnaires may be used as proxy markers for vitamin D status (Ardestani et al., 2011). However the current studies did not support these measures as no associations were observed between 25(OH)D and self-reported physical activity or time sun exposure.

6.2 In Conclusion
In 2004 Vieth discussed how progress was slow in relation to vitamin D research with a lack of evidence from RCT’s in relation to health outcomes, aside from osteoporosis. Much of the research has reported that 25(OH)D concentrations that correlate with desirable effect extend to at least 70 nmol/l with no clear threshold (Vieth, 2004). In the present studies there were fewer significant relationships observed between 25(OH)D and markers of glycaemia when concentrations were 70 nmol/l or above. This is to say that sub-optimal 25(OH)D concentrations have a negative effect on measures of glycaemia, but above these values, other variables may become predominant in glucose control. In study 3 both vitamin D status and measures of glycaemia were optimal, hence the absence of any improvement in measures of glucose control as a consequence of supplementation.
In T2D poor glycaemic control is a major risk factor for the development of diabetic complications (Chen et al., 2004). Data from the present study demonstrated that participants with poorer glucose control had significantly lower 25(OH)D concentrations when compared to those with normal glucose control. These findings are similar to those of Scragg et al. (2004) who reported that over 50% of patients with T2D have low vitamin D levels (< 50 nmol/l), and low vitamin D status in T2D has been reported to predict future micro-vascular events (Cigolini et al., 2006).

When combined with exercise vitamin D supplementation resulted in a reduced sub maximal exercising HR. This may be as a consequence of the effect of 25(OH)D on endothelial function which would reduce peripheral resistance (Zittermann et al., 2003) and HR for a given work rate. This is of clinical significance as micro-vascular events are the cause of many of the complications of diabetes that reduce quality of life and increase the risk of mortalities (Penckofer et al., 2010). However, it is still unclear whether low vitamin D status is causal or merely a marker of poor health (George et al., 2012). It has been suggested that diabetes could be the cause of vitamin D deficiency (and the subsequent changes in calcitropic hormones that lead to osteopenia in diabetes) (Scragg et al., 1995). Based on the evidence to date in relation to vitamin D and glucose homeostasis, it seems plausible that there is in fact a reciprocal relationship (Scragg et al., 1995; Wortsman et al., 2000), though the majority of evidence suggests that vitamin D deficiency would contribute to the development of T2D rather than vice versa (Hurst et al., 2010; Forouhi et al., 2008; Danescu et al., 2009).

An important finding of the current thesis was the confirmation of a seasonal variation in 25(OH)D concentrations with mean 25(OH)D increasing from 42.6 nmol/l
to 71.3 nmol/l. This demonstrates how during the summer months sun exposure in Mid-Wales can raise 25(OH)D concentrations to levels that are considered by many (Vieth, 2004; Bischoff-Ferrari et al., 2006) as adequate, however during the winter vitamin D therapy should be considered to avoid sub optimal 25(OH)D. This is an area that warrants further investigation as there are other factors that contribute towards seasonal variations in human conditions. It may be that it is not the level of vitamin D that is important but the rise and fall as a consequence of season. During the winter months in many countries people tend to be less physically active and calorie intake is often increased, which results in weight gain and reduced glucose control. In addition to the seasonal reduction in vitamin D status, the increased weight gain could also increase the risk of vitamin D deficiency, as vitamin D may be sequestered in the adipose tissue and consequently be unavailable for biological processes within the body (Wortsman et al., 2000). When exposed to an identical amount of UVB irradiation the increase in blood vitamin D₃ concentrations was 57% less in obese participants when compared to non-obese participants (Wortsman et al., 2000). However, when correcting vitamin D deficiency, there was no significant difference in the bioavailability of orally ingested vitamin D in obese and non-obese participants (Wortsman et al., 2000), highlighting the importance of oral supplementation during the winter months in obese individuals.

Diabetes is the fourth leading cause of death worldwide, and in addition to health care costs, there is the personal cost due to the development of diabetes during the more economically productive years of people’s lives (Khuwaja, Khowaja, & Cosgrove, 2010). Based on the data from the observational studies within this thesis and the existing body of literature it seems plausible that the increasing prevalence
of sub-optimal vitamin D status could be contributing to an increased incidence of T2D.
References


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